

Propriétés nutritionnelles de la citrulline : un nouvel acteur dans la régulation du métabolisme protéino-énergétique

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Arthur Goron. Propriétés nutritionnelles de la citrulline : un nouvel acteur dans la régulation du métabolisme protéino-énergétique. Sciences agricoles. Université Grenoble Alpes, 2017. Français. NNT : 2017GREAV016 . tel-01685287

HAL Id: tel-01685287 https://theses.hal.science/tel-01685287

Submitted on 16 Jan 2018 $\,$

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Communauté UNIVERSITÉ Grenoble Alpes

THÈSE

Pour obtenir le grade de

DOCTEUR DE LA COMMUNAUTE UNIVERSITE GRENOBLE ALPES

Spécialité : Biologie cellulaire

Arrêté ministériel : 25 mai 2016

Présenté par

Arthur GORON

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Propriétés nutritionnelles de la citrulline : un nouvel acteur dans la régulation du métabolisme protéino-énergétique

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Remerciements

A Christophe Pison, Moïse Coëffier, Dominique Dardevet, Julien Fauré, Anne-Laure Borel et Michel Rigoulet pour avoir acceptés de juger mon travail en tant que jury de thèse.

A Christophe « maître Jedi » Moinard, pour votre encadrement, votre rigueur, votre soutien, vos encouragements et vos conseils avisés qui m'ont permis de réaliser à bien cette thèse. Votre padawan sort grandi de ces années passées en votre compagnie.

A Eric Fontaine pour ton aide précieuse lors ces années de doctorat, pour ton entrain et ton savoir scientifique qui m'ont réconciliés avec la mitochondrie.

A « Agir pour les maladies chroniques » de m'avoir offert la possibilité de réaliser cette thèse grâce à leur financement.

A l'entreprise SEB et en particulier à Mariette Sicard et Paul Dancer, qui m'ont permis de prolonger mon financement afin de terminer sereinement ma thèse.

A la fondation UGA qui, grâce au mécénat de Findus, m'a permis de financer un des travaux de cette thèse dans le cadre de la Chaire « Nutrition des seniors ».

A Uwe Schlattner pour m'avoir si chaleureusement accueilli au sein du laboratoire.

A Frédéric Lamarche pour ta bonne humeur. Tu as toujours été volontaire lorsque j'avais besoin de ton aide.

A Hervé Dubouchaud pour ton savoir autour de l'exercice qui m'a été grandement utile.

A Sarah Hamant qui a toujours été là quand j'en avais besoin. Tes mails vont me manquer.

A Régis Montmayeul pour ton soutien technique et logistique qui m'a sorti d'affaires bon nombre de fois.

A Gérard « Camarade » Larmurier pour ta gentillesse et ta bonne humeur.

A Charlotte Breuillard pour ta bonne humeur et pour ces bons moments passés ensembles (notamment en congrès).

A tous les doctorants et ex-doctorants que j'ai côtoyé et qui sont aujourd'hui plus que des collègues mais des amis précieux. Merci pour ces merveilleux moments passés tous ensemble.

A Cindy Tellier pour ton aide à l'animalerie.

A Cécile Cottet pour ta bonne humeur, ton aide en imagerie. Merci d'avoir été à l'écoute quand j'en avais besoin.

A Stéphane Attia pour les discussions footballistiques qui m'ont permis de faire passer le temps lors de mes ennuyeux broyages.

A Michel Seve, Sandrine Bourgoin, Valérie Cunin et Sylvie Michelland pour votre accueil au sein de la plateforme Prométhée, votre gentillesse et votre aide en protéomique.

A Christelle Corne pour avoir si gentiment accepté de faire mes dosages d'acides aminés.

A Christophe Hourdé et Philippe Noirez pour votre aide en histologie.

A Denis Rousseau pour nos discussions philosophico-footballistiques. En revanche, ton café ne restera pas un souvenir impérissable.

A toutes les autres personnes du laboratoire, merci pour votre accueil et pour les bons moments passés en votre compagnie. A Servane Le Plénier, qui m'a encadré lors de mes stages de Master. Si je termine mon cursus universitaire sur une thèse, c'est aussi grâce à toi.

A mes parents, qui m'ont toujours soutenu dans mes choix.

Enfin, à Aurélie, pour ton soutien dans les moments difficiles. Tu as accepté de vivre une relation à distance afin que je puisse mener à bien cette thèse. Cela a été difficile pour nous deux mais contrairement au proverbe qui dit « loin des yeux, loin du cœur », ces années passées loin de toi n'ont fait que renforcer l'amour que je te porte.

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INTRODUCTION GÉNÉRALE

La citrulline est un acide aminé non essentiel et non protéinogène qui tient son nom de la pastèque (en latin, citrullus vulgaris). Durant de nombreuses décennies, cet acide aminé a été considéré comme un intermédiaire du cycle de l'urée et comme un simple acide aminé parmi d'autres. L'intérêt pour cet acide aminé s'est ravivé lorsque Winmueller et Spaeth ont montré que l'intestin produisait de la citrulline de façon notable (1). Cette particularité a tout d'abord été mise à profit à des fins de diagnostics, car la citrulline se révèle être le meilleur marqueur de la fonction intestinale. Par la suite, les propriétés métaboliques de cet acide aminé ont été mises en évidence. Tout d'abord sur le plan cardiovasculaire (en tant que précurseur de monoxyde d'azote (NO)), puis en tant que modulateur de l'homéostasie azotée. Ainsi, en 2004, dans un travail princeps, Osowska et al. (2) ont mis en évidence un rôle majeur dans la régulation du métabolisme protéique. En effet, il a été démontré dans ce travail que la citrulline est capable de restaurer le bilan azoté dans un modèle murin de syndrome de grêle court (2). A partir de ce travail, diverses études ont tenté de déterminer les effets de la citrulline sur le métabolisme protéique, notamment au niveau musculaire. Ainsi Osowska et al. (3) ont montré dans un modèle de malnutrition protéino-énergétique, que la citrulline est capable de stimuler la synthèse protéique. Cet effet de la citrulline a été confirmé dans plusieurs travaux. Le plénier et al. (4) ont notamment montré que cet effet stimulateur de la synthèse protéique musculaire de la citrulline est direct dans des muscles isolés périfusés. Cependant, il est bien connu que le turn-over protéique musculaire est hétérogène et qu'une augmentation globale de la synthèse protéique n'implique pas forcément une augmentation de la synthèse de toutes les protéines. Cela a bien été mis en évidence avec la citrulline puisque Moinard et al. (5) ont démontré, grâce à une approche protéomique différentielle, que la citrulline est capable de surexprimer certaines protéines et de sous-exprimer d'autres protéines. Cette hétérogénéité du turn-over protéique a également été démontrée par Faure et al. (6) qui ont montré que la citrulline induit un switch métabolique dans le muscle. En effet, la citrulline est capable d'induire une surexpression d'enzymes impliquées dans la glycolyse et la glycogénolyse, tandis que certaines enzymes impliquées dans le cycle de Krebs sont sous-exprimées avec la citrulline. Bien que cette étude se soit focalisée sur le métabolisme énergétique, elle illustre bien cette hétérogénéité du turn-over protéique malgré une stimulation globale de la synthèse protéique. Le métabolisme énergétique est logiquement intimement lié au métabolisme protéique puisque la synthèse protéique est un poste de dépense énergétique très important, lié au fait que la formation d'une liaison peptidique nécessite beaucoup d'énergie. Ainsi, la part de la dépense énergétique consacrée à la synthèse protéique est estimée à au moins 20% de la dépense énergétique totale de la cellule, en fonction du type cellulaire considéré (7). Outre l'étude de Faure et al. suggérant un switch métabolique musculaire induit par la citrulline, d'autres études ont déterminé que la citrulline agit sur le métabolisme énergétique. Ainsi, Moinard et al. (5) ont mis en évidence que la citrulline stimule l'expression génique de TFAM, un facteur de biogenèse mitochondriale. Par ailleurs, l'effet de la citrulline sur le métabolisme énergétique ne se limite pas au muscle puisque Joffin et al. (8–10) ont ainsi démontré dans des explants adipeux, que la citrulline stimule la β-oxydation ainsi que l'expression génique musculaire de TFAM, d'UCP1 et de CPT1. Ces effets pourraient expliquer, du moins partiellement, d'autres travaux montrant que la citrulline est capable d'augmenter la dépense énergétique (11). Ainsi toutes ces études ont permis de mettre en évidence l'implication de la citrulline dans la régulation du métabolisme énergétique. Cependant, ces données restent parcellaires et surtout, ne permettent pas de démontrer l'inter-relation entre les effets sur métabolisme protéique et les effets sur le métabolisme énergétique.

Le but de notre travail a été de préciser les mécanismes d'action de la citrulline sur la synthèse protéique musculaire et d'explorer le rôle du métabolisme énergétique dans cet effet.

Dans une première étude (Publication n°1) nous avons étudié *in vivo* la relation entre métabolisme protéique et énergétique et l'action de la citrulline sur ces derniers. Par ailleurs, concernant la citrulline, si son action sur la synthèse protéique a bien été prouvée, en revanche l'impact de cet acide aminé sur le sécrétome musculaire est totalement méconnu. En effet, le muscle est aujourd'hui considéré comme un organe endocrine (12). Ainsi, une augmentation de la synthèse protéique par la citrulline dans la cellule musculaire pourrait moduler certaines protéines sécrétées. Pour répondre à cette question, nous avons réalisé une deuxième étude (Publication n°2) afin de déterminer la modulation par la citrulline de l'expression des protéines sécrétées par la cellule musculaire *in vitro*. Enfin, de façon complémentaire à ces deux études montrant la modulation du métabolisme protéique par la citrulline, nous avons essayé de préciser les mécanismes impliqués dans la régulation simultanés des métabolismes protéique et énergétique en utilisant une approche *in vitro* sur des cellules musculaires (Publication n°3).

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DONNÉES BIBLIOGRAPHIQUES

1 Propriétés physico-chimiques

1.1 Propriétés physiques

La citrulline (C₆H₁₃N₃O₃) est un solide incolore à température et pression ambiante dont la masse molaire est de 175 g.mol⁻¹. Son point de fusion est de 222°C. C'est un acide α -aminé avec une chaîne carbonée asymétrique ; de ce fait, il existe deux énantiomères, sa forme naturelle étant la forme L (pouvoir rotatoire: α_{20}^{D} =3,7°, avec une configuration absolue S). Sa formule développée est présentée fig.1.



Fig. 1 : Formule développée de la citrulline

1.2 Propriétés chimiques

1.2.1 Solubilité et propriétés acido-basiques

Du fait de sa chaîne latérale polaire, la citrulline est relativement soluble dans l'eau mais très peu dans l'alcool (13). Comme tous les autres acides aminés, la citrulline présente deux acidités avec le groupement acide carboxylique (pKa \approx 2,4) et le groupement amine (pka \approx 9,4). La citrulline étant un diacide, il est important de préciser que la citrulline peut subir des réactions de protonation et déprotonation. Malgré cela, en condition physiologique, la presque totalité (plus de 99,9%) de la citrulline est sous forme zwitterion (14).

1.2.2 Propriétés de complexation de la citrulline

A pH alcalin, à la fois le groupe carboxylique et le groupe amine peuvent jouer un rôle en tant que donneurs d'électrons. Ils peuvent ainsi se complexer pour former des sels de citrulline tels que le chlorohydrate de citrulline ou le malate de citrulline pour ne citer que les plus connus. La chaîne latérale est moins susceptible de se complexer car aucun électron des atomes d'azote ne sont conjugués à la double liaison. Il a également été rapporté des complexations avec plusieurs cations bivalents, formant ainsi des sels. Ces sels ont été répertoriés par Curis et al. (15). Cependant, à ce jour, aucune structure cristalline d'un

complexe de la citrulline avec un cation bivalent n'a été répertoriée. Par conséquent, ces complexations ne sont supportées que par des données indirectes en solution.

1.2.3 Réactivités moléculaires de la citrulline

La citrulline présente les réactivités moléculaires communes à la famille des acides α -aminés, en particulier la possibilité de former des liaisons peptidiques et ainsi être présente dans les protéines. Cependant, le fait qu'il n'existe aucun codon génique pour cet acide aminé explique que sa présence dans des protéines résulte de modifications post-traductionnelle et non pas d'une incorporation lors de la traduction (le rôle biologique des protéines citrullinées sera décrit ultérieurement).

La citrulline se distingue de la plupart des autres acides aminés par la présence d'une fonction urée. Il semble naturel que le rôle biologique de la citrulline soit basé sur la réactivité de ce groupe, et particulièrement de sa proche relation au groupe fonctionnel de l'arginine. Cette réactivité est due à l'atome de carbone du groupe uréide qui est très électrophile, dont ses électrons sont attirés par les atomes d'azote et d'oxygène proches. De telles réactions sont biologiquement cruciales. Pour exemple, la réaction avec l'aspartate en tant que composé nucléophile constitue une étape clé du cycle de l'urée puisque que cette réaction forme l'argininosuccinate (16).

1.2.4 Propriétés antioxydantes

La citrulline est présente en grande quantité dans la pastèque, dans laquelle elle a un rôle de défense anti-oxydante et de résistance au stress hydrique (17). Ainsi, Akashi et al. (18) se sont intéressés à caractériser ces propriétés anti-oxydantes. Pour cela, les auteurs ont étudié *in vitro* la capacité de la citrulline, ainsi que celle d'autres molécules, à réagir avec les radicaux hydroxyles. A titre de comparaison, dans cette étude la citrulline agit en tant *scavenger* des radicaux hydroxyles (avec une constante de vitesse de la réaction mesurée à $3,9 \times 10^9 \text{ M}^{-1}.\text{s}^{-1}$) de manière plus importante que le mannitol, connu pour être un *scavenger* efficace d'espèces réactives de l'oxygène (ROS) (19), à toutes les concentrations mesurées (2.5 mM à 60mM). De plus, dans la même étude, la citrulline diminue fortement les dommages oxydatifs de l'ADN par ajout d'H₂O₂ *in vitro*. Quelques données ont mis en évidence ces propriétés antioxydantes chez l'animal. Moinard et al. (5) ont ainsi démontré chez des rats âgés sains, qu'une complémentation en citrulline permet de diminuer l'oxydation lipidique des VLDL et LDL induite par une réaction au cuivre mesuré par la

formation de 7-ketocholesterol (le principal produit résultant de l'oxydation du cholestérol). Ce résultat sur l'effet de la citrulline sur la peroxydation lipidique a été également démontré par Miyashita et al. (20) chez des souris. En effet, ces auteurs ont mis en évidence qu'un traitement à la doxorubicine, un traitement de chimiothérapie anticancéreuse, a comme effet secondaire une augmentation de la peroxydation lipidique dans le cœur et qu'une complémentation en citrulline permet de limiter ce phénomène. L'effet antioxydant de la citrulline ne se limite pas aux lipides puisqu'il a été montré chez des rats dénutris et renourris avec un régime complémenté en citrulline que la carbonylation des protéines dans le muscle est fortement diminué (21). Il a également été montré in vitro dans l'érythrocyte de poisson qu'un apport de citrulline diminue l'apoptose grâce notamment à une diminution de la fragmentation de l'ADN, une diminution de l'hémolyse, une diminution des concentrations d'ions superoxyde et de H₂O₂ et une diminution des protéines carbonylées suite à une réaction radicalaire de ces cellules par apport de FeSO₄ et de H₂O₂ dans le milieu de culture (22). Enfin, très récemment, il a été démontré que la citrulline est capable de diminuer in vitro le stress nitrosatif induit par L'ADMA (asymetric dimethylarginine) et déterminé par la formation de 3-nitrotyrosine dans des cultures primaires humaines de cellules épithéliales pulmonaires (23).

2 Métabolisme de la citrulline

2.1 Transport de la citrulline

Le transport de la citrulline dans les cellules est peu documenté du fait que cet acide aminé était peu étudié jusqu'à présent. Il a été montré dans des entérocytes *in vitro* (Caco-2), que la citrulline emprunte les systèmes de transport B⁰⁺ et L (24). En effet, les auteurs ont démontré que ces deux systèmes de transport captent la citrulline dans le *lumen* avec des activités comparables. Par ailleurs, une autre équipe a étudié le transport de la citrulline dans les cellules des tubules rénaux proximaux (25). Elle a rapporté que le transport de la citrulline a lieu grâce à deux systèmes de transport sodium-dépendant et un transporteur sodium-indépendant, et que la citrulline emprunte également des transporteurs classiques des acides aminés cationiques et neutres.

Dans les macrophages, le transport de la citrulline semble être saturable, insensible au pH et partiellement dépendant du sodium (26). En intracellulaire, la citrulline traverse la membrane mitochondriale *via* un échangeur ornithine/citrulline, permettant sa sortie de la mitochondrie après sa synthèse dans le cycle de l'urée (27) (voir le chapitre Métabolisme hépatique de la citrulline). Le transport de la citrulline a également été caractérisé dans plusieurs autres types cellulaires, notamment dans les cellules de muscle lisse (28), dans des cultures de cellules neuronales (29), et dans des cellules endothéliales aortiques (30). En revanche, à notre connaissance, il n'existe aucune donnée quant au transport de la citrulline dans les cellules musculaires striées.

2.2 Synthèse et métabolisme de la Citrulline

2.2.1 Synthèse de la citrulline via l'ornithine transcarbamylase

L'ornithine transcarbamylase (OTC, également appelée ornithine Carbamoyl Transférase), est une enzyme de 40 kDa impliquée dans le cycle de l'urée puisqu'elle catalyse la conversion de l'ornithine en citrulline par la réaction suivante :

Ornithine + Carbamoyl phosphate → Citrulline + Phosphate

Chez la plupart des eucaryotes, tels que les mammifères, cette enzyme est située dans la matrice mitochondriale (31). Chez les mammifères, l'OTC est principalement présente dans

le foie, d'où sa fonction dans la cycle de l'urée, et dans la muqueuse intestinale dans une moindre mesure (Tableau 1) (32–35).

Tissus	Activité de l'OTC -1 -1 (µmol.g .h)
Intestin grêle	100
Foie	4110
Rein	5
Thymus	2
Poumons	2
Rate	0
Muscle	0

Tableau 1. Activité de l'enzyme OTC dans les tissus (32)

Bien que cette réaction soit réversible, les propriétés thermodynamiques de celle-ci favorisent fortement la formation de citrulline (36). En revanche, il est à noter que dans certaines espèces de bactéries, cette réaction peut intervenir aisément dans l'autre sens, c'est-à-dire que la citrulline est le substrat (37). L'OTC peut également utiliser la lysine comme substrat : dans ce cas le produit de la réaction est l'homocitrulline. Cependant, l'affinité de l'OTC pour la lysine est très faible comparée à celle de l'ornithine et à l'état physiologique cette réaction est quantitativement négligeable (38).

2.2.2 Synthèse de la citrulline via les NO synthases

Les enzymes NO-synthases (NOS) sont responsables de la synthèse ubiquitaire de NO. Il existe trois familles de NOS qui diffèrent au niveau de leur expression et de leur localisation : la nNOS est principalement présente dans les cellules nerveuses et l'eNOS dans les cellules endothéliales. Ces NOS sont des formes constitutives. La dernière famille de NOS est l'iNOS principalement présente les macrophages mais que l'on retrouve dans presque tous les tissus. Cette enzyme, contrairement aux deux autres est inductible. Toutes ces enzymes partagent un mécanisme commun pour la synthèse de NO et de citrulline à partir d'arginine, et nécessitant du NADP⁺, FMN et bioptérine comme cofacteurs , suivant la réaction suivante (39) :

Il faut noter que la production de citrulline par la NOS est négligeable par rapport à la production de citrulline par l'OTC, de l'ordre de 1% (40,41), et ne constitue donc pas une source de citrulline significative pour influencer la disponibilité en citrulline quand celle-ci est affectée.

2.2.3 Catabolisme de la citrulline via l'argininosuccinate synthase

L'argininosuccinate synthase (ASS) est une enzyme cytosolique de 47kDa qui catalyse la réaction suivante :

Citrulline + Aspartate + ATP → Argininosuccinate + Pyrophosphate + AMP Dans des conditions biologiques standards, thermodynamiquement, la réaction est très légèrement favorable au métabolisme de la citrulline (42). Ainsi, dans ce cas, la thermodynamique dicte peu le sens de la réaction et celle-ci est principalement contrôlée par l'utilisation des produits de la réaction. Par conséquent, la citrulline est principalement utilisée en tant que substrat dans cette réaction enzymatique (16). L'ASS est pratiquement ubiquitaire, avec des niveaux élevés dans le foie et le rein comparés à l'intestin qui possède des niveaux en ASS faibles à l'âge adulte (Tableau 2) (43). Cette disparité est liée essentiellement à une régulation différente de l'expression de l'ASS puisque il n'existe aucune modification post-traductionnelle ni de contrôle significatif de la cinétique d'action (16). Toutefois, Husson et al. (16) ont monté que l'activité de l'ASS peut être régulée par différents facteurs, incluant les hormones, les nutriments et des stimuli pro-inflammatoires, mais que ces facteurs agissent principalement au niveau transcriptionnel. Par exemple dans le rein, l'expression génique de l'ASS est sensible aux analogues d'AMP cycliques (44), à un déficit nutritionnel (44) ou au lypopolysaccharides (45). Seule une inactivation de l'ASS par nitrosylation a été mise en évidence par Hao et al. (46).

L'activité de l'ASS est couplée à celle de l'enzyme cytosolique argininosuccinate lyase (ASL) puisque l'argininosuccinate, produite par l'ASS à partir de citrulline, est transformée directement en arginine par l'ASL selon la réaction suivante (47) :

Argininosuccinate \rightarrow Arginine + Fumarate

La distribution tissulaire de l'ASL est comparable à celle de l'ASS : l'expression est prédominante dans le foie et les reins (Tableau 2) (48,49).

Tissus	Activité de l'ASS -1 -1 (μmol.g .h)	Activité de l'ASL -1 -1 (μmol.g .h)
Intestin grêle	68	6
Foie	1386	308
Rein	677	41
Cerveau	51	10
Poumons	22	10
Rate	11	14

Tableau 2. Activité des enzymes ASS et ASL dans les tissus (43,48)

Chez les plantes et les bactéries, comme l'arginine, la citrulline peut également être métabolisée en N-Carbamoylputrescine, une polyamine ayant de nombreux rôles notamment dans la croissance et le développement cellulaire (50). Cependant, chez le mammifère, Ramani et al. (50) ont démontré que cette polyamine ne semble pas être synthétisée à partir de citrulline. En effet, avant ou après une complémentation en citrulline (1g/kg/jour) pendant 12 semaines chez des rats, la N-Carbamoylputrescine reste indétectable.

2.3 Citrullination des protéines

La citrulline étant un acide aminé non protéinogène, il n'existe pas de codon pour la citrulline. Pourtant il existe des protéines contenant des résidus citrullyl. Ce phénomène est exclusivement lié à des modifications post-traductionnelles des protéines. Ces résidus citrullyl sur les protéines citrullinées est cependant totalement indépendant de la citrulline libre circulante. En effet, cette modification post-traductionnelle résulte d'une déamination de résidus arginyl par l'enzyme peptidylarginine déiminase suivant la réaction suivante :

Arginyl + $H_20 \rightarrow Citrullyl + NH_4^+$

Il existe 5 isoformes de l'enzyme qui se situent toutes dans le cytoplasme. Cependant, une translocation dans le noyau de l'isoforme peptidylarginine déiminase 4 est possible (51). Cette translocation permet de citrulliner certains histones, modulant la décondensation de la chromatine (52).

Les véritables rôles biologiques de ces citrullinations restent aujourd'hui peu connus. Au niveau de la peau, la principale protéine citrullinée connue est la filaggrine qui pourrait se lier à la kératine seulement quand la filaggrine est citrullinée (53). Au niveau des cheveux, la trichohyaline est connue pour être citrullinée mais le rôle de cette citrullination n'est pas connu.

La citrullination de la kératine dans la peau pourrait jouer un rôle biologique important. En effet, une dysfonction de ce phénomène de citrullination au niveau de la kératine pourrait être associée au psoriasis car cette modification post-traductionnelle est beaucoup moins présente dans la peau affectée que dans la peau saine (54). Mais les données sont trop parcellaires pour déterminer si ce phénomène est une cause ou une conséquence de la pathologie.

Au niveau du système nerveux, il est connu que la myéline peut être citrullinée. Le rôle exact de cette citrullination n'est pas connu. cette dernière permettant notamment une modification de la charge de la myéline, il a été proposé que la sclérose en plaque pourrait être liée à une dysfonction de la citrullination de la myéline (55). En effet, puisque la citrullination modifie la charge de la myéline, une citrullination trop importante de la myéline pourrait modifier les interactions avec les lipides, et ainsi causer une dégradation de la myéline, observée dans la sclérose en plaques. De plus, une citrullination trop importante

de la myéline pourrait également révéler des sites antigéniques reconnus par les anticorps plasmatique, ce qui pourrait expliquer le caractère auto-immun de la maladie.

D'autres citrullinations ont déjà été mises en évidences dans certains noyaux de leucocytes au moment de la différenciation en granulocyte ou monocyte, suggérant une implication dans le processus de différenciation mais le rôle reste inconnu. Enfin, on peut noter que le dosage de peptides citrullinés est un excellent marqueur de la polyarthrite rhumatoïde (liée à une accumulation de protéines citrullinées de façon anormale dans le cas de cette pathologie) (56–59).

2.4 Métabolisme hépatique de la citrulline

La citrulline est connu, depuis longtemps pour être un intermédiaire du cycle de l'urée (Fig. 2) dans l'hépatocyte.



Fig. 2 : Cycle de l'urée.

CP : Carbamoylphosphate, CPS : Carbamoylphosphate synthétase, OTC : Ornithine transcarbamylase, ASS: Argininosuccinate synthase, ASL : Argininosuccinate lyase

Cependant, ce *pool* de citrulline est particulièrement labile car toute la citrulline synthétisée est convertie en argininosuccinate par l'ASS cytoplasmique, et il n'y a aucune libération de citrulline dans la circulation générale (1). De plus, il a été montré que les hépatocytes impliqués dans le cycle de l'urée sont incapables de capter de la citrulline provenant de la circulation portale, et que l'absorption de citrulline issue de la circulation artérielle est minimale (1). Cette particularité a été confirmée par Jourdan et al. (60) en utilisant un modèle de foie isolé périfusé et par Agarwal et al. (61) chez des souris complémentées en citrulline. Par conséquent, le métabolisme de la citrulline dans le foie est un métabolisme strictement compartimenté, déconnecté des autres voies métaboliques liées à la citrulline, du moins en condition physiologique. Cette compartimentation de la citrulline dans le foie pourrait être altérée dans certaines situations pathologiques. Van de Poll et al. (62) ont ainsi rapporté une captation et une libération de citrulline par le foie chez des patients ayant subis une chirurgie gastro-intestinale et chez des patients porteurs de métastases

colorectales dans le foie (63). Cependant, cette captation et libération de citrulline dans ce cas peut être liée à la métabolisation de la citrulline par la cellule tumorale, puisque deux études du même groupe de recherche ont montré que les cellules tumorales pouvaient métaboliser cet acide aminé(64,65).

2.5 Métabolisme intestinal de la citrulline

L'intérêt de la citrulline a été ravivé en 1981 lorsque Windmueller et Spaeth ont démontré que de la citrulline est produite de façon conséquente par l'intestin (1). Cette citrulline provient d'une synthèse endogène à partir du métabolisme de l'arginine et/ou de la glutamine (1,66), mais le principal précurseur parmi ces deux acides aminés est encore aujourd'hui un sujet de controverse (67–69). Dans l'entérocyte, une fraction de l'arginine alimentaire (~ 40% (70)) est catabolisée par l'arginase en ornithine et une fraction de la glutamine alimentaire et au niveau portal est catabolisée par la glutaminase et l'ornithine aminotransferase (OAT) en ornithine. L'ornithine ainsi produite par ces deux acides aminés est métabolisée en citrulline par l'OTC (71). De plus, bien que l'arginine et la glutamine soient les deux principaux précurseurs de citrulline, ils ne sont pas les seuls. En effet, Wu et al. (72) ont montré pour la première fois que la proline peut également être un précurseur de citrulline dans l'intestin grêle de porcelets. Ces résultats ont été confirmés chez l'Homme à la fois chez le nouveau-né et chez l'adulte sain (73,74). La réaction conduisant à la synthèse de citrulline a été décrite par Wu et Morris (75). La proline est métabolisée en Δ 1-l-Pyrroline-5-Carboxylate (P5C) par la proline oxydase puis en ornithine par l'OAT :

Proline \rightarrow P5C \rightarrow Ornithine \rightarrow Citrulline

Une fois que la citrulline est synthétisée par l'entérocyte, elle est libérée dans la veine portale où elle échappe à la captation hépatique. Elle est ensuite très largement captée par le rein (1).

2.6 Métabolisme inter-organes de la citrulline

La découverte du cycle inter-organe de la citrulline a été initiée pour la première fois par Windmueller et Spaeth (1) qui ont établi que la citrulline était synthétisée par l'intestin à partir de glutamine et d'arginine. Il a ensuite été démontré qu'il existe une forte corrélation entre l'apport de glutamine et la libération de citrulline par l'intestin grêle chez l'adulte (76). Le principal organe métabolisant la citrulline est le rein qui exprime l'ASS et l'ASL mais pas les autres enzymes du cycle de l'urée (16). Ainsi, le rein libère de l'arginine en fonction de l'apport en citrulline (63), et la concentration plasmatique en citrulline est le principal facteur déterminant la production rénale d'arginine puisque la production rénale d'arginine est proportionnelle à la concentration circulante en citrulline (77). Ces fonctions métaboliques expliquent qu'une insuffisance intestinale induit une hypocitrullinémie tandis qu'une défaillance rénale induit une hypercitrullinémie (78). La particularité de ce cycle inter-organe est l'absence de captation et de libération de la citrulline par le foie, le métabolisme hépatique de la citrulline étant strictement compartimentée (79). L'intérêt de ce cycle inter-organes de la citrulline a été proposé par Morimoto et al. (80) qui ont montré que la production de citrulline par l'intestin augmente dans l'entérocyte quand l'apport protéique est faible. Ainsi, il a pu être suggéré que ce cycle inter-organes de la citrulline joue un rôle dans la modulation de l'homéostasie azotée et que cette modulation est liée à la disponibilité en acides aminés. En effet, lorsque la disponibilité des acides aminés est importante comme après l'ingestion de protéines, le cycle de l'urée est activé par la forte concentration en arginine, puisque cet acide aminé est un activateur de l'uréogenèse, à la fois via son rôle d'intermédiaire de ce cycle métabolique mais également car c'est un activateur de la N-acétylglutamate synthase, dont le produit N-acétylglutamate active la carbamoyl-phosphate synthase (étape limitante du cycle de l'urée) de façon allostérique. Ainsi, plus la concentration en arginine augmente dans la circulation portale et plus le cycle de l'urée est activé (81). Par ailleurs, il faut noter que le seul autre acide aminé capable de produire de la citrulline dans l'intestin est la glutamine (82). Finalement, l'arginine dans l'aire splanchnique joue le rôle de « marqueur » d'une prise alimentaire excessive de protéines. Dans cette condition, l'activation de l'uréogenèse par l'arginine protège l'organisme des niveaux circulants excessifs en acides aminés dont l'accumulation en périphérie est potentiellement neurotoxique. L'autre intérêt de l'activation de l'uréogenèse par l'arginine est justement de diminuer les niveaux en arginine et ainsi éviter une production de NO excessive.

En revanche, lorsque la disponibilité en acides aminés est faible, l'induction de l'OAT et de l'arginase (avec notamment une stimulation de leur expression) dans l'entérocyte induit une libération de citrulline (à la place de l'arginine) (83). Cela permet de limiter l'activation du cycle de l'urée et ainsi de limiter les pertes azotées. En effet, la citrulline n'étant pas captée par le foie, les besoins en arginine sont couverts par la resynthèse d'arginine par la citrulline dans le rein. Finalement, la citrulline permet de maintenir l'homéostasie de l'arginine en limitant son catabolisme. Cette voie de synthèse de l'arginine à partir de citrulline via l'ASS du rein représente environ 60% de la synthèse *de novo* d'arginine chez l'adulte (75).

Ce cycle inter-organes (schématisé Fig. 3) contribue donc à la stricte homéostasie de l'arginine. Quelques études ont pu mettre en évidence cette dernière puisque Castillo et al. (84) ont démontré qu'une restriction à court terme en arginine n'a aucun effet sur le taux de synthèse de NO au niveau corps entier malgré une diminution de 38% du flux plasmatique d'arginine. Par ailleurs, Lassala et al. (85) ont mis en évidence chez la brebis gestante, qu'une administration d'arginine n'augmente pas la concentration en arginine chez le fœtus, preuve une nouvelle fois que l'organisme se protège lui-même contre un excès d'arginine.



Fig. 3 Métabolisme inter-organes de la citrulline adapté de Breuillard et al. (86). *P5C* : Δ1-Pyrroline-5-carboxylate, OAT : ornithine aminotransférase, OTC : ornithine transcarbamylase, ASS : argininosuccinate synthétase, ASL : argininosuccinate lyase

Cependant, un flux métabolique ne permet pas de faire un lien direct avec un phénomène biologique. L'utilisation d'inhibiteurs permet de préciser la relation métabolisme/phénomène biologique. Ainsi, afin de mieux comprendre l'importance du rôle de la citrulline intestinale, Hoogenraad et al. (87) ont démontré chez le rat, qu'un régime dépourvu en arginine et complémenté avec un inhibiteur spécifique de l'OTC, la glycylglycine N-phosphonacetyl-L-ornithine, induit une forte diminution de l'arginine plasmatique, une perte de poids et une mortalité plus élevée. Cependant, l'addition de 1% d'arginine au régime permet de restaurer partiellement les concentrations plasmatiques en arginine et de limiter partiellement la perte de poids. De la même façon, l'apport de 1% de citrulline au régime permet de restaurer totalement la concentration plasmatique en arginine et le poids des animaux. Cette étude prouve donc le caractère essentiel de la synthèse intestinale de citrulline dans l'homéostasie de l'arginine, et l'importance de cet axe arginine-citrullinearginine. En revanche, il faut souligner la limite de ce travail puisque la glycylglycine Nphosphonacetyl-L-ornithine inhibe l'OTC intestinale mais également l'OTC hépatique (de façon partielle) qui est d'une importance capitale dans le cycle de l'urée.

2.7 Citrulline et métabolisme du NO

Du fait que la citrulline est facilement convertie en arginine via l'ASS et l'ASL, la citrulline est un précurseur de NO. En effet, de nombreuses cellules qui peuvent métaboliser l'arginine en NO sont ainsi capables d'utiliser la citrulline circulante pour produire du NO. La citrulline peut également être produite par conversion de l'arginine en NO. Dans des macrophages activés, la citrulline ainsi recyclée en arginine contribuerait à 20% du NO produit (88,89). Un tel recyclage existe également dans les cellules endothéliales. Quand les besoins en NO sont particulièrement importants, comme dans les macrophages activés, la citrulline peut même être synthétisée à partir du glutamate. Cependant, l'utilisation de la citrulline pour produire du NO n'est pas ubiquitaire. Par exemple, dans les cellules de muscle lisse, la citrulline n'est pas capable de remplacer l'arginine pour produire du NO (28). Egalement, dans le cerveau, les neurones produisant du NO ne sont pas capables de reconvertir la citrulline en arginine car ces cellules n'expriment pas les enzymes ASS et ASL. Ainsi la citrulline est libérée par les neurones et captée par les cellules gliales qui peuvent convertir la citrulline en arginine. L'arginine nouvellement formée est ensuite libérée et captée par les neurones pour former plus de NO (29). Au niveau des cellules endothéliales, l'arginine utilisée pour la synthèse de NO provient d'une néosynthèse à partir de la citrulline. En effet Goowin et al. (90) ont démontré in vitro dans des cellules endothéliales que lorsque l'ASS est inhibée par des siRNA, la production de NO est fortement diminuée, malgré une concentration saturante en arginine dans le milieu. Ceci confirme que le pool d'arginine utilisé pour la synthèse de NO provient strictement d'une néosynthèse à partir de la citrulline.

3 Citrulline : biomarqueur des fonctions rénale et intestinale

3.1 Citrulline et fonction rénale

La citrullinémie est un bon marqueur de la fonction rénale, notamment des tubules proximaux. En effet, chez le rat, une grave défaillance rénale est caractérisée par une hypercitrullinémie (91), qui apparaît même être plus sensible à la dysfonction rénale que la créatinémie (92). En effet, Levillain et al. (91) ont démontré, chez des rats ayant subis différents degrés de néphrectomie (10 à 90%), que la citrullinémie augmente de façon proportionnelle au degré de néphrectomie avec un niveau maximal, et stable dans le temps, 48h après la chirurgie. Malheureusement, le rôle de la citrulline en tant que biomarqueur de la fonction rénale n'a jamais été démontré chez l'Homme. Ainsi, en clinique, la citrulline n'est pas utilisé en tant que biomarqueur de la fonction rénale (même si les patients présentant une insuffisance rénale majeure ont une hypercitrullinémie caractérisée).

3.2 Citrulline et fonction de l'intestin grêle

L'intestin grêle est la principale source de citrulline circulante, il n'est ainsi pas surprenant que la citrullinémie puisse être utilisée en tant que marqueur de la fonction de l'intestin grêle. Cela a été démontré par Crenn et al. (93) dans un travail princeps, chez des patients atteints d'un syndrome de grêle court, que la concentration en citrulline était fortement diminuée avec une relation directe entre les concentrations en citrulline et la masse entérocytaire fonctionnelle avec un haut niveau de sensibilité (92%) et de spécificité (90%) (Fig. 4). Suite à cette étude pionnière, d'autres études ont établis l'utilité de ce marqueur dans différents contextes où la fonction de l'intestin grêle avait besoin d'être surveillée (contrôle de l'implantation de transplants après une chirurgie de l'intestin (94–96), pathologies intestinales (97–99)). Chez les patients de réanimation, une faible concentration plasmatique en citrulline est associée à une augmentation de la CRP et de la mortalité (100). La concentration plasmatique en citrulline en tant que marqueur de la masse entérocytaire à également été démontré chez l'enfant atteint du syndrome de grêle court (101). Par ailleurs, Blijlevens et al. (102) ont mis en évidence que la citrullinémie était plus sensible et spécifique pour détecter les dommages intestinaux induits par la chimiothérapie que le test de perméabilité basé sur l'assimilation de sucres non digestibles. Outre l'action de marqueur de la fonction intestinal, des études montrent que la citrullinémie pourrait être utilisée de manière prédictive, en particulier pour prédire le risque de rejet de transplants d'intestin grêle (103–105).



L'équation de régression est : citrulline plasmatique (μ mol/L) = 0,23 x taille intestin grêle (cm) + 5,68 (μ mol/L. •, patients avec défaillance intestinale permanente ; \circ , patients avec défaillance intestinale transitoire

4 Aspects thérapeutiques de la citrulline

De part ces nombreuses propriétés métaboliques, la citrulline a été étudiée à de multiples reprises pour déterminer si son utilisation pouvait être thérapeutique dans le cas de certaines pathologies. Ainsi, un bénéfice potentiel ou avéré d'une prise de citrulline lors de pathologies intestinales ou cardiovasculaires variées a pu être démontré.

4.1 Pharmaco-cinétique et tolérance

La pharmaco-cinétique de la citrulline a été déterminée au cours de différentes études cliniques. Chez des volontaires sains, Moinard et al. (106) ont démontré qu'après ingestion de citrulline à différentes doses, les concentrations plasmatiques en citrulline et en arginine augmentent rapidement et de façon proportionnelle à la dose de citrulline ingérée, puis retournent aux valeurs de base après 5 à 8h en fonction de la dose (Fig. 5).



Cependant, à la plus forte dose (15g), la production d'arginine n'est plus proportionnelle à la dose de citrulline administrée, suggérant que la conversion de la citrulline en arginine dans le rein pourrait être limitante, et que la saturation commence à apparaitre autour de cette dose de citrulline. Cette complémentation en citrulline n'affecte pas la concentration

plasmatique d'acides aminés autres que l'ornithine et l'arginine. Rougé et al. (107) ont démontré que l'administration orale de citrulline permet d'augmenter la disponibilité systémique en citrulline et en arginine ainsi que la balance azotée (phénomène qui a également été observé dans l'étude Moinard et al. (106)). Dans ces études, la citrulline a une très forte biodisponibilité car la concentration en citrulline augmente très rapidement et que les pertes urinaires en citrulline sont très faibles (106,107). Ces résultats sur la pharmacocinétique de la citrulline sont en phase avec les résultats obtenus par Collins et al. (108) après une ingestion chronique de pastèque, riche en citrulline, chez des volontaires sains. De plus, il a été démontré à de nombreuses reprises que la prise orale de citrulline est plus effective pour augmenter les niveaux circulants en arginine que la prise d'arginine ellemême (61,109–115) que ce soit chez l'adulte ou la personne âgée. Cette observation s'explique par le fait que la citrulline n'est pas métabolisée par le foie et est donc métabolisée en arginine pour augmenter sa biodisponibilité circulante.

Concernant la tolérance, la complémentation orale en citrulline est sans danger (116). En effet, contrairement à l'arginine ou l'ornithine qui entrainent des effets gastro-intestinaux indésirables à hautes doses (i.e. > bolus de 10g) (117), la citrulline est très bien tolérée (106,109,115,118–120). Cela peut s'expliquer par la rapide saturation de l'absorption intestinale lors de la prise orale à forte dose d'arginine et d'ornithine, induisant des diarrhées osmotiques (117). Cette différence entre ces acides aminés suggère que l'absorption intestinale de citrulline n'est pas une étape limitante dans la biodisponibilité de la citrulline, même à hautes doses. En effet, une étude réalisée sur des anses de cochon isolées perfusées a montré que la citrulline est absorbée beaucoup plus rapidement que l'arginine par exemple, et permettrait d'expliquer la réduction du risque de diarrhées osmotiques (Rasmussen & Baracos, données non publiées). Enfin, la complémentation chronique en citrulline est sans danger puisqu'aucun effet secondaire n'a jusque-là été recensé (109,121–123).

4.2 Citrulline et pathologies intestinales

Les effets de la citrulline en tant qu'agent thérapeutique lors de pathologies intestinales ont été peu étudiés. Pourtant quelques données montrent que la citrulline pourrait être de première importance. En effet, Osowska et al. (2) ont démontré dans un modèle de grêle court (par résection intestinale massive (80%)) chez le rat que la balance azotée est fortement diminuée avec notamment une chute des concentrations plasmatiques en arginine et en citrulline. Or, quand ces rats sont complémentés en citrulline (1g/kg/jour) pendant 10 jours, la balance azotée est restaurée dès le 5^{ème} jour par la citrulline, contrairement à la l'arginine qui n'améliore cette balance azotée qu'à partir du 10^{ème} jour sans restauration complète (Fig. 6). De plus, la prise de citrulline augmente de façon très importante le *pool* d'arginine plasmatique et musculaire (de façon plus importante que la complémentation en arginine).



Fig. 6 Balance azotée cumulée durant 10 jours de nutrition entérale enrichie ou non en citrulline ou arginine (1g/kg/jour) après une résection intestinale massive (80%) chez le rat (2) Groupes : sham (Rats ayant subis une laparotomie et nourris par nutrition entérale), control (rats ayant subis une résection intestinale et nourris par nutrition entérale), arginine (rats ayant subis une résection intestinale et nourris par nutrition entérale enrichie en arginine), et citrulline (rats ayant subis une résection intestinale et nourris par nutrition entérale enrichie en citrulline). Les résultats sont exprimés comme la différence entre l'apport azotée et l'excrétion azotée urinaire journalière. Les résultats sont présentés comme la valeur moyenne (SEM). *p<0,05 vs. Sham, †p<0,05 vs. Control, ‡p<0,05 vs. Arginine.

Dans le même modèle de grêle court, ces auteurs ont également étudié l'effet d'une complémentation en citrulline ou en arginine par voie parentérale (124). Par cette voie d'administration, la citrulline et l'arginine sont incapables d'améliorer la balance azotée, avec en plus un effet délétère de l'arginine sur ce paramètre. Cependant, dans cette étude,

les auteurs ont mesuré la balance azotée seulement durant 3 jours. Or, lors de la précédente étude, l'amélioration de la balance azotée par la citrulline par voie entérale a été mise en évidence seulement à partir du 5^{ème} jour de complémentation. Il est donc possible que l'étude par voie parentérale n'ait pas été assez longue pour mettre en évidence un effet positif de la citrulline. En revanche, les auteurs ont démontré que la complémentation en citrulline par voie parentérale après résection intestinale est capable d'augmenter le contenu protéique intestinal ainsi que la masse musculaire, probablement *via* l'action de la citrulline sur la synthèse protéique musculaire. Par ailleurs, en utilisant le même modèle, Filippi et al. (125) ont déterminé un effet dose en évaluant l'efficacité de doses allant de 0.5g/kg à 5g/kg. Il apparait que l'effet de la citrulline était bien dose-dépendante, notamment sur le poids des muscles et sur le gain pondéral des animaux.

Ces résultats montrent globalement des effets positifs de la citrulline lors d'un syndrome de grêle court mais des études cliniques sont nécessaires afin de confirmer ces résultats chez l'Homme. L'effet de la citrulline sur le contenu protéique intestinal pourrait être en phase avec les données de la littérature concernant les effets de la citrulline sur la perméabilité intestinale. En effet, Batista et al. (126) ont montré qu'une complémentation en citrulline chez la souris permet d'améliorer la perméabilité intestinale et de limiter la translocation bactérienne lors d'une obstruction intestinale. Cet effet sur la perméabilité intestinale a été retrouvé par Antunes et al. (127) qui montrent qu'une complémentation en citrulline chez la souris avant l'induction d'une inflammation de la muqueuse intestinale permet de limiter la perméabilité intestinale avec notamment une atténuation des dommages au niveau de l'architecture de la muqueuse intestinale.

De plus, il a été montré chez la souris que lors d'une endotoxémie, la microcirculation intestinale est diminuée suite à une altération de la production de NO (par diminution de la concentration plasmatique et intracellulaire en arginine). Or, une complémentation en citrulline (contrairement à une complémentation en arginine) permet d'améliorer la microcirculation *via* une amélioration de la production de NO probablement due à l'augmentation du *pool* plasmatique et intracellulaire en arginine (128). Tous ces résultats sur les effets de la citrulline sur l'intestin lors de pathologies induites ont été obtenus chez le rongeur et il est encore difficile à ce jour, de transposer ces résultats chez l'Homme. Une seule étude a montré des résultats similaires chez l'Homme lors de l'exercice, connu pour endommager la muqueuse intestinale et diminuer la perfusion intestinale. En effet, Van

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Wijck et al. (129) ont démontré chez l'homme qu'une complémentation en citrulline avant un exercice permet de diminuer les dommages intestinaux et d'améliorer la circulation sanguine intestinale lors d'un exercice, probablement *via* une augmentation de la production de NO puisque la concentration en arginine est fortement augmentée.

4.3 Citrulline et pathologies cardiovasculaires

Du fait de la capacité de la citrulline à générer de l'arginine, et donc d'être précurseur de NO, plusieurs travaux ont mis en évidence que la citrulline pourrait être bénéfique pour les patients atteints de certaines maladies cardiovasculaires. Il a notamment été démontré qu'une complémentation en citrulline pendant 4 mois chez des patients atteints d'insuffisance cardiaque permettait d'améliorer l'éjection systolique du ventricule gauche et la fonction endothéliale (130). Cette amélioration de la fonction endothéliale a été retrouvée chez des patients atteints d'insuffisance cardiaque complémentés avec de la citrulline durant deux mois (131). De plus, un traitement de 6 semaines à base de poudre de pastèque, comme source de citrulline (environ 6g/jour), a permis à des sujets obèses avec de l'hypertension d'améliorer leur fonction artérielle avec notamment une réduction de la pression artérielle (132). L'apport de citrulline, sous forme de jus de pastèque, a également montré des effets bénéfiques sur la fonction cardiovasculaire de rats diabétiques de type 2 et obèses, via l'amélioration de la vasodilatation l'acétylcholine-dépendante (133). Chez des personnes cinquantenaires, la citrulline était également capable de réduire l'onde de pouls mesurée à la cheville, permettant d'évaluer la compliance artérielle (134). De plus, Morita et al. (135) ont démonté chez des patients souffrant d'angine de poitrine, angine caractérisée par des spasmes au niveau des coronaires, qu'une complémentation en citrulline (800mg/jour pendant 8 semaines) augmentent la production de NO et diminue l'oxydation de lipoprotéines (LDL, ApoB) conduisant à une amélioration de la fonction endothéliale. L'intérêt de la citrulline sur certaines pathologies cardiovasculaires a été mis en évidence à tout âge puisque qu'une étude clinique a démontré que la citrulline permettait de diminuer les complications vasculaires chez l'enfant atteint de drépanocytose (119). Une autre étude clinique chez des enfants sous bypass cardiopulmonaire a permis de montrer que l'habituelle hypertension pulmonaire inhérente à ce genre d'opération n'était pas développée lorsque les enfants atteignaient une concentration plasmatique en citrulline d'environ 40µM après une complémentation orale en cet acide aminé (118). De plus la complémentation est très bien tolérée et sans danger chez ce type de patient (120). Enfin, la citrulline pourrait être intéressante contre l'athérosclérose puisque Berthe et al. (136) ont démontré in vitro que la citrulline sur des cellules aortiques stimule la production de NO et l'expression de l'eNOS. De plus, chez des souris hypercholestérolémiques déficientes en

récepteurs aux LDL, une athérosclérose sévère liée à la très forte concentration plasmatique en cholestérol est présente. Or, la complémentation en citrulline sous forme d'extrait de pastèque permet de diminuer les taux de cholestérol, d'améliorer l'homéostasie cytokinique (avec notamment une diminution de cytokines pro-inflammatoires (INF-γ et MCP-1) et une augmentation de cytokines anti-inflammatoires (IL-10)) et d'atténuer le développement de l'athérosclérose sans modification de la pression systolique (137).
4.4 Citrulline et sepsis

Le sepsis est connu comme étant une cause d'admission très fréquente en unité de réanimation. Lors du sepsis, la concentration plasmatique de lipopolysaccharides (LPS) est très élevée entrainant une endotoxémie qui peut aboutir à une défaillance multiviscérale. Le sepsis induit également des altérations du métabolisme des acides aminés et notamment de l'arginine qui est fortement diminuée (128,138,139). Cette déficience est le résultat de la diminution de la captation d'arginine et un ralentissement de la production *de novo* d'arginine à partir de citrulline, en combinaison d'un catabolisme de l'arginine élevé du fait de l'augmentation de l'activité de l'arginase et l'iNOS lors de la réponse immunitaire. De ce constat, plusieurs stratégies pour augmenter la disponibilité en arginine dans ces conditions inflammatoires ont vu le jour lors des deux dernières décennies.

Plusieurs causes sont responsables de la chute de la production d'arginine et l'augmentation du catabolisme de cet acide aminé. La baisse de production peut être le résultat d'une disponibilité limitée de la citrulline (138,140), d'une diminution de la captation des protéines lors d'une défaillance intestinale (139) ou d'une altération de la conversion de glutamine en citrulline (malgré une extraction splanchnique de la glutamine qui reste inchangée) (141). Enfin, une insuffisance rénale peut limiter la production d'arginine à partir de citrulline.

Outre la diminution de la concentration en arginine lors du sepsis (128,139,142–144), l'endotoxémie et les conditions inflammatoires qui en découlent sont caractérisées par une production et une biodisponibilité réduite de la citrulline (111,139,145,146). Ces altérations contribuent à la baisse de la synthèse *de novo* d'arginine durant le sepsis et l'endotoxémie (128,139,145). Ces faibles concentrations en citrulline ont été associées à un taux de mortalité plus élevé dans cette population (147–149). De plus, les faibles concentrations en citrulline sont également associées à une diminution de la production de NO (139).

La complémentation en citrulline pourrait être une intervention thérapeutique afin de restaurer la production d'arginine. En effet, durant l'endotoxémie et l'inflammation associée, la complémentation en citrulline permet d'augmenter les concentrations plasmatiques en citrulline et en arginine (109,128). Lors d'une inflammation, cette complémentation en citrulline est même plus efficiente pour augmenter la concentration plasmatique en arginine qu'une complémentation en arginine, du fait que la citrulline n'est pas capté par le foie (111). Il a également été démontré que la complémentation en

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citrulline, contrairement à l'arginine, augmente la concentration en NO et améliore la microcirculation lors d'une endotoxémie au niveau des villosités jéjunales (128), ce qui pourrait induire une meilleure absorption. Enfin, l'utilisation de citrulline plutôt que d'arginine comme agent thérapeutique est renforcé par le fait que certaines études ont suggéré que l'arginine pourrait induire une mortalité plus importante chez les malades de réanimation au cours du sepsis, en renforçant les effets d'une production excessive de NO sur la défaillance multiviscérale. Ce point reste controversé et bien qu'aucune preuve clinique ou expérimentale ne permet de le démontrer, le principe de précaution s'impose et il n'est pas recommandé de complémenter ces malades en arginine (150).

La complémentation en citrulline pourrait donc être une intervention thérapeutique intéressante mais d'autres études cliniques sont encore nécessaires pour en faire un premier choix.

4.5 Citrulline et syndrome MELAS

Le syndrome MELAS (pour *Mitochondrial Encephalomyopathy, Lactic Acidosis, and Strokelike episodes*) est l'une des plus fréquentes mitochondriopathies. Elle résulte d'une altération de la synthèse protéique mitochondriale (incluant la synthèse des sous-unités de la chaîne respiratoire) induisant une altération de la production d'énergie. Cette incapacité à produire suffisamment d'énergie pour subvenir aux besoins cellulaires induit une dysfonction multiviscérale, amplifiée par une déficience en NO observée dans cette pathologie. L'arginine et la citrulline étant des précurseurs de NO, il a été proposé que leur administration puisse augmenter la disponibilité en NO et ainsi avoir des bénéfices thérapeutiques pour éviter certaines complications, notamment les accidents vasculaires liés au déficit en NO lors du syndrome MELAS (151). Cette hypothèse est supportée par des études cliniques. Il a notamment été montré que la complémentation orale en arginine diminue la fréquence et la sévérité des accidents vasculaires (152,153).

L'effet thérapeutique de l'arginine lors d'accidents vasculaires est associé à l'augmentation de la disponibilité en NO, conduisant à améliorer la vasodilatation intracérébrale et le flux sanguin. Ce potentiel mécanisme est supporté par la démonstration qu'une complémentation en arginine chez des patients atteints du syndrome MELAS est associée à une production de NO augmentée (112,154).

Concernant la citrulline, il a été démontré que la complémentation en citrulline augmente la production de NO chez des patients atteints du syndrome MELAS. L'augmentation de la production de NO est même plus importante avec une complémentation en citrulline qu'avec une complémentation en arginine (112). Ceci est lié au fait que la complémentation en citrulline augmente de manière plus importante la concentration plasmatique en arginine que l'arginine elle-même à un dosage équivalent chez les patients atteints du syndrome MELAS (112,113). Les raisons de cette augmentation plus importante de l'argininémie par la citrulline comparée à l'arginine elle-même ont été abordées dans la partie 4.1. De ce fait, la complémentation en citrulline pourrait être plus intéressante que la complémentation en arginine car les effets bénéfiques de l'arginine sont essentiellement liés à l'augmentation du NO. De plus, il a été rapporté que la complémentation en arginine ou en citrulline diminue les concentrations plasmatiques en alanine et en lactate, suggérant qu'une telle

complémentation pourrait améliorer l'hyperlactatémie présente chez la personne atteinte du syndrome MELAS (112,155).

Des études cliniques sur l'effet d'une complémentation en citrulline sur les différents aspects du syndrome MELAS sont nécessaires pour supporter l'utilisation d'une telle complémentation en tant que traitement pour le syndrome MELAS.

5 Citrulline et métabolisme cérébral

La citrulline a également la propriété de moduler le métabolisme cérébral. De façon étonnante, cet acide aminé passe sans problème la barrière hématoencéphalique (chez des rats adultes, elle est non détectable et elle passe à 1,5 mM après 5 jours de complémentation en citrulline) (156). Ainsi, il a été démontré qu'une complémentation orale en citrulline pendant 3 mois chez des rats âgés permet d'améliorer les changement liés à l'âge des rafts lipidiques au niveau de l'hippocampe (157). Plus précisément, avec l'âge la structure des rafts lipidiques est modifiée. En effet, avec l'âge, la concentration en citrulline dans le cerveau est diminuée (avec une altération de l'activité arginase et NO synthase) et les rafts s'enrichissent en cholestérol, en cavéoline 1 et en l'amyloid precursor protein (APP) (cette dernière étant associée à une augmentation des plaques amyloïde, impliquées dans la maladie d'Alzheimer). Or, la complémentation en citrulline est capable de modifier la structure de ces rafts, se rapprochant de celle d'un rat adulte avec la citrulline. Pour vérifier le potentiel effet protecteur de la citrulline sur l'accumulation de peptides amyloïde, les mêmes auteurs ont étudié in vitro l'effet de la citrulline (5mM) sur une lignée de neuroblastes sur-exprimant l'APP. Ils ont ainsi démontré que la citrulline diminue significativement la production d'APP confirmant ainsi l'effet protecteur de la citrulline sur le métabolisme de l'APP (158). Ce résultat a des conséquences réelles in vivo puisque Ginguay et al. (159) ont démontré, chez des souris transgéniques surexprimant l'APP, qu'une complémentation en citrulline diminue les plaques amyloïdes dans l'hippocampe et dans le cortex. De plus, il est connu que chez les personnes souffrant de la maladie d'Alzheimer, l'expression et l'activité de l'OTC est augmentée au niveau des neurones (160). Cette augmentation pourrait être un mécanisme protecteur des cellules mais cela reste à démontrer. Mais, il a été montré que posséder l'allèle récessif rs5963409 de l'OTC est associé à un risque plus important de développer la maladie d'Alzheimer (161). Ainsi, les données restent parcellaires et ne permettent pas à ce jour de déterminer le vrai impact des changements cellulaires au niveau de l'hippocampe par la citrulline.

Par ailleurs, il a été démontré récemment que la citrulline est capable de stimuler la voie dopaminergique dans le cerveau. En effet, Moinard et al. (162) ont mis en évidence qu'une complémentation en citrulline chez des rats âgés stimule la motricité des rats, évaluée par le test du labyrinthe en Y. Or, cette augmentation de la motricité avec la citrulline pourrait être

liée à une action sur le système nerveux central notamment *via* une stimulation de la voie dopaminergique. Il a ainsi été démontré que l'augmentation de la motricité par la citrulline est associée à une augmentation de la forme totale et de la forme phosphorylée de la tyrosine hydroxylase (+125%), l'enzyme limitante de la voie dopaminergique (156). En revanche, de façon surprenante, au niveau de la jonction neuromusculaire, il a été démontré chez le rat que la citrulline inhibe la libération d'acétylcholine, neurotransmetteur impliqué dans le signal nerveux induisant la contraction musculaire (163). Ces effets de la citrulline sur le métabolisme cérébral pourraient être liés au fait que la citrulline est capable de moduler l'expression de l'eNOS dans l'hippocampe et réduire la perte de vascularisation cérébrale chez le rongeur lors d'une ischémie (164). Ainsi, la citrulline pourrait affecter le système vasculaire cérébral et améliorer l'oxygénation. Cependant, les mécanismes précis d'action de la citrulline restent encore à explorer.

6 Citrulline et immunité

La citrulline a également été rapportée comme ayant un rôle modulateur de l'immunité. En effet, le NO est connu pour être un immuno-modulateur *via* une action directe sur les cellules immunitaires ou une action indirecte *via* une modulation des cytokines pro- et antiinflammatoires. Ainsi, du fait que la citrulline est un précurseur de NO, il a été démontré *in vitro* par Breuillard et al. (165) que la citrulline stimule la production de NO, diminue la sécrétion de TNF- α et stimule la libération d'IL-6 par les macrophages de rats diabétiques. En revanche, les effets de la citrulline sur la production de NO et la production de TNF- α n'ont pas été retrouvé dans des macrophages de rats âgés (166).

Par ailleurs, il a été montré *in vitro* que lors d'une carence en arginine, l'apport de citrulline permet de rétablir la prolifération des cellules T, de stimuler l'expression de l'ASS dans ces cellules et de rétablir la fonction immunitaire en prévenant la diminution de l'expression de la chaine zêta de CD3, nécessaire à la fonction immunitaire (167). Enfin, la citrulline est capable d'agir directement sur les macrophages en augmentant l'efflux de cholestérol par ces derniers *via* une augmentation de l'expression des transporteurs de cholestérol ABCA1 et ABCG1 (168).

7 Citrulline et métabolisme protéique musculaire

L'intérêt de la citrulline dans la régulation du métabolisme protéique musculaire a été proposé lorsque Osowska et al. (2) ont montré, dans un modèle de grêle court, qu'une complémentation avec de la citrulline améliore le bilan azoté. Ainsi, l'effet stimulateur de la synthèse protéique musculaire a été mis en évidence pour la première fois en 2006 par Osowska et al. (3) chez des rats mâles âgés dénutris, en utilisant un modèle de malnutrition protéino-énergétique. Ce modèle de malnutrition protéino-énergétique consiste à nourrir les rats avec seulement 50% des ingesta spontanés pendant 12 semaines. Puis, les rats sont renourris une semaine avec un régime correspondant à 90% de leur ingesta spontanés et enrichis en citrulline ou en acides aminés non essentiels (afin que les régimes soient isoazotés). Il a ainsi été démontré que les rats recevant le régime enrichi en citrulline ont une synthèse protéique musculaire augmentée de 80% et un gain protéique musculaire net de 20% (Fig. 7) et sans affecter la protéolyse myofibrillaire (estimée par l'excrétion urinaire de 3-MH). En utilisant le même modèle, il a été démontré que l'accrétion protéique liée à la prise de citrulline s'accompagne d'une augmentation de la force maximale ainsi que d'une augmentation de la motricité des animaux permettant d'établir un continuum entre une action métabolique et un retentissement clinique (162). Les effets de la citrulline sur la synthèse protéique musculaire et sur la force ne se limitent pas au rat âgé puisque ces résultats ont été confirmés par Ventura et al. (169) chez des rates adultes complémentées et soumises à une restriction alimentaire modérée (60% des ingesta spontanés) pendant 2 semaines. En effet, chez les rats complémentés en citrulline, la synthèse des protéines myofibrillaires est plus importante et la force musculaire est préservée contrairement aux rates restreintes sans complémentation en citrulline.





Les trois groupes ont été subis une restriction alimentaire (50% des ingesta spontanés) pendant 12 semaines. Groupes : R, rats âgés tués à la fin de la période de restriction ; CIT, rats âgés renourris (90% des ingesta spontanés) une semaine avec un régime enrichi en citrulline (5g/kg/jour) ; NEAA, rats âgés renourris (90% des ingesta spontanés) une semaine avec un régime enrichi en acides aminés non-essentiels (régime isoazoté et isocalorique avec le groupe CIT). ANOVA + test Duncan : *p<0,05 vs. R et NEAA.

Par ailleurs, il apparait que l'effet positif de la citrulline sur le gain musculaire s'inscrit dans la durée puisqu'une complémentation en citrulline pendant 3 mois chez le rat âgé (20 mois) permet un gain de masse musculaire moyen de 25% (et lié spécifiquement à une accrétion protéique et à une augmentation de la taille des fibres musculaires) (5). Cependant, l'action de la citrulline sur la synthèse protéique musculaire ne se limite pas au rat âgé, car dans un modèle de jeûne court chez des rats adultes (mis à jeun pendant 18 heures), le jeûne se traduit par une chute de 40% de la synthèse protéique musculaire qui est totalement restaurée par un bolus oral de citrulline (171). Enfin, dans un modèle de restriction de croissance intra-utérine induite par une restriction alimentaire maternelle chez le rat, Bourdon et al. (172) ont démontré qu'une complémentation en citrulline permet de stimuler la croissance fœtale. Cet effet pourrait être lié à l'augmentation de la synthèse protéique fœtale et/ou à l'augmentation de la production de NO observée.

Il a également été montré que cette capacité de la citrulline à moduler la synthèse protéique musculaire existe chez l'Homme : en effet, une complémentation orale en citrulline (10 g vs placebo isoazoté) permet d'améliorer la synthèse protéique musculaire de 25% chez des adultes sains soumis à un régime hypo-protéiné (8%) durant 3 jours (173). De plus, cette amélioration de la synthèse protéique musculaire semble insuline-indépendante puisque l'insulinémie n'est pas différente entre les différents groupes. Plus récemment, Bouillane et al. (123) ont démontré pour la première fois qu'une complémentation orale en citrulline (10g/jour) pendant 21 jours chez des patients dénutris augmente la masse maigre et réduit la masse grasse. Enfin, l'effet de la citrulline sur la synthèse protéique semble spécifique au muscle puisque 3 études cliniques ont étudié les effets d'une complémentation en citrulline dans différentes conditions sur la synthèse protéique au niveau corps entier et aucune d'entre-elles n'a pu montrer un effet positif de la citrulline (122,173,175). Cependant, si la capacité de la citrulline à moduler la synthèse protéique musculaire a été prouvée, les mécanismes précis de son action restent peu connus. Une nouvelle étude a permis de remédier partiellement à ces lacunes. En effet, Le Plénier et al. (4) ont montré en utilisant un modèle de muscles de rats isolés périfusés (epitrochlearis), avec ou sans citrulline, que la synthèse protéique musculaire est plus élevée avec citrulline démontrant une action directe de la citrulline sur la synthèse protéique musculaire. De plus, cette augmentation de la synthèse protéique musculaire pourrait être liée à une stimulation de la voie mTORC1, principale voie de régulation de la synthèse protéique, car il a été montré, dans cette étude que, dans le même modèle de malnutrition protéino-énergétique décrit précédemment, les rats renourris avec de la citrulline ont une activation de S6K1 et 4E-BP1 musculaire plus importante. Ce résultat est confirmé dans le modèle de muscle isolé périfusé de rat adulte puisque l'ajout de rapamycine, un inhibiteur de mTORC1, annule l'effet positif de la citrulline sur la synthèse protéique musculaire (81). Par conséquent, ces résultats semblent tous relier l'augmentation de la synthèse protéique musculaire par la citrulline à une action stimulatrice sur la voie mTORC1, via une activation de S6K1 et 4E-BP1 par phosphorylation. De plus cette stimulation de mTORC1 est partiellement Akt/PI3K indépendante car la citrulline maintient l'activation de 4E-BP1 par phosphorylation lorsque la voie Akt/PI3K, en amont de mTORC1, est inhibée. Enfin, bien que la citrulline stimule la synthèse protéique musculaire globale, elle agit de façon plus complexe sur l'expression des protéines. En effet, la citrulline est capable de stimuler l'expression de protéines spécifiques et d'inhiber l'expression d'autres protéines. La modulation spécifique par la citrulline de certaines protéines a été récemment répertoriée dans une revue générale par Bourgoin-Voillard et al. (176). Plus précisément, la citrulline stimule plus particulièrement l'expression de protéines myofibrillaires et module des enzymes du métabolisme énergétique (voir chapitre 8). Concernant les effets de la citrulline sur la protéolyse, à ce jour peu de données sont disponibles dans la littérature. Ham et al. ont notamment démontré chez des souris immobilisées qu'une complémentation en citrulline diminue l'expression génique de Bnip3, un gène pro-autophagique, fortement stimulé par l'immobilisation. De plus, le rapport protéique LC3BII : LC3BI, un marqueur du nombre d'autophagosome, est augmenté lors d'une immobilisation mais restauré quand la souris est complémentée en citrulline (177). Ce résultat pourrait être relié au travail de Faure et al. (6). En effet, ces auteurs montrent qu'une complémentation en citrulline pendant 5 jours permet de diminuer le proteasome activator complex subunit 1. Cependant, malgré des données intéressantes mais fragmentaires, la régulation de la protéolyse musculaire par la citrulline reste encore à explorer.

8 Citrulline et métabolisme énergétique

L'action de la citrulline ne se limite pas au métabolisme protéique et affecte aussi le métabolisme énergétique. En effet, afin d'explorer les mécanismes d'action de la citrulline, une étude protéomique différentielle a montré, dans le même modèle de malnutrition protéino-énergétique précédemment décrit qu'une renutrition enrichie en citrulline chez des rat âgés entraîne, au niveau musculaire, une surexpression des enzymes impliquées dans la glycogénolyse (i.e., glycogène phosphorylase) et la glycolyse (i.e., phosphoglucomutase 1, 6-phosphofructokinase, triosephosphate isomérase, ß-enolase et pyruvate kinase isozymes M1/M2) et une sous-expression de certaines enzymes du cycle de Krebs (i.e., isocitrate déshydrogénase et succinate déshydrogénase) et de la chaîne respiratoire mitochondriale (i.e., complexe NADH déshydrogénase, NADH ubiquinone oxydoréductase, et ATP synthase) (6). Dans une autre étude protéomique, cette fois-ci chez des rats âgés, il a été démontré qu'une complémentation en citrulline stimule l'expression de TFAM (facteur de biogenèse mitochondriale) ainsi que l'activité du complexe I au niveau des mitochondries (5). Dans ce même travail, les auteurs ont observé une fonte massive du tissu adipeux chez les animaux âgés (près de -50% de la masse grasse abdominale). Ce travail a suggéré que la modulation du métabolisme énergétique ne se limitait pas au muscle puisque Joffin et al. (8) ont démontré dans des explants de tissus adipeux de rats âgés, qu'une exposition à de la citrulline induit une augmentation de la libération de glycérol et d'acides gras. Ce résultat n'est pas observé dans des explants de rats jeunes. Ce résultat est dû au fait que chez les rats âgés la citrulline diminue la néoglycérogenèse (diminution de l'expression de la PEPCK) tandis que la β -oxydation est inchangée, permettant la libération d'acides gras.

De manière surprenante, dans des explants de tissus adipeux de rats jeunes, la β -oxydation est augmentée ainsi que l'expression génique de CPT1 (9). Ces résultats ont été confirmés dans des explants adipeux de rats adultes obèses (8) (Fig. 8).



Fig. 8 Effet de la citrulline sur la β-oxydation sur des explants adipeux *in vitro* issus de rats adultes sains (CD : *control diet*) ou obèses (HFD : *High-fat diet*).

Les explants ont été incubés ou non avec de la citrulline (CIT) (2,5 mM) pendant 24h avant analyse. L'oxydation du palmitate (marqué avec ${}^{3}H_{2}O$) a été évaluée sur 100 mg de tissus adipeux retropéritonéal et déterminée par la libération d' ${}^{3}H_{2}O$. Les résultats sont exprimés en pourcentage du contrôle et chaque valeur représente la moyenne ± SEM.

Enfin, la citrulline est également capable de stimuler l'expression génique d'UCP1 (10). Ces effets de la citrulline pourraient ainsi expliquer la diminution de la masse adipeuse de souris obèses complémentées avec de citrulline (11) (et qui a également été observée chez l'Homme). De plus dans cette étude, l'homéostasie glucidique est restaurée avec une amélioration de la tolérance au glucose et de la sensibilité à l'insuline, et l'activité lipogénique hépatique est diminuée. L'effet sur l'homéostasie glucidique confirme le travail de Wu et al. (133) chez des rats diabétiques puisque ils ont mis en évidence que l'apport de citrulline par une complémentation en jus de pastèque diminue les concentrations plasmatiques en glucose, mais également en acides gras (une diminution du tissu adipeux blanc et une augmentation du tissu adipeux brun est également observé). Malgré un rôle évident de la citrulline sur le métabolisme énergétique, les données restent parcellaires et il n'est pas possible de comprendre de façon intégrée comment la citrulline agit sur ce métabolisme.

9 Citrulline et exercice

Certaines propriétés de la citrulline semblent communes à celles de l'exercice, notamment la capacité à stimuler la synthèse protéique musculaire et à affecter le métabolisme énergétique. Ainsi, la citrulline pourrait être un acide aminé ergogénique. Cependant, peu d'études ont à ce jour étudier les effets d'une complémentation en citrulline sur la performance.

Hickner et al. (178) ont été les premiers à étudier une complémentation aigue en citrulline (3 à 9g) avant un exercice à intensité croissante jusqu'à épuisement chez des jeunes sujets sains. De façon surprenante, la complémentation en citrulline a diminué la performance des athlètes en comparaison avec le placebo. Cutrufello et al. (179) n'ont pas non plus réussi à montrer un effet bénéfique de la citrulline sur la performance à la fois dans une filière aérobie ou dans une filière anaérobie avec des doses similaires de citrulline. Par conséquent, ces deux études n'ont montré aucun effet bénéfique d'une complémentation aigue en citrulline sur la performance. En revanche, les résultats semblent différents lorsque la citrulline est ingérée de façon chronique. En effet, deux études ont évalué la complémentation en citrulline pendant 7 jours. Dans la première, Bailey et al. (121) ont démontré que la complémentation en citrulline (6g/jour) améliore la durée d'effort jusqu'à épuisement de 12% lors d'un test de performance à haute intensité sur vélo. Ce résultat sur la performance pourrait être lié à la plus faible VO₂ moyenne observée ou à la diminution de la pression artérielle avec la complémentation en citrulline. La seconde étude a démontré, avec une complémentation chronique en citrulline inférieure (2,4g/jour), une réduction du temps nécessaire pour effectuer 4 km en vélo de 1,5% (180). De plus, dans cette étude, au niveau de la fatigue musculaire, le ressenti des sujets était meilleur après la complémentation en citrulline.

Les effets de la citrulline ne se limitent pas à la performance puisque la citrulline permet d'augmenter la perfusion musculaire et également de préserver la perfusion splanchnique. En effet, une hypoperfusion splanchnique apparait durant un exercice physique intense, due à une redistribution sanguine de l'aire splanchnique aux muscles actifs et au système cardiopulmonaire (181). Cette hypoperfusion splanchnique est associée à une diminution de la fonction de barrière de l'intestin et ainsi à une performance athlétique pouvant être diminuée (182). Cependant, Van Wijck et al. (129) ont démontré qu'une ingestion de citrulline (10g) préserve la perfusion splanchnique et atténue les désordres intestinaux liés à l'exercice.

Cependant, la citrulline est souvent consommée sous forme de malate de citrulline par les athlètes. Pour cette raison ; plusieurs auteurs ont utilisés ce sel de citrulline pour évaluer les propriétés de la citrulline lors d'un exercice. Ainsi, il a été démontré qu'une consommation chronique de malate de citrulline (6g/jour) pendant 16 jours induit une augmentation de 34% de la production oxydative d'ATP durant l'exercice, et une augmentation de 20% du taux de récupération de la phosphocréatine après un exercice (183). En revanche, cette étude a de nombreux points limitants (i.e. pas de groupe placebo et étude non effectuée en aveugle). Une autre étude a montré qu'une unique ingestion de malate de citrulline (8g) augmente la capacité de travail de 19% (mesuré par le nombre de répétitions effectuées jusqu'à épuisement lors d'une exercice en résistance) (184). Plus récemment, Glenn et al. (185) ont mis en évidence une augmentation de la performance lors d'exercices en résistance après la prise de malate de citrulline (8g) par des jeunes femmes.

Des résultats similaires sur la performance en filière anaérobie ont été retrouvés par Wax et al. (186) chez des hommes entrainés et par Glenn et al. (187) chez des femmes adultes (d'environ 50 ans) entrainées après une prise aiguë de malate de citrulline (8 g). Ainsi le malate de citrulline semble être intéressant pour améliorer la performance, en particulier lors d'exercice en résistance. Cependant, ces résultats doivent être considérés avec précaution puisqu'il n'est pas possible de déterminer si les effets sont liés à la citrulline ou au malate (qui est notamment impliqué dans la production d'énergie en tant qu'intermédiaire du cycle de Krebs (178,188)). Par conséquent, bien que les résultats d'une complémentation en citrulline soient plutôt prometteurs lors d'un exercice, d'autres études sont nécessaires pour confirmer l'effet ergogénique de la citrulline. Par ailleurs, concernant les autres études disponibles, elles n'ont généralement pas de placebo isoazoté ce qui en limite la portée clinique.

BUT DE L'ETUDE

Le but de notre travail a été de préciser l'action de la citrulline sur la synthèse protéique musculaire et d'explorer le rôle du métabolisme énergétique dans cet effet.

Dans une première étude (Publication n°1), nous avons étudié *in vivo* la relation entre les métabolismes protéique et énergétique, et l'action de la citrulline sur ces derniers. En parallèle et au vu de propriétés qui semblent communes, nous avons évalué les potentiels effets synergiques d'un entrainement physique et d'une complémentation en citrulline. Pour se faire, nous avons exploré les métabolismes protéique (notamment la synthèse protéique musculaire) et énergétique (notamment le métabolisme mitochondrial) et évalué la performance chez des rats Wistar adultes sains sédentaires ou entrainés pendant 4 semaines. Ces rats ont été complémentés en citrulline (1g/kg/jour) durant cette même période.

Par ailleurs, concernant la citrulline, si son action sur la synthèse protéique a bien été prouvée, en revanche l'impact de cet acide aminé sur le sécrétome musculaire est totalement méconnu et une augmentation de la synthèse protéique par la citrulline dans la cellule musculaire pourrait moduler certaines protéines sécrétées. Pour répondre à cette question, nous avons réalisé une deuxième étude (Publication n°2) afin de déterminer, par une approche protéomique différentielle, la modulation par la citrulline (5mM) de l'expression des protéines sécrétées par la cellule musculaire *in vitro* dans des myotubes dérivés de cultures primaires de myoblastes.

Enfin, de façon complémentaire à ces travaux, nous avons essayé de préciser, dans le même modèle de cellules musculaires, les mécanismes impliqués dans la régulation simultanée des métabolismes protéique et énergétique, et notamment comment le métabolisme énergétique est modulé par la citrulline pour supporter l'activation de la synthèse protéique musculaire (Publication n°3).

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TRAVAUX EXPÉRIMENTAUX

Résumé et publication N°1

Effets synergiques d'une complémentation en citrulline et de l'exercice sur la performance chez le rat :

Evidences d'une implication des métabolismes protéique et énergétique

Clinical Science. Sous presse

Goron A., Lamarche F., Cunin V., Dubouchaud H., Hourde C., Noirez P., Corne C., Couturier K, Sève M., Fontaine E., Moinard C.

Introduction :

L'exercice ou la citrulline sont deux régulateurs du métabolisme protéique musculaire et du métabolisme énergétique. Cependant, la combinaison des deux a été très peu étudiée et pourrait avoir des effets synergiques sur le métabolisme musculaire et la performance.

<u>But de l'étude</u> :

Le but de l'étude a été d'explorer (i) l'action de la citrulline sur les métabolismes protéique et énergétique ainsi que sur leur inter-relation, et (ii) d'évaluer une possible action synergique de la citrulline avec l'exercice.

<u>Méthodes</u> :

Des rats adultes sains Wistar ont été complémentés ou non en citrulline (1g/kg/jour) et entrainés ou non en endurance pendant 1h, 5 jours par semaine à une vitesse de 25m.min⁻¹ pendant 2 mois (i.e. Ctrl, CIT, Ctrlex, CITex). La capacité d'endurance maximum a été mesurée afin d'évaluer la performance, et les métabolismes protéique (e.g. synthèse protéique, typage de fibres, exploration de la voie mTORC1, analyse protéomique musculaire) et énergétique (e.g. dépense énergétique et fonction mitochondriale) ont été explorés.

<u>Résultats</u> :

L'entrainement physique a amélioré la capacité d'endurance des rats avec un effet additif lorsque les rats ont été complémentés en citrulline mais sans effet de la citrulline chez les rats non entrainés (Ctrl : 29 min \pm 1, CIT : 35 min \pm 2, Ctrlex : 122 min \pm 6, CITex : 139 min \pm 4 ; p<0,05 Ctrl vs. Ctrlex, Ctrl ex vs. CIT ex). Cet effet synergique sur la capacité d'endurance n'est pas lié à une modification de la masse corporelle puisque qu'il n'y a pas de modification ni de la masse corporelle, ni de la masse musculaire ou adipeuse avec la citrulline. La synthèse protéique a en revanche été augmentée par la complémentation en citrulline (+33% ; CIT vs. Ctrl, p<0,05) et par l'exercice (+27% ; Ctrlex vs. Ctrl, p<0,05) avec un effet synergique lorsque combinés (+11% ; CITex vs. Ctrlex). Cette stimulation semble liée à une activation de la voie mTORC1 même si l'effet synergique de la citrulline et de l'exercice n'a pas été retrouvé. Concernant le métabolisme énergétique, l'entrainement physique augmente la dépense énergétique des rats (effet global de l'exercice, p<0,05) sans effet de la citrulline. Cet effet de l'exercice peut être expliqué par l'augmentation de la densité mitochondriale chez les rats entrainés. La fonction mitochondriale (respiration mitochondriale et activité des complexes de la chaîne respiratoire) n'est pas directement modifiée ni par la complémentation en citrulline ni par l'exercice. En revanche, l'analyse protéomique montre que l'expression de nombreuses enzymes impliquées dans l'apport de substrats pour la chaîne respiratoire est augmentée.

Conclusion :

La complémentation en citrulline et l'entrainement en endurance chez des rats adultes sains modulent à la fois le métabolisme protéique et le métabolisme énergétique, avec notamment des effets synergiques des deux sur la performance et la synthèse protéique.

Synergistic effects of citrulline supplementation and exercise on

performance in male rats: evidences for implication of

protein and energy metabolisms

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Conflict of interest: C. Moinard is a shareholder in Citrage[®]. No other authors have any conflict of interest.

Funding: A. Goron received grant support from Agir pour les maladies chroniques.

Acknowledgments: We thank Mrs M. Osman for providing valuable technical expertise, Mrs C. Quiclet and C. Tellier for their contributions to tissue removal, Mr H. Djemai for his contribution to the immunohistology analysis, and Mr G. Fouet for his contribution to the mitochondrial metabolism measurements.

Abstract

Background: Exercise and citrulline are both regulators of muscle protein metabolism. However, the combination of both has been under-studied yet may have synergistic effects on muscle metabolism and performance.

Methods: Three-month-old healthy male rats were randomly assigned to be fed *ad libitum* for 4 weeks with either a citrulline-enriched diet (1g/kg/day) (*CIT*) or an isonitrogenous standard diet (by addition of nonessential amino acid) (*Ctrl*) and trained (running on treadmill 5d/wk) (*ex*) or not. Maximal endurance activity and body composition were assessed, and muscle protein metabolism (protein synthesis, proteomic approach) and energy metabolism (energy expenditure, mitochondrial metabolism) were explored.

Results: Body composition was affected by exercise but not by citrulline supplementation. Endurance training was associated with a higher maximal endurance capacity than sedentary groups (p<0.001), and running time was 14% higher in the *ClTex* group than the *Ctrlex* group (139±4 min vs. 122±6 min, p<0.05). Both endurance training and citrulline supplementation alone increased muscle protein synthesis (by +27% and +33%, respectively, vs. *Ctrl*, p<0.05) with an additive effect (+48% vs. *Ctrl*, p<0.05). Mitochondrial metabolism was modulated by exercise but not directly by citrulline supplementation. However, the proteomic approach demonstrated that citrulline supplementation was able to affect energy metabolism, probably due to activation of pathways generating acetyl-CoA.

Conclusion: Citrulline supplementation and endurance training in healthy male rats modulates both muscle protein and energy metabolisms, with synergic effects on an array of parameters, including performance and protein synthesis.

Introduction

It is classically accepted that preserving and/or developing muscle mass is essential to preserve health and quality of life (1). Exercise is well known as an efficient way to improve muscle mass and muscle function (in particular via an increase of muscle protein synthesis and a stimulation of mitochondria biogenesis) (2,3). Nutrition is a universally-recognized strategy to preserve and/or improve muscle mass and to repair exercise-induced physiological injury (for recent review, see (4)). However, although the bulk of research focuses on nutrients as substrates in energy metabolism, a number of recent studies stress the role of nutrients as signalling molecules that can potentially exert ergogenic effects (5–7). Amino acids, for instance, are not only used for protein synthesis but also show important regulatory properties and may improve performance and recovery (5). Among them, citrulline (CIT), a non-proteinogenic amino acid, has emerged as a major regulator of muscle function. Evidence has been building for years that CIT is able to modulate nitrogen homeostasis (see (8,9) for reviews) and increase muscle protein synthesis (10) and muscle strength (11,12). This latter effect could be explained by the specific effect of CIT on the myofibrillar proteins synthesis and expression in the muscle, which is the protein fraction involved in the muscle contractility (12,13). Finally, it has been recently demonstrated that CIT regulates muscle protein synthesis directly through the mTORC1/PI3K/MAPK pathways (14).

The effect of CIT on muscle protein synthesis has been confirmed in humans: in a princeps work, Jourdan et al. show that an oral administration of CIT in healthy subjects with low protein diet is able to increase the muscle protein synthesis by 25% (15). In the same way, a chronic CIT supplementation improves endurance performance in athletes. In particular, seven days of CIT supplementation helps improve time-to-exhaustion in cycling exercise and increase velocity (16,17). On another side, CIT is also able to modulate mitochondrial metabolism. In healthy aged rats, CIT supplementation increases muscle mitochondrial density and Tfam gene expression (a mitochondrial biogenesis factor) (18). However, these studies did not explore the coordinated regulation of both energy and protein metabolisms by CIT. It is classically accepted that protein synthesis is closely related to energy production (around 20% of energy consumption in cells (19)). Here we tested the hypothesis that CIT directly activate muscle protein synthesis and this stimulation is supported via a regulation of mitochondrial function. Moreover, we proposed that CIT actions could be synergistically improved by exercise in order to enhance performance in healthy adult rats. The aim of this work is i) to explore the action of CIT on both protein and energy metabolism and their interrelation, and ii) to evaluate a possible synergistic effect of CIT with exercise.

Materials and Methods

Animals:

Eighty 3-month-old male Wistar rats (Charles River, L'Arbresle, France) were used in this study. The rats were maintained on a 12-h light/dark cycle with *ad libitum* access to standard diet A04 (Safe, Augy, France) and water for a two-week acclimatization period during which daily spontaneous intake was determined as 25g/day. The protocol was reviewed and approved by the regional ethics committee and authorized by the French Ministry of Research for animal testing (under number 225/02541).

Study design:

At the end of the acclimatization period, the rats were weighed and randomized into four groups:

- *CIT* and *CITex* groups: rats (n=10 for each group) were fed *ad libitum* on a CIT-enriched diet with 1g/kg/day CIT during four weeks.
- Ctrl and Ctrlex groups: rats (n=10 for each group) were fed ad libitum on an isonitrogenous diet (using non-essential amino acids: histidine, serine, alanine and glycine in equimolar ratio) during four weeks.

For the first two weeks, the rats in the Ctrlex and CITex groups were gradually trained for 5 days per week on a motorized treadmill (Bioseb, Vitrolles, France) until reaching a speed of 25 m.min-1 during 1 h (20). For the last 2 weeks of conditioning, the rats were continually trained at this same training intensity. After the four-week experiment period, the rats were weighed in post-absorptive state and euthanized by beheading.

For study feasibility reasons, rats were divided into two experimental groups: 40 rats were used for all experiments except those on mitochondrial metabolism (10 per conditioning situation) while the other 40 rats were used for the mitochondrial metabolism study (see fig. 1 for summarized study design).

Experiment 1

Maximum endurance capacity

One day before euthanasia (in order to evaluate the combined effect of CIT on the muscle protein synthesis stimulus involved by exercise (21, 22)), rat maximum endurance capacity was measured at a treadmill speed of 25 m.min-1. Exhaustion was determined as inability of the rat to avoid touching

the electrical grid located at the rear of the motorized treadmill five times in a 30-second interval, as described in (20).

Body composition

Directly after beheading, the abdominal cavity was quickly opened and organs, muscles and fat deposits were excised and weighed (liver, heart, spleen, pancreas, thymus, kidneys, adrenals, muscles (soleus, plantaris, tibialis, and gastrocnemius), mesenteric fat, epididymal fat, retroperitoneal fat and subcutaneous adipose tissue). Retroperitoneal, epididymal, and mesenteric fat pad were taken from the right side of each animal (23). All tissues were weighed, and the muscles were immediately frozen in liquid nitrogen and stored at 80°C until further analyses.

Muscle protein content

Frozen muscles (plantaris) were homogenized in 10 % trichloroacetic acid (TCA) (10 volumes) using a motor-driven glass/glass potter at 4°C. After centrifugation, the supernatant was collected for amino acids determination (see below) and the precipitate was used for protein determination. After delipidation with acetone, the precipitate was dissolved in 1N NaOH (4 mL/100 mg tissue) for 12 h at 40°C. Total protein content was assayed by the method of Gornall adapted by Fleury (24) and expressed in mg proteins.muscle-1.

Plasma and muscle amino acids

Muscle and plasma amino acid concentrations were determined using a Hitachi L8800 amino acid analyzer. Blood was collected in heparin tubes for amino acids analysis as described in Neveux et al. (25).

Muscle protein synthesis by SUnSET method

The SUNSET method is a new technique to measure relative protein synthesis and it has been compared to classical tracers' method. Indeed, Goodman et al. demonstrated that puromycin labelling compared to the flooding dose method (using 3H-phenylalanine) displays identical result (26, 27). Briefly, 30 min before euthanasia, 0.040 mol/g of puromycin dissolved in 1ml of PBS was injected in intraperitoneal. One plantaris was extracted and frozen for WB analysis (see below). In a preliminary set of data, we verified that endurance test 24h prior the MPS (vs. rats without test) does not impact MPS (data on shown).

Western blotting

All antibodies were purchased from Cell Signaling Technology (Ozyme, France), i.e. Phospho-4E-BP1 (ser65) (1:500), 4E-BP1 (1:1000), phosphor-S6K1 (Thr389) (1:500), S6K1 (1:500), very long-chain acyl-CoA dehydrogenase (1:1000), phospho-ACC (ser79) (1:1000) and anti-mouse IgG HRP-linked antibody

(1:2000), except IgG2a monoclonal anti-puromycine antibody (clone 12D10, 1:5000, from Millipore, France) and anti-mouse IgG Fc2a HRP-linked antibody (1:50,000, from Jackson Immuno Research Labs Inc., West Grove, PA). Muscle protein synthesis was determined by western blot as described by Goodman et al. (27).

Immunohistology

Histology analyses were performed on the plantaris muscles. Transverse serial muscle sections (10 μ m) were obtained at -20°C using a cryostat microtome, mounted on glass coverslips, and air-dried at room temperature. Frozen muscle sections were treated with 5% BSA and 1% sheep serum for 2 hours following incubation with primaries antibodies anti-MHC2x (6H1 developmental studies hybridoma bank) or anti-MHC2b (BFF3 developmental studies hybridoma bank) and anti-Laminin (Z0097, Dako; dilution 1:300) overnight at 4°C. Secondary antibodies (goat anti-mouse IgGM alexa 647 and goat anti-rabbit alexa 488, dilution 1:400) were then incubated in blocking solution for 1 h at room temperature. A second incubation with other primary antibodies (anti-MHC1 (BAD5 developmental studies hybridoma bank) or anti-D10, 1:5000; Millipore, France) was performed for 2 h at room temperature. Secondary antibodies (goat anti-mouse IgG2b or IgG1 alexa 350 and goat anti-IgG2a mouse alexa 546, dilution 1:400) were incubated in blocking solution for 1 h at room temperature.

Muscle fiber diameter (minimal Feret's diameter) corresponding to the minimum separation, for any fiber orientation, of parallel lines that just touch each side of the fiber was determined. All muscle images were captured using a Leica DMi8 Inverted Microscope (Leica, Germany). Images analyses were performed with ImageJ using a specific self-developed macro-template muscle fiber (18). See supplementary data for a pictures example of immuno-staining.

Muscle proteome analysis

Muscle (*gastrocnemius*) proteome analysis using MALDI TOF/TOF mass-spectrometry was performed as previously described (28), with modifications: 1) Protein digestion was by a modified FASP Protocol (29) using Microcon 30 kDa centrifugal ultrafiltration units (Millipore) operated at 14,000 g. Aliquots containing 1 mg of proteins were mixed with 200 µL of 8M urea in 0.1M Tris/HCl, pH 8.5. 2) 100 µg of each sample digest (Ctrl, CIT, Ctrlex, CITex) was labeled with iTRAQ 8-plex reagents as per the manufacturer's instructions (Sciex, Les Ulis, France) in order to perform analyses in duplicate.3) Proteins were identified and quantified with ProteinPilot software v4.5 (Sciex, Les Ulis, France) using the Rat Uniprot Refseq database downloaded in January 2016. Proteins with at least 1 peptide above 95% confidence level were retained, and the list of identified proteins was limited to a 1% False Discovery Rate (FDR). For quantification, bias and background correction was applied. The data were analyzed using the R package Isobar (30) with a Cauchy fit, which allows the determination of statistical significance of protein/peptide regulation (p<0.05).

Plasma and muscle oxidative stress markers

Ferric reducing antioxidant power (FRAP), thiol groups and glutathione peroxidase (GPx) were determined as described in Le Guen et al. (31).

Inflammatory markers measurement

Plasma α -2-macroglubulin concentration was assayed by Elisa following the kit manufacturer's protocol (Euromedex, Souffelweyersheim, France)

Experiment 2

Energy expenditure

Energy expenditure was measured using an indirect open circuit calorimeter in individual cages over 24 hours, at day 3 before euthanasia. Oxygen consumption (VO2), carbon dioxide production (VCO2) and respiratory quotient (RQ) were monitored using a gas analyzer (LE 405, Panlab-Bioseb, France). Airflow rate was obtained from a gas pump and flow meter (Panlab-Bioseb). Data were recorded using a computer-assisted data acquisition program (Metabolism Calculation Software v.2.0.2). Daily energy expenditure (EE) was calculated as EE = (3.815 + 1.232 × VO2/VCO2) × VO2 × 1.44, expressed in kcal.day-1.kg-1 (31).

Citrate synthase activity

Citrate synthase activity was measured in plantaris muscle as performed by Le Guen et al. (31).

Isolated mitochondria preparation

After euthanasia, the gastrocnemius muscle of both legs was collected and transferred into the cold buffer containing sucrose 150 mM, KCl 75 mM, Tris 50 mM, KH2PO4 1 mM, MgCl2 5 mM, EGTA 1 mM, and lipid-free serum albumin 0.2% pH 7.4 was used for mitochondrial extraction. Mitochondria were prepared according to Fontaine et al. (32).

Myofiber isolation and permeabilization

Permeabilized skeletal muscle fibers were prepared from plantaris muscle using the method described by Kuznetsov et al. (33).

Mitochondrial parameters measurement

Rates of oxygen consumption by permeabilized myofibers and isolated mitochondria were measured using a Clark-type O2 electrode (Oxygraph; Hansatech Instruments). Mitochondria (0.2 mg/mM)

were incubated at 37°C in a respiration buffer containing 125 mM KCl, 5 mM Pi, 20 mM Tris-HCl, 0.1 mM EGTA and 0.1% fat-free BSA (pH 7.2). The suspension was stirred constantly with a built-in electromagnetic stirrer and bar flea. Mitochondria were energized with various substrates, i.e. glutamate-malate (GM; 5 mM/2.5 mM) or succinate (S; 5 mM), under ADP stimulation (1 mM) (state III) and oxygen consumption was recorded before and after adding oligomycin (0.25 mg.mL–1), an inhibitor of ATP synthase (state IV). When succinate was used, rotenone was added in the respiratory chamber. Oxygen consumption (state III) of permeabilized myofibers was measured after adding the various substrates, i.e. GM (5/2.5 mM) or S (5 mM) under ADP stimulation (1 mM), and ATP-independent oxygen consumption rate (state IV) was measured by adding oligomycin (0.25 mg.mL–1). After every measurement, fiber bundles were dried overnight at 100°C and weighed. Myofiber respiration rates were expressed as nmol O•min–1.mg dry wt–1. Mitochondrial respiration rates were expressed as nmol O•min–1.mg dry wt–1. Mitochondrial respiration rates were expressed as nmol O•min–1.mg dry wt–1. Mitochondrial respiration rates were expressed as nmol O•min–1.mg dry wt–1. Mitochondrial respiration rates were expressed as nmol O•min–1.mg dry wt–1. Mitochondrial respiration rates were expressed as nmol O•min–1.mg dry wt–1. Mitochondrial respiration rates were expressed as nmol O•min–1.mg dry wt–1. Mitochondrial respiration rates were expressed as nmol O•min–1.mg dry wt–1. Mitochondrial respiration rates were expressed as nmol O•min–1.mg dry wt–1. Mitochondrial respiration membrane potential changes compared to mitochondrial respiration was performed in isolated mitochondria. Wastes (proton leak and/or redox slipping) were thus measured according to membrane potential as in Nicholls et al. (34).

Mitochondrial oxygen-free radical production

ROS production was estimated by measuring H_2O_2 release in a stirred 2 mL-chamber containing 0.1 mg of mitochondria and filled with a respiration buffer containing 6 UI horseradish peroxidase and 1 μ mol.L⁻¹ Amplex Red[®] (excitation: 560 nm; emission: 584 nm) plus the same substrates as for respiration. Measurements were carried out both in basal conditions and after sequential additions of 2 μ mol.L⁻¹ rotenone and 2 μ mol.L⁻¹ antimycin A. Results were expressed in pmol H₂O₂.min⁻¹.mg proteins⁻¹ using H₂O₂ standard solution.

β-hydroxyacyl-CoA dehydrogenase activity

Measurement of β -hydroxyacyl-CoA dehydrogenase activity was adapted from Lowry et al. (35).

Acylcarnitine profiles

Acylcarnitine profiles were measured as described by Vianey-Saban et al. (36).

Statistics

All results were compared between groups using two-way Anova and Bonferroni post-hoc tests for all results on SigmaPlot 11.0 software except for muscle immunohistology (18) and proteome analysis (28). Differences at p < 0.05 were considered significant.

Results

Maximum endurance capacity (Table 1)

Endurance training was associated with a higher running time until exhaustion than sedentary groups (+302%, p<0.001, global effect of exercise), and running time was higher in the *CITex* group than the *Ctrlex* group (+14%; p<0.05).

Body composition (Table 1)

Endurance training was associated with lower body mass (-9%, p<0.05) and lower visceral (retroperitoneal, epididymal, and mesenteric fat) (-30%, p<0.05) and subcutaneous fat mass (-17%, p<0.05) than sedentary groups (global effect of exercise). There was no effect of CIT or endurance training on muscle mass or muscle (*tibialis*) protein content.

Plasma and muscle amino acids (Table 1)

Plasma and muscle (*tibialis*) CIT concentrations are reported in table 1. Plasma and muscle CIT concentrations were greater in CIT-supplemented rats than non-supplemented rats (p<0.05). Similar patterns were observed for arginine and ornithine (data not shown).

Muscle protein synthesis (Fig. 2 and 3)

Both endurance training and CIT supplementation alone increased muscle protein synthesis (by +27% and +33%, respectively, *vs. Ctrl*, p<0.05) with an additive effect (+48% *vs. Ctrl*, p<0.05; +11%, *CITex vs. Ctrlex*, p<0.05). A similar pattern of results was observed in immunohistology (Fig. 3)

Muscle fiber size and type (Fig. 3)

Fiber size distribution was affected by CIT supplementation with a shift to the greater fiber diameters. The endurance training effect was the most potent in type IIx fiber. In type I, IIa and IIb fiber, there was an additive effect of endurance training and CIT supplementation in the shift to the greater fiber diameters.

Transduction pathways (Fig. 4)

Phosphorylation status of S6K1 and 4E-BP1, as transduction regulators of mTORC1, was evaluated in the *tibialis* by Western blot. A marked increase in both S6K1 and 4E-BP1 phosphorylation status were observed with both endurance training (*Ctrlex*) and CIT supplementation (*CIT*) alone (respectively +49% and +57% for S6K1 and 45% and 46% for 4E-BP1 *vs. Ctrl*, p<0.05) and combined (*ClTex*) (+66% for S6K1 and 41% for 4E-BP1 *vs. Ctrlex*, *p* < 0.05) without significant difference between these groups. We also evaluated the phosphorylation status of ACC, a good marker of AMPK activation. No difference between groups was observed.

Muscle proteome analysis (Table 4)

Around 100 proteins were significantly modulated by either CIT supplementation or exercise or both. A partial summarized list is given in table 4. The full table can be found in supplementary data.

Oxidative stress markers (Table 2)

There was a positive global effect of CIT supplementation on plasma FRAP, a global marker of nonenzymatic antioxidant capacity, which was significantly increased in the *CITex* group compared to the *Ctrlex* group (+18%, *CITex vs. Ctrlex*, p<0.05). Training and/or CIT supplementation has no influence on plasma GPx and thiol groups.

Muscle GPx was decreased by exercise alone (-36%, *Ctrlex vs. Ctrl*, p<0.05) but this decrease by exercise was totally blunted by CIT supplementation (*CITex vs. Ctrlex*, p<0.05). Exercise and/or CIT supplementation had no influence on muscle thiols and FRAP. In muscle-isolated mitochondria, H_2O_2 production remains unchanged in all condition.

Inflammatory markers (Table 2)

Plasma α -2-macroglobulin was slightly increased by exercise alone (17.8±0.7 *vs.* 14.6±0.9 µg.mL⁻¹, *Ctrlex vs. Ctrl*, p<0.05) but combined CIT supplementation blunted this increase (p<0.05).

<u>Energy expenditure</u> (Table 3)Energy expenditure and respiratory quotient did not differ between specific groups. However, there was a significant global effect of endurance training which increased daily energy expenditure (+7% vs. non-trained groups, p<0.005) and decreased respiratory quotient (0.89±0.01 vs. 0.86±0.01, p<0.05).

Citrate synthase activity (Table 3)

Maximal citrate synthase activity was increased in trained groups (+41% vs. sedentary groups, p<0.05, global effect of exercise).

Mitochondrial metabolism (Table 3)

In muscle-isolated mitochondria (*gastrocnemius*), mitochondrial respiration in complex I and II were unchanged by exercise or CIT supplementation. Moreover, respiration compared to proton-motive force (see suppl. data) was similar whatever the tested conditions. The absence of change in isolated complex I and II activities confirmed our earlier results. However, in permeabilized muscle fibers (*plantaris*), exercise increased mitochondrial respiration in complex I and II at states III and IV (sedentary groups *vs.* trained groups, p<0.05) but mitochondrial respiration remained unchanged between all groups when compared to mitochondrial density $\left(\frac{\text{Respiration}}{\text{Citrate synthase activity}}\right)$.

<u>*B-oxidation markers*</u> (Table 3)

Muscle maximal β -hydroxyacyl-CoA dehydrogenase activity was not modulated in any condition. However, there was a positive global effect of exercise on protein expression of very-long-chain acyl-CoA dehydrogenase (+26% vs. sedentary groups; global effect of exercise, p<0.05) without specific effect between groups. Note that we also profiled acylcarnitines and found that CIT supplementation increased acylcarnitine C8 by 39% in trained rats (p<0.05) (Supplementary data).

Discussion

Amino acids are commonly used as ergogenic substrates but there is little hard evidence that amino acids actually improve performances. Here we tested the combined effect of CIT supplementation and physical training on both protein metabolism and energy metabolism in muscle (for the sake of clarity, data are summarized in Table 5). CIT supplementation was found to increase endurance capacity by 14% in trained rats. This result is in line with reports from experimental and clinical trials (16, 17, 37) evaluating a chronic specific CIT supplementation but unfortunately the authors did not explore the mechanisms involved. In contrast, some studies did not show any enhancement of muscle protein synthesis following exercise but these studies were made in aged men with reduced physical activity or in response to resistance exercise. Moreover, these authors compared the effect of a very high dose of protein (45 g of whey protein) or high dose of protein (15 g or 20 g of whey protein) plus CIT. In these conditions, they were studying how replacement of some protein by CIT would have the same effect rather the specific effect of CIT supplementation. The improvement of performance by CIT cannot be linked to modification of body composition or muscle protein content as CIT does not affect them. This result on body composition may seem disconcerting, since it has already been shown that the CIT supplementation can increase muscle mass and/or decrease fat mass (11,18). But, these two studies were done in other conditions, in well-fed or malnourished old rats. However, when CIT was administered during moderate dietary restriction in healthy adult rats, muscle strength was preserved whereas muscle mass and protein content remained unchanged (12). Here, CIT and exercise synergistically increased muscle protein synthesis but not muscle mass and protein content. This result is independent to the higher endurance capacity since sedentary CITsupplemented rats do not have an improvement of endurance capacity but a better muscle protein synthesis compared to sedentary control rats. The positive result on the muscle protein synthesis by CIT could be related to an activation of muscle proteolysis, as illustrated by the increase of cathepsin D and proteasome 26S subunit observed by the proteomic approach. This finding may have major repercussions in terms of muscle functionality. Proteins are continuously exposed to several damages (like oxidative stress) that alter their biological roles, so any increase in protein turn-over improves the continuous remodeling of proteins, maintaining their quality and efficacy. Our proteomic approach also revealed a specific action of CIT on myofibrillar proteins which likely would help improve performance. This result is in line with Faure et al. (13) who described similar results in malnourished old rats.

As expected, both exercise and CIT also activated the mTORC1 pathway (the main regulatory pathway of protein synthesis) but without additive effect. However, mTORC1 is mainly activated in

post-prandial state or after exercise, whereas we explored the mTORC1 pathway in fasted animals (i.e. 24 h after the endurance test), which could explain why the additive effect seen with muscle protein synthesis was not reproduced here.

The increase of muscle protein synthesis should be supported by a modulation of energy metabolism. Indeed, protein synthesis is an important driver of energy expenditure (i.e. accounting for around 20% of energy consumption in cells) (19). Therefore, the increase of muscle protein synthesis could be associated to an increase of the energy expenditure. Thus, we explored energy metabolism and found that exercise increased energy expenditure and reduced respiratory quotient, as expected, but without effect of CIT supplementation. However, this analysis was too global to detect very specific adaptations of energy metabolism, especially at mitochondrial level. In isolated mitochondria, we found no effect of CIT and/or exercise on many parameters (complex I and II respiration, relationship between respiration and proton-motive force, energy efficiency). This result is confirmed at specific isolated complex level. However, mitochondrial behavior could be influenced by cellular environment. Here, mitochondrial respiration in permeabilized fibers demonstrated that only exercise enhanced fiber respiration. It is well known that despite some enhancement of mitochondrial functions, the main effect of exercise on energy metabolism is the increase of the mitochondrial density (3,38). This is in line with our finding that exercise increased citrate synthase activity, a marker of mitochondrial density. Thus, the effect of exercise is not related to a functional modulation of mitochondria because fiber respiration was not increased by exercise when related to citrate synthase activity. Unfortunately, we found no effect of CIT supplementation on these mitochondrial parameters, but all our experiments on isolated mitochondria and permeabilized fibers were performed under conditions where substrate concentration was saturating. Thus, CIT and/or exercise would play a role in substrate availability for mitochondria and could thus modify substrate fluxes. Interestingly, such hypothesis was supported by the proteomic approach as summarized in Fig. 5. Briefly, our results clearly show that CIT would activate pathways generating acetyl-CoA from both glucose and fatty acids and their associated transport. Moreover, CIT is able to profoundly affect the proteins involved in the electron transport chain that could influence ATP production. To confirm this assertion, further studies are required to elucidate the regulation of muscle energy metabolism.

Finally, we report effects of CIT on antioxidant enzymes expression and GPx activity that could help optimize mitochondrial functions (since oxidative alterations result in mitochondrial dysfunctions) and reduce inflammation. These results are in line with the literature, as direct CIT antioxidant properties have already been reported to be linked to the ability of CIT to scavenge hydroxyl radicals (39). This antioxidant property could favorably affect muscle protein synthesis, already shown in old rats (40).

Concerning the possible anti-inflammatory effect of CIT, our findings are in agreement with van Waardenburg et al. (41) showing an inverse relationship between plasma CIT concentration and CRP concentration in critically-ill children. More recently, Breuillard et al. (42) observed a direct anti-inflammatory effect of CIT in isolated macrophages. Our findings are therefore of major interest, since inflammation and oxidative stress could worsen performance (43).

In conclusion, fragmentary data suggested that CIT could improve performance during exercise. Our work clearly demonstrates that CIT effectively enhances endurance capacity which could be related to an activation of muscle protein synthesis (even such relationship is not clearly established in literature). Moreover, we demonstrate that the increase in energy required to sustain muscle protein synthesis and performance is not related to direct modifications of mitochondrial functions but rather to a modulation of substrates fluxes and likely also to anti-inflammatory and antioxidant effects of CIT. Thus, the mechanisms underlined in the enhancement of endurance capacity by CIT seem to be multifactorial. These observations remain to be confirmed in humans.

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	Ctrl	CIT	Ctrlex	CITex	Global effect
Maximal endurance capacity (min)	29.4±1.3 ^a	34.7±2.1ª	122.1±6.1 ^b	139.1±4.0°	Ex
Body weights					
Initial body weight (g)	358±4	353±6	351±5	353±5	-
Final body weight (g)	401±4 ^a	399±7 ^a	368±4 ^b	367±4 ^b	Ex
Organ masses					
Liver (g)	12.1±0.3	11.2±0.4	11.1±0.3	11.0±0.3	-
Spleen (mg)	603±20	602±23	573±17	610±31	-
Thymus (mg)	442±27	403±20	346±18	365±30	-
Pancreas (g)	$1.54{\pm}0.08$	1.58±0.11	1.36±0.09	1.41±0.06	-
Kidneys (g)	1.17 ± 0.02	1.21±0.03	1.11±0.03	1.15 ± 0.04	-
Adrenals (mg)	33±3	37±3	37±4	37±3	-
Heart (mg)	930±21	936±23	887±12	923±18	-
Fat masses					
Subcutaneous fat mass (g)	3.0±0.3 ^a	3.4±0.3 ^a	2.7 ± 0.2^{b}	2.6±0.2 ^b	Ex
Visceral fat mass (g)	12.9±1.1 ^a	13.8±1.3 ^a	9.0±0.6 ^b	9.9±0.9 ^b	Ex
Total fat mass (g)	$16.0{\pm}1.2^{a}$	17.2 ± 1.6^{a}	11.7 ± 0.7^{b}	$12.5{\pm}1.0^{b}$	Ex
Muscle masses					
Tibialis (mg)	717±11	752±45	630±64	702±22	-
Soleus (mg)	161±6	157±8	157±5	157±7	-
Gastrocnemius (g)	2.27 ± 0.05	2.035 ± 0.07	2.05 ± 0.04	1.98 ± 0.06	-
Plantaris (mg)	364±8	357±12	354±9	336±13	-
Total (g)	3.27±0.06	3.30±0.10	3.14 ± 0.10	3.18±0.10	-
Muscle protein contents	02.4 ± 1.1	025 ± 1.2	24.6 ± 1.9	25.2 1.4	
(mg proteins. muscle ⁻¹)	23.4±1.1	∠3.3±1.3	24.0±1.8	23.3±1.4	-
Plasma citrulline (µmol.l ⁻¹)	20±2 ^a	97±6 ^b	21 ± 2^{a}	97 ± 8^{b}	CIT
Muscle citrulline (nmol.g ⁻¹)	71 ± 3^{a}	262±21 ^b	79 ± 6^{a}	313±24 ^b	CIT

Table 1. Anthropometric data and maximal endurance capacity in control vs CIT-supplemented rats that were exercise-trained (5d/w during 4 weeks) vs not.

Means \pm SEM. Significant differences are grey-backgrounded. Results with different superscript letters are significantly different (p<0.05) (two-way Anova + Bonferroni test).

	Ctrl	CIT	Ctrl ex	CIT ex	Global effect
Plasma markers of oxidative stress					
FRAP (µM.g proteins ⁻¹)	401±22 ^{ab}	431±21 ^{ab}	371±22 ^a	439±25 ^b	CIT
Thiols (µM.g proteins ⁻¹)	239±6	246±9	238±6	249±8	-
GPx (U.g ⁻¹ proteins)	8049±227	8066±299	7976±156	8440±257	-
Muscle markers of oxidative stress					
FRAP (µM.g proteins ⁻¹)	37.4±1.7	34.2±2.7	36.5±1.3	34.8±2.3	-
Thiols (µM.g proteins ⁻¹)	71.5±2.0	69.9±1.7	69.3±0.6	68.1±1.4	-
GPx (U.g ⁻¹ proteins)	178±25 ^a	167±24 ^a	114±11 ^b	194±25 ^a	-
Mitochondria complexes					
H_2O_2 production (pmol.min ⁻¹ .mg proteins ⁻¹)					
Complex I	116±5	119±4	117±14	117±17	-
Complex II	2449±250	2286±188	2271±151	2362±246	-
Plasma α -2-macroglobulin (μ g.ml ⁻¹)	14.6±0.9 ^a	15.1±0.6 ^a	17.8±0.7 ^b	15.3±0.8 ^a	-

Table 2. Plasma and muscle markers of oxidative stress and inflammatory status in control vs CIT-supplemented rats that were exercise-trained (5d/w during 4 weeks) vs not.

Means \pm SEM. Significant differences are grey-backgrounded. Results with different superscript letters are significantly different (p<0.05) (two-way Anova + Bonferroni test).

		Ctrl	CIT	Ctrlex	CITex	Global effect
Energy exp (kcal day	enditure	156±4	157±3	167±6	169±5	Ex
Respiratory	quotient	0.90±0.01	0.89±0.01	0.86±0.01	0.86±0.01	Ex
Mitochondr Citrate syntha (µmol.min ⁻¹	ial density ase activity .g tissue ⁻¹)	59±5 ^a	57±3 ^a	83±3 ^b	80±3 ^b	Ex
Muscle isolated respira (nmol O.min ⁻¹ .n	mitochondrial ation ng proteins ⁻¹)					
Complex I	State III	78.0±5.3	76.3 ± 6.5	77.8 ± 4.2	80.7 ± 5.1	-
respiration	State IV	8.7±0.5	7.9±0.6	7.8±0.6	7.7±0.5	-
Complex II	State III	54.2±4.8	52.8±4.0	53.1±3.4	51.6±2.8	-
respiration	State IV	12.9±0.9	12.8±0.8	13.6±0.9	13.1±0.9	-
Muscle permea respira (nmol O.min ⁻¹ .	abilized fiber ation mg fibers ⁻¹)					
Complex I	State III	4.41 ± 0.63^{a}	4.02 ± 0.39^{a}	6.50±0.39 ^b	6.02 ± 0.47 ^b	Ex
respiration	State IV	0.97±0.10	1.05 ± 0.15	1.52±0.9	1.46±0.13	Ex
				h	h	
Complex II	State III	4.17±0.43 "	4.20±0.19 °	6.44 ± 0.80^{-6}	6.81±0.90°	Ex
respiration	State IV	2.06±0.19	2.15±0.11	3.42±0.43	3.25±0.39	Ex
(nmol.min ⁻¹ .m	g proteins ⁻¹)					
Complex I	activity	541±20	546±27	590±25	596±30	-
Complex I	I activity	159±13	162±10	187±11	192±12	-
Muscle β-H A (µmol.min ⁻¹ .m	D activity g proteins ⁻¹⁾	3.95±0.34	3.96±0.42	3.82±0.33	3.94±0.46	-
Muscle VLCA (A.U	D expression J.)	6334±256 ^a	5918± 505 ^a	7699 ± 789^{a}	7710± 686 ^a	Ex

Table 3. Muscle energy metabolism data in control *vs* CIT-supplemented rats that were exercise-trained (5d/w during 4 weeks) *vs* not.

Means \pm SEM. Significant differences are grey-backgrounded. Results with different superscript letters are significantly different (p<0.05) (two-way Anova + Bonferroni test).

supplem	ented fats that were exercise-trained (50/w during 4 we	CIT	Ctrlex	CITex
Uniprot accessions	Deregulated proteins	Ctrl	Ctrl	Ctrlex
	Carbohydrate metabolic process			
P04642	Lactate dehydrogenase A	1.37	1.01	1.13
P42123	Lactate dehydrogenase B	1.54	1.19	1.60
P07953	Fructose-2,6-biphosphatase 1	1.81	1.16	1.40
P09117	Fructose-bisphosphate aldolase C	0.28	0.50	0.55
P53534	Glycogen phosphorylase	1.31	0.97	1.30
	Lipid metabolic process		_	
P18163	Acyl-CoA synthetase long-chain family member 1	2.63	0.96	1.82
P07483	Fatty acid binding protein 3	1.69	1.17	1.60
	Krebs cycle and respiratory electron chain		-	
B2RZD6	Ndufa4 (Complex I subunit)	0.66	1.24	0.80
Q561S0	Ndufa10 (Complex I subunit)	1.20	0.95	1.16
B2RYW3	Ndufb9 (Complex I subunit)	1.53	1.10	1.22
Q641Y2	Ndufs2 (Complex I subunit)	0.68	0.87	0.69
Q5XIH3	Ndufv1 (Complex I subunit)	1.45	1.16	1.32
P19234	Ndufv2 (Complex I subunit)	1.08	0.86	1.26
D4A0T0	Ndufb10 (Complex I subunit)	1.11	0.91	1.20
Q6PDU7	ATP synthase F0, subunit C	1.15	0.90	1.28
D3ZAF6	ATP synthase F0, subunit F	1.90	0.95	1.97
P10719	ATP synthase F1, subunit β	1.02	0.82	1.13
P35434	ATP synthase F1, subunit δ	1.28	1.84	1.18
A0A0G2K8Q8	Uqcr10 (Complex III subunit)	1.66	1.17	1.52
P21913	Complex II, subunit B	1.18	0.87	1.46
P13086	Succinate-CoA ligase, subunit α	0.94	0.81	1.11
P00406	Cytochrome c oxidase, subunit 2	1.60	0.99	1.51
P19804	NME/NM23 nucleoside diphosphate kinase 2	1.53	0.88	1.15
P10759	AMP deaminase 1	1.91	1.20	1.38
P38718	Mitochondrial pyruvate carrier 2	1.48	0.87	1.11
Q6AXV4	SAMM50	1.40	0.96	1.29
P08461	Dihydrolipoamide S-acetyltransferase	1.15	0.80	1.12
P49432	Pyruvate dehydrogenase E1 component, subunit β	1.09	0.75	1.12
P41565	Isocitrate dehydrogenase 3, subunit γ	1.14	0.90	1.43

Table 4. Modulated proteins involved in energy metabolism evaluated by proteomic control *vs* CIT-supplemented rats that were exercise-trained (5d/w during 4 weeks) *vs* not.

Proteins were quantified by a differential proteomic approach using MALDI TOF/TOF mass-spectrometry. Validation of the quantitative analysis was conducted with a noise model using the R bioconductor package Isobar based on technical replicates of the different conditions. Protein spot abundance between 0.9 and 1.1 was not considered different. Significantly different proteins abundances are grey-backgrounded (p<0.05).

	СІТ	Exercise	CIT + Exercise
Physical performance			
Endurance capacity	Ø	\uparrow	$\uparrow\uparrow$
Body composition			
Fat mass	Ø	\checkmark	\checkmark
Muscle parameters			
Muscle protein synthesis	\uparrow	\uparrow	$\uparrow\uparrow$
mTOR pathway	\uparrow	\uparrow	\uparrow
GPx	Ø	\checkmark	Ø
Acylcarnitine C8	Ø	\checkmark	Ø
Energy metabolism			
Energy expenditure	Ø	\uparrow	\uparrow
Mitochondrial density	Ø	\uparrow	\uparrow
Mitochondrial enzymes expression	$\wedge \downarrow$	$\wedge \downarrow$	$\wedge \downarrow$
Plasma parameters			
Citrulline	\uparrow	Ø	\uparrow
FRAP	Ø	Ø	\uparrow
α -2-macroglobulin	Ø	\uparrow	Ø

Table 5. Summary of the effects of CIT, exercise or both in a model of trained rats.



Fig. 1. Summarized study design



Fig. 2. Muscle protein synthesis evaluated by puromycin-incorporated protein staining in Western **Blot.** Results are presented as means ± SEM. Values with different superscript letters are statistically different (p<0.05; two-way Anova + Bonferroni test).





Fig.3. Muscle immunohistology data. (A) Muscle protein synthesis evaluated by puromycinincorporated protein staining in *plantaris* using inverted microscope represented as box plots. Values with different superscript letters are statistically different (p<0.05). (B) Frequency distribution of plantaris fibers size diameter represented as diameter distribution. (C) Frequency distribution of plantaris type IIx fibers size diameter represented as diameter distribution. For Fig.3B and 3C, all groups are significantly different (p<0.05).





Fig. 4. Effect of CIT and/or exercise on phosphorylation status of mTORC1 pathway and ACC (as AMPK activation marker) markers by Western Blot. Phosphorylation status of S6K1 (A), 4E-BP1 (B) and ACC (C) in skeletal muscle. Results are presented as means ± SEM. Values with different superscript letters are statistically different (p<0.05; two-way Anova + Bonferroni test).





Supplementary data

		CIT	Ctrlex	CITex
Uniprot accessions	Deregulated proteins	Ctrl	Ctrl	Ctrlex
	Carbohydrate metabolic process			
P04642	Lactate dehydrogenase A	1.37	1.01	1.13
P42123	Lactate dehydrogenase B	1.54	1.19	1.60
P07953	Fructose-2,6-biphosphatase 1	1.81	1.16	1.40
P09117	Fructose-bisphosphate aldolase C	0.28	0.50	0.55
P38652	Phosphoglucomutase-1	1.26	1.51	1.01
P62161	Calmodulin	1.16	1.56	0.87
P53534	Glycogen phosphorylase	1.31	0.97	1.30
	Lipid metabolic process			
P18163	Acyl-CoA synthetase long-chain family member 1	2.63	0.96	1.82
P07483	Fatty acid binding protein 3	1.69	1.17	1.60
P17764	Acetyl-CoA acetyltransferase	0.87	0.70	0.99
Q64428	Trifunctional enzyme subunit α	0.90	0.71	1.01
Q60587	Trifunctional enzyme subunit β	1.13	0.73	1.06
P33124	Long-chain fatty acid CoA ligase 6	0.92	0.72	1.01
P08503	Medium-chain acyl-CoA dehydrogenase	0.92	0.74	1.08
P23965	Enoyl-CoA delta isomerase 1	1.37	2.04	1.23
	Krebs cycle and respiratory electron chain			
B2RZD6	Ndufa4 (Complex I subunit)	0.66	1.24	0.80
Q561S0	Ndufa10 (Complex I subunit)	1.20	0.95	1.16
B2RYW3	Ndufb9 (Complex I subunit)	1.53	1.10	1.22
Q641Y2	Ndufs2 (Complex I subunit)	0.68	0.87	0.69
Q5XIH3	Ndufv1 (Complex I subunit)	1.45	1.16	1.32
P19234	Ndufv2 (Complex I subunit)	1.08	0.86	1.26
D4A0T0	Ndufb10 (Complex I subunit)	1.11	0.91	1.20
Q6PDU7	ATP synthase F0, subunit C	1.15	0.90	1.28
D3ZAF6	ATP synthase F0, subunit F	1.90	0.95	1.97
P10719	ATP synthase F1, subunit β	1.02	0.82	1.13
P35434	ATP synthase F1, subunit δ	1.28	1.84	1.18
P35435	ATP synthase F1, subunit γ	0.83	0.66	1.00
Q06647	ATP synthase F1, subunit O	0.95	0.73	0.99
Q5M9I5	Uqcr8 (Complex III subunit)	0.97	2.37	1.41
A0A0G2K8Q8	Uqcr10 (Complex III subunit)	1.66	1.17	1.52
P20788	Uqcr5 (Complex III subunit)	0.96	0.68	1.01
P21913	Complex II, subunit B	1.18	0.87	1.46
P13086	Succinate-CoA ligase, subunit α	0.94	0.81	1.11
P00406	Cytochrome c oxidase, subunit 2	1.60	0.99	1.51
P19804	NME/NM23 nucleoside diphosphate kinase 2	1.53	0.88	1.15

Table 1. Modulated proteins evaluated by proteomic control vs CIT-supplemented rats that were exercise-trained (5d/w during 4 weeks) vs not.

P38718 Mitochondrial pyruvate carrier 2 1.48 0.87 1.11 Q6AXV4 SAMM50 1.40 0.96 1.29 Q01205 Dihydrolipoamide S-acetyltransferase 0.99 0.74 1.03 P98461 Dihydrolipoamide S-acetyltransferase 1.15 0.80 1.12 P49432 Pyruvate dehydrogenase E1 component, subunit β 0.97 2.13 1.25 P41565 Isocitrate dehydrogenase 3, subunit γ 1.14 0.90 1.43 Oxidative stress P07895 Superoxide dismutase [Cu-Zn] 1.22 1.41 1.12 Q92339 Glutathione S-transferase, ma 7 1.55 1.08 1.46 Q6P7Q4 Glyoxalase 1 1.33 1.02 1.22 Q6301 1-macroglobulin 1.76 1.20 1.51 P11232 Thioredoxin 1 1.19 0.81 1.27 Q63010 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q921P2 Actini, alpha 1 0.73 0.78 0.93 </th <th>P10759</th> <th>AMP deaminase 1</th> <th>1.91</th> <th>1.20</th> <th>1.38</th>	P10759	AMP deaminase 1	1.91	1.20	1.38
Q6AXV4 SAMM50 1.40 0.96 1.29 Q01205 Dihydrolipoanide S-succinytransferase 0.99 0.74 1.03 P08461 Dihydrolipoanide S-acetytransferase 1.15 0.80 1.12 P49432 Pyruvate dehydrogenase E1 component, subunit β 1.09 0.75 1.12 QU2X7 Timm21 0.97 2.13 1.25 P41565 Isocitrate dehydrogenase 3, subunit γ 1.14 0.90 1.43 Otidative stress Diffection of the dismutase [Cu-Zr] 1.22 1.41 1.12 Q0752 Superoxide dismutase [Cu-Zr] 1.22 1.41 1.12 Q0752 Superoxide dismutase [Cu-Zr] 1.22 1.41 1.12 Q0753 Glutathione S-transferase morg 1 1.33 1.02 1.22 Q69704 Glyoxalase 1 1.33 1.02 1.22 Q3524 Peroxinedoxin 6 1.25 0.84 1.27 Q3524 Peroxinedoxin 6 1.25 0.84 1.23	P38718	Mitochondrial pyruvate carrier 2	1.48	0.87	1.11
Q01205 Dihydrolipoamide S-succip/transferase 0.99 0.74 1.03 P08461 Dihydrolipoamide S-succip/transferase 1.15 0.80 1.12 P449432 Pyruvate dehydrogenase T component, subunit β 1.09 0.75 1.12 Q5U2X7 Timm21 0.97 2.13 1.25 P41565 Isocitrate dehydrogenase 3, subunit γ 1.14 0.90 1.43 Oxidative stress P07895 Superoxide dismutase [Cu-Zn] 1.22 1.44 1.12 Q02339 Glutathione S-transferase, mag 1 1.33 1.02 1.27 P08009 Glutathione S-transferase, ma 7 1.55 1.08 1.46 Q67704 Giyoxalase 1 1.33 1.02 1.21 P11232 Thioredoxin 1 1.19 0.81 1.27 Q3544 Peroxinedoxin 6 1.25 0.84 1.23 Q81D3 Nicotinamide nucleotide transhydrogenase 0.95 0.82 1.22 Q304F3 Troponyosin alpha 3 chain 0.34 1.59	Q6AXV4	SAMM50	1.40	0.96	1.29
P08461 Dihydrolipounide S-acetyltransferase 1.15 0.80 1.12 P49432 Pyrovate dehydrogenase EI component, subunit β 1.09 0.75 1.12 Q5U2X7 Timm21 0.97 2.13 1.25 P41565 Isocitrate dehydrogenase 3, subunit γ 1.14 0.90 1.43 Oxidative stress Oxidative stress P07895 Superoxide dismutase [Cu-Zn] 1.22 1.41 1.12 Q9Z339 Glutathione S-transferase omega 1 1.33 1.02 1.22 Q63041 1-macroglobulin 1.76 1.20 1.51 P11232 Thioredoxin 1 1.19 0.81 1.77 Q53244 Peroxinedoxin 6 1.25 0.84 1.23 Q5BIZ3 Nicotinamide mucleotide transhydrogenase 0.95 0.82 1.22 Q63610 Troponyosin alpha-3 chain 0.34 1.99 0.56 Q9Z1P2 Actinin, ulpha 1.07 0.78 0.93 P63259 Actinin, ulpha 1 0	Q01205	Dihydrolipoamide S-succinyltransferase	0.99	0.74	1.03
P49432 Pyruvate dehydrogenase EI component, subunit β 1.09 0.75 1.12 QSU2X7 Timm21 0.97 2.13 1.25 P41565 Isocitrate dehydrogenase 3, subunit γ 1.14 0.90 1.43 Oxidative stress P07895 Superoxide dismutase [Mn], mitochondrial 1.38 1.17 1.21 Q92339 Glutathione S-transferase, mu 7 1.55 1.08 1.46 Q6P7Q4 Glyoxalase 1 1.33 1.02 1.22 Q63041 1-macroglobulin 1.76 1.20 1.51 P11232 Thioredoxin 1 1.19 0.81 1.27 Q53243 Nicotinamide nucleotide transhydrogenase 0.95 0.82 1.22 Q6310 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q921P2 Actin, apha 1 0.75 0.78 0.93 Q63259 Actin, apha 1 1.43 1.18 1.10 P4466 Myosin regulatory light chain 2 1.81 1.10 1.30 <tr< td=""><td>P08461</td><td>Dihydrolipoamide S-acetyltransferase</td><td>1.15</td><td>0.80</td><td>1.12</td></tr<>	P08461	Dihydrolipoamide S-acetyltransferase	1.15	0.80	1.12
Q5U2X7 Timm21 0.97 2.13 1.25 P41565 Isocitrate dehydrogenase 3, subunit y 1.14 0.90 1.43 Oxidative stress P07895 Superoxide dismutase [Mn], mitochondrial 1.38 1.17 1.21 P07632 Superoxide dismutase [Cu-2n] 1.22 1.41 1.12 Q92339 Glututhione S-transferase omega 1 1.34 0.90 1.22 Q93041 1-macroglobulin 1.76 1.20 1.51 P11232 Thioredoxin 1 1.19 0.81 1.27 Q35244 Peroxiredoxin 6 1.25 0.84 1.23 Q5B1Z3 Nicotinamide nucleotide transhydrogenase 0.95 0.82 1.22 Q51010 Tropomyosin alpha-3 chain 0.75 0.78 0.93 P63259 Actini, alpha 1 0.75 0.78 0.93 P63259 Actini, alpha cardiac muscle 0.53 0.44 0.72 P68136 Actin, alpha cardiac muscle 0.53 0.44 0.72 <t< td=""><td>P49432</td><td>Pyruvate dehydrogenase E1 component, subunit β</td><td>1.09</td><td>0.75</td><td>1.12</td></t<>	P49432	Pyruvate dehydrogenase E1 component, subunit β	1.09	0.75	1.12
P41565 Isocitrate dehydrogenase 3, subunit y 1.14 0.90 1.43 Oxidative stress P07895 Superoxide dismutase [Cu-Zn] 1.22 1.41 1.12 Q07632 Superoxide dismutase [Cu-Zn] 1.22 1.41 1.12 Q07632 Superoxide dismutase [Cu-Zn] 1.22 1.41 1.12 Q0704 Gituathione S-transferase, nu 7 1.55 1.08 1.46 Q6P7Q4 Gityoxalase 1 1.33 1.02 1.22 Q63041 1-macroglobulin 1.76 1.20 1.51 P11232 Thioredoxin 1 1.19 0.81 1.27 O35244 Peroxiredoxin 6 1.25 0.84 1.23 Q63610 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q921P2 Actinin, alpha 1 0.75 0.78 0.93 P633259 Actin, alpha 2 chain 1.43 1.18 1.10 P04466 Myosin regulatory light chain 2 1.81 1.01 1.30 P13413<	Q5U2X7	Timm21	0.97	2.13	1.25
Oxidative stress P07895 Superoxide dismutase [Cu-Za] 1.38 1.17 1.21 Q92339 Glutathione S-transferase omega 1 1.34 0.90 1.27 Q98009 Glutathione S-transferase, nu 7 1.55 1.08 1.46 Q67Q4 Glyoxalase 1 1.33 1.02 1.22 Q63041 1-macroglobulin 1.76 1.20 1.51 P11232 Thioredoxin 1 1.19 0.81 1.27 Q35244 Peroxiredoxin 6 1.25 0.84 1.23 Q63101 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q92/1P2 Actinin, alpha 1 0.75 0.78 0.93 P63259 Actinin, alpha 1 0.75 0.78 0.93 P34133 Troponin C, fast skeletal type 0.68 0.68 0.99 Q304F3 Troponin C, fast skeletal type 1.47 0.93 1.28 P68035 Actini, alpha sketul muscle 0.86 0.73 0.96 Q4640	P41565	Isocitrate dehydrogenase 3, subunit γ	1.14	0.90	1.43
Oxidative stress P07895 Superoxide dismutase [Mn], mitochondrial 1.38 1.17 1.21 P07632 Superoxide dismutase [Cu-Zn] 1.22 1.41 1.12 Q97333 Gilutathione S-transferase omega 1 1.34 0.90 1.22 Q6704 Gilyoxalase 1 1.33 1.02 1.22 Q63041 1-macroglobulin 1.76 1.00 1.51 P11232 Thioredoxin 6 1.25 0.84 1.23 Q5B1Z3 Nicotinamide nucleotide transhydrogenase 0.95 0.82 1.22 Q63610 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q921P2 Actinin, alpha 1 0.75 0.78 0.93 P63259 Actin, agmma 1 1.43 1.18 1.10 P04466 Myosin regulatory light chain 2 1.81 1.10 1.30 P13413 Troponin C2, fast skeletal type 0.68 0.63 0.99 Q304F3 Troponin C2, fast skeletal type 0.86 0.73 0.99 <td></td> <td></td> <td></td> <td></td> <td></td>					
P07895 Superoxide dismutase [Mn], mitochondrial 1.38 1.17 1.21 P07632 Superoxide dismutase [Cu-Zn] 1.22 1.41 1.12 Q2Z339 Glutathione S-transferase omega I 1.34 0.90 1.27 P0809 Glutathione S-transferase, mu 7 1.55 1.08 1.46 Q6P7Q4 Glyoxalase I 1.33 1.02 1.22 Q63041 I-macroglobulin 1.76 1.20 1.51 P11232 Thioredoxin 6 1.25 0.84 1.23 Q5B1Z3 Nicotinamide nucleotide transhydrogenase 0.95 0.82 1.22 Q63610 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q921P2 Actini, gamma 1 1.43 1.18 1.10 P04466 Myosin regulatory light chain 2 1.81 1.10 1.30 P13413 Troponin C2, fast skeletal type 0.68 0.86 0.99 Q304F3 Troponin C2, fast skeletal type 0.86 0.73 0.96 P0835 Actin		Oxidative stress			
P07632 Superoxide dismutase [Cu-Zn] 1.22 1.41 1.12 Q97339 Glutathione S-transferase omega 1 1.34 0.90 1.27 P08009 Glutathione S-transferase omega 1 1.35 1.08 1.46 Q67Q4 Glyoxalase 1 1.35 1.02 1.22 Q63041 1-macroglobulin 1.76 1.20 1.51 P11232 Thioredoxin 6 1.25 0.84 1.23 Q5B173 Nicotinamide nucleotide transhydrogenase 0.95 0.82 1.22 Muscular functionality Q63610 Tropomyosin alpha 3 chain 0.34 1.59 0.56 Q9Z1P2 Actini, alpha 1 0.75 0.78 0.93 P63259 Actin, apha 1 1.43 1.18 1.10 1.30 P13413 Troponin C2, fast skeletal type 0.68 0.86 0.99 Q304F3 Troponin C2, fast skeletal muscle 0.36 0.72 0.96 P68035 Actin, alpha skeletal muscle 0.36 0.73 0.	P07895	Superoxide dismutase [Mn], mitochondrial	1.38	1.17	1.21
Q9Z339 Glutathione S-transferase omega 1 1.34 0.90 1.27 P08009 Ghutathione S-transferase, nu 7 1.55 1.08 1.46 Q67Q4 Glyoxalase 1 1.33 1.02 1.22 Q63041 1-macroglobulin 1.76 1.20 1.51 P11232 Thioredoxin 1 1.19 0.81 1.27 O35244 Peroxiredoxin 6 1.25 0.84 1.23 Q5BIZ3 Nicotinamide nucleotide transhydrogenase 0.95 0.82 1.22 Muscular functionality Q63610 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q9Z1P2 Actinin, alpha 1 0.75 0.78 0.93 P63259 Actin, gamma 1 1.43 1.18 1.10 P13413 Troponin C2, fast skeletal type 0.66 0.99 0.304F3 Troponin C2, fast skeletal type 1.47 0.93 1.28 P68035 Actin, alpha sketetal muscle 0.86 0.73 0.96 0.74 0.72	P07632	Superoxide dismutase [Cu-Zn]	1.22	1.41	1.12
P08009 Glutathione S-transferase, mu 7 1.55 1.08 1.46 Q677Q4 Glyoxalase 1 1.33 1.02 1.22 Q63041 1-macroglobulin 1.76 1.20 1.51 P11232 Thioredoxin 1 1.19 0.81 1.27 O35244 Peroxiredoxin 6 1.25 0.84 1.23 Q63610 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q971P2 Actinin, alpha 1 0.75 0.78 0.93 P63259 Actin, gamma 1 1.43 1.18 1.10 P04466 Myosin regulatory light chain 2 1.81 1.10 1.30 P13413 Troponin C2, fast skeletal type 0.68 0.86 0.99 Q304F3 Troponin C2, fast skeletal type 1.47 0.93 1.28 P68035 Actin, alpha skeletal muscle 0.86 0.73 0.96 P04692 Tropomyosin alpha-1 chain 0.91 1.55 0.99 D3ZSG3 Tropomodulin 4 0.87 0.75 <td>Q9Z339</td> <td>Glutathione S-transferase omega 1</td> <td>1.34</td> <td>0.90</td> <td>1.27</td>	Q9Z339	Glutathione S-transferase omega 1	1.34	0.90	1.27
Q6P7Q4 Glyoxalase 1 1.33 1.02 1.22 Q63041 1-macroglobulin 1.76 1.20 1.51 P11232 Thioredoxin 1 1.19 0.81 1.27 O35244 Peroxiredoxin 6 1.25 0.84 1.23 Q5BJZ3 Nicotinamide nucleotide transhydrogenase 0.95 0.82 1.22 Muscular functionality V Muscular functionality Q6310 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q9Z1P2 Actinin, aganma 1 1.43 1.18 1.10 P04466 Myosin regulatory light chain 2 1.81 1.10 1.30 P13413 Troponin C2, fast skeletal type 0.68 0.86 0.99 Q304F3 Troponin C2, fast skeletal muscle 0.86 0.73 0.96 P08136 Actin, alpha skeletal muscle 0.86 0.73 0.96 P08733 Myosin regulatory light chain 2, muscle isoform 0.96 1.44 1.11 P	P08009	Glutathione S-transferase, mu 7	1.55	1.08	1.46
Q63041 1-macroglobulin 1.76 1.20 1.51 P11232 Thioredoxin 1 1.19 0.81 1.27 O35244 Peroxiredoxin 6 1.25 0.84 1.23 Q5BJZ3 Nicotinamide nucleotide transhydrogenase 0.95 0.82 1.22 Muscular functionality Q63610 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q9Z1P2 Actinin, alpha 1 0.75 0.78 0.93 P63259 Actin, gamma 1 1.43 1.18 1.10 1.30 P13413 Troponin C2, fast skeletal type 0.68 0.86 0.99 0304F3 Troponin C2, fast skeletal type 1.47 0.93 1.28 P68035 Actin, alpha skeletal muscle 0.86 0.73 0.96 1.44 1.11 P04692 Tropomyosin alpha-1 chain 0.91 1.55 0.99 032SG3 Tropomyosin alpha-1 chain 0.91 1.38 A0A042K7B6 Dysferlin 0.62 0.70 0.71 D4AA	Q6P7Q4	Glyoxalase 1	1.33	1.02	1.22
P11232 Thioredoxin 1 1.19 0.81 1.27 035244 Peroxiredoxin 6 1.25 0.84 1.23 Q5BJZ3 Nicotinamide nucleotide transhydrogenase 0.95 0.82 1.22 Muscular functionality Q63610 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q9Z1P2 Actinin, alpha 1 0.75 0.78 0.93 P63259 Actin, gamma 1 1.43 1.18 1.10 P04466 Myosin regulatory light chain 2 1.81 1.10 1.30 P13413 Troponin 11, slow skeletal type 0.68 0.86 0.99 Q304F3 Troponin C2, fast skeletal type 1.47 0.93 1.28 P68035 Actin, alpha ardiac muscle 1 0.53 0.44 0.72 P68136 Actin, alpha-1 chain 0.91 1.55 0.99 D32SG3 Tropomodulin 4 0.87 0.78 0.78 Q9ER30 Kelch-like family member 40 1.64 0.90 1.38 <	Q63041	1-macroglobulin	1.76	1.20	1.51
O35244 Peroxiredoxin 6 1.25 0.84 1.23 Q5BJZ3 Nicotinamide nucleotide transhydrogenase 0.95 0.82 1.22 Muscular functionality Q63610 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q9Z1P2 Actinin, alpha 1 0.75 0.78 0.93 P63259 Actin, agamma 1 1.43 1.18 1.10 P04466 Myosin regulatory light chain 2 1.81 1.10 1.30 P13413 Troponin C2, fast skeletal type 0.68 0.86 0.99 Q304F3 Troponin C2, fast skeletal muscle 0.86 0.73 0.96 P68035 Actin, alpha scardiac muscle 1 0.53 0.44 0.72 P68136 Actin, alpha scletal muscle 0.86 0.73 0.96 P08733 Myosin regulatory light chain 2, muscle isoform 0.96 1.44 1.11 P04692 Tropomodulin 4 0.87 0.75 0.78 Q9ER30 Kelch-like family me	P11232	Thioredoxin 1	1.19	0.81	1.27
QSBJZ3 Nicotinamide nucleotide transhydrogenase 0.95 0.82 1.22 Muscular functionality	O35244	Peroxiredoxin 6	1.25	0.84	1.23
Muscular functionality Q63610 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q9Z1P2 Actinin, alpha 1 0.75 0.78 0.93 P63259 Actin, gamma 1 1.43 1.18 1.10 P04466 Myosin regulatory light chain 2 1.81 1.10 1.30 P13413 Troponin I1, slow skeletal type 0.68 0.86 0.99 Q304F3 Troponin C2, fast skeletal type 1.47 0.93 1.28 P68035 Actin, alpha cardiac muscle 1 0.53 0.44 0.72 P68136 Actin, alpha skeletal muscle 0.86 0.73 0.96 P08733 Myosin regulatory light chain 2, muscle isoform 0.96 1.44 1.11 P04692 Tropomoyosin alpha-1 chain 0.91 1.55 0.99 D3ZSG3 Tropomodulin 4 0.87 0.75 0.78 QPER30 Kelch-like family member 41 1.37 1.00 1.30 D4AA43 Kelch-like family member 40 1.64 0.90 1.38 <td>O5BJZ3</td> <td>Nicotinamide nucleotide transhydrogenase</td> <td>0.95</td> <td>0.82</td> <td>1.22</td>	O5BJZ3	Nicotinamide nucleotide transhydrogenase	0.95	0.82	1.22
Muscular functionality Q63610 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q921P2 Actinin, alpha 1 0.75 0.78 0.93 P63259 Actin, gamma 1 1.43 1.18 1.10 P04466 Myosin regulatory light chain 2 1.81 1.10 1.30 P13413 Troponin 11, slow skeletal type 0.68 0.86 0.99 Q304F3 Troponin C2, fast skeletal type 0.63 0.44 0.72 P68035 Actin, alpha cardiac muscle 1 0.53 0.44 0.72 P68136 Actin, alpha skeletal muscle 0.86 0.73 0.96 P08733 Myosin regulatory light chain 2, muscle isoform 0.96 1.44 1.11 P04692 Tropomyosin alpha-1 chain 0.91 1.55 0.99 D3ZSG3 Tropomodulin 4 0.87 0.75 0.78 Q9ER30 Kelch-like family member 41 1.37 1.00 1.30 D4A6M0 Mitsugumin 29 1.84 0.84 1.86					
Q63610 Tropomyosin alpha-3 chain 0.34 1.59 0.56 Q9Z1P2 Actinin, alpha 1 0.75 0.78 0.93 P63259 Actin, gamma 1 1.43 1.18 1.10 P04466 Myosin regulatory light chain 2 1.81 1.10 1.30 P13413 Troponin I1, slow skeletal type 0.68 0.86 0.99 Q304F3 Troponin C2, fast skeletal type 1.47 0.93 1.28 P68035 Actin, alpha acardiac muscle 1 0.53 0.44 0.72 P68136 Actin, alpha skeletal muscle 0.86 0.73 0.96 P08733 Myosin regulatory light chain 2, muscle isoform 0.91 1.55 0.99 D3ZSG3 Tropomodulin 4 0.87 0.75 0.78 Q9ER30 Kelch-like family member 41 1.37 1.00 1.30 D4AA43 Kelch-like family member 40 1.64 0.90 1.38 Q2PS20 Junctophilin-2 0.68 0.44 0.70 A0A069KMIB5 unc-45 m		Muscular functionality			
Q9Z1P2 Actinin, alpha 1 0.75 0.78 0.93 P63259 Actin, gamma 1 1.43 1.18 1.10 P04466 Myosin regulatory light chain 2 1.81 1.10 1.30 P13413 Troponin II, slow skeletal type 0.68 0.86 0.99 Q304F3 Troponin C2, fast skeletal type 1.47 0.93 1.28 P68035 Actin, alpha cardiac muscle 1 0.53 0.44 0.72 P68136 Actin, alpha skeletal muscle 0.86 0.73 0.96 P08733 Myosin regulatory light chain 2, muscle isoform 0.91 1.55 0.99 D3ZSG3 Tropomodulin 4 0.87 0.75 0.78 Q9ER30 Kelch-like family member 41 1.37 1.00 1.30 D4AA43 Kelch-like family member 40 1.64 0.90 1.38 Q2PS20 Junctophilin-2 0.68 0.44 0.70 A0A062K7B6 Dysferlin 0.75 0.65 0.84 D3ZU20 Protein Sht3bgr	Q63610	Tropomyosin alpha-3 chain	0.34	1.59	0.56
P63259 Actin, gamma 1 1.43 1.18 1.10 P04466 Myosin regulatory light chain 2 1.81 1.10 1.30 P13413 Troponin I1, slow skeletal type 0.68 0.86 0.99 Q304F3 Troponin C2, fast skeletal type 1.47 0.93 1.28 P68035 Actin, alpha cardiac muscle 1 0.53 0.44 0.72 P68136 Actin, alpha skeletal muscle 0.86 0.73 0.96 P08733 Myosin regulatory light chain 2, muscle isoform 0.96 1.44 1.11 P04692 Tropomyosin alpha-1 chain 0.91 1.55 0.99 D3ZSG3 Tropomodulin 4 0.87 0.75 0.78 Q9ER30 Kelch-like family member 41 1.37 1.00 1.30 D4AA43 Kelch-like family member 40 1.64 0.90 1.38 A0A0G2K7B6 Dysferlin 0.62 0.70 0.71 D4A6M0 Mitsugumin 29 1.84 0.84 1.86 Q2PS20 Junctophilin-2 <td>Q9Z1P2</td> <td>Actinin, alpha 1</td> <td>0.75</td> <td>0.78</td> <td>0.93</td>	Q9Z1P2	Actinin, alpha 1	0.75	0.78	0.93
P04466 Myosin regular ylight chain 2 1.81 1.10 1.30 P13413 Troponin I1, slow skeletal type 0.68 0.86 0.99 Q304F3 Troponin C2, fast skeletal type 1.47 0.93 1.28 P68035 Actin, alpha cardiac muscle 1 0.53 0.44 0.72 P68136 Actin, alpha skeletal muscle 0.86 0.73 0.96 P08733 Myosin regulatory light chain 2, muscle isoform 0.96 1.44 1.11 P04692 Tropomyosin alpha-1 chain 0.91 1.55 0.99 D3ZSG3 Tropomodulin 4 0.87 0.75 0.78 Q9ER30 Kelch-like family member 41 1.37 1.00 1.30 D4AA43 Kelch-like family member 40 1.64 0.90 1.38 A0A0G2K7B6 Dysferlin 0.62 0.70 0.71 D4A6M0 Mitsugumin 29 1.84 0.84 1.86 Q2FS20 Junctophilin-2 0.68 0.64 0.58 0.64 D3ZU20 <t< td=""><td>P63259</td><td>Actin, gamma 1</td><td>1.43</td><td>1.18</td><td>1.10</td></t<>	P63259	Actin, gamma 1	1.43	1.18	1.10
P13413 Troponin I1, slow skeletal type 0.68 0.86 0.99 Q304F3 Troponin C2, fast skeletal type 1.47 0.93 1.28 P68035 Actin, alpha cardiac muscle 1 0.53 0.44 0.72 P68136 Actin, alpha skeletal muscle 0.86 0.73 0.96 P08733 Myosin regulatory light chain 2, muscle isoform 0.96 1.44 1.11 P04692 Tropomyosin alpha-1 chain 0.91 1.55 0.99 D3ZSG3 Tropomodulin 4 0.87 0.75 0.78 Q9ER30 Kelch-like family member 41 1.37 1.00 1.30 D4AA43 Kelch-like family member 40 1.64 0.90 1.38 A0A0G2K7B6 Dysferlin 0.62 0.70 0.71 D4A6M0 Mitsugumin 29 1.84 0.84 1.86 Q2PS20 Junctophilin-2 0.68 0.44 0.70 A0A096MJB5 unc-45 myosin chaperone B 0.64 0.58 0.64 D3ZU20 Protein Smtnl1 0.75 0.65 0.87 D3ZVB7 Osteoglycin </td <td>P04466</td> <td>Myosin regulatory light chain 2</td> <td>1.81</td> <td>1.10</td> <td>1.30</td>	P04466	Myosin regulatory light chain 2	1.81	1.10	1.30
Q304F3 Troponin C2, fast skeletal type 1.47 0.93 1.28 P68035 Actin, alpha cardiac muscle 1 0.53 0.44 0.72 P68136 Actin, alpha skeletal muscle 0.86 0.73 0.96 P08733 Myosin regulatory light chain 2, muscle isoform 0.96 1.44 1.11 P04692 Tropomyosin alpha-1 chain 0.91 1.55 0.99 D3ZSG3 Tropomodulin 4 0.87 0.75 0.78 Q9ER30 Kelch-like family member 41 1.37 1.00 1.30 D4AA43 Kelch-like family member 40 1.64 0.90 1.38 A0A0G2K7B6 Dysferlin 0.62 0.70 0.71 D4A6M0 Mitsugumin 29 1.84 0.84 1.86 Q2PS20 Junctophilin-2 0.68 0.44 0.70 A0A096MJB5 unc-45 myosin chaperone B 0.64 0.58 0.64 D3ZWP4 Protein Smtnl1 0.75 0.65 0.84 D3ZVB7 Osteoglycin 0.86 <td>P13413</td> <td>Troponin I1, slow skeletal type</td> <td>0.68</td> <td>0.86</td> <td>0.99</td>	P13413	Troponin I1, slow skeletal type	0.68	0.86	0.99
P68035 Actin, alpha cardiac muscle 0.53 0.44 0.72 P68136 Actin, alpha skeletal muscle 0.86 0.73 0.96 P08733 Myosin regulatory light chain 2, muscle isoform 0.96 1.44 1.11 P04692 Tropomyosin alpha-1 chain 0.91 1.55 0.99 D3ZSG3 Tropomodulin 4 0.87 0.75 0.78 Q9ER30 Kelch-like family member 41 1.37 1.00 1.30 D4AA43 Kelch-like family member 40 1.64 0.90 1.38 A0A0G2K7B6 Dysferlin 0.62 0.70 0.71 D4A6M0 Mitsugumin 29 1.84 0.84 1.86 Q2PS20 Junctophilin-2 0.68 0.44 0.70 A0A069MJB5 unc-45 myosin chaperone B 0.64 0.58 0.64 D3ZU20 Protein Smtn11 0.75 0.65 0.87 D3ZVB7 Osteoglycin 0.86 0.66 1.08 Q3KRE8 Tubulin alpha-4A chain 0.94 <	Q304F3	Troponin C2, fast skeletal type	1.47	0.93	1.28
P68136Actin, alpha skeletal muscle0.860.730.96P08733Myosin regulatory light chain 2, muscle isoform0.961.441.11P04692Tropomyosin alpha-1 chain0.911.550.99D3ZSG3Tropomodulin 40.870.750.78Q9ER30Kelch-like family member 411.371.001.30D4AA43Kelch-like family member 401.640.901.38A0A0G2K7B6Dysferlin0.620.700.71D4A6M0Mitsugumin 291.840.841.86Q2PS20Junctophilin-20.680.440.70A0A096MJB5unc-45 myosin chaperone B0.640.580.64D3ZVP4Protein Smtnl10.750.650.84D3ZU20Protein Sh3bgr0.960.650.87D3ZVB7Osteoglycin0.860.661.08Q3KRE8Tubulin beta-2B chain1.150.660.75Q5XIF6Tubulin alpha-4A chain0.940.701.15Q9EPC6Profilin-20.940.760.98P62161Calmodulin1.161.560.87	P68035	Actin, alpha cardiac muscle 1	0.53	0.44	0.72
P08733Myosin regulatorMyosin regulatorQuestion </td <td>P68136</td> <td>Actin, alpha skeletal muscle</td> <td>0.86</td> <td>0.73</td> <td>0.96</td>	P68136	Actin, alpha skeletal muscle	0.86	0.73	0.96
P04692 Tropomyosin alpha-1 chain 0.91 1.55 0.99 D3ZSG3 Tropomodulin 4 0.87 0.75 0.78 Q9ER30 Kelch-like family member 41 1.37 1.00 1.30 D4AA43 Kelch-like family member 40 1.64 0.90 1.38 A0A0G2K7B6 Dysferlin 0.62 0.70 0.71 D4A6M0 Mitsugumin 29 1.84 0.84 1.86 Q2PS20 Junctophilin-2 0.68 0.44 0.70 A0A096MJB5 unc-45 myosin chaperone B 0.64 0.58 0.64 D3ZWP4 Protein Smtnl1 0.75 0.65 0.84 D3ZVB7 Osteoglycin 0.86 0.66 1.08 Q3KRE8 Tubulin beta-2B chain 1.15 0.66 0.75 Q5XIF6 Tubulin alpha-4A chain 0.94 0.76 0.98 Q9EPC6 Profilin-2 0.94 0.76 0.98 P62161 Calmodulin 1.16 1.56 0.87	P08733	Myosin regulatory light chain 2, muscle isoform	0.96	1.44	1.11
D3ZSG3Tropomodulin 40.870.750.78Q9ER30Kelch-like family member 411.371.001.30D4AA43Kelch-like family member 401.640.901.38A0A0G2K7B6Dysferlin0.620.700.71D4A6M0Mitsugumin 291.840.841.86Q2PS20Junctophilin-20.680.440.70A0A096MJB5unc-45 myosin chaperone B0.640.580.64D3ZWP4Protein Smtnl10.750.650.84D3ZVB7Osteoglycin0.860.661.08Q3KRE8Tubulin beta-2B chain1.150.660.75Q5ZVIF6Profilin-20.940.701.15Q9EPC6Profilin-20.940.760.98P62161Calmodulin1.161.560.87	P04692	Tropomyosin alpha-1 chain	0.91	1.55	0.99
Q9ER30Kelch-like family member 411.371.001.30D4AA43Kelch-like family member 401.640.901.38A0A0G2K7B6Dysferlin0.620.700.71D4A6M0Mitsugumin 291.840.841.86Q2PS20Junctophilin-20.680.440.70A0A096MJB5unc-45 myosin chaperone B0.640.580.64D3ZWP4Protein Smtnl10.750.650.84D3ZVB7Osteoglycin0.860.6661.08Q3KRE8Tubulin beta-2B chain1.150.6660.75Q5XIF6Tubulin alpha-4A chain0.940.701.15Q9EPC6Profilin-20.940.760.98P62161Calmodulin1.161.560.87	D3ZSG3	Tropomodulin 4	0.87	0.75	0.78
D4AA43Kelch-like family member 401.640.901.38A0A0G2K7B6Dysferlin0.620.700.71D4A6M0Mitsugumin 291.840.841.86Q2PS20Junctophilin-20.680.440.70A0A096MJB5unc-45 myosin chaperone B0.640.580.64D3ZWP4Protein Smtnl10.750.650.84D3ZU20Protein Sh3bgr0.960.650.87D3ZVB7Osteoglycin0.860.661.08Q3KRE8Tubulin beta-2B chain1.150.660.75Q5XIF6Tubulin alpha-4A chain0.940.701.15Q9EPC6Profilin-20.940.760.98P62161Calmodulin1.161.560.87	Q9ER30	Kelch-like family member 41	1.37	1.00	1.30
A0A0G2K7B6Dysferlin0.620.700.71D4A6M0Mitsugumin 291.840.841.86Q2PS20Junctophilin-20.680.440.70A0A096MJB5unc-45 myosin chaperone B0.640.580.64D3ZWP4Protein Smtnl10.750.650.84D3ZU20Protein Sh3bgr0.960.6550.87D3ZVB7Osteoglycin0.860.661.08Q3KRE8Tubulin beta-2B chain1.150.660.75Q5XIF6Tubulin alpha-4A chain0.940.701.15Q9EPC6Profilin-20.940.760.98P62161Calmodulin1.161.560.87	D4AA43	Kelch-like family member 40	1.64	0.90	1.38
D4A6M0Mitsugumin 291.840.841.86Q2PS20Junctophilin-20.680.440.70A0A096MJB5unc-45 myosin chaperone B0.640.580.64D3ZWP4Protein Smtnl10.750.650.84D3ZU20Protein Sh3bgr0.960.650.87D3ZVB7Osteoglycin0.860.6661.08Q3KRE8Tubulin beta-2B chain1.150.660.75Q5XIF6Tubulin alpha-4A chain0.940.701.15Q9EPC6Profilin-20.940.760.98P62161Calmodulin1.161.560.87	A0A0G2K7B6	Dysferlin	0.62	0.70	0.71
Q2PS20Junctophilin-20.680.440.70A0A096MJB5unc-45 myosin chaperone B0.640.580.64D3ZWP4Protein Smtnl10.750.650.84D3ZU20Protein Sh3bgr0.960.650.87D3ZVB7Osteoglycin0.860.6661.08Q3KRE8Tubulin beta-2B chain1.150.660.75Q5XIF6Tubulin alpha-4A chain0.940.701.15Q9EPC6Profilin-20.940.760.98P62161Calmodulin1.161.560.87	D4A6M0	Mitsugumin 29	1.84	0.84	1.86
A0A096MJB5unc-45 myosin chaperone B0.640.580.64D3ZWP4Protein Smtnl10.750.650.84D3ZU20Protein Sh3bgr0.960.650.87D3ZVB7Osteoglycin0.860.661.08Q3KRE8Tubulin beta-2B chain1.150.660.75Q5XIF6Tubulin alpha-4A chain0.940.701.15Q9EPC6Profilin-20.940.760.98P62161Calmodulin1.161.560.87	Q2PS20	Junctophilin-2	0.68	0.44	0.70
D3ZWP4 Protein Smtnl1 0.75 0.65 0.84 D3ZU20 Protein Sh3bgr 0.96 0.65 0.87 D3ZVB7 Osteoglycin 0.86 0.66 1.08 Q3KRE8 Tubulin beta-2B chain 1.15 0.66 0.75 Q5XIF6 Tubulin alpha-4A chain 0.94 0.70 1.15 Q9EPC6 Profilin-2 0.94 0.76 0.98 P62161 Calmodulin 1.16 1.56 0.87	A0A096MJB5	unc-45 myosin chaperone B	0.64	0.58	0.64
D3ZU20 Protein Sh3bgr 0.96 0.65 0.87 D3ZVB7 Osteoglycin 0.86 0.66 1.08 Q3KRE8 Tubulin beta-2B chain 1.15 0.666 0.75 Q5XIF6 Tubulin alpha-4A chain 0.94 0.70 1.15 Q9EPC6 Profilin-2 0.94 0.76 0.98 P62161 Calmodulin 1.16 1.56 0.87	D3ZWP4	Protein Smtnl1	0.75	0.65	0.84
D3ZVB7 Osteoglycin 0.86 0.66 1.08 Q3KRE8 Tubulin beta-2B chain 1.15 0.66 0.75 Q5XIF6 Tubulin alpha-4A chain 0.94 0.70 1.15 Q9EPC6 Profilin-2 0.94 0.76 0.98 P62161 Calmodulin 1.16 1.56 0.87	D3ZU20	Protein Sh3bgr	0.96	0.65	0.87
Q3KRE8 Tubulin beta-2B chain 1.15 0.66 0.75 Q5XIF6 Tubulin alpha-4A chain 0.94 0.70 1.15 Q9EPC6 Profilin-2 0.94 0.76 0.98 P62161 Calmodulin 1.16 1.56 0.87	D3ZVB7	Osteoglycin	0.86	0.66	1.08
Q5XIF6 Tubulin alpha-4A chain 0.94 0.70 1.15 Q9EPC6 Profilin-2 0.94 0.76 0.98 P62161 Calmodulin 1.16 1.56 0.87	O3KRE8	Tubulin beta-2B chain	1.15	0.66	0.75
Q9EPC6 Profilin-2 0.94 0.76 0.98 P62161 Calmodulin 1.16 1.56 0.87	O5XIF6	Tubulin alpha-4A chain	0.94	0.70	1.15
P62161 Calmodulin 1.16 1.56 0.87	O9EPC6	Profilin-2	0.94	0.76	0.98
	P62161	Calmodulin	1.16	1.56	0.87
G3V8S6 Histidine-rich calcium binding protein, isoform CRA b 0.60 1.71 0.70	G3V8S6	Histidine-rich calcium binding protein, isoform CRA b	0.60	1.71	0.70

A0A0G2KAQ5	Myozenin 2, isoform CRA_a	1.17	1.07	1.45
	Protein turnover			
P55063	Heat shock protein 1-like	2.04	1.08	1.55
O08839	Bridging integrator 1	0.55	1.41	0.74
F1LYF3	Protein Rps1911	1.84	1.33	1.43
P24268	Cathepsin D	1.53	0.84	1.52
Q5U2S7	Proteasome 26S subunit, non-ATPase, 3	1.11	0.75	1.19
	Others			
P04276	Vitamin D-binding protein	1.40	1.27	1.62
Q497B0	Omega-amidase NIT2	1.56	1.15	1.23
P06907	Myelin protein P0	1.79	0.50	0.55
P61983	14-3-3 protein gamma	1.56	1.05	1.36
P40329	ArgininetRNA ligase, cytoplasmic	2.03	0.80	1.19
Q9R1T1	Barrier-to-autointegration factor	2.49	1.28	1.39
P50399	Rab GDP dissociation inhibitor beta	0.64	0.46	0.73
G3V848	ACTH receptor	0.76	0.49	0.45
P31977	Ezrin	0.97	0.62	0.99
Q62920	PDZ and LIM domain protein 5	0.80	0.65	0.82
B0BN46	Glyoxylate reductase/hydroxypyruvate reductase	0.90	0.69	0.97
Q01129	Decorin	0.84	0.76	1.00
M0R629	Adenylosuccinate synthetase isozyme 1	0.92	0.76	0.94
M0RCL5	Histone H2A	0.87	0.77	0.89
P06214	Aminolevulinate dehydratase	1.38	0.94	1.64
P35281	Ras-related protein Rab-10	0.63	1.17	0.57
P97532	3-Mercaptopyruvate sulfurtransferase	1.47	0.87	1.50

Proteins were quantified by a differential proteomic approach using MALDI TOF/TOF mass-spectrometry. Validation of the quantitative analysis was conducted with a noise model using the R bioconductor package Isobar based on technical replicates of the different conditions. Significantly different proteins abundances are grey-backgrounded (p<0.05).

	Ctrl	CIT	Ctrlex	CITex
Free Carnitine (µmol.l ⁻¹)	77.6±3.4	75.8±3.2	77.4±3.2	81.7±3.1
$\frac{C2}{(\mu mol.l^{-1})}$	36.7±5.9	41.2±5.5	35.4±5.5	35.4±5.3
$\begin{array}{c} \text{C3} \\ \text{(nmol.1-1)} \end{array}$	951±76	846±71	550±71	925±68
C4 (nmol.1 ⁻¹)	945±126	1139±119	1089±119	1265±113
C5(nmol.l ⁻¹)	239±16	260±15	213±15	249±14
C6 (nmol.l ⁻¹)	104±22	105±21	98±21	98±20
C8 (nmol.l ⁻¹)	58±7 ^a	45±7 ^{ab}	37±7 ^b	51±6 ^a
C10 (nmol.l ⁻¹)	33±4	26±4	28 ± 4^{a}	31±4
C12 (nmol.l ⁻¹)	31±5	28±4	30±4	28±4
C14 (nmol.l ⁻¹)	32±7	38±6	33±6	35±6
C16 (nmol.l ⁻¹)	84±16	103±15	86±15	86±14
C18 (nmol.l ⁻¹)	25±4	26±4	26±4	24±4

Table 2. Muscle acylcarnitine profiles in control vs CIT-supplemented rats that were exercise-trained (5d/w during 4 weeks) vs not.

Means \pm SEM. Significant differences are grey-backgrounded. Results with different superscript letters are significantly different (p<0.05) (two-way Anova + Bonferroni test).



Fig.1. Immunofluorescence detection of the plantaris muscle cross-section (A) with four layers: myosin heavy chain (MyHC) 1 (blue, B), laminin (green, C), puromycine (red, D) and MyHC2b (purple, E).



Fig.2. Oxygen consumption plotted against proton-motive force in control vs CIT-supplemented rats that were exercise-trained (5d/w during 4 weeks) vs not. Means ± SEM.

Résumé et publication N°2

Modulation de la synthèse protéique musculaire par les acides aminés : Quelles conséquences pour le sécrétome ? – Etude *in vitro* préliminaire

A. Goron, C. Breuillard, V. Cunin, S. Bourgoin-Voillard,

M. Seve, C. Moinard

Soumis pour publication

Introduction :

Le muscle est maintenant reconnu comme un organe endocrine capable de sécréter des myokines mais la régulation de ce processus est peu décrite (excepté pour l'exercice). En particulier, les effets des acides aminés (activateurs de la synthèse protéique musculaire) sur le sécrétome musculaire ont été très peu étudiés.

But de l'étude :

Le but de cette étude a été de déterminer *in vitro* les effets d'un état reflétant une « hyperaminoacidémie » ou les effets d'acides aminés spécifiques (citrulline ou leucine) sur la synthèse protéique et sur la modulation du sécrétome musculaire (par une approche protéomique) dans des myotubes. Nous pourrons ainsi mieux caractériser l'action de la citrulline sur cet aspect du métabolisme protéique musculaire.

<u>Méthodes</u> :

Des myotubes (dérivés d'une culture primaire de myoblastes) ont été mis dans un milieu dépourvu en sérum et en acides aminés pendant 14h ± citrulline ou leucine (5mM) ou dans un milieu riche en acides aminés et dépourvu de sérum (i.e. *No AA, CIT, LEU, AAs*). Après ces 14 d'incubation, (i) le milieu a été récupéré afin de déterminer l'expression de certaines protéines sécrétées *via* une approche protéomique différentielle, (ii) et les cellules ont été lysées afin de mesurer la synthèse protéique par la méthode SUNSET.

<u> Résultats</u> :

Les résultats démontrent que l'hyperaminoacidémie ou l'addition spécifique de citrulline ou de leucine dans le milieu de culture stimulent tous la synthèse protéique musculaire (respectivement +54%, +36%, +31 *vs. No AA, p<0,05*). Le sécrétome a également été modulé par ces différentes conditions mais de façons différentes. L'hyperaminoacidémie a permis d'augmenter principalement l'expression de protéines impliquées dans le développement cellulaire et l'angiogenèse (e.g. Sema3c, Sema3d, *follistatin-related protein 1, angiopoietin*-

like 2), et de diminuer l'expression de protéines impliquées dans l'homéostasie calcique (e.g. FKBP1A, metastasine, phosphohistidine phosphatase 1). L'addition de leucine a quant à elle principalement diminuée l'expression de protéines relatives au cytosquelette (e.g. cofilin-2 and calponin-3). Enfin, la citrulline semble moduler des protéines principalement impliquées dans l'homéostasie cardiovasculaire (e.g. calumenine, cystatine C).

Conclusion :

Malgré un effet commun de l'hyperaminoacidémie ou de l'ajout d'acides aminés spécifiques, tels que la citrulline ou la leucine, sur la synthèse protéique dans les cellules musculaires, l'action de ces différents effecteurs est très différente sur le sécrétome musculaire. Cette étude a permis de démontrer que le sécrétome est modulé de manière nutrimentspécifique. Ainsi, ce travail démontre la complexité de la régulation de l'homéostasie protéique par les acides aminés.

Modulation of muscle protein synthesis by amino acids:

What consequences for the secretome? - A preliminary in vitro study

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Conflict of interest: C. Moinard and C. Breuillard are shareholders in the Citrage company. Other authors have no conflicts of interest.

Funding: A. Goron was supported by a grant from *Agir pour les maladies chroniques.* C. Breuillard and this work were supported by the UGA Foundation ("Chaire Nutrition des Séniors") thanks to the sponsorship of Findus.

Contributions: Arthur Goron, Charlotte Breuillard contributed to all parts of this work. Valérie Cunin contributed to the experimental proteomics part of this work. Sandrine Bourgoin-Voillard and Michel Sève contributed to the analytic proteomics part of this work. Christophe Moinard contributed to the lead of this work.

Abstract

Muscle is an endocrine organ able to secrete myokines, but the regulatory processes (except exercise) are still largely unknown. In particular, how the muscle secretome is modulated by amino acids (activators of muscle protein synthesis) is unclear. The purpose of this study was to investigate the effect of hyperaminoacidemia or of adding specific amino acids (citrulline or leucine) on protein synthesis, using the SUnSET method, and the modulation of the muscle secretome using a proteomic approach in myotubes. The results demonstrate that hyperaminoacidemia, leucine or citrulline addition all stimulate muscle protein synthesis, respectively by 54%, 36% and 31% compared with a control without amino acid (p < 0.05). The secretome is also modulated but in different ways. Hyperaminoacidemia mostly upregulated proteins involved in cell development and angiogenesis, but downregulated proteins involved in calcium homeostasis. The modulation of the secretome by leucine addition is different, with mostly downregulated proteins related to the cytoskeleton. Finally, the proteins modulated by citrulline are mostly involved in cardiovascular homeostasis. In conclusion, besides the positive effect of hyperaminoacidemia or specific amino acid addition on protein synthesis in muscle cells, the secretome is modulated in different ways, underlining the complexity of the regulation of protein homeostasis by nutrients.

Keywords: Hyperaminoacidemia, Citrulline, Leucine, Proteomics.

Introduction

Muscle is recognized as the largest store of proteins at whole body level. The maintenance of muscle protein homeostasis requires tightly controlled protein intake, proteolysis and amino acid oxidation (1–3). Importantly, in the fed state, it is recognized that postprandial hyperaminoacidemia is a major factor in activating muscle protein synthesis (MPS) (4, 5). However, the role of muscle in nitrogen homeostasis is not limited to its capacity to store proteins. Over the last decade, a new view has emerged on muscle functions, in particular the capacity of muscle to secrete proteins, especially cytokines (myokines) (6). These secreted proteins act on the muscle itself, but also at the systemic level. For example, it has been proved that the muscular secretome is able to modulate adipose tissue or liver metabolism (for reviews, see Pedersen et al. (6, 7)). Muscle contraction is recognized to activate MPS and to be the main signal for the release of myokines. The fed state is also known to be a major regulator of MPS. It is therefore reasonable to expect nutritional regulation of MPS to affect the muscle secretome, thus enabling nutrients to act indirectly outside the muscle. The nutrient effect has not yet been explored. The purpose of this study was to investigate the in vitro effect of hyperaminoacidemia on protein synthesis, and the modulation of the muscle secretome in myotubes from a primary culture of mouse myoblasts. Secondly, we tested the specific effect of leucine (LEU), known to be the major inducer of MPS in the fed state (8,9) and known to activate the MPS in myotubes (10), and citrulline (CIT), which has emerged in the last decade as an important modulator of MPS (11-13).

Materials and methods

Cell culture and preparation of secreted protein samples

Primary cultures were derived from gastrocnemius and tibialis anterior muscles of 4-week-old male mice as previously described (14). Cells were plated at low density (100 cells/cm2) on 0.02% gelatincoated dishes (cold water fish skin; Sigma, Saint-Quentin-Fallavier, France) and grown in complete medium composed of DMEM-Ham's F12 (Gibco®, Invitrogen, Saint-Aubin, France), 2% Ultroser G (Biosepra, Pall Corporation, Saint-Germain-en-Laye, France), 20% fetal calf serum (Sigma-Aldrich), and antibiotic-antimycotic solution (10000 U/mL penicillin G sodium, 10000 µg/mL streptomycin sulfate, and 25 µg/mL amphotericin B, Gibco, Invitrogen, Saint-Aubin, France). To differentiate muscle cells into myotubes, myoblasts were plated on Matrigel-coated dishes (Dutscher, Brumath, France) in complete medium, and after 12 h adhesion switched to DMEM-Ham's F12 + 2% horse serum (Sigma-Aldrich) for 3 days. Myotubes were then incubated in the same medium without serum or in the same medium without serum or amino acid \pm CIT (5 mM) (Citrage, Créteil, France) or LEU (5mM) (Merck Millipore, Darmstadt, Germany) for 14 h (respectively AAs, No AA, CIT and LEU groups (see Supplementary Data for the study design). These culture conditions are adapted from Le Plénier et al. (13). The different 14 h-conditioned media from 120,000,000 (12 x 10, 000, 000 in 150 cm² flasks) cells were then collected and cooled on ice. Floating cells and cell debris were removed by centrifugation (200 × g, 10 min, 4 °C) followed by sterile filtration (pore size 0.2 μ m). Proteins were then concentrated and desalted by ultrafiltration using a 5 kDa molecular mass cutoff spin column (Amicon Ultra-15, Millipore) according to the manufacturer's instructions. The total protein amount was determined using a standard Bradford protein assay (Thermo Scientific).

Protein synthesis

Similarly myotube protein synthesis was determined by the SUNSET method (15). Briefly, after the different treatments, puromycin (1 μ M) (Merck) was added to the medium for 30 min. Cells were then collected and lyzed in an RIPA buffer, and centrifuged at 3,000 × g for 10 min at 4 °C. The pellet was collected. Sample protein concentrations were determined with a standard Bradford protein assay (Thermo Scientific), and equivalent amounts of protein (50 μ g) from each sample were dissolved in Laemmli buffer and subjected to electrophoretic separation on 10% SDS-PAGE acrylamide gels. Electrophoresis was terminated before the dye front reach the bottom of the gel. Proteins were transferred to a nitrocellulose membrane in transfer buffer. Membranes were stained with Ponceau red to verify equal loading in all lanes, and then blocked with 5% powdered milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 1 h followed by overnight incubation at 4 °C with mouse IgG2a monoclonal anti-puromycin antibody (clone 12D10, 1:5000) dissolved in TBST

containing 1% BSA. Membranes were washed for 30 min in TBST and then incubated for 1 h at room temperature in 5% milk-TBST containing horseradish peroxidase conjugated anti-mouse IgG Fc 2a antibody (1:50,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). After 30 min of washing in TBST, the blots were developed on film using ECL plus reagent (Amersham, Piscataway, NJ, USA). Densitometry measurements were performed by determining the density of each whole lane (incorporating the entire molecular weight range of puromycin-labeled peptides) using the public domain ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/nih-image/).

Proteome analysis of secretome

Digestion and peptide isobaric labeling with iTRAQ reagents

Twice 100 µg of each protein sample (*No AA, AAs, CIT,* and *LEU* groups) was reduced with 2.5 mM TCEP (60 °C for 1 h), and cysteine residues were blocked with 10 mM MMTS at room temperature for 20 min. Trypsin/lysine C mix solution (Mass Spec Grade, Promega, Charbonnières-les-Bains, France) was added in a 1:10 ratio (w/w) and incubated overnight at 37 °C. Each sample digest was then labeled with iTRAQ 8-plex reagents according to the manufacturer's instructions (iTRAQ Reagents 8 plex Applications kit; Sciex, les Ulis, France) in order to perform analysis in duplicate.

OFFGEL Isoelectrofocusing

To perform peptide fractionation according to pl, a 3100 OFFGEL Fractionator and OFFGEL Kit 3-10 (both from Agilent Technology) were used following the user protocol. OFFGEL had already been validated as an efficient tool for fractionating peptides from complex proteomes (such as the secretome) and was compatible with iTRAQ labeling. The device was set up to separate peptides in 24 liquid fractions using a 24 cm IPG gel strip with a linear pH gradient ranging from 3 to 10. 800 μ g of iTRAQ-labeled peptide mix was dried by vacuum centrifugation and suspended with a focusing buffer (1.2% ampholytes 3-10 in water (v/v)). This sample solution was loaded in each of the 24 wells placed above the IPG strip, and peptides were focused with a constant current of 50 μ A until the total voltage reached 50 kVh. After focusing, the sample was recovered from each well, transferred to individual tubes, and dried in a vacuum concentrator before LC-MALDI-MS/MS analysis.

Reversed-phase liquid chromatography (RP-LC)

Peptide separation was performed on an Ultimate-3000 chromatography system (Dionex /ThermoScientific/LC Packings, Amsterdam, The Netherlands) equipped with a Probot MALDI spotting device (Dionex/Thermo Scientific/LCPackings, Amsterdam, The Netherlands). Each dried

OFFGEL fraction was dissolved in 15 μ L of 0.1% TFA (v/v), and 5 μ L was injected and captured on a trapping column (PepMap, C18, 300 μ m i.d. × 5 mm, LC Packings). Peptides were eluted onto an analytical column (PepMap, C18, 75 μ m i.d. × 15 cm, LCPackings) using an automated binary gradient (300 nL/min) from 100% buffer A (2% (v/v) acetonitrile (ACN); 0.05% (v/v) TFA) to 55% buffer B (80% (v/v) ACN; 0.04% (v/v) TFA) over 45 min. For MALDI-MS/MS analysis, column effluent was mixed in a 1:3 ratio with MALDI matrix (2 mg/mL α -cyano-4-hydroxy-cinnamic acid in 70% ACN, 0.1% TFA) through a 25 nL mixing tee, and spotted on a MALDI sample plate (Opti-TOF LC/MALDI Insert 123×81 mm plate, AB Sciex) at a frequency of one spot per 15 s.

MALDI MS/MS analysis

MS and MS/MS analyses were performed using a 4800 MALDI-TOF/TOF analyzer (Sciex, Les Ulis, France) controlled by 4000 Series Explorer software v. 3.5. After screening all LC-MALDI sample plate positions in MS-positive reflector mode using 3000 laser shots, the fragmentation of automatically selected precursors was performed at collision energy of 1 kV using air as collision gas (pressure $\sim 2 \times 10^{-6}$ Torr). MS spectra were acquired between *m/z* 750 and 3500. The 30 most intense ion signals per spot position were selected as precursors for MS/MS acquisition. MS and MS/MS spectra were externally calibrated using the Peptide Calibration Standard II (Bruker Daltonics, Bremen, Germany) with a peptide mass tolerance at 50 ppm.

Protein identification and quantification

The proteins were identified and quantified with ProteinPilot software v. 4.5 (AB Sciex, les Ulis, France) using the Rat Uniprot Refseq database downloaded in January 2016. Proteins with at least 1 peptide above 95% confidence level were retained and the list of identified proteins was limited to a 1 % False Discovery Rate (FDR). For quantification, bias and background correction was applied. Only quantified proteins with at least one peptide at the 95 % peptide confidence level were considered. The statistical significance of protein dysregulation (p < 0.05) was determined by analyzing the quantitative data using the R package Isobar (16) with a Cauchy sequence.

Results

Protein synthesis (Fig.1)

Addition of a complete medium strongly activated protein synthesis (+54%, AAs vs. No AA, p < 0.05). Similar effects were observed by addition of LEU (+31%, LEU vs. No AA, p < 0.05) or CIT (+36%, CIT vs. No AA, p < 0.05). However, this effect was less pronounced with the LEU than with the AAs group (AAs vs. LEU, p < 0.05).

Muscle proteome analysis (Fig.2)

Proteomic analysis allowed the identification of 620 secreted proteins by applying an FDR of 1%. Among these proteins 615 were quantified with at least one peptide at the 95% peptide confidence level. 24, 16 and 9 proteins were significantly modulated by complete medium, and by LEU or CIT additions, respectively. With complete medium, 9 proteins were significantly downregulated and 14 upregulated (Fig. 2A). LEU downregulated 9 proteins and upregulated 8 (Fig. 2B). Finally, CIT significantly downregulated only 5 proteins and upregulated 4 proteins (Fig. 2C). Interestingly, some proteins shared a common regulation in the different treatments (two proteins in all three tested conditions, two proteins in the AAs and LEU groups, one protein in the AAs and CIT groups, and two proteins in the *LEU* and CIT groups, see Supplementary Data).

Discussion

Until recently, the regulation of the muscle secretome by nutrients has been largely underestimated, while in the last decade muscle has emerged as an endocrine organ with systemic effects (6). Interestingly, MPS is largely regulated by nutrients and in particular by amino acids in the fed state. As classically described, we observed that a complete medium, CIT or LEU directly stimulated muscle protein synthesis in similar ways. Our proteomic data showed that the regulation of the secretome could be more complex. Depending on the amino acid considered, some biological processes may be favored and others not, as the expression of some proteins increased and that of other proteins decreased. A second important finding was that CIT and LEU had similar enhancing effects on intracellular protein synthesis, but the secreted proteins modulated by the two amino acids were very different.

Looking closer at the effect of hyperaminoacidemia (which mimics a fed state), most upregulated proteins were involved in cell development (angiogenesis, growth, cell cohesion, etc.). This result is not surprising since it is well known that food intake and amino acids regulate growth and development of cells (17–23). By contrast, proteins downregulated by hyperaminoacidemia were mainly involved in calcium homeostasis. The scientific literature on the relationship between calcium homeostasis and hyperaminoacidemia is scant, and our finding cannot be readily explained. One possible clue might be the ability of amino acids to bind Ca2+ and form a salt (24, 25): it is known that after ingestion of amino acids in large amounts, part is excreted in urine, and this favors urinary Ca2+ loss. It has also been shown that the dose of amino acids administered is correlated with renal excretion of Ca²⁺ (26). These modulations may thus be seen as a protective adaptation to preserve calcium homeostasis. However, this hypothesis needs further research.

With LEU, downregulated proteins were mainly related to the cytoskeleton. Among these, cofilin-2 is known to interact with LIM protein. The interaction between these two proteins has direct implications in actin cytoskeleton dynamics in regulating F-actin depolymerization. A deregulation of this interaction by many different mechanisms is observed in a range of cardiac and skeletal myopathies, and could impair F-actin depolymerization, leading to sarcomere dysfunction (27). However, at this level, the true impact of downregulation by LEU cannot be appraised, and further studies are needed to explore its consequences. The proteins upregulated by LEU are very diverse, which and no hypothesis can be advanced concerning their significance.

Finally, CIT addition displays some wide-ranging results, and some proteins modulated by CIT are of special interest: CIT addition downregulated calumenin, which is involved in vascular homeostasis, especially in thrombosis via a stimulation of coagulation factor synthesis (28). CIT also downregulated

another protein involved in the vascular system, cystatin C, whose role is not well known but which is associated with an increased risk of cardiovascular events (29). These results are in line with the literature, it being well known that CIT has beneficial effects on the cardiovascular system (30–35), especially via its NO precursor role (33). This proteomic study therefore suggests that the effect of CIT on the cardiovascular system could be not only linked to this NO precursor role but be more complex.

Among the proteins upregulated in all our conditions, transcobalamin is the transporter of cobalamin (also known as vitamin B12), which is involved in cell growth (36). Vitamin B12 is present exclusively in animal-derived food (usually rich in proteins): thus an increase in amino acid availability could be a cell signal to adapt cobalamin transport, which may need to be improved in the post-prandial state. Lastly, it could suggest an improvement in growth mechanisms via a better transport of cobalamin into the cells.

The second protein upregulated in all our conditions was fetuin-A. Until now, this protein was known to be secreted by the liver. To our knowledge, this study shows for the first time that fetuin A can be released by muscle cells. The potential effect of the upregulation of this protein is still unclear, but a proteomic study of the cerebrospinal fluid of Alzheimer's disease patients has suggested involvement of fetuin A. In these patients, fetuin A concentrations were lower than in controls (37). Although the pathophysiological significance of this observation remains to be determined, this finding suggests that fetuin A could be linked to Alzheimer's disease and neurodegeneration. Concerning CIT addition, a study has demonstrated that CIT supplementation in old rats is able to decrease the amyloid precursor protein concentration, whose aggregation is well known to be associated with Alzheimer's disease (38). Thus the upregulation of fetuin A by CIT could be an explanation of this effect of CIT: this hypothesis remains to be explored. Concerning LEU addition, the literature does not let us advance any hypothesis on the action of this upregulation of fetuin A.

In conclusion, this study demonstrates that hyperaminoacidemia or specific amino acid (CIT or LEU) additions stimulate MPS. This result is in line with the literature (4, 8, 9, 11–13). These different cell treatments are also able to modulate the secretome in different ways. Thus hyperaminoacidemia mostly upregulates proteins involved in cell development, and downregulates proteins involved in calcium homeostasis. The modulation of the secretome by LEU addition is very different, mostly downregulating proteins related to the cytoskeleton. Finally, the modulation of the secretome by CIT addition is harder to interpret, but could be related to some systemic effects of CIT reported in the literature. This study yields preliminary findings suggesting that modulation of the muscle secretome by a mimetic fed state or specific amino acid addition could have systemic effects, and so argues for

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continued work on the muscle secretome and its relationships with other organs. This pioneering work also underlines the importance of muscle secretome regulation by nutrients, and offers an approach to further elucidating the mechanisms involved in some of the systemic effects of amino acids.

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Fig.1. Modulation of intracellular protein synthesis by hyperaminoacidemia (AAs group) or specific amino acid addition (*LEU* or *CIT* groups) compared with the amino acid-deprived condition (*No* AA group) in myotubes evaluated by puromycin-incorporated protein staining. Results are expressed in arbitrary units and presented as means \pm SEM. Values with different superscript letters are statistically different (p < 0.05; One-way Anova + Bonferroni test).

AAs/No AA



Α





С



Fig.2. Modulation of proteins by hyperaminoacidemia (AAs group) or specific amino acid addition (LEU or CIT groups) compared with acid-deprived condition (No AA group) evaluated using iTRAQ labeling and LC-MALDI-MS/MS analysis. All proteins represented are significantly modulated by at least 10% compared with No AA group (p < 0.05, Cauchy fit). A. Modulated proteins by hyperaminoacidemia (AAs group) compared with the condition without amino acids (No AA group); B. Modulation of proteins by LEU (LEU group) compared with the condition without amino acids (No AA group); C. Modulation of proteins by CIT (CIT group) compared with the condition without amino acids (No AA group). Supplementary data



Fig.2. Number of modulated proteins and common secreted proteins modulation between all conditions

Résumé et publication N°3

Stimulation de la synthèse protéique musculaire par la citrulline: une régulation bioénergétique?

A. Goron, F. Lamarche, S. Blanchet, P. Delangle, U. Schlattner, E. Fontaine, C. Moinard

Introduction :

La citrulline est bien connu pour stimuler la synthèse protéique musculaire *via* une activation de la voie de transduction mTORC1. Cependant, la modulation du métabolisme énergétique pour supporter cette augmentation de la synthèse protéique musculaire n'est pas connue. <u>But de l'étude</u> :

Le but de ce travail est d'explorer l'implication du métabolisme énergétique pour supporter l'augmentation de la synthèse protéique musculaire par la citrulline.

<u>Méthodes</u> :

Des myotubes dérivés d'une culture primaire de myoblastes de souris ont été utilisés et les effets de la citrulline sur la synthèse protéique ont été déterminés par la méthode SUnSET. En parallèle, le métabolisme énergétique a été exploré avec notamment des mesures du statut énergétique, de la respiration mitochondriale totale ou allouée à la synthèse protéique, etc.

<u>Résultats</u> :

Nos résultats ont confirmé l'effet stimulateur de la citrulline sur la synthèse protéique musculaire dans des conditions de stress (respectivement +22% et +11% en condition de privation en acides aminés et en sérum pendant 16h ou par ajout d'un découplant à faible dose). L'augmentation de la synthèse protéique musculaire par la citrulline n'était pas supportée par une amélioration du statut énergétique (diminué par nos conditions de culture) ni par une augmentation de la respiration mitochondriale. En revanche, la citrulline a permis de réorienter les flux énergétiques au profit de la synthèse protéique (mais aux dépens d'autres postes de dépense énergétique encore à déterminer) puisque la respiration allouée à la synthèse protéique (déterminée à l'aide de cycloheximide, un inhibiteur de la synthèse protéique) était augmentée avec cet acide aminé (respectivement +28% et +21% en condition de privation en acides aminés et en sérum pendant 16h ou par ajout d'un découplant à faible dose). Cet effet de la citrulline n'est pas associé à une amélioration de la fonction mitochondriale.

Egalement, la citrulline a permis de stimuler la β -oxydation de 34% en condition de privation en acides aminés et en sérum.

Conclusion :

La citrulline stimule la synthèse protéique musculaire en condition de stress (privation en acides aminés et en sérum ou addition d'un découplant à faible dose) et cette augmentation est supportée par une réorientation des flux énergétiques au profit de la synthèse protéique. Les mécanismes de cette réorientation énergétique restent toutefois inconnus.

Stimulation of muscle protein synthesis by citrulline:

A bioenergetics regulation?

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Conflict of interest: C. Moinard is shareholder of the Citrage Company. Others authors do not have any conflict of interest.

Funding: A. Goron was supported by a grant from Agir pour les maladies chroniques.

Acknowledgments: We thank Dr. M. Tokarska-Schlattner and Mr S. Attia for their contribution in the adenylate measurements and in the biochemical analysis. We thank Dr B. Morio for his contribution in the script of this paper.

Abstract

Introduction: Citrulline is known to stimulate muscle protein synthesis, but the modulation of energy metabolism to support this protein synthesis is not understood. The aim of this work was to explore the implication of energy metabolism in the capacity of citrulline to enhance muscle protein synthesis. Methods: Myotubes derived from primary culture of mouse myoblast were used to study the effect of citrulline addition on protein synthesis was by the SUnSET method. In parallel, energy metabolism was explored (energy status, mitochondrial respiration, respiration allocated to protein synthesis, etc). Results: Citrulline stimulated muscle protein synthesis under stress conditions, either serum/amino acid deficiency or energy stress (using mild uncoupling), by +22% and +11%, respectively. The increase of protein synthesis by citrulline was not due to enhanced energy status or mitochondrial respiration, but supported by an increased share of mitochondrial respiration allocated to generate ATP, amounting to +28% or +21% for protein synthesis under serum/amino acid deficiency or energy stress, respectively. The effect of citrulline on the mitochondrial metabolism was not associated with an improvement of mitochondrial function, but with a change in the metabolic pathway providing substrates for mitochondrial respiration, since β-oxidation was increased by 34%.. Conclusion: Citrulline increases muscle protein synthesis under stress conditions (amino acid/serum deficiency or energy stress) and this increase is supported by reallocation of mitochondrial fuel to the protein synthesis machinery. The underlying mechanisms remain to be established.

Introduction

Citrulline (CIT), a non proteinogenic amino acid, has emerged as a major regulator of muscle protein synthesis. In a pioneering work, Osowska et al. (1) demonstrated that CIT supplementation was associated with an increase of muscle protein synthesis in malnourished rats. Several years later, using an in vitro model, Le Plénier et al. (2) showed a direct positive effect of CIT on protein synthesis in muscle. Moreover, the same authors demonstrated in vitro in myotubes that CIT addition during amino acid/serum deficiency was able to stimulate the mTORC1 pathway, a key activator of protein synthesis.

Several works confirmed the positive effect of CIT on muscle protein synthesis, especially in the malnourished state. As protein synthesis has a very high energy requirement (3), an activation of protein synthesis by CIT should be supported by a regulation of the energy metabolism. Indeed, some indirect evidence suggests that CIT may also affect energy metabolism. CIT ingestion by healthy aged rats increased muscle mitochondrial density and Tfam expression (a mitochondrial transcription factor) (4). CIT supplementation increased energy expenditure in obese mice (5) and, as a malate salt, improved phosphocreatine regeneration after exercise in humans (6). Moreover, CIT has been shown to affect lipid metabolism (7–9).

As mentioned above, protein synthesis is one of the numerous highly energy-dependent biochemical pathways. In face of potentially high ATP consumption rates, cellular ATP content is relatively low and there is little ATP reserve., Thus, ATP turnover has to be extremely fast, and ATP generation and consumption have to be constantly and closely matched to avoid energy failure, Generally, ATP-consuming processes decrease when ATP supply becomes limiting. However, it has been shown that the different ATP-consuming pathways are not affected equally by a decrease in ATP availability. The ATP-consuming processes are, in fact, inhibited in a hierarchical order, with protein synthesis and RNA/DNA synthesis falling rapidly as energy supply becomes limiting, while Na+/K+ pumping and Ca2+ cycling being largely preserved.

While ATP production can be indirectly estimated by oxygen consumption, it must be kept in mind that the energy effectively released during ATP hydrolysis varies according to its Gibbs free energy which depends on the ATP/ADP ratio. When different ATP-consuming processes are in competition, both ATP turnover and ATP Gibbs free energy may affect ATP allocation. For example, when the ATP Gibbs free energy decreases, the pathways that require high

ATP/ADP ratio tend to be inhibited, thus favouring the availability of ATP for the pathways that can run at low ATP/ADP ratio.

Thus, the aim of this study was to decipher the mechanisms underlying the relationship between protein and energy metabolisms and its modulation by CIT. In this work, we report that protein synthesis decreases when ATP/ADP ratio decreases, but that CIT can re-allocate ATP to protein synthesis without affecting the ATP/ADP ratio.

Materials and methods

Primary cultures were derived from gastrocnemia and tibialis anterior muscles of 4-week-old male mice as previously described (10). Cells were plated at low density (100 cells/cm2) on 0.02% gelatin-coated dishes (cold water fish skin; Sigma, Saint-Quentin-Fallavier, France) and grown in complete medium composed of DMEM-Ham's F12 (Gibco[®], Invitrogen, Saint-Aubin, France), 2% Ultroser G (Biosepra, Pall corporation, Saint-Germain-en-Laye, France), 20% fetal calf serum (Sigma-Aldrich), and antibiotic-antimycotic solution (10000 U/ml penicillin G sodium, 10000 µg/ml streptomycin sulfate, and 25 µg/ml amphotericin B, Gibco, Invitrogen, Saint-Aubin, France). To differentiate muscle cells into myotubes, myoblasts were plated on Matrigel-coated dishes (Dutscher, Brumath, France) in complete medium, and after 6 h adhesion, switched to DMEM-Ham's F12 + 2% horse serum (Sigma-Aldrich) at day 2. At day 5, myotubes were then serum- and amino acid-deprived or not for 16 h (Eurobio, Courtaboeuf, France) containing 0.2% BSA (Sigma-Aldrich, Saint-Quentin-Fallavier, France).

Cells treatments

Myotubes were then incubated in different conditions:

- serum- and amino acid-deprived myotubes for 16h:
 - No addition (AA/serum⁻)
 - LEU addition (5mM) (AA/serum⁻ + LEU)
 - CIT addition (5mM) (AA/serum⁻ + CIT)
 - ARG addition (5mM) (AA/serum⁻ + ARG)
- Standard condition:
 - No addition (Ctrl+)

- LEU addition (5mM) (LEU)
- CIT addition (5mM) (CIT)
- ARG addition (5mM) (ARG)
- \circ DNP (10 μ M) addition (DNP)
- $\circ~$ LEU (5mM) and DNP (10 μ M) additions (DNP + LEU)
- \circ CIT (5mM) + DNP (10 μ M) additions (DNP + CIT)
- \circ ARG (5mM) + DNP (10 μ M) additions (DNP + ARG)
- Rapamycin (200nM) (only for measure of energy status and protein synthesis)

Myotubes were incubated with specific amino acid for 90 min whereas cells were incubated with DNP for 15 min (60 min after specific amino acid incubation). In our conditions, LEU was used as a positive control (LEU is known to be a strong activator of muscle protein synthesis) and ARG as a negative control (which is not known to stimulate muscle protein synthesis and allowing us to evaluate nitrogen load effect) (11).

Myotubes protein synthesis

Myotubes protein synthesis was determined by the SUnSET method (12). Briefly, after the different treatments, puromycin (1µM) was added to the medium for 30 min. Cells were then collected and lysed in a RIPA buffer and centrifuged at 3,000 g for 10 min, and the pellet was collected. Sample protein concentration was determined with a DC protein assay kit (Bio-Rad, Hercules, CA, USA), and equivalent amounts of protein (50µg) from each sample were dissolved in Laemmli buffer and subjected to electrophoretic separation on 10% SDS-PAGE acrylamide gels. Electrophoresis was terminated before the dye front reach the bottom of the gel. Proteins were transferred to a nitrocellulose membrane in transfer buffer. Membranes were stained with Ponceau Red to verify equal loading in all lanes then membranes were blocked with 5% powdered milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 1 h followed by an overnight incubation at 4°C with mouse IgG2a monoclonal anti-puromycin antibody (clone 12D10, 1:5000) dissolved in TBST containing 1% BSA. Membranes were washed for 30 min in TBST and then incubated for 1 h at room temperature in 5% milk-TBST containing horseradish peroxidase conjugated anti-mouse IgG Fc 2a antibody (1:50,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA).

After 30 min of washing in TBST, the blots were developed on film using ECL plus reagent (Amersham, Piscataway,NJ, USA). Densitometric measurements were performed by determining the density of each whole lane (incorporating the entire molecular weight range of puromycin-labeled peptides) using the public domain ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/nih-image/).

Energy status

Adenine nucleotides were extracted from the myotubes with ice-cold 0.6 N perchloric acid, centrifuged at 3,000 g for 10 min at 4°C, neutralized to pH 6–7 with 5 M K2CO3, and centrifuged again at 3,000 g for 10 min (4°C). [ATP] and [ADP] were then measured by HPLC as described in (13).

Myotubes mitochondrial respiration

Cellular respiration was measured using the XF-96 analyzer (Seahorse Bioscience). Mitochondrial bioenergetics assays was performed based on published protocols [25]. The XF assay medium (HCO free modified DMEM, Seahorse Bioscience) was supplemented with 2 mM L-glutamine and 1 mM pyruvate and with further additions relevant to the experiment. The pH was adjusted to 7.4 at 37°C. 30 000 myoblastes/well were grown to confluence and differentiated into myotubes for 5 days in Seahorse assay plates as described above.

Mitochondrial respiration test was then performed under basal condition followed by sequential additions of 1 μ M oligomycin A (in order to inhibit the ATP synthesis), and 1 μ M rotenone/antimycin A (in order to inhibit the respiratory chain). The following mitochondrial parameters were determined: basal respiration, basal mitochondrial respiration (basal cellular respiration minus non-mitochondrial respiration), ATP turnover-driven respiration (basal respiration minus oligomycin-inhibited respiration), resting respiration (oligomycin-inhibited respiration) and non-mitochondrial respiration (inhibited respiration by rotenone/antimycin A). The results were expressed in pmol O₂.min⁻¹.well⁻¹.

ATP demand

Oxygen consumption rate of intact myotubes was also measured, as described above, before and after the injection of cycloheximide (40μ M), actinomycin D (10μ M) or ouabain (380μ M), in order to estimate how much energy was allocated to protein synthesis, DNA and RNA synthesis, or Na+/K+ ATPase pumps activity, respectively. The percentage of mitochondrial respiration allocated to protein synthesis (determined by basal mitochondrial respiration divided by mitochondrial respiration with protein synthesis inhibition). The percentage of mitochondrial respiration allocated to others items of energy expenditure (DNA and RNA synthesis or Na+/K+ ATPase pumps activity) was determined from the same way.

Interaction of CIT and ARG with phosphate

³¹P NMR (Phosphorous Nuclear Magnetic Resonance) spectra of samples containing phosphate (2mM) and various amounts of either CIT or ARG (0 to 50mM) in HEPES buffer (100mM, pH 7.4) were registered on a Bruker Avance 400 spectrometer (³¹P frequency 162 MHz) equipped with a QNP probe, at 298 K. Deuterium oxide (10% vol.) was added to the samples for lock. Spectra were acquired with no proton decoupling, 16k acquisition points, a spectral window of 40 ppm, and a resolution of 0.81 Hz/point.

AMPK exploration

AMPK activity was estimated by determining the phosphorylation status of ACC, a direct target from AMPK. Cells were collected and lysed in a RIPA buffer and centrifuged at 3,000 g for 10 min at 4°C and the pellet was collected. Sample protein concentrations were determined with a standard Bradford protein assay (Thermo Scientific), and equivalent amounts of protein (40µg) from each sample were dissolved in Laemmli buffer and subjected to electrophoretic separation on 10% SDS-PAGE acrylamide gels. Proteins were transferred into a nitrocellulose membrane in transfer buffer. Membranes were stained with Ponceau Red to verify equal loading in all lanes then membranes were blocked with 5% powdered milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 1 h followed by an overnight incubation at 4°C with mouse anti-phospho-ACC (Ser79) or anti-ACC antibodies (Santa Cruz) dissolved in TBST containing 1% BSA. Membranes were washed for 30 min in TBST and then incubated for 1 h at room temperature in 5% milk-TBST containing horseradish peroxidase conjugated anti-mouse IgG antibody (1:50,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). After 30 min of washing in TBST, the blots were developed on film using ECL plus reagent (Amersham, Piscataway,NJ, USA). Densitometry measurements were performed by determining the density of each whole lane (incorporating the entire molecular weight range of puromycin-labeled peptides) using the public domain ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/nih-image/).

Isolated mitochondria preparation:

To explore mitochondria metabolism, it was not possible to use cell culture since the quantity of mitochondria would not be sufficient. We explored mitochondria isolated from rats muscle. Briefly, the gastrocnemius muscle of both legs from 3-mo-old Wistar male rats were collected, and transferred in the cold buffer containing sucrose 150 mM, KCl 75 mM, Tris 50 mM, KH2PO4 1 mM, MgCl2 5 mM, EGTA 1 mM, and lipid-free serum albumin 0.2%, pH 7.4, was used for mitochondrial extraction. Mitochondria were prepared according to Fontaine et al. (14).

Myofiber isolation and permeabilization

Permeabilized skeletal muscle fibers were prepared from plantaris muscle using a method described by Kuznetsov et al. (15).

Mitochondrial parameters in isolated mitochondria and permeabilized fibers:

The oxygen consumption rate of permeabilized myofibers and isolated mitochondria was measured using a Clark-type O_2 electrode (Oxygraph; Hansatech Instruments). Mitochondria (0.2 mg/ml) were incubated at 37°C in a respiration buffer containing 125 mM KCl, 5 mM P_i, 20 mM Tris·HCl, 0.1 mM EGTA, and 0.1% fat-free BSA (pH 7.2). The suspension was stirred constantly with a built-in electromagnetic stirrer and bar flea. Mitochondria were energized with either glutamate-malate (GM; 5 mM/2.5 mM) or succinate (S; 5 mM) and oxygen consumption was recorded before and after 1 mM ADP was added. Then, oligomycin $(1 \mu g/ml)$ was added in order to measure the oxygen consumption in the absence of ATP synthesis. When succinate was used, rotenone was added in the respiratory chamber in order to inhibit Complex I activity. After the addition of the various substrates GM (5/2.5 mM) or S (5 mM), maximal oxygen consumption rate (V_{max}) was measured under ADP stimulation (1 mM). Then, oligomycin addition allowed measuring oxygen consumption ATP synthase-independent. After each measurement, fiber bundles were carefully removed, dried overnight at room temperature, and then heated at 100°C for 10 min and weighed. Myofiber respiration rates were expressed as nmol O·min⁻¹.mg dry wt⁻¹, and mitochondrial respiration rates were expressed as nmol O.min⁻¹.mg protein⁻¹.

The yield of oxidative phosphorylation (P/O) was measured using isolated mitochondria as described in (16). Briefly, ATP production was measured by HPLC and divided by the corresponding oxygen consumption. Finally, muscle activities of complex I and complex II

were also determined as described in (17). Enzymatic activities were expressed as µmol.min-1.mg protein-1.

<u>**B-oxidation**</u>

The fatty acid oxidation (FAO) was measured in myotubes following manufacturer's instructions. Briefly, the myotubes oxygen consumption rate (OCR) was analyzed using a Seahorse 96 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA, USA), in XF media containing 11 mM glucose, 1 mM sodium pyruvate and 1mM Palmitate sodium conjugated to 0.17 mM BSA (6:1 molar ratio), under basal condition and after Etomoxir (40µM) addition, an inhibitor of CPT1. The difference between respiration under basal condition and respiration under CPT1 inhibition represented the respiration from FAO substrates. The percentage of respiration from β -oxidation has been determined by the ratio basal mitochondrial respiration: Respiration with CPT1 inhibition.

Anaerobic glycolysis

The anaerobic glycolysis was determined following the manufacturer's instruction. Briefly, myotubes were incubated in the assay medium (XF base medium DMEM with 10mM glucose). Extracellular acidification rate was measured under basal condition (to determine basal anaerobic glycolysis) and after successive injections of 0.5 mM oligomycin A (to determine the maximum anaerobic glycolysis capacity by blocking ATP synthase) and 50 mM 2-deoxyglucose (to determine the non-glycolytic acidification by blocking glycolysis).

Statistics

All results are presented as means ± SEM. For cell measurements, statistics were performed between different culture conditions (i.e. *Ctrl+, AA/serum⁻* and *DNP*) and between groups in the same culture condition (e.g. *Ctrl+, LEU, CIT, ARG*). The statistical significance of differences was analyzed using a One-way Anova + Bonferroni test when three groups or more were compared or a Student t-test when only two groups were compared. Values were considered to be different from one another when *P*-values were lower than 0.05.

Results

Protein synthesis and energy status

As expected, LEU addition (as positive control) stimulated protein synthesis (+9%, LEU vs. Ctrl+, p<0.05) in standard condition, whereas ARG addition (as negative control) did not affected protein synthesis. In this particular condition of incubation, CIT addition had no significant effect on protein synthesis (Figure 1, left panel).

As compared with the control condition, amino acid and serum deficiency decreased protein synthesis (-29%, AA/serum⁻ vs. Ctrl+, p<0.05). In condition of amino acid and serum deficiency, LEU or CIT addition stimulated cell protein synthesis (+18%, AA/serum⁻+LEU vs. AA/serum⁻, p<0.05, +22%, AA/serum⁻+CIT- vs. AA/serum⁻, p<0.05)), whereas ARG addition remained ineffective (Figure 1, middle panel).

In order to test whether amino acid and serum deficiency affected energy status, we measured the ATP/ADP ratio in control condition and after amino acid/serum deficiency. As shown in figure 2, the ATP/ADP ratio decreased by 13% (AA/serum⁻ vs. Ctrl+, p<0.05) after 16-hours amino acid and serum deprivation. Interestingly, within each condition of incubation, the addition of LEU, CIT or ARG did not affect the ATP/ADP ratio.

We next selected the concentration of the mitochondrial uncoupler DNP that led to the same decrease in ATP/ADP ratio (figure 2, right panel) and measured protein synthesis (figure 1, right panel) in this particular condition of incubation. As can be appreciated in figure 1, uncoupling treatment decreased protein synthesis (-21%, DNP vs. Ctrl+, p<0.05). In this condition, CIT stimulated protein synthesis (+11%, DNP+CIT vs. DNP, p<0.05), whereas LEU and ARG addition failed to stimulate protein synthesis. Note that in this condition of incubation also, LEU, CIT or ARG did not affect the ATP/ADP ratio (figure 2, right panel). We also showed that inhibition of mTORC1 (using rapamycin (200 nM)) in standard condition decreased protein synthesis without any change of the energy status (supplementary data).

AMPK exploration (supplementary data)

As expected, AMPK activity (evaluated by ACC phosphorylation status) was enhanced by amino acid/serum deficiency (+33%, AA/serum⁻ vs. Ctrl+, p<0.05), while CIT addition did not prevent this effect.

Interaction of CIT and ARG with phosphate

Because the Gibbs free energy of ATP depends on both the ATP/ADP ratio and the concentration of free concentration of Pi, we next measured whether CIT could physically interact with Pi. The phosphorous chemical shift (δ) of phosphate (2 mM) did not show any significant change upon CIT addition at pH 7.4, even in a 25-fold excess of CIT, indicating no interaction: $\delta_{(CIT 50 \text{ mM})} - \delta_{(CIT 0 \text{ mM})} = 0.01$ ppm for a resolution of 0.005 ppm/point). ARG studied in the same conditions for comparison induced a significant change in the phosphorous chemical shift of phosphate, suggesting an interaction of the phosphate anion with the guanidinium function of ARG: $\delta_{(ARG 50 \text{ mM})} - \delta_{(ARG 0 \text{ mM})} = 0.11$ ppm. The stability of the arginine-phosphate electrostatic interaction, that is proposed to be H-bond driven, was indeed demonstrated to be large (18).

Myotubes mitochondrial respiration and ATP demand

As shown in Table 1, whatever the culture condition (Standard, amino acid and serum deficiency or mild uncoupling) the basal respiration remained unchanged, and was not affected by the addition of LEU, CIT or ARG. However, the respiration allocated to protein synthesis was significantly decreased by the amino acid and serum deficiency (36% vs. 41%, AA/serum⁻ vs. Ctrl+, p<0.05), as well as by mild uncoupling (34% vs. 41%, DNP vs. Ctrl+, p<0.05). Interestingly, the addition of LEU or CIT (but not that of ARG) normalized the percentage of the respiration allocated to protein synthesis.

In order to further understand which expense item(s) was (were) affected by amino acid and serum deficiency, we completed the experiment by measuring the respiration allocated to other energy expenditure items. As shown in table 2, the respiration allocated to DNA synthesis and Na⁺/K⁺ ATPase pumps activity were not affected by amino acid and serum deficiency, leading to an increase the percentage of respiration linked to still unknown items.

Mitochondrial metabolism (Table 3)

The isolated mitochondrial complexes activities (complex I and II) were not modified by CIT addition (5mM).

In isolated skeletal muscle mitochondria, CIT addition (5mM) had no effect on respiration, neither during ATP synthesis (state III) or after ATP synthesis had been inhibited by oligomycine (state IV). CIT did not change the relationship between electrical membrane potential and the resting oxygen consumption rate. Moreover, CIT did not affect the oxidative phosphorylation efficiency (as assessed by P/O ratio).

Similar results on the respiration were observed using permeabilized fibers from Wistar rat's plantaris (Supplementary data).

Anaerobic Glycolysis (Table 4)

In order to test whether CIT could stimulate the anaerobic production of ATP, basal anaerobic glycolysis and maximum anaerobic glycolysis capacity were measured in myotubes. As shown in Table 4, CIT did not change anaerobic production of ATP.

<u>β-oxidation (Table 4)</u>

The yield of oxidative phosphorylation in vivo depends on the substrate used (it is lower with lipids than with carbohydrates). In order to test whether CIT could increase oxidative phosphorylation efficiency by decreasing β -oxidation, we next measured the effect of CIT on the percentage of respiration linked to β -oxidation. As shown in table 4, 12.1% of the respiration provided from β -oxidation_in control condition and this percentage increased to 16.2% (p<0.05) in the presence of CIT. Note that contrary to CIT, ARG did not change the proportion of respiration linked to β -oxidation.

Discussion

In this work we have reported that a decrease in ATP/ADP ratio led to a decrease in protein synthesis in rat myotubes. Such decrease in ATP/ADP ratio was not due to a decrease in oxygen consumption in our experimental conditions, but less oxygen consumption was devoted to protein synthesis. CIT re-allocated oxygen consumption to protein synthesis without affecting total oxygen consumption, oxidative phosphorylation efficiency, anaerobic ATP production or ATP Gibbs free energy.

Among the ATP-consuming processes, protein synthesis represents an important item of ATP consumption (approximately 40% in this work as estimated by oxygen consumption). We reported that protein synthesis falls rapidly as energy supply becomes limiting, while other items of ATP consumption remain largely preserved. The distribution of ATP among the ATP-consuming pathways can be modulated enzymatically by the enzymes controlling each pathway. However, enzymes only activate thermodynamically favorable reactions by decreasing the activation energy of the catalyzed reaction. In ATP consuming reactions, the ATP Gibbs free energy directly affects the energy activation of the reaction. Therefore, because both the Gibbs free energy and the activation energy required are different for each ATP-consuming process, the kinetics of reaction can differently be affected by a same decrease in $\frac{[ATP]}{[ADP] \times [Pi]}$ ratio. The fact that protein synthesis decreases when ATP/ADP ratio

decreases suggests that protein synthesis remains thermodynamically favorable (otherwise, the reaction would not occur) but that the energy activation of at least one step increases (thus slowing down the entire pathway). Finally, mTORC1 pathway is well known to regulate protein synthesis. But, our results showed that the regulation of protein synthesis by mTORC1 is downstream compared to energy status regulation since inhibition of mTORC1 (using rapamycin) decreased the protein synthesis without any change of energy status.

Furthermore, nutrients such as amino acids are not only used for protein synthesis but also show important regulatory properties. Among them, CIT is well known to stimulate muscle protein synthesis via an activation of mTORC1 pathway (1, 2, 19, 20). However, the mechanism by which CIT stimulates protein synthesis is not fully understood. Interestingly, most of the studies showing a positive effect of CIT on muscle protein synthesis have been observed after protein-energy deficiency (1, 2, 21). We also confirmed this result in the present work, but failed to observe a significant effect of CIT in normal condition, whereas CIT stimulated protein synthesis when ATP/ADP ratio had been decreased by mild uncoupling.

Together, these observations lead us to propose that CIT stimulates protein synthesis by decreasing the energy activation of one or several ATP-consuming reaction(s) involved in protein synthesis. In control condition, CIT probably decreases the energy activation, but failed to significantly stimulate protein synthesis. This is most probably because the enzyme(s) regulated by CIT run at their Vmax and cannot be further activated by a decrease in energy activation. At present, whether this (these) enzyme(s) participate(s) or regulate(s) protein synthesis is not known.

It is usually admitted that amino acid availability is the major limiting step in protein synthesis. It has been thus proposed that CIT may stimulate protein synthesis after proteinenergy deficiency due to the fact that CIT may represent a nitrogen source. This hypothesis can be ruled out, since ARG (the only known metabolite of CIT) did not stimulate protein synthesis in that condition of incubation, while CIT stimulated protein synthesis after mild uncoupling (a condition in which all the amino acids are present). Interestingly, LEU stimulated protein synthesis in control condition and after protein-energy deficiency, but not after mild uncoupling. This difference between these amino acids suggests a different mechanism from CIT and LEU in the regulation of protein metabolism.

Beside the mechanism of action proposed above, we tested whether CIT could stimulate ATP production. We observed that CIT did not affect mitochondrial physiology (in particular it did not increase the oxidative phosphorylation efficiency), and did not stimulate cell oxygen consumption and anaerobic glycolysis, suggesting that it did not increase cell ATP production.

It has been reported that CIT stimulates β -oxidation in adipose tissue (7, 8). Here we also observed that CIT stimulated fatty acid β -oxidation in myotubes. The mechanism by which CIT stimulates β -oxidation remains to be solved.

Conclusion

This work further confirms that the ATP-consuming processes are arranged in a hierarchy, with protein synthesis being very sensitive to a decrease in ATP/ADP ratio. It also demonstrates that the hierarchy (i.e. the allocation of ATP to the different ATP-consuming processes) can occur without changes in ATP production, suggesting that if some ATP-consuming processes decrease others increase. In this framework, CIT reallocates ATP to

protein synthesis when ATP/ADP ratio has been decreased. We propose that this is due to a decrease in energy activation of one or several enzymatic step involved or controlling protein synthesis. More generally, this kind of thermokinetic control can explain why regulators are effective or ineffective at controlling a given step depending on the energy status.

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Fig.1. Myotubes protein synthesis evaluated by puromycin-incorporated protein staining in Western Blot. Results are presented as means ± SEM. Only significant amino acid effects are presented here. Significant differences between culture conditions (Standard condition, amino acid/serum deficiency and energy stress) are presented in results. In the same condition culture, values with different superscript letters are statistically different (p<0.05; One-way Anova + Bonferroni test).



Fig.2. Myotubes energy status determined by the ratio ATP: ADP measured by HPLC. Results are presented as means ± SEM. Only significant amino acid effects are presented here. Significant differences between culture conditions (Standard condition, amino acid/serum deficiency and energy stress) are presented in results. In the same condition culture, values with different superscript letters are statistically different (p<0.05; One-way Anova + Bonferroni test).

onditions different	en culture cc , values with	ences betwe	ificant differ	ed here. Signi results. In the	are present presented in	o acid effects • stress) are p	ificant aminc y and energy	VI. Only sign Im deficienc	means ± SEN 10 acid/seru	esented as indition, amir	sults are pr andard con	Re (St
36 ^{ab} ±1	41 ^c ± 2	39 ^{bc} ± 1	34 ^a ±1	37 ^a ±1	46 ^b ± 2	41 ^b ± 1	36 ^a + 1	39 ^a ± 2	43 ^a ±1	42 ^a ±1	41 ^a ± 1	% respiration allocated to protein synthesis (1/2)
48 ± 3a	44 ^ª ±3	47 ^a ± 3	51 ^ª ± 2	44 ^a ± 1	39 ^b ± 2	43 ^{ab} ± 2	***** 46 ^a ± 2	44 ^a ± 2	42 ^a ± 2	43 ^a ± 2	43 ^a ± 2	Respiration with protein synthesis inhibition 2
76 ± 3ª	74 ^a ±3	77 ^a ± 3	77 ^a ± 2	71 ^a ± 2	72 ^a ± 3	73 ^a ± 3	72 ^a ± 3	73 ^a ± 2	73 ^a ± 3	74 ^a ± 3	73 ^a ± 3	Basal mitochondrial respiration 1
DNP + ARG	DNP + CIT	DNP + LEU	DNP	AA/serum [`] +ARG	AA/serum ⁻ +CIT	AA/serum ⁻ +LEU	AA/serum ⁻	ARG	CIT	LEU	Ctrl+	
	M))	J or ARG (5m	on (CIT, LEU	no acid additi	specific amii	.gy stress)±s	ency or ener	erum defici	nino acid/s	condition, a	(standard c	n

superscript letters are statistically different (p<0.05; One-way Anova + Bonferroni test).

Table 1. Myotubes basal respiration and respiration allocated to protein synthesis evaluated in different culture conditions

	Ctrl+	AA/serum	AA/serum +CIT	
% respiration allocated to protein synthesis	41 ^a ± 1	36 ^b ± 1	46 ^a ±2	
% respiration allocated to DNA synthesis	16 ^ª ± 1	13 ^ª ± 1	14 ^a ± 1	
% respiration allocated to Na ⁺ /K ⁺ ATPase pumps activity	17 ^ª ± 1	15ª±1	15 ^a ± 1	
Estimated % respiration allocated to others energy expenditure items	26	36	25	

Table. 2 Energy allocation to protein synthesis or others important items of energy expenditure

Results are presented as means ± SEM. Values with different superscript letters are statistically different (p<0.05; One-way Anova + Bonferroni test).

Table 3.	Direct	effect of	f citrulline	(5mM) on	mitochondrial function
				(

		Ctrl	СІТ		
Muscle is	olated				
mitochondria	respiration				
(nmol O.min ⁻¹ .	mg proteins				
-1)					
Complex I	State III	92.6 ^a ± 6.5	86.8 ^ª ± 1.6		
respiration	State IV	$6.6^{a} \pm 0.4$	6.4 ^a ± 0.3		
Complex II	State III	60.7 ^a ± 2.7	60.6 ^a ± 3.4		
respiration	State IV	13.9 ^ª ± 0.3	13.7 ^ª ± 0.8		
Isolated comp	olex activity				
(µmol.min⁻¹.m	g proteins⁻¹)				
Complex	activity	$520^{a} + 21$	$540^{a} + 24$		
Complex I	activity	552 <u>1</u> 21	540 ± 24		
Complex II activity		150 ^a ± 10	150 ^a ± 15		
		155 ± 12	136 ± 15		
Energy ef	ficiency				
$\left(\frac{\mathbf{P}}{\mathbf{r}}\right)$					
.0.					
_		2.16 ^ª ±	2.12 ^ª ±		
Comp	lex l	0.05	0.06		

Results are presented as means \pm SEM. Values with different superscript letters are statistically different (p<0.05; Student t-test).

_				
-	Ctrl+	AA/serum	AA/serum +CIT	AA/serum +ARG
Anaerobic glycolysis				
(∆pH/min)				
Basal anaerobic glycolysis	20.2 ^a ± 1.2	19.7 ^a ± 1.1	19.6 ^a ± 0.9	19.1 ^a ± 0.9
Maximum capacity	2	2	2	2
of anaerobic glycolysis	40.2° ± 2.4	38.8° ± 2.2	39.1° ± 1.9	38.0° ± 1.9
β-oxidation				
% respiration	12 4 L 0 0 ^a	12.1 . 0.08	16 2 L 0 0 ^b	12 2 L 0 7 ^a
from β-oxidation	12.4 ± 0.8	12.1 ± 0.8	10.2 ± 0.8	12.3 ± 0.7

Table 4. Citrulline or arginine (5mM) effects on anaerobic glycolysis and β -oxidation in amino/acid/serum deficiency

Results are presented as means \pm SEM. Values with different superscript letters are statistically different (p<0.05; One-way Anova + Bonferroni test).

Supplementary data

Direct effect of citrulline (5mM) on mitochondrial respiration

in permeabilized muscle fibers

		Ctrl	CIT
Muscle peri fibers res (nmol O. min	meabilized piration ⁻¹ .mg fibers ⁻		
Complex I	State III	4.65 ± 0.64	4.84 ± 0.39
respiration	State IV	0.86±0.09	0.88 ± 0.07
Complex II respiration	State III State IV	4.04 ± 0.97 1.84 ± 0.17	4.10 ± 1.01 1.78 ± 0.18

Results are presented as means \pm SEM. Values with different superscript letters are statistically different (p<0.05; Student t-test).


Myotubes protein synthesis without or with mTORC1 inhibition evaluated by puromycinincorporated protein staining in Western Blot. Results are presented as means ± SEM. Values with different superscript letters are statistically different (p<0.05; Student t-test).



Myotubes energy status without or with mTORC1 inhibition determined by the ratio ATP: ADP measured by HPLC. Results are presented as means \pm SEM. Values with different superscript letters are statistically different (p<0.05; Student t-test).



Phosphorylation status of ACC determined by Western Blotting. Results are presented as means ± SEM. Values with different superscript letters are statistically different (p<0.05; One-way Anova + Bonferroni test).

DISCUSSION GENERALE

Depuis une dizaine d'années, la citrulline est apparue comme un nouveau nutriment particulièrement prometteur, et qui semble jouer un rôle fondamental dans la régulation de l'homéostasie métabolique. En effet, différents travaux ont montré la capacité de cet acide aminé à stimuler l'accrétion protéique musculaire ainsi qu'à moduler le métabolisme énergétique. Cependant, en l'état de l'art, il n'est pas possible de comprendre de façon intégrée comment la citrulline régule de façon précise l'homéostasie énergétique au niveau corps entier et les données sur les mécanismes d'action restent encore parcellaires. Malgré quelques données sur une modulation du métabolisme énergétique par la citrulline, il est encore difficile de comprendre comment la citrulline est capable de moduler le métabolisme énergétique pour supporter l'activation de la synthèse protéique musculaire, coûteuse en énergie. Ainsi, le but de nos travaux a été de préciser l'inter-relation existante entre le métabolisme protéique d'une part et le métabolisme énergétique d'autre part et l'action de la citrulline sur ces métabolismes.

Tout d'abord, nous avons pu confirmer l'effet activateur de la citrulline sur la synthèse protéique musculaire. Cependant, nos travaux se démarquent de ceux de la littérature par deux aspects :

- Notre étude *in vivo* a été réalisée chez des animaux sains alors que l'ensemble des données de la littérature a été obtenu en situation de carence protéique ou de malnutrition (3,4,189)
- C'est la première fois, à notre connaissance qu'un effet chronique de la citrulline est évalué sur la synthèse protéique musculaire.

Outre la nutrition, l'exercice est reconnu comme étant un puissant régulateur du *turn-over* protéique musculaire. En effet, de nombreux travaux de la littérature indiquent qu'un entrainement physique s'accompagne d'une activation de la synthèse protéique musculaire et d'une adaptation du métabolisme énergétique, notamment *via* une augmentation de la densité mitochondriale. Cependant, l'exercice et la citrulline ont-ils des voies communes dans l'adaptation du métabolisme protéino-énergétique ? Il semblerait que non puisque l'exercice augmente à la fois la synthèse protéique musculaire et la densité mitochondriale (sans affecter directement la fonction mitochondriale). En revanche, la citrulline augmente bien la synthèse protéique musculaire sans affecter ni la densité ni la fonction mitochondriale. Mais l'approche protéomique suggère bien un effet de la citrulline sur le métabolisme énergétique en favorisant l'ensemble des voies métaboliques fournissant des

substrats à la chaine respiratoire. Ce résultat paradoxal peut s'expliquer par le fait que toutes les mesures de la fonction mitochondriale ont été réalisées en conditions saturantes en substrats, permettant d'évaluer la fonction mitochondriale maximale. Or, in vivo, nous ne sommes pas dans de telles conditions et les flux métaboliques dépendent de la disponibilité en substrats. Ainsi, nous pouvons proposer l'hypothèse suivante : l'action synergique de la citrulline et l'exercice sur la synthèse protéique musculaire et la performance serait liée d'une part à la capacité de l'exercice à stimuler la densité mitochondriale, et d'autre part, à celle de la citrulline à favoriser l'apport en substrats pour la respiration mitochondriale. Si l'action synergique de la citrulline et de l'exercice sur la performance a pu être rapportée chez l'Homme (121,180), à ce jour aucune étude n'avait pu proposer un mécanisme d'action. Par ailleurs, il nous a semblé important de mieux comprendre les effets de la citrulline sur le *turn-over* protéique musculaire. Si des approches mécanistiques ont pu mettre en évidence les voies de transductions impliquées (4), différentes études avaient pu montrer que la citrulline ne semblait pas affecter de la même manière l'ensemble des protéines musculaires. Ainsi, Ventura et al. (169) avaient montré un effet spécifique de la citrulline sur la synthèse des protéines myofibrillaires (résultat retrouvé par Faure et al. (6) sur l'expression de protéines myofibrillaires). Cet effet hétérogène de la citrulline est confirmé par notre approche protéomique.

Par ailleurs, nous avons voulu explorer une autre facette de la synthèse protéique musculaire qui est encore peu étudié et qui concerne les protéines musculaires sécrétées. En effet, il est bien connu que l'exercice stimule la synthèse protéique musculaire ainsi que la sécrétion de myokines. En revanche, qu'en est-il de la régulation du sécrétome musculaire par la citrulline (et de façon plus générale, par les acides aminés) ? En effet, à notre connaissance il n'existe aucune donnée sur la modulation des nutriments sur le sécrétome musculaire permettant de mimer une situation de « jeûne ». Conformément aux données de la littérature, l'apport d'acides aminés (mimant une hyperaminoacidémie), de leucine ou de citrulline permet de restaurer la synthèse protéique musculaire dans des myotubes.

Nous avons ainsi pu mettre en évidence pour la première fois que cet effet s'accompagnait d'une modulation du sécrétome. En revanche, l'effet sur le sécrétome n'est pas le même en fonction de l'acide aminé considéré. Ainsi, un mélange complet en acides aminés augmente principalement l'expression de protéines impliquées dans le développement cellulaire et

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l'angiogenèse (e.g. Sema3c, Sema3d, follistatin-related protein 1, angiopoietin-like 2), et diminue l'expression de protéines impliquées dans l'homéostasie calcique (e.g. FKBP1A, metastasine, phosphohistidine phosphatase 1). De tels résultats pourraient être expliqués par le fait que l'hyperaminoacidémie liée à la prise alimentaire pourrait contribuer au développement musculaire. L'addition de leucine quant à elle diminue principalement l'expression de protéines relatives au cytosquelette (e.g. cofilin-2 et calponin-3). Enfin, la citrulline semble moduler des protéines principalement impliquées dans l'homéostasie cardiovasculaire (e.g. caluménine, cystatine C). Bien que nos résultats démontrent une modulation spécifique du sécrétome musculaire, quelques protéines sont modulées de façon commune. Parmi elles, l'expression de la transcobalamine est stimulée par l'hyperaminoacidémie, la citrulline ou la leucine. La transcobalamine est le transporteur de la vitamine B12 qui est impliquée dans le développement cellulaire (191). Or, la vitamine B12 est presque exclusivement présente dans la nourriture animale, riche en protéines. Ainsi, il est possible qu'une augmentation de la disponibilité en acides aminés puisse être un signal cellulaire afin d'adapter la capacité de transport de la vitamine B12, qui nécessiterait d'être augmentée à l'état post-prandial. Cette explication reste hypothétique et des travaux sur le sujet sont requis pour la confirmer. Ce travail préliminaire confirme la complexité de la régulation du métabolisme protéique et que, même si des molécules ont des effets similaires sur la synthèse protéique musculaire globale, celle-ci reste hétérogène et cette étude montre qu'un nouveau champ de recherche sur le métabolisme protéique musculaire reste encore à explorer.

Enfin, nos travaux réalisés *in vivo* ont permis de confirmer notre hypothèse de travail proposant une adaptation coordonnée des métabolismes protéique et énergétique par la citrulline. Cependant, si cette approche *in vivo* nous permet d'avoir une vision intégrée de la problématique, elle ne nous permet pas de déterminer de façon précise les mécanismes impliqués. Seule une approche *in vitro* le permettrait.

Ainsi, nous avons déterminé l'effet aigu de la citrulline sur la mitochondrie isolée ou sur des fibres musculaires. De la même façon que lors de l'étude *in vivo*, nous n'avons pas pu mettre en évidence un effet de la citrulline sur les paramètres mitochondriaux. Or, la citrulline pourrait agir sur le métabolisme mitochondrial en modulant les flux énergétiques. Ainsi, nous avons pu montrer que malgré une respiration mitochondriale globale inchangée, la respiration allouée à la synthèse protéique est fortement augmentée par citrulline, aux

dépens d'autres postes de dépense énergétique (mais qui ne sont ni la synthèse d'ADN ni l'activité des pompes Na^+/K^+). Pour expliquer ce résultat, notre hypothèse est que la citrulline stimule la synthèse protéique en diminuant l'énergie d'activation d'une ou plusieurs réactions ATP-dépendantes impliquées dans la synthèse protéique.

En effet, l'énergie libérée durant l'hydrolyse de l'ATP varie en fonction de son enthalpie libre, également appelée énergie libre de Gibbs, qui dépend du statut énergétique de la cellule. Ainsi, lorsque différentes réactions consommant de l'ATP sont en compétition, à la fois le *turnover* de l'ATP et l'énergie libre de Gibbs de l'ATP pourraient être affectés. Par exemple, lorsque l'énergie libre de Gibbs de l'ATP diminue, les voies métaboliques qui requièrent un haut niveau d'énergie de l'ATP tendent à être inhibées, favorisant la disponibilité de l'ATP pour des voies métaboliques qui peuvent fonctionner avec un ATP « moins énergétique ». Lors de nos travaux, cette notion a été vérifiée puisque nous avons montré pour la première fois que la synthèse protéique diminue lorsque le statut énergétique est diminué (malgré une respiration mitochondriale inchangée), tandis que d'autres postes de dépenses énergétiques (tels que la synthèse protéique diminue lorsque le statut énergétique diminue lorsque le statut énergétique diminue suggère que :

- la synthèse de protéines reste un processus thermodynamiquement favorable (sinon la synthèse protéique serait totalement inhibée)
- l'énergie d'activation d'au moins une étape de la synthèse protéique augmente, entrainant un « ralentissement » de cette voie métabolique.

Or, le fait que la citrulline stimule la synthèse protéique sans modifier le statut énergétique (et donc l'énergie libre de Gibbs de l'ATP) suggère que la citrulline diminue l'énergie d'activation nécessaire à une ou plusieurs des réactions (nécessitant de l'ATP) impliquées dans la synthèse de protéines.

De plus, dans ces travaux, nous avons démontré que la citrulline est capable de stimuler la β -oxydation. Ce résultat est en accord avec les résultats de Joffin et al. (8,9) qui ont montré *in vitro* que la citrulline stimule cette voie métabolique dans des explants de tissus adipeux. En revanche, ce résultat est paradoxal au vu des résultats montrant une augmentation de la production d'ATP au profit de la synthèse protéique puisqu'il est bien connu que la β -oxydation est une voie métabolique avec un rendement énergétique inférieur à la glycolyse pour produire de l'énergie. Mais il est important de le relativiser car, dans nos

conditions, la contribution de la β -oxydation pour la respiration mitochondriale reste faible (13% de la respiration totale). Maintenant que nos travaux ont pu mettre clairement en évidence une régulation de métabolisme énergétique par la citrulline, les mécanismes précis de cette régulation restent à élucider.

CONCLUSION ET PERSPECTIVES

En conclusion, nos travaux in vivo et *in vitro* ont permis de confirmer l'effet stimulateur de la citrulline sur la synthèse protéique musculaire. Cet effet n'est cependant pas global et reste complexe. En effet, cela a déjà été démontré avec notamment une stimulation plus importante de la synthèse des protéines myofibrillaires. Nos travaux ont ainsi permis de préciser davantage la modulation de la synthèse des protéines intracellulaires (Publication n°1). Nos travaux ont également permis d'explorer pour la première fois les effets des acides aminés sur le sécrétome musculaire et de mettre en évidence la complexité de cette régulation due à des effets spécifiques sur celui-ci en fonction des acides aminés (citrulline et leucine) (Publication n°2).

L'autre découverte majeure est que cette augmentation de la synthèse protéique musculaire est associée à une modification en temps réel du métabolisme énergétique avec notamment une première démonstration d'un lien direct entre, d'une part, une modulation du métabolisme protéique musculaire et d'autre part, une adaptation du métabolisme énergétique par la citrulline afin de soutenir le coût énergétique de la synthèse protéique. Ces travaux montrent ainsi une nouvelle facette de la citrulline en étudiant pour la première fois la relation étroite entre les métabolismes protéique et énergétique.

Malgré ces avancées, des questions restent en suspens :

- Sur l'aspect énergétique, par quels mécanismes la citrulline est-elle capable de réorienter les flux énergétiques au profit de la synthèse protéique et comment cette réorientation permet de stimuler la synthèse protéique malgré un statut énergétique inchangé ? Notre hypothèse est que la citrulline stimule la synthèse protéique en diminuant l'énergie d'activation d'une ou plusieurs réactions ATP-dépendantes impliquées dans la synthèse protéique.
- Pour nos travaux sur les effets de la citrulline sur le sécrétome musculaire, nous avons opté pour une approche protéomique globale en déterminant les protéines les plus modulées par la citrulline. Cependant, il serait également intéressant d'étudier la régulation de protéines spécifiques par la citrulline afin de répondre à des questions précises sur les effets systémiques de cet acide aminé. Par exemple, il est connu que le métabolisme musculaire et le métabolisme cérébral peuvent interagir, notamment par l'exercice. Nos travaux ont notamment démontré que la citrulline stimule l'expression de la Fetuine A. Or, chez les patients atteints de la maladie d'Alzheimer,

cette protéine est diminuée dans le cerveau. Du fait des propriétés de la citrulline sur le métabolisme cérébral, notamment les données suggérant un effet inhibiteur de la citrulline sur la formation de plaques amyloïdes, se pourrait-il que la diminution de la Fetuin A chez les patients atteints de la maladie d'Alzheimer soit liée à une sécrétion moins importante de cette protéine par le muscle ? Ainsi, les effets positifs de la citrulline sur le métabolisme cérébral pourraient être liés à la modulation de l'expression de Fetuine A.

De façon générale, il serait intéressant d'étudier la synthèse spécifique de protéines d'intérêts. Pour cela, il suffirait de coupler la méthode SUnSET à une immuno-précipitation préalable des protéines d'intérêts. Egalement, par imagerie, la méthode SUnSET peut également être utilisée afin de co-localiser la synthèse protéique et une protéine d'intérêt. Cela a déjà été réalisé par Goodman et al. (192) sur des coupes de muscles où les auteurs ont colocalisés la synthèse protéique et différents types de chaîne lourdes de myosine et mis en évidence de cette façon qu'une modulation de la synthèse protéique musculaire globale ne reflète pas nécessairement des changements au niveau de fibres spécifiques. Ceci permettrait de compléter les mécanismes de régulation du métabolisme protéique par la citrulline.

En conclusion, nos travaux sont une contribution à la meilleure compréhension de la régulation du métabolisme protéino-énergétique musculaire par la citrulline.

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LISTE DES ABREVIATIONS

3-MH: 3-Methylhistidine

- 4E-BP1 : Eukaryotic translation initiation factor 4E-binding protein 1
- ADMA : asymmetric dimethylarginine
- ADP : Adénosine diphosphate
- AMP : Adénosine monophosphate
- APP : l'amyloid precursor protein
- ASL : Argininosuccinate lyase
- ASS : Argininosuccinate synthase
- ATP : Adénosine triphosphate
- CPT1 : Carnitine palmitoyltransferase 1
- IL-10 : Interleukine 10
- INF-γ : Interféron gamma
- LDL : Low density lipoprotein
- LPS : Lipopolysaccharide
- mTORC1 : Mammalian target of rapamycin complex 1
- MCP-1 : Monocyte chimoattractant protein 1
- NADH : Nicotinamide adénine dinucléotide
- NO : Monoxyde d'azote
- NOS : NO synthase
- OAT : Ornithine aminotransferase
- OTC : Ornithine transcarbamylase ROS : Reactive oxygen species
- P5C : Δ1-I-Pyrroline-5-Carboxylate
- PEPCK : Phosphoénolpyruvate carboxykinase
- PI3K : Phosphoinositide 3-kinase
- S6K1 : S6 kinase 1
- SUnSET : Surface sensing of translation
- TFAM : Mitochondrial transcription factor A
- TNF- α : Tumor necrosis factor α
- UCP1 : Uncoupling protein 1
- VLDL : Very low density lipoprotein

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Synergistic effects of citrulline supplementation and exercise on performance in male rats: evidences for implication of protein and energy metabolisms. <u>Goron A</u>, Lamarche F, Cunin V, Dubouchaud H, Hourde C, Noirez P, Corne C, Couturier K, Sève M, Fontaine E, Moinard C. *Clinical Science (sous presse).2017*

Modulation of muscle protein synthesis by amino acids: What consequences for the secretome? - A preliminary in vitro study. <u>Goron A</u>, Breuillard C, Cunin V, Bourgoin-Voillard S, Seve M., Moinard C. *Soumis pour publication*.

Stimulation of muscle protein synthesis by citrulline: A bioenergetics regulation? <u>Goron A</u>, Lamarche F, Blanchet S, Delangle P, Fontaine E, Moinard C. *Soumis pour publication*.

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Citrulline directly modulates muscle protein synthesis via the PI3K/MAPK/4E-BP1 pathway in a malnourished state: evidence from in vivo, ex vivo, and in vitro studies. Le Plénier S, <u>Goron A</u>, Sotiropoulos A, Archambault E, Guihenneuc C, Walrand S, Salles J, Jourdan M, Neveux N, Cynober L, Moinard C. *Am J Physiol Endocrinol Metab. 2017.*

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La complémentation en citrulline améliore la performance physique au cours de l'exercice. <u>Goron A</u>, Dubouchaud H, Hourdé C, Noirez P, Djemai H, Corne C, Van Noolen L, Couturier K, Fontaine E, Moinard C. *Congrès JFN (Marseille), 2015*.

Combined effects of citrulline supplementation and physical training on muscle protein metabolism in healthy adult rats. <u>Goron A</u>, Cunin V, Sève M, Fontaine E, Moinard C. *Congrès ESPEN (Copenhague), 2016*.

Modulation nutritionnelle du sécrétome musculaire : évaluation par une approche in vitro. <u>Goron A</u>, Breuillard C, Cunin V, Bourgoin-Voillard S, Seve M., Fontaine E., Moinard C. *Congrès JFN (Montpellier), 2016*.

Effets combinés d'une complémentation en citrulline et d'un entrainement physique sur le protéome musculaire chez des rats adultes sains. <u>Goron A</u>, Cunin V, Sève M, Fontaine E, Moinard C. *Congrès JFN (Montpellier), 2016*.

Communication orale

La citrulline réoriente les flux énergétiques au profit de la synthèse protéique musculaire. <u>Goron A</u>, Blanchet S, Fontaine E, Moinard. *Congrès JFN (Montpellier), 2016*.

ANNEXES

RESEARCH ARTICLE

Citrulline directly modulates muscle protein synthesis via the PI3K/MAPK/ 4E-BP1 pathway in a malnourished state: evidence from in vivo, ex vivo, and in vitro studies

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Submitted 24 May 2016; accepted in final form 18 October 2016

Le Plénier S, Goron A, Sotiropoulos A, Archambault E, Guihenneuc C, Walrand S, Salles J, Jourdan M, Neveux N, Cynober L, Moinard C. Citrulline directly modulates muscle protein synthesis via the PI3K/MAPK/4E-BP1 pathway in a malnourished state: evidence from in vivo, ex vivo, and in vitro studies. Am J Physiol Endocrinol Metab 312: E27-E36, 2017. First published November 8, 2016; doi:10.1152/ajpendo.00203.2016.—Citrulline (CIT) is an endogenous amino acid produced by the intestine. Recent literature has consistently shown CIT to be an activator of muscle protein synthesis (MPS). However, the underlying mechanism is still unknown. Our working hypothesis was that CIT might regulate muscle homeostasis directly through the mTORC1/PI3K/MAPK pathways. Because CIT undergoes both interorgan and intraorgan trafficking and metabolism, we combined three approaches: in vivo, ex vivo, and in vitro. Using a model of malnourished aged rats, CIT supplementation activated the phosphorylation of S6K1 and 4E-BP1 in muscle. Interestingly, the increase in S6K1 phosphorylation was positively correlated (P < 0.05) with plasma CIT concentration. In a model of isolated incubated skeletal muscle from malnourished rats, CIT enhanced MPS (from 30 to 80% CIT vs. Ctrl, P < 0.05), and the CIT effect was abolished in the presence of wortmannin, rapamycin, and PD-98059. In vitro, on myotubes in culture, CIT led to a 2.5-fold increase in S6K1 phosphorylation and a 1.5-fold increase in 4E-BP1 phosphorylation. Both rapamycin and PD-98059 inhibited the CIT effect on S6K1, whereas only LY-294002 inhibited the CIT effect on both S6K1 and 4E-BP1. These findings show that CIT is a signaling agent for muscle homeostasis, suggesting a new role of the intestine in muscle mass control.

eukaryotic initiation factor 4E-binding protein 1; mitogen-activated protein kinase; phosphatidylinositol 3-kinase; muscle; myotube; amino acids; protein synthesis; mammalian target of rapamycin

CITRULLINE (CIT) is a nonprotein amino acid. It takes its name from the watermelon (*citrullus vulgaris*). CIT is known mainly as an intermediary of ureagenesis in periportal hepatocytes (24)

and at the whole body level is produced almost exclusively by enterocytes (4). Intestinal CIT production is controlled largely by dietary protein supply (37). CIT has also emerged as an important regulator of nitrogen homeostasis in both humans and animals, as reviewed recently (6). For example, in pioneering work, we used a model of aged malnourished rats to demonstrate that CIT-enriched diet stimulated muscle protein synthesis (MPS) (+80%), and led to a net muscle protein gain (+20%) (10, 33). Interestingly, this metabolic effect was accompanied by muscle histological change and an increase in maximum strength as well as increased traction in treated animals (11). Similar results were obtained in healthy aged rats, in which CIT increased muscle protein content (28). This was also found in other situations such as fasting or caloric restriction in adults rats (22, 38). Finally, our experimental work showing the ability of CIT to modulate MPS was confirmed in humans; in a crossover trial, Jourdan et al. (20) showed that CIT administration to healthy volunteers fed a hypoprotein diet increased MPS compared with a nonessential amino acid mixture. Thus all these studies confirm the ability of CIT to modulate MPS in vivo, but no work has yet determined the underlying mechanisms of CIT action (an increase in nitrogen disposal for muscle or a pharmacological effect) directly or indirectly through arginine and/or nitric oxide production (21). The transductional properties of this amino acid are not known, and many molecular targets may be involved. Amino acids induce anabolic responses through several transductional pathways, such as phosphatidylinositol 3-kinase (PI3K)/Akt, MAPK, and the major mammalian target of rapamycin complex 1 (mTORC1) (15).

We hypothesized that CIT might act directly on MPS through activation of these signaling pathways. To investigate the mechanisms by which CIT modulates muscle protein synthesis, we studied *1*) the involvement of transductional pathways (mTORC1, PI3K/Akt, and MAPK pathways) in the regulation of MPS by CIT and *2*) its possible direct action on these pathways.

For this purpose, we used a systematic approach using experiments conducted in vivo (in malnourished aged rats), ex

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vivo (in rat epitrochlearis muscle), and in vitro (on myotubes in culture).

EXPERIMENTAL PROCEDURES

In vivo studies. We used a validated rat model of malnutrition (40). In brief, Sprague-Dawley male rats aged 23 mo were used. Thirty rats were subjected to a dietary restriction for 12 wk (50% of spontaneous food intake with a standard diet; UAR 04, Villemoisson-sur-Orge, France) and then assigned to three groups; one group was euthanized (R), and the others were refed for 1 wk (90% of spontaneous food intake) with either a citrulline-supplemented diet (CIT) or an isonitrogenous standard diet (Ctrl). The other rats (10 of them) were fed ad libitum (AL). Rats in the postabsorptive state were anesthetized with isoflurane and euthanized by beheading. Blood was sampled, and muscles of the hindlimbs (tibialis anterior) were rapidly removed, weighed, and frozen in liquid nitrogen. Soluble proteins were extracted, and immunoblotting was performed as described previously (22). Plasma was also collected to measure CIT concentration as described below (33).

Ex vivo studies. Our protocol was reviewed and approved by the Ile-de-France Regional Ethics Committee (no. P2.CM.032.07) for the use of animals in research. Eighty 3-mo-old male Sprague-Dawley rats (Charles River, L'Arbresle, France) were used. They were maintained in a 12-h light-dark cycle with a standard rodent diet (UAR, Villemoisson-sur-Orge, France), with ad libitum access to water. Spontaneous intakes were determined daily. At the end of the acclimatization period, the rats were randomized into two groups. A healthy group (n = 40) was fed ad libitum for 6 wk with the standard diet. A malnourished group (n = 40) was subjected to dietary restriction {50% of the spontaneous food intake with a 5% protein diet [5% (wt/wt) proteins, 3% (wt/wt) lipids, 59% (wt/wt) carbohydrates, and 33% (wt/wt) water, fiber, vitamins, and minerals];UAR} for 6 wk, as described previously (40). Animal weights and spontaneous intakes were recorded daily. After 6 wk of experimentation, rats in the postabsorptive state were euthanized by beheading after general anesthesia with isoflurane (Minerve, Esternay, France).

Muscle incubation. All of the chemicals named below were purchased from Sigma Aldrich Chemical (Saint-Quentin-Fallavier, France), except for L-citrulline (L-CIT), which was a gift from Kyowa Hakko. (Tokyo, Japan). The inhibitors used were purchased from Tebu-Bio (Le Perray-en-Yvelines, France). Muscles were incubated described as previously (26, 27). Briefly, epitrochlearis muscles were dissected intact for incubation. They were immediately preincubated for 30 min at 37°C in Krebs-Henseleit buffer (KHB; 120 mM NaCl, 4.8 mM KCl, 25 mM NaHCO₃, 1 mM CaCl₂, 1.2 mM NaH₂PO₄ and 1.2 mM MgSO₄) supplemented with 2 mM HEPES, pH 7.4, 8 mM glucose, and 10 mU/ml insulin, and saturated with 95% O₂-5% CO₂ gas mixture. The muscles were then transferred for 120 min in fresh media of the same composition containing 1 mM L-[ring-¹³C]phenylalanine (99 atom%; Cambridge Isotope Laboratories, Andover, MA) with and without 2.5 mM L-CIT. The dose was chosen according to an in vivo study (33) showing that CIT supplementation in aged rats led to a CIT plasma concentration of ~2.5 mM. In additional experiments, the same studies were performed with selective inhibitors to explore the involvement of mTORC1/PI3K/MAPK pathways. Briefly, epitrochlearis muscles were preincubated for 30 min in KHB supplemented with 2 mM HEPES, pH 7.4, 8 mM glucose, 10 mU/ml insulin, and a selective inhibitor (INH) as described in Table 1. The muscles were then transferred for 120 min in fresh medium of the same composition with supplementation of 2.5 mM CIT and 1 mM L-[ring-¹³C₆] phenylalanine. After incubation, the two epitrochlearis muscles were removed, blotted on filter paper, weighed, and frozen in liquid nitrogen, and the medium was aliquoted and stored at -20°C until analysis.

Determination of whole muscle protein synthesis. Tissue protein synthesis rates were evaluated by the flooding dose method, as
 Table 1. Treatment and experimental conditions of muscle incubations

	Ctrl-	CIT	CIT + R	CIT + W	CIT + P
KHB, glc (8 mM), insulin (10 mU/ml),					
and 1 mM L-[ring- ¹³ C]phenylalanine	+	+	+	+	+
CIT (2.5 mM)	_	+	+	+	+
R (200 nM)	_	_	+	_	-
W (200 nM)	-	_	_	+	_
P (20 μM)	-	-	-	-	+

Ctrl, control; CIT, citrulline; R, rapamycin; W, wortmannin; P, PD-98059; KHB, Krebs-Henseleit buffer; glc, glucose.

described previously (35), measuring the incorporation of labeled L-[*ring*-¹³C₆]phenylalanine in the protein pool. Fractional synthesis rates (FSR) of proteins were calculated using the following equation:

$$FSR = (Ei \times 100)/(Ep \times t)$$

where Ei is the enrichment as atom percentage excess of L-[*ring*- $^{13}C_6$] phenylalanine derived from phenylalanine from proteins at time *t* (minus basal enrichment), Ep is the mean enrichment in the precursor pool (tissue fluid L-[*ring*- $^{13}C_6$]phenylalanine), and *t* is the incorporation time in hours. FSR data are expressed in percentage per hour (%/h).

Absolute synthesis rate (ASR) is calculated as $ASR = P \times FSR$, where P is the protein content. ASR data are expressed as milligrams of proteins per 24 h (mg/24h).

Determination of subcellular protein synthesis. Protein synthesis of each protein fraction (sarcoplasmic, mitochondrial, and myofibrillar) was also assessed as described previously (16). Only FSR was measured, because all the samples were used up for the determination of enrichment, and no material was left for the determination of protein content. Data (FSR) are expressed in %/h.

Determination of free amino acid levels. Media were deproteinized with a 30% (wt/vol) sulfosalicylic acid solution. Frozen epitrochlearis muscles were homogenized in ice-cold 10% trichloroacetic acid containing 0.5 mM EDTA and 125 μ M norvaline as a sample preparation internal standard. Amino acids were assayed as described previously (31). Muscle amino acid concentrations are expressed in micromoles per gram tissue.

In vitro studies. Primary cultures were derived from gastrocnemia and tibialis anterior muscles of 4-wk-old male mice, as previously described (32). Cells were plated at low density (100 cells/cm²) on 0.02% gelatin-coated dishes (cold water fish skin; Sigma, Saint-Quentin-Fallavier, France) and grown in complete medium composed of DMEM-Ham's F-12 (GIBCO, Invitrogen, Saint-Aubin, France), 2% Ultroser G (Biosepra, Pall corporation, Saint-Germain-en-Laye, France), 20% fetal calf serum (Sigma-Aldrich), and antibiotic-antimycotic solution (10,000 U/ml penicillin G sodium, 10,000 µg/ml streptomycin sulfate, and 25 µg/ml amphotericin B; GIBCO, Invitrogen). To differentiate muscle cells into myotubes, myoblasts were plated on Matrigel-coated dishes (Dutscher, Brumath, France) in complete medium and, after 6-h adhesion, switched to DMEM-Ham's F-12 + 2% horse serum (Sigma-Aldrich). Myotubes were then serumand amino acid-deprived for 16 h on day 2 of differentiation in serumand amino acid-free DMEM (Eurobio, Courtaboeuf, France) containing 0.2% BSA (Sigma-Aldrich, Saint-Quentin-Fallavier, France).

Experiment 1. To characterize the kinetic effect of CIT on the mTORC1 pathway, serum- and amino acid-deprived myotubes were incubated in serum- and amino acid-free DMEM with and without 5 mM CIT for 60, 90, 120, 180, 240, and 300 min. Incubation in a standard DMEM (DMEM-Ham's F-12; Gibco) was used as positive control. The dose of CIT was determined according to previous studies evaluating the effect of amino acids (i.e., leucine or glutamine) in vitro (2, 5, 14).

Experiment 2. Serum- and amino acid-deprived cells were pretreated with and without selective inhibitors, as described in Table 2, for 30 min in serum- and amino acid-free DMEM. After a preincubation period, cells were incubated for 120 min (i.e., optimal conditions as determined in *experiment 1*) in the conditions described in Table 2.

Extraction of soluble proteins. Cells were washed twice with cold PBS and then scraped from the culture dish into lysis buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 5 mM EDTA, phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium fluoride), and protease inhibitors (complete protease inhibitor cocktail; Roche, Meylan, France)]. After lysis (30 min, 4°C), the samples were centrifuged at 10,000 g for 20 min at 4°C. The protein concentration of the clarified lysates was determined using the Lowry assay (DC Protein Assay Kit 2; Bio-Rad, Marne-La-Coquette, France).

Immunoblotting. All antibodies were purchased from Cell Signaling Technology (Ozyme, St. Quentin-en-Yvelines, France): phosphorylated eukaryotic initiation factor 4E-binding protein 1 (4E-BP1; Ser⁶⁵; no. 9451, 1:500), phospho-4E-BP1 (Thr^{37/46}; no. 2855, 1:500), 4E-BP1 (no. 9644, 1:1,000), phosphorylated p70 ribosomal protein S6 kinase 1 (S6K1; Thr³⁸⁹; no. 9234, 1:500), S6K1 (no. 2708, 1:500), and anti-rabbit IgG horseradish peroxidase-linked antibody (no. 7074, 1:2,000). Samples were then standardized to 0.5 mg/ml by dilution with 3× Laemmli SDS sample buffer (Ozyme, Saint-Quentin-en-Yvelines, France) containing 30% glycerol, 0.625 M Tris (pH 6.8), 20% (wt/vol) SDS, 0.5% (wt/vol) bromophenol blue, dH₂O, and 1:9 dilution β -mercaptoethanol and heated at 95°C for 10 min. Protein at 20 µg/lane was loaded onto TGX 12% SDS-PAGE precast gels (Bio-Rad) and transferred to nitrocellulose membranes (Hybond-C Extra; GE Healthcare, Aulnay-sous-Bois, France) at 120 mA for 2 h. After membranes were blocked for 1 h in TBS-T buffer (10 mM Tris·HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) with 5% nonfat skimmed milk, blots were incubated overnight at 4°C with primary antibodies recognizing phosphorylated forms. After washing, the membranes were incubated with horseradish peroxidaseconjugated secondary antibodies for 1 h in TBS-T with gentle agitation. Proteins were then visualized using enhanced chemiluminescence (ECL Prime; GE Healthcare) on ImageQuant Las 4000 system (GE-Healthcare) using a CCD camera. Band density was quantified using ImageJ software. For normalization, blots were stripped using antibody stripping buffer (Gene Bio-Application, Paris, France) and then reprobed for total proteins to verify the relative amount analyzed.

Statistics. In the in vivo and in vitro studies, nonparametric tests were used, avoiding Gaussian assumptions.

In ex vivo studies, relationships between absolute synthesis rate (ASR) with and without CIT and ASR under inhibitor were modeled by linear approaches. Bayesian inferences were chosen because they were well suited to small samples.

In the in vivo studies, differences in phosphorylation state of the mTORC1 targets were studied using nonparametric ANOVA among four groups (AL, R, Ctrl, and CIT) using a Kruskal-Wallis test.

Table 2. Treatment and experimental conditions of primarymyotube cultures

	Ctrl-	Ctrl+	CIT	CIT + R	CIT + L	CIT + P
DMEM-Ham's F-12	_	+	_	_	_	_
Serum- and amino acid-free						
DMEM	+	_	+	+	+	+
CIT (5 mM)	_	_	+	+	+	+
R (20 nM)	_	_	_	+	_	_
L (50 µM)	_	_	_	_	+	-
P (10 µM)	-	—	-	-	_	+

Ctrl, control; CIT, citrulline; R, rapamycin; L, LY-294002; P, PD-98059.

Correlations between CIT concentrations and phosphorylation level of S6K1 were estimated and tested by a Spearman correlation test.

Ex vivo studies were analyzed using a Bayesian linear model (13). This model assesses associations between ASR with CIT and ASR under control for *group 1* and between ASR with CIT and ASR under inhibitor for *group 2*. Two models were implemented by a Bayesian approach: model 1 without constraints on slopes and model 2 assuming an inverse relationship between slopes for the two groups. More precisely, for rat i:

Model 1:
$$ASR^{(CIT,G1)}_{i} = \alpha ASR^{(ctrl,G1)}_{i} + \varepsilon_{i}$$
 and $ASR^{(CIT+inh,G2)}_{i} = \beta ASR^{(CIT,G2)}_{i} + \varepsilon_{i}$
Model 2: $ASR^{(CIT,G1)}_{i} = \alpha ASR^{(ctrl,G1)}_{i} + \varepsilon_{i}$ and $ASR^{(CIT+inh,G2)}_{i} = (1/\alpha) ASR^{(CIT,G2)}_{i} + \varepsilon_{i}$

where ASR^(T, G) is ASR for rats under "treatment" T (t = CIT, Ctrl, or CIT + inh) in group G (G = group 1 or group 2).

For each model, positivity of slopes was assumed with a null intercept and Gaussian residuals with different variances between groups. These models were estimated for healthy and malnourished rats separately. Vague positive prior distributions were chosen for slopes (exponential with parameter equal to 0.01) and for variances (inverse- γ with parameters equal to 0.01 and 0.01). Bayesian inference was done via Marko Chain Monte Carlo algorithms with 50,000 iterations and 1,000 iterations for burn-in, using Winbugs software (23). Convergence was assessed by checking good mixing between iterations and accurate approximations by MC error. Results are expressed as posterior means of slopes and a 95% posterior credibility interval. The advantage of a Bayesian approach is that it offers the possibility of estimating posterior distribution not only of parameters but also of all quantities of interest directly deduced from parameters. In particular, for *model 1*, we were interested in the product $\alpha\beta$; its posterior mean and 95% posterior credibility interval will also be given.

In the in vitro studies, the effects of CIT with and without inhibitors on phosphorylation status were tested with nonparametric rank sum tests. The effect of CIT on the phosphorylation state of S6K1 and 4E-BP1 was appraised via the study of the distribution of relative phosphorylated values: the median was then compared with value 1 with a Wilcoxon test. Concerning the effects of inhibitors, two groups were compared (1 group with CIT and another with CIT and inhibitor) with a Wilcoxon-Mann-Whitney test.

RESULTS

Effect of a CIT-enriched diet on muscular mTORC1 activation in aged malnourished rats. A previous study (33) had shown that in this aged rat model, CIT was able to increase MPS. We thus evaluated the phosphorylation status of S6K1 and 4E-BP1 in the tibialis of these rats by Western blot. Following caloric restriction, a marked decrease in S6K1 phosphorylation status (AL vs. R, P < 0.05; Fig. 1A) and a nonsignificant decrease in 4E-BP1 phosphorylation status (AL vs. R, P = 0.116; Fig. 1B) were observed. This latter result probably lacked power, as the figure clearly shows the decrease. Interestingly, only CIT-supplemented rats activated the phosphorylation status of those proteins in skeletal muscle to normal levels.

Correlations between citrullinemia and ratio of phosphorylation/total level of S6K1. As reported previously (33), CIT was largely increased by CIT supplementation (CIT: 2394 \pm 279 vs. R: 104 \pm 7, P < 0.05), and only ornithine and arginine patterns were modified. The increase in phosphorylation level of S6K1 was positively correlated with the CIT concentration in plasma from CIT-treated rats (Spearman

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Fig. 1. Effect of a citrulline (CIT)-enriched diet on muscular mammalian target of rapamycin complex 1 (mTORC1) activation in aged, malnourished rats. One group of aged rats (20 mo old) was healthy [ad libitum (AL)]. The other 3 groups were restricted for 12 wk; 1 group was euthanized at the end of the restricted period (R), and 1 group was refed for 5 days with a control diet (Ctrl) or with a CIT-supplemented diet (CIT). Phosphorylation status of p70 ribosomal protein S6 kinase 1 (S6K1; *A*) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1; *B*) in skeletal muscles was measured by Western blot. Nonparametric ANOVA on S6K1 phosphorylation values and on 4E-BP1 phosphorylation values among 4 groups (AL, R, Ctrl, and CIT) rejected significantly means equality between groups (Kruskal-Wallis test, P = 0.023 and P = 0.0289, respectively). Posterior comparison was made by a nonparametric Wilcoxon test. *P < 0.05 vs. R. AU, arbitrary units.

correlation coefficient between S6K1 and [CIT] in plasma was 0.72, P < 0.05; Fig. 2A). We found no correlation between CIT concentration in muscle and S6K1 phosphorylation level (Fig. 2B).

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Effect of CIT on protein synthesis in isolated epitrochlearis muscle. We investigated whether the muscle was the direct target of CIT for regulation of muscle protein synthesis using an isolated incubated muscle model. For this purpose, epitrochlearis muscles from healthy or malnourished rats were incubated for 2 h with and without CIT. Whole muscle protein synthesis, fractional protein synthesis (i.e., myofibrillar, mitochondrial, and sarcoplasmic proteins), and amino acid patterns were determined. CIT treatment increased by 80% (α^{M} = 1.89) on average the absolute synthesis rate (ASR) of malnourished rats (Fig. 3*B*), whereas FSRs were not modified (data not shown). However, although total FSR was not affected by CIT, it was increased specifically in subcellular fractions from healthy (Fig. 3*C*) and malnourished rats (Fig. 3*D*).

Moreover, incubation with CIT elevated muscle concentration of this amino acid 12-fold (CIT: 7.50 ± 1.00 vs. Ctrl: $0.65 \pm 0.10 \ \mu$ mol/g, means \pm SE, *t*-test: P < 0.001 vs. Ctrl), whereas concentrations of other amino acids in muscle, in particular CIT-related amino acids (i.e., ornithine and arginine), were not influenced by CIT incubation.



Fig. 2. Correlations between citrullinemia and ratio of phosphorylation to total level of S6K1 from CIT-treated rats (*A*) and between citrulline in tibialis muscle and phosphorylation level of S6K1 from CIT-treated rats (*B*). Correlations were estimated and tested by a Spearman correlation test.



Fig. 3. Effect of CIT treatment on muscle protein synthesis in isolated epitrochlearis muscle. Epitrochlearis muscles of healthy and malnourished rats were incubated for 120 min with citrulline (CIT; 2.5 mM) and without CIT (Ctrl). Synthesis rates were estimated using rate of L-[*ring*-¹³C]phenylalanine incorporation into proteins. *A* and *B*: box plot charting the distribution of absolute synthesis rate (ASR) in epitochlearis muscle of healthy (*A*) and malnourished rats (*B*) incubated with and without CIT. ASR is expressed in mg/24 h. The effect of CIT was analyzed by a Bayesian approach (model 1 without constraint), with Ctrl to CIT being estimated by the parameter- α is expressed as posterior means of slopes and a 95% posterior credibility interval, and it is significantly different from whether one excluded from the interval. α^{H} , healthy; α^{M} , malnourished. *C* and *D*: fractional synthesis rate (FSR) of each protein fraction of epitrochlearis from healthy (*C*) and malnourished rats (*D*). Light gray bars, control; dark gray bars, CIT. FSR is expressed in %/h. Differences were studied using a nonparametric Mann-Whitney test. **P* < 0.05 vs. Ctrl; #*P* = 0.07 vs. Ctrl.

Effect of selective inhibitors on protein synthesis of muscle incubated with citrulline. To investigate mechanisms by which CIT modulates MPS, we explored transductional pathways (i.e., mTORC1, PI3K/Akt, and MAPK pathways) in epitrochlearis muscle by an inhibitor-based approach. For this purpose, epitrochlearis of healthy and malnourished rats were incubated for 2 h with CIT and with CIT and selective inhibitors. Whole muscle protein synthesis was determined by the flooding dose method. We postulated that the action of the selective inhibitors used (rapamycin, wortmannin, and PD-98059) would lead to a return to baseline; i.e., we assumed that $\beta = 1/\alpha$. To test this assumption, we studied the product $\alpha\beta$ under *model 1* (Table 3). Because one is generally included in the 95% posterior credibility interval, the assumption that $\beta = 1/\alpha$ seems reasonable. Further results were obtained under model 2 (with constraints), enabling a statistical power increase.

The presence of rapamycin (inhibitor of mTORC1 pathway; Fig. 4A) decreased absolute synthesis rate by 44% on average in muscle of malnourished rats. A decrease in 25% for healthy rats was weakly significant. The same results were obtained for wortmannin (inhibitor of the PI3K path-

way; Fig. 4B) and for PD-98059 (inhibitor of the MAP kinase pathway) (Fig. 4C).

CIT-induced stimulation was completely inhibited in the presence of the inhibitors, demonstrating that the activation of

Table 3. Comparison of passages from control to citrullineand from citrulline to citrulline with inhibitors

	$\alpha\beta^R$	$\alpha\beta^{P}$	$\alpha\beta^{W}$
	CIT + INH of mTORC1	CIT + INH of MAPK kinase	CIT + INH of PI3K
Healthy rats Malnourished	0.92 (0.24, 1.53)	1.26 (0.75, 1.83)	0.51 (0.06,0.92)
rats	0.98 (0.13, 2.20)	1.03 (0.20, 2.10)	1.04 (0.12, 2.35

 $\alpha\beta$ -product of each inhibitor is defined as follows: R, rapamycin; P, PD-98059; W, wortmannin; CIT, citrulline; INH, inhibitor; mTORC1, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase. The parameter-α corresponds to the passage Ctrl – to CIT and parameter-β to the passage CIT to CIT + INH. Results are expressed as posterior means of αβ product slopes and a 95% posterior credibility interval. When one is included in the interval, then the assumption α = 1/β is not rejected [Bayesian approach (model 1 without constraint) as described above].

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Fig. 4. Selective inhibitors totally blunted CIT effect in incubated epitrochlearis muscles. Epitrochlearis muscles of healthy and malnourished rats were incubated with CIT (2.5 mM) for 120 min with selective inhibitor (CIT + INH) and without selective inhibitor (CIT). Synthesis rates were estimated using rate of L-[*ring*-¹³C]phenylalanine incorporation into proteins. Box plot charting the distribution of ASR in epitochlearis muscle of healthy and malnourished rats incubated with and without rapamycin (20 nM; *A*), with and without wortmannin (200 nM; *B*), or with and without PD-98059 (20 μ M; *C*). ASR is expressed in mg/24 h. As described above, the effects were analyzed by a Bayesian approach (model 2 with constraint where $\beta = 1/\alpha$). Parameter- β is expressed as posterior means of slopes and a 95% posterior credibility interval, and it is significantly different from whether one is excluded from the interval. H, healthy; M, malnourished; R, rapamycin; W, wortmannin; P, PD-98059.

these pathways was necessary for the CIT-induced stimulation of muscle protein synthesis.

CIT activated mTORC1 pathway in culture of primary myotubes. To investigate the effect of CIT on the mTORC1 pathway, we used culture of primary muscle cells (Fig. 5). Thus serum- and amino acid-deprived myotubes were treated with 5 mM CIT for 120 min following a Western blot test to measure the phosphorylation status of 4E-BP1 and S6K1. Amino acid deprivation is associated with a decrease in mTORC1 activation (see Fig. 6). As shown in Fig. 5, CIT supplementation led to a 2.5-fold increase in the phosphorylation status of S6K1 (Fig. 5A) and a 1.5-fold increase in the phosphorylation status of 4E-BP1 (Fig. 5B) compared with untreated control. However, after 120 min of treatment of CIT, phosphorylation of 4E-BP1 at Thr^{37/46} was unaffected (data not shown).

Effects of inhibitors on phosphorylation status of S6K1 and 4E-BP1. To explore the possible involvement of the mTORC1, PI3K/Akt, and MAP kinases/ERK1/2 pathways, cells were incubated with the specific inhibitors rapamycin, LY-294002, and PD-98059, respectively. We observed that both rapamycin and PD-98059 inhibited the CIT effect on S6K1 (Mann-Whitney, P = 0.0039 for both tests; Fig. 7, A1 and C1). Only LY-294002 inhibited CIT effect on the two targets of mTORC1 (Mann-Whitney, P = 0.0039 and 0.0038; Fig. 7, B1 and B2, respectively).



Fig. 5. CIT stimulated mTORC1 pathway in myotubes in culture. Primary myotubes were serum and amino acid deprived for 16 h and treated with CIT (5 mM) and without CIT (Ctrl) for 2 h. Phosphorylation status of mTORC1 targets (4E-BP1 and S6K1) was measured by Western blot. Box plot charting the distribution of relative phosphorylated values of S6K1 (*A*) and 4E-BP1 (*B*) in serum- and amino acid-deprived myotubes after 120 min of stimulation with CIT (5 mM). *P < 0.05 (n = 12) for nonparametric Wilcoxon test.

DISCUSSION

Citrulline has emerged as an important regulator of nitrogen homeostasis (6, 9). Over the last 10 yr, the ability of CIT to modulate protein metabolism has been shown in different situations (short bowel syndrome, fasting, aging, etc.) and in both animals and humans. However, the underlying mechanism of this action remains poorly understood. The aim of this study was to determine the precise mechanism by which CIT administration influences MPS.

The mTORC1 signaling pathway is considered as a nitrogen-sensing pathway regulating skeletal MPS (17) and the recent work by Guridi et al. (18) confirms that regulation of mTORC1 determines lean body mass. Experimental and clinical studies have shown that essential amino acids, particularly



Fig. 6. Effect of amino acid deprivation on mTORC1 signaling pathway in myotubes in culture. Primary myotubes were serum and amino acid deprived for 16 h and treated without serum with a standard DMEM containing amino acids (DMEM-Ham's F-12) or with amino acid-free DMEM for 2 h. Phosphorylation status of mTORC1 targets (4E-BP1 and S6K1) was measured by Western blot.

leucine, activate the mTORC1 pathway and consequently stimulate MPS (12).

In the present study, we show that CIT supplementation in malnourished aged rats is able to stimulate the mTORC1 pathway in the skeletal muscle. This experimental result is in line with those of others using a model of short fasting (22) but seems to conflict with those of Churchward-Venne et al. (7). These authors did not observe any effect of CIT on muscle protein synthesis or mTORC1 activation in elderly men. This discrepancy could be linked to marked differences between our work and the design of this study. First, Churchward-Venne et al. (7) compared the effect of a very high dose of protein (45 g of whey protein) or high dose of protein (15 g of protein) plus CIT. In these conditions, they were not exploring the effect of CIT but rather, how replacement of some protein by CIT would have the same effect (not the same question). Second, considering the effect on the mTORC1 pathway, they did not observe any effect of CIT (in combination with whey protein) between the two groups studied, but the authors did not evaluate mTORC1 activation in basal conditions, which does not allow any conclusion to be drawn about the possible effect of CIT. However, in vivo, the anabolic effect of CIT on muscle could be indirect and mediated by hormonal change, such as stimulation of insulin secretion. Insulin is known to stimulate MPS through activation of the PI3K/Akt pathway. However, this is unlikely because no effect of CIT was observed on insulin secretion in either animal (22) or human studies (20, 29). This hypothesis is supported at the transductional level, since we observed no CIT-related activation of Akt (the main insulin target). Taken together, these data show that the anabolic action of CIT is independent of insulin and the Akt pathway. Interestingly, we observed a positive correlation between plasma CIT concentration (but not muscle CIT concentration) and S6K1 phosphorylation. Similar results were obtained in a previous study (34) in a model of short bowel syndrome rats in

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Fig. 7. Effect of selective inhibitors of CIT activation on mTORC1 in deprived myotubes. Primary myotubes were serum and amino acid deprived for 16 h and treated with CIT (5 mM), CIT + rapamycin (20 nM), CIT + LY-294002 (50 μ M), and CIT + PD-98059 (10 μ M) for 2 h. Phosphorylation status of mTORC1 targets (4E-BP1 and S6K1) was measured by Western blot. Box plot charting the distribution of relative phosphorylated values of S6K1 (*A1*, *B1*, and *C1*) and 4E-BP1 (*A2*, *B2*, and *C2*). **P* < 0.05 for nonparametric Mann-Whitney test comparing CIT and CIT + INH.

which the increase in muscle weight was correlated with CIT concentration in plasma but not in muscle. These correlations suggest that the CIT effect could be direct on the muscle signaling pathway. However, measurement of MPS and phosphorylation state of targets of mTORC1 does not prove causality. In particular, CIT is subject to intense interorgan trafficking, generating glutamine, arginine, or other amino acids (8) that could be the true activation of MPS at the muscle level. For this reason, we used an ex vivo approach, enabling us to significantly improve our knowledge. As observed in vivo, CIT increases MPS in incubated epitrochlearis, suggesting a direct effect of this amino acid. In addition, the amino acid pattern was not affected by CIT in either the incubation medium or muscle. This result was expected because muscle does not possess the enzymatic equipment (i.e., argininosuccinate synthetase and argininosuccinate lyase) for the metabolization of CIT into arginine (39). A limitation of our work is that only CIT properties were explored. Thus, it would be of interest to compare CIT with its other metabolites (i.e., arginine, ornithine, or glutamine) to evaluate their respective effect.

Muscle is composed of several groups of proteins differing in their function: myofibrillar proteins (for contraction), mitochondrial proteins (for energy production), and sarcoplasmic proteins involved in numerous other functions (26). Interestingly, we demonstrate that CIT action did not affect all protein fractions. This result is in line with previous data (10) obtained in vivo indicating that CIT supplementation increases the expression of protein involved in muscle contraction. In this ex vivo study, we explored the possible involvement of the mTORC1 pathway with the use of metabolic inhibitors. Interestingly, pretreatment of incubated muscle with rapamycin clearly demonstrated that MPS activation by CIT is an mTORC1-dependent mechanism, confirming in vivo results. To better characterize the mechanism of action, the use of LY-294002 and PD-98059 supports the possible involvement of PI3K and MAP kinase in CIT action, since we observed that they were all efficient in suppressing CIT-induced increase in MPS. However, this model is not best suited to determining the precise relationship between MAP kinase, PI3K, and mTORC1. Also, muscle is a complex structure made up of different cell types, and our results based on the ex vivo model are not sufficient to assert that CIT directly affects MPS. For this reason, we used an in vitro approach. Consistent with our ex vivo studies, we report that CIT directly stimulates mTORC1 signaling via an overactivation of S6K1 and 4E-BP1 in myotubes. This last experiment confirms that CIT activates mTORC1 by a myotube autonomous system.

By characterizing this CIT effect at the molecular level, we show that rapamycin affects S6K1 but not 4E-BP1 phosphorylation status. This result is surprising since S6K1 and 4E-BP1 are downstream targets of mTORC1. However, this type of mTORC1-independent regulation of 4E-BP1 was recently described by others (41) and showed in a model of C_2C_{12} myoblasts that mTORC1 inhibition did not alter the phosphorylation state of 4E-BP1 (30). In particular, these authors showed that 4E-BP1 could be regulated by the PI3K/Akt pathway and S6K1 by both the PI3K/Akt and MAPK/ERK1/2 pathways. Such mechanisms would be consistent with our ex vivo observations and our in vivo observation (higher activation of 4E-BP1 than S6K1).

Thus PI3K inhibition abolishes the CIT action on 4E-BP1 and S6K1 and supports a CIT-mediated regulation of S6K1 and 4E-BP1 by this transduction pathway. To refine this result, the MAPK/ERK1/2 pathway was also studied (using PD-98059 as an inhibitor) because this pathway interacts with the PI3K/Akt pathway. Our results suggest that S6K1 activation is linked to both PI3K and MAPK/ERK1/2, whereas 4E-BP1 activation involves only PI3K.

Taken as a whole, our data suggest an mTORC1-dependent mechanism of CIT action on S6K1 involving MAPK/ERK1/2 and PI3K/Akt pathways. Whereas the relationship between PI3K and MAPK/ERK1/2 has been demonstrated (1, 25), their interrelationship is still not clear because it often depends on the cell type under study and on the experimental conditions.

Finally, our results show a relationship between the activation of PI3K/MAPK/4E-BP1 pathways by CIT and its action on MPS, because the inhibition of PI3K and MAP kinase profoundly disturbed the enhancing effect of CIT, confirming our in vitro observations.

In recent years, a new concept has emerged to explain some specific properties of amino acids, that of nutrient transceptors (36), i.e., amino acid transporters, which may regulate cellular trafficking and are also called amino acid transceptors (19). However, to date, very little is known about CIT membrane transporters (3), and further work will be necessary to confirm this last hypothesis.

To conclude, our combined data obtained in vivo, ex vivo, and in vitro clearly establish for the first time the role of CIT as an important signaling amino acid for the control of MPS.

GRANTS

This work was supported by a grant from the French Ministry of Research (contract quadriennal EA 4466).

DISCLOSURES

S. Le Plénier, L. Cynober, and C. Moinard are shareholders of Citrage.

AUTHOR CONTRIBUTIONS

S.L.P., A.G., E.A., C.G., J.S., and M.J. performed experiments; S.L.P., A.G., A.S., J.S., and N.N. analyzed data; S.L.P., A.S., S.W., N.N., and C.M. interpreted results of experiments; S.L.P. prepared figures; S.L.P., L.C., and C.M. drafted manuscript; S.L.P., A.G., A.S., E.A., C.G., S.W., J.S., M.J., N.N., L.C., and C.M. edited and revised manuscript; A.G., A.S., C.G., S.W., J.S., M.J., N.N., L.C., and C.M. approved final version of manuscript

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REVIEW

Regulation of the proteome by amino acids

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Besides their main contribution as substrates for protein synthesis, amino acids as signaling molecules could exert some regulatory functions on protein synthesis and/or proteolysis that have been emphasized in a number of recent studies. Several publications have highlighted supplemental roles of those amino acids in protein metabolism as well as in immunity, heat shock response, or apoptosis processes. In this way, via their regulatory properties, selected amino acids (such as leucine, glutamine, arginine, citrulline, or methionine) directly influence the proteome. In this review, we are proposing an overview of the regulation of the proteome by amino acids in mammals.

Received: August 26, 2015 Revised: December 30, 2015 Accepted: January 12, 2016

Keywords:

Animal proteomics / Arginine / Citrulline / Glutamine / Leucine / Methionine



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1 Introduction

Proteins are among the most abundant macromolecules in humans with about 10 kg of the whole body weight. The maintenance of such a protein mass requires the existence of a strictly regulated homeostasis (Supporting Information Fig. 1). The maintenance of protein homeostasis results from

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Abbreviations: 4E-BP, eukaryotic translation initiation factor 4E binding protein; AMPK, adenosine monophosphate-activated protein kinase; ARG, arginine; ATP, adenosine triphosphate; BCAA, branched-chain amino acid; BCKA, branched-chain keto acid; CIT, citrulline; eIF4, eukaryotic translation initiation factor 4; GCN2, general control nonderepressible 2; GLN, glutamine; IL, interleukin; iNOS, inducible nitric oxide synthase; LEU, leucine; MAP kinase, mitogen-activated protein kinase; MET, methionine; MPS, muscle protein synthesis; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; PA, phosphatidic acid; PI3K, phosphoinositide 3-kinase; rpS6, ribosomal protein S6; S6K, S6 kinase; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor a strictly controlled balance between protein intake, protein synthesis, proteolysis, protein metabolism, and amino acids oxidation [1]. The existence of a rapid protein turnover allows a quick adaptation to a variation of nutritional and/or pathophysiological conditions. Interestingly, protein synthesis is performed from a very small free amino acids pool (around 70 g, which represents <1% of the total amino acids). That pool is itself compartmentalized into two pools: an extracellular and an intracellular one containing 95% of the free amino acids, being the true precursors of protein synthesis. The impact of the free amino acid pool on protein synthesis was shown decades ago, especially when it was discovered to be a substrate to regulate protein synthesis for the growth and maintenance of body tissues (skin, tendons, muscles, organs) as well as for enzyme and other protein regulation (proteins that are essential to maintain the volume and composition of body fluids, the acid-base balance, protein transport, and energy balance). Nevertheless, over the last 15 years, new concepts have emerged concerning amino acid properties, particularly on their role as regulators of the proteome. Amino acids have a broad impact that encompasses many events affecting cellular proteins ranging from the regulation of gene expression or transcription to protein synthesis, posttranslational modifications, the immune response, autophagy, or



Figure 1. Structures of the main free amino acids known to alter the proteome.

apoptosis. Besides their contribution as substrates for protein synthesis, amino acids-as signaling molecules-could exert some regulatory function on the protein turnover (i.e. protein synthesis, modifications and/or proteolysis that have been emphasized in a number of recent studies [2-4]). Interestingly, some amino acids have also been reported to play a substantial role in inflammation through pro- or antiinflammatory effects. In this review, we are proposing an overview of the regulation of the proteome by amino acids. Most of the studies have investigated the effects of amino acids on protein expression while very few of them have highlighted their effects on post-translational modifications, localization, and interactions of proteins. In the present review, we are focusing on the effects of leucine (LEU), glutamine (GLN), arginine (ARG), citrulline (CIT), and methionine (MET) on which several proteomic studies have been published. Some other amino acids could also exert noticeable effects, yet very few data are available as for them. The last part of this review highlights the mechanisms through which these amino acids regulate the mTOR complex 1 (mTORC1) signaling pathway, a signaling pathway involved in the control of the protein turnover.

2 Leucine

LEU (Fig. 1) is the most abundant amino acid in many dietary proteins (around 20% of all the dietary proteins in a human diet) and is one of the three proteinogenic branched-chain amino acids (BCAAs), a family that also includes isoleucine and valine. LEU is metabolized by BCAA aminotransferase into branched-chain keto acid (BCKA) prior to an oxidative decarboxylation by BCKA dehydrogenase and/or a release into the bloodstream [5]. The release can be distributed into different tissues (liver or adipose and muscular tissues) where it can be oxidized or resynthesized from BCKA through a cycle between the liver and muscles [6]. Among BCAAs, LEU seems to have the greatest impact on protein synthesis, protein degradation, glucose homeostasis, leptin secretion, and food intake (Table 1) [7].

Not only is LEU a substrate for protein synthesis but since the 1970s, it has been recognized as a main promoter of muscle protein synthesis (MPS) in the fed state in skeletal muscle and adipose tissues [8, 9]. The stimulatory effect of LEU on MPS is mediated via an activation of the mTORC1 pathway [10-12]. Indeed, LEU is able to stimulate the phosphorylation of mTORC1 and downstream proteins-namely eukaryotic translation initiation factor 4E binding protein (4E-BP1), S6 kinase (S6K1), and eukaryotic translation initiation factor 4E (eIF4E)—in this signaling pathway. The activation of protein synthesis via the activation of mTORC1 has not been well defined yet, but both insulin-dependent and independent mechanisms appear to be involved. Indeed, LEU causes a transient slight increase of insulin and it has been shown that in the presence of somatostatin, the phosphorylation of S6K1 and 4E-BP1 by LEU is attenuated, suggesting an insulin-dependent mechanism. However, the association of eIF4E-eIF4G, an important step in the initiation of translation, remains unchanged, suggesting an insulinindependent mechanism [13]. Meanwhile, Fujita et al. [14] showed that the effect of LEU on human MPS could be intimately related to the phosphorylation status of both adenosine monophosphate-activated protein kinase (AMPK) and mTORC1, two associated signaling proteins. More precisely, they showed that an LEU-enriched essential amino acidcarbohydrate mixture activated AMPK and mTORC1 in association with an increase in protein synthesis not only via an enhanced translation initiation but also by promoting translation elongation. As reported by Buse and Reid [8] and Fulks et al. [9] from rat diaphragm investigations, LEU also inhibits protein degradation in skeletal muscle as well as in liver, and therefore is considered as essential in/to protein turnover. This decrease of proteasome activity was also reported in human duodenum by Coëffier et al. [15] and Goichon et al. [4]. They provided evidence that this proteasome inhibition was due to an increase of the phosphorylation states of phosphoinositide 3-kinase (PI3K), Akt, AMPK, p38 MAPK, c-Jun N-terminal kinase, glycogen-synthase kinase (GSK)-3a/b, signal transducer and activator of transcription (STAT) 3, and STAT5 and increased cyclin D1 mRNA. Further investigations performed with a 2D-gel proteomic approach helped to assess the effects of an LEU supplementation on the whole duodenal mucosal proteome [15]. Data showed that LEU supplementation dysregulated the expression of four proteins involved in lipid metabolism (downregulation of

Table 1. Some proteomic changes mediated by LEU

Biological processes	Modulated proteins by LEU	Up- (+) or down (–)- change by LEU
Protein synthesis protein turnover	mTORC1	+
	phosphorylation of 4E-BP1	+
	phosphorylation of S6K1	+
	phosphorylation of eIF4E	+
	phosphorylation of PI3K	+
	phosphorylation of Akt	+
	phosphorylation of AMPK	+
	phosphorylation of p38 MAPK	+
	phosphorylation of JNK	+
	phosphorylation of GSK-3a/b	+
	phosphorylation of STAT3	+
	phosphorylation of STAT5	+
	cyclin D1 (mRNA)	+
	HADHA	_
	ACADVL	_
	CPT2	_
	FABP1	+
Glucose homeostasis	mTORC1	+
	GCN2	+
	phosphorylation of S6K1	+
	AMPK	_
	insulin	+
	insulin granule transport	+
	GTP-binding nuclear protein Ran	_
	dihydropyrimidinase-related protein 2	+
	proteasome subunit a type 6	+/- (unclear)
	cathepsin D	+
	serine/threonine-protein phosphatase PP1	_
	phosphorylation of chaperones GRP58	_
	phosphorylation of BiP (GRP78)	_
	phosphorylation of eIF-4B	+
	phosphorylation of RNA helicase DEAD box polypeptide 3	+
Leptin secretion	mTORC1	+
	phosphorylation of elF4G	+
	phosphorylation of S6K1	+
	phosphorylation of rpS6	+
	phosphorylation of 4E-BP1/ PHAS-1	+
	rpS6	+

ACADVL: acyl-coenzyme A dehydrogenase very long-chain; CPT2: carnitine O-palmitoyltransferase 2; FABP: fatty acid-binding protein; GSK: glycogen-synthase kinase; HADHA: trifunctional enzyme subunit alpha; JNK: c-Jun *N*-terminal kinase; PHAS:= phosphorylated heatand acid-stable protein.

trifunctional enzyme subunit alpha, acyl-Coenzyme A dehydrogenase very long-chain, and carnitine O-palmitoyltransferase 2; and upregulation of fatty acid-binding protein 1) suggesting a slowdown effect on fatty acid beta-oxidation in human duodenal mucosa. As previously mentioned, LEU also regulates glucose homeostasis mostly by providing substrates for gluconeogenesis; but LEU can also regulate the oxidative use of glucose by stimulating glucose recycling via the glucose-alanine cycle in skeletal muscle [16]. Yang et al. [17] showed that LEU could also be considered as a potential nutrient strategy for insulin secretion disorders due to its significant regulation of insulin secretion of pancreatic β cells. Based on the LEU role in glucose homeostasis and insulin secretion, several studies debated on the role of insulin in LEU-mediated protein synthesis [10, 13, 18–20]. They concluded that LEU affects protein synthesis through insulinindependent (the mTORC1 pathway) rather than insulindependent mechanisms. Nevertheless, LEU-mediated protein synthesis is still favored with basal insulin levels since it is well known that amino acids and insulin are both required to regulate the assembly of the eIF4E/eIF4G complex [21]. More recently, in isolated mouse islets and INS-1E cells [22], an increase of basal insulin secretion was reported by Liu et al. to be correlated to the alteration of proteins and genes involved in insulin granule transport, insulin trafficking (a decrease of GTP-binding nuclear protein Ran protein expression and an increase of dihydropyrimidinase-related protein 2 protein expression), insulin signal transduction (proteasome subunit a type 6), autophagy-related enzymes (cathepsin D), and the oxidative phosphorylation pathway (cytochrome c oxidase gene). LEU altered the expression of serine/threonine-protein phosphatase PP1 negatively, which was related to the phosphorylation/dephosphorylation states of proteins. Moreover, insulin 1 gene expression was downregulated by the LEU treatment while pancreas duodenum homeobox 1 and insulin 2 mRNA expressions were upregulated. The authors also evidenced that LEU induced a concomitant accumulation of cholesterol in INS-1E cells with an upregulation of enzymes involved in cholesterol biosynthesis (3-hydroxy-3-methylglutaryl-CoA reductase, mevalonate (diphospho) decarboxylase, and squalene epoxidase) and low-density lipoprotein receptors. LEU is also known to help reduce food intake and body weight. Cota et al. [23] evidenced that this effect was related to the mammalian target of rapamycin (mTOR) signaling pathway. In the rat, they reported an increase of hypothalamic mTOR signaling after the administration of LEU without investigating the expression of the whole proteome. However, such results were not observed in aged rats [24]. Interestingly, a few studies on LEU deprivation allowed to list proteins targeted by LEU. For instance, Talvas et al. [25] submitted C2C12 mouse myoblast muscle cells to LEU deprivation to evaluate its effect on phosphorylation levels of proteins. The 2D-PAGE proteomic approach combined to an IMAC enrichment allowed to demonstrate that molecular chaperone glucose-regulated protein 58 kDa (GRP58) and BiP (GRP78), RNA helicase DEAD box polypeptide 3 and eIF4B were phosphorylated protein targets of LEU deprivation. Meanwhile, Xiao et al. [26] investigated the alterations in metabolic proteins involved in the regulation of insulin sensitivity from mice, human HepG2 cells, and mouse primary hepatocytes under LEU deprivation. They concluded that LEU deprivation increased hepatic insulin sensitivity through the general control nonderepressible (GCN) 2, mTORC1/S6K1, and AMPK pathways. Besides, Zhang et al. [27] reported that dietary LEU intake did not only decrease hyperglycemia or increased insulin secretion but also decreased diet-induced obesity and hypercholesterolemia in mice. Fafournoux and co-workers [28] also reported an interesting study using a large-scale approach (transcriptomics) to figure out the impact of LEU deprivation on gene expression. They evidenced a dysregulation of 731 genes involved in several biological processes such as amino acid metabolism, lipid metabolism, and signal regulation. Thanks to MEF cells, either expressing or not expressing GCN2- and rapamycin treatments, the authors highlighted the fact that those dysregulations occurred not only through the mTORC1 pathway, but mostly through the GCN2 pathway. It also appeared that the GCN2-dependent regulation of these genes was accomplished via the function of the ATF4 transcription factor.

LEU also affects satiety by stimulating leptin secretion. Roh et al. [29] evidenced that leptin biosynthesis in rat adipocyte cells was favored by an administration of LEU without increasing the amount of leptin mRNA in adipocytes. Their study suggested that LEU activated the expression of leptin in adipose cells at the level of translation via the mTORC1mediated pathway through ribosomal protein S6 (rpS6), translational inhibitor 4E-BP/phosphorylated heat- and acidstable protein-1. Later, Lynch et al. [30] confirmed the implication of the mTORC1 signaling pathway in rat adipose tissue in leptin production by identifying other similar or additional targets (phosphorylation of eIF4G, S6K1, rpS6, and 4E-BP1).

To sum up, LEU is a major regulator of protein synthesis and turnover using mostly the downstream signaling pathways of 4E-BP1, S6K1 and eIF4E/mTORC1 in skeletal muscle and adipose tissue, but also in liver. Other studies evidenced that other signaling proteins such as APMK, PI3K, or Akt could be affected as well as proteins involved in lipid metabolism. Effects of LEU on glucose homeostasis, leptin secretion, and food intake were clearly evidenced. Although its mechanism of action in such cases remains to be fully elucidated, a few investigations highlighted the role of proteins and genes involved in insulin granule transport, insulin trafficking, signal transduction and oxidative phosphorylation pathways, chaperone glucose-regulated protein 58 kDa, RNA helicase DEAD box polypeptide 3, and eIF4B. We cannot conclude this LEU section without mentioning the relations in protein metabolism between GLN, alanine, and LEU: BCAAs including LEU are essential donors of nitrogen in synthesis of GLN and alanine in skeletal muscle [31].

3 Glutamine

GLN (Fig. 1) is the amide of glutamic acid, a dicarboxylic amino acid. In healthy humans, GLN is de novo synthesized in sufficient quantity to meet specific requirements, even if absent from the diet [32]. This amino acid is the most common one in plasma (0.6 mmol/L) and represents 61% of muscle-free amino acids [33]. Most importantly, in a pioneering paper, Jepson et al. [34] demonstrated, in several pathophysiological situations, a close relationship between muscle GLN concentration and MPS. Their results were confirmed in humans since the decrease in concentration of free GLN at muscular level is correlated with protein synthesis in this tissue [35]. Finally, in healthy individuals, the oral intake of GLN stimulates protein synthesis without modifying catabolism [36]. According to this result, it has been proposed that such a relationship could either be linked to a direct action of GLN on protein synthesis or purely coincidental. A possible explanation could be related to the ability of GLN to regulate cell volume by promoting cell swelling. GLN could activate transduction pathways (integrins, Src, and MAP-kinases) that could promote protein synthesis [37-39]. Another study evidenced a link between GLN and signaling pathways in intestinal protein synthesis. Boukhettala et al. [40] observed that GLN supplementation increased the phosphorylation level of 4E-BP1, a downstream protein in the mTORC1 signaling pathway.

Like LEU, GLN is an essential actor in protein turnover as it significantly affects proteolysis by specifically inhibiting the ubiquitin-adenosine triphosphate (ATP)-dependent proteolytic pathway, more especially [3, 41, 42] by promoting the expression of ubiquitin-conjugating enzyme E2 or (SUMO1) and ubiquitin-like protein SMT3A (SUMO2), but also by preventing the expression of ubiquitin, ubiquitinlike protein FAT10, or ubiquitin-conjugating enzyme E2 variant 2 in proinflammatory conditions. Another study claimed that GLN had an effect on signaling pathways in protein turnover by showing that GLN supplementation increased the phosphorylation level of S6K1, a downstream protein in the mTORC1 signaling pathway [43]. Moreover, GLN is required for extracellular LEU to activate mTORC1 [44] and recently, GLN metabolism has been shown to control mTORC1 [45, 46]. GLN could also act in other metabolic processes as a carbon and nitrogen interorgan transporter, gluconeogenic precursor, in the ammoniogenesis in kidney and may improve glucose metabolism by reducing insulin resistance [47, 48]. Although GLN is the most abundant-free amino acid in human blood, metabolic, and critical illnesses of patients in intensive care units after sepsis, surgery, trauma, or other injuries make them run a higher risk of GLN depletion [47, 49, 50]. In such cases, GLN is a conditionally essential amino acid and its role is vital in metabolism (as a major substrate for immune cells and enterocytes), cellular protection, inflammatory response, stress state, apoptosis, and prevention of organ injury as well as in the maintenance of intestinal protein metabolism by regulating gut homeostasis and the gut barrier function [49]. In addition, GLN may also improve glucose metabolism by reducing insulin resistance. The mechanism related to GLN protective effect in critical illnesses is still debated. While Preiser and Wernerman [48] claimed that GLN protective effects were related to improvements in tissue protection, immune regulation, preservation of glutathione, and antioxidant capacities as well as the preservation of cellular metabolism after injury, Curi et al. [51] indicated that GLN activated intracellular signaling pathways and regulated the expression of genes related to signal transduction, apoptosis, and metabolism. Sakiyama et al. [52] reported a link between GLN protective effects and signaling pathways. In their study, they evidenced that GLN regulated the mTORC1 and p38 mitogen-activated protein kinase (MAP kinase) pathways to induce autophagy under basal and stressed conditions and prevented apoptosis under heat stress. Evans et al. [53, 54] reported that GLN protected human colonic epithelial cells against cytokine-induced apoptosis characterized by nuclear condensation and the activation of caspase-8 and caspase-3. The authors evidenced that this GLN protection occurred through metabolic pathways independent from antioxidant glutathione (GSH) production, but mostly dependent on the pyrimidine pathway. It is also largely admitted that GLN beneficial effects are due to the induction of HSPs [55-59], such as HSP25/27, HSP32 (also named HO-1), HSP72, and more specially HSP70 whose expression depends upon the GLN concentration and is known to protect

the intestines against cellular, lung, ischemia/reperfusion injuries, and sepsis shock. For instance, Coëffier et al. [58] revealed in 2002 that GLN contributed to the protection of the intestines by enhancing HO-1 (HSP32) mRNA and protein expression in the human mucosa in (states of) intestinal inflammation. In 2005, Uehara et al. [59] reported that GLN administration as a single dose (0.75 g/kg) reduced gut cell apoptosis as well as gut injury via histology post endotoxemic shock and more specifically by enhancing hemeoxygenase-1 (HO-1) in the colon of rats exposed to endotoxemia, but not in the duodenum. Interestingly, GLN has also a substantial role in the inflammatory response. Coëffier et al. [60] reported in 2003 that GLN reduced the production of proinflammatory cytokines interleukin (IL)-6 and IL-8, and enhanced the production of antiinflammatory cytokine IL-10 in the human gut. In the lung after sepsis, Singleton et al. [61] figured out that GLN acted on the inflammatory response through the inhibition of lung tissue NF-KB activation and p38 MAPK/extracellular signal-regulated kinases/MKP-1 phosphorylation, $I\kappa B - \alpha$ degradation, and an attenuation of the cytokine release by significantly decreasing tumor necrosis factor (TNF)- α and IL-6. Furthermore, GLN was found to prevent inducible nitric oxide synthase (iNOS) expression in postsepsis lungs. Several years later, Thébault et al. [42] explored the antiinflammatory effects of GLN in intestinal cells with a nontargeted proteomic approach to obtain a more complete list of proteins affected by GLN in proinflammatory conditions. They evidenced that 27 proteins were up- or downregulated by GLN. Those proteins are involved in biosynthesis or proteolysis (40%; e.g. Ubiquitin-like protein FAT10, Ubiquitin, Proteasome subunit alpha type 4), membrane trafficking (16%; e.g. adenosine diphosphateribosylation factor-like protein 1), cell cycle, and apoptosis mechanisms (8%; e.g. p53 and DNA damage-regulated protein 1), nucleic acid metabolism (8%; e.g. Thioredoxin-like protein 4B), and a few proteins in lipid metabolism (Microsomal dipeptidase), in the mitochondrial function (Ubiquinolcytochrome c reductase complex 11 kDa protein) or in carbohydrate metabolism (Transketolase-like 1). Patients suffering from intestinal inflammation are also often exposed to oxidative stress and micronutrition depletion. Therefore in 2010, Thébault et al. [62] explored the proteome modifications induced by GLN combined to antioxidant micronutrients. They reported that in antioxidative conditions, GLN acted mostly on carbohydrate, lipid, protein and xenobiotic metabolisms, cell growth, and differentiation. Most of the proteins modulated by this oral nutritional supplementation are implicated in processes such as intestinal inflammation and cancer (e.g. enolase 1, fatty acid-binding protein 1, manganese superoxide dismutase, and ribosomal protein SA). GLN has also been known to influence the immune function for a long time. [63]. Indeed, GLN deprivation reduces the proliferation of lymphocytes by activating the expression of CD25, CD71, CD45RO, and TNF- α or stimulating IFN- γ secretion and alters the activity of monocytes and macrophages by stimulating IL-1 secretion and RNA synthesis [64]. In addition to the

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Table 2.	Some	proteomic	changes	mediated	by	GLN
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Biological processes	Modulated proteins or other factors by GLN	Up- (+) or down (–)- change by GLN
Metabolism	integrins	+
Protein synthesis	Src	+
Proteolysis carbon and nitrogen interorgan	phosphorylation of MAP-kinases	+
transporter Gluconeogenetic precursor	phosphorylation of 4E-BP1	+
Ammoniogenesis (kidney)	ubiquitin-like protein FAT10	_
	small ubiquitin-related modifier 1 (SUMO1)	+
	ubiquitin	_
	ubiquitin like protein SMT3A (SUMO2)	+
	ubiquitin-conjugating enzyme E2	+
	ubiquitin-conjugating enzyme E2 variant 2	_
	matrilysin	_
	proteasome subunit alpha type 4	_
	proteasome subunit alpha type 1	+
	G-rich sequence factor-1	+
	phosphorylation of S6K1	++
	ADP-ribosylation factor-like protein 1	_
Immunological response	mTORC1	+
Inflammatory response Replication of immune cells	p38 MAP kinases	++
T cells helper function and responsiveness	Phosphorylation of Erk	+
Synthesis of immunoglobulins A	Phosphorylation of MKP-1	+
	NF-κB	
	CD25, CD71, CD45RO	_
	TNF-α	_
	IFN-γ	_
	Proinflammatory cytokines (IL-6, IL-8) Antiinflammatory cytokines (IL-4 and IL-10)	+
Heat shock response	HSP70, HSP25/27, HSP32 (HO-1), and HSP72	+
Gut protection Replication of enterocytes	cytokine	_
Maintenance of gut-associated lymphoid tissue	microsomal dipeptidase	_
function and cellularity	occludin	+
	ZO-1	+
Others (antioxidant, apoptosis,)	caspase recruitment domain protein 9	_
	corticotropin-releasing factor-binding protein	+

ADP: adenosine diphosphate; IFN: interferon.

previous data, Lenaerts et al. [65] investigated the substancial health-promoting effects of GLN on human intestinal Caco-2 cells evidencing that GLN supplementation in catabolic states altered the expression of other proteins: plasma Retinol-Binding Protein, Ornithine AminoTransferase, apolipoprotein AI (ApoA-I), mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, and long-chain fatty acid-CoA ligase 5. As previously mentioned, GLN is well known for its protective effect in the gut barrier function. Several investigations were indeed carried out in such fields in addition to the papers on the effects of GLN in the duodenum, which are related mostly to the modulation of intestinal protein metabolism [42, 49, 66–70]. In a similar context, Li et al. [71] evidenced that the deprivation of GLN decreased the expression of claudin-1, occludin, and zona occulden-1 (ZO-1), three proteins involved in the intestinal epithelial tight junction barrier function that may lead to inflammation and mucosal injury. Similar results were obtained using GLN supplementation both in vitro or in vivo using different models [72–75].

Finally, only one publication explored the effect of GLN supplementation in apoptotic conditions on a whole intestinal proteome [76]. They identified 24 proteins as dysregulated by GLN supplementation. They were mostly involved in cell cycle and apoptosis mechanisms, signal transduction, autophagy, and cytoskeleton organization. Several of those proteins are very interesting when it comes to explaining the apoptosis suppressive capacities of GLN, e.g. proteins involved in the apoptosis pathway (MAP3K7 plus downstream effectors and Aven), autophagy (Atg5), and cytoskeleton regulation (stathmin).

To sum up, GLN is a nonessential free amino acid in healthy patients although it plays a key role in several biological processes (Table 2) and especially in protein turnover. Those effects could be related to the ability of GLN to regulate cell volume by promoting cell swelling and activate transductional (pathways) or signaling pathways. GLN is also known to regulate protein turnover by acting on the ubiquitin ATP-dependent proteolytic pathway and the mTORC1 signaling pathway. Thanks to its important role in protein metabolism including in cancer cells, GLN is subjected to several studies that evaluate the efficacy of therapies based on the reduction of GLN levels in the context of metabolic reprogramming in cancer [77, 78]. GLN also plays a major role in critical illnesses of patients in intensive care units after sepsis, surgery, trauma, or other injuries who could suffer from GLN depletion. In such conditions, GLN is shown to be a conditional essential-free amino acid by acting on metabolism, cellular protection, inflammatory response involving lymphocyte and cytokines, stress state, apoptosis, prevention of organ, gut homeostasis, and gut barrier function. While several studies evaluated the effects of GLN deprivation or supplementation on targeted proteins involved in protein synthesis, protein degradation, signaling pathways, inflammatory response (such as cytokines), or stress state (such as HSPs), only very few have investigated the whole proteome, mostly in the duodenum. Thus, our knowledge on how the whole proteome is modulated by GLN deprivation or supplementation in any tissue requires further investigation using a nontargeted proteomic approach.

4 Arginine

ARG is a dibasic AA that is considered as (a) nonessential (amino acid) in healthy humans (Fig. 1) while it is considered as (a) conditionally essential (amino acid) to immunity and during metabolic stress [79]. ARG is obtained from dietary sources, endogenous synthesis, and protein turnover [80]. It is synthesized from CIT especially by the kidneys [81]. It is also synthesized in the liver but only as an intermediary of the urea cycle. It is degraded either by arginase, which releases ornithine and urea, or by NO synthase causing the formation of CIT and nitric oxide [82]. Besides, ARG may be the precursor of ornithine (which is recognized as a polyamine precursor), or agmatine (a polyamine which is a major regulator of cell functions). Moreover, ARG is an important element in muscle (or muscular) energy; after reacting with glycine and MET, it allows the formation of creatine. Finally, ARG acts as a secretagogue (like insulin, glucagon, growth hormones, prolactin, and catecholamines). All these properties of ARG could make it be considered a regulator of protein metabolism [82]. It also influences protein turnover. As described by Bruins et al. [83], ARG supplementation in pigs decreased protein synthesis and degradation in the liver while, across rat hindquarters, it enhanced protein synthesis and degradation. But, no effect on kidneys was reported. Cui et al. also reported effects of ARG on protein turnover as well as an improvement of protein anabolism and an attenuation of muscle protein catabolism after dietary ARG supplementation [84]. Other investigations evidenced that supplemental ARG in septic rats led to a significant increase of fibrinogen, histone, and albumin synthesis [85]. ARG was also reported to affect some enzymes of its (own) biosynthesis by repressing the level of argininosuccinate synthetase and argininosuccinase [86]. Besides metabolism and biosynthesis processes, ARG also plays a critical role in the immune system by not only having functions as a substrate for iNOS but as it may also influence iNOS protein levels in cytokine-stimulated macrophages or cytokine-stimulated astrocytes without affecting the iNOS mRNA level, the production of TNF- α , and the overall protein synthesis [87, 88]. Lee et al. [88] also demonstrated that ARG prevented the activation of GCN2 kinase, and therefore decreased the phosphorylation status of the eukaryotic initiation factor (eIF2), which, in turn, regulated the translation of iNOS mRNA. ARG could also act on T-cell proliferation by regulating the expression of T cell antigen receptor ζ chain (CD3 ζ) [89, 90]. It should be noted that ARG also acts on the expression of cat-1 protein through the regulation of transcription, mRNA stability, and mRNA translation [91, 92]. In their studies, the authors confirmed the effect of ARG on eIF2 phosphorylation induced by GCN2 kinase, a phenomenon required to stimulate CAT-1 transcription and CAT-1 mRNA translation. An interesting study explored the whole proteome with a better molecular insight of preconfluent intestinal Caco-2 proteome in response to ARG depletion/supplementation [93]. Their study showed that ARG deprivation significantly decreased the proteins involved in proliferation (e.g. protein SET, dUTPase) and heat shock response (e.g. 47-kDa HSP, heat shock 70-kDa protein 1, heat shock cognate 71-kDa protein, HSP 90-b, and endoplasmin). ARG deprivation also regulates proteins involved in apoptosis (e.g. Histone H2B, splicing factor ARG/serinerich 1) and protein synthesis (e.g. eIF2a, glucosidase II b subunit, and reticulocalbin-1). As mentioned below, Lee et al. [88] had already shown that ARG prevented the activation of GCN2 kinase leading to a decrease of the phosphorylation status on eIF2. Other studies confirmed the effect of ARG in the phosphorylation status. For instance, Nakajo et al. and Ban et al. [94,95] evidenced a positive effect of ARG to increase the phosphorylation status of S6K1 and 4E-BP1 in rat intestinal epithelial cells. However, it seems that the enhancing effect of ARG is conditioned by the environment of other amino acids. Nakajo et al. [94] showed that GLN had an inhibitory effect on LEU- or the ARG-induced activation of the mTORC1 signaling pathway. Yet, the experiment was performed in vitro and we cannot exclude a potential competition for amino acid transporters.

To sum up, ARG is a conditionally essential amino acid that plays critical roles in protein synthesis, protein turnover, protein metabolism and the immune system, heat shock response, the apoptosis process, or the secretion of proteins (Table 3) by modulating the expression of several proteins directly related to those processes but also involved in the mTORC1 signaling pathway. Besides those studies, not all the mechanisms involved in such protein alterations have been totally elucidated and data are still missing to fully understand how ARG acts on the proteome.

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Table 3.	Some	proteomic	changes	mediated	by	ARC
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Biological processes	Modulated proteins by ARG	Up- (+) or down (–)- change by ARG
Metabolism Protein synthesis	argininosuccinate synthetase	_
Protein turnover Gluconeogenesis	argininosuccinase	_
	glucosidase II b subunit	_
	reticulocalbin-1	_
	phosphoenolpyruvate carboxykinase (GTP), mit.	_
	mTOB downstream (phosphorylation of S6K1_elF2_elF4B)	$\pm /-$ (unclear)
Immune system	NO synthase	_ (unoiour)
	CD3 ζ	+
Heat shock response	47-kDa HSP	
	heat shock 70-kDa protein 1	+
	heat shock cognate 71-kDa protein	+
	HSP 90-β	+
	endoplasmin	+
Apoptosis	Histone H2B	_
	splicing factor ARG/serine-rich 1	+
	albumin	+
	fibrinogen	+
	insulin	+
Secretion	glucagon	+
	growth hormones	+
	prolactin	+
	catecholamines	+
Nuclear protein	histone	+

5 Citrulline

CIT (Fig. 1) is a nonessential and nonproteinogenic amino acid, which is found in watermelons especially at concentrations ranging from 0.7 to 3.6 mg/g of fresh fruit where it plays a major antioxidant role [96, 97]. This amino acid had not drawn much attention before the last decade because biologists were unaware of its metabolic properties. Indeed, CIT was known to be an intermediate in the urea cycle and was also present in proteins due to posttranslational modifications through deamination-which can alter protein functions-but these citrullinated proteins are not related to the metabolism of this amino acid. However, over the last decade, CIT has garnered much interest since the discovery of its implication in the regulation of nitrogen homeostasis. Significantly, in a pioneering paper, Osowska et al. [98] showed that CIT supplementation could restore nitrogen homeostasis in a short bowel syndrome model. To extend the concept, the same authors evaluated the impact of CIT on aging. Thus, Osowska et al. [99]-using a model of proteinenergy malnutrition-showed, that a CIT-enriched diet in old rats stimulated MPS (+80%) and allowed a net muscle protein gain (+20%). Using the same model, the authors were able to show that muscle protein accretion related to CIT ingestion was accompanied by an increase in maximum force and promoted a continuum between biological or metabolic actions and a clinical impact [100]. In the wake of this work, various studies have confirmed these results in fasted [101] and food-restricted adult rats [102]. Finally, Moinard and coworkers [103] showed that CIT had a direct effect on protein synthesis in muscles.

Furthermore, it appears that the positive effect of CIT on MPS and muscle gain is a long-term one since a CIT supplementation of 3 months in old rats allowed an average muscle gain of 25%, which could be explained by the greater fiber-size diameter in the CIT group as compared to the isonitrogenous-control group [104]. More recently, it has also been shown that the ability of CIT to modulate MPS existed in humans. Indeed, CIT oral supplementation (10 g vs. isonitrogenous placebo) was shown to improve MPS by 25% in healthy adults subjected to low-protein diets (8%) for 3 days [105]. This work also demonstrated that the effects of CIT were muscle-specific. Indeed, the authors found an increase of MPS with no difference in the whole-body protein synthesis. This study was in accordance with another one that demonstrated CIT had no effect on the whole-body protein synthesis [106]. Recent data have shown that these effects of CIT on MPS are direct and can be due to the direct activation of mTORC1 [101, 103], which have been confirmed by an in vitro study in cultured myotubes [107].

Interestingly, the stimulation of MPS by CIT is really specific and seems to be focused on the myofibrillar protein. Indeed, the results of Ventura et al. [102] on the improvement of MPS by CIT in female adult rats submitted to a restricted diet were associated to an increase of myofibrillar protein synthesis with no modification of the sarcoplasmic synthesis. These results can be related to the CIT improvement of the observed muscle function. In the same way, Faure et al. [2], in a

Biological processes	Modulated proteins by CIT	Up- (+) or down (–)-change by CIT
Mvofibrillar proteins	Myosin-binding protein C, slow-type, isoform CRA b	
	Tropomyosin α -1 and α -3 chains	+
	α-Actin	+
	Myosin light chain 1	+
	Myosin regulatory light chain 2	+
Cytoskeletal proteins	Gelsolin	_
Energetic metabolism proteins	Hexaprenyldihydroxybenzoate methyltransferase	_
	Chaperone activity of bc1 complex-like	+
	Glycogen phosphorylase muscle form	+
	Triosephosphate isomerase	+
	β-enolase	+
Other proteins	Ferritin heavy chain	_
	Complement C3	_
	APOBEC-2	+

proteomic approach, showed that CIT administration (in aged malnourished rats) was able to increase the expression of key components of myofibrils including isoforms of myosin and actin, while the expression of proteins involved in the depolymerization of actin filaments was decreased. Therefore, the effect of CIT on protein synthesis is proved.

However, the effects of CIT on muscle catabolism pathways such as proteolysis have more rarely been explored. Indeed, in Osowska et al.'s work [99], the 3-MH to creatinine ratio (a whole body level reflection of muscle proteolysis) was unaffected by CIT supplementation. But, in the same model, CIT supplementation led to a decrease in protein carbonylation in the muscle and it is well known that posttranslational oxidative protein modifications are associated to a loss of the protein function related to an increase of protein degradation [108]. Additionally, Ham et al. [109] demonstrated in limb-casted mice studied over a period of 14 days that CIT supplementation did not alter the mRNA expression of genes involved in the proteasome muscle protein breakdown system (Fbxo32, tripartite-motif-containing 63 gene, Foxo1, and Foxo4) but CIT could modulate the autophagosome system: The same authors showed that CIT induced an upregulation of the mRNA expression of the Bnip3 gene-which mediates the recruitment of the growing autophagosome to damaged mitochondria [110]-but not on the LC3BII:LC3B1 ratio, a reliable marker of the number of autophagosomes [111]. Interestingly, in another model [2], data showed a probable downregulation of the expression of a proteolysis activator protein (subunit 1 of the proteasome activator complex) by CIT, which could lead to a decrease in muscle protein catabolism. Finally, these contradictions illustrate the complexity of proteolysis regulation-which largely depends upon the pathophysiological state of the subject-and the study of a clear relationship between CIT and proteolysis remains to be pursued further.

Moreover, CIT can specifically modulate the expression of targeted proteins like many proteins involved in energy metabolism. Indeed, CIT is able to enhance the protein expression of enzymes involved in glycogenolysis and glycolysis while enzymes involved in the Krebs cycle are probably downregulated by CIT in skeletal muscles [2]. However, all the proteins modulated by CIT in vivo were unaffected by this amino acid in cultured myotubes, suggesting an indirect effect of CIT. Moinard et al.'s recent study [104] seems to go in the same direction as they showed that the muscle expression of specific proteins to slow type fibers such as myosin-binding protein C slow-type isoform was downregulated by CIT. This plausible/probable property of CIT could be linked to its capacity to modulate muscle gene expression of APOBEC2 because Sato et al. [112] demonstrated that APOBEC2 deficiency was associated with a metabolic shift in muscle fiber toward fast fiber type especially since the authors demonstrated, in the same work, a probable effect of CIT on fiber typology with an increase of glycolitic fibers and a decrease of intermediate type fibers. Other data in this study confirmed the role of CIT on mitochondrial metabolism because CIT supplementation in 3month-old rats was able to modulate the expression of muscle genes involved in energy metabolism. Moreover, both muscle gene expression of Tfam-a transcription factor involved in mitochondrial biogenesis [113, 114]-and of various complex I subunits—coded by either the mitochondria (Nd1 and Nd6) or the nucleus (Ndufb8 and Ndufb6)-were upregulated by CIT. These data showed CIT could be able to modulate the mitochondrial metabolism. These observations were corroborated by a proteomic approach that showed an increase in the CIT-supplemented group of proteins involved in mitochondrial metabolism—such as the chaperone activity of bc1 complex-like enzyme—or a decrease of hexaprenyldihydroxybenzoate methyltransferase, two proteins that play a role in ubiquinone biogenesis [115-117].

As a conclusion, the data clearly show that CIT is able to modulate both muscle transcriptome and proteome (See summary in Table 4) that could not only partially explain its key role in the regulation of protein metabolism but also in the regulation of the mitochondrial function and energy metabolism. Yet, the too few studies on the subject do not allow to assess accurately the potential impact of CIT and other studies are needed to confirm its action on the muscle proteome.

6 MET and homocysteine

MET is a nonpolar AA-the most common one-that is considered as an essential amino acid in humans (Fig. 1). In mRNA, MET is encoded by a unique codon (AUG) and is generally the first AA to be incorporated in protein synthesis. MET is obtained from dietary sources or protein turnover and can be converted to S-adenosylmethionine, an important cofactor acting as a methyl donor in various enzymatic reactions. S-adenosylmethionine is also the precursor of homocysteine (HCY), which can regenerate MET using vitamin B12-dependent MET synthase or betaine-homocysteine methyltransferase. HCY is also a precursor of cysteine via the transsulfuration pathway. MET effects on protein expression are complex to investigate as it plays several roles at different levels: at the transcriptional level by a modification of the histone methylation [118], at the translational level as it is frequently the first amino acid to be incorporated in the proteins in the ribosome, and at the signaling level as it acts on some important pathways (P38 MAP kinase pathway [119] and oxidative stress dependent pathways).

HCY is a thiol-containing amino acid that is produced in all cells as an intermediate of the MET cycle [120]. Induced Hyperhomocysteinemia alters the abundance of several liver proteins involved in homocysteine or MET (metabolisms), nitrogen and lipid metabolisms, and oxidative stress [121]. Hyperhomocysteinemia was obtained by a mutation in cystathionine β -synthase or a high-MET diet in mice, and hepatic proteins were studied by quantitative proteomics (2D-DIGE and identification by MS). The very few similarities between the proteomes as compared to the numerous differences that could be observed represented a major issue. Particularly, several proteins involved in the response to oxidative stress were upregulated in wild-type mice in response to diet while this was not observed in mutants. This effect on the expression of proteins involved in oxidative stress was confirmed in another 2D gel electrophoresis proteomic study on mice liver supplemented with MET in drinking water [122]. Dysregulated proteins are involved in oxidative stress, inflammation, and cell death regulation. This link between the MET level and a global overexpression of proteins involved in oxidative stress and cellular defenses was also observed in a model of mice fed an MET and choline deficient diet mimicking steatohepatitis. The proteomic experiments revealed a downregulation of the proteins responsible for cellular defensive strategies against oxidative stress [123]. More recently, Wen et al. [124] demonstrated that the growth of breast muscles by MET in broilers involved myostatin, mTOR, and FoxO4. High MET diets increased mTOR phosphorylation and decreased FoxO4 phosphorylation.

In cancer cells, MET dependence is a unique feature characterized by growth and cell cycle arrest (typically between S and G2/M) under conditions of MET depletion [125, 126]. A differential proteomic approach using 2D electrophoresis and MALDI-TOF/TOF MS identified ten proteins in a gastric cancer cell cultured medium with MET restriction [119]. These proteins were associated to cell cycle arrest, apoptosis and the response to stress signals such as P38 MAP kinase, peroxiredoxin 6, glutathione S-transferase Mu,3 and Ras-related proteins. Although the mechanisms linking MET and the cell cycle remain uncertain, this effect was investigated as a possible therapeutic target and a strategy to optimize the effects of cancer chemotherapies [127].

7 Amino acids and the mTORC1 signaling pathway

As described in this review, amino acids such as LEU, GLN, ARG, CIT, and MET act significantly on protein expression as well as on the immune response by activating the mTORC1 signaling pathway. Although several mechanisms of this regulation have been described in the literature, they are quite elusive [128]. The mTORC1 signaling pathway is a major metabolic crossroads of the protein turnover as it regulates protein synthesis. It is widely known that this transduction pathway is regulated by amino acids. Indeed, amino acid depletion reduces mTORC1 signaling and an increase of amino acid availability boosts mTORC1 signaling. The mechanism through which AAs regulate mTORC1 signaling has long remained poorly understood though. For example, many studies demonstrated that Ras homologue enriched in brain (Rheb), as a growth factor, was necessary for amino acids to activate the mTORC1 pathway [129-131]. However, the regulating mechanism of the mTORC1 pathway by amino acids is distinct from (the regulating mechanism of) growth factors, even if it also involves Rheb [132]. Recently, Sancak et al. [133] have demonstrated that amino acids regulate the association between mTORC1 and the late lysosome/endosome system (LEL), which seems essential for its activation. This association occurs via a mechanism that is dependent on another Ras-related GTPases family called the Rag GTPases. Indeed, the importance of the association with LEL in the activation of mTORC1 has recently been reported in several studies particularly because LEL is enriched with phosphatidic acids (PAs) and Rheb, both direct activators of mTORC1 [133-135]. Specifically, amino acids were shown to be able to promote the association of mTORC1 with LEL (enriched in Rheb and PA) via an activation of the Rags [131, 136]. Finally, AAs were shown to improve the concentration of PA in the LEL. Another component implicated in amino acid signaling mTORC1 is linked to leucyl-tRNA synthetase (LeuRS). Indeed, it had been demonstrated in two studies that this enzyme charging LEU to its corresponding tRNA, also acted as an LEU sensor in the activation of mTORC1 [137, 138]. Yet, the regulatory mechanism details differ considerably from one study to the other and complementary works are mandatory to validate for good this possible regulatory component of the mTORC1 signaling pathway. To conclude, current

evidence shows that amino acids regulate the mTORC1 pathway through the modulation of the concentration of PA and Rheb activity in the LEL. However, these new discoveries about mTORC1 activation mechanism at LEL by amino acids do not allow distinguishing between the effects of some specific amino acids.

8 Conclusion

This review allowed us to show more clearly how some AAs (LEU, GLN, ARG, CIT, and MET) did alter the protein expression. The data currently available in the literature showed that those free amino acids act on a panel of proteins involved in protein synthesis/proteolysis by directly dysregulating the expression of the proteins involved in those processes or in mTOR and MAPK signaling pathways. However, it would be oversimplistic to limit the specific effect of these amino acids to protein turnover. Indeed, only a few publications used a proteomic approach allowing a more complete view of the proteome. Moreover, those approaches were still limited compared to the current common proteomic approaches (identification, quantitation, posttranslational modifications, interactions, localization) used to identify and to characterize dysregulated proteins. Hence, further investigation using these novel proteomic approaches should allow us to obtain more data and improve our knowledge in protein turnover modulation-dependent amino acids. Moreover, except for some studies that focused their analysis on the targeted phosphorylation of proteins related to mTOR or MAPK signaling pathways, no study explored the phosphorylation status in the whole proteome in deep. Therefore, a further large-scale investigation on the phosphorylation of the whole proteome should be carried out to better understand how it is influenced by amino acids like LEU, ARG, GLN, CIT or MET and to determine the missing link between amino acids and mTORC1 activation. Furthermore, no research article has explored the relationship between the proteins dysregulated by amino acids and other PTMs that often occur in tissue and significantly regulate the protein function and activity. As the free amino acids were shown to act strongly in the ubiquitin ATP-dependent proteolytic pathway or cellular metabolism in which acetylation plays a key role, investigation of both PTMs, ubiquitination and acetylation could be of great interest to explore whether amino acids act on protein stability and activity through the alteration of those PTMs. Finally, as amino acids are small molecules related to a lot of metabolites in the cell and in the extracellular compartments of the body, a more systemic approach with metabolomics and proteomics together could also help a better understanding of the relationship between the level of amino acids and the content and regulation of the different proteomes.

A.G. is supported by les Fonds Agir pour les Maladies Chroniques.

The authors have declared the following potential conflict of interest: C.M. is the shareholder of $Citrage^{\mathbb{R}}$.

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BIOCHIMIE Statut nutritionnel de la personne âgée

Préservation du statut nutritionnel de la personne âgée : un atout pour un vieillissement réussi ?

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RÉSUMÉ

La malnutrition protéino-énergétique (communément appelée dénutrition) résulte d'un déséquilibre entre les apports et les besoins nutritionnels d'un individu. La conséquence directe de ce déséquilibre est une perte tissulaire (en particulier de la masse musculaire qui représente la plus grande masse protéique de l'organisme). Chez la personne âgée, cette dénutrition est reconnue comme un véritable indicateur de fragilité et a des conséquences fonctionnelles et des répercussions cliniques majeures : augmentation de la morbi-mortalité, accroissement du risque de chutes, perte d'autonomie et de qualité de vie, etc. Il convient donc de dépister et diagnostiquer la dénutrition le plus précocement possible chez la personne âgée et de proposer la stratégie de prise en charge la plus adaptée afin de préserver au mieux la qualité de vie et l'indépendance des personnes âgées.

MOTS-CLÉS : malnutrition protéino-énergétique, dénutrition, sarcopénie.

I. - INTRODUCTION

L'augmentation de l'espérance de vie, résultant de l'évolution de la structure sociale et du progrès médical, explique l'accroissement de la population âgée. Les facteurs essentiels, permettant d'assurer un vieillissement réussi, sont la préservation d'un statut nutritionnel satisfaisant et la conservation de la fonction musculaire, garants de l'autonomie et de la qualité de vie. En effet, toute perte de la masse musculaire s'accompagne d'une augmentation du risque de troubles de la marche, de chutes, de fractures, de dépendance, d'une diminution de la qualité de vie et d'une moindre capacité de réponse à un stress. Enfin, la diminution de la mobilité est reconnue comme étant un facteur prédictif de mortalité chez les personnes âgées.

II. - LA MALNUTRITION PROTÉINO-ÉNERGÉTIQUE CHEZ LA PERSONNE ÂGÉE

La malnutrition protéino-énergétique (MPE, communément appelée dénutrition) résulte d'un déséquilibre entre les apports azotés et énergétiques et les besoins nutritionnels d'un individu. La conséquence directe de ce déséquilibre sera un changement mesurable des fonctions et/ou de la composition corporelle, et notamment une perte tissulaire (en particulier de la masse musculaire qui

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représente la plus grande masse protéique de l'organisme) avec des répercussions cliniques majeures chez la personne âgée. Il en résulte une perte de poids corporel involontaire, de 5 à 10 % sur les 6 à 12 derniers mois par rapport au poids habituel (1, 2).

Il est établi que le risque de MPE augmente fortement avec l'âge. En effet, cette MPE pourra être un révélateur et/ou un élément d'aggravation d'altérations propres au vieillissement (ex : fragilité du système immunitaire, perturbations de la fonction digestive ou présence d'une sarcopénie). Si la MPE a une prévalence relativement limitée chez les personnes vivant à domicile (de l'ordre de 4 à 10 %), elle augmente fortement chez les sujets institutionnalisés (de 15 à 38 %) et encore plus lorsqu'ils sont hospitalisés (jusqu'à 70 %). Des causes multiples (environnementales, psychiques, physiopathologiques, iatrogéniques, etc.), concourent à réduire la prise alimentaire et à favoriser l'installation d'une MPE (Tableau) (3).

Chez les personnes vivant à leur domicile, la MPE augmente la morbi-mortalité, les risques de chutes, la dépendance. Pour celles qui sont hospitalisées, la MPE se traduit, entre autre, par une augmentation de la durée de séjour ainsi que des risques d'infections nosocomiales, indépendamment de la pathologie causale (4), d'escarres et d'un accroissement de la morbi-mortalité.

III. - DÉTECTION ET DÉPISTAGE DE LA MPE CHEZ LA PERSONNE ÂGÉE

La MPE étant un facteur de morbi-mortalité, il est crucial de dépister les personnes âgées dénutries ou à risque de dénutrition en évaluant régulièrement leur statut nutritionnel (même chez ceux vivant à domicile). Il est établi qu'une personne âgée est dénutrie si elle présente au moins un des critères suivants :

- une perte de poids > 5 % en 1 mois ou > 10 % en 6 mois,
- un indice de masse corporelle (IMC) < 21,
- un taux sérique d'albumine < 35 g/l.

Si ces critères semblent relativement simples à mesurer, certaines précautions doivent cependant être prises. En effet, pour évaluer la perte de poids, il faut disposer d'un poids de référence (qui fait souvent défaut), d'où la nécessité de peser régulièrement les personnes âgées. Par ailleurs, chez les personnes obèses ou ayant une surcharge pondérale, l'IMC peut être tout à fait normal alors même qu'elles ont eu une perte de poids importante et qu'elles sont dénutries (obésité sarcopénique). Enfin, l'albuminémie peut être modifiée par des paramètres sans rapport avec la nutrition (inflammation, entre autres) et est, chez le sujet âgé, un marqueur de morbi-mortalité plutôt que du statut nutritionnel (5).

Tableau	-	Facteurs concourant	à	l'installation	d'une	e MPE	chez	la	personne	âgé	èe.
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Facteurs de risques	
Environnementaux	Isolement Solitude Difficultés financières Institutionnalisation
Psychiques	Dépression Perte du conjoint Troubles cognitifs (démences vasculaires, maladie d'Alzheimer, etc.)
Physiopathologiques	Troubles de la mastication Troubles de la déglutition Altérations du goût Constipations Diarrhées Incontinence Modifications métaboliques Maladies chroniques
Régimes restrictifs	Régimes amaigrissants Régimes pour diabétiques Régimes hyposodés Régimes sans résidus Régimes pauvres en cholestérol
Iatrogéniques	Médicaments anorexigènes Anti-inflammatoires entraînant des troubles digestifs Médicaments constipants, etc.

Par ailleurs, il existe de nombreux outils permettant de dépister la MPE chez la personne âgée :

- Des marqueurs anthropométriques : poids, taille, IMC, plis cutanés, circonférence des membres. Faciles à mesurer et peu coûteux, ces marqueurs peuvent être étudiés au lit du malade ; en revanche, leur mesure nécessite une très grande expertise de la part du clinicien, en particulier pour les plis cutanés.
- Des marqueurs biologiques : les plus utilisés sont les protéines marqueurs de l'état nutritionnel : albumine, transthyrétine - anciennement appelée préalbumine -, Retinol binding Protein (RBP) et transferrine. En effet, les concentrations plasmatiques de ces protéines produites par le foie reflètent leur biogenèse et sécrétion. Or, leur taux de renouvellement étant extrêmement élevé, leur synthèse est très largement dépendante des apports protéiques de l'alimentation. Ainsi, il est considéré que les variations de ces protéines sont de bons marqueurs de la « perte protéique » par le corps entier. Globalement sensibles, spécifiques, facilement disponibles et peu onéreux, ces marqueurs sont donc avantageux (6). Toutefois, des indices combinant des paramètres anthropométriques et biologiques ont été proposés durant ces dernières décennies. L'un des plus connu est le Nutritional Risk Index (NRI), développé par Buzby et al. (7) :

NRI = 1,519 x [Albumine] + 0,417 x (poids actuel/poids usuel)

En fonction de la valeur de l'indice, le risque de dénutrition peut être défini comme suit :

> 100 > NRI > 97,5 : faible 97,5 > NRI > 83,5 : modéré 83,5 > NRI : sévère

Plus récemment, cet indice a été spécifiquement adapté à la personne âgée sous le terme de GNRI (*Geriatric Nutritional Risk Index*) (8), le poids usuel étant remplacé par le poids « idéal » calculé selon la formule de Lorentz (Figure 1). Par ailleurs, le classement du risque de dénutrition est légèrement différent :

> 98 > GNRI > 92 : faible 82 > GNRI > 92 : modéré 82 > GNRI : sévère

La valeur de l'indice étant influencée par la méthode d'analyse utilisée pour doser les marqueurs biochimiques de dénutrition (9), il est impératif que celle-ci soit inchangée durant le suivi des patients.

Enfin, au début des années 90, un autre outil a été dé-

Homme = Taille (cm) - 100 - [Taille (cm) - 150] / 4 Femme = Taille (cm) - 100 - [Taille (cm) - 150] / 2,5

Figure 1 - Formule de Lorentz.

veloppé par Guigoz et Vellas afin de dépister rapidement la dénutrition : le *Mini Nutritional Assessment* (MNA) (Figure 2). Très utilisé par les gériatres, notamment en milieu hospitalier, c'est un questionnaire composé de 18 items combinant des données anthropométriques et des questions sur les habitudes alimentaires, dont le remplissage ne prend que quelques minutes et ne demande pas d'expertise particulière.

IV. - MODIFICATIONS MÉTABOLIQUES CHEZ LA PERSONNE ÂGÉE

Le vieillissement se traduit par un ensemble de modifications métaboliques et physiologiques qui, si elles sont sans conséquence chez une personne âgée en bonne santé, vont favoriser l'installation d'une MPE. Ce vieillissement s'accompagne d'une dysrégulation de la prise alimentaire et d'une incapacité à s'adapter à des variations d'apports alimentaires (10). Par ailleurs, les individus âgés présentent des modifications du goût et, de façon plus générale, de leurs perceptions olfacto-gustatives : leur seuil étant plus élevé, les aliments leur paraissent donc moins goûteux. De plus, les personnes âgées ont une dentition modifiée, en particulier une rétractation des gencives favorisant le déchaussement dentaire. La conséquence est une mastication des aliments moins efficace, et ceux perçus comme trop durs (viandes ou légumes fibreux) seront exclus de l'alimentation. Mais l'une des modifications majeures chez la personne âgée, qui impactera directement les risques de MPE, concerne le métabolisme protéique.

A) L'homéostasie azotée au cours du vieillissement

Le vieillissement est associé à de nombreuses modifications du métabolisme azoté. Le fait le plus marquant est une fonte importante de la masse musculaire, appelée sarcopénie. De très nombreux travaux ont été consacrés à ce sujet et de multiples facteurs en sont responsables (11), mais l'un des plus notables est la modification du métabolisme azoté au niveau de l'aire splanchnique.

B) Métabolisme azoté splanchnique chez la personne âgée

Quantitativement, l'aire splanchnique (intestin, foie, pancréas, rate) ne représente que 4 à 6 % du poids corporel, mais elle assure 20 à 30 % de son renouvellement protéique. Physiologiquement, il est reconnu que près de 50 % de l'azote d'origine alimentaire est utilisé par l'aire splanchnique (synthèse protéique et oxydation). Cependant, il semblerait qu'au cours du vieillissement, l'extraction splanchnique des acides aminés augmente fortement, conduisant au phénomène de séquestration splanchnique des acides aminés (12). Ainsi, la quantité d'acides aminés disponibles en périphérie serait insuffisante pour assurer une synthèse protéique musculaire satisfaisante, ce qui concourrait à la lente érosion du capital musculaire de la personne âgée. Par ailleurs, ces modifications du métabo-

Nom: Prénom: Sexe : Age : Prénom : Date : Sexe : Age : Prénom : Date : Répondez à la promière partie du questionnaire en indiquant le score approprié pour chaque question. Additionez les ponts de la parte Depistage : Date : Origitage I o patient prénont-: Depistage : Taille , cm : Date : Origitage I o patient prénont-: Depistage : I o mainter pour othenir Tapprécision précise de l'état nutritionnel. Origitage I o patient prénont-: I o mainter pour othenir apprécision précise de l'état nutritionnel : I o mainter pour othenir apprécision précise de l'état nutritionnel : I o patient prénont : I o patient de point : I o mainter pour othenir apprécision précise de précise alimentaires I o mainter de point : I o mainter de pointer : I o mainter de pointer : I o a sourd : I o a soure : I o a a sourd : I o a a soure :	Mini Nutritional Assessmer MNA [®]			nt Nestlé NutritionInstitute		
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12-14 points: à risque de dénutrition 6-11 points: à risque de dénutrition 0-7 points: dénutrition avérée Pour une évaluation approfondie, passez aux questions G-R P Le patient vit-il de façon indépendante à domicile ?	Sco (sou	re de dépistage		0 = se considère comme dénutri 1 = n'est pas certain de son état nutritionnel 2 = se considère comme n'ayant pas de problème de nutrition		
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Figure 2 - Le Mini Nutritional Assessement (MNA®).

lisme protéique pourraient contribuer au défaut de réponse de la personne âgée dénutrie à la réalimentation.

V. - PRISE EN CHARGE NUTRITIONNELLE DE LA MPE CHEZ LA PERSONNE ÂGÉE

Le traitement de la MPE devra prendre en compte d'une part, les causes de la dénutrition (revenus insuffisants, problèmes dentaires, solitude, etc.) et d'autre part, la prise en charge nutritionnelle par elle-même. De façon générale, plusieurs solutions simples peuvent permettre d'augmenter les apports alimentaires des personnes âgées (13). Il convient, en premier lieu, de tenir compte de leurs préférences alimentaires, de leur assurer un maximum de convivialité et de confort au moment des repas. Pour les sujets ayant des problèmes articulaires, l'utilisation de couverts ergonomiques et l'assistance à la prise du repas peuvent s'avérer nécessaires. Il faut également respecter le temps nécessaire au repas afin de permettre à la personne de manger un maximum d'aliments. Par ailleurs, la personne âgée étant très sensible à la dénutrition, les périodes de jeûne doivent être évitées, en particulier le jeûne nocturne qui ne doit pas dépasser 12 h, et des petites collations peuvent être envisagées lors de réveil(s) durant la nuit. Enfin, il convient de soustraire, autant que faire se peut, les médicaments anorexigènes ou alors de les donner après le repas.

Quant à la prise en charge nutritionnelle, elle peut être

difficile en raison d'une réponse défectueuse du sujet âgé à la réalimentation, liée aux modifications de son métabolisme protéique. Ceci a pu être prouvé chez des adultes jeunes et âgés (d'âge moyen respectivement de 30 et 72 ans) en bonne santé, en situation post-absorptive et au décours de l'administration d'acides aminés et de glucose. Si le renouvellement musculaire basal était similaire dans les deux groupes, en revanche, après le repas, la synthèse protéique musculaire était augmentée seulement chez les individus jeunes (elle était inchangée chez ceux qui étaient âgés) (14). L'une des conséquences directes est que cette altération métabolique favorise, chez les personnes âgées, une résistance à la renutrition. Ce phénomène, bien connu des gériatres, a pu être démontré dans différentes études, en particulier celle réalisée par Hébuterne et coll. (15) dans laquelle les auteurs ont confirmé que les effets de la réalimentation sont moins marqués chez le patient âgé : après 27 jours de nutrition entérale, le groupe adulte (moins de 65 ans) avait un gain pondéral de 6,3 kg alors qu'il n'était que de 4,7 kg dans le groupe âgé (plus de 65 ans).

VI. - AUTRES PISTES À L'ÉTUDE : MANIPULATIONS NUTRITIONNELLES CHEZ LA PERSONNE ÂGÉE

Du fait de la difficulté à renourrir les personnes âgées dénutries, plusieurs stratégies ont été proposées.



A) Saturation de la séquestration splanchnique

Différentes études cliniques et expérimentales ont démontré que la séquestration splanchnique est une des raisons de la résistance à la renutrition et il a donc été envisagé de saturer celle-ci. Un régime hyperprotéique le permet (16), mais il a généralement une faible compliance et présente l'inconvénient d'être satiétogène. L'alternative proposée a été, non pas d'augmenter l'apport protéique total, mais de modifier sa répartition dans la journée. Ainsi est né le concept de nutrition pulsée où 80 % de l'apport protéique est concentré sur le repas du midi. L'étude princeps de Arnal et coll. (17), menée chez le sujet âgé sain, a révélé que la saturation du secteur splanchnique avec un tel régime assurait l'augmentation du renouvellement protéique au niveau du corps entier et favorisait une rétention plus importante d'azote qu'un régime standard. Des résultats analogues ont été constatés chez des personnes âgées dénutries hospitalisées recevant une alimentation contrôlée, isocalorique et isoazotée (1,3 g de protéines/kg poids/jour), pendant 42 jours (18), la ration protéique étant répartie sur le petit déjeuner, le déjeuner, la collation et le dîner pour le groupe témoin, et centrée majoritairement (72 %) sur le repas du midi pour le groupe pulsé. Il a ainsi été montré que ce régime protéique pulsé permettait d'accroître la masse maigre et la masse musculaire squelettique. Par conséquent, le régime protéique pulsé serait un moyen intéressant de saturer la séquestration splanchnique des acides aminés, en apportant en périphérie ces éléments nécessaires à la synthèse protéique (19). Cependant, si la nutrition pulsée est efficace pour restaurer le statut nutritionnel des personnes âgées dénutries, elle est difficilement transposable à grande échelle car la réalisation des repas pose un certain nombre de problèmes techniques (repas du midi très riche en protéines et autres repas pauvres en protéines) et semble difficilement réalisable à domicile.

B) Autres stratégies

L'alternative aux approches précédentes est d'utiliser une nutrition qui serait enrichie en un nutriment spécifique qui pourrait avoir une action anabolique en périphérie (protéines rapides (20), leucine (21), citrulline (22), vitamine D (23). Si certaines études expérimentales et cliniques sont encourageantes, les travaux de confirmation sont encore, pour la plupart, en cours de réalisation.

VII. - CONCLUSION

La MPE est un fléau qui a des conséquences majeures chez la personne âgée. Si son incidence reste limitée à domicile, en revanche elle concerne une part importante de la population âgée institutionnalisée ou hospitalisée. Son diagnostic et son dépistage régulier sont essentiels afin de proposer aux personnes dénutries une stratégie de renutrition adaptée et ainsi assurer un vieillissement réussi et une qualité de vie la meilleure possible.

Conflit d'intérêt : C. Moinard : actionnaire de la société Citrage® ; A. Goron : aucun.

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BIOCHIMIE Statut nutritionnel de la personne âgée

Amino acids and sport: a true love story?

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Conflicts of interest: Christophe Moinard is shareholder of Citrage[®] Company. Arthur Goron does not have any conflict to declare.

Abstract

Among ergogenic dietary supplements, the consumption of amino acids or derived is very popular in athletes for many reasons (to prevent nutritional deficiency, to improve muscle function, to decrease exercise side-effects, to improve performance, etc.). However, it is difficult to have a clear idea about which amino acids have a real ergogenic impact. In this review, we propose to analyse the clinical studies evaluating specific amino acids (glutamine, arginine, leucine etc.) in athletes. In conclusion, despite promising results, many studies failed to totally convince du to methodologic limits or due to specific flaw and many research are still mandatory to have evidence-based data to recommend the use of a specific amino acid in athlete.

Key words: Glutamine, arginine, branched chain amino acids, β -alanine, leucine, citrulline athletes
Food supplements are very popular through the world, especially in Western population (Europa and USA). However, there is often misunderstanding about the exact definition of food supplements. According to European Food Safety Authority (EFSA) definition, food supplements are concentrated sources of nutrients or other substances with a nutritional or physiological effect, whose purpose is to supplement the normal diet. Food supplements are marketed 'in dose' form, for example as pills, tablets, capsules or liquids in measured doses etc. Supplements may be used to correct nutritional deficiencies or maintain an adequate intake of certain nutrients and for Food and Drug Administration (FDA), a dietary supplement is a product intended for ingestion that contains a "dietary ingredient" intended to add further nutritional value to (supplement) the diet. [...] Dietary supplements may be found in many forms such as tablets, capsules, softgels, gelcaps, liquids, or powders. Some dietary supplements can help ensure that you get an adequate dietary intake of essential nutrients; others may help you reduce your risk of disease. These definitions are closed and similar in many countries. However, the spectrum of dietary supplement commercialization has derived from their nutritional properties to their physiological properties, then derived from physiological properties to their ergogenic effects. Among population, athletes are particularly concerned (1,2). Hence, there is a dual interest for athletes to consume dietary supplements in order i) to avoid any nutritional deficiency that are associated to a reduced performance ii) to affect the competitiveness (performance, recovery, immunity, etc).

Among ergogenic dietary supplements, the consumption of amino acids or derived is very popular because some of them may have ergogenic effects. Almost all athletes are convinced that amino acids may help them to gain competitiveness and many companies propose some amino acids (alone or in mixture) to help sportsmen. Actually, the scientific rationale for the use of such amino acids is often thin and is not necessary based on scientific studies. In such situation, it is difficult for consumers to discard fact from fiction.

The purpose of this review is to provide a critical analysis of the literature about the potential ergogenic effects of amino acids in athletes. In this review, only clinical data has been considered.

Arginine

Arginine (ARG) is a dibasic aliphatic amino acid. Due to its structure, ARG is at a crossroad of numerous metabolic pathways of major active molecules: nitric oxide (vasodilation and molecular signalization, glutamine (Cf. supra), proline (involved in wound healing), polyamines (DNA stabilization, gene regulation, immunity, and intestinal trophicity), creatine (muscular energy), and agmatine (cellular signalization). Among these properties, sports community has retained that ARG could be an ergogenic support via its capacity to generate creatine, to increase anabolic hormones secretion and especially to promote vasodilation. And ARG as NO precursor would favor muscle blood perfusion and this latter would have two impacts i) First, an increase of nutrients availabilities for muscle ii) secondly, a greater release of metabolites, such as lactate and ammonia, which are related to the muscle fatigue, especially in resistance exercise.

Some studies have shown the effects of ARG on NO production and muscle blood perfusion (REF). However, in trained athletes (both endurance and resistance exercise), almost all studies did not observed a such effect of ARG (3–10) except Alvares et al. (11). In this study, fifteen men were supplemented with 6g of ARG (HCl) before performing resistance exercise of the biceps. The result is that ARG supplementation increased (around 2-fold) the muscle blood volume in muscle during recovery from sets of resistance exercise. Despite the increase in muscle blood flow, the authors failed to observe any beneficial effect of ARG supplementation to improve muscle blood perfusion seems weak or inconsistent, especially as this parameter does not appear to be related to improving sports performance.

ARG is recognize for its secretagogue properties (i.e. Insulin and Growth hormone (GH)). This property is used since several decades to detect GH deficiency. In practice, ARG is administrated by intravenous (IV) route (30 g) and GH secretion is monitored allowing to detect responders and non-responders (GH deficiency patients). However, a 30g oral ARG dosage is not realistic since it induces severe hyper-osmotic diarrhea (12). Moreover, at lower dosage (<15g) ARG is well tolerated in healthy subjects. However, this amino acid is largely metabolized by splanchnic area leading to a decrease of its peripheral bioavailability. This fact was well demonstrated by Bode-Böger et al. (13). Indeed, authors observed that after 6 g

arginine IV infusion, plasma ARG concentration peaked to 822 ± 59 µM while plasma ARG concentration peaked to 310 ± 152 µM after 6 g oral ARG administration.

Taking into account these data, several authors explored the influence of oral ARG administration on hormonal pattern and almost all studies failed to retrieved the ARG secretagogue effect observed after IV ARG infusion both in healthy sedentary or trained population (14–17)

Considering the clinical data evaluating the effect of ARG on muscle perfusion and hormone secretion, it should be noted that few evidences support a positive effect of ARG. However, despite disappointing results, an ergogenic effect of ARG on performance cannot be exclude and a large literature explored this research field.

A large number of studies have investigated the ergogenic effect of ARG. However, the majority of these works combined ARG with others active components. In these conditions, it is not possible to discard the specific ergogenic effect of ARG. In this review, only studies evaluated ARG alone were considered. To the best of our knowledge, only nine studies have investigated the acute specific effect of ARG supplementation on athletic performance in endurance or resistance exercises (3,7–9,11,18–21). Among them, only Yavuz et al. (20) showed a positive effect on performance. In a crossover design study, nine elite male wrestlers consumed a single high dose of 0.15 g.kg⁻¹ or a placebo 1 hour before to perform an incremental bicycle ergometer test to exhaustion. Time to exhaustion increased by 5.8% in the ARG trial compared to the placebo trial. This result is surprising since none of the eight other studies have shown an effect of ARG administration on the aerobic or anaerobic performance. But the study of Yavuz et al. has a major limit since it was not a double-blinded study, and in this condition, the performance test could be influenced. However, does such results are observed during chronic ARG supplementation?

Unfortunately, only two studies investigated the chronic effect of ARG supplementation on performance. Alvares et al. (17) did not demonstrate any effect of 4 weeks of ARG supplementation in trained male runners on performance. In contrast, Fricke et al. (22) have shown that postmenopausal women consuming 18g ARG (HCl) or a placebo (18g dextrose) daily for six months had a better peak jump force relative to bodyweight. Others performance measures (absolute peak jump power and peak jump force) were not improved. The major limit of this study is that placebo consumed for 6 months contained 18 g dextrose and such amount cannot be negligible especially in a non-crossover study.

In conclusion, based on available literature, it is difficult to recommend ARG supplementation for sportsmen.

Glutamine

Glutamine (GLN) is recognized as the most abundant free α -amino acid at whole body level and it plays several important metabolic roles: GLN is the major fuel for rapidly dividing cells (like enterocytes and immune cells), it is the main carrier of α -amino nitrogen between tissues and is therefore important in inter organ traffic and acid-base homeostasis. Finally, since princeps work of Millward et al. (23) showing a close relationship between muscle glutamine concentration and muscle protein synthesis, many studies had underlined the role of GLN as a regulator of protein turnover (24–26). All these data are a good rationale to propose a systematic use of GLN in athletes. Indeed, for this population, it has been proposed that GLN could i) limit dysimmunity associated to exercise ii) have ergogenic effects via activation of muscle protein synthesis. Considering these potential benefits of GLN, it is not surprising that this amino acid has become a popular supplement among athletes, including elite college athletes (27).

In order to explore anabolic properties of GLN, Welbourne et al. (28) evaluating the effect of GLN supplementation on growth hormone release. The aim of the work was to evaluate growth hormone secretion after oral GLN load (2g) in middle-aged volunteers. The mean value of plasma growth hormone, 90 min after a 2-g oral dose of GLN, was 4-fold higher than the control value, but sharp rises in growth hormone level after GLN occurred in only 4 subjects, and the results were not statistically significant (28). Moreover, it should be noted that 1 h of strenuous exercise could increase plasma growth hormone level 20-fold. Thus, there is no rationale to athletes in training to take GLN to increase growth hormone release (7). Moreover, GLN is recognized to be the major neoglucogenic amino acid in fasting state (29,30). The popular opinion of athletes is that GLN, by this way, could restore muscle glycogen. To test this hypothesis, Wilkinson et al. (31) evaluated the effect of glutamine supplementation (0.3g/ kg BW) on muscle glycogen recovery (using muscle biopsies) and muscle protein synthesis. Unfortunately, authors failed to demonstrate a positive effect of GLN. However, despite these disappointing results, does GLN may positively affect performance in athletes? In a first paper, Haub et al. (32) evaluated the combined effects of GLN (0.03g/kg BW) and exercise in trained males (i.e. five exercise bouts on a cycle ergometer at 100% of VO₂ peak). Time to fatigue for last bout was used as the major criterion. None effect on performance was reported. A possible explaination could be linked to the low dose of GLN used (around 2-3g and splanchnic extraction of GLN is very high). However, such interpretation can be exclude since Khorshidi-Hosseini et al. (33), using a large dose of GLN (0.25g/kg), observed a similar result on anaerobic power. Finally, several authors tested chronic GLN supplementation. In the first one, young adults received daily GLN ingestion (0.9 g/kg of lean mass/day) during 6 weeks. It was unsuccessfully to improve muscle mass and strength during resistance training (34). Even when combined with creatine and ribose, GLN supplementation and 8 weeks of resistance training showed no additional increases in lean muscle mass, one-repetition maximum resistance, or the maximal number of endurance repetitions performed as compared with placebo (35). At last, even with a long term supplementation (0.03g/kg during 3 months), in military police officers, GLN was not able to show an ergogenic effect. Therefore, any data support an acute or chronic ergogenic effect of GLN.

Finally, a last issue to observe any beneficial effect of GLN could be related to its capacity to modulate immunity. Indeed, a relationship between highly strenuous exercise, such as a marathon, and increased incidence of upper respiratory tract infections (URTI) has been highlighted by several authors (36,37). This relationship is most commonly attributed to changes in circulating leukocytes after exercise: leukocytosis, neutrophilia, and lymphopenia. Moreover, it was described that hypoglutaminemia, observed after a strenuous exercise, was associated to immunosuppression (38) suggesting a possible relationship between these both phenomena. Such interpretation seems logical because GLN does serve as a major substrate for immune cells. For these reasons, it was proposed that GLN supplementation would limit or counter the exercise-induced lymphopenia, resulting in fewer URTI after marathons or other high-demand activities. However, almost all studies examinating the potential effects of GLN supplementation on altered immune function secondary to exercise failed to report a consistent effect (37,39–41). Only one study evaluating the effect of GLN supplementation (3g/day during 2 weeks) in male judoists, suggest a modest effect of GLN on neutrophil function (42).

In conclusion, based on available literature, it is difficult to recommend ARG supplementation for sportsmen.

Citrulline and malate citrulline

Citrulline (CIT) is a non-essential amino acid considered only as a urea cycle intermediate during several decades. In 80's, Windmuller and Spaeth demonstrated that intestine was a major CIT producer. Finally, since 2000, CIT has emerged as a highly promising metabolite with many regulatory properties (i.e. NO precursor and muscular protein synthesis regulation) (43). Hence, ingestion of CIT (10g) resulted in a selective increase in mixed muscle protein synthesis (+25%) (44). In brief, CIT could be a good candidate as an ergogenic molecule for athletes.

In this way, in a pioneering study, Hickner et al. (45) evaluated, in young healthy subjects, the specific effect of CIT supplementation (3g to 9g) before an incremental treadmill test until exhaustion. As an unexpected result, CIT supplementation impaired exercise performance compared with placebo. To explain this surprising response, it was indicated that CIT ingestion might reduce nitric-oxide-mediated pancreatic insulin secretion or increase insulin clearance. This hypothesis was based on the lower plasma insulin levels found after CIT ingestion (45). However, such hypothesis is not supported by literature i) in vitro studies performed on isolated pancreatic islets demonstrated secretagogue properties of CIT (46) ii) in malnourished aged rats, CIT supplementation increase basal insulin concentration (47). In the same way, Cutrufello et al. (48) failed to demonstrate an ergogenic effect of CIT supplementation in anaerobic and aerobic exercise performance. These two studies demonstrated no effect of acute CIT supplementation on any performance parameters with CIT (ranging of 3g to 9g). However, results changed when CIT is chronically ingested. Indeed, two studies evaluated the CIT supplementation during 7 days. In the first one, Bailey et al. (49) demonstrated that CIT supplementation (6g/day) improved time to exhaustion by 12% and the total amount of work completed (vs. placebo) in high-intensity cycling exercise. These ergogenic results could be explained by a lower VO_2 mean response time and a mean arterial blood pressure observed with the CIT supplementation. The second one demonstrated, with lower supplementation (2.4g/day), that CIT chronic ingestion significantly reduced completion time of 4-km cycling trial by 1.5% (vs. placebo) (50). Moreover, subjective feelings of muscle fatigue were improved immediately after exercise with CIT supplementation. This last result could be linked to the Bailey et al. (49) results on blood pressure.

Moreover, CIT would not only improve muscle perfusion but also preserve splanchnic perfusion. Indeed, intestinal hypoperfusion appears during strenuous physical activity, due to

blood redistribution from splanchnic area toward active muscles and cardiopulmonary system (51). It has been associated with intestinal disorders such as an impairment of gut barrier function and a worsen athletic performance (52). In this way, Van Wijck et al. (53) demonstrated that acute CIT ingestion (10g) preserved splanchnic perfusion in the gut and attenuated intestinal disorders during exercise in athletes. Thus, CIT could be a promising intervention to help athletes who often have intestinal problems related to exercise.

However, in dietary habits of athletes, CIT is usually consumed as malate salt. For this reason, several authors used this chemical form to evaluate CIT properties. The first of these studies examined the rate of muscle ATP production during an exercise of finger flexions (54). This study concluded that 6 g.day⁻¹ of citrulline malate (CM) for 16 days resulted in a significant increase (34%) in the rate of oxidative ATP production during exercise, and a 20% increase in the rate of phosphocreatine recovery after exercise. However, this study has several methodological flaws (i.e. no placebo group and no blinded condition). Two others studies conducted by the same research group showed an increase in plasma NO metabolites in well trained endurance athletes after a cycling competition; these athletes were supplemented with only one dose of CM (6 g) 2 hours before exercise (55,56). Interestingly, CIT preserved oxidative burst of polymorphonuclear neutrophils (PMNs) after exercise (55). This result could be of interest since dysimmunity is a classical symptom observed after intensive physical activity. Unfortunately, performance was not evaluated is this study. Another recent study by Pérez-Guisado and Jakeman (57) showed that a single dose of CM (8 g) increased work capacity by an average of 19%, measured as the number of repetitions performed until exhaustion during a resistance exercise. More recently, in a randomized and double-blind trial, Glenn et al. (58) demonstrated that acute CM (8g) supplementation in young females increased upper- and lower-body resistance exercise performance (respectively by 6% and 20%) and decreased rating of perceived exertion. Similar results on anaerobic performance were found by Wax et al. in resistance-trained males (59) and Glenn et al. (60) in middle-aged trained females after an acute CM supplementation (8g). However, results on CM supplementation must be considered with cautious since it is not possible to discard CIT effect to malate effect (i.e. malate may be involved in the beneficial effects on energy production as TCA intermediate (45,61)).

In conclusion, CIT seems to be a good candidat to help athletes. However, further studies are required to definitively conclude on the possible ergogenic effect of this amino acid.

Taurine

Taurine (TAU) is a sulfur molecule considered as an amino acid whereas the carboxylic function is in β of amine function. TAU is present in many tissues and the most abundant free amino acid in skeletal muscle and heart (mmol/l) but is not oxidized or incorporated into proteins (62,63). His role is very large because TAU is involved in cell signaling, volume regulation, cell development, membrane stability, antioxidant defense against stress and many metabolic effects (62). TAU is also a regulator of Ca²⁺ release from the sarcoplasmic reticulum in muscle and is involved in the contractile ability (64) and thus it could be interesting for the athlete.

In this line, its ergogenic effect was first explored in experimental level, and was then continued in human. The TAU effect was explored in many works with positive effects. Unfortunately, in almost cases, TAU was given in combination of several nutrients and it was not possible to distinguish the specific effect of TAU in such combination. Finally, to the best of knowledge, only four clinical trials explored the specific ergogenic effect of TAU.

In particular, Rutheford et al. (65) examined the acute effects of oral TAU supplementation (1.66g) given 1h before a 90 min submaximal cycle and subsequent endurance time trial in endurance trained male cyclists. Whatever the parameters concerned (time trial performance, rate of perceived exertion (RPE), heart rate and oxygen consumption (VO₂)), TAU was without effect compared to the control group. In contrast, a more recent study demonstrated that TAU ingestion (1g given 2h prior the time of performance testing) in well trained middle-distance runners was able to induce a slight improvement in performance of a 3 km treadmill time trial (66). Similar to the work of Rutherford et al. (65), TAU ingestion did not induce changes in RPE, heart rate, VO₂ and blood lactate concentration. These conflicting results on the performance could be related by several factors such as the intensity of the exercise performed or kinetic TAU administration. Indeed, in the study of Rutherford et al. (65) that shows no improvement in performance with acute TAU supplementation, the exercise intensity was moderate, since it represents 60% of the subjects VO_{2max} should be higher than the previous study.

Thus, in the study of Rutherford, moderate intensity physical effort involves almost entirely aerobic energy component while in the other study the physical exercise could involve both

aerobic and anaerobic energy components. The increase in performance on the 3-km race could therefore be linked to an effect of TAU on the anaerobic muscular metabolism. Such hypothesis is supported by study of Dutka et al. (64) which demonstrated that TAU differently affects the sarcoplasmic reticulum Ca^{2+} sensitivity of type I or II muscle fibers. Another explanation could be related to the TAU bioavaibility. Indeed, it has been demonstrated that the peak of TAU plasma is reached 2 hours after the ingestion of the amino acid. Thus, in the study of Rutherford et al. (65), despite highest dose of TAU (but delivered 1h before test), its plasma concentration would not be sufficient to obtain conclusive results to improve the physical performance.

Concerning TAU chronic supplementation, the literature is also scarce. Hence, only two trials have been performed. First, Ra et al. (67) did not demonstrated a specific effect of 2 weeks TAU supplementation (6g/day) on delayed onset muscle soreness (DOMS) (i.e. no changes of serum lactate dehydrogenase, creatine kinase and aldolase activities) 2 days after eccentric exercise. Unfortunately, the authors did not explore any performance parameters. In contrast, da Silva et al. (19) demonstrated that 3 weeks-TAU chronic supplementation (50md/kg/jour, around 4g/day) was able to improve the leg concentric and isometric strength and to decrease DOMS and muscle damages (i.e. decrease of serum lactate dehydrogenase and creatine kinase activities) during recovery period after eccentric exercise. They also found a decrease of markers of oxidative stress (markers of lipoperoxidation, protein carbonylation) but no change in cytokines pattern (TNF_a, IL1_β and IL-10). This positive result on strength would support the fact that TAU effect would be more effective in anaerobic condition.

Despite some positive results of TAU, due to the lack of robust randomized control trials aiming to demonstrate the ergogenic effect of TAU, consensual recommendations cannot be stated.

Beta-alanine

Beta-alanine (β -ALA) is a non-essential and non-proteinogenic β -amino acid mainly produced in the liver from the degradation of uracil. This amino acid is a constituent of anserine, penthotenic acid (B5 vitamin) and carnosine. Hence, β -ALA, combined with histidine, is the carnosine precursors (a reaction catalyzed by the carnosine synthetase) (68). Carnosine is abundantly found in excitable tissues, especially in skeletal muscle (69). Among its physiological roles, its main function is intracellular pH regulation, making carnosine a physicochemical buffer (69). It is usually considered that β -ALA availability is the ratelimiting factor for the endogenous synthesis of carnosine within skeletal muscle (69). This assertion is based on the fact that β -ALA concentration in tissue is lower than histidine (70). Moreover, the carnosine synthetase would be less sensitive to a variation of muscle histidine concentration ([histidine] >> Km = 16 μ M) than a variation of muscle β -ALA availability ([β -ALA] << Km=1.0-2.3 mM) (71,72). In brief, carnosine synthetase activity would be unaffected by increase of histidine concentration whereas it would be sensitive to an increase of β -ALA concentration. These metabolic specificities allowed to explore the potential interest of β -ALA supplementation in order to preserve the carnosine homeostasis.

In a princeps study, Harris et al. (69) demonstrated that β -ALA supplementation (i.e. ≥ 2 weeks) induces a significant increase of carnosine content in muscle. This result had been also reported by several authors (73,74). As example, β -ALA supplementation (minimum 2 weeks with around 6g/day) is able to increase the carnosine content in muscle by 60-80%, which is estimated to elevate the contribution of carnosine to whole muscle H⁺ buffering by an ~3–5 % increase in muscle buffering capacity (69).Such buffering capacity, during high-intensity exercise, could be an important contributor to performance and tolerance to exercise. The interest of β -ALA supplementation in athletes until 2011 is summarized in a meta-analysis (15 published studies) of Hobson et al. (75).

Briefly, they showed that β -ALA improved exercise to a greater extent than placebo. A highly significant effect was shown for exercises lasting 60-240 seconds, which strengthens the suggestion that the primary role of muscle carnosine is pH buffering. With the growing popularity of β -ALA, numerous investigations have been published since this meta-analysis and have shed further light on the ergogenic potential of this nutritional supplement. At the time of the meta-analysis, there was clear evidence that exercises shorter than one minute are unaffected by β -ALA (76–78). Since 2011, Hoffman et al. (79) and Cochran et al. (80) found a similar results on anaerobic performance. This was confirmed by Hannah et al. (81), who

showed no effect of β -ALA on maximum voluntary contractions. To the best our knowledge, only Kresta et al. (82) demonstrated that 4 weeks supplementation (6,1g/day), in active women, improved the repeated sprint power (normalized to bodyweight) and reduced the rate of fatigue with multiple Wingate anaerobic capacity tests. These studies seems to confirm the lack or the slightly effect of β -ALA on anaerobic performance. However, some recent studies seem to demonstrate that β -ALA could be efficient to improve explosive performance. First, Gross et al. (83) showed that 5 weeks of supplementation (4.8g/day) is able to improve maximal and mean countermovement jump variables (both 7%). This result on explosive performance has been confirmed by Carpentier et al. (84) showing a lower decline in jump heights during a fatigue test, after 8 weeks of β -ALA supplementation in young athletes. However, the countermovement jump and squat jump tests were not affected by the supplementation unlike the study of Gross et al. (83). Moreover, Hannah et al. (81) did not show any improvement of explosive voluntary contractions. The whole of these three studies are promising but more studies are needed to confirm a positive effect of β -ALA supplementation on explosive capacities.

Despite these results on anaerobic performances, several studies have shown the efficacy of increased muscle carnosine content through β -ALA supplementation on high-intensity exercises lasting 1–4 min. Indeed, exercise performance and capacity have been improved in a variety of cycling (73,85–88) , running (89,90), and repeated-bout upper- and lower-body protocols (91,92). The majority of research reporting an ergogenic effect of β -ALA supplementation is on exercise lasting 1–10 min, although not all agree (93–96). Using a high-intensity cycling capacity test at 110 % of maximum power output (designed to last between 120-240 s) (97), β -ala supplementation. improved the time-to-exhaustion from 12% to 14% (73) (88) (98) in recreationally active participants.

These studies highlight the consistency in responses across individuals following supplementation during a high-intensity test limited by increasing acidosis. Hobson et al. (75) showed an effect of β -ALA on exercise lasting more than 240 s, although this result was likely due to the incremental nature of many of the tests employed, which are very low in intensity in the earlier stages (85,86). Three studies did not show any improvement on prolonged cycling time trial performance (87,96,99), which is in line with the role of carnosine as an intramuscular buffer since fatigue during exercise of this duration is not associated with modification of acidosis. Some doubts has been raised concerning the efficacy of β -ALA on athletes since sprint-trained individuals have been shown to have an elevated

buffering capacity compared with their non-trained and endurance-trained counterparts (100,101). It has been argued that previously elevated muscle buffering capacity could minimize any improvements brought about via increased carnosine. This has gained some support from indirect evidence in studies with well-trained athletes who supplemented with β-ALA and showed no improvements in performance (76,78,93,94). However, a recent study investigated the effects of β-ALA supplementation on high-intensity cycling performance in both trained cyclists and nontrained individuals. Both groups showed a similar improvement in total work done (≈ 3 %) following supplementation (92), indicating that β-ALA is equally efficient in untrained and athletic populations. Indeed, there is now a growing evidence to support the effective use of β-ALA among the elite athletic populations, especially on 100-and 200-m freestyle swimming (102), 2000-m rowing (89,103,104) and 800-m running (89).

Concerning the known side effets from the use of β -ALA, the only currently one reported in the literature is paraesthesia, which has been described as a prickly sensation on the skin that starts within 10–20 min following ingestion and lasts up to 1 h (69). These symptoms typically arise from high doses of β -ALA and are associated with the peak plasma β -ALA level (69). Although harmless, paraesthesia is unpleasant and may compromise the blinding of a research investigation; therefore, dosing strategies aiming to avoid paraesthesia are employed. To circumvent the occurrence of paraesthesia, studies have consistently staggered dosing protocols throughout the day. Future research should ascertain as to whether longer-term supplementation is free of any other side effects.

In conclusion, the literature on ergogenic effects of β -ALA is the most consistent. It seems that β -ALA is an good ergogenic aid for exercise of 1 min to 4 min intensity. Otherwise, for resistance exercise or endurance exercise, β -ALA seems ineffective.

Leucine

Besides his role as an energy substrat, LEU has several important properties, in particular the capacity to stimulate muscle protein synthesis (105). Thus, LEU could be a major supplement for the athletes and many studies worked on the interest of LEU as an ergoneic aid for athletes. However, among this large literature, most of these studies did not evaluate the direct action of LEU on the performance or associated-parameters. Because the aim of this review is to evaluate the specific action of amino acids as ergogenic aid, we will only discuss of papers evaluating the specific effect of LEU wich could help athletes.

Three works determined the specific acute affect of LEU on performance. Stock et al. (106) showed, in resistance trained adults (mostly men) performing sets of squats to fatigue (75% of the 1-RM) and consuming a carbohydrate beverage 30 min before and immediately after exercise, that addition of LEU (45 mg.kg⁻¹, 22.5 mg before and 22.5 mg after exercise, around 3.5g overall) in the beverage did not affect the delayed-onset muscle soreness nor performance. In a second study evaluating the acute LEU effect (107), untrained males performed 100 depth jump from 60 cm and 6 sets of ten repetitions of eccentric-only leg presses with a high supplementation of LEU (250 mg.kg⁻¹, the upper limit of leucine safe intake in healthy adults being stated at 0.53g.kg⁻¹.day⁻¹ as reported by Cynober et al. (108)) or placebo 30 min before, during and immediately post-exercise. Then, muscle function was determined by peak force during an isometric squat and by jump height during a static jump at pre-exercise and 24,48 ,72 and 96 h post-exercise. Peak force was significantly decreased across all time points for both experimental groups. However, the LEU group experienced an attenuated drop in mean peak force across all post-exercise time points compared to the placebo group. Muscle soreness increased across all time points for the both groups but the LEU group experienced a significantly higher increase in mean muscle soreness postexercise. Therefore, following exercise-induced muscle damage, acute very high-dose leucine supplementation may contribute to help the athlete by decreasing muscle fatigue. However, this study has a major limitation because LEU supplementation is very high and placebo is not isonitrogenous. Thus, the positive effect of the LEU suppelementation observed could be related to an important nitrogen ingestion and not to a specific effect of LEU. Finally, a third study from Thomson et al. (109) demonstrated a positive effect of acute LEU supplementation on performance. In this crossover study, 10 male cyclists performed 2 to 2.5 h interval training bouts on 3 consecutive evenings, ingesting either LEU-protein, high-carbohydrate nutrition $(0.1/0.4/1.2/0.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1};$ leucine, protein, carbohydrate, fat, respectively) or isocaloric control ($0.06/1.6/0.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$; protein, carbohydrate, fat, respectively) nutrition for 1.5 h after exercise. Following 39 h of recovery, cyclists then performed a repeat-sprint performance test. Mean sprint power was improved by 2.5% and perceived overall tiredness was reduced during the sprints by 13%, but without changes of perceptions of leg tiredness and soreness. Therefore, high acute dose of LEU seems to positively improve the performance in trained and untrained subjects.

Two studies evaluated the chronic effect of a LEU supplementation. Crowe et al. (110) demonstrated in competitive outrigger canoeists supplemented with 45 mg.kg⁻¹.day⁻¹(around 3.5g) or not during 6 weeks, that rowing time to exhaustion was increased, perceived exertion was decreased and upper body power was better in LEU-supplemented group. Ispoglou et al. (111) showed similar result in untrained men ingesting 4 g LEU per day or a placebo (lactose) during 12 weeks and following a resistance training program. Strength on each exercise was assessed by 5 reptition maximum (5-RM) and body composition was assessed by DXA.The LEU group demonstrated significantly higher gains (40.8% vs. 31.0% for placebo group) in total 5-RM strength (sum of 5-RM in eight exercises) and in particular 5-RM strength in five out of the eight exercises without any changes in body composition.

In conclusion, LEU seems to be a very good ergogenic aid. Indeed, almost all works studying specific effect of LEU on performance showed a positive effect. However, although many studies worked on the LEU capacity to improve performance, only few studies (all reported in this review) highlighted the specific ergogenic effect of LEU. Thus, more studies are required to definitively confirmed the interest of LEU supplementation as ergogenic aid.

Conclusion

Since the last decades, the ergogenic properties of amino acids fascinate many athletes and provoke many clinical trials to confirm their properties. However, despite many studies, the results are often unclear. Indeed, most of the clinical trials studied amino acids effects in combination with others products. Therefore, it is not possible to determine the real specific effect of an amino acid considered. Thus, our review focused only on studies determining specific effects of the most used amino acids by athletes. To answer whether an amino acid could be an ergogenic aid, we tried to dissect on particularities of each studies; in particular, the training of subjects, the administration timing, the dose, the acute or chronic effect. Thus, considering all these particularities, it was difficult to definitively conclude on the possible ergogenic effect on amino acid reported in this review. Moreover, despite the intention of all studies cited in this review, almost all studies have limits. Indeed, in almost all cited studies, the supplementation is not isonitrogenous and isocaloric. Therefore, when a study shows positive results from an amino acids, it is often difficult to discare the specific effect of the amino acid or whether it is an effect related to a improvement of the nitrogen or energy homeostasis. Finally, a part of studies are not performed in double-blind way.

Despite these limits, for some amino acids, results seem interesting for the athletes. Among all amino acids considered in this review, β -ALA is the only one with a consistent literature and enough evidences to consider β -ALA as an ergogenic aid for athletes in particular exercise intensities. LEU , CIT and, to a lesser extent, TAU seem to have ergogenic effects. Unfortunately, the literature is to light to clearly recommend these amino acids as ergogenic aids. Further studies sould be required to support the likely ergogenic effect from these amino acids. Finally, ARG and GLN are both important amino acids useb by athletes. And yet, despite a literature relatively consistent, no evidences show a possible ergogenic effect of these amino acids. Therefore, a supplementation with ARG or GLN is clearly ineffective to improve performance or associated-factors.

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Propriétés nutritionnelles de la citrulline :

un nouvel acteur dans la régulation du métabolisme protéino-énergétique.

Outre son rôle dans le métabolisme du cycle de l'urée, la citrulline possède de nombreuses propriétés notamment celle de stimuler la synthèse protéique musculaire. Or, la synthèse protéique est un poste de dépense énergétique important de la cellule. Nos travaux ont ainsi exploré, à la fois *in vivo* et *in vitro*, les effets de la citrulline sur le métabolisme énergétique afin de comprendre comment l'activation de la synthèse protéique musculaire par cet acide aminé est coordonnée avec le métabolisme énergétique. Nos résultats ont permis de mettre en évidence que la citrulline module bien le métabolisme énergétique, notamment *via* une réorientation des flux énergétiques au profit de la synthèse protéique. De plus, nos travaux ont précisé les effets de la citrulline sur le métabolisme protéique avec notamment un effet synergique de la citrulline et de l'exercice sur la synthèse protéique et sur la performance. Enfin, ces travaux ont permis d'explorer pour la première fois *in vitro* les effets de la citrulline (et de la leucine) sur le sécrétome musculaire. Nous avons ainsi démontré que la citrulline module le sécrétome musculaire et mis en évidence la complexité de régulation des protéines sécrétées par les acides aminés. En conclusion, nos travaux sont une contribution à la meilleure compréhension de la régulation musculaire du métabolisme protéino-énergétique par la citrulline.

Mots clés : mitochondrie, muscle, exercice, synthèse protéique, bioénergétique, protéomique, sécrétome

Nutritional properties of citrulline: a new actor in the protein-energy metabolism regulation

Besides his role in the metabolism of the urea cycle, citrulline has many properties including the ability to stimulate muscle protein synthesis. However, protein synthesis is an important item of cell energy expenditure. Our work has explored both the in vivo and in vitro effects of citrulline on energy metabolism in order to understand how the activation of muscle protein synthesis by this amino acid is coordinated with energy metabolism. Our results have shown that citrulline modulates energy metabolism via a reorientation of energy flux in favor of protein synthesis. Moreover, our work has clarified citrulline effects on protein metabolism with a synergistic effect of citrulline and exercise on protein synthesis and performance. Finally, this work allowed to explore for the first time citrulline (and leucine) effects on muscle secretome. We have thus demonstrated that citrulline modulates muscle secretome and highlighted how complex is the regulation of secreted proteins by amino acids. In conclusion, our work contributes to a better understanding on muscle regulation of protein-energy metabolism by citrulline.

Keywords: mitochondria, muscle, exercise, protein synthesis, bioenergetics, proteomics, secretome