



Optimized EPA/DHA 6/1 formulation prevents Angiotensin-II induced hypertension and endothelial dysfunction in rats

Zahid Rasul Niazi

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DE STRASBOURG**



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Optimized EPA: DHA 6:1 formulation prevents Angiotensin-II induced hypertension and endothelial dysfunction in rats

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Dedication

This thesis is dedicated to my beloved father, late mother, my lovely wife and children for their love, patience and understanding.

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List of publications

1. Publications:

Rasul Zahid Niazi, Silva G.C, Porto Ribeiro T, Zgheel F, Auger C, Schini-Kerth V. Chronic oral Intake of the omega 3 formulations EPA:DHA 6:1 prevents the angiotensin II-induced hypertension and endothelial dysfunction in rats (submitted to the British Journal of Pharmacology).

Mohamad Kassem, **Zahid Rasul Niazi**, Malak Abbas, Ali El Habhab, Guillaume Kreutter, Sonia Khemais-Benkhiat, Cyril Auger, Maria-Cristina Antal, Valerie B. Schini-Kerth, Florence Toti, Laurence Kessler. The Endocrine Pancreas, an Early Sensor of Senescence in Middle-aged Rats with Still Normal Vascular Function. (Under revision)

Alain N'guessan Yao, **Zahid Rasul Niazi**, Iveta Najmanová, Mamadou Kamagaté, Amissi Said, Henri Die-Kacou, Cyril Auger, Philippe Chabert, Valérie Schini-Kerth. Chronic treatment with an aqueous extract of *Phyllanthus amarus* prevents hypertension and endothelial dysfunction in Doca-salt rats. (paper in preparation)

Amissi Said, **Zahid Rasul Niazi**, Mélanie Burban, Romain Kessler, Mathieu Canuet, Florence Toti, Laurent Monassier, Nelly Boehm, Cyril Auger, Ferhat Meziani and Valérie B. Schini-Kerth. The omega-3 EPA:DHA 6:1 superior formulation prevents the monocrotaline-induced pulmonary hypertension, endothelial dysfunction and vascular remodeling, and right ventricular failure in rats. (paper in preparation)

2. Poster Communications:

1) **Niazi Z**, Silva G.C, Porto Ribeiro T, Zgheel F, Auger C, Schini-Kerth V. Chronic oral Intake of the omega 3 formulation EPA:DHA 6:1 prevents the angiotensin II-induced hypertension and endothelial dysfunction in rats, **JCI** (Campus Illkirch day) 13-14 April Illkirch France 2015.

2) **Niazi Z**, Silva G.C, Porto Ribeiro T, Zgheel F, Auger C, Schini-Kerth V. Chronic oral Intake of the omega 3 formulation EPA:DHA 6:1 prevents the angiotensin II-induced hypertension and endothelial dysfunction in rats, **CPBI** (Congrès de la société de physiologie et de biologie intégrative) 4-6 May Strasbourg-France 2015.

3) **Niazi Z**, Silva G.C, Porto Ribeiro T, Zgheel F, Auger C, Schini-Kerth V. Chronic oral Intake of the omega 3 formulation EPA:DHA 6:1 prevents the angiotensin II-induced hypertension and endothelial dysfunction in rats, **FMTS** (Fédération de médecine translationnelle de Strasbourg) 16-17 April, Strasbourg-France 2015.

4) Kassem M, **Niazi Z**, Abbas M, Samama B, Auger C, Constantinescu A, Vauchelles R, Lessinger JM, BOEHM N, Schini-kerth V, Toti F, Kessler L. Morphological and functional abnormalities of the endocrine pancreas in middle age rat: Impact for transplantation, **CPBI** (Congrès de la Société de Physiologie et de Biologie Intégrative) 4-6 may, Strasbourg-France. La résumé est publié au journal Acta Physiologica Volume 214, Issue Supplement S700, Pages 1-92, May 2015.

5) **Niazi Z**, Silva G.C, Porto Ribeiro T, Zgheel F, Auger C, Schini-Kerth V. Chronic oral Intake of the omega 3 formulation EPA:DHA 6:1 prevents the angiotensin II-induced hypertension and endothelial dysfunction in rats, (Printemps de la cardiologie) 2-3 April Toulouse-France 2015.

6) **Niazi Z**, Silva G.C, Porto Ribeiro T, Zgheel F, Auger C, Schini-Kerth V. Chronic oral Intake of the omega 3 formulation EPA:DHA 6:1 prevents the angiotensin II-induced hypertension and endothelial dysfunction in rats, **ESC** (European society of cardiology) 23-26 May Seville-Spain 2015.

7) **Niazi Z**, Silva G.C, Porto Ribeiro T, Zgheel F, Auger C, Schini-Kerth V. Chronic oral Intake of the omega 3 formulation EPA:DHA 6:1 prevents the angiotensin II-induced hypertension and endothelial dysfunction in rats, **CIC** (French society of pharmacology and therapeutics) 21-23 April Caen-France 2015.

3. Oral Communications

1) **Niazi Z**, Silva G.C, Porto Ribeiro T, Zgheel F, Auger C, Schini-Kerth V. Chronic oral Intake of the omega 3 formulation EPA:DHA 6:1 prevents the angiotensin II-induced hypertension and endothelial dysfunction in rats, **CIC** (French society of pharmacology and therapeutics) 21-23 April Caen-France 2015.

2) **Niazi Z**, Silva G.C, Porto Ribeiro T, Zgheel F, Auger C, Schini-Kerth V. Chronic oral Intake of the omega 3 formulation EPA:DHA 6:1 prevents the angiotensin II-induced hypertension and endothelial dysfunction in rats, **JCI** (journées scientifiques du campus universitaire d'Illkirch) 21-13 May Strasbourg-France 2016.

3) Kassem M, **Niazi Z**, Abbas M, Vauchelles R, Auger C, Antal MC, Schini-kerth V, Toti F, Kessler L. The endocrine pancreas an early sensor of senescence in middle-aged rats: Impact for transplantation, **ADA** (American Diabetes Association) 10-14 June New Orleans, LA- USA 2016.

List of Abbreviations

AA	Arachidonic Acid
AC	Adenylyl cyclase
ACE	Angiotensin Converting Enzyme
Ach	Acetylcholine
ADM	Adenosine Monophosphate
ADMA	Asymmetric Dimethylarginine
ADP	Adenosine Di-Phosphate
ALA	Alpha-Linolenic Acid
ANOVA	Analysis of Variance
ARAs	Angiotensin Receptor Antagonists
ARBs	Angiotensin Receptor Blockers
AT1R	Angiotensin II Type 1 Receptor
AT2R	Angiotensin II Type 2 Receptor
ATP	Adenosine Tri-Phosphate
BH4	Tetrahydrobiopterin
BP	Blood Pressure
BW	Bodyweight
CAD	Coronary Artery Disease
cAMP	Cyclic Adenosine-3',5'-Mono-Phosphate
CBDL	Common Bile Duct Ligation
cGMP	Cyclic Guanosine 3'-5' Monophosphate
CHD	Coronary Heart Disease
CHF	Congestive Heart Failure
COX	Cyclooxygenases
CRP	C-reactive protein
CVD	Cardiovascular Disease
Cx37	Connexin 37
CYP	Cytochrome
DHA	Docosahexaenoic Acid
DM	Diabetes Mellitus
DPA	Docosapentaenoic Acid
EC	Endothelial Cell
EDCF	Endothelium-Derived Contracting Factor
EDH	Endothelium-Dependent Hyperpolarization
EDHF	Endothelium-Derived Hyperpolarizing Factor
EDRF	Endothelium-Derived Relaxing Factor
EETs	Epoxyeicosatrienoic Acids
EFA	Essential Fatty Acids
eNOS	Endothelial Nitric Oxide Synthase

EPA	Eicosapentaenoic Acid
EPCs	Endothelial Progenitor Cells
ER	Estrogen Receptor
FAME	Fatty Acid Methyl-Esters
FAs	Fatty Acids
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GC/MS	Gas Chromatography/Mass Spectrometry
GLA	Gamma-Linolenic Acid
GLC	Gas Liquid Chromatography
GPx	Glutathione Peroxidase
GTP	Guanosine Triphosphate
H₂O₂	Hydrogen Peroxide
HDL	High Density Lipoprotein
HETEs	Hydroxyeicosatetraenoic Acids
HO-1	Hemeoxygenase-1
HSP-90	Heat Shock Protein 90
IK_{Ca}	Intermediate conductance calcium-activated potassium channel
IL-6	Interleukin-6
Inos	Inducible Nitric Oxide Synthase
JAKs	Janus kinases
LA	Linoleic Acid
LDL	Low Density Lipoprotein
Lev	Levcromakalim
L-NA	N^ω-Nitro-L-arginine
L-NAME	N^ω-Nitro-L-Arginine Methyl Ester
LOX	Lipoxygenases
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LTs	Leukotrienes
MAPKs	Mitogen activated protein kinases
MCP-1	Monocyte Chemoattractant Protein-1
MGJ	Myoendothelial Gap Junction
MMPs	Matrix Metalloproteinase
MnTMPyP	Mn (III) Tetrakis(1-methyl-4 pyridyl) porphyrin, Superoxide Dismutase Mimetic
MUFA	Mono-Unsaturated Fatty Acids
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
O²⁻	Superoxide Anions
OA	Oleic Acid

OH[·]	Hydroxyl Groups
ONOO⁻	Peroxynitrite
PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PDGF	Platelet-Derived Growth Factor
PEG-SOD	Polyethylene Glycol-Superoxide Dismutase
PGE	Prostaglandins E2
PGG₂	Prostaglandin G₂
PGH₂	Prostaglandins H₂
PGI₂	Prostacyclin
PI3K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PLA-2	Phospholipase A-2
PLD	Phospholipase D
PUFA	Poly-Unsaturated Fatty Acids
RAAS	Renin-Angiotensin-Aldosterone-System
RAS	Renin-Angiotensin System
RIPA	Radioimmunoprecipitation assay
RLP	Remnant lipoprotein
ROS	Reactive Oxygen Species
SEM	Standard error mean
SFA	Saturated fatty acids
Sgc	Soluble Guanylyl Cyclase
SHR	Spontaneously hypertensive rat
Skca	Small conductance calcium-activated potassium channel
SNP	Sodium nitroprusside
SOD	Superoxide Dismutase
TGF-B1	Transforming Growth Factor-B1
TNF-α	Tumor Necrosis Factor-alpha
TXA₂	Thromboxane A₂
VCAM	Vascular Cell Adhesion Molecule-1
VEGF-A	Vascular Endothelial Growth Factor A
VEGFR	Vascular Endothelial Growth Factor Receptor
VLDL	Very Low Density Lipoprotein
VSMC	Vascular Smooth Muscle Cell
WHO	World Health Organization

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Abstract

Cardiovascular diseases are the leading cause of death worldwide, both in developed and developing countries. The incidence of cardiovascular diseases is linked to several risk factors that are either non-modifiable (age, gender, genetic background) or modifiable. The modifiable risk factors include lifestyle (western diet, tobacco use, alcohol abuse, physical inactivity) and treatable diseases such as hypertension, dyslipidemia, obesity, and diabetes. Many preclinical and clinical studies have shown that development of cardiovascular diseases and risk factors are associated early with an endothelial dysfunction.

The endothelium, the monocellular layer lining all blood vessels, represents the largest cell compartment in contact with blood flow. Endothelial cells contribute to the vascular tone and constitute a protective surface with anti-thrombotic properties, mainly through the release of potent vasoprotective factors such as nitric oxide (NO). In cardiovascular diseases and also during physiological ageing, the endothelial dysfunction is characterized by a decreased formation of vasoprotective factors and an increased formation of vasoconstricting factors, resulting in an imbalance leading towards the accelerated development of vascular pathologies. Endothelial dysfunction is involved in atherosclerotic lesion formation by the promotion of both the early and late mechanisms of atherosclerosis including up-regulation of adhesion molecules, increased chemokine secretion and leukocyte adherence, increased cell permeability, enhanced low-density lipoprotein oxidation, platelet activation, cytokine elaboration, and vascular smooth muscle cell proliferation and migration.

In addition, several studies have demonstrated that endothelial dysfunction and cardiovascular diseases development are also associated with an increased vascular oxidative stress and an up-regulation of the local angiotensin system. Angiotensin II (Ang II) contributes to the pathophysiology of atherosclerosis and vascular diseases not only via its role in hypertension but also via its direct effects on vascular cell growth and migration. Ang II also contributes to the development of cardiovascular diseases through the induction of oxidative stress by up-regulating NADPH oxidase, the main producer of reactive oxygen species (ROS) in the vascular wall.

Diets could play a role in the development of cardiovascular diseases. Indeed, while western diet rich in saturated fatty acids has been associated with an increased risk of cardiovascular diseases, other diets such as the Mediterranean diet rich in unsaturated fatty acids have been associated with a reduced incidence of cardiovascular diseases up to 30 %. Several epidemiological studies and clinical trials have shown that dietary intake of fish, fish oil or omega-3 polyunsaturated fatty acids (n-3 PUFAs) have beneficial effects against coronary heart disease, stroke, and hypertension. Moreover, the dietary consumption of the major n-3 PUFAs, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been related to a reduced risk of cardiovascular disease morbidity/mortality. The exact mechanism by which n-3 PUFAs inhibit atherosclerosis is still unclear, but it may relate to the modulation of lipid metabolism, decrease in pro-inflammatory cytokine production, and inhibition of inflammatory processes.

It has been demonstrated in endothelial cells that EPA stimulates the endothelial nitric oxide synthase (eNOS) activation by inducing its detachment from the inhibitory supportive protein caveolin, while DHA stimulates eNOS activity by increasing the interaction between eNOS and heat shock protein 90 (HSP-90) which activates PKB/Akt pathway resulting in eNOS phosphorylation and activation. In conditions like hypertension or renal failure, n-3 PUFA could reduce the increased level of asymmetric circulating dimethylarginine (ADMA, an endogenous inhibitor of eNOS) resulting in an increase of eNOS activity.

Moreover, our research team recently demonstrated that the stimulation of the endothelial function by n-3 PUFAs is dependent on both the purity and the ratio of EPA and DHA. Indeed, an optimized EPA:DHA 6:1 formulation is a potent stimulator of the endothelial formation of NO, and to a lesser extent, of an increased endothelium-dependent hyperpolarisation (EDH) response. The induction of the endothelial formation of NO by omega-3 fatty acids is mediated by redox-sensitive activation of the Src/PI3-kinase/Akt and MAPKs pathways leading to eNOS activation, which is dependent on the ratio and amount of the EPA:DHA in the formulation.

In humans, a direct vasodilatory effect has been demonstrated following intake of DHA, which inhibits the vasoconstrictor response produced by angiotensin and norepinephrine. A large body of studies demonstrated that n-3 PUFA are able to reduce systemic blood pressure and a recent

meta-analysis confirmed that a consumption of more than 2 g/d of EPA + DHA can reduce systolic and diastolic blood pressure in humans.

The aim of the present study was to determine whether chronic oral intake of the optimized EPA:DHA 6:1 formulation is able to prevent the hypertension and endothelial dysfunction induced by Ang II in rats.

Male Wistar rats received by daily gavage 500 mg/kg BW of either corn oil (control) or the EPA:DHA 6:1 formulation. After one week, the rats underwent either sham surgery or implantation of an osmotic mini-pump infusing 0.4 mg/kg/day of angiotensin II. Systolic blood pressure was measured twice weekly using the tail cuff sphygmomanometry method. After 4 weeks of gavage, the animals were euthanized and the organ was collected. The secondary branch of mesenteric artery were used for vascular reactivity studies using a wire myograph, for immunofluorescence and fluorescence histochemistry studies on frozen section, and for western blot analysis of protein expression.

The major results of our study show that infusion of Ang II (0.4 mg/kg/day) caused a significant increased in systolic blood pressure, which was reaching 194 ± 5.9 mmHg compared to 120 ± 5.6 mmHg in control rats. Oral intake of EPA:DHA 6:1 (500 mg/kg/day) significantly prevented the Ang II-induced hypertension (147 ± 5.9 mmHg), while having no effect on basal systolic blood pressure (110 ± 3.9 mmHg).

The chronic intake of the optimized EPA:DHA 6:1 formulation is associated with significantly increased plasmatic presence in omega-3 fatty acids, mainly as EPA, DHA and the intermediate elongated metabolite of EPA, the docosapentaenoic acid (DPA), resulting in a decreased omega-6/omega-3 ratio. The reduction of this ratio have been associated with a shift towards beneficial health effects of omega-3, including reduced cardiovascular and cancer risk, whereas increased ratios such as the Western diet has been associated with increased prevalence of cardiovascular and chronic diseases. Vascular reactivity studies in the secondary branch of mesenteric artery indicate that Ang II induced an endothelial dysfunction characterized by reduced relaxations in response to acetylcholine affecting both the NO- and EDH-mediated component, and increased formation of endothelium-derived contractile factors (EDCFs) in response to acetylcholine. The

chronic intake of EPA:DHA 6:1 normalized both the NO, EDH and EDCF responses in secondary branch of mesenteric artery (Simopoulos 2002).

To better characterize the molecular mechanisms involved in the protective effects of EPA:DHA 6:1 intake, we performed quantitative analysis of protein expression in the secondary branch of the mesenteric artery by immunofluorescence.

Firstly, we studied the expression of eNOS, arginase-1, SK_{Ca} and Cx37 (EDH component of relaxation), and cyclooxygenases (COXs, involved in EDCFs). Compared to the controls, Ang II significantly up-regulate the expression of eNOS, arginase 1, COX-1 and COX-2, the inducible isoform of COXs, while down-regulating the expression of SK_{Ca} and Cx37. The intake of EPA:DHA 6:1 also normalized the expression levels of eNOS, arginase 1, SK_{Ca}, Cx37, COX-1 and COX-2.

As endothelial dysfunction is associated with a vascular oxidative stress, we measured the level of oxidative stress in the vascular wall of the secondary branch mesenteric artery using the redox-sensitive fluorescent probe dihydroethidium (DHE). Ang II induced a significant increases of DHE fluorescence throughout the vascular wall as compared to control rats, which was significantly prevented by the EPA:DHA 6:1 intake. As the increased vascular oxidative stress in Ang II-induced hypertension has been attributed, at least in part, to the up-regulation of NADPH oxidase expression through the activation of the Ang II type 1 receptor (AT1R), we then determined that expression levels of both AT1R and NADPH oxidase sub-units p22^{phox} and p47^{phox}. Compared to control rats, the secondary branch of the mesenteric artery of rats infused with Ang II exhibit a significantly increased expression level of AT1R, p22^{phox} and p47^{phox}. The EPA:DHA 6:1 treatment significantly improves the Ang II-induced vascular oxidative stress, up-regulation of AT1R and NADPH oxidase.

To confirm the results obtained by immunofluorescence in the secondary branch of the mesenteric artery, we performed Western blot analysis of the expression levels of eNOS, COX-2, and the NADPH oxidase subunit p22^{phox} in the main mesenteric artery. The Ang II group presented a significantly increased expression of eNOS, COX-2, and the NADPH oxidase subunit p22^{phox}, that was prevented by the chronic oral intake of EPA:DHA 6:1.

Altogether, the present findings indicate that chronic intake of the optimized EPA:DHA 6:1 formulation prevented the development of hypertension and endothelial dysfunction induced by the infusion of Ang II in rats. The Ang II-induced endothelial dysfunction is associated to an up-regulation of the local angiotensin system and an increased vascular oxidative stress. The beneficial effect of EPA:DHA 6:1 is mediated by an improvement of both the NO- and the EDH-mediated relaxations and a reduction of endothelium-dependent contractile response, most likely by preventing the oxidative stress induced by the up-regulation of the local angiotensin system.

Résumé

Les maladies cardiovasculaires représentent la première cause de mortalité dans le monde, que cela soit dans les pays développés ou ceux en cours de développement. L'incidence des maladies cardiovasculaires est associée à de nombreux facteurs de risques pouvant être non-modifiables (âge, sexe, patrimoine génétique ...) ou modifiables. Parmi ces derniers, on trouve des facteurs liés au style de vie (régime occidental, tabagisme, alcoolisme, sédentarité) ou des pathologies pouvant être traitées comme l'hypertension, les dyslipidémies, l'obésité ou les diabètes. De plus, de nombreuses études précliniques et cliniques ont montré que les maladies cardiovasculaires sont précocement associées à une dysfonction endothéliale.

L'endothélium, la monocouche cellulaire tapissant l'intérieur des vaisseaux sanguins, est le plus grand organe en contact direct avec le flux sanguin. Les cellules endothéliales ont un rôle clé dans le maintien du tonus vasculaire et constituent une couche protectrice exerçant des effets anti-thrombotiques, principalement grâce à la formation et libération de puissants facteurs vasoprotecteurs tels que le monoxyde d'azote (NO). Dans les maladies cardiovasculaires ou au cours du vieillissement physiologique, apparaît une dysfonction endothéliale caractérisée par une diminution de la formation des facteurs protecteurs et une augmentation de la formation des facteurs vasoconstricteurs, le tout engendrant un déséquilibre menant au développement accéléré des pathologies vasculaires. La dysfonction endothéliale est impliquée dans la formation de lésions athéromateuses en favorisant les mécanismes précoces et tardifs du développement de l'athérosclérose dont l'augmentation de l'expression des molécules d'adhésion, de la sécrétion de

chimiokines, de l'adhésion des leucocytes, de l'oxydation des LDL, de l'activation plaquettaire, et de la prolifération et de la migration des cellules musculaires lisses vasculaires. De plus, plusieurs études ont montré que la dysfonction endothéliale et le développement des maladies cardiovasculaires sont associés à une augmentation du stress oxydant vasculaire et à une surexpression du système angiotensine local. L'angiotensine II (Ang II) participe à la physiopathologie de l'athérosclérose et des maladies vasculaires non seulement de par son rôle dans l'hypertension, mais aussi de par son effet direct sur la prolifération et la migration des cellules vasculaires. L'Ang II contribue aussi au développement des maladies cardiovasculaires de par l'augmentation du stress oxydant vasculaire induit par la surexpression de la NADPH oxydase, la principale source des espèces réactives de l'oxygène dans la paroi vasculaire.

L'alimentation peut jouer un rôle dans le développement des maladies cardiovasculaires. Ainsi, alors que le régime occidental riche en graisses saturées a été associé à une augmentation du risque de maladies cardiovasculaires, d'autres types d'alimentation tels que le régime Méditerranéen riche en graisse non-saturées ont montrées une réduction de l'incidence de maladies cardiovasculaires allant jusqu'à 30 % de réduction. De nombreuses études épidémiologiques ou d'intervention ont montré que la consommation alimentaire de poisson, d'huile de poisson ou d'acides gras polyinsaturés omega-3 (n-3 PUFAs) exerçait des effets bénéfiques vis-à-vis de la maladie coronarienne, des accidents vasculaires cérébraux et de l'hypertension. De plus, la consommation des acides gras n-3 PUFAs majeurs, à savoir l'acide eicosapentaénoïque (EPA) et l'acide docosahexaénoïque (DHA), est associée à une réduction de la morbi-mortalité cardiovasculaire. Les mécanismes par lesquels les n-3 PUFAs inhibent le développement de l'athérosclérose restent à éclaircir, mais pourraient être dû, au moins partiellement, à une modulation des métabolites lipidiques, une diminution de la production de cytokines pro-inflammatoires et une réduction des processus inflammatoires. Il a été montré que dans les cellules endothéliales, l'EPA stimule l'activation de la NO synthase endothéliale (eNOS) en induisant sa dissociation d'avec la protéine inhibitrice cavéoline, alors que le DHA stimule la formation endothéliale de NO en augmentant les interactions entre la eNOS et la protéine chaperonne HSP-90 qui active la voie PKB/Akt conduisant à la phosphorylation activatrice de la eNOS. Dans les situations physiopathologiques comme l'hypertension ou l'insuffisance rénale, les n-3 PUFAs

peuvent réduire l'augmentation des niveaux circulants de diméthylarginine (ADMA, un inhibiteur endogène de la eNOS), ce qui induit une augmentation de l'activité de la eNOS.

De plus, notre équipe de recherche a récemment démontré que la stimulation de la fonction endothéliale par les n-3 PUFAs dépend à la fois du ratio et du degré de pureté de la formulation en EPA et DHA. En effet, la formulation optimisée EPA:DHA 6:1 est un puissant activateur de la formation endothéliale de NO, et de façon moindre de l'augmentation de la réponse d'hyperpolarisation dépendante de l'endothélium (EDH). L'induction par les n-3 PUFAs de la formation endothéliale de NO due à l'activation de la eNOS via les voies de signalisation redox-sensibles Src/PI3-kinase/Akt et MAPKs, est dépendante du ratio et de la quantité de EPA et DHA dans la formulation. Un grand nombre d'études clinique montre que la consommation des n-3 PUFAs réduit la pression artérielle systolique chez l'homme, et une récente méta-analyses a confirmé que la consommation de EPA plus DHA supérieure à 2 g/j pouvait réduire les pressions artérielles systolique et diastolique.

L'objectif de la présente étude est de déterminer si la consommation chronique de la formulation optimisée EPA:DHA 6:1 est capable de prévenir l'hypertension et la dysfonction endothéliale induites par l'Ang II chez le rat.

Des rats Wistar males ont reçu quotidiennement par gavage 500 mg/kg soit d'huile de maïs (contrôle) soit de la formulation EPA:DHA 6:1. Après une semaine, les rats subissent soit une procédure simulée, soit l'implantation d'une mini-pompe osmotique infusant 0,4 mg/kg/j d'Ang II. La pression artérielle est mesurée deux fois par semaine pendant l'ensemble de la procédure expérimentale à l'aide de la méthode de sphingomanométrie par brassard caudal. Après 4 semaines de gavage, les animaux sont euthanasiés et les organes sont prélevés. Les branches secondaires de l'artère mésentérique sont utilisées pour l'étude de la réactivité vasculaire à l'aide d'un myographe à fil, pour des études en immunofluorescence et histochimie fluorescente sur coupes congelées, et pour des analyses en Western blot de l'expression de protéines.

Les principaux résultats de notre étude indiquent que l'infusion d'Ang II (0,4 mg/kg/j) à des rats induit une augmentation significative de la pression artérielle systolique, qui atteint $194 \pm 5,9$ mmHg par rapport au $120 \pm 5,6$ mmHg chez les rats contrôles. La consommation orale de

EPA:DHA 6:1 (500 mg/kg/j) prévient significativement l'hypertension induite par l'Ang II ($147 \pm 5,9$ mmHg) mais n'a aucun effet sur la tension artérielle normale ($110 \pm 3,9$ mmHg).

Les études de réactivité vasculaire dans les branches secondaires de l'artère mésentérique montrent que l'Ang II induit une dysfonction endothéliale caractérisée à la fois par une diminution des relaxations en réponse à l'acétylcholine affectant les composantes NO et EDH de la relaxation, et par une augmentation de la formation des facteurs constricteurs dérivés de l'endothélium (EDCFs) en réponse à l'acétylcholine. La consommation chronique de EPA:DHA 6:1 normalise les réponses NO, EDH et EDCFs dans les branches secondaires de l'artère mésentérique.

Afin de mieux caractériser les mécanismes moléculaires impliqués dans l'effet protecteur de EPA:DHA 6:1, des analyses quantitatives des niveaux d'expression de protéines ont été effectués dans les branches secondaires de l'artère mésentérique par immunofluorescence sur coupes congelées.

Dans un premier temps, nous avons étudié l'expression de la eNOS et de l'arginase 1 (composante NO de la relaxation), de SK_{Ca} et Cx37 (composante EDH de la relaxation), et de cyclooxygénases (COXs, impliquées dans les réponses EDCFs). Par rapport aux animaux contrôles, l'Ang II induit une augmentation significative de l'expression de la eNOS, d'arginase 1, et de COX-1 et COX-2, la forme inducible des COXs, et une diminution significative de l'expression de SK_{Ca} et Cx37. La prise chronique de EPA:DHA 6:1 prévient significativement les effets de l'Ang II sur l'expression des protéines cibles.

Comme la dysfonction endothéliale est associée à un stress oxydant vasculaire, nous avons évalué le niveau de stress oxydant dans les branches secondaires d'artère mésentérique à l'aide de la sonde fluorescente redox-sensible dihydroéthidium (DHE). L'Ang II induit une augmentation significative de la fluorescence dans l'ensemble de la paroi vasculaire en comparaison des rats contrôles, qui est significativement prévenue par la prise chronique de EPA:DHA 6:1. Du fait que l'augmentation de stress oxydant dans l'hypertension induite par l'Ang II est due, du moins partiellement, à une surexpression de la NADPH oxydase liée à l'activation du récepteur de l'angiotensine II de type 1 (AT1R), les niveaux d'expression d'AT1R et des sous-unités p22^{phox} et p47^{phox} de la NADPH oxydase ont été déterminés. En comparaison des branches secondaires

d'artère mésentérique de rat contrôles, les rats recevant l'Ang II montrent une augmentation significative des niveaux d'expression d'AT1R, p22^{phox} et p47^{phox}. Le traitement avec EPA:DHA 6:1 prévient significativement cette augmentation de stress oxydant vasculaire et de l'expression d'AT1R et des deux sous-unités de la NADPH oxydase.

Afin de confirmer ces résultats obtenus par immunofluorescence et histochimie fluorescente sur les branches secondaires d'artère mésentérique, l'analyse par Western blot de l'expression des protéines eNOS, COX-2, et la sous-unité p22^{phox} de la NADPH oxydase a été réalisée dans l'artère mésentérique principale. L'Ang II induit une augmentation significative de l'expression de eNOS, COX-2, et p22^{phox}, et cette surexpression est significativement prévenue par la prise de EPA:DHA 6:1.

L'ensemble des résultats obtenus lors de la présente étude indique que la prise chronique de la formulation optimisée EPA:DHA 6:1 prévient le développement de l'hypertension et de la dysfonction endothéliale induites par l'infusion d'Ang II chez le rat. La dysfonction endothéliale induite par l'Ang II est associée à une régulation positive du système angiotensine local et une augmentation du stress oxydant vasculaire. Les effets bénéfiques de la consommation chronique de EPA:DHA 6:1 impliquent une amélioration des composantes de relaxations NO et EDH, et à une diminution des réponses contractiles dépendantes de l'endothélium, probablement via la prévention du stress oxydant vasculaire induit par la régulation positive du système angiotensine local.

Chapter 1

Physiology of the endothelium

1.1 Cardiovascular diseases

Cardiovascular diseases are a group of several pathologies including coronary heart diseases (CHD) such as myocardial infarction, cerebrovascular diseases (stroke), hypertension, peripheral artery diseases, rheumatic heart diseases, congenital heart diseases and heart failure (Cheng, Austin et al. 2002). Cardiovascular diseases are the leading cause of mortality and morbidity worldwide, with an estimated 17 million annual deaths (WHO 2011) (Towfighi and Saver 2011). Amongst cardiovascular diseases, CHD accounts for 7.2 million deaths and 5.7 million are due to stroke (Lloyd-Jones, Adams et al. 2009). It is now well established that endothelial dysfunction is an early hallmark of major cardiovascular and other diseases (Figure 1), which is thought to contribute to the initiation and the development of these diseases (Austin, Lentz et al. 2004).

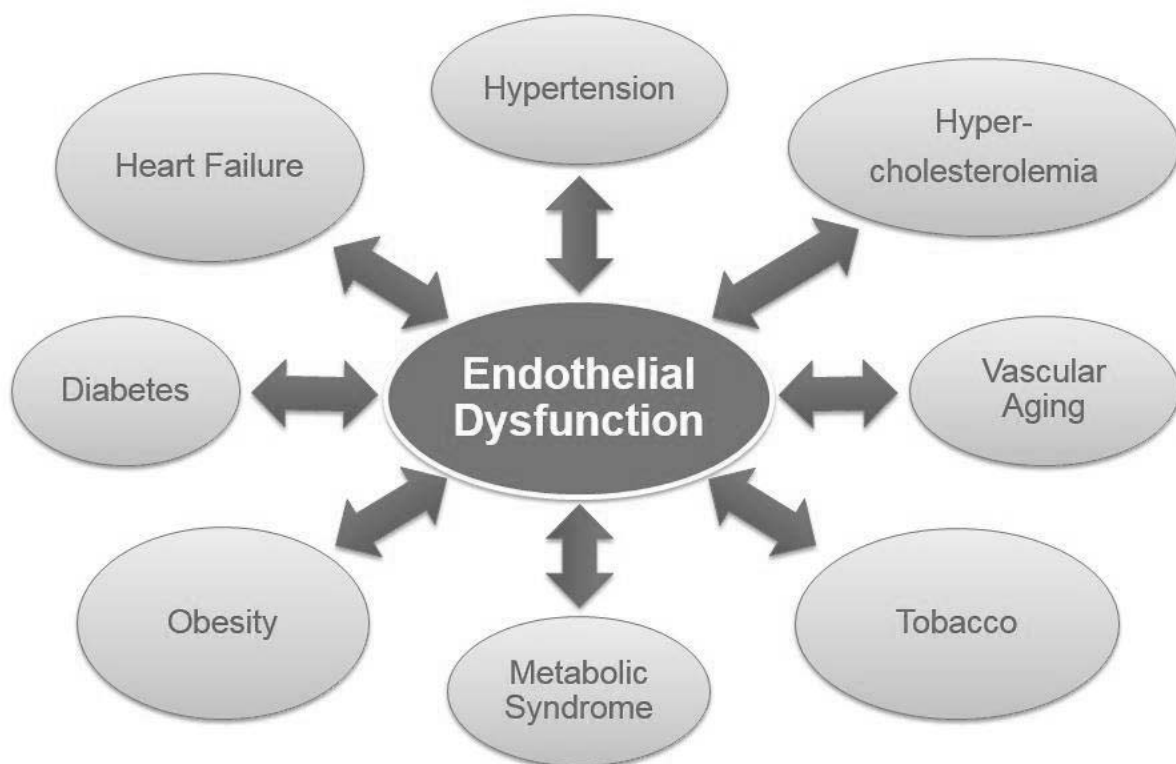


Figure 1. Endothelial dysfunction: A hallmark of major cardiovascular and other related diseases.

1.2 Vascular endothelium

All blood vessels including arteries, arterioles, capillaries, veins and venules are the part of the circulatory system. The wall of the blood vessels can be divided into three distinguished layers which are the intima, the media and the adventitia (Pugsley and Tabrizchi 2000) (Figure 2). The intima (tunica interna/intima) is the innermost monocellular layer called endothelium which is supported by connective tissue and the internal elastic lamina (Krstic 2013). The endothelium is located at the interface between the blood flow and the vessel wall. The media (tunica media) is mainly buildup of smooth muscle cells, collagen fibers and elastic lamina. (Severs and Robenek 1992).

The adventitia (tunica externa or tunica adventitia) is the outermost layer comprising of collagen, elastin, fibroblasts, macrophages, *vasa vasorum*, nerve endings and fibers for the protection of the blood vessels (Mulvany 1990). The importance of each layer depends on the size and location of the arteries. In large conducting arteries, a high number of elastic fibers are present in the media. Muscular arteries contain more smooth muscles cells while only few smooth muscle cells along the internal elastic lamina are found in arterioles. Capillaries only contain endothelial cell and the basement membrane with connective tissues (Pais, Meiselman et al. 2010).

Endothelial cells line the whole circulatory system starting from large arteries arising from the heart to the capillaries and veins. They regulate the flow of nutrient substances and blood cells (Mangge, Becker et al. 2014) and act as a selective barrier between the lumen of blood vessel and surrounding tissues (Galvão, Araújo et al. 2006). Covering a largest surface area, the endothelium plays an important role in the regulation of blood flow and is a chief regulator of body homeostasis through the synthesis and secretion of various active molecules, including vasodilating factors and vasoconstricting factors finely controlling vascular tone. Endothelial cells also regulate smooth muscle cells (SMC) proliferation, exchanges of molecules between the plasma and the interstitial fluid. They also play a vital role in the balance between pro- and anticoagulant mechanisms and in immunity (Klein 2013).

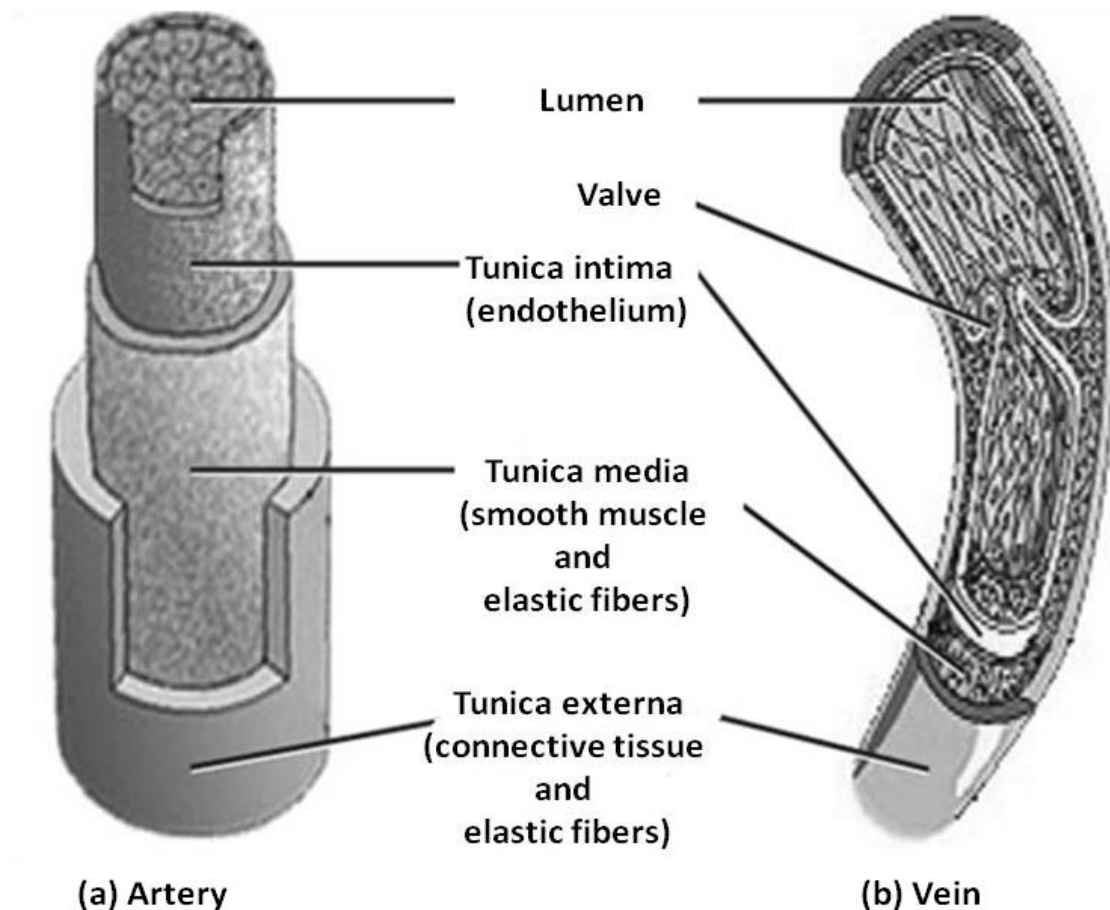


Figure 2. Structure of the blood vessel (adapted from Boston University School of Public Health).

1.3 Endothelial regulation of vascular tone

The vascular endothelium plays a major role in the regulation of vascular tone through a variety of mechanisms. Several mediators which can modify vascular tone are derived from the endothelium (Schalkwijk and Stehouwer 2005; Klein 2013). In 1980, Furchgott and Zawadzki demonstrated the phenomenon of endothelium-dependent arterial relaxation. Acetylcholine induces relaxation in arterial rings by releasing endothelium-derived relaxing factor (EDRF) which stimulates soluble guanylyl cyclase responsible for the conversion of GTP to cyclic GMP. Later on, EDRF was identified as the radical gas nitric oxide (NO) (Arnal, Dinh-Xuan et al. 1999). NO diffuses from the endothelium to the underlying smooth muscle where it activates soluble guanylyl cyclase to cause a rise in intracellular cyclic GMP and relaxation of the vessel wall (Gryglewski, Palmer et al. 1986; Rubanyi and Vanhoutte 1986).

The endothelium has the capacity to regulate the local vascular homeostasis by maintaining the balance between vasodilation and vasoconstriction, by controlling vascular smooth muscle cell (VSMC) proliferation and migration, and by acting on thrombosis and fibrinolysis via the release of various factors (Davignon and Ganz 2004). Imbalance of these different mechanisms promotes endothelial dysfunction, which may lead to serious cardiovascular diseases such as hypertension, a major CVD risk factors.

In response to physical and chemical stimuli such as changes in pressure, shear stress, and pH as well as to substances released by autonomic and sensory nerves and circulating hormones, autacoids, and cytokines, the vascular endothelium synthesizes relaxing and contractile factors responsible for the modulation of VSMC tone. Relaxation factors include nitric oxide (NO), prostacyclin (PGI₂), endothelium-derived hyperpolarization (EDH), and contractile factors includes thromboxane A₂, isoprostanes, superoxide anions (ROS), endothelin-1, and angiotensin-II factors (Figure 3) (Mombouli and Vanhoutte 1999; Feletou and Vanhoutte 2006).

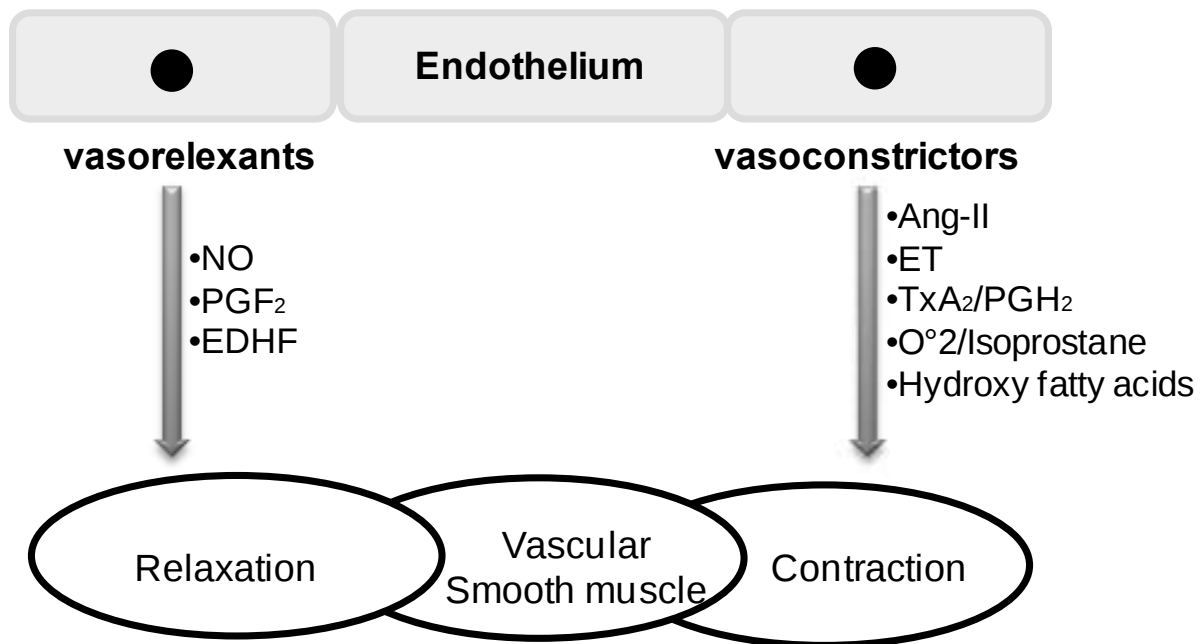


Figure 3. Endothelium-dependent release of vasoconstricting and vasorelaxing factors.

NO, nitric oxide; PGI₂, prostacyclin; EDHF, endothelium-derived hyperpolarizing factor; A-II, angiotensin II; ET, endothelin; TxA₂/PGH₂, thromboxaneA₂/prostaglandin H₂O₂, hydrogen peroxide (Abeywardena and Head 2001).

1.3.1 The endothelium-derived vasorelaxing factors

1.3.1.1 Nitric oxide (NO)

NO is a key cellular signaling molecule involved in a number of physiological and pathological processes. In the beginning, it was identified as a factor capable of activating soluble guanylyl cyclase responsible for the relaxation of vascular smooth muscle cells (Katsuki, Arnold et al. 1977). In 1980, Furchgott and Zawadzki revealed that the endothelium causes vasorelaxation by the production of endothelium-derived relaxing factor (EDRF) which was later identified as NO (Furchgott and Zawadzki 1980; Palmer, Ferrige et al. 1987; Palmer, Ashton et al. 1988; Palmer and Moncada 1989). Besides its role in regulation of vascular tone, NO inhibits leukocytes adhesion, platelet aggregation, and has anti-apoptotic and antithrombotic effects. Moreover, NO is an important factor of endothelium viability, longevity and cardiovascular health (Morello, Perino et al. 2009).

The endothelium-derived NO is produced by the endothelial NO synthase (eNOS) from L-arginine and plays a critical role in normal vascular biology and pathophysiology (Cai and Harrison 2000) (Figure 4).

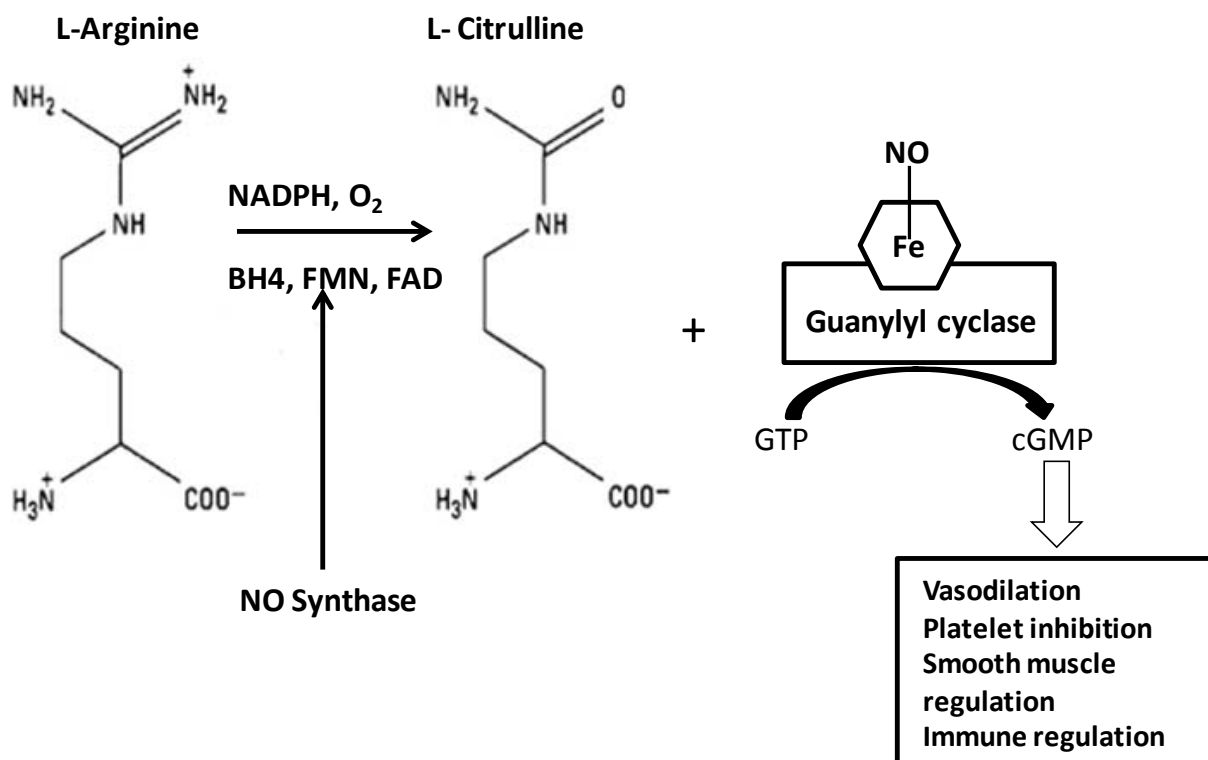


Figure 4. NO generation from L-Arginine and its functional properties (Ghalayini 2004)

There are three different isoforms of NO synthase; the neuronal NOS (NOS1 or nNOS), the inducible NOS (NOS2 or iNOS) and the endothelial NOS (NOS3 or eNOS) (Weiming, Liu et al. 2002). iNOS is an inducible isoform whereas nNOS and eNOS are being constitutively expressed (Mombouli and Vanhoutte 1999; Stuehr 1999). Under normal conditions, the majority of eNOS is bound to the protein caveolin-1, which inactivates eNOS, and this complex is located in micro domains in the cell membrane named caveolae (Michel, Feron et al. 1997; Bucci, Gratton et al. 2000). eNOS can be activated by Ca²⁺ dependent and independent pathways. eNOS can be activated by substituting caveolin-1 by Ca²⁺/CaM in response to Ca²⁺-mobilizing agonists. When

intracellular Ca^{2+} levels increase, calmodulin detaches eNOS from caveolin-1 thus permitting the enzyme to become active. Furthermore, eNOS has been shown to be regulated by the interaction with positive and negative protein modulators such as heat shock protein 90 (Ju, Zou et al. 1997; Garcia-Cardena, Fan et al. 1998; Pritchard, Ackerman et al. 2001) (Figure 5).

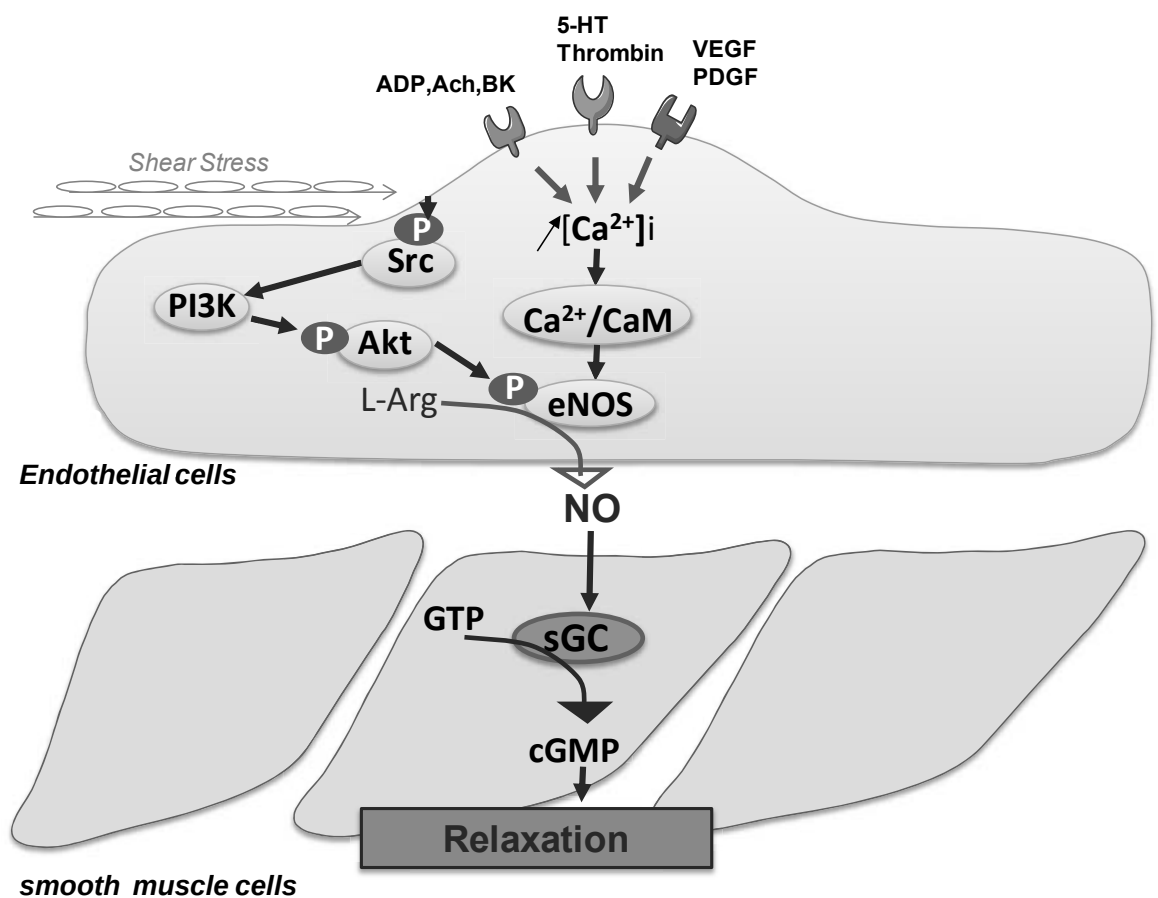


Figure 5. Nitric oxide synthesis pathway in the endothelial cell and its actions in the vascular smooth muscle cell.

ACh, acetylcholine; BK, bradykinin; ADP, adenosine diphosphate; 5-HT, serotonin; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; Src, Sarcoma-family kinases; PI3/kinase, phosphoinositide 3-kinase; Akt, Protein kinase B; Ca^{2+} / CaM, calcium calmodulin; L-Arg, L-arginine; eNOS, endothelial Nitric Oxide Synthase; NO, nitric oxide; sGC, soluble guanylyl cyclase; GTP, Guanosine 5'-Triphosphate; cGMP, cyclic Guanosine 3'-5' monophosphate

NO freely diffuses to the underlying VSMC where it activates the soluble guanylyl cyclase converting GTP into cyclic guanosine 3'-5'-monophosphate (cGMP), which leads to vascular smooth muscle cells relaxation.

In addition to vasorelaxation, NO exerts several vasoprotective and anti-atherogenic effects including inhibition of platelets aggregation, monocyte adhesion, vascular smooth muscle cell migration and proliferation, oxidation of LDL, and of the expression of pro-inflammatory and pro-atherothrombotic mediators such as monocyte chemoattractant protein-1 (MCP-1), adhesion molecules and tissue factor (Tsao, Buitrago et al. 1996; Dimmeler, Haendeler et al. 1997; Hermann, Zeiher et al. 1997). (Figure 6)

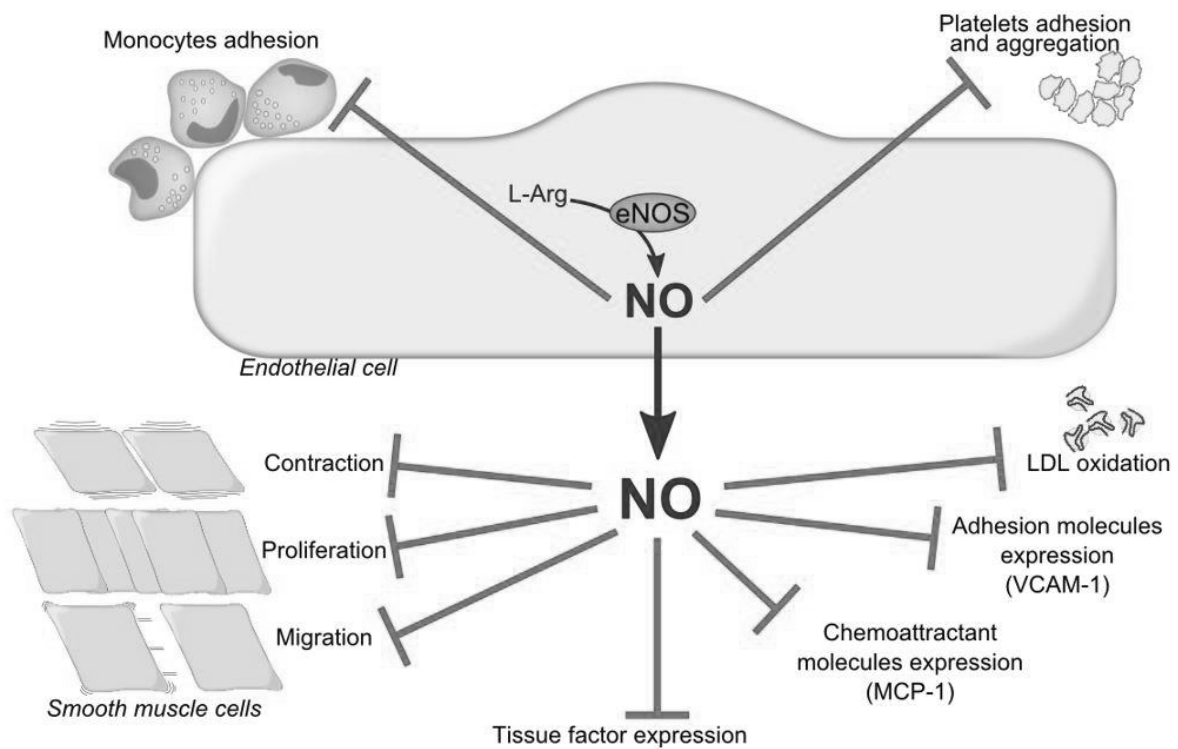


Figure 6. The pleiotropic effects of NO.

VCAM-1; Vascular cell adhesion molecule-1, MCP-1; Monocyte chemoattractant protein-1, LDL; Low density lipoproteins.

eNOS can also be regulated in endothelial cells at a post-translational level primarily through multisite phosphorylations and protein/protein interactions. Residues Ser1177 and Ser 615 are the activation sites and the residues Thr495 and Ser114 are the inhibition sites on eNOS (Dimmeler, Haendeler et al. 1997; Bohm, Ahlborg et al. 2002; Bauer, Fulton et al. 2003; Fleming 2010) (Figure 7). In response to several physiological stimuli, phosphorylation of eNOS across key regulatory sites plays an important role in the regulation of the enzymatic activity (Ju, Zou et al. 1997; Newby, Hess et al. 2012). Phosphorylation of eNOS at Ser1177 is associated with an increased enzyme activity (Gallis, Corthals et al. 1999; McCabe, Fulton et al. 2000). Akt, one of the major regulatory targets of PI3-kinase, has been shown to directly phosphorylate eNOS at Ser117 and activate the enzyme in response to vascular endothelial growth factor (VEGF), sphingosine-1-phosphate, and estrogen (Dimmeler, Haendeler et al. 1997; Fulton, Gratton et al. 1999). Furthermore, eNOS can be also activated by phosphorylation on Ser1177 by AMP-activated protein kinase, protein kinase A (PKA), and protein kinase G (PKG) (Busse, Edwards et al. 2002; Flemming and Wingender 2010).

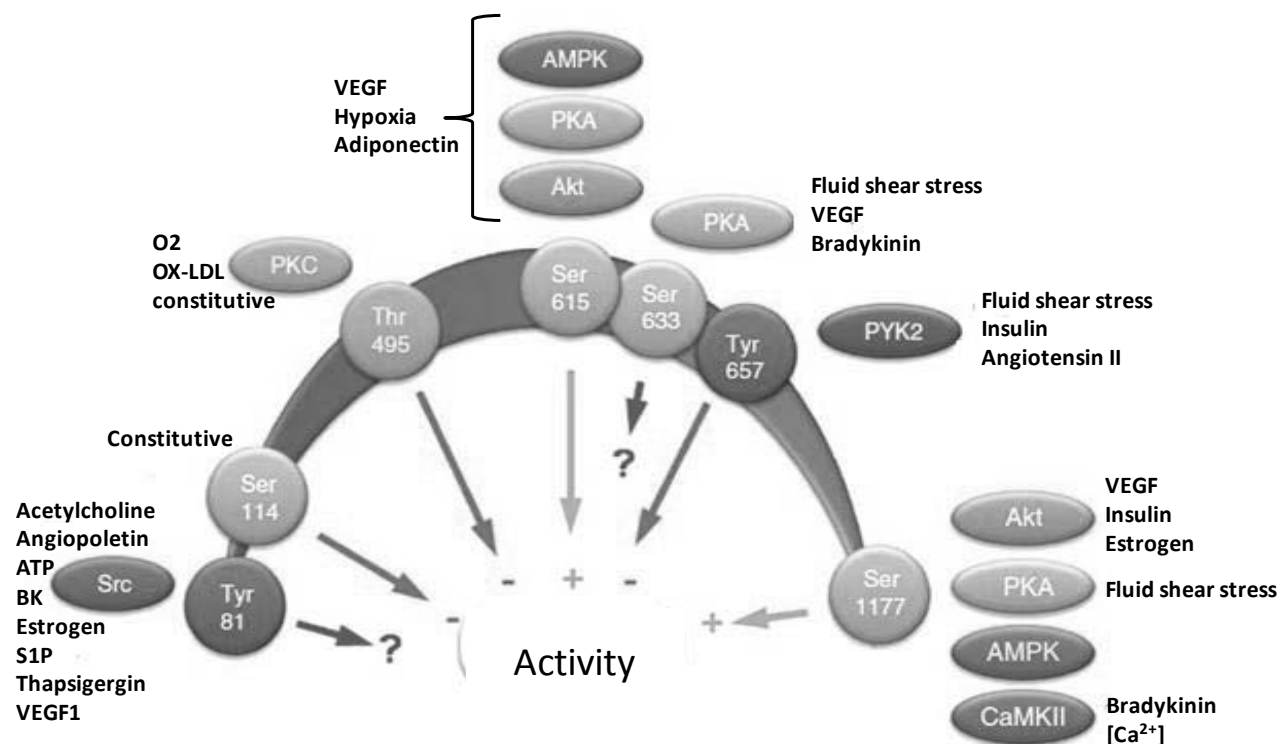


Figure 7. The activation and inhibition sites of eNOS.

Green arrows for activation, red arrows for inhibition, black arrow for no direct effect on enzyme activity. The numbers refer to the human sequence (Fleming 2010).

There are various assumed phosphorylation sites, but the most extensively studied eNOS residues, are serine residue in the reductase domain (human eNOS sequence: Ser1177; bovine sequence Ser1179), which positively regulates NO production, and a threonine residue within the CaM-binding domain (human eNOS sequence: Thr495; bovine sequence Thr497) (Boo, Hwang et al. 2002). Ischemia-reperfusion injury is another eNOS regulator which leads to the eNOS phosphorylation at Ser1177 and Ser 633 through the activation of PKA pathway (Li, Yang et al. 2010). Furthermore, there are numerous kinases reported to be involved in the phosphorylation of eNOS following cell activation by different stimuli such as shear stress, vascular endothelial growth factor (Butt, Bernhardt et al. 2000), hypoxia (Michell, Griffiths et al. 1999; Chen, Liu et al. 2008), including extracellular signal-regulated kinase 1/2 which alters eNOS protein expression

and activity (Ramasamy, Parthasarathy et al. 1998). eNOS can be phosphorylated on serine, tyrosine, and threonine residues leading to eNOS activation or inactivation (Figure 8).

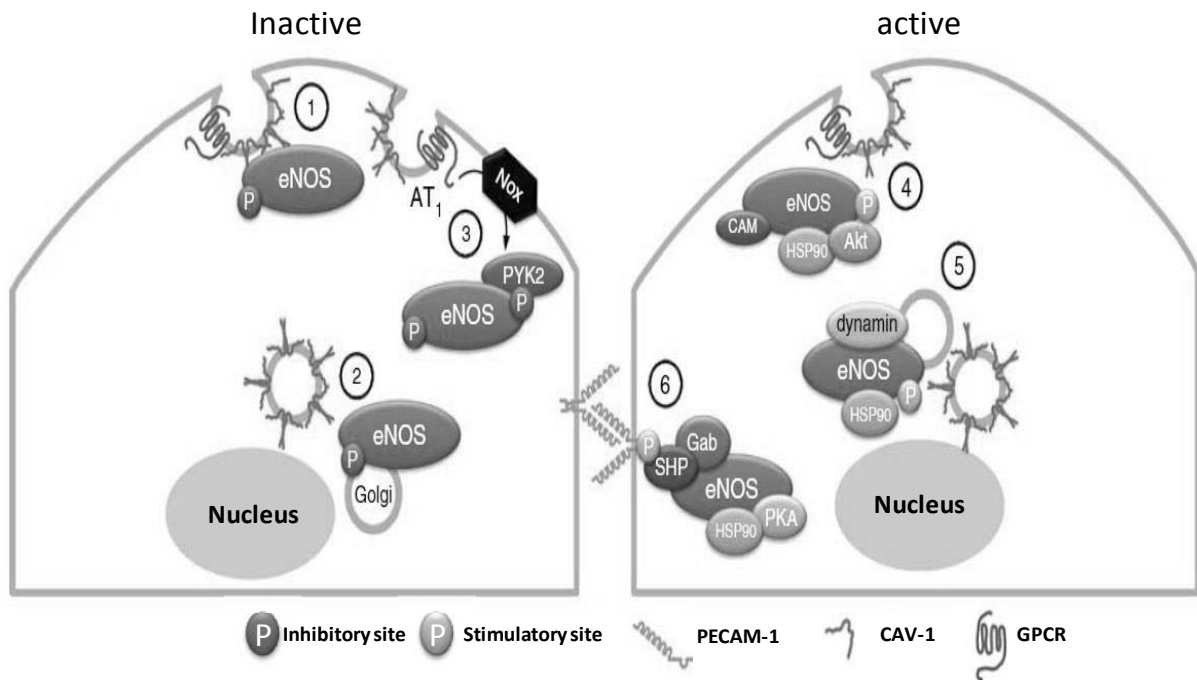


Figure 8. Regulation of eNOS activity.

(1) At rest, the eNOS is coupled to cav-1 (caveolin-1, a structural protein of caveolae) that decreases its activity. (2) eNOS is constitutively phosphorylated at Thr 495 preventing its activation by the $\text{Ca}^{2+}/\text{CaM}$. (3) eNOS may be inhibited in response to oxidative stress by tyrosine phosphorylation by PYK2 (proline-rich tyrosine kinase). (4) eNOS can be activated by both $\text{Ca}^{2+}/\text{CaM}$ (calcium / calmodulin) and phosphorylation of Ser1177. Hsp90 (heat shock protein) facilitates the recruitment of Akt responsible for the phosphorylation of eNOS (Fleming 2010).

1.3.1.2 Endothelium-derived hyperpolarization (EDH)

EDH is defined as a hyperpolarization of endothelial origin that is transmitted to the vascular smooth muscle leading to its relaxation. The EDH was formerly known as the Endothelium-Derived Hyperpolarizing Factor (EDHF). Beside NO and prostacyclin, the EDH mediated component of relaxation plays a major role in endothelium-dependent relaxation in most of the medium to small calibre resistance arteries, small arteries and arterioles such as second and third-branch mesenteric artery as well as in coronary arteries (Feletou and Vanhoutte 1996; Shimokawa, Yasutake et al. 1996). The role of the EDH component of relaxation is more important in resistance blood vessels as compare to that of NO and prostacyclin, including in humans (Nakashima, Mombouli et al. 1993; Shimokawa, Yasutake et al. 1996).

The EDH component of the relaxation is evaluated in the presence of the combination of inhibitors of eNOS like L-NAME and of COXs like indomethacin (Gerber, Anwar et al. 1998). In the EDH mediated response, SK_{Ca} and IK_{Ca} are activated so that potassium ions move from the intracellular compartment to the extracellular space of endothelial cells, which leads to their hyperpolarization (Figure 9). This higher concentrations of potassium ions in the extracellular space can activate inwardly rectifying K⁺ (K_{IR}) channels and Na⁺/K⁺-ATPase to cause potassium ions efflux from VSMC leading to hyperpolarization and hence, relaxation (Edwards, Dora et al. 1998; Félétou and Vanhoutte 2006). In 1998, Edwards et al reported that hyperpolarization can also be transferred from endothelial cells to VSMC via myo-endothelial gap junctions. Myo-endothelial gap junctions are intracellular channels which can transfer signals from the endothelial cells to the underling vascular smooth muscle cells (Sandoo, van Zanten et al. 2010). Hyperpolarization of VSMC leads to the reduction in cytosolic calcium concentration following closure of voltage-activated calcium channels leading to relaxation. Endothelial hyperpolarization can also be mediated by hydrogen peroxide (H₂O₂) (Matoba, Shimokawa et al. 2002) or arachidonic acid-derived metabolites including epoxyeicosatrienoic acids (Quilley and McGiff 2000).

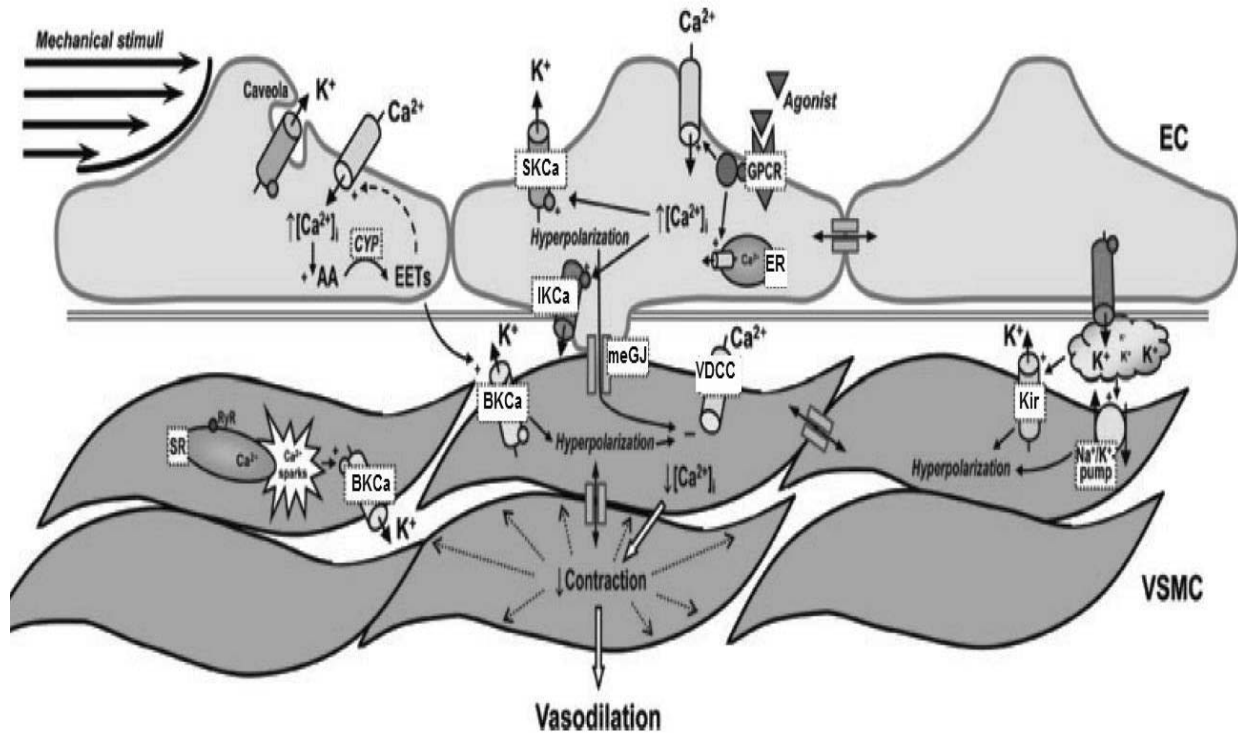


Figure 9. Hypothesis describing the endothelium-derived hyperpolarizing pathway.

AA, arachidonic acid; ACh, acetylcholine, $[Ca^{2+}]_i$, intracellular calcium concentration; CYP, cytochrome P450 epoxygenase; EC, endothelial cell; EETs, epoxyeicosatrienoic acids; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; BKCa, large conductance Ca^{2+} -activated K^+ channel; SKCa, small-conductance Ca^{2+} -activated K^+ channel subtype 3; IKCa, intermediate-conductance Ca^{2+} -activated K^+ channel; Kir, inwardly rectifying K^+ channel; meGJ, myo-endothelial gap-junction; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; VDCC, voltage-dependent Ca^{2+} channel; VSMC, vascular smooth muscle cell (Grgic, Kaistha et al. 2009).

1.3.1.3 Prostacyclin

Prostacyclin (PGI₂) is the major product of cyclooxygenase (COX) catalyzed metabolism of arachidonic acid in the endothelium (Cheng, Austin et al. 2002). Prostacyclin is produced from prostaglandin under the action of the enzyme prostacyclin synthase (Dogne, de Leval et al. 2004). PGI₂, PGG₂ and PGH₂ are major products of vascular cyclooxygenase (COX). There are two isoforms of COX encoded by two separate genes. COX-1 is constitutively expressed and is present in many tissues, including endothelial cells (Gryglewski, Uracz et al. 2002). COX-2 is not constitutively expressed, but can be induced rapidly and transiently in many cells, including vascular endothelial cells and smooth muscle cells, under the effect of physical stimuli and pro-inflammatory agents. PGI₂ stimulates smooth muscle relaxation by stimulating adenylyl cyclase and formation of cyclic adenosine -3', 5'- monophosphate (cAMP) that activates protein kinase A, which reduces intracellular Ca²⁺ by decreasing Ca²⁺ release from the endoplasmic reticulum and by stimulating its uptake by it. The vasodilator activity of PGI₂ is determined by the expression of specific receptors which are prostaglandin I₂ receptors of the G-protein coupled receptor family in vascular smooth muscle cells. PGI₂ is a potent vasodilator, and an effective endogenous inhibitor of platelet aggregation (Coleman, Smith et al. 1994). In addition, PGI₂ facilitates the release of NO by endothelial cells (Shimokawa, Flavahan et al. 1988) and in turn, the action of PGI₂ in vascular smooth muscle cells and platelets is potentiated by NO (Delpy, Coste et al. 1996). PGI₂ synthase preferentially couples with COX-2 rather than COX-1 in coexpression systems (Figure 10) (Ueno, Takegoshi et al. 2005).

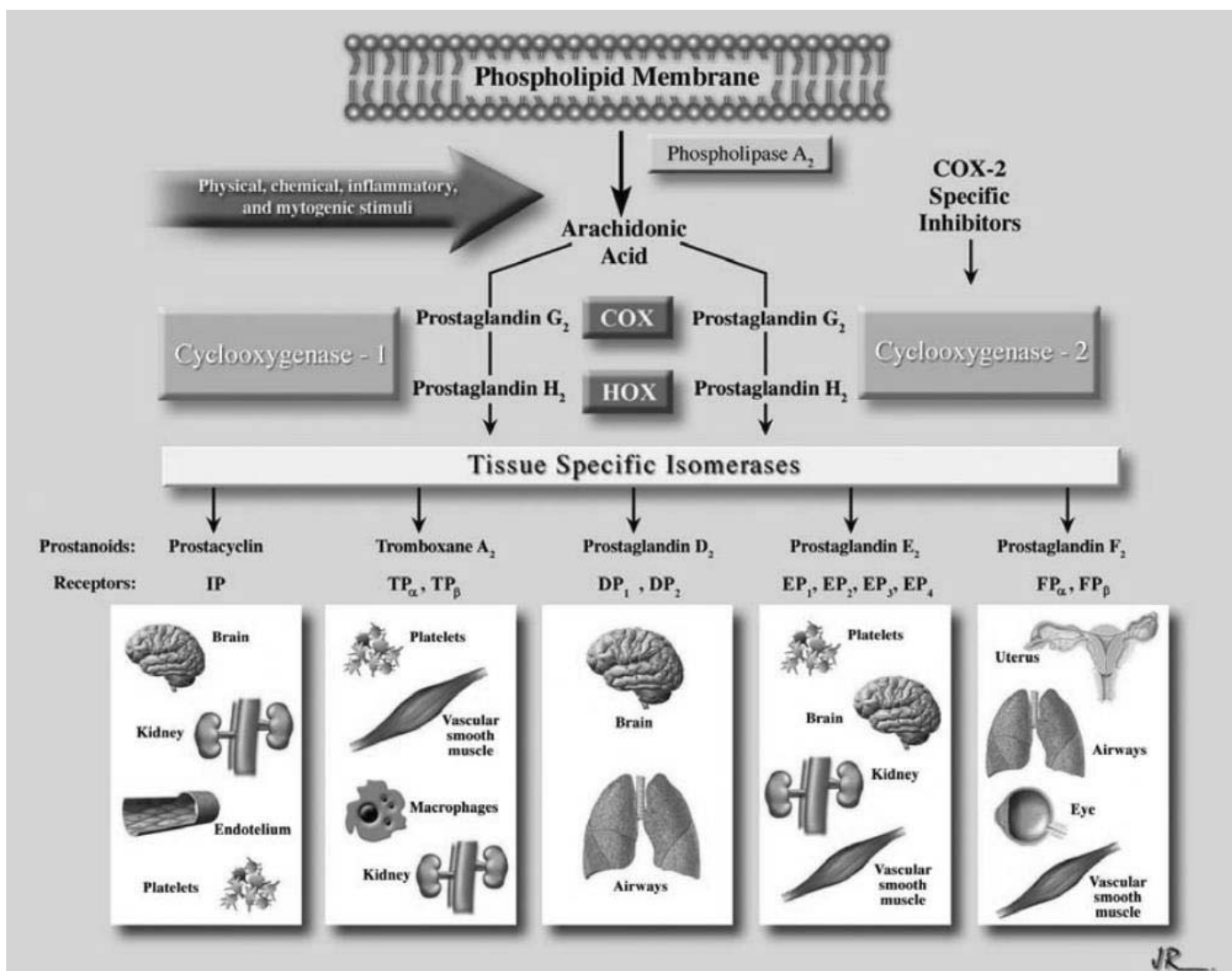


Figure 10. Production and action of prostaglandins (Araujo, Soeiro et al. 2005).

1.3.2 The endothelium-derived vasoconstricting factors

1.3.2.1 Angiotensin II

The octapeptide Angiotensin II (Ang II) is a potent vasoconstrictor hormone of the renin-angiotensin system (RAS) that is formed following the conversion of Ang I into Ang II by Angiotensin I Converting Enzyme (ACE) (Baker, Chernin et al. 1990). Angiotensin II (Ang II) is a multifunctional peptide hormone that regulates blood pressure (BP), plasma volume, as well as cardiac, renal and neuronal function, and controls thirst responses. This peptide is of central importance in hypertension and myocardial remodeling, and is the main effector of the renin-angiotensin system (RAS) (Weber and Brilla 1991). Taken as a whole, the RAS is involved in different cardiovascular pathologies such as left ventricular hypertrophy, post-infarct remodeling, or neointima formation (Li, McTiernan et al. 2000). The classical effects of Ang II on its target organs are mostly mediated by two membrane receptors, the Ang II type 1 receptors (AT1R) and type 2 receptors (AT2R), which mediate tissue-specific functions (Horiuchi, Akishita et al. 1999). AT1R and AT2R are a G-protein coupled receptors involved in the regulation of vascular cell proliferation and cell death (Kaschina and Unger 2003). AT1R are expressed in all organs, including heart, kidney, liver, adrenal glands, brain, lung and in all cells of the cardiovascular system, namely endothelial cells, smooth muscle cells, fibroblasts, monocytes, macrophages and cardiac myocytes and, thus, is important in cardiovascular pathobiology. While AT2R is highly expressed in fetal heart and fetal aorta, lung and liver (Dasgupta and Zhang 2011), AT2R expression declines fast after birth, but can be induced later in adult life under pathological conditions (Figure 11).

The acute vasoconstrictor function of Ang II is primarily mediated through AT1R by classical G-protein-dependent signaling mechanisms. Depending on cell types, Ang II activates AT1R that can in turn activate at least four different effector, namely voltage-gated Ca^{2+} channels, phospholipase C, phospholipase D and phospholipase A-2 (PLA-2) and can inhibit adenylyl cyclase (Greco 2007). In addition, Ang II stimulation of AT1R activates the extracellular-signal-regulated kinase (ERK) cascade, platelet-derived growth factor, epidermal growth factor receptor (EGFR), insulin receptor pathways and non-receptor tyrosine kinases belonging to the c-Src family,

proline-rich tyrosine kinase 2, focal adhesion kinase and janus kinases (JAKs) (Berry, Touyz et al. 2001).

Unlike AT1R, the AT2R contributes to the maintenance of blood pressure by controlling the vascular tone through vasodilatation (Dasgupta and Zhang 2011). AT2R stimulation by Ang II leads to an increase in cGMP levels through a mechanism involving bradykinin B₂ receptor, causing endothelial formation of NO (Abadir, Periasamy et al. 2006). Although AT2R expression decreases after birth, it can increase again in some pathophysiological conditions. Stimulation of *de novo* AT2R expression may inhibit neointima formation, cell proliferation, and inflammation in vascular injury, myocardial infarction and ischemic diseases, suggesting its protective role (Ichiki, Takeda et al. 2001).

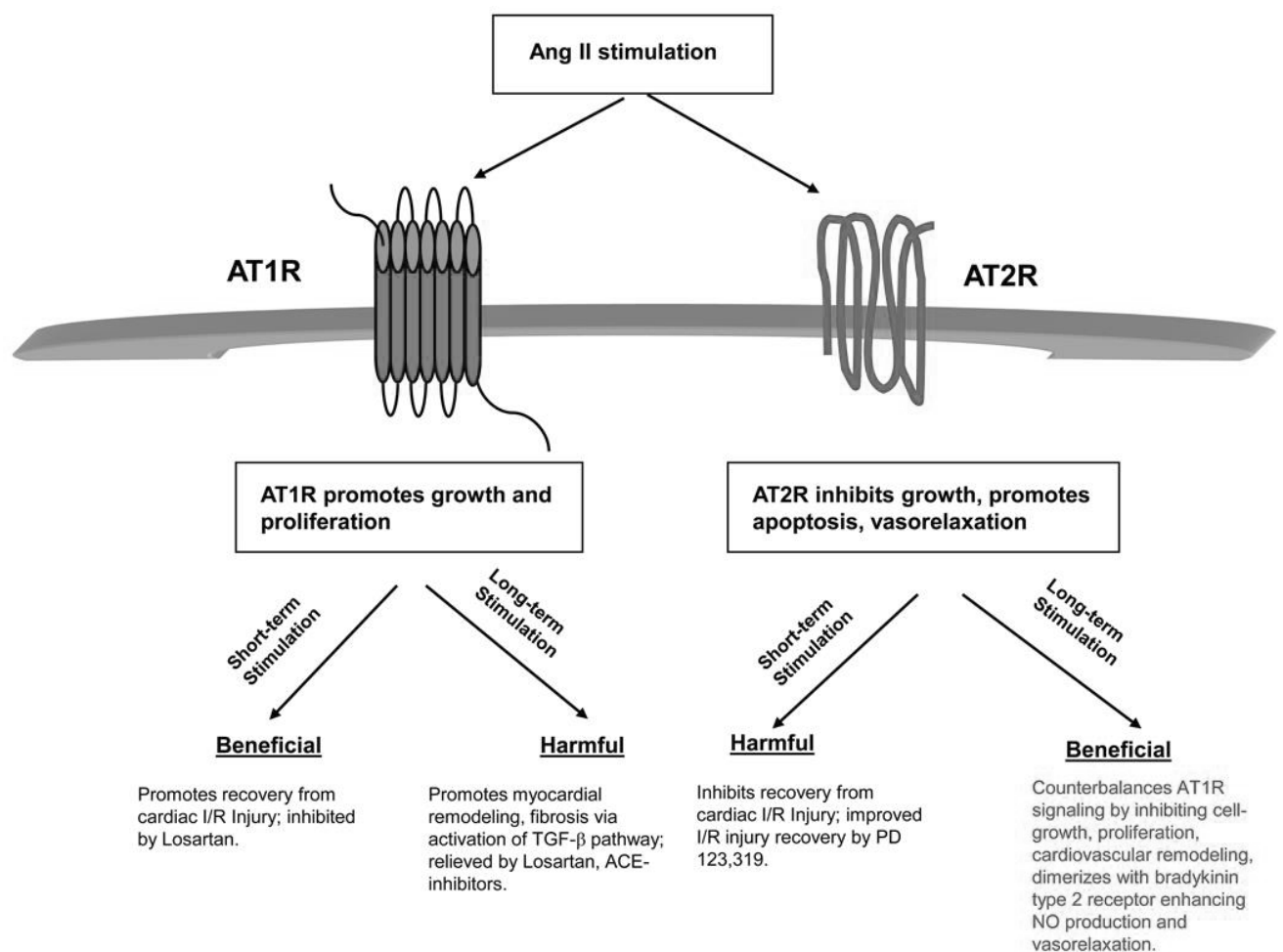


Figure 11. Summary of acute and chronic stimulation of angiotensin II receptors (Dasgupta and Zhang 2011).

1.3.2.2 Endothelin-1

Endothelin (ET)-1 is a potent vasoconstrictor peptide originally isolated from endothelial cells. There are three structurally different ET isoforms (i.e. ET-1, ET-2, ET-3) as well as a vasoactive intestinal constrictor (Böhm and Pernow 2007). Amongst the three ET isopeptides, the 21-amino acid peptide ET-1 is regarded as the most prominent isoform in the cardiovascular system, accounting for the majority of pathological effects exerted by ETs (Barton, Traupe et al. 2003).

Under physiological conditions, ET-1 is produced in small amounts mainly in endothelial cells, primarily acting as an autocrine/paracrine mediator (Pernow, Shemyakin et al. 2012). Under pathophysiological conditions, however, the production is stimulated in a large number of different cell types, including endothelial cells, vascular smooth muscle cells, cardiac myocytes, and inflammatory cells such as macrophages and leukocytes (Grieve, Byrne et al. 2004).

The biological effects of ET-1 are transduced by two distinguishable receptor subtypes, ET_A and ET_B receptors, respectively (Hunley and Kon 2001). In the vasculature, the ET_A receptor is mainly located on vascular smooth muscle cells and mediates potent vasoconstriction. ET-1 may also induce indirect vasoconstrictor effects due to the generation of endothelium-derived thromboxane A₂ (Marasciulo, Montagnani et al. 2006). The ET_B receptor is primarily located on endothelial cells, but may also be present on vascular smooth muscle cells (Schneider, Boesen et al. 2007). Stimulation of the endothelial ET_B receptor results in release of NO and prostacyclin which cause vasodilatation, whereas stimulation of the vascular smooth muscle cell ET_B receptor results in vasoconstriction (Seo, Oemar et al. 1994). Thus, the net effect produced by ET-1 is determined on the receptor localization and the balance between ET_A and ET_B receptors (Davie, Haleen et al. 2002) (Figure 12).

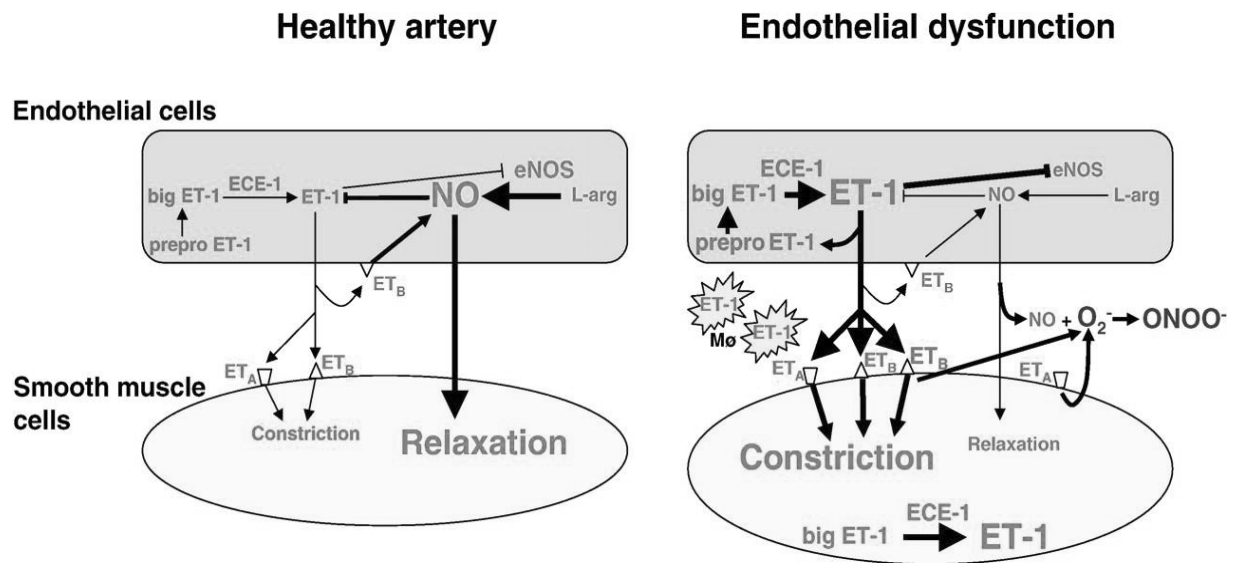


Figure 12. The biological effect of endothelin-1 on arteries in physiological and pathophysiological conditions.

In healthy arteries the production of ET-1 is small and the bioavailability of NO is preserved. In endothelial dysfunction there is increased expression of ET-1 in smooth muscle cells and macrophages (MØ). Both the ET_A and the ET_B receptor on smooth muscle cells may mediate formation of superoxide (O₂⁻). Collectively the balance of effects is shifted towards more vasoconstriction, inflammation and oxidative stress in endothelial dysfunction (Böhm and Pernow 2007).

1.3.2.3 Thromboxane A₂ & Prostacyclin I₂ (TxA₂ & PGI₂)

The prostanoids prostacyclin (PGI₂) and thromboxane A₂ (TXA₂) play an essential role in the maintenance of vascular homeostasis. PGI₂ is a vasodilator and an inhibitor of platelet aggregation, whereas TXA₂ is a vasoconstrictor and a promoter of platelet aggregation (Gamble, James et al. 2001).

PGI₂ and TXA₂ are products of arachidonic acid (AA) metabolism by cyclooxygenase (COX), followed by metabolism of the COX product, PGH₂, by the terminal synthase enzymes, prostacyclin or TX synthase, respectively (Ruan, So et al. 2011). Two isoforms of COX have been identified: COX-1 is expressed constitutively in most cell types, whereas COX-2 is induced by inflammatory stimuli such as bacterial endotoxin and cytokines (Caughey, Cleland et al. 2001). It is considered that PGI₂ is the main prostanoid synthesized by vascular endothelium and TXA₂ is the main prostanoid produced by platelets (Smith, Borgeat et al. 1991). However, the endothelium has been reported to synthesize TXA₂ in addition to PGI₂, and both COXs isoforms have been observed, with only COX-1 being detectable in unstimulated cells (Morteau 2001). Endothelial COX-2 can be up-regulated *in vitro* by inflammatory stimuli and shear stress (Brown and DuBois 2005). Because the balance between PGI₂ and TXA₂ production is central in the maintenance of vascular tone and platelet aggregation (Konturek and Pawlik 1986; Sobrino, Oviedo et al. 2010), determination of the roles of endothelial COX isozymes, particularly with regard to the contribution of COX-2 in the regulation of prostanoids biosynthesis by the endothelium, is important (Figure 13) (Caughey, Cleland et al. 2001).

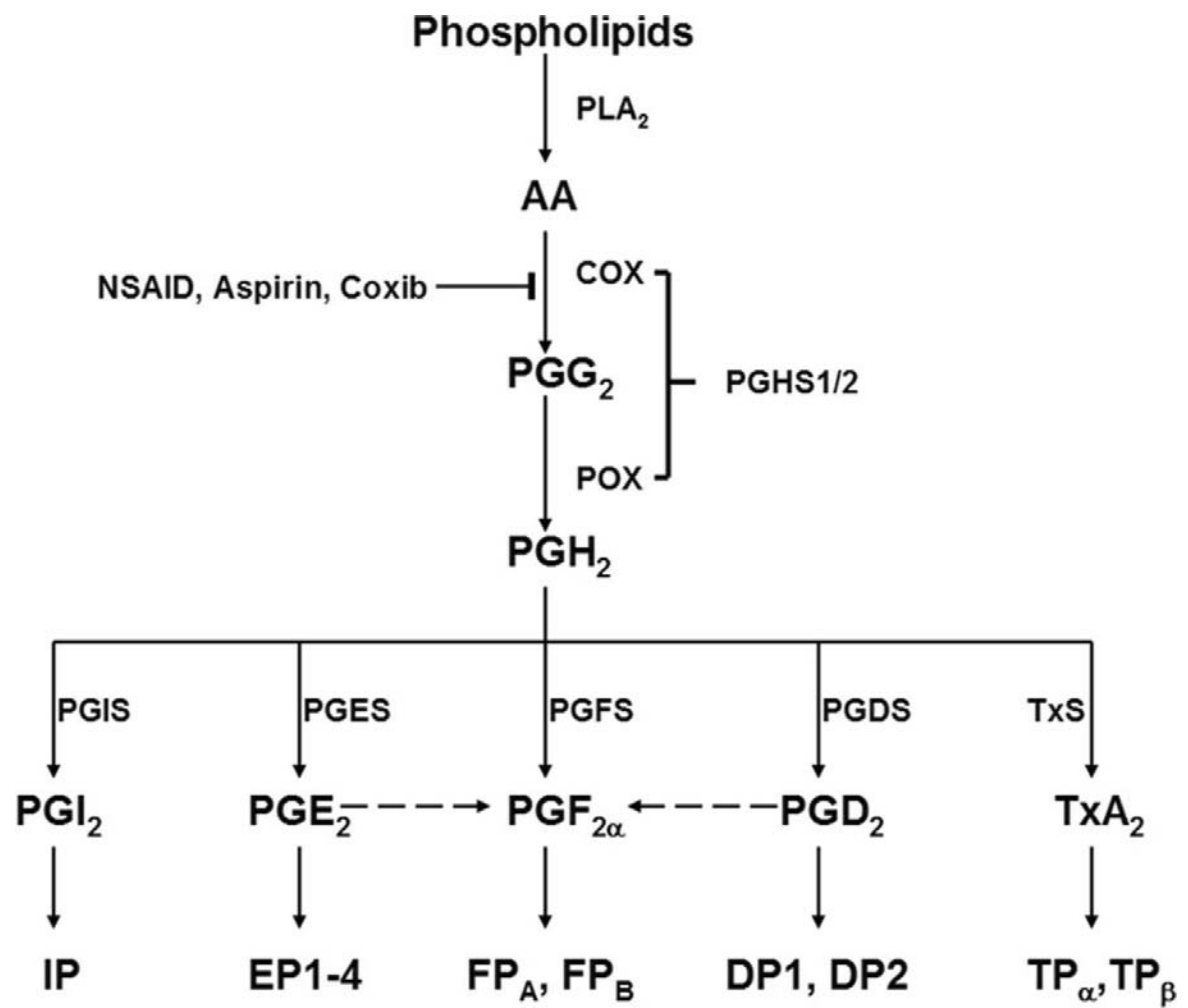


Figure 13. Prostanoids biosynthesis and response pathways (Zhang, Gong et al. 2010).

1.3.2.4 Oxidative stress and reactive oxygen species (ROS)

Reactive oxygen species (ROS) are recognized as important signaling molecules in the cardiovascular system and are released by vascular cells during pathophysiological conditions like hypertension, diabetes mellitus, atherosclerosis and in acute and chronic inflammatory diseases (Eisenberg and Ghigliotti 1999). NO, ($O_2^{\bullet-}$) the hydroxyl radical ($\cdot OH$), H_2O_2 , and peroxynitrite ($ONOO^-$) are produced in the vasculature under both normal and stress conditions such as inflammation or injury. Superoxide anions ($O_2^{\bullet-}$) can be generated by different enzymes (e.g., NADPH oxidase, xanthine oxidase, cyclooxygenases, NO synthases, cytochrome P450 monooxygenases, and enzymes of the mitochondrial respiratory chain) in virtually all cell types, including vascular smooth muscle and endothelial cells (Féletou and Vanhoutte 2006). ROS and in particular superoxide anions can also act directly or indirectly as potent contracting agents via the reduction of the NO bioavailability or by activating COXs in vascular smooth muscle cells (Hibino, Okumura et al. 1999), leading to attenuated endothelium-dependent relaxations (Aubin, Carrier et al. 2006; Liu, You et al. 2007). Moreover, ROS can also impair EDH-mediated endothelium-dependent relaxations through the reduction of calcium-activated potassium channels activity (Kusama, Kajikuri et al. 2005) or by modifying the transmission of the hyperpolarization from endothelial cells to the underlying smooth muscle cells through myoendothelial gap junctions (Griffith, Chaytor et al. 2005). Several studies have shown the beneficial effects of antioxidants on the deleterious effect of oxidative stress on the endothelial function (Kanani, Sinkey et al. 1999; Aubin, Carrier et al. 2006; Liu, You et al. 2007).

1.4 Endothelial dysfunction

Endothelial dysfunction is a broad term which implies dysregulation of endothelial cell functions, including impairment of the barrier functions of endothelial cells, vasodilation, disturbances in proliferative capacities, migratory as well as tube formation properties, angiogenic properties, attenuation of synthetic function, and deterrence of white blood cells from adhesion and diapedesis. Several factors contribute to endothelial dysfunction including smoking, high blood pressure, diabetes, high cholesterol levels, obesity, hyperglycemia, advance glycation end products (AGEs), and genetic factors. Endothelial dysfunction has been associated with an impairment of endothelium-dependent relaxations involving a reduced bioavailability of NO in major CV diseases such as hypertension, atherosclerosis, chronic renal failure, and diabetes (Griendling and FitzGerald 2003; Rush, Denniss et al. 2005). The mechanism underlying endothelial dysfunction has been linked to increased oxidative stress which is associated with a reduced NO bioavailability and the formation of inflammatory mediators such as vascular cell adhesion molecule-1 (VCAM-1) expression (Figure 14) (Khan, Harrison et al. 1996; Libby 2002). In addition, different enzymes have been involved in the arterial oxidative stress involving NADPH oxidases, xanthine oxidases, COX-1 and COX-2, cytochrome P450 monooxygenases, enzymes of the mitochondrial respiratory chain, and uncoupled eNOS. Superoxide anion can react with NO to form the radical peroxynitrite (Koppenol, Moreno et al. 1992), leading to the oxidation of the eNOS cofactor tetrahydrobiopterin (BH₄) and the subsequent uncoupling of eNOS, thereby further promoting oxidative stress (Cai and Harrison 2000).

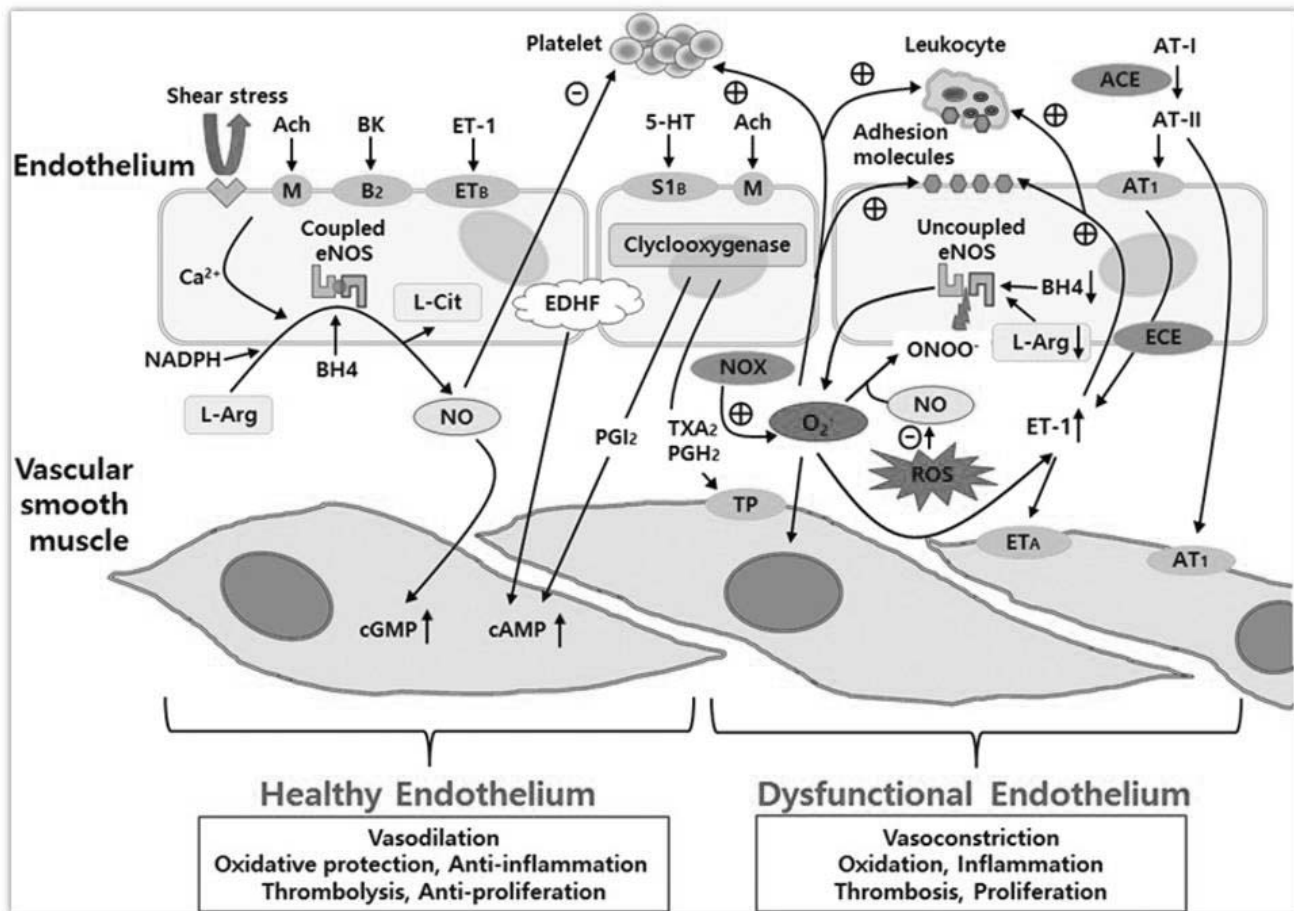


Figure 14. The effects of vascular endothelial factors on the function of vascular smooth cells in healthy and pathological conditions.

In the healthy endothelium, the eNOS is responsible for most of the vascular NO production. However, eNOS becomes a potential ROS generator when in the pathological uncoupled state, due to oxidative stress. ACE, angiotensin-converting enzyme; Ach, acetylcholine; AT-I, angiotensin I; AT-II, angiotensin II; AT₁, angiotensin 1 receptor; BH₄, tetrahydrobiopterin; BK, bradykinin; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; ECE, endothelin converting enzyme; eNOS, endothelial nitric oxide synthase; EDHF, endothelium derived hyperpolarizing factor; ET_A and ET_B, endothelin A and B receptors; ET-1, endothelin-1; L-Arg, L-arginine; L-Cit, L-citrulline; M, muscarinic receptor; O₂⁻, superoxide anion; ONOO⁻, peroxynitrite; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOX, nicotinamide adenine dinucleotide phosphate oxidase; PGH₂, prostaglandin H₂; PGI₂, prostaglandin I₂; ROS, reactive oxygen species; S1_B, serotonin receptor; TP, thromboxane prostanoid receptor; TXA₂, thromboxane; 5-HT, serotonin; ⊖, inhibition; ⊕, stimulation. (Park and Park 2015).

1.5 Endothelial dysfunction and hypertension

Hypertension is associated with endothelial dysfunction, where the delicate balance between vasodilators and vasoconstrictors produced by the endothelium is disrupted, with disturbance in the NO pathway leading to predominance of vasoconstrictors like ET-1, which contribute to high blood pressure (Sandoo, Veldhuijzen van Zanten et al. 2010). Even though it is still unclear whether endothelial dysfunction is the cause or the consequence of elevated blood pressure (Program 2000), it appears to be an essential factor in hypertension. Studies in humans have reported a significant impairment of the vasodilator response of small resistance vessels to acetylcholine, but not to sodium nitroprusside (SNP), in hypertensive patients (Endemann and Schiffrin 2004). Importantly, investigations have indicated a larger incidence of cardiovascular events in hypertensive patients with more severe endothelial dysfunction compared to hypertensive patients with less severe endothelial dysfunction, and therefore it is suggested as a marker for future cardiovascular events in hypertensive patients (Calhoun, Jones et al. 2008). Treatment with angiotensin-converting enzyme (ACE) inhibitors has been shown to improve endothelial function (Mancini, Henry et al. 1996). ACE inhibitors reduce oxidative stress and stimulate bradykinin to help increase NO bioavailability (Hornig, Landmesser et al. 2001). Products blocking Ang II type 1 receptor (AT1R), known as Ang II receptor blockers (ARBs), are successful primarily in the therapy of hypertension, but may also be beneficial in patients with intolerance to angiotensin-converting enzyme (ACE) inhibitors for the treatment of several cardiovascular diseases, such as stable coronary heart disease and heart failure (Dézsi 2014). Moreover, the renin–angiotensin–aldosterone system (RAAS), as well as AT1R and AT2R, play an important role in the regulation of cell proliferation and neoplastic progression (Ager, Neo et al. 2008).

In particular, endothelial dysfunction leading to diminished NO bioavailability impairs endothelium-dependent vasodilation in patients with essential hypertension and may also lead to premature development of atherosclerosis (Figure 15) (Vallance and Chan 2001). Different mechanisms of reduced NO bioavailability have been shown both in hypertensive states and several cardiovascular diseases, and endothelial dysfunction is likely to occur prior to vascular dysfunction (Cai and Harrison 2000). Thus, the strategies currently used to improve endothelial dysfunction may result in the improved outcome for hypertensive patients (Quyyumi 1998).

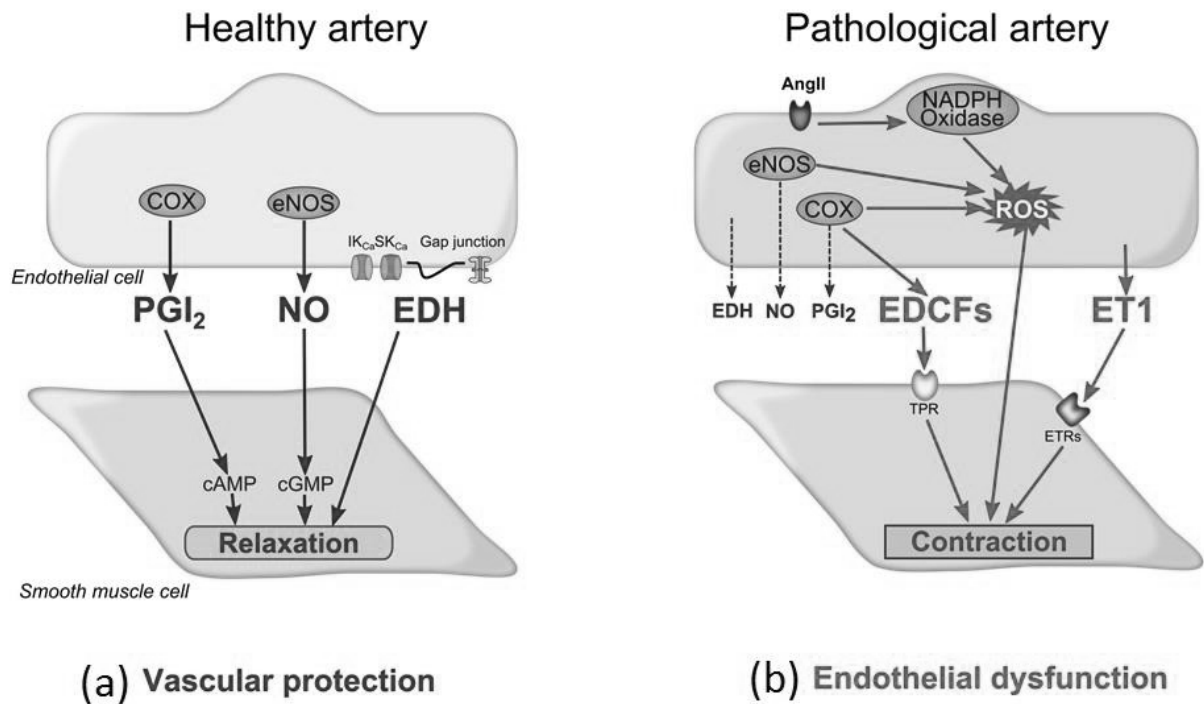


Figure 15. Mechanisms implicated in essential hypertension-associated endothelial dysfunction.

(a): In healthy artery endothelium produces vasoprotectors (PGI₂, NO and EDH) which induces relaxation in vascular smooth muscle cells. (b) In pathological artery, the release and activity of vasoprotectors is decreased while the local angiotensin system is activated resulting in the increased production of vasoconstrictors (ROS, EDCFs and ET1) which induces contraction in the vascular smooth muscle cells leading to endothelial dysfunction and hypertension.

1.5.1 Models of Hypertension

The Spontaneously Hypertensive Rat (SHR) is the most commonly used model of cardiovascular disease, with over 4000 Medline references in the last 10 years. The male SHR is usually used as a model of established human hypertension, for example to define hypertension-induced changes in signaling mechanisms and to test new antihypertensive drugs. One of the major advantages of the SHR is lack of inter-individual variation, with the limitation that the SHR can only model one of many possible causes of human hypertension. The SHR is a useful model as compounds that are able to lower blood pressure in SHR are likely to also be effective in hypertensive humans (Ching 2008).

Different experimental protocols have been described to induce DOCA-salt hypertension in the literature, including subcutaneous implantation of DOCA pellets (Churchill, Churchill et al. 1997). DOCA administration (synthetic mineralocorticoid derivative), in combination with salt loading in the diet, to young adult Wistar rats followed by surgical removal of one kidney is associated with hypertension with cardiovascular remodeling, hypertrophy, fibrosis, conduction abnormalities and endothelial dysfunction. Similar cardiovascular remodeling occurs in patients with hypertension and heart failure but these patients are usually not young, nor on a high salt diet, nor taking salt-retaining compounds nor functioning with a single kidney (Iyer, Chan et al. 2010). The DOCA-salt model markedly depressed renin-angiotensin system and hence has been extensively used in hypertension research as an angiotensin-independent model (Schenk and McNeill 1992).

NO synthesis can be blocked by inhibitors such as L-NAME (N^o-nitro-L-arginine methyl ester) and nitro-L-arginine (Roche, Cook et al. 1996). Chronic administration of L-NAME increased systolic blood pressure and heart weight, and decreased renal function. Chronic administration of L-NAME to rats during gestation induces the development of a pre-eclamptic syndrome similar to humans (Hropot, Grötsch et al. 1994). The role of reduced NO production in human hypertension is still unclear; therefore it is too early to decide whether NO synthase inhibition is an appropriate model. However, this model deserves more attention as it is technically easy with low mortality rate (Richer, Boulanger et al. 1996).

Ang II induced hypertension is one of the most widely used pharmacological model of hypertension in rats. Blockade of the renin-angiotensin system (RAS) with ACE inhibitors or angiotensin II type

1 receptor (AT1R) antagonists has become one of the most successful therapeutic approaches in medicine. This has led to the concept of combined RAS blockade. RAS is a complex system which plays a major role in the maintenance of hemodynamics by regulation of arterial pressure and water and electrolyte balance. Ang II increases the risk of cardiovascular event by increasing arterial blood pressure and directly acts on cardiac and renal tissues (Schmieder, Hilgers et al. 2007). It induces endothelial dysfunction and stimulates inflammatory, proliferative, fibrotic and thrombotic processes in the vasculature, and is a key regulator of vascular remodeling and inflammation. Ang II increase vascular tone, constricts smooth muscle cells, regulates vascular cell growth, apoptosis, fibrosis, matrix metalloproteinase production and degradation of extracellular matrix (Schiffrin, Park et al. 2000; Touyz 2005). These effects are observed often in arterial hypertension and atherosclerosis (Kane, Etienne-Selloum et al. 2010). Ang II may also affect blood pressure by its effects on kidney, brain and sympathetic nervous system (Reid 1992).

1.5.2 Excessive Reactive Oxygen Species Production in Hypertension

ROS play a major role as intracellular signaling molecules to regulate normal biological cellular responses (Griendling, Sorescu et al. 2000). In pathological conditions, loss of redox homeostasis contributes to vascular oxidative damage (Gao and Mann 2009). Multiple sources of oxidative stress have been implicated in the pathogenesis of hypertension-related endothelial dysfunction. Recently, evidences have indicated that specific enzymes, the NOX family of NADPH oxidases, have an important function in generating ROS in a highly regulated fashion in physiological conditions, and that in disease states, hyper activation of NOXs contributes to oxidative stress and consequent cardiovascular diseases (Figueira, Barros et al. 2013). Investigations have gone further to demonstrate the potential mechanisms controlling two important sources of hypertension-associated oxidative stress, NADPH oxidase and mitochondria (Montezano and Touyz 2012). Taken together with recent reports demonstrating the coordinated formation of reactive oxygen species (ROS) from NADPH oxidase and mitochondria in hypertensive states, a model of hypertension-induced ROS originating from coordinated mitochondrial sources and NADPH oxidase appears to be promising (Dikalov and Ungvari 2013).

1.5.2.1 Inflammatory Regulation of Hypertension-Associated Endothelial Dysfunction

Inflammatory mechanisms appear to play a significant role in some types of pulmonary hypertension (PH), including monocrotaline-induced PH in rats and pulmonary arterial hypertension of various origins in humans, such as connective tissue diseases (Sadoughi, Zhang et al. 2011). Inflammation in adipose tissue is associated with impaired endothelial function in obese patients. Adipose tissue also plays a major role in regulating metabolism and inflammation through the production of both pro-inflammatory and anti-inflammatory adipokines (Fantuzzi 2005). Though most investigations relating the process of adipose inflammation to vascular endothelial function concentrate on insulin resistance and obesity, recent studies have evaluated the effect of perivascular adipose tissue on vascular homeostasis in hypertension. Adipose tissue from hypertensive rats applied to thoracic aorta segments failed to suppress phenylephrine-induced vasoconstriction, in contrast to adipose tissue from normotensive animals (Baranowska-Kuczko, Kozłowska et al. 2016).

Recent data also define novel roles for elements of both innate and adaptive immune responses in regulating endothelial function under hypertensive conditions (Pauletto and Rattazzi 2006). Activation of innate immunity's complement pathway may negatively impact vascular endothelial function in hypertension, whereas increased anti-inflammatory interleukin-10 expression from the adaptive immune response blunts the adverse effects of angiotensin II-associated hypertension on endothelial function (Ferri, Croce et al. 2007). Circulating endothelial progenitor cells (EPCs), derived from myeloid pluripotent stem cells that also give rise to mature mononuclear cells, also play significant roles in maintaining endothelial homeostasis through their regenerative and repair mechanisms (Urbich and Dimmeler 2004). Overall, these newer data suggest that hypertension-associated vascular endothelial dysfunction relates to local vascular inflammation as well as to systemic inflammation (Cottone and Cerasola 2008).

Animal studies have shown that oxidative stress and renal tubulointerstitial inflammation are associated with, and have major roles in, the pathogenesis of hypertension (Vaziri 2008). This relation is supported by the observations that increase level of oxidative stress and renal tubulointerstitial inflammation increase arterial pressure in animal models (Vaziri and Rodriguez-

Iturbe 2006). Conversely, hypertension has been shown to cause oxidative stress and inflammation in renal and cardiovascular tissues in experimental animals. All together, these observations indicate that oxidative stress, inflammation and arterial hypertension participate in a self-perpetuating cycle which can lead to progressive cardiovascular disease (Brasier, Recinos et al. 2002)

1.5.3 Antihypertensive treatments

In clinical settings today, a variety of antihypertensive medicines are being used alone and in combination with other drugs to reach target goal of blood pressure.

Calcium channel blockers

Calcium channel blockers inhibits the entrance of calcium ions via voltage-operated calcium channel in cells of the heart and blood vessel walls, resulting in lower blood pressure (Epstein and Braunwald 1982) and hence are also called calcium antagonists. They relax and dilate blood vessels by affecting the smooth muscle cells in the arterial walls.

Beta-blockers

The exact mechanisms of action of beta-blockers as anti-hypertensive drugs is still largely unknown. The proposed mechanism is that beta-blockers inhibit the effects of the sympathetic nervous system on beta-adrenergic receptor of the heart (Parati and Esler 2012). They reduce the work of the heart and requirement blood and oxygen. As a result, the heart doesn't have to work as hard, which decreases blood pressure. Also, they help to control heart rate and are used in the treatment of abnormal heart rhythms that may be too fast or irregular. Beta-blockers are also widely used to treat hypertension, although they are no longer a first choice for initial treatment of most patients according to current guidelines (Hunt, Abraham et al. 2005).

Diuretics

Diuretics acts on kidneys to remove more sodium and water from the body, which helps to relax the blood vessel walls, thereby lowering blood pressure. Moreover, they are combined with other blood pressure medicines as they can enhance the effect of the other antihypertensive drugs and prevent the fluid retention (Weber, Schiffrin et al. 2014). Thiazide diuretics are recommended as the first line of treatment for hypertension and are usually prescribed as one of at least two medicines to control hypertension.

Vasodilators

Vasodilators are medications that cause dilatation of blood vessels predominantly by the release of NO. They act directly on the VSMC in the walls of arteries resulting in vasorelaxation and preventing vasoconstriction. As a result, blood flows more easily through arteries.

ACE inhibitors

ACE inhibitors block the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor and mitogenic. Therefore, they lower arteriolar resistance and increase venous capacity; decrease cardiac output, cardiac index, stroke work, and decrease resistance in blood vessels in the kidneys; and lead to increased natriuresis.

Angiotensin II receptor antagonists (AT1R blockers)

The angiotensin II receptor antagonists (AT1R blockers, ARBs, sartans) are a group of antihypertensive drugs that act by blocking the effects of Ang II-mediated via AT1R activation in the body, thereby lowering blood pressure. Their structure is similar to Ang II and they bind to Ang II receptors as inhibitors. AT1 blockers are widely used drugs for mild to moderate hypertension, chronic heart failure, secondary stroke prevention and diabetic nephropathy.

AT1 blockers and ACE inhibitors directly inhibit RAS and are the most effective for the treatment of hypertension (Taal and Brenner 2000).

1.6 Endothelial Dysfunction and Diabetes

Diabetes Mellitus (DM) is a major health problem worldwide, associated with morbidity and mortality. DM is characterized by persistent elevation of the blood glucose level (Monesi, Baviera et al. 2012). There are two types of diabetes: the type 1 diabetes (T1DM), which occurs due to the absence of the formation of insulin, and type 2 diabetes (T2DM), which is characterized by insulin insensitivity as a result of insulin resistance usually associated with metabolic syndrome and obesity (Figure 16) (Sharma, Bernatchez et al. 2012). Several clinical studies with both types 1 and type 2 diabetic patients have shown the presence of an endothelial dysfunction (McVeigh, Brennan et al. 1992; Nathan, Lachin et al. 2003). In addition, studies report that endothelial dysfunction appears early in the development of DM, which may suggest a role of impaired endothelium-dependent vasodilatation in the initiation and development of both macro-vascular and micro-vascular complications of diabetes. Individuals with type I and type II diabetes have evidence of both microvascular and macrovascular endothelial dysfunction (Caballero, Arora et al. 1999). Endothelial dysfunction can even be evident in healthy individuals with a family history of diabetes, suggesting a genetic link (Alvarado-Vásquez, Zapata et al. 2007). Patients with diabetes often have reduced NO bioavailability which results from increased oxidative stress, and oxidation of LDL due to hyperglycaemia (Endemann and Schiffrin 2004). Patients with type 1 diabetes have shown improved endothelial function when taking ACE inhibitors, through a reduction in oxidative stress, and an increase in NO bioavailability (Heitzer, Schlinzig et al. 2001).

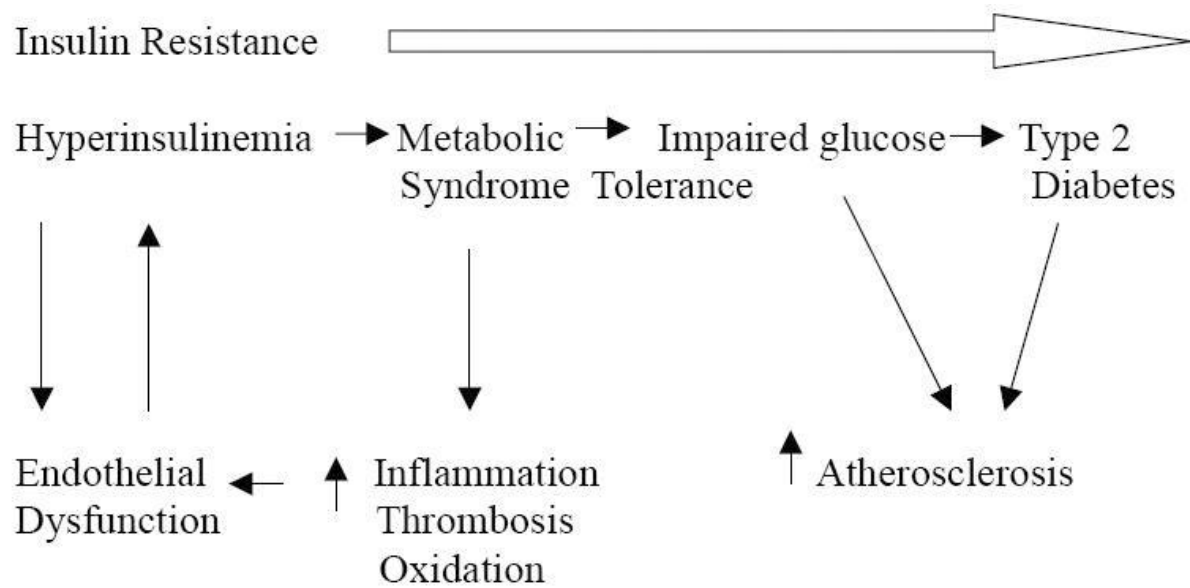


Figure 16. Progression of endothelial dysfunction in relation to the progression of insulin resistance (Cosentino and Lüscher 1997).

Chapter 2 Omega-3 and cardiovascular effects

2. Lipids

Lipids are defined as small hydrophobic or amphipathic (or amphiphilic) molecules that may originate entirely or, in part, through condensations of thioesters and/or isoprene units (Fahy, Subramaniam et al. 2005)

Lipids are a chemically diverse group of substances that are poorly soluble or insoluble in water, but soluble in a polar organic solvents such as chloroform, hydrocarbons, alcohols or ethers (Rane and Anderson 2008). They are mostly composed of carbon, hydrogen, oxygen and also sometimes nitrogen and phosphorous. Triglyceride, phospholipids sterols and waxes are the main types of lipids (McDonald 2002). The triglycerides exists in both foods and in the body, and usually serve as energy sources and can be stored in the adipose tissue for later use within the body (Turchini, Francis et al. 2011)

The lipid classification system enables categorization of lipids and their properties in a way that is compatible with other macromolecular databases. Using this approach, lipids from biological tissues are divided into eight categories: fatty acids (Table 1), glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides (derived from condensation of ketoacyl subunits); sterol lipids and prenol lipids (derived from condensation of isoprene subunits). Each category contains distinct classes and subclasses of molecules (Fahy, Subramaniam et al. 2005).

Table 1. Structure and taxonomy of fatty acids (Simopoulos 1998)

Structure	Taxonomy	Common name
C4:0	Butanoic acid	Butyric acid
C6:0	Hexanoic acid	Caproic acid
C8:0	Octanoic acid	Caprylic acid
C10:0	Decanoic acid	Capric acid
C12:0	Dodecanoic acid	Lauric acid
C14:0	Tetradecanoic acid	Myristic acid
C16:0	Hexadecanoic acid	Palmitic acid
C16:1 ω-7	<i>Cis</i> -9 hexadecaenoic acid	Palmitoleic acid
C18:0	Octadecanoic acid	Stearic acid
C18:1 ω-9	<i>Cis</i> -9 octadecaenoic acid	Oleic acid (OA)
C18:2 ω-6	<i>Cis</i> -9,12 octadecadienoic acid	Linoleic acid (LA)
C18:3 ω-6	<i>Cis</i> -6,9,12 octadecatrienoic acid	γ -linolenic acid (GLA)
C18:3 ω-3	<i>Cis</i> -9,12,15 octadecatrienoic acid	α -linolenic acid (ALA)
C20:0	Eicosanoic acid	Arachidic acid
C20:4 ω-6	<i>Cis</i> -5,8,11,14 eicosatetraenoic acid	Arachidonic acid (AA)
C20:5 ω-3	<i>Cis</i> -5,8,11,14,17 eicosapentaenoic acid	Eicosapentaenoic acid (EPA)
C22:0	Docosanoic acid	Behenic acid
C22:5 ω-3	<i>Cis</i> -7,10,13,16,19 docosapentaenoic acid	Lupanodonic acid (DPA)
C22:6 ω-3	<i>Cis</i> -4,7,10,13,16,19 docosahexaenoic acid	Cervonic acid (DHA)
C24:0	Tetracosanoic acid	Lignoceric acid

2.1 Saturated fatty acids (SFA)

SFA only have carbon-carbon single bonds in their chain and these carbons are fully occupied with hydrogen atoms, thus forming straight chains giving strength to the structure (Brownstein 1959). They are a useful source of energy and also have some important physical properties including poorly soluble in water in their undissociated (acidic) form, whereas they are relatively hydrophilic as potassium or sodium salts. (Fuhrhop and Endisch 2000). The most prevailing SFA are palmitic acid (C16), and stearic acid (C-18), found most commonly in animal products (Table 2). Vegetable derivatives of SFA such as palm oil, palm kernel oil, and coconut oil are produced from vegetables (Healy, Pfeifer et al. 1994). High consumption of SFA is associated with high LDL levels which is an independent risk factors of cardiovascular diseases (CVD) (Siri-Tarino, Sun et al. 2010; Colquhoun, Ferreira-Jardim et al. 2011).

Table 2. Structure of different unbranched fatty acids with a methyl end and a carboxyl (acidic) end (Hagan 2015).

Number of C atoms	Common Name	Systemic Name	Formula
2	Acetic acid	Ethanoic acid	CH ₃ COOH
4	Butyric acid	Butanoic acid	CH ₃ (CH ₂) ₂ COOH
6	Caproic acid	Hexanoic acid	CH ₃ (CH ₂) ₄ COOH
8	Caprylic acid	Octanoic acid	CH ₃ (CH ₂) ₆ COOH
10	Capric acid	Decanoic acid	CH ₃ (CH ₂) ₈ COOH
12	Lauric acid	Dodecanoic acid	CH ₃ (CH ₂) ₁₀ COOH
14	Myristic acid	Tetradecanoic acid	CH ₃ (CH ₂) ₁₂ COOH
16	Palmitic acid	Hexadecanoic acid	CH ₃ (CH ₂) ₁₄ COOH
18	Stearic acid	Octadecanoic acid	CH ₃ (CH ₂) ₁₆ COOH
20	Arachidic acid	Eicosanoic acid	CH ₃ (CH ₂) ₁₈ COOH
22	Behenic acid	Docosanoic acid	CH ₃ (CH ₂) ₂₀ COOH

2.2 Monounsaturated fatty acids (MUFA)

Upon losing one or more pairs of hydrogen atoms from the carbon chain, the fatty acids are called unsaturated fatty acids. The fatty acids containing one double bond are MUFA (Figure 17). Some common MUFA are palmitoleic acid (16:1 n-7), cis-vaccenic acid (18:1 n-7) and oleic acid 19 (18:1 n-9). Palmitoleic acid has 16 carbon atoms with the first double bond occurring 7 carbon atoms away from the methyl group and 9 carbons from the carboxyl end, which can be lengthened to the 18-carbon cis-vaccenic acid. Oleic acid has 18 carbon atoms with the first double bond occurring after the ninth carbon atom from the methyl end of fatty acid chain. Vegetable oils such as olive oil and canola oil are good sources of MUFA. MUFA consumption is strongly associated with a decrease in LDL cholesterol (Corrao, Bagnardi et al. 1999).



Oleic acid (MUFA)

Figure 17. Monounsaturated fatty acids MUFAs

2.3 Polyunsaturated fatty acids (PUFA)

PUFA contain more than one double bonds in their chains (Figure 18). According to the international nomenclature, the positioning of the first double bond is given by (n-x) notation, counting the number of carbon atoms from the methyl end (Keller, Dreyer et al. 1993). For example, in omega-3 (n-3) and omega-6 (n-6) fatty acids, the first double bond starts at 3 and 6 carbons from the methyl end, respectively. Thus α -Linoleic acid symbol 18:3 n-3 identifies a fatty acid having 18 carbon atoms and 3 double bonds, the first double bond occurring after the third carbon atom from the methyl end of the fatty acid chain, known as the *n* end (Mateos 2012).

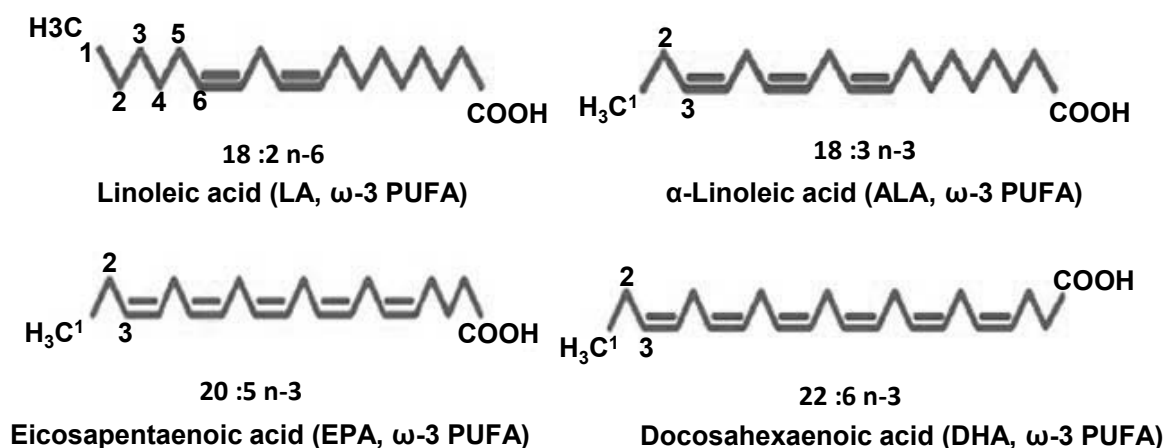


Figure 18. Major Polyunsaturated fatty acids (PUFA). (Nair, Leitch et al. 1997).

2.3.1 Trans fatty acids (TFA)

Trans fatty acids are unsaturated fatty acids of plant origin that have a *trans* arrangement of the carbon atoms adjacent to its double bonds resulting from the hydrogenation process, which gives a more rigid molecule close to a saturated fatty acids. The two hydrogen atoms of the carbons adjacent to the double bond point to opposite directions, which is different from the double bond of *cis*-configuration in the fatty acids of mammals. Furthermore, the location of the double bond is not fix and it may appear anywhere along the molecule, so that many positional isomers may exist. In *trans* fatty acids, angle of the double bond is smaller than the *cis*-isomeric configuration and hence acyl chain is more linear, resulting in a more rigid molecule with different physical properties such as greater thermodynamic stability and a higher melting point. The *trans* configuration is designated by a *t*-; the number preceding the *t*- indicates the position of the *trans* bond acids counted from the carboxyl end of the molecule, and *c*- designates the *cis* isomers. Consumption of such acids is thought to increase the risk of atherosclerosis.

2.3.2 n-3 polyunsaturated fatty acids (n-3 PUFAs)

Long-chain polyunsaturated fatty acids with the first double bond at the third position from the methyl terminal that are found in plants and some types of fish. n-3 PUFAs are found in short and long-chain varieties. The short chain form is alpha-linolenic acid, 18:3 n-3 (ALA) which contains 18 carbons having 3 double bonds and is considered as essential fatty acids because it can't be synthesized within the body (Sen 2013). The long chain n-3 PUFA includes EPA, DPA (docosapentaenoic acid) and DHA, and as compared to ALA, these fatty acids are elongated and highly unsaturated; EPA has 20 carbons with 5 double bonds while DPA and DHA has 22 carbons with 5 and 6 double bonds, respectively (Figure 19).

Main vegetal sources of n-3 PUFA include flax seed, camelina seed, perilla and chiaseed oils which contain the 18-carbon ALA as the major n-3 PUFA (Turchini, Francis et al. 2011). The carbon-20 and carbon-22 n-3 PUFA such as EPA and DHA are abundantly found in seafood such as fish and shellfish. These fatty acids are also considered as essential fatty acids. Fish such as tuna, sardines, salmon, mackerel and herring contains higher concentration of these fatty acids. Shellfish like abalone, oyster, mussel and scallop are also good sources of these long chain n-3 PUFA (Su, Wiltshire et al. 2004). The National Heart Foundation of Australia recommends the consumption

of 500 mg per day of combined DHA and EPA, which is associated with a reduction in the risk of coronary heart disease (Colquhoun, Ferreira-Jardim et al. 2011) .

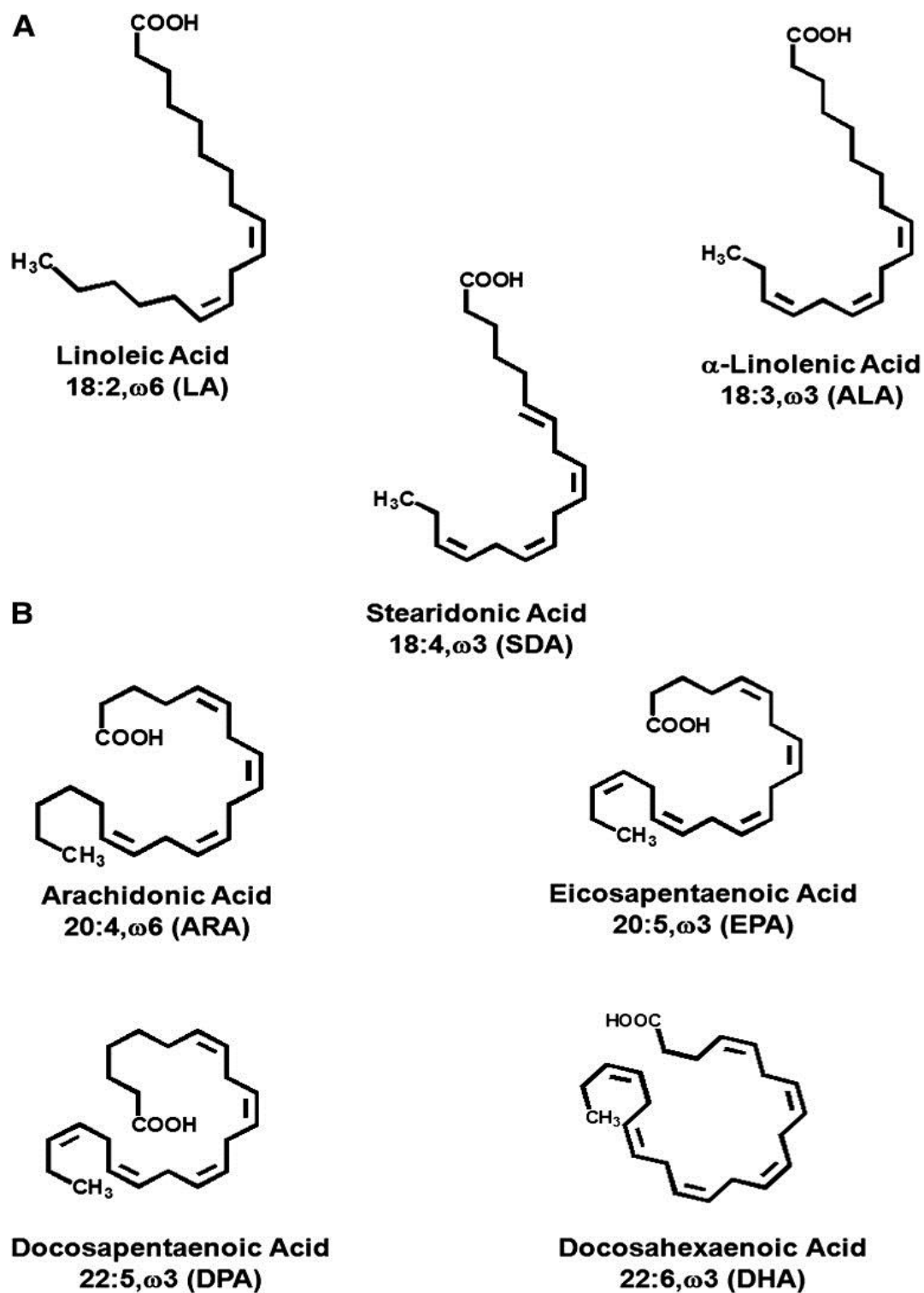


Figure 19. Structures of dietary ω 3 and ω 6 polyunsaturated fatty acids. A: C18 ω 3 and ω 6 PUFA. B: C20–22 ω 3 and ω 6 PUFA (Jump, Depner et al. 2012).

2.3.3 n-6 polyunsaturated fatty acids

Long-chain polyunsaturated fatty acids with the first double bond at the sixth position from the methyl terminal that are found in plants and in animal muscles and organ meat. n-6 PUFA has its short-chain representative, linoleic acid, which is an essential fatty acid and the most prevalent one in western diets. Linoleic acid, is most abundantly found in nature, having high proportion in most of the vegetable oils such as sunflower, safflower, corn, soybean and canola oils (Mateos 2012). Evening primrose and borage oil are also enriched in linoleic acid. Animal products also serve as a major source of n-6 PUFA in the form of arachidonic acid predominantly found in both muscle and organ meats (Sinclair 1991).

2.4 The importance of the ratio of omega-6/omega-3 essential fatty acids

Western diets are deficient in omega-3 fatty acids, and have excessive amounts of omega-6 fatty acids. Excessive amounts of omega-6 polyunsaturated fatty acids (PUFA) and a very high omega-6/omega-3 ratio promote the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases, whereas increased levels of omega-3 PUFA (a low omega-6/omega-3 ratio) exert suppressive effects (Simopoulos 2008) (Figure 20).

Mammalian cells cannot convert omega-6 to omega-3 fatty acids because they lack the converting enzyme, Δ^3 desaturase. Linoleic acid, α -Linoleic acid and their long-chain derivatives are important components of animal and plant cell membranes (Barceló-Coblijn and Murphy 2009). These two classes of essential fatty acids, are metabolically and functionally distinct, and often have important opposing physiological functions. An optimal balance of EFA is important for good health and normal development (Simopoulos 2006). When humans ingest fish or fish oil, the EPA and DHA from the diet partially substitute the omega-6 fatty acids, especially AA, in the membranes of probably all cells, but especially in the membranes of platelets, erythrocytes, neutrophils, monocytes, and liver cells (Simopoulos 2009).

AA and EPA are the parent compounds for eicosanoid production. Due to the increased amounts of omega-6 fatty acids in the Western diet, the eicosanoid metabolic products from AA, specifically prostaglandins, thromboxanes, leukotrienes, hydroxy fatty acids, and lipoxins, are formed in larger quantities than those formed from omega-3 fatty acids, specifically EPA (Simopoulos 2008). The eicosanoids from AA are biologically active in very small quantities, and, they promote to the

formation of thrombus and atheromas, allergic and inflammatory disorder, particularly in susceptible people (Simopoulos 2011). Thus, a diet rich in omega-6 fatty acids shifts the physiological state to one that is prothrombotic and proaggregatory, with increases in blood viscosity, vasospasm, and vasoconstriction and decreases in bleeding time (Hussein 2013).

Omega-3 fatty acids inhibit the production of platelet-derived growth factor (PDGF) in bovine endothelial cells (De Caterina, Cybulsky et al. 1994). Thus, the reduction in its production by endothelial cells, monocytes/macrophages, and platelets could inhibit both the migration and proliferation of smooth muscle cells, monocytes/macrophages, and fibroblasts in the arterial wall. Omega-3 fatty acids also increase endothelium-derived relaxing factor (EDRF) which facilitates relaxation in large arteries and vessels (Nicolosi and Stucchi 1990). Supplementing the diet with omega-3 fatty acids (3.2 g EPA and 2.2 g DHA) in normal subjects increased the EPA content in neutrophils and monocytes more than sevenfold without changing the quantities of AA and DHA (Nicolosi and Stucchi 1990). The antiinflammatory effects of fish oils are partly mediated by the inhibition of the 5-lipoxygenase pathway in neutrophils and monocytes (Lee, Hoover et al. 1985). Moreover, several studies show that omega-3 fatty acids influence interleukin metabolism by decreasing IL-1 and IL-6, suggesting an important role in the prevention of atherosclerosis (Jung, Torrejon et al. 2008)

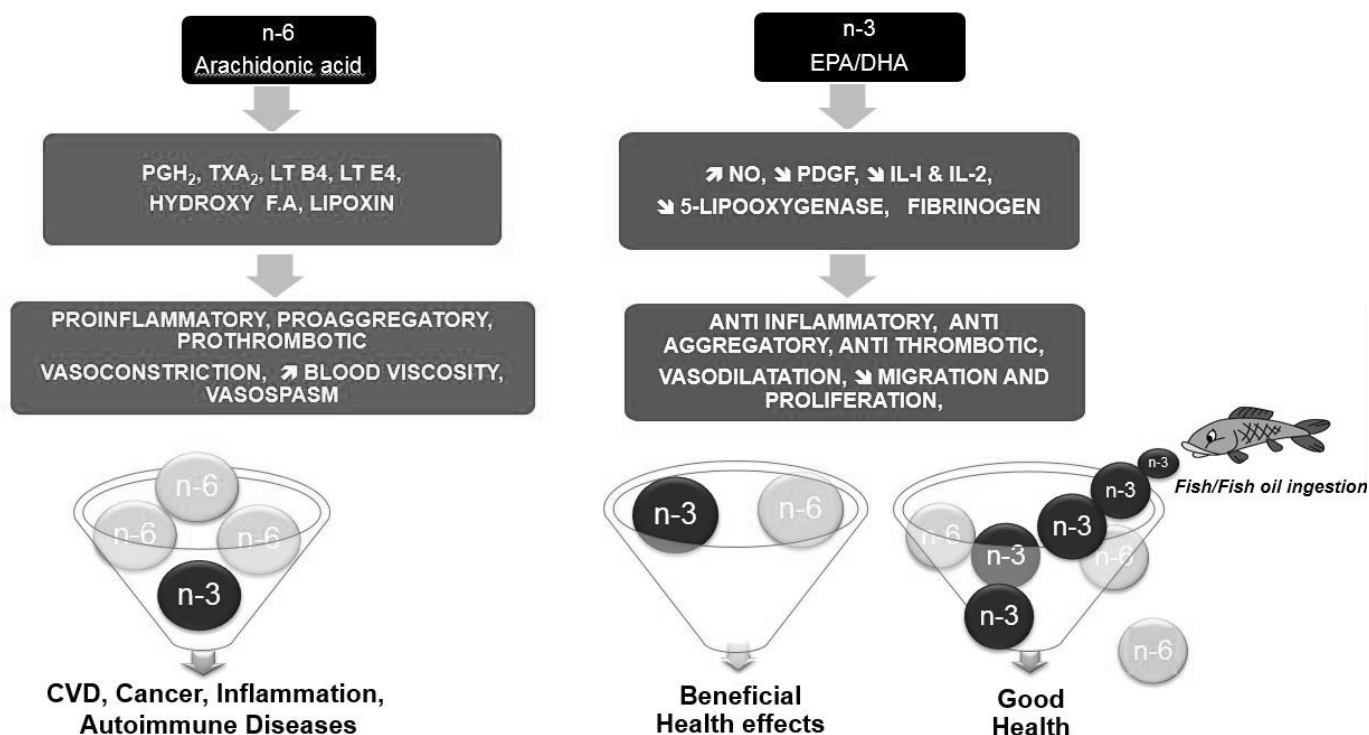


Figure 20. Importance of omega-6/omega-3 ratio.

A very high omega-6/omega-3 ratio promotes the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases, whereas increased levels of omega-3 PUFA (a low omega-6/omega-3 ratio) exert suppressive effects.

PGH₂, Prostaglandin H₂; TXA₂, Thromboxane A₂; LT E₄, Leukotriene E₄; LT B₄, Leukotriene B₄; PDGF, Platelet derived growth factor; IL-1, Interleukin-1; IL-2, Interleukin-2.

2.5 Metabolism of PUFA

Free arachidonic acid (AA) is oxidized through three major metabolic routes: (i) the cyclooxygenase (COX) pathway producing prostaglandins and thromboxanes, (ii) the lipoxygenase (LOX) pathway leading to leukotrienes, hydroxyeicosatetraenoic acids (HETEs)

and lipoxins, whereas (iii) the cytochrome P450 (CYP) pathway implies oxidation by monooxygenases to produce hydroxylated and epoxidised fatty acids (Hong, Bose et al. 2004). α -linolenic acid (ALA) can be metabolized to some extent by mechanisms including desaturation and elongation to yield EPA, DHA, while linoleic acid (LA) is the metabolic precursor of arachidonic acid (AA) (Holub 2002). In the omega-6 fatty acids pathway, linoleic acid can be first converted into gamma-linolenic acid (GLA, 18:3, omega-6 fatty acid) by the enzyme Δ 6-desaturase before elongation leading to (DGLA) dihomo-GLA (DHGLA, 20:3, omega-6 fatty acids) (Das 2008). The dihomo-GLA can be further converted into arachidonic acid (AA, 20:4, omega-6 fatty acids) by the Δ 5-desaturase. The omega-3 fatty acids pathway uses the same series of enzymes for converting α -linolenic acid (ALA) into EPA and then into docosapentaenoic acid (DPA) by elongase (Figure 21). The conversion of ALA to EPA and DPA occurs primarily in the liver in the endoplasmic reticulum, whereas the final conversion of DPA to DHA requires a translocation to the peroxisome for a β -oxidation reaction (Arterburn, Hall et al. 2006).

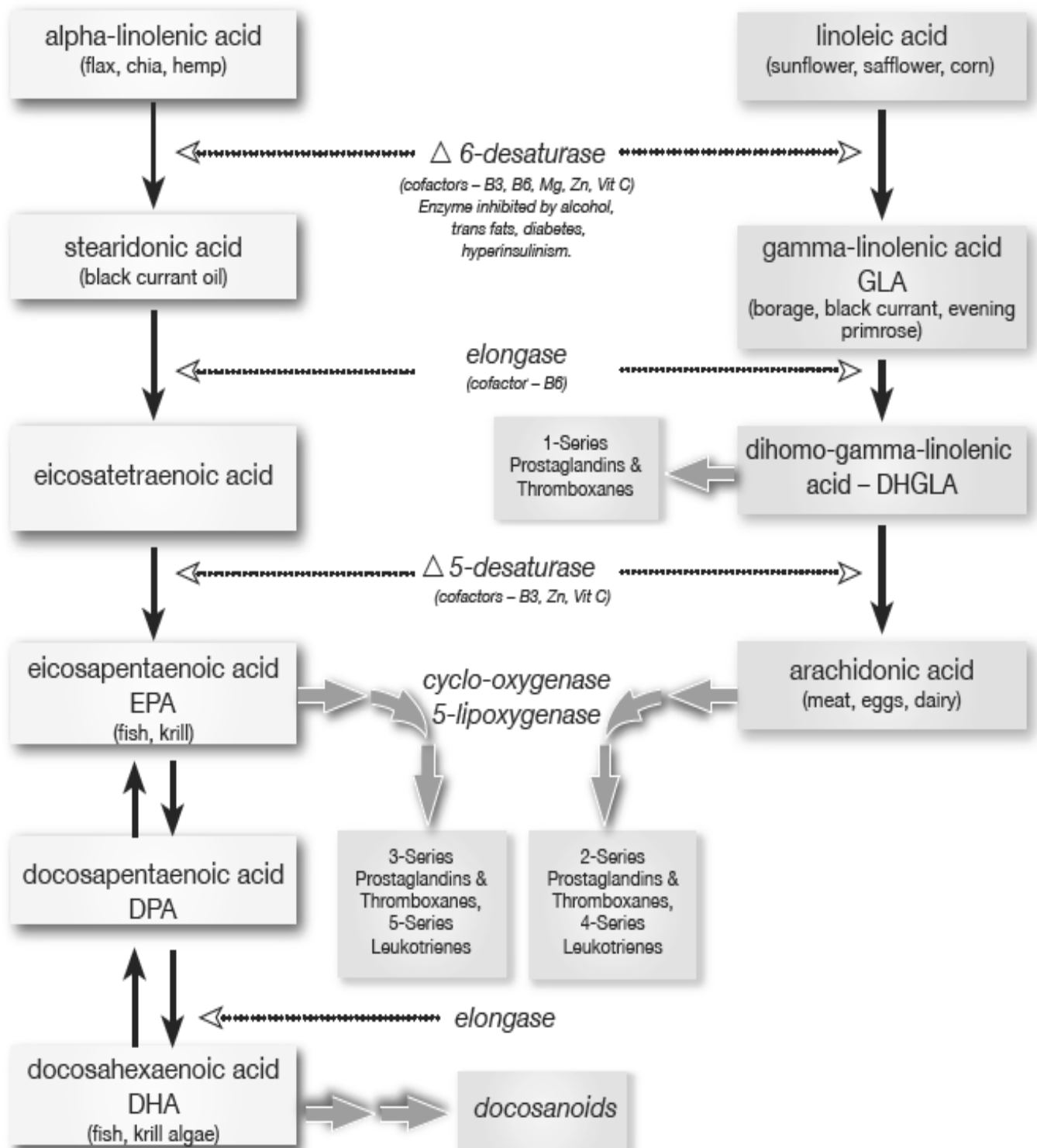


Figure 21. Synthesis pathway of omega-6 and omega-3 fatty acids in mammals.

2.6 Health benefits of n-3 polyunsaturated fatty acids

2.6.1 n-3 polyunsaturated fatty acids and diabetes

Intake of n-3 fatty acids, either as fish oil or ethyl ester formulations is related with a variety of biochemical changes that might be beneficial in diabetes: reduced triglyceridemia, mainly through enhanced triglyceride lipolysis and enhanced fatty acid oxidation (Sirtori and Galli 2002).

Increasing the consumption of n-3 long chain PUFAs improves several cardiovascular risk factors in persons with diabetes and may reduce the risk of conversion from impaired glucose tolerance to type 2 diabetes (Carpentier, Portois et al. 2006). Many epidemiologic studies reported that type 2 diabetes was less prevalent among Japanese as compared to their mainland counterparts. Lower prevalence was attributed mainly to diets rich in n-3 long chain PUFAs (Montmayeur, le Coutre et al. 2010).

n-3 long chain PUFAs have beneficial effects in lowering triglyceride levels and reducing remnant lipoprotein (RLP) levels in subjects with type 2 diabetes or hypertriglyceridemia and may increase high-density lipoprotein (HDL) cholesterol levels (Okumura, Fujioka et al. 2002). RLPs are highly atherogenic lipoproteins produced in the hydrolysis of chylomicrons and very low density lipoprotein (VLDL) (Shin, Kim et al. 2004). Modest amounts of purified EPA 0.9 to 1.8 g/day reduced the RLP levels significantly by 77% in patients with type 2 diabetes treated for 3 months (Nettleton and Katz 2005).

It is of interest that in *in vitro* studies EPA has been shown to increase glucose-induced insulin secretion from beta-TC3 insulinoma cells (Dubnov and Berry 2004). Other potentially beneficial aspects of n-3 treatment in diabetes may be related to the role of these dietary components in providing a source of vasoactive compounds, potentially leading to reduced blood pressure and improved peripheral perfusion (Hornstra 2012).

2.6.2 n-3 polyunsaturated fatty acids and cardiovascular diseases

Long chain PUFAs including EPA and DHA are the key nutrients in fish responsible for the potential cardioprotective effects of fish consumption (Kris-Etherton, Harris et al. 2002). A beneficial effect of fish consumption on CVDs has been suggested to be related to overall favorable

effects on lipid profile, threshold for arrhythmias, platelets activity, inflammation, endothelial function, atherosclerosis and hypertension (Figure 22).

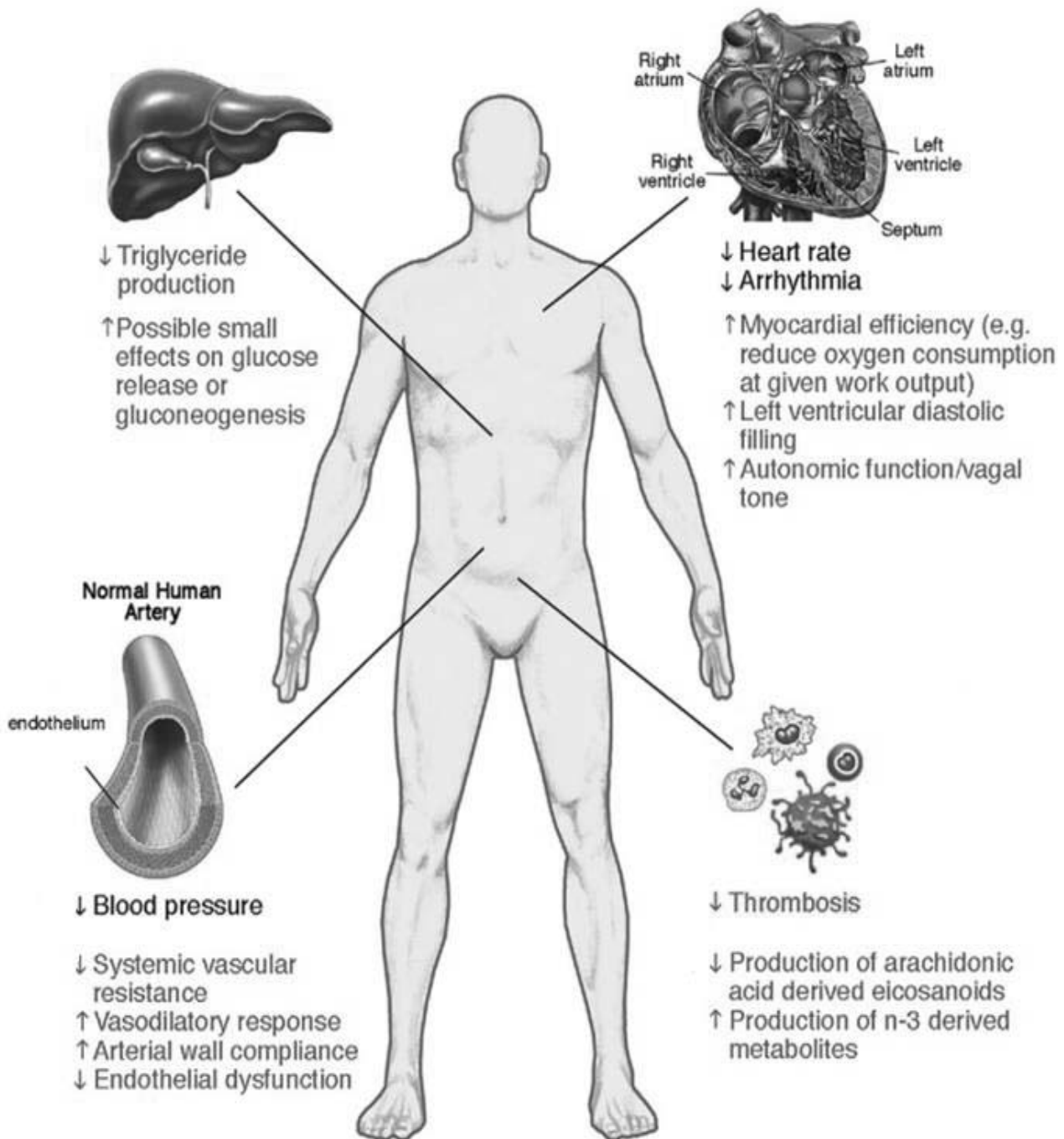


Figure 22. Physiological effects of n-3 PUFA that might influence CVD Risk (Mozaffarian and Wu 2011).

2.6.2.1 Lipid Profile

Hypertriglyceridemia is one of the components of metabolic syndrome. Several studies reported that long chain n3 PUFAs reduce the blood triglyceride levels (Kris-Etherton, Harris et al. 2002). Recently, 47 trials showed that fish oil supplementation with 1 g/day was effective in reducing triglyceride levels by 0.34 mmol/L over an average treatment period of 24 weeks in participants with hypertriglyceridemia (He 2009).

PUFA have multiple CVD-related physiological effects such as lowering of plasma triglycerides including reduced fatty acid availability for triglyceride synthesis (Reddy and Katan 2004). Moreover, they reduced delivery of nonesterified fatty acids to the liver reduced, hepatic enzyme activity for triglyceride synthesis; and increased hepatic synthesis of phospholipids rather than triglycerides (Nakamura and Nara 2003).

2.6.2.2 Inflammation and endothelial function

Several epidemiological studies reported that n-3 PUFA fatty acids have beneficial effects on inflammation and endothelial function (Mori and Beilin 2004). Omega-3 fatty acids are anti-inflammatory. Indeed inverse association has been found between Omega-3 or fish consumption and circulating levels of C-reactive protein, interleukin-6, endothelial-leukocytes adhesion molecule-1, soluble intercellular adhesion molecule-1, tumor necrosis factor, INF- α soluble receptor 1, and matrix metalloproteinases-3 (He 2009).

Omega 3 fatty acids have recognized anti-inflammatory actions that may contribute to their beneficial cardiac effects (Russo 2009). Omega 6 fatty acids can be converted into arachidonic acid and then metabolised into the omega 6 eicosanoids. Consumption of omega 3 fatty acids increases eicosapentanoic acid in the cell membrane. This competes with arachidonic acid for enzymatic conversion into its own metabolites, the omega-3-derived eicosanoids (Wall, Ross et al. 2010). Independent of the effects on the metabolism of eicosanoids, fish oils suppress pro-inflammatory cytokines and reduce expression of cell adhesion molecules (Calder 2006) which are critical in recruiting circulating leucocytes to the vascular endothelium, an important event in the

pathogenesis of atherosclerosis and inflammation. Omega-3 fatty acids also have direct effects on endothelial vasomotor function. Higher plasma concentrations are associated with improved dilatation of the brachial artery in young adults with cardiovascular risk factors, which implies a protective effect on endothelial function (Din, Newby et al. 2004). In hyperlipidaemic men, omega-3 fatty acid supplementation improved systemic arterial compliance, and supplementation with docosahexanoic acid increased vasodilator responses in the human forearm arteries. These effects may be mediated through actions on intracellular signalling pathways, leading to reduced activation of transcription factors such as NF- κ B (Egert and Stehle 2011). However, the precise effects of omega 3 fatty acids on these fundamental cellular processes and their potential impact on coronary heart disease are yet to be delineated completely.

2.6.2.3 Atherosclerosis

Although high dose supplementation of omega-3 fatty acids exerts a hypotriglyceridemic effects, these fatty acids also increase and enhance oxidation of LDL cholesterol (Larsson, Kumlin et al. 2004). However, data from both animal models and humans are inconsistent. It is unclear whether omega-3 fatty acids have a direct effect on the pathogenesis of atherosclerosis. Consumption of 6g/d EPA and DHA for 2 years had no major favorable effects on the thickness of atherosclerotic coronary arteries (Woodman 2003). Several other studies showed that dietary intake of omega-3 fatty acids or nonfried fish is associated with a lower prevalence of subclinical atherosclerosis classified by significant changes in common carotid intima-media thickness, in percent stenosis, while no modification in coronary artery calcium score, and ankle-brachial index were observed (Von Schacky, Angerer et al. 1999).

2.6.2.4 Platelets aggregation

Omega-3 FAs compete with omega-6 FAs for prostaglandin and leukotrienes synthesis at the cyclooxygenase and lipoxygenase level. omega-3 fatty acids modulate prostaglandin metabolism by increasing prostaglandin E₃, an active vasodilator and inhibitor of platelets aggregation, thromboxane A₃, leukotrienes B₅, and by decreasing production of thromboxane A₂, a potent platelet aggregation and vasoconstrictor, and leukotrienes B₄ formation (an inducer of inflammation and a powerful inducer of leukocyte chemotaxis and adherence) (Jha 2004). In addition, omega-3 fatty acids may react with reactive oxygen species because of their double bonds and lead to

decreased production of hydrogen peroxide, which is a critical activator of the nuclear NF- κ B. Other studies also indicate that high intake of omega-3 fatty acids would produce a lower platelet count, less platelet aggregation, a longer bleeding time and lower concentrations of thromboxane metabolites.

2.7 Clinical implications of omega-3 fatty acids

Omega 3 fatty acids from fish or fish oil supplements should be considered in the secondary prevention regimen of patients after myocardial infarction (Holub and Holub 2004). Patients should consume about 1 g/day of eicosapentanoic acid and docosahexanoic acid, preferably by increasing their intake of oily fish to at least two servings per week (Harris and Von Schacky 2004). Fish oil capsules may be considered for those unable to tolerate fish or change their diet effectively. Approved pharmaceutical grade capsules should be prescribed rather than encouraging over the counter supplements (Din, Newby et al. 2004). Recent guidelines from the American Heart Association have gone further, supporting the use of fish oil supplements for patients with “documented” coronary heart disease. However, they believe that more evidence is required before considering fish oil supplements for patients with coronary heart disease outside the specific indication of myocardial infarction. Others have argued that fish oil supplements should not be recommended routinely for patients after myocardial infarction until more definitive evidence is available (Rahman, Haque et al. 2005). No trial has assessed the effects of fish oils on risk of coronary heart disease in primary prevention, and therefore explicit recommendations for this group cannot be made currently. Such a trial may prove impractical in terms of the numbers required. However, on the basis of evidence from epidemiological and observational studies the consumption of (preferably oily) fish at least twice weekly should be encouraged as part of a balanced diet (Din, Newby et al. 2004). Any recommendations regarding fish and fish oils should be balanced against safety issues. Side effects such as fishy aftertaste are uncommon, and gastrointestinal upset is infrequent at moderate intakes. Some reports show that fish oil may worsen glycemic control in diabetes, but two meta-analyses found no adverse effect. Furthermore, a recent prospective cohort study found that a higher consumption of omega 3 fatty acids was associated with a lower incidence of coronary heart disease and mortality in diabetic women (Din, Newby et al. 2004; Borghi and Cicero 2005). Concerns have been raised regarding adverse effects on low density lipoprotein (LDL) cholesterol and oxidative stress, but increases in LDL cholesterol are

modest and studies into oxidative stress have been contradictory. Overall these effects are unlikely to be dominant given the apparent cardiac benefits of omega 3 fatty acids. More specific concerns regarding dietary fish relate to environmental contaminants, and a recent study showed that mercury in fish may attenuate their cardioprotective effects (Cicero, Ertek et al. 2009).

AIM OF THE STUDY

The endothelial cells are the major regulator of vascular homeostasis and play a key role by synthesis and secretion of various potent vasodilating and vasoconstricting factors. In addition, they also inhibit platelet aggregation, decrease the endothelial expression of adhesion molecules and smooth muscle cell proliferation, thus reducing the risk of cardiovascular diseases. Endothelial dysfunction is an early hallmark for the development and progression of most of the cardiovascular diseases, like hypertension, atherosclerosis, myocardial infarction, cerebrovascular diseases (stroke), peripheral artery diseases, rheumatic heart diseases, congenital heart diseases and heart failure, which are the leading cause of mortality and morbidity worldwide. Endothelial dysfunction is associated with an impairment of endothelium-dependent relaxations involving a reduced NO bioavailability, EDH component of relaxation and an increased production of endothelium derived contractile factors (EDCF). Endothelial dysfunction could lead to hypertension.

Several naturally occurring constituents like red wine polyphenols, caffeine, omega-3 fatty acids, carotenoids, vitamins E and C have received significant consideration because of their potential antioxidant activity. Consuming a diet rich in these natural antioxidants has been associated with prevention and treatment of endothelial dysfunction and associated cardiovascular diseases (Potashkin 2014).

Antihypertensive treatments affecting RAS (AT1R and ACE inhibitors) contributes to 60 % to 65 % of overall hypertensive therapy and, therefore, Ang II-induced hypertension is one of the most widely used pharmacological model of hypertension in rats. In several experimental models of endothelial dysfunction, an overexpression of the local angiotensin system associated to a vascular oxidative stress has been described. Red wine polyphenols (RWPs) are able to prevent the Ang II-induced endothelial dysfunction mostly due to their antioxidant properties (Kane, Etienne-Selloum et al. 2010). RWPs intake caused a persistent improvement of the endothelial function, particularly the EDH component of relaxation, in middle-aged rats and this effect seems to involve the normalization of the expression of IK_{Ca} , SK_{Ca} and the angiotensin system (Khodja, Chataigneau et al. 2012). Various studies demonstrates that regular intake of fish products and dietary consumption of fish or fish oil rich in omega-3 PUFAs, particularly EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) has been related to a reduced risk of cardiovascular disease morbidity/mortality.

In a previous study of our group different ratio of EPA and DHA (EPA:DHA 1:1, EPA:DHA 3:1, EPA:DHA 6:1, EPA:DHA 1:3, EPA:DHA 1:6) and alone EPA and DHA were studied and they found that omega-3 EPA:DHA 6:1 is the most potent formulation for the induction of both NO-mediated and EDH-mediated response in porcine arteries by the activation of eNOS through Src PI3K Akt Pathway (Zgheel, Alhosin et al. 2014). Being the most superior formulation of omega-3 EPA:DHA 6:1 was selected to treat angiotensin II induced endothelial dysfunction and hypertension in rats. The major goal of this thesis was to determine whether chronic intake of EPA:DHA 6:1 affects hypertension and endothelial dysfunction induced by angiotensin II infusion in rats.

More specifically the aims were

1. To study the effect of EPA:DHA 6:1 in angiotensin induced hypertension.
2. To study the impregnation of EPA:DHA 6:1 in plasma and its effects on omega6/omega3 ratio.
3. To study the effect of EPA:DHA 6:1 in the endothelium -dependent relaxation in second branch mesenteric arterial rings.
4. To characterize the mechanisms underlying EPA:DHA 6:1 induced endothelium-dependent relaxation mediated by NO and EDH, in second branch mesenteric arteries.
5. Evaluate the ability of EPA:DHA to reduce contractile responses in second branch mesenteric arterial rings.
6. To study Ang II induced oxidative stress responses (ROS) in second branch mesenteric arteries.
7. To find the source of ROS.

RESULTS

ARTICLE I

EPA:DHA 6:1 prevents angiotensin II-induced hypertension and endothelial dysfunction in rats: role of NADPH oxidase and COX-derived oxidative stress

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Cardiovascular diseases are the major of death worldwide and should remain the leading cause of mortality and morbidity over the next few decades. Hypertension and other risk factors of cardiovascular diseases are associated early with the development of an endothelial dysfunction. The endothelial dysfunction is generally characterized by a reduced formation of vasoprotective factors including nitric oxide (NO) and endothelium-dependent hyperpolarization (EDH), and an increased production of vasoconstricting factors such as cyclooxygenase (COX)-derived metabolites of arachidonic acid involved in the endothelium-dependent contractile factors.

While current antihypertensive treatment are able to potently reduce the blood pressure, they seems to have a limited ability to protect and/or improve the endothelial dysfunction, a pivotal event in the protection of the cardiovascular system. Several epidemiological and both primary and secondary prevention studies have indicated that dietary intake of omega-3 polyunsaturated fatty acids (PUFAs), including the two major compounds eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), reduces the risk of cardiovascular diseases. Moreover, studies have reported that purified formulations of EPA and DHA are able to induce potent and sustained endothelium-dependent relaxations of isolated artery rings via an increased formation of NO and EDH. A previous study of the research team has shown that the endothelium-dependent vasorelaxant effect of omega-3 PUFAs is dependent on both the purity and ratio of EPA:DHA, with EPA:DHA ratio of 6:1 and 9:1 being superior formulations (Zgheel et al., 2014).

The aim of the present study was to determine whether chronic oral intake of the optimized EPA:DHA 6:1 formulation is able to prevent the hypertension and endothelial dysfunction induced by Ang II infusion in rats.



EPA:DHA 6:1 prevents angiotensin II-induced hypertension and endothelial dysfunction in rats: role of NADPH oxidase and COX-derived oxidative stress

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Major area of pharmacology:	Vascular pharmacology
Cross-cutting area:	Hypertension
Additional area(s):	Natural Product

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EPA:DHA 6:1 prevents angiotensin II-induced hypertension and endothelial dysfunction in rats: role of NADPH oxidase and COX-derived oxidative stress

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Running title: EPA:DHA 6:1 prevents Ang II-induced hypertension and endothelial dysfunction

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Statement of author contributions: V.S.K. and C.A contributed to the design of the experiments. Z.R., G.C.S., T.P.R., A.J.L.-G., M.K., M.A. and F.Z. performed the experiments. Z.R., G.C.S., V.S.K. and C.A. analyzed the data. Z.R., V.S.K. and C.A. contributed to the writing of the manuscript. V.S.K. and C.A. supervised the research.

For Peer Review

Abstract

Background and Purpose: EPA:DHA 6:1 has been shown to be a superior omega-3 formulation inducing a sustained endothelial NO synthase-derived formation of nitric oxide (NO). This study examined whether chronic intake of EPA:DHA 6:1 prevents hypertension and endothelial dysfunction induced by angiotensin II (Ang II) in rats.

Experimental approach: Wister rats received orally corn oil or EPA:DHA 6:1 (500 mg/kg/day) before chronic infusion of Ang II (0.4 mg/kg/day). Systolic blood pressure was determined by tail cuff sphygmomanometry, vascular reactivity using a myograph, oxidative stress using dihydroethidium and protein expression by immunofluorescence and Western blot.

Key results: Ang II-induced hypertension was associated with blunted acetylcholine-induced relaxations of secondary branch mesenteric artery rings affecting the endothelium-dependent hyperpolarization (EDH) and the NO components, both of which were improved by the NADPH oxidase inhibitor VAS-2870. The Ang II treatment induced also endothelium-dependent contractile responses (EDCFs), which were abolished by the cyclooxygenase inhibitor indomethacin. An increased level of vascular oxidative stress, and expression of NADPH oxidase subunits (p47^{phox} and p22^{phox}), COX-1 and COX-2, eNOS, and Ang II type 1 receptor were observed in the Ang II group whereas SK_{Ca} and connexin 37 were down-regulated. Intake of EPA:DHA 6:1 prevented the Ang II-induced hypertension and endothelial dysfunction improving both the NO and EDH components, and reducing EDCFs and the expression of target proteins.

Conclusion and implications: The present findings indicate that chronic intake of EPA:DHA 6:1 prevented the Ang II-induced hypertension and endothelial dysfunction in rats, most likely by preventing NADPH oxidase- and cyclooxygenase-derived oxidative stress.

Abbreviations

Ang II: angiotensin II; AT1R: angiotensin II type 1 receptor; AT2R: angiotensin II type 2 receptor; COX: cyclooxygenase; Cx37: connexin 37; DHA: docosaheaxaenoic acid; EDH: endothelium-dependent hyperpolarization; eNOS: endothelial NO synthase; EPA: eicosapentaenoic acid; IK_{Ca}: intermediate conductance calcium-activated potassium channel; NO: nitric oxide; PUFA: polyunsaturated fatty acid; SK_{Ca}: small conductance calcium-activated potassium channel.

Introduction

Cardiovascular diseases including coronary heart disease and stroke remain the leading cause of death worldwide both in developed and developing countries, with hypertension being a major risk factor (Mozaffarian *et al.*, 2016). The development of an endothelial dysfunction often characterized by a reduced formation of vasoprotective factors such as nitric oxide (NO) and endothelium-dependent hyperpolarization (EDH) associated with an increased production of vasoconstricting factors such as cyclooxygenase (COX)-derived metabolites of arachidonic acid, is thought to be a pivotal early event in the initiation and development of most types of cardiovascular diseases. Several experimental data have indicated a key role of the angiotensin systems, both the circulating and the local ones, in the alteration of the endothelial function in hypertension, atherosclerosis and diabetes, and also in ageing-related endothelial dysfunction (Dal-Ros *et al.*, 2012; Dal-Ros *et al.*, 2009; Idris Khodja *et al.*, 2012; Kane *et al.*, 2010; Lee *et al.*, 2013b; Lee *et al.*, 2011). Ang II is thought to contribute to endothelial dysfunction by inducing vascular oxidative stress subsequent to the up-regulation of the expression of NADPH oxidase (Harrison *et al.*, 2003; Sunggip *et al.*, 2013), which, in turn, promotes the uncoupling of eNOS (Lee *et al.*, 2013a), the alteration of calcium-dependent K channels involved in EDH (Behringer *et al.*, 2013), and an increased expression of COXs involved in endothelium-dependent contractile responses (Feletou *et al.*, 2011; Ohnaka *et al.*, 2000).

Current antihypertensive treatments effectively reduce blood pressure but seem to have limited ancillary effect to improve the endothelial dysfunction and hence to protect the cardiovascular system (Ghiadoni *et al.*, 2012). Indeed, some antihypertensive drugs can improve endothelial dysfunction, particularly calcium channel antagonists in the microcirculation, angiotensin-converting enzyme inhibitors and angiotensin type 1 receptor antagonists mostly in conduit arteries (Ghiadoni *et al.*, 2012; Li *et al.*, 2014). Several epidemiological and both primary and secondary prevention studies have indicated that dietary intake of omega-3 polyunsaturated fatty acids (PUFAs), including the two major compounds eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), reduces the risk of cardiovascular diseases (Delgado-Lista *et al.*, 2012; DiNicolantonio *et al.*, 2014). Moreover, dietary supplementation with omega-3 PUFAs-rich fish or fish oil reduced significantly blood pressure in several clinical studies with hypertensive patients (Appel *et al.*, 1993; Knapp *et al.*, 1989; Miller *et al.*, 2014), and improved endothelium-dependent vasodilatation in patients with coronary artery diseases (Tagawa *et al.*,

1999), in heart transplanted patients (Fleischhauer *et al.*, 1993) and in hypertensive type 2 diabetic patients (Woodman *et al.*, 2003). We as well as several other groups have shown that purified formulations of EPA and DHA are potent and sustained inducers of endothelium-dependent relaxations of isolated artery rings via an increased formation of NO and EDH (Omura *et al.*, 2001; Stebbins *et al.*, 2008; Zgheel *et al.*, 2014). The endothelium-dependent vasorelaxant effect of omega-3 PUFAs is dependent on both the purity and ratio of EPA:DHA, with EPA:DHA ratio of 6:1 and 9:1 being superior formulations (Zgheel *et al.*, 2014). Moreover, the characterization of the signal transduction pathway has indicated that the redox-sensitive Src/PI3-kinase/Akt pathway leads to eNOS activation by phosphorylation of the activator site Ser 1177 (Zgheel *et al.*, 2014).

Therefore, the aim of the present study was to determine whether chronic intake of the superior EPA:DHA 6:1 formulation is able to prevent the Ang II-induced hypertension in rats, and that this effect is associated with an improved endothelial function and to characterize the role of the NO, EDH and EDCFs pathways.

Materials and Methods

Ethics statement

This study conforms to the Guide of care and the use of laboratory animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996). The present protocol was approved by the local Ethics Committee (Comité Régional d’Ethique en Matière d’Expérimentation Animale de Strasbourg, approval AL/01/09/09/05).

Preparation of Omega-3 PUFAs products

Purified EPA and DHA were provided by Pivotal Therapeutics, Inc (Woodbridge, ON, Canada). The EPA:DHA 6:1 ratio mixture was prepared and adjusted to the relative purity of both the EPA and the DHA solutions under nitrogen flux to avoid oxidation of the omega-3 PUFAs. The formulation is then aliquoted in amber glass vials under nitrogen, and stored at 4 °C until use.

In vivo treatment of rats

Male Wistar rats (10 weeks old) were randomly divided into four groups of eight rats each. Rats received daily by gavage 500 mg/kg/day of either EPA:DHA 6:1 or corn oil (control) for 5 weeks. After 1 week, rats underwent sham surgery (sham rats) or surgery with implantation of an osmotic mini-pump infusing Ang II (0.4 mg/kg/day) for 4 weeks as described previously (Dal-Ros *et al.*, 2009). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Centravet, Velaine-en-Haye, France). A 1 cm incision was made in the midscapular region, and an osmotic mini-pump (Alzet 2004, Charles River Laboratories, Saint-Germain-sur-l’Arbresle, France) was implanted. Pumps contained Ang II (Enzo Life Sciences, Villeurbanne, France), which was dissolved in saline solution to obtain an infusion rate of 0.4 mg/kg/day. Sham-operated rats underwent an identical surgical procedure without pump implantation. After surgery, rats were housed in a thermo-neutral environment, on a 12:12 h photoperiod and were provided food and drinking water *ad libitum*.

Determination of the plasma level of lipids

Analyses of total blood fatty acids levels, and calculation of Omega Score (EPA + DHA + Docosapentaenoic acid), AA/EPA ratio, and n-6/n-3 ratio were performed at a central laboratory of the University Health Network (Specialty Laboratory, Toronto, Ontario, Canada), accredited by the College of American Pathologists' Laboratory Accreditation Program. The fatty acid compositions of whole blood were determined on 200 μ l of sample after lipid extraction by a modification of the method of Bligh and Dyer (Bligh *et al.*, 1959). The total lipid fraction was then methylated with 12 % (w/w) boron trifluoride in methanol by incubation at 90 °C for 25 min to produce fatty acid methyl-esters (FAME). After cooling, the FAME were extracted with hexane, washed with water, dried under nitrogen and dissolved in hexane. The fatty acid composition was then determined by GLC performed on a 100 m Varian Select™ FAME CP7420 capillary column (0.25 mm i.d.), using an Agilent Technologies 6890 N series gas chromatograph equipped with a split/splitless mode injector, and a flame ionization detector. The injector and detector were maintained at 280 and 300 °C, respectively, and samples were analyzed by multilevel temperature programming in the range of 90–265 °C with ultra-high purity grade helium as the carrier gas. The percent composition of fatty acids was calculated from the individual peak areas using appropriate standards. The procedure was routinely validated by proficiency testing using GC/MS.

Blood pressure measurements

Systolic blood pressure was measured in conscious rats twice a week for a total of four weeks, by using a tail-cuff sphygmomanometer connected to a computerized system (BP-2000 Blood pressure analysis system, Visitech Systems, Inc., Apex, NC, USA).

Vascular reactivity studies

Vascular reactivity studies of secondary branch mesenteric artery rings were performed using a DMT wire myograph (Danish Myo Technology A/S, Aarhus, Denmark). Briefly, secondary branch mesenteric arteries of rats were cleaned of connective tissue, cut into rings (2–3 mm in length), mounted onto two stainless steel wires and suspended in organ baths containing oxygenated Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.18, CaCl₂ 1.25, NaHCO₃ 25 and D-glucose 11, pH 7.4, 37 °C) for the determination of changes in isometric tension. After the equilibration period, rings were

exposure to Krebs bicarbonate solution containing high concentration of potassium (80 mM) until reproducible contractile responses were obtained. Thereafter, rings were contracted with phenylephrine (PE, 1 μ M) to approximately 80% of the maximal contraction by the high potassium solution before addition of acetylcholine (Ach, 1 μ M) to test the endothelial function. After washout and a further 30-min equilibration period, rings were again contracted with PE before the construction of a concentration-relaxation curve to either Ach, levcromakalim (Lev, an ATP-sensitive K⁺ channel opener) or sodium nitroprusside (SNP, a NO donor). In some experiments, rings were exposed to an inhibitor for 30 min before being contracted with PE. Relaxations were expressed as the percentage of the reversal of the contraction to PE. For the determination of endothelium-dependent contractile responses, rings with endothelium were exposed to inhibitors of EDH- and NO-mediated relaxations (0.1 μ M of each charybdotoxin and apamin and 300 μ M of N^o-nitro-L-arginine, respectively) for 30 min before the construction of a concentration-contraction curve to Ach.

Immunofluorescence studies

Frozen secondary branch mesenteric arteries embedded in Tissue-Tek OCT (Sakura 4583, Leiden, Netherlands) were cryosectioned at 14 μ m. Sections were air dried for 15 min and stored at -80 °C until use. Sections were first fixed with paraformaldehyde at 4% (Electron microscopy sciences, Hatfield, PA, USA), washed and treated with either 10% milk or 5% goat serum in PBS containing 0.1% Triton X-100 for 1 h at room temperature to block non-specific binding. Mesenteric artery sections were then incubated overnight at 4 °C with an antibody directed against either eNOS (1/50, cat: 610297, BD Transduction Laboratories, Le Pont de Claix, France), arginase-1 (1/100, cat: 610708, BD Transduction Laboratories), small and intermediate conductance calcium-activated potassium channels (SK_{Ca}, 1/200, cat: APC-025; IK_{Ca}, 1/200, cat: APC064, Alomone Labs, Jerusalem, Israel), connexin 37 (Cx37, 1/100 to 1/200, cat: CX37B12-A, Alpha Diagnostic International, San Antonio, TX, USA), angiotensin II type 1 receptor (AT1R, 1/ 400, sc-1177, Santa Cruz Biotechnology, Clinisciences, Nanterre, France), AT2R (1/50, sc-9040, Santa Cruz Biotechnology), p47^{phox} (1/200, sc-14015, Santa Cruz Biotechnology), p22^{phox} (1/200, sc-20781, Santa Cruz Biotechnology), cyclooxygenase-1 or 2 (COX-1, 1/250, ab109025, Abcam, Paris, France ; COX-2, 1/200, sc-1745, Santa Cruz Biotechnology). For negative controls, the primary antibody was omitted. Sections were then

washed with PBS, incubated with the fluorescent secondary antibody (1/400, Alexa 633-conjugated goat anti-rabbit or anti-mouse IgG, A-21070 and A-21050, Thermo Fisher, Illkirch, France) for 2 h at room temperature in the dark before being washed with PBS and mounted in Dako fluorescence mounting medium (Dako S3023, Les Ulis, France) and cover-slipped before being evaluated by confocal microscopy using a confocal laser-scanning microscope (Leica TSC SPE-Mannheim, Germany). Quantification of fluorescence levels was performed using Image J software (version 1.49p for Windows, US National Institutes of Health, Bethesda, MD, USA).

Determination of the *in situ* vascular level of oxidative stress

The redox-sensitive fluorescent dye dihydroethidium (DHE, 2.5 μ M) was applied to 25 μ m unfixed cryosections of secondary branch mesenteric artery for 30 min at 37 °C in a light-protected humidified chamber. The sections are then mounted in Dako fluorescence mounting medium and cover-slipped before being evaluated by confocal microscopy using a confocal laser-scanning microscope (Leica TSC SPE-Mannheim, Germany). Quantification of fluorescence levels was performed using Image J software (version 1.49p for Windows, US National Institutes of Health, Bethesda, MD, USA).

Western blot analysis

Proteins were extracted from secondary branch mesenteric artery segments with RIPA lysis and extraction buffer containing phosphatase and protease inhibitors (composition in mM: Tris/HCl 50 (pH 7.5, Euromedex, Souffelweyersheim, France), NaCl 150, Na_3VO_4 2, sodium pyrophosphate 5, NaF 10, a tablet of protease inhibitor (Complete[®], Roche, Meylan, France), N-ethylmaleimide 20, and 0.1 % SDS, 0.5 % sodium deoxycholate and 1% Triton X-100). Total proteins (20 μ g) were separated on 10% SDS-polyacrylamide (Euromedex) gels, using prestained markers (Euromedex) for molecular mass determinations. Separated proteins were transferred onto nitrocellulose membranes (Amersham, GE Healthcare, Velizy-Villacoublay, Franc) at 100 V for 120 min. Membranes were blocked with 5% bovine serum albumin on Tris-buffered saline solution (Euromedex) and 0.1% Tween 20 (Euromedex) (TBS-t) for 1 h. Membranes were incubated with the respective primary antibody (COX-2, p22^{phox}, and eNOS; dilution of 1:1000) overnight at 4 °C. After washing, membranes were incubated with their corresponding peroxidase-labeled secondary antibody (Cell Signaling Technology) at room

temperature for 60 min. Immunoreactive bands were detected by enhanced chemiluminescence substrate solution (Bio-Rad, Marnes-la-Coquette, France). Chemiluminescence signal was recorded using an ImageQuant™ LAS4000 system (GE healthcare) and analyzed using ImageQuant™ TL software (version 8.1, GE healthcare). Membranes were stripped subsequently and reprobed with a mouse polyclonal anti-glyceraldehyde-3-phosphate deshydrogenase (GAPDH) antibody (Abcam, Paris, France) for normalization purposes.

Statistical analysis

Values are expressed as means $\pm$ SEM. Statistical analysis was performed using an analysis of variance (ANOVA) followed by the Bonferroni post-hoc test as appropriate using GraphPad Prism (version 5 for Microsoft windows, GraphPad software, Inc., San Diego, CA, USA). Values of $P < 0.05$ were considered to be statistically significant.

Results

Chronic oral intake of EPA:DHA 6:1 treatment increases plasma levels of omega-3 PUFAs

After 5 weeks of chronic feeding of rats with EPA:DHA 6:1, the plasma contained significantly higher levels of PUFAs including EPA, DHA and docosapentaenoic acid (DPA), resulting in an increased total omega-3 PUFAs content and omega score (Table 1). Moreover, the total omega-6 PUFAs proportion was significantly reduced in groups receiving EPA:DHA 6:1 (Table 1). Thus, the omega-6/omega-3 ratio was significantly reduced in rats receiving the EPA:DHA 6:1 formulation compared to those receiving corn oil (Table 1).

EPA:DHA 6:1 prevents the Ang II-induced hypertension

Chronic administration of 0.4 mg/kg/day of Ang II to rats caused an increase in systolic blood pressure, which was significant after 3 days and remained elevated throughout the experiment (190.6 ± 8.5 and 120.7 ± 4.2 mmHg for the Ang II and control groups at week 4, respectively; Figure 1). Intake of EPA:DHA 6:1 (500 mg/kg/day) significantly prevented the Ang II-induced hypertension, whereas it was without effect in normotensive rats (149.0 ± 7.8 and 115.6 ± 2.8 mmHg at week 4 for the Ang II + EPA:DHA 6:1 group and the EPA:DHA 6:1 group, respectively; Figure 1).

EPA:DHA 6:1 treatment prevents the Ang II-induced endothelial dysfunction

To determine the effect of the antihypertensive EPA:DHA 6:1 treatment on the endothelial function, vascular reactivity studies were performed on secondary branch mesenteric artery rings. In rings precontracted by PE (1 μ M), Ach induced concentration-dependent relaxations, which were significantly reduced at 0.1 to 1 μ M in the Ang II-treated group (Figure 2A). To study NO-mediated relaxations, rings were incubated in the presence of indomethacin (an inhibitor of COXs) and TRAM34 plus apamin (inhibitors of IK_{Ca} and SK_{Ca} , respectively) to prevent the formation of vasoactive prostanoids and EDH-mediated relaxation, respectively. The EDH-mediated relaxation was studied in rings incubated with indomethacin and N^G -nitro-L-arginine (L-NA, an eNOS inhibitor) to prevent the formation of vasoactive prostanoids and NO, respectively. Compared to the control group, the NO-mediated relaxation was slightly but significantly reduced in the Ang II group (Figure 2B), whereas the EDH-mediated relaxation was

markedly reduced (Figure 2C). The EPA:DHA 6:1 treatment prevented the Ang II-induced endothelial dysfunction as indicated by NO- and EDH-mediated relaxations similar to those of the control group (Figures 2 B and C). Moreover, the incubation of rings with VAS-2870, a selective NADPH oxidase inhibitor, prevented the Ang II-induced blunted NO and EDH components, indicating a key determinant role of NADPH oxidase-derived oxidative stress (Figures 2 D and E).

To study the potential role of endothelium-dependent contractile responses in the Ang II-induced endothelial dysfunction, rings were subjected to increasing concentrations of Ach in the presence of L-NA, apamine and TRAM34 to prevent the formation of NO and EDH, respectively. Ach induced small concentration-dependent contractile responses in the rings with endothelium from the control group, which were significantly increased in the Ang II group (Figure 3A).

EPA:DHA 6:1 treatment prevented the Ang II-induced increase in endothelium-dependent contractile responses but was without effect in the normotensive group (Figure 3A). The Ang II-induced increased contractile response to Ach was abolished in the presence of indomethacin (Figure 3B), indicating the involvement of the COXs-derived vasocontractile prostanoids.

Neither the Ang II treatment nor the EPA:DHA 6:1 treatment significantly affected the function of the vascular smooth muscle as indicated by similar concentration-dependent contractile responses to PE, and concentration-dependent relaxations to SNP, a NO donor, in arterial rings without endothelium (Figure 4).

EPA:DHA 6:1 treatment prevents the Ang II-induced vascular oxidative stress and expression of target proteins involved in NO, EDH and EDCFs responses

To characterize the mechanisms underlying the Ang II-induced endothelial dysfunction and the protective effect of the EPA:DHA 6:1 treatment, the expression level of several proteins involved in NO, EDH and EDCFs responses were determined by immunofluorescence in sections of secondary branch mesenteric arteries. The Ang II treatment was associated with an increased expression level of eNOS, indicating most likely a compensatory mechanism, and of arginase 1, an enzyme that can limit the bioavailability of the eNOS substrate arginine thereby promoting eNOS uncoupling (Figure 5). The Ang II treatment was also associated with a reduced expression of connexin 37 (Cx37) and SK_{Ca}, and an increased expression of both COX-1 and COX-2 (Figure 5). The EPA:DHA 6:1 treatment prevent the Ang II-induced alterations of

the target proteins involved in the NO, EDH and EDCFs pathways (Figure 5). Since Ang II-induced hypertension and endothelial dysfunction are mediated by an increased vascular level of oxidative stress subsequent to the AT1R-mediated activation and expression of NADPH oxidase (Harrison *et al.*, 2003), the vascular level of oxidative stress and the expression of NADPH oxidase subunits (p22^{phox} and p47^{phox}) and Ang II receptors were determined by fluorescent histochemistry and immunofluorescence in secondary branch of mesenteric arteries. The Ang II treatment was associated with an increased vascular level of oxidative stress associated with an increased expression of NADPH oxidase subunits p22^{phox} and p47^{phox} and of both AT1R and AT2R; all these effects were prevented by the EPA:DHA 6:1 treatment (Figure 6).

In order to confirm the immunofluorescence signals and due to the limited amount of tissue, Western blot analysis was performed to determine the expression level of eNOS, COX-2 and p22^{phox} in the secondary branch of the mesenteric artery. The Ang II treatment increased the expression level of eNOS, COX-2 and p22^{phox} whereas no such effect was observed in the Ang II + EPA:DHA 6:1 group and in the EPA:DHA 6:1 group (Figure 7).

Discussion

The major findings of the present study indicate that the chronic oral intake of EPA:DHA 6:1, a superior omega-3 PUFAs formulation, prevents the hypertension and endothelial dysfunction induced by Ang II infusion in rats. The Ang II-induced endothelial dysfunction in the resistance secondary branch of the mesenteric artery ($< 100 \mu\text{m}$ in diameter) involves mainly a blunted EDH component and, to a lesser extent, a NO component, and also an increased endothelium-dependent contractile response. The EPA:DHA 6:1 treatment preserved both NO- and EDH-mediated relaxations and also prevented endothelium-dependent contractile responses. The beneficial effect of EPA:DHA 6:1 on the Ang II-induced endothelial dysfunction involves the blunting of the overexpression of eNOS and arginase 1 both involved in the NO component, of the decreased expression of Cx37 and SK_{Ca} involved in the EDH component, and of the increased expression of COX-1 and COX-2 involved in the endothelium-dependent contractile responses and is best explained by the prevention of the NADPH oxidase-derived oxidative stress and the overexpression of the vascular AT1R.

The chronic oral administration of EPA:DHA 6:1 to rats is associated with significantly increased plasma levels of omega-3 PUFAs including EPA, DHA and DPA, the intermediate elongated metabolite of EPA. In addition, the plasma level of omega-6 PUFAs is decreased by about 17 % by the EPA:DHA 6:1 treatment, especially through a decrease in the proportion of arachidonic acid, resulting in a significantly decreased omega-6/omega-3 ratio in the groups treated by EPA:DHA 6:1. The reduction of the omega-6/omega-3 ratio has been associated with beneficial health effects including reduced cardiovascular and cancer risk, whereas an increased ratio such as that induced by a Western diet has been associated with an increased prevalence of cardiovascular and chronic diseases (Simopoulos, 2002).

Consistent with previous studies, the continuous infusion of 0.4 mg/kg/day of Ang II in rats induced a rapid increase in systolic blood pressure that remained elevated throughout the study (Dal-Ros *et al.*, 2009; Kane *et al.*, 2010). The chronic intake of the EPA:DHA 6:1 partially but significantly prevented the Ang II-induced increased systolic blood pressure by about 60 %. A previous study by Ulu *et al.* indicated that intake of EPA and DHA (0.75 % each in the diet) in combination with an inhibitor of the soluble epoxide hydrolase significantly prevented the Ang II-induced hypertension in mice, and that this effect was associated with a reduced renal expression of the pro-inflammatory cytokine MCP-1 (Ulu *et al.*, 2013). Similarly, chronic intake

of DHA (1 or 5 % in the diet for 14 weeks) dose-dependently prevented the increase in systolic blood pressure and the hypertension-related vascular dementia in spontaneously hypertensive stroke-prone rats (Kimura *et al.*, 2002). Altogether, these observations are in good agreement with meta-analyses and clinical trials reporting that intake of omega-3 PUFAs is associated with reduced blood pressure in Humans, both in hypertensive patients and normotensive subjects (Appel *et al.*, 1993; Knapp *et al.*, 1989; Miller *et al.*, 2014). Indeed, the meta-analysis by Appel *et al.* reported that chronic intake of more than 3 g/day of omega-3 PUFAs for less than 3 months reduced systolic blood pressure by 1.5 and 5.5 mmHg in normotensive subjects and untreated hypertensive patients, respectively (Appel *et al.*, 1993). Similarly, the randomized clinical trial by Knapp *et al.* indicated that chronic intake of 15 g/day of omega-3 PUFAs for 4 weeks reduced systolic blood pressure by 6.5 mmHg in mildly hypertensive patients (Knapp *et al.*, 1989). Moreover, Miller *et al.* reported in a meta-analysis that an intake of EPA+DHA superior to 2 g/day reduced systolic blood pressure by about 4.51 and 1.25 mmHg in hypertensive and normotensive subjects, respectively (Miller *et al.*, 2014). In the present study, EPA:DHA 6:1 was administered at the dose of 500 mg/kg/day to rats, which is equivalent to about 5.67 g/day for a 70 kg Human (Reagan-Shaw *et al.*, 2008). This dose is in line with clinical trials and meta-analyses reporting a beneficial effect of omega-3 PUFAs on the cardiovascular system with doses ranging from 0.18 up to 10 g/d of omega-3 products with various degrees of purity and ratios (Delgado-Lista *et al.*, 2012; Enns *et al.*, 2014).

The Ang II-induced hypertension and endothelial dysfunction in the aorta and main mesenteric artery is associated with an increased level of vascular oxidative stress, mainly through the AT1R-mediated activation and overexpression of NADPH oxidase, a major source of reactive oxygen species in the arterial wall (Dal-Ros *et al.*, 2012; Dal-Ros *et al.*, 2009; Harrison *et al.*, 2003; Rajagopalan *et al.*, 1996). A pivotal role of NADPH oxidase in the Ang II-induced endothelial dysfunction in the secondary branch of the mesenteric artery is also indicated by the fact that VAS-2870, a specific inhibitor of NADPH oxidase, restored both the NO- and EDH-mediated relaxations and by the increased vascular expression level of NADPH oxidase subunits and AT1R in the Ang II group. The increased formation of NADPH oxidase-derived superoxide anions will reduce the bioavailability of NO by chemical reaction leading to peroxynitrite, which, in turn, promotes the uncoupling of eNOS to further increase the level of oxidative stress (Förstermann, 2010). Such a concept is supported by the fact that inhibition of eNOS decreased

the formation of superoxide anions in the arterial wall of Ang II-treated hypertensive rats (Mollnau *et al.*, 2002). In addition, the increased expression level of arginase 1, an enzyme competing with eNOS for the substrate, in the second branch mesenteric artery of the Ang II group suggests that arginase 1 might also contribute to eNOS uncoupling (Kim *et al.*, 2009; Shatanawi *et al.*, 2015). Furthermore, the Ang II-induced increased expression level of eNOS protein in the second branch mesenteric artery might indicate the initiation of a compensatory mechanism to preserve to some extent the formation of NO. NADPH oxidase-derived oxidative stress accounts for the blunted EDH component most likely by impairing the electrical conduction and signaling of the redox-sensitive Ca^{2+} -activated K^+ channels SK_{Ca} and IK_{Ca} (Behringer *et al.*, 2013). Furthermore, it might also explain the Ang II-induced increased indomethacin-sensitive EDCFs response in the secondary branch of the mesentery artery possibly as a consequence of the upregulation of the expression of both COX-1 and COX-2 in the arterial wall, two redox-sensitive genes (Feletou *et al.*, 2011; Hu *et al.*, 2002; Ohnaka *et al.*, 2000). Besides contributing to the exaggerated vascular tone, COXs-derived prostanoids have also been shown to contribute to vascular remodeling in Ang II-treated rats (Virdis *et al.*, 2012), and to promote oxidative stress in SHR rats (Virdis *et al.*, 2010). COX-derived EDCFs have also been shown to contribute to endothelial dysfunction in hypertensive Humans since indomethacin improved the blunted endothelium-dependent vasodilatation to Ach in the forearm of hypertensive patients but of normotensive subjects (Taddei *et al.*, 1993).

The present findings suggest that the beneficial effect of EPA:DHA 6:1 on the Ang II-induced endothelial dysfunction is best explained by their antioxidant properties. Indeed, the EPA:DHA 6:1 treatment prevented the Ang II-induced increased vascular oxidative stress and overexpression of the pro-oxidant enzymes NADPH oxidase and COXs thereby restoring both the NO and EDH components and blunting EDCFs responses. In addition to their ability to prevent the vascular expression of NADPH oxidase (Gortan Cappellari *et al.*, 2013), omega-3 PUFAs have also been reported to directly inhibit NADPH oxidase (Morre *et al.*, 2010) and to scavenge ROS (Richard *et al.*, 2008). Previous studies have also shown that antioxidant treatments such as polyphenolic compounds improved the endothelial function in patients with cardiovascular diseases including hypertensive patients (for review, see Schini-Kerth *et al.*, 2010). Moreover, vitamin C improved the endothelium-dependent vasodilatation in hypertensive patients whereas it was without effect in normotensive subjects (Taddei *et al.*, 2001).

Besides limiting oxidative stress, the beneficial effect of EPA:DHA 6:1 might also involve the improved plasma omega-6/omega-3 ratio. Indeed, the reduction of the omega-6/omega-3 ratio favours the production of omega-3-derived anti-inflammatory metabolites such as resolvins (Calder, 2010; Hong *et al.*, 2003; Seki *et al.*, 2009). Such metabolites may contribute to improve vascular tone since resolving D1 prevented the hyperreactivity induced by endothelin-1 and pro-inflammatory cytokines (TNF α and IL-6) in human pulmonary artery rings (Hiram *et al.*, 2014). Omega-3 PUFAs have also been shown to reduce the expression of the pro-inflammatory COX-2 in vascular smooth muscle cells whereas an increased expression was observed in response to arachidonic acid, an omega-6 PUFA (Bousserouel *et al.*, 2003). A reduced pro-inflammatory responses of EPA:DHA 6:1 is likely to contribute to its antihypertensive and vasoprotective effect since Ang II-induced T cell activation and the subsequent vascular inflammatory response have been identified as key mechanisms in Ang II-induced hypertension and endothelial dysfunction (Marvar *et al.*, 2010; Trott *et al.*, 2014).

In conclusion, the present findings indicate that chronic intake of EPA:DHA 6:1 is able to blunt the Ang II-induced hypertension associated with an improved endothelial dysfunction. The beneficial effect of the EPA:DHA 6:1 treatment is characterized by sustained NO and EDH components and the prevention of EDCFs responses most likely as a consequence of the improved vascular level of oxidative stress by targeting NADPH oxidase and COXs.

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Conflict of interest

The authors report no conflict of interest.

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Table 1: Fatty acids levels in plasma (expressed in percent of total fatty acids).

Fatty Acid Common Name	Formula	Control	Ang II	EPA:DHA 6:1	Ang II + EPA:DHA 6:1
<i>Saturated Fatty Acids</i>					
Lauric acid	12:0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Myristic acid	14:0	0.39±0.03	0.33±0.08	0.49±0.16	0.49±0.07
Pentadecanoic acid	15:0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Palmitic acid	16:0	26.96±0.56	26.38±0.71	28.02±1.46	25.22±0.60
Heptadecanoic acid	17:0	0.04±0.04	0.27±0.08	0.09±0.09	0.25±0.07
Stearic acid	18:0	16.55±0.52	17.24±1.07	15.33±1.04	17.70±0.46
Arachidic acid	20:0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Behenic acid	22:0	0.00±0.00	0.05±0.05	0.00±0.00	0.00±0.00
Lignoceric acid	24:0	0.00±0.00	0.00±0.00	0.00±0.00	0.06±0.06
<i>Monounsaturated Fatty Acids</i>					
Myristoleic acid	14:1	0.11±0.05	0.14±0.06	0.00±0.00	0.03±0.03
Myristoleic acid	14:1T	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
10-Pentadecenoic acid	15:1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Palmitoleic acid	16:1w7	0.35±0.07	0.16±0.09	0.40±0.21	0.11±0.05
Palmitoleic acid	16:1w9	1.43±0.18	1.47±0.20	1.93±0.21	1.79±0.16
10-Heptadecenoic acid	17:1	0.04±0.04	0.11±0.09	0.00±0.00	0.03±0.03
Petrolaidic acid	18:1w5	0.00±0.00	0.09±0.09	0.00±0.00	0.00±0.00
Vaccenic acid	18:1w7	3.83±0.21	3.06±0.51	3.48±0.29	3.15±0.21
Oleic acid	18:1w9	6.18±0.46	6.26±0.49	6.63±0.51	6.62±0.24
11-Eicosenoic acid	20:1w7	0.06±0.04	0.18±0.07	0.15±0.10	0.26±0.10
5-Eicosenoic acid	20:1w9	0.08±0.06	0.00±0.00	0.00±0.00	0.00±0.00
8-Eicosanoic acid	21:1w9	0.09±0.07	0.21±0.07	0.00±0.00	0.00±0.00
Erucic acid	22:1w9	0.28±0.09	0.22±0.08	0.27±0.16	0.13±0.10
Nervonic acid	24:1w9	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
<i>Polyunsaturated Fatty Acids</i>					
<i>Omega 3</i>					
Alpha-linolenic acid	18:3w3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Steridonic acid	18:4w3	0.23±0.07	0.27±0.06	0.10±0.07	0.14±0.07
Eicosatrienoic acid	20:3w3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Eicosatetraenoic acid	20:4w3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Eicosapentaenoic acid (EPA)	20:5w3	0.28±0.15	0.38±0.17	3.34±0.54*	3.00±0.75* [#]
Docosatrienoic acid	22:3w3	0.20±0.07	0.32±0.08	0.00±0.00	0.00±0.00
Docosapentaenoic acid (DPA)	22:5w3	0.49±0.04	0.35±0.08	2.34±0.31*	1.86±0.16* [#]
Docosahexaenoic acid	22:6w3	3.56±0.28	3.95±0.16	5.20±0.35*	6.02±0.30* [#]

(DHA)					
Omega 6					
Linoleic acid	18:2w6	21.00±0.69	20.15±0.84	20.97±0.68	18.31±0.70
Gamma-linolenic acid	18:3w6	0.00±0.00	0.13±0.1	0.00±0.00	0.00±0.00
Eicosadienoic acid	20:2w6	0.00±0.00	0.00±0.00	0.00±0.00	0.07±0.07
Dihomogamma-linolenic acid	20:3w6	0.72±0.07	0.67±0.05	0.85±0.14	0.83±0.16
Arachidonic acid (AA)	20:4w6	16.66±1.19	16.93±0.82	10.12±1.25*	13.64±1.24
Docosadienoic acid	22:2w6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Docosatetraenoic acid	22:4w6	0.39±0.08	0.42±0.10	0.30±0.12	0.29±0.11
Docosapentaenoic acid	22:5w6	0.11±0.05	0.26±0.10	0.00±0.00	0.00±0.00
Omega 9					
Mead's acid	20:3w9	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Total		100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
Omega Status					
Omega Score (EPA+DPA+DHA)		4.33±0.42	4.69±0.24	10.88±0.62*	10.88±0.68* [#]
Total Saturated fatty acids		43.94±0.83	44.26±1.21	43.93±1.01	43.71±0.71
Total Monounsaturated fatty acids		12.44±0.72	11.91±1.22	12.87±0.53	12.12±0.25
Total Polyunsaturated fatty acids		43.62±1.24	43.83±0.66	43.21±0.89	44.16±0.74
Total Omega-3 fatty acids		4.75±0.50	5.28±0.31	10.98±0.60*	11.02±0.72* [#]
Total Omega-6 fatty acids		38.87±0.90	38.56±0.54	32.23±1.02*	33.14±1.15* [#]
Omega-6/Omega-3 ratio		8.67±0.71	7.48±0.42	3.02±0.24*	3.11±0.27* [#]
AA/EPA ratio		45.10±11.04	41.48±11.29	4.38±1.44*	6.23±1.29* [#]

Ang II: Angiotensin II; AA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid. Results are expressed as percent of total plasma fatty acid level and shown as means ± SEM of 8 rats per group.* P < 0.05 vs Ctrl, [#] P < 0.05 vs Ang II.

Figure legend

Figure 1: Chronic intake of EPA:DHA 6:1 prevents the Ang II-induced hypertension in rats. Rats received daily by gavage 500 mg/kg/day of either EPA:DHA 6:1 or corn oil (control) for 1 week before administration of Ang II (0.4 mg/kg/day) using mini osmotic pumps for 3 weeks. Blood pressure was monitored by tail-cuff sphygmomanometry. Results are expressed as means $\pm$ SEM of 8 rats per group. * $P < 0.05$ vs. Control, # $P < 0.05$ vs. Ang II.

Figure 2: EPA:DHA 6:1 prevents the Ang II-induced endothelial dysfunction in the secondary branch of the mesenteric artery. (A-C) Rings with endothelium were contracted with 1 μ M PE before the addition of increasing concentrations of Ach. (B) NO-mediated relaxations were studied in presence of indomethacin (10 μ M) and charybdotoxin plus apamin (100 nM each) to prevent the formation of vasoactive prostanoids and EDH-mediated relaxations, respectively. (C) EDH-mediated relaxations were studied in presence of indomethacin and N^o-nitro-L-arginine (300 μ M) to prevent the formation of vasoactive prostanoids and NO, respectively. (D-E) To assess the role of oxidative stress in Ang II-induced endothelial dysfunction, NO- (D) and EDH-mediated (E) relaxations were performed in the presence of VAS-2870, a selective NADPH oxidase inhibitor (1 μ M). Results are expressed in % relaxations as means $\pm$ SEM of 8 rats per group. * $P < 0.05$ vs. Control, # $P < 0.05$ vs. Ang II.

Figure 3: EPA:DHA 6:1 prevents the Ang II-induced increased endothelium-dependent contractile responses in the secondary branch of the mesenteric artery. Rings with endothelium were subjected to increasing concentrations of Ach to construct a concentration-contractile responses curve in the absence (A) or the presence (B) of indomethacin (10 μ M) to prevent the formation of vasoactive prostanoids. Endothelium-dependent contractile responses

were studied in the presence of charybdotoxin plus apamin (100 nM each) and N^o-nitro-L-arginine (300 μ M) to prevent the formation of EDH and NO, respectively. Results are expressed in % relaxations as means $\pm$ SEM of 8 rats per group. * $P < 0.05$ vs. Control, [#] $P < 0.05$ vs. Ang II.

Figure 4: Neither the Ang II treatment nor the EPA:DHA 6:1 treatment affected the function of the vascular smooth muscle in the secondary branch of the mesenteric artery.

(A) Rings with endothelium were subjected to increasing concentrations of PE to construct a concentration-contractile responses curve. Results are expressed in mN/mm as means $\pm$ SEM of 8 rats per group. (B) Rings with endothelium were contracted with 1 μ M PE before the addition of increasing concentrations of SNP to construct a concentration-relaxation curve. Results are expressed in % relaxations as means $\pm$ SEM of 8 rats per group. All experiment were performed in the presence of charybdotoxin plus apamin (100 nM each), N^o-nitro-L-arginine (300 μ M) and indomethacin (10 μ M) to prevent the EDH- and NO-mediated relaxations and the formation of vasoactive prostanoids, respectively.

Figure 5: EPA:DHA 6:1 prevents the Ang II-induced vascular oxidative stress and the up-regulation of NADPH oxidase and Ang II receptors in the secondary branch of the mesenteric artery. Vascular oxidative stress and protein immunoreactive signals were determined in unfixed cryosections of the secondary branch of the mesenteric artery. The determination of vascular oxidative stress was done by fluorescence histochemistry using the redox-sensitive probe dihydroethidium, and the expression level of NADPH oxidase subunits p22^{phox} and p47^{phox} (adventitia), and AT1R and AT2R (media) by immunofluorescence and analysed by a confocal laser-scanning microscope (Leica SP2 UV DM IRBE). Quantification of

fluorescence levels was performed using FIJI GPL v2 software (<http://fiji.sc/Fiji>). Results are expressed as means $\pm$ SEM of 4-5 rats per group. * $P < 0.05$ vs. Control, # $P < 0.05$ vs. Ang II.

Figure 6: EPA:DHA 6:1 prevents the Ang II-induced up-regulation of eNOS, arginase 1, COX-1 and COX-2, and down-regulation of SK_{Ca} and Cx37 in the vascular wall of the secondary branch of the mesenteric artery. Protein expression levels were determined in unfixed cryosections of the secondary branch of the mesenteric artery. The determination of expression levels of target proteins was done by immunofluorescence. Immunofluorescence signals were observed using a confocal laser-scanning microscope. Results are expressed as means $\pm$ SEM of 4-5 rats per group. * $P < 0.05$ vs. Control, # $P < 0.05$ vs. Ang II.

Figure 7: EPA:DHA 6:1 prevents the Ang II-induced overexpression of eNOS, COX-2 and p22^{phox} in the secondary branch of the mesenteric artery. Protein expression levels of eNOS, COX-2 and the NADPH oxidase subunit p22^{phox} were determined in the secondary branch of the mesenteric artery by Western blot. Chemiluminescence signals were analysed using an ImageQuant™ LAS4000 system. Results are expressed as means $\pm$ SEM of 4-5 rats per group. * $P < 0.05$ vs. Control, # $P < 0.05$ vs. Ang II.

Figure 1

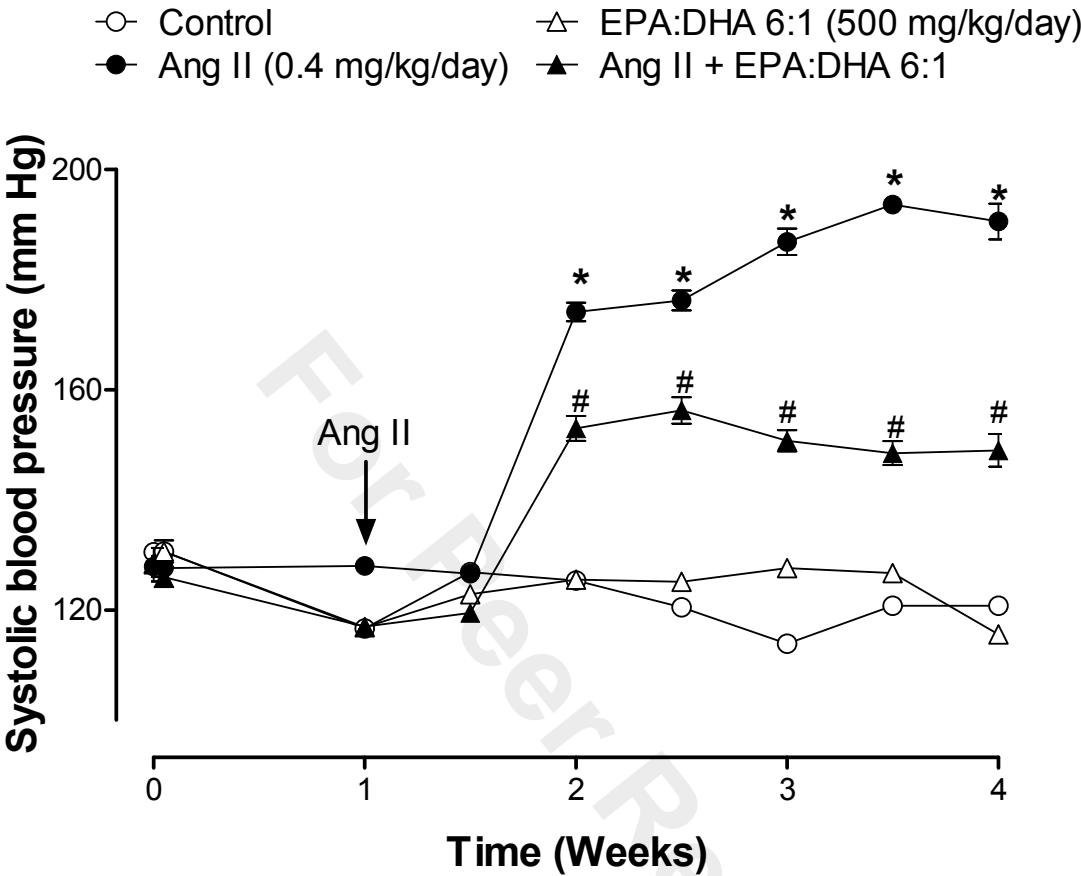


Figure 2

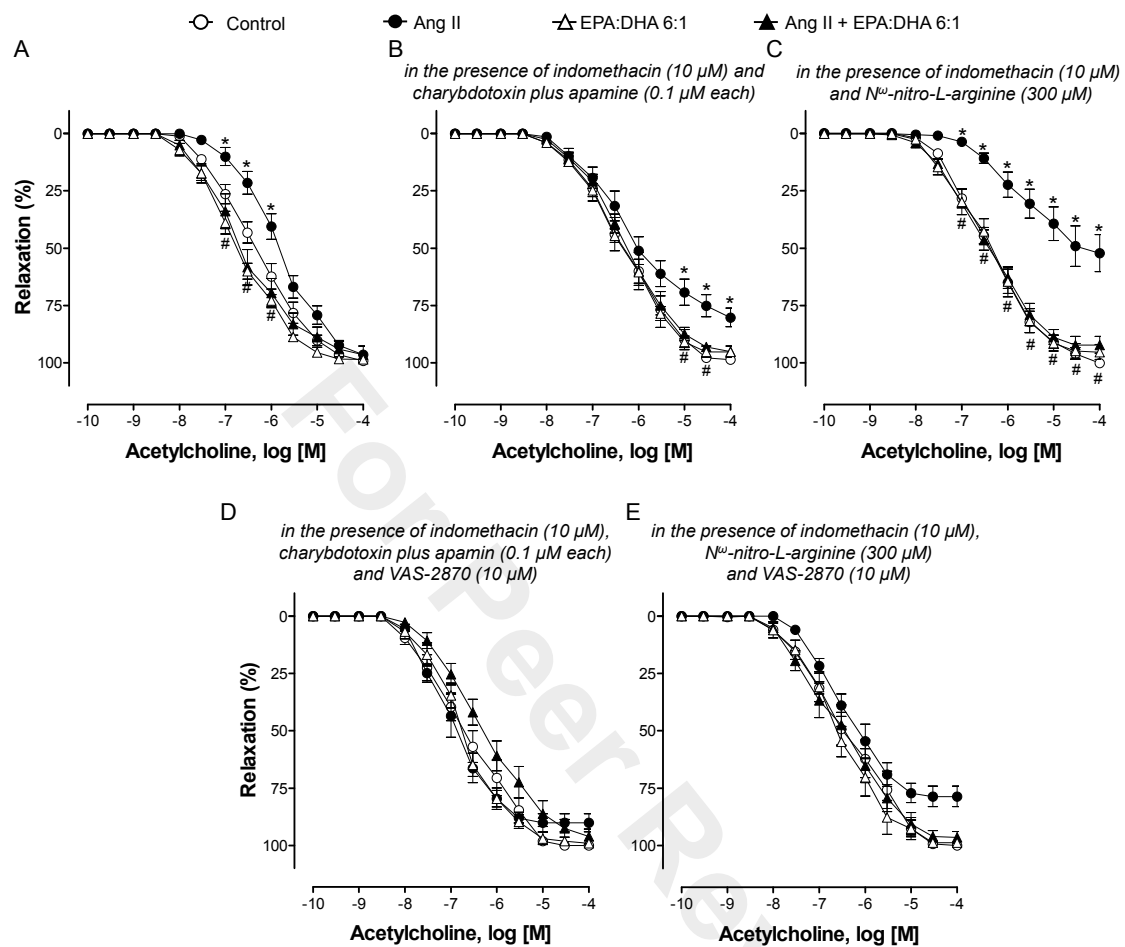


Figure 3

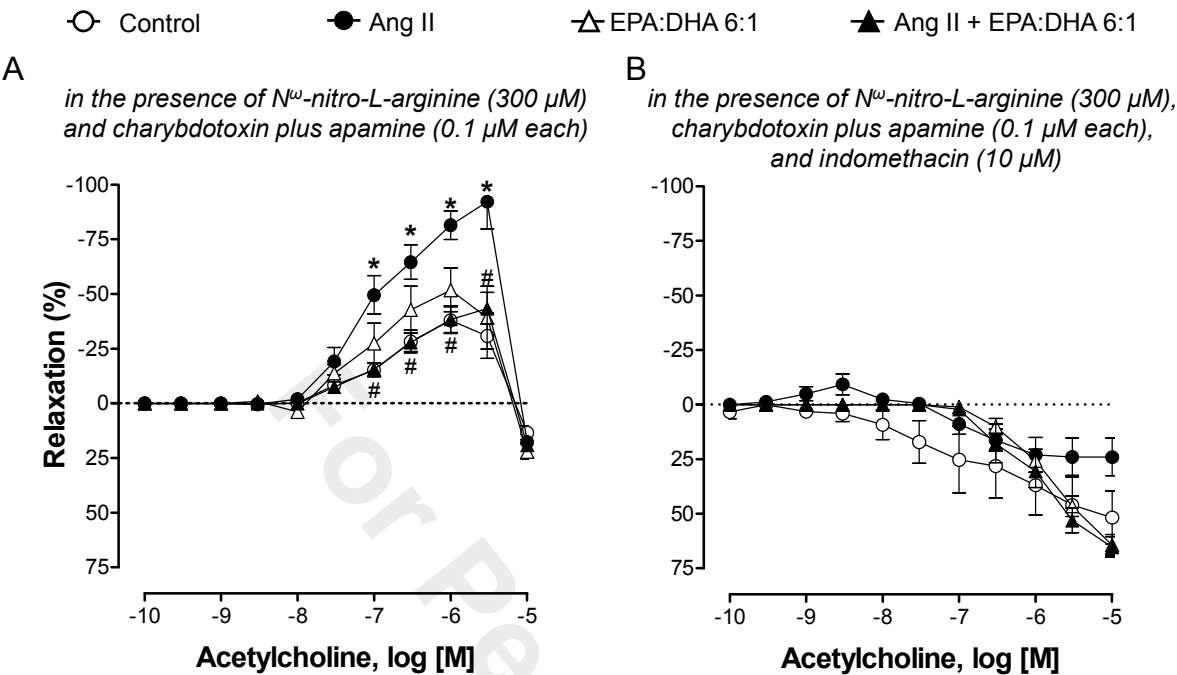


Figure 4

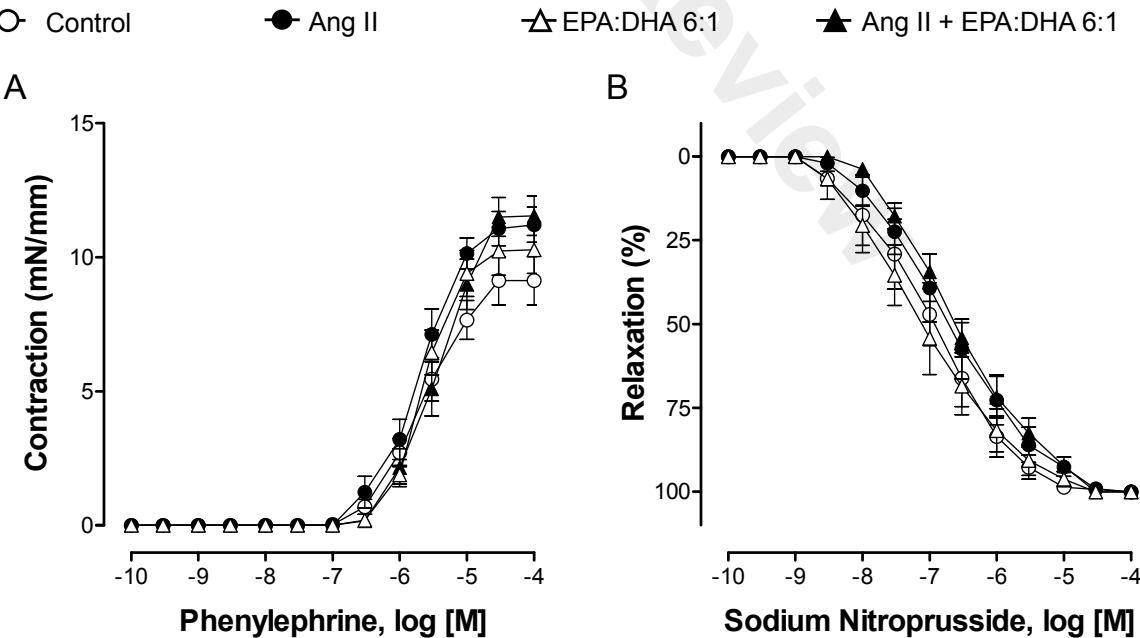


Figure 5

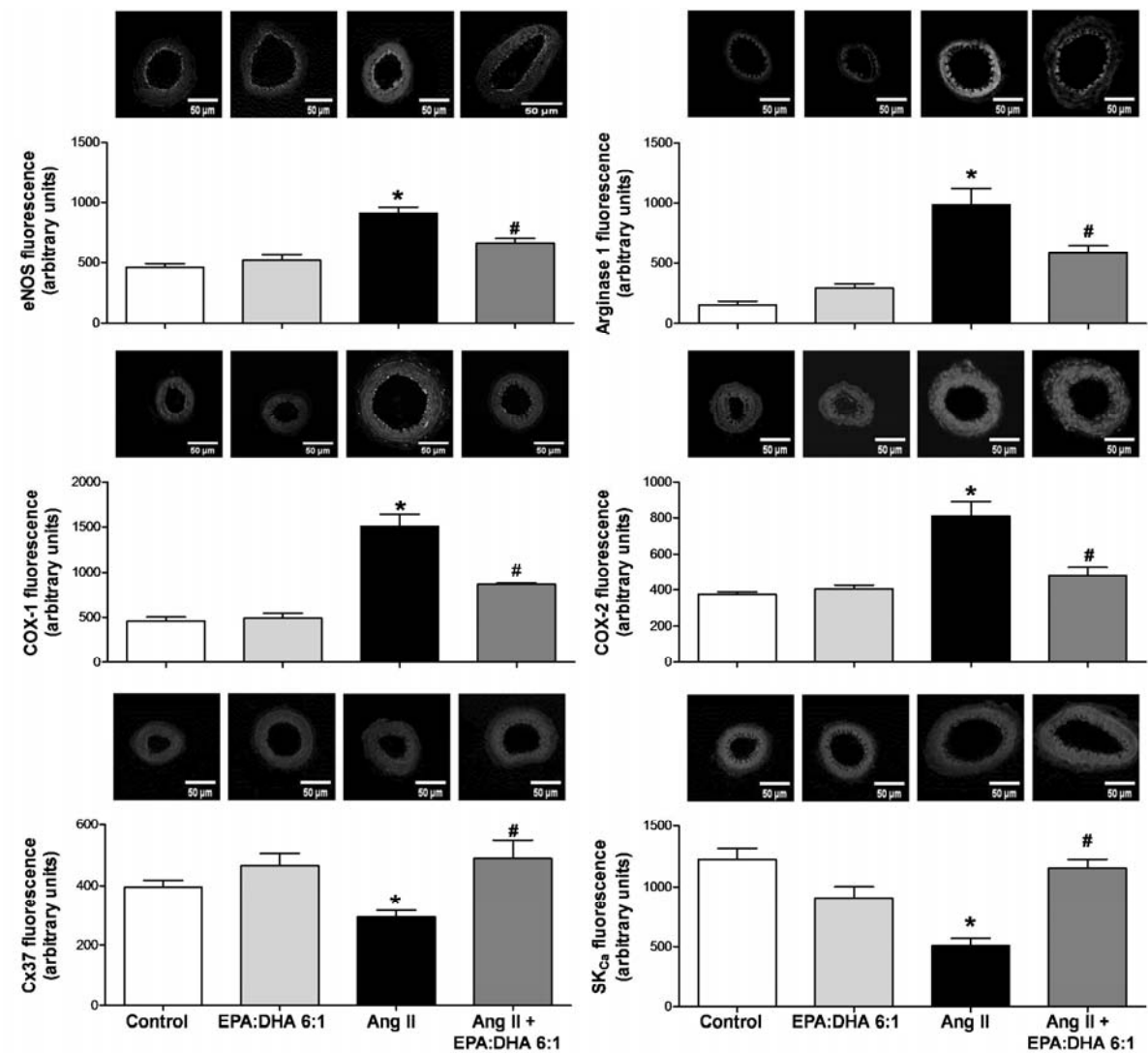


Figure 6

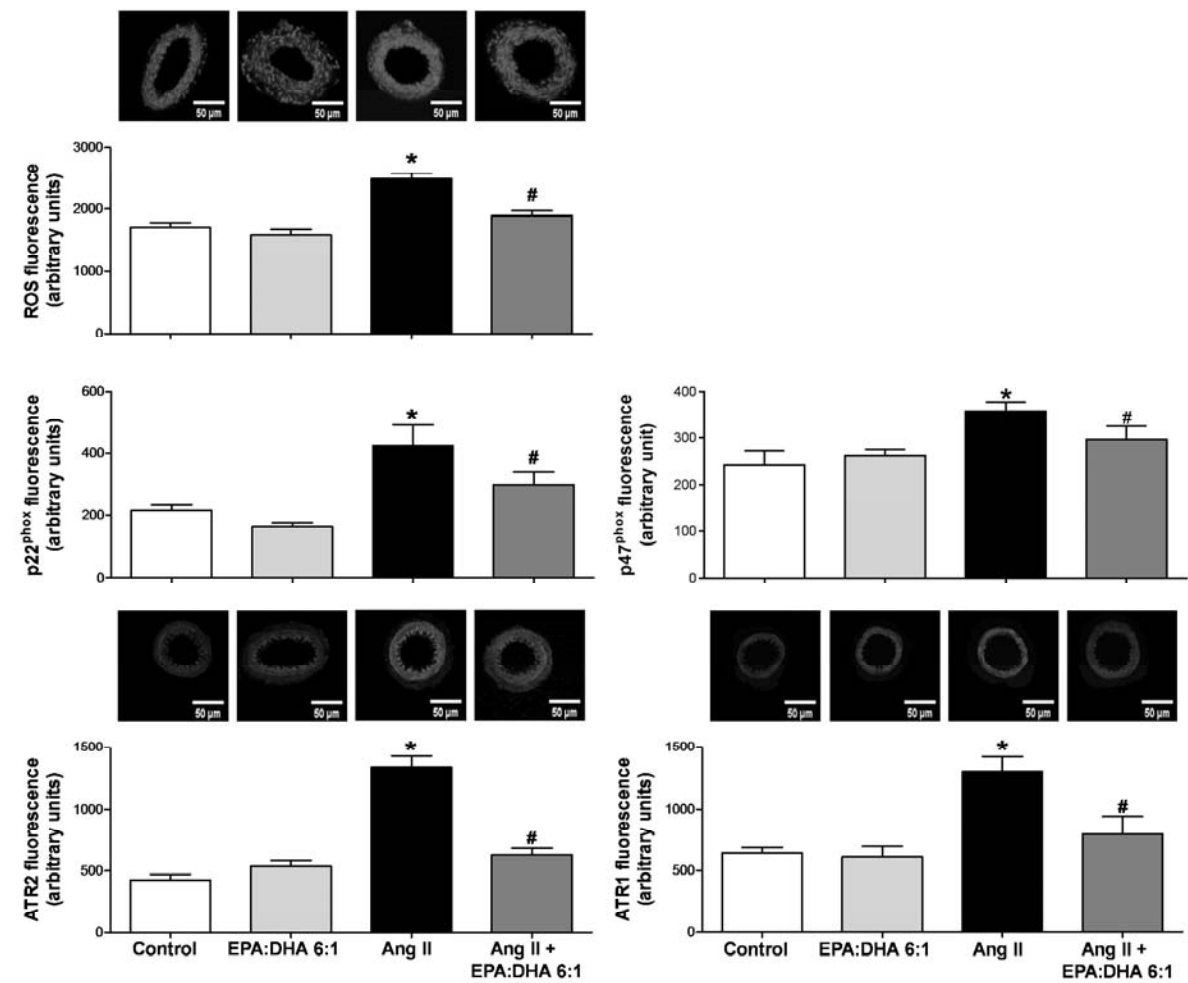
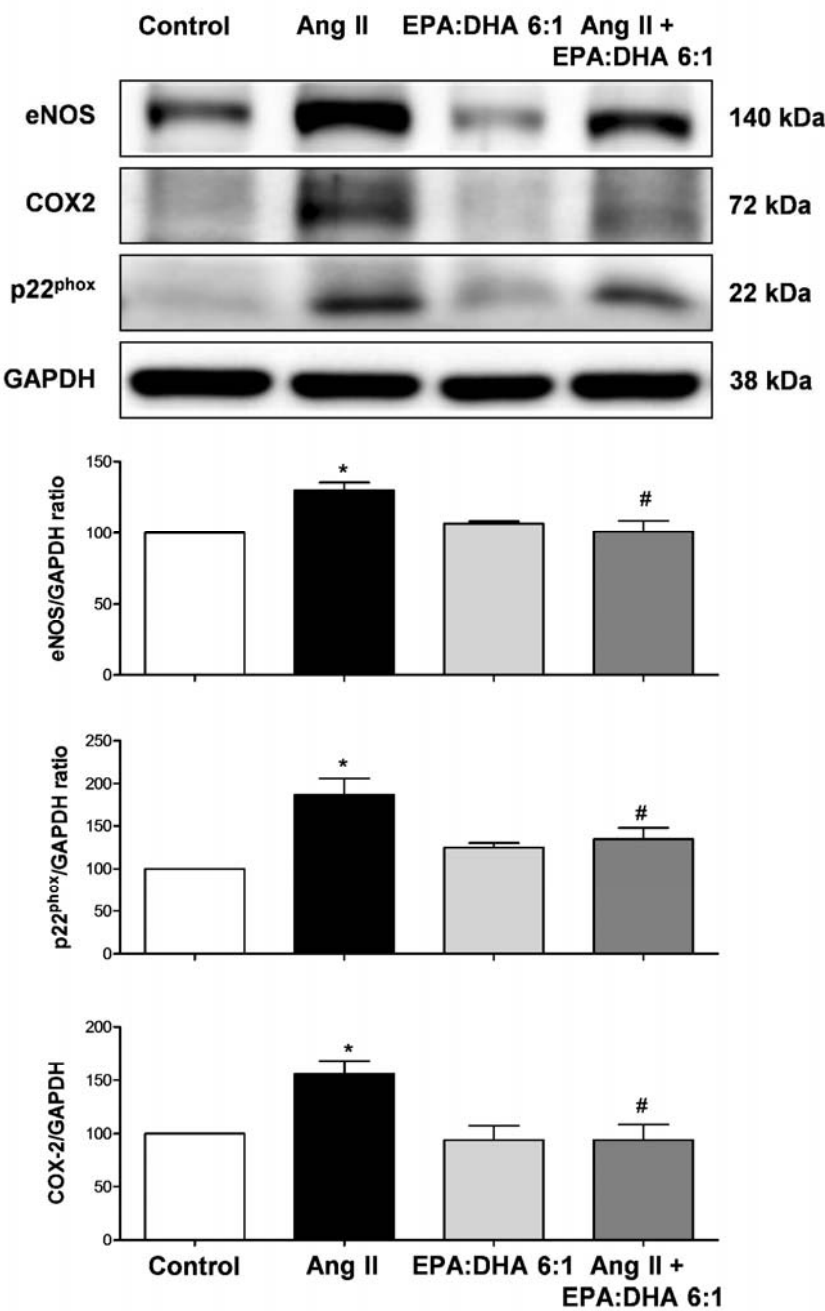


Figure 7



Results

The major results of our study show that infusion of Ang II caused a significant increase in systolic blood pressure, as compared to control rats. EPA:DHA 6:1 treatment significantly prevented the increase in systolic blood pressure by about 57%, while having no effect on the systolic blood pressure of normotensive rats. The chronic intake of the optimized EPA:DHA 6:1 formulation is associated with significantly increased plasma levels of omega-3 fatty acids, mainly as EPA, DHA and the intermediate elongated metabolite of EPA, the docosapentaenoic acid (DPA), resulting in a decreased omega-6/omega-3 ratio. The reduction of this ratio has been associated with a shift towards beneficial health effects of omega-3, including reduced cardiovascular and cancer risk. Vascular reactivity studies in the secondary branch of the mesenteric artery indicate that Ang II induced an endothelial dysfunction characterized by reduced relaxations in response to acetylcholine affecting both the NO- and EDH-mediated components, and increased formation of endothelium-derived contractile factors (EDCFs) in response to acetylcholine. The chronic intake of EPA:DHA 6:1 normalized both the NO, EDH and EDCF responses in the secondary branch of the mesenteric artery. To better characterize the molecular mechanisms involved in the protective effects of EPA:DHA 6:1 intake, we performed quantitative analysis of protein expression in the secondary branch of the mesenteric artery by immunofluorescence. The Ang II treatment increased the level of vascular oxidative stress and the expression of NADPH oxidase subunits p22^{phox} and p47^{phox}, AT1R, AT2R, eNOS, arginase-1, COX-1 and COX-2, and decreased the expression of SK_{Ca} and connexin 37, which were improved by the omega-3 treatment. To confirm the results obtained by immunofluorescence in the secondary branch of the mesenteric artery, we performed Western blot analysis of the expression levels of eNOS, COX-2, and the NADPH oxidase subunit p22^{phox} in the secondary branch mesenteric artery. The Ang II group presented a significantly increased expression of eNOS, COX-2, and the NADPH oxidase subunit p22^{phox}, that was prevented by the chronic oral intake of EPA:DHA 6:1.

Altogether, the present findings indicate that chronic intake of the optimized EPA:DHA 6:1 formulation prevented the development of hypertension and endothelial dysfunction induced by the infusion of Ang II in rats. The Ang II-induced endothelial dysfunction is associated to an up-regulation of the local angiotensin system and an increased vascular oxidative stress. The beneficial effect of EPA:DHA 6:1 is mediated by an improvement of both the NO- and the EDH-mediated

relaxations and a reduction of endothelium-dependent contractile response, most likely by preventing the oxidative stress induced by the up-regulation of the local angiotensin system.

Conclusions and perspective

In conclusion, the present work has assessed the potency of an optimized EPA:DHA 6:1 formulation to protect the endothelial function *in vivo*. The present findings shows that Ang II infusion is associated with the development of endothelial dysfunction in the secondary branch of the mesenteric arteries, which affects markedly the EDH-mediated component of relaxation and also, to some extent, the NO-mediated component of relaxation. The Ang II-induced endothelial dysfunction involves a redox-sensitive mechanism implicating NADPH oxidase, COX-1 and COX-2. The chronic oral intake of the optimized EPA:DHA 6:1 formulation prevents Ang II-induced endothelial dysfunction and hypertension most likely by decreasing oxidative stress-mediated impairment of both NO and EDH components as well as a reduction of endothelium-dependent contractile response.

There are numerous reports that support an important role of omega-3 fatty acids in preventing endothelial dysfunction, hypertension and cardiovascular diseases. The impregnation of omega-3 fatty acids in vascular tissues is also important, and needs to be clarified. However, the active molecules involved in the protective effects of omega-3 intake on endothelial function remains to be identified. Recent studies have reported that metabolites of omega-3 fatty acids, such as the resolvins, are key elements in the anti-inflammatory effect of omega-3. They also have been reported to exert beneficial vascular effect such as reducing the hyperreactivity induced by endothelin-1 and pro-inflammatory cytokines in pulmonary arteries. Since Ang II-induced T cell activation and the subsequent vascular inflammatory response have been identified as key mechanisms in Ang II-induced hypertension and endothelial dysfunction, a reduced pro-inflammatory responses due to production of anti-inflammatory omega-3 metabolites is likely to contribute to the EPA:DHA 6:1 antihypertensive and vasoprotective effect. Thus, further work is needed for the identification of active metabolites of omega-3 fatty acids in the vascular wall including resolvins, protectins, thromboxane A₃, leukotriene 5 and lipoxins.

The present study has assessed the potency of the EPA:DHA 6:1 formulation to prevent the Ang II-induced hypertension and endothelial dysfunction. However, the curative potential of the

optimized EPA:DHA 6:1 formulation on an established hypertension and associated endothelial dysfunction needs to be determined. Indeed, while the current antihypertensive treatments effectively reduce blood pressure, they seem to have limited ancillary effect on the endothelial dysfunction and hence to protect the cardiovascular system. Thus, the potency of the EPA:DHA 6:1 formulation to improve the endothelial dysfunction associated with an established hypertension could be an interesting novel therapeutic approach to reduce the cardiovascular mortality risk of hypertension.

Finally, on the basis of the beneficial vasoprotective effect of the EPA:DHA 6:1 formulation demonstrated in the present study and the reported beneficial effect of omega-3 intake in humans, a clinical study can be designed to determine the potential of EPA:DHA 6:1 to reduce cardiovascular risk factors in hypertensive patients.

GENERAL DISCUSSION AND PERSPECTIVES

Arterial hypertension

Arterial hypertension is a major problem of public health, being associated with end organ damage and cardiovascular morbidity and mortality. Arterial hypertension is the most important risk factor for a number of cardiovascular diseases including CAD, CHF, peripheral vascular disease, stroke and chronic kidney disease (Sarnak, Levey et al. 2003). Prevalence of arterial hypertension in United States is very high and continues to increase with a control rate of just 30 % in treated patients (Ong, Cheung et al. 2007). At the age of 75 years or older, about 70 % of men and women develop arterial hypertension (Chobanian, Bakris et al. 2003). Although effective antihypertensive drugs such as AT1R antagonists (ARAs), angiotensin-converting enzyme inhibitors (ACEI), beta-blockers, and calcium channel blockers, diuretics, and vasodilators exist, the control rate of hypertension remains low possibly due to poor observance and side effects. Treatments blocking pathological effects of the RAS at different levels have been shown to limit target-organ damage in hypertension and to decrease cardiovascular morbidity and mortality. AT1R and ACE inhibitors based treatments contributed to 60 % and 65 % of overall hypertensive therapy.

Omega-3 polyunsaturated fatty acids

Interest in natural products research is strong and can be attributed to several factors, including unmet therapeutic needs, the remarkable diversity of both chemical structures and biological activities of naturally occurring secondary metabolites. In this context, the adverse effects associated with synthetic molecules, decreased patients acceptability, contributed to shift the interest into natural products.

Numerous experimental and clinical studies have documented that omega-3 fatty acids can benefit the cardiovascular system, and particularly in patients diagnosed with CAD (Harris, Mozaffarian et al. 2009). The American Heart Association recommends the intake of 1 g/day of the two omega-3 fatty acids EPA and DHA for cardiovascular disease prevention, treatment after a myocardial infarction, prevention of sudden death, and secondary prevention of cardiovascular disease (Von Schacky and Harris 2007).

Omega-3 fatty acid lower plasma triglycerides mainly through reduced fatty acid availability for triglyceride synthesis due to decreased *de novo* lipogenesis, increased fatty acid beta-oxidation (Kusunoki, Kanatani et al. 2006), reduced delivery of nonesterified fatty acids to the liver, reduced

hepatic enzyme activity for triglyceride synthesis, and increased hepatic synthesis of phospholipids rather than triglycerides (Nakamura and Nara 2003). Omega-3 fatty acids have anti-inflammatory properties and an inverse association has been found between regular intake of fish oil or fish and C-reactive protein, interleukin-6, E-selectin, soluble intercellular adhesion molecule-1, tumor necrosis factor, IFN- α soluble receptor 1, and matrix metalloproteinase-3 (He 2009). Fish oils inhibit pro-inflammatory cytokines production and reduce expression of cell adhesion molecules (Calder 2006). These factors are critical in recruiting circulating leucocytes to the vascular endothelium, an important early event in the pathogenesis of atherosclerosis and inflammation.

Omega-3 fatty acids compete with omega-6 fatty acids for prostaglandin and leukotrienes synthesis at the cyclooxygenase and lipoxygenase level. Omega-3 fatty acids modulate prostaglandin metabolism by increasing synthesis of prostaglandin E₃, an active vasodilator and inhibitor of platelets aggregation, thromboxane A₃, leukotrienes B₅, and by decreasing production of thromboxane A₂, a potent inducer of platelet aggregation and vasoconstrictor, and leukotriene B₄ formation (an inducer of inflammation and a powerful inducer of leukocyte chemotaxis and adherence). Other studies also indicate that high intake of fish oil would produce a lower platelet count, less platelet aggregation, a longer bleeding time and lower concentrations of thromboxane metabolites. The omega-3 fatty acids-induced hypotriglyceridemia effect requires doses of DHA and EPA of 3 to 4 g/day. In American population such doses reduce triglyceride levels by 30 % to 40 % (Harris, Ginsberg et al. 1997; Lavie, Milani et al. 2009). However, data from both animal models and humans on cardiovascular protection are inconsistent. Consumption of 6 g/d EPA and DHA for 2 years by 59 patients had no major favorable effects on the diameter of atherosclerotic coronary arteries (Woodman 2003). Several studies showed that dietary intake of fish oil or non-fried fish is associated with a lower prevalence of subclinical atherosclerosis classified by significant changes in common carotid intima-media thickness and in percent stenosis while no modification in coronary artery calcium score, and ankle-brachial index were observed (Von Schacky, Angerer et al. 1999). DHA is the precursor to a newly described metabolite called 10,17S-docosatriene, which is part of a family of compounds called resolvins. These have firstly been described as being released in the brain in response to an ischemia and respond to the proinflammatory actions of infiltrating leukocytes by blocking interleukin-1-beta-induced NF- κ B activation and cyclooxygenase-2 expression (Layé 2010).

The previous studies of our research team have shown that EPA and DHA, the major omega-3 fatty acids, induced concentration-dependent relaxations in coronary artery rings (Zgheel, Alhosin et al. 2014). The relaxation in response to EPA was slightly but significantly greater than those to DHA at 0.4 % (v/v) (77.8 ± 10.3 and 64.7 ± 12.8 %, respectively). Then, the ability of optimized omega-3 ratios to induce endothelium-dependent relaxation was determined and the results showed that EPA:DHA at ratio of either 6:1 or 9:1 induced relaxations significantly more potent than ratio of 3:1, 1:1, 1:3, 1:6, and 1:9. Similarly, the role of the purity of the EPA:DHA ratio was determined. The endothelium-dependent relaxation in porcine coronary artery rings in response to a product with a high purity of omega-3 EPA:DHA (694:121 mg/g) was significantly greater than those in response to a product with a lower purity of omega-3 EPA:DHA (352:65 mg/g). Thus, the biological activity of omega-3 products is critically dependent on the consumption, ratio and purity.

Beneficial effects of omega-3 fatty acid on endothelial function

Dietary omega-3 fatty acids have a variety of anti-inflammatory and immune-modulating effects that may be of relevance to atherosclerosis and its clinical manifestations of myocardial infarction, sudden death, and stroke (Mori and Beilin 2004). The omega-3 fatty acids that appear to be most potent in this respect are the long-chain polyunsaturated derived from marine oils, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Riediger, Othman et al. 2009). Several studies have indicated an inverse correlation between the risk of cardiovascular diseases and the increased consumption of omega-3 fatty acids, such as eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), that are found mainly in fatty fish (e.g., salmon, trout, herring, sardines, and mackerel) (Lavie, Milani et al. 2009). The beneficial cardiovascular effects of dietary supplementation with omega-3 fatty acids include decreased arrhythmias, decreased triglycerides plasma concentrations, decreased blood pressure, and decreased platelet aggregation, all leading to reduced risk of cardiovascular mortality in patients with cardiovascular diseases (Harris, Miller et al. 2008). In addition, the protective effect of omega-3 fatty acids could also be explained by their ability to improve endothelium-dependent relaxation of the arteries by stimulating the formation of the endothelium vasoprotective factors NO and EDH, as well as reduction in endothelium-dependent contractile responses (Ribeiro, Oliveira et al. 2016).

Since EPA:DHA 6:1 and 9:1 are more efficient than 1:1 (which is the only available prescribed drug) for endothelium-dependent relaxations in porcine coronary artery rings (Zgheel, Alhosin et

al. 2014), the EPA:DHA 6:1 formulation was selected for the present study and given at the dose of 500 mg/kg/d to rats, which is equivalent to 5.67 g/d for a 70 kg Human (Reagan-Shaw, Nihal et al. 2008). This dose is in line with reported clinical trials and meta-analysis, where doses range from 0.18 up to 10 g/d of various omega-3 products (Delgado-Lista, Perez-Martinez et al. 2012; Enns, Yeganeh et al. 2014).

Ang II can be infused to mice or rats subcutaneously by Alzet osmotic mini pumps over a period of 4 weeks (Zimmerman, Lazartigues et al. 2004). Ang II doses of 0.7-1 mg/kg/day in mice and of 0.3-10 mg/kg/day for rats can induce hypertension rapidly in 7-10 days or slowly in 4-8 weeks (Edgley, Kett et al. 2001). Rats develop cardiac and renal fibrosis as well as aortic and cardiac hypertrophy after two weeks of infusion (Huentelman, Grobe et al. 2005)..

Ang II was infused at 0.4 mg/kg/day to male Wistar rats subcutaneously by Alzet osmotic mini pumps over a period of 4 weeks. Ang II is a multifunctional peptide hormone that regulates blood pressure, plasma volume, cardiac, renal and neuronal functions, and also controls thirst responses. This peptide is of central importance in hypertension and myocardial remodeling and is the principle effector molecule of the renin-angiotensin system (RAS) playing an important role in the regulation of arterial blood pressure (Zhuo and Li 2011). In the present study Ang II infusion induced a rapid increase in systolic blood pressure of rats within a couple of days that remained elevated throughout the study. The chronic intake of the EPA:DHA 6:1 formulation was able to partially but significantly prevent the Ang II-induced increased systolic blood pressure. This result is in line with previously published studies reporting that omega-3 intake 3.3 to 7 g/d is associated with decreased blood pressure by 2.9 and 1.6 mm Hg among hypertensive patients (He 2009). Indeed, the recent meta-analysis by Miller et al. indicates that EPA+DHA provision is associated with reduced systolic blood pressure, whereas diastolic blood pressure is reduced for EPA+DHA provision exceeding 2 g/d (Miller, Van Elswyk et al. 2014).

In our experimental model, we observed that the continuous infusion of 0.4 mg/kg/d of Ang II in rats induced endothelial dysfunction in secondary branch of the mesenteric artery that is characterized by reduced endothelium-dependent relaxing responses, involving mainly blunted EDH-mediated responses and to a lesser extent NO-mediated relaxations, and an increased formation of EDCFs. As a result, omega-3 prevented Ang II induced endothelial dysfunction as

indicated by a significant improvement of both components of the relaxation and a decreased in endothelium-dependent contractile response. The chronic oral intake of the optimized EPA:DHA 6:1 significantly reduced Ang II induced over expression of eNOS, arginase-1, COX-1 and COX-2 as well as prevented the down-regulation of SK_{Ca} and Cx₃₇. Supplementation with omega-3 fatty acids also significantly improved the endothelial function in Humans without affecting endothelium-independent dilation (Wang, Liang et al. 2012). Endothelial dysfunction has been associated with an impairment of endothelium-dependent relaxations involving a reduced bioavailability of NO in major CV diseases such as hypertension, atherosclerosis, chronic renal failure, and diabetes (Griendling and FitzGerald 2003; Rush, Denniss et al. 2005; Félétou and Vanhoutte 2006). The mechanism underlying endothelial dysfunction has been linked to increased oxidative stress which is associated with a reduced bioavailability of (NO), an alteration of the production of prostanoids, including prostacyclin, thromboxane A₂, and/or isoprostanes, an impairment of endothelium-dependent hyperpolarization, these phenomena being able to contribute to endothelial dysfunction individually or in association.

In the present study, infusion of Ang II in rats induced an increase in vascular oxidative stress, mainly through the AT1R-mediated overexpression and activation of NADPH oxidase, in the vascular wall, which, in turn, affects the endothelial function by reducing both NO and EDH mediated relaxations and an increased in the formation of EDCF_s (Figure 23). Indeed, the increased formation of NADPH oxidase-derived superoxide anions most likely reduces the bioavailability of NO by chemical reaction leading to peroxynitrite formation, which, in turn, promotes the uncoupling of eNOS that increases further the oxidative stress (Förstermann 2010; Rochette, Zeller et al. 2014). Increased oxidative stress also impairs functioning of the Ca²⁺-activated K⁺ channels, SK_{Ca} and IK_{Ca}, resulting in an impaired electrical conduction and electrical signaling, leading to reduced EDH-mediated relaxation (Behringer, Shaw et al. 2013; Ellinsworth, Sandow et al. 2016). This result highlights the key role of the oxidative stress in the Ang II-induced endothelial dysfunction by reducing both EDH- and NO-mediated relaxations subsequent to the upregulation of NADPH oxidase subunits (p47^{phox} and p22^{phox}). The chronic oral intake of the optimized EPA:DHA 6:1 formulation significantly decreased the level of vascular oxidative stress, at least in part, by decreasing the expression of both the NADPH oxidase subunits p22^{phox} and p47^{phox} and AT1R. Our results are well in line with clinical studies showing a decreased level of oxidative

vascular stress following omega-3 treatment in atherosclerotic patients (Eftekhari, Aliasghari et al. 2013).

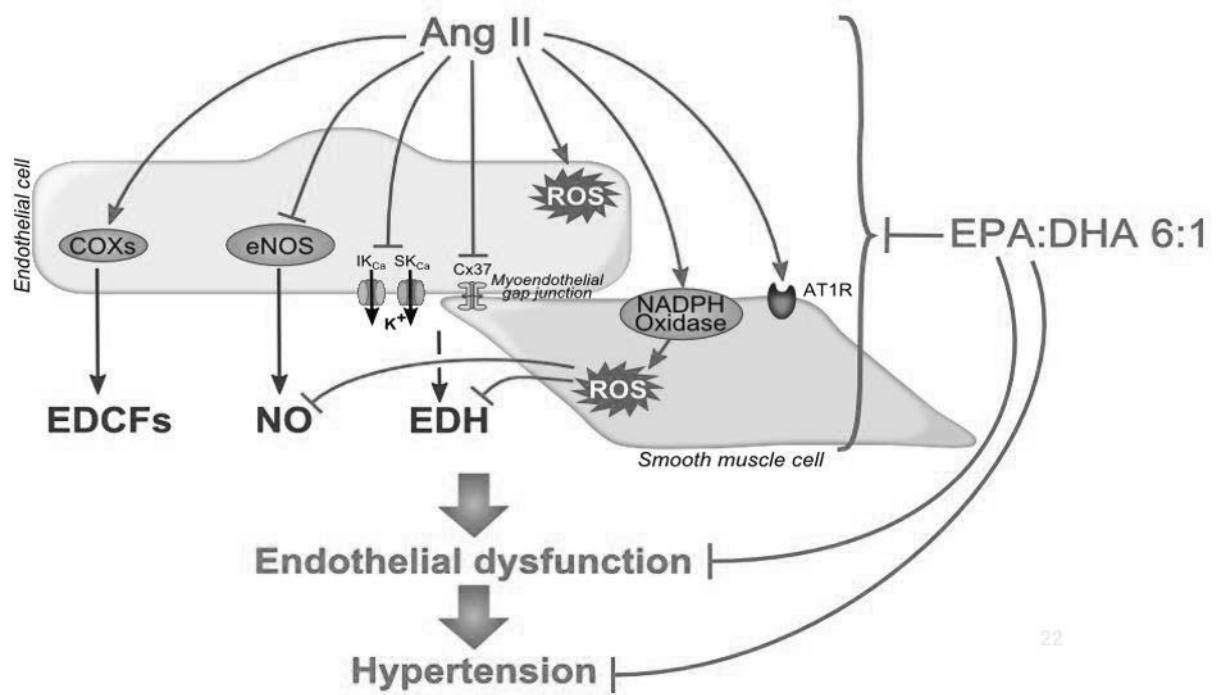


Figure 23. Omega-3 EPA:DHA 6:1 prevents Ang II-induced endothelial dysfunction and hypertension in rats.

In the cardiovascular system, Ang II activates NADPH oxidase through AT1R activation which increases vascular oxidative stress that is strongly associated with the progression of cardiovascular disease (Manrique, Lastra et al. 2009). Several signaling pathways in response to Ang II are mediated by ROS (Mehta and Griendling 2007). Different enzymes have been involved in the increased arterial oxidative stress involving AT1R, NADPH oxidases, xanthine oxidases, COX-1 and COX-2, cytochrome P450 monooxygenases, enzymes of the mitochondrial respiratory chain, and eNOS uncoupling. ROS ($O_2^{\bullet}$) can react with NO to form peroxynitrite (Koppenol, Moreno et al. 1992), leading to the oxidation of the eNOS cofactor tetrahydrobiopterin (BH_4) and the subsequent uncoupling of eNOS thereby further promoting oxidative stress (Cai and Harrison 2000).

The chronic intake of the optimized EPA:DHA 6:1 formulation is associated with significantly increased plasma level of omega-3 fatty acids, mainly as EPA, DHA and the intermediate elongated metabolite of EPA, the docosapentaenoic acid (DPA), resulting in a decreased omega-6/omega-3 ratio. Excessive amounts of omega-6 polyunsaturated fatty acids (PUFA) and a very high omega-6/omega-3 ratio promote the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases, whereas increased levels of omega-3 fatty acids (a low omega-6/omega-3 ratio) exert protective effects.

The reduction of this ratio has been associated with a shift towards beneficial health effects of omega-3, including reduced cardiovascular and cancer risk (Simopoulos 2008). Human beings have evolved on a diet with a ratio of omega-6 to omega-3 essential fatty acids of approximately 1, whereas in Western diets this ratio is 10/1-22.5/1 (Simopoulos 2011). Western diets are excessive in omega-6 fatty acids and deficient in omega-3 fatty acids as compared with the diet on which human beings evolved (Simopoulos 2008).

In the present study, chronic oral intake of the optimized EPA:DHA 6:1 formulation significantly decreases Ang II-induced over-expression of AT1R, COX-1 and COX-2. Omega-3 fatty acids have recognized anti-inflammatory actions in humans that may contribute to their beneficial cardiac effects (Wall, Ross et al. 2010). Omega-6 fatty acids can be converted into arachidonic acid and

then metabolized into the omega-6 eicosanoids. Consumption of omega-3 fatty acids increases EPA in the cell membrane. This PUFA competes with arachidonic acid for enzymatic conversion into its own metabolites, the omega-3 derived eicosanoids (Wall, Ross et al. 2010).

Increased concentration and activity of Ang II is strongly associated with inflammation. Ang II has a key proinflammatory effect on the cardiovascular system by stimulating vascular damage, inducing adhesion molecule-1 expression, recruiting inflammatory cells, increasing cytokine expression and tissue repairing (Brasier, Recinos et al. 2002). The physiological effects of Ang II are mediated by Ang II receptor subtype 1 (AT1R), which is widely distributed in many organs. In a rat experimental model Ang II infusion caused hypertension accompanied by marked monocyte infiltration as well as VCAM-1 and MCP-1 expression in the microvessels wall or perivascular tissue (Cheng, Vapaatalo et al. 2005). In endothelial, VSMC and mononuclear cells, Ang II is associated with an increased expression of MCP-1, the main chemokine for monocyte/macrophages and of interleukin-8 (IL-8), which are potent chemoattractants and activators of neutrophils (Yadav, Saini et al. 2010). Ang II also increased IL-6 production in macrophages and vascular cells, and upregulated TNF- α and IL-6 gene expression (Libby, Ridker et al. 2002). ACE inhibitors and AT1 antagonists reduced the expression of inflammatory markers, adhesion molecules, and also cytokines (Mezzano, Ruiz-Ortega et al. 2001). Ang II activates vascular and inflammatory cells to secrete proinflammatory mediators that assist to recruit new mononuclear cells, and, hence, results in additional inflammatory response contributing to the progression of vascular damage.

Independent of their effects on the metabolism of eicosanoids fish oils suppress pro-inflammatory cytokines and reduce expression of cell adhesion molecules in humans (Calder 2006).

Conclusions and perspective

In conclusion, the present work has assessed the potency of optimized EPA:DHA 6:1 formulation to protect the endothelial function *in vivo*. The present findings shows that Ang II infusion is associated with the development of endothelial dysfunction in second branch mesenteric arteries, which affects markedly EDH-mediated component and also, to some extent, the NO-mediated component of relaxation. The Ang II-induced endothelial dysfunction involves a redox-sensitive mechanism implicating NADPH oxidase, COX-1 and COX-2. The chronic oral intake of the optimized EPA:DHA 6:1 formulation prevents Ang II-induced endothelial dysfunction and hypertension most likely by decreasing oxidative stress-mediated impairment of both NO and EDH components as well as a reduction of endothelium-dependent contractile response.

There are numerous reports that support an important role of omega-3 fatty acids in preventing endothelial dysfunction, hypertension and cardiovascular diseases. Further work is needed for the determination of active metabolites of omega-3 fatty acids other than resolvins, protectins, thromboxane A₃, leukotriene 5 and lipoxins.

Further studies are needed to determine the curative potential of the optimized EPA:DHA 6:1 formulation in treatment of endothelial dysfunction and hypertension in Ang II-induced hypertensive models of rats (Figure 24). The impregnation of omega-3 fatty acids in vascular tissues is also important, which needs to be clarified. Furthermore, additional investigations are required to determine the role of the omega-3 purity and ratio to improve hypertension-related dysfunction of the vascular system including endothelial dysfunction and vascular remodeling.

On the basis of vasoprotective results established by the current study a clinical study can be designed to determine the potential of EPA:DHA 6:1 to reduce cardiovascular risk factors in hypertensive patients (Figure 25).

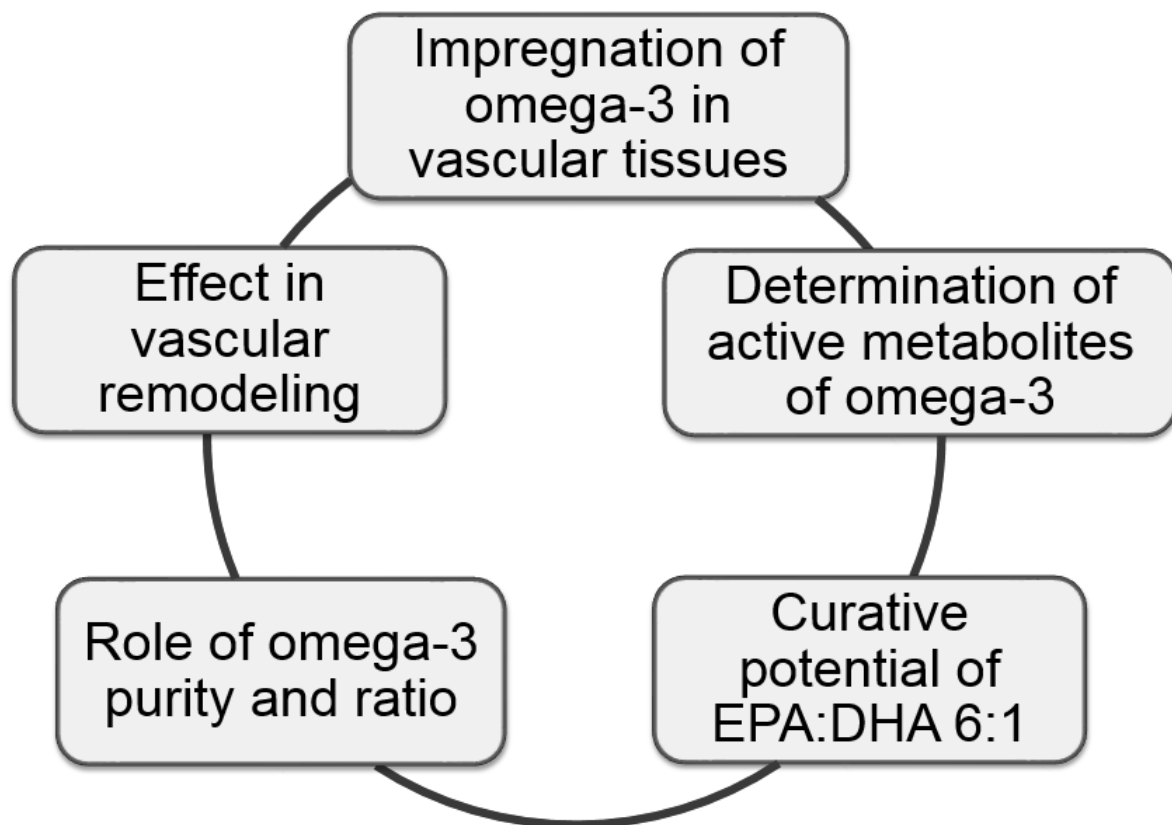


Figure 24. Perspectives in animal models.

Determination of active metabolites of omega-3 fatty acids, curative potential of the optimized EPA:DHA 6:1 formulation, importance of the omega-3 purity and ratio and impregnation of omega-3 fatty acids in vascular tissues.

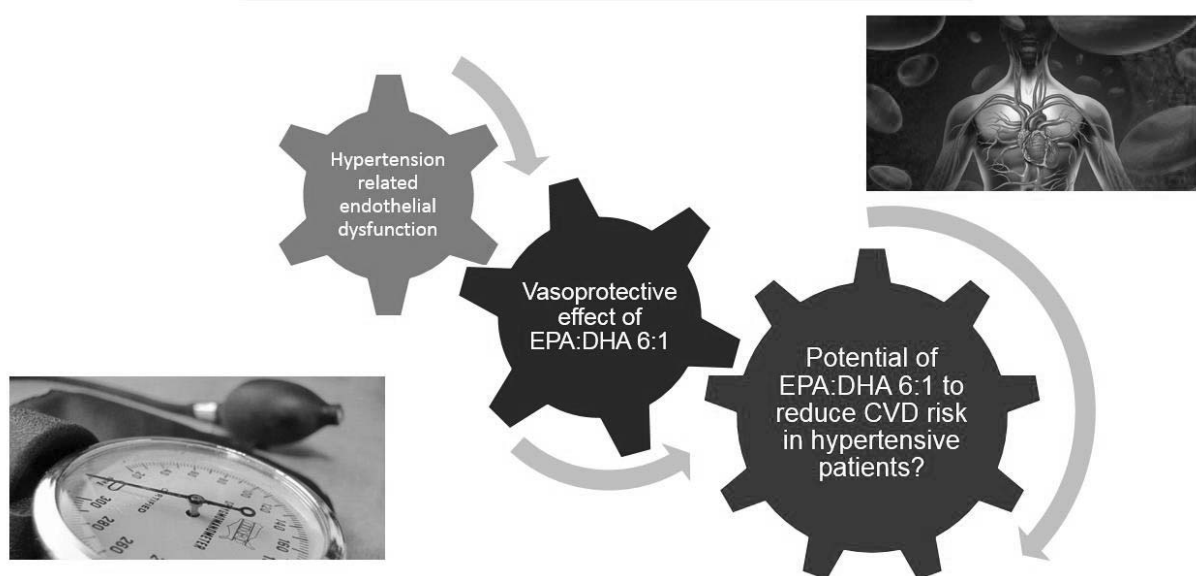


Figure 25. Clinical perspective.

Determination of EPA:DHA 6:1 potential to reduce cardiovascular risk factors in hypertensive patients.

DISCUSSION ET PERSPECTIVES

Hypertension artérielle

L'hypertension artérielle est un problème de santé publique majeur car associé à des lésions hypertensives d'organes cibles et à une morbi-mortalité cardiovasculaire. L'hypertension artérielle est ainsi le facteur de risque principal pour un nombre de maladies cardiovasculaires dont la maladie coronarienne, l'insuffisance cardiaque, les maladies vasculaires périphériques, les accidents vasculaires cérébraux et la néphropathie chronique (Sarnak, Levey et al. 2003). La prévalence de l'hypertension artérielle aux Etats-Unis est très élevée et continue d'augmenter, avec de plus un taux de stabilisation d'à peine 30 % chez les patients traités (Ong, Cheung et al. 2007). Après l'âge de 75 ans, environ 70 % des hommes et des femmes vont développer une hypertension artérielle (Chobanian, Bakris et al. 2003). Il a été montré que les traitements visant à bloquer le système rénine-angiotensine à divers niveaux peuvent limiter les lésions hypertensives aux organes cibles et diminuent la morbidité et la mortalité cardiovasculaires. Parmi ces traitements, les antagonistes des AT1R (ARA II ou sartans) et les inhibiteurs de l'enzyme de conversion de l'angiotensine (IEC) représentent entre 60 et 65 % de l'ensemble des traitements antihypertenseurs.

Les acides gras polyinsaturés omega-3

La recherche sur les produits naturels présente un fort intérêt du fait de plusieurs critères dont la non-couverture des besoins thérapeutiques, et la diversité remarquable des métabolites secondaires naturels au niveau à la fois des structures chimiques et des activités biologiques. Dans ce contexte, les effets indésirables associés aux molécules de synthèse et la diminution de l'acceptabilité des patients contribuent à augmenter l'intérêt pour les produits naturels.

De nombreuses études expérimentales et cliniques ont montré que les acides gras omega-3 peuvent avoir une répercussion bénéfique sur le système cardiovasculaire, et plus particulièrement chez les patients souffrant de maladie coronarienne (Harris, Mozaffarian et al. 2009). Ainsi, l'American Heart Association recommande la prise quotidienne de 1 g des deux acides gras omega-3 EPA et DHA dans le cadre de la prévention primaire et secondaire des maladies cardiovasculaires, après un infarctus du myocarde, et pour la prévention des morts subites cardiovasculaires (Von Schacky and Harris 2007).

Les acides gras omega-3 réduisent le taux plasmatique en triglycérides principalement en réduisant la disponibilité des acides gras pour la synthèse de triglycérides par diminution de la lipogenèse *de*

novo, augmentation de la bêta-oxydation des acides gras (Kusunoki, Kanatani et al. 2006), réduction de l'influx hépatique d'acides gras non-estérifiés, diminution des activités enzymatiques hépatiques de synthèse des triglycérides, et augmentation de la synthèse hépatique des phospholipides au dépend des triglycérides (Nakamura and Nara 2003). De plus, les acides gras omega-3 ont des propriétés anti-inflammatoires, et une corrélation négative a été montrée entre la consommation régulière de poisson ou d'huile de poisson et les taux circulants de protéine C-réactive, d'interleukine 6, d'E-selectin, de sICAM-1, de TNF, de la forme soluble du récepteur à l'interferon alpha, et de la métalloprotéase matricielle 3 (He 2009). Les huiles de poisson inhibent la production des cytokines pro-inflammatoires et réduisent l'expression de molécules d'adhésion cellulaire (Calder 2006). Ces facteurs sont critiques dans le recrutement des leucocytes circulants sur l'endothélium vasculaire, un événement précoce clé dans la pathogenèse de l'athérosclérose.

Les acides gras omega-3 entrent en compétition avec les acides gras omega-6 au niveau des cyclooxygénases et des lipoxygénases pour la synthèse des prostaglandines et des leucotriènes. Ainsi, les acides gras omega-3 modulent le métabolisme des prostaglandines en augmentant la synthèse de prostaglandine E₃, un vasodilatateur et inhibiteur de l'agrégation plaquettaire, du thromboxane A₃, de leucotriène B₅ et en diminuant la synthèse de thromboxane A₂, un puissant vasoconstricteur et inducteur de l'agrégation plaquettaire, et de leucotriène B₄ (un inducteur de l'inflammation et la chimiotaxie et de l'adhérence des leucocytes). D'autres études ont aussi montré que la consommation élevée d'huile de poisson conduisait à une diminution du taux de plaquettes, de l'agrégation plaquettaire, de la concentration des métabolites du thromboxane, ainsi qu'à une augmentation du temps de saignement. Les effets hypotriglycémisants des acides gras omega-3 requièrent des doses d'EPA et DHA de 3 ou 4 g par jour. Dans les populations américaines, ces doses peuvent réduire le taux de triglycérides de l'ordre de 30 à 40 % (Harris, Ginsberg et al. 1997; Lavie, Milani et al. 2009). Cependant, la consommation quotidienne de 6 g d'EPA et DHA pendant 2 ans par 59 patients n'a pas montré d'effet favorable sur l'évolution du diamètre d'artères coronaires athéromateuses (Woodman 2003). A l'inverse, plusieurs études ont montré que la consommation d'huile de poisson ou de poissons non frits était associée à une diminution de la prévalence de l'athérosclérose subclinique caractérisée par un changement significatif de l'épaisseur intima-media de l'artère carotide commune et du pourcentage de sténose, alors qu'aucun changement n'ont été observé pour la quantification du calcium dans l'artère coronaire

ou pour l'index de pression systolique (Von Schacky, Angerer et al. 1999). Le DHA est le précurseur d'un métabolite récemment décrit sous le nom de 10,17S-docosatriène, qui est un membre de la famille des résolvines. Ces molécules ont d'abord été décrites comme relâchées par le cerveau en réponse à l'ischémie, et elles s'opposent à l'effet pro inflammatoire des infiltrats leucocytaires en bloquant l'activation de NF- κ B et l'expression de la cyclooxygénase-2 induites par l'interleukine 1-bêta (Layé 2010). Les études précédentes de notre équipe de recherche ont montré que l'EPA et le DHA, les acides gras omega-3 majeurs, induisent des relaxations dépendantes de la concentration dans les anneaux d'artères coronaires de porc (Zgheel, Alhosin et al. 2014). La relaxation en réponse à l'EPA est légèrement mais significativement plus importante que celle en réponse au DHA (77.8 ± 10.3 et 64.7 ± 12.8 % à 0.4 % v/v, respectivement). Ensuite, la capacité de ratios optimisés d'omega-3 à induire la relaxation dépendante de l'endothélium a été déterminée, et les résultats indiquent que les ratios EPA:DHA de 6:1 et 9:1 induisent des relaxations significativement plus importantes que celles en réponse aux ratios 3:1, 1:1, 1:3, 1:6, et 1:9. De plus, la relaxation dépendante de l'endothélium obtenu en réponse à un produit ayant un degré de pureté en EPA:DHA élevé (694:121 mg/g) était significativement plus importante que celle obtenue en réponse à un produit ayant un degré de pureté plus faible (352:65 mg/g). Ainsi, les effets bénéfiques des omega-3 dépendent fortement de la dose consommée, de la composition et de la pureté des omega-3.

Effets bénéfiques des acides gras omega-3 sur la fonction endothéliale

Les acides gras omega-3 de l'alimentation présentent divers effets anti-inflammatoire et de modulation du système immunitaire qui pourrait jouer un rôle dans le ralentissement du développement de l'athérosclérose et de l'apparition de ses signes cliniques comme l'infarctus du myocarde, la mort subite et l'accident vasculaire cérébral (Mori and Beilin 2004). Les acides gras omega-3 qui semblent les plus efficaces dans ce cadre sont les polyinsaturés à longues chaînes des huiles d'origine marine, c'est-à-dire l'acide eicosapentaénoïque (EPA) et l'acide docosahexaénoïque (DHA) (Riediger, Othman et al. 2009). Plusieurs études ont montré une corrélation inverse entre le risque de maladies cardiovasculaires et une augmentation de la consommation d'acides gras omega-3, comme l'EPA et le DHA, qui sont principalement retrouvés dans les poissons gras (saumon, truite, hareng, sardines, maquereau, etc.) (Lavie, Milani et al. 2009). Les effets bénéfiques d'une augmentation de la consommation alimentaire en acides gras

omega-3 sur le système cardiovasculaire incluent une baisse des arythmies, de la triglycémie, de la pression artérielle et de l'agrégation plaquettaire, l'ensemble de ces effets résulte en une baisse de risque de mortalité cardiovasculaire chez les patients atteints de maladies cardiovasculaires (Harris, Miller et al. 2008). De plus, les effets protecteurs des omega-3 peuvent être attribués à leur capacité à améliorer la relaxation dépendante de l'endothélium dans les artères en stimulant la formation endothéliale des facteurs vasoprotecteurs NO et EDH, ainsi qu'en réduisant les réponses contractiles dépendantes de l'endothélium (Ribeiro, Oliveira et al. 2016).

Du fait que les formulations EPA:DHA 6:1 et 9:1 sont plus efficaces que le ratio 1:1 (qui correspond à la seule spécialité avec AMM sur le marché) pour l'induction des relaxations dépendantes de l'endothélium dans des anneaux d'artère coronaire de porc (Zghele, Alhosin et al. 2014), la formulation EPA:DHA 6:1 a été retenue pour l'étude présente et donnée à des rats à la dose de 500 mg/kg/j, ce qui équivaut à 5,67 g/j pour un homme de 70 kg (Reagan-Shaw, Nihal et al. 2008). Cette dose est cohérente avec les études cliniques et les méta-analyses, où les doses rapportées vont de 0,18 à 10 g/j sous forme de divers produits à base omega-3 (Delgado-Lista, Perez-Martinez et al. 2012; Enns, Yeganeh et al. 2014).

L'angiotensine II (Ang II) peut être infusée à des rats ou des souris grâce à des mini pompes osmotiques sur une durée allant jusqu'à 4 semaines (Zimmerman, Lazartigues et al. 2004). L'infusion d'Ang II à la dose de 0,7-1 mg/kg/j chez la souris ou de 0,3-10 mg/kg/j chez le rat va induire une hypertension de façon rapide en 7 à 10 jours ou de façon lente en 4 à 8 semaines (Edgley, Kett et al. 2001). Les rats recevant l'Ang II en infusion vont développer en 2 semaines des fibroses rénales et cardiaques ainsi qu'une hypertrophie aortique et cardiaque (Huentelman, Grobe et al. 2005).

Dans notre étude, l'Ang II a été infusée pendant 4 semaines à la dose de 0,4 mg/kg/j à des rats Wistar males à l'aide de mini-pompes osmotiques Azlet. L'ang II est une hormone peptidique multifonctionnelle qui régule la pression sanguine, le volume plasmatique, les fonctions cardiaques, rénales et neuronales, et qui contrôle aussi la soif. Ce peptide joue un rôle central dans le développement de l'hypertension artérielle et dans le remodelage cardiaque, et il est l'effecteur

principal du système rénine-angiotensine qui joue un rôle majeur dans la régulation de la tension artérielle (Zhuo and Li 2011). Dans la présente étude, l'infusion d'Ang II a induit une augmentation rapide de la pression artérielle chez les rats en quelques jours et qui resté élevée tout au long de l'étude. La prise quotidienne de la formulation EPA:DHA 6:1 a permis de prévenir partiellement mais significativement l'augmentation de tension artérielle induite par l'Ang II. Ce résultats est en accord avec les études publiées préalablement qui montraient que la consommation d'acides gras omega-3 à des doses de 3,3 à 7 g/j était associée à une diminution de la tension artérielle de 2,9 et 1,6 mmHg chez les patients hypertendus (He 2009). Ainsi, la méta-analyse de Miller et al. rapporte que la consommation de EPA+DHA est toujours associée à une diminution de la pression sanguine systolique, alors que la pression sanguine diastolique est diminuée significativement pour des consommations d'EPA+DHA supérieures à 2 g/j (Miller, Van Elswyk et al. 2014).

Dans notre modèle expérimental, nous avons observé que l'infusion d'Ang II à la dose de 0,4 mg/kg/j induisait l'apparition dans les branches secondaires de l'artère mésentérique d'une dysfonction endothéliale caractérisée par une diminution des réponses vasorelaxantes dépendantes de l'endothélium, impliquant principalement une réduction de la composante EDH et dans une moindre mesure de la composante NO, et d'une augmentation de la formation des EDCF. La consommation chronique de la formulation EPA:DHA 6:1 a eu pour effet de prévenir la dysfonction endothéliale induite par l'Ang II comme l'indique l'amélioration significative des deux composantes de la relaxation et la réduction des réponses contractiles dépendantes de l'endothélium. La consommation chronique de la formulation EPA:DHA 6:1 a significativement diminué la surexpression induite par l'Ang II de la eNOS, de l'arginase-1, des COX-1 et COX-2, et a prévenu la sous-expression de SK_{Ca} et Cx₃₇. La supplémentation alimentaire en acides gras omega-3 améliore aussi la fonction endothéliale chez l'homme, sans pour autant affecter les vasodilatations indépendantes de l'endothélium (Wang, Liang et al. 2012). La dysfonction endothéliale a été associée à une atteinte des relaxations dépendantes de l'endothélium impliquant une réduction de la biodisponibilité du NO dans la plupart des maladies cardiovasculaires dont l'hypertension, l'athérosclérose, la néphropathie chronique ou le diabète (Griendling and FitzGerald 2003; Rush, Denniss et al. 2005; Félétou and Vanhoutte 2006). Le mécanisme sous-jacent à la dysfonction endothéliale a été lié à une augmentation du stress oxydant vasculaire lui-même associé à une diminution de la biodisponibilité du NO, une altération de la production des

prostanoïdes tels que la prostacycline, le thromboxane A2 ou les isoprostanes, une diminution de la composante EDH de relaxation ainsi qu'à une augmentation de formation d'endothéline-1, l'ensemble de ces phénomènes pouvant contribuer à la dysfonction endothéliale de manière isolée ou ensemble.

Dans la présente étude, l'infusion d'Ang II est associée à une augmentation du stress oxydant vasculaire chez les rats, principalement par la surexpression et l'activation de la NADPH oxydase induite par AT1R dans la paroi vasculaire, ce qui va à son tour affecter la fonction endothéliale en réduisant les deux composantes de la relaxation NO et EDH, et en augmentant la formation d'EDCFs (Figure 23). En effet, l'augmentation de la formation par la NADPH oxydase d'anions superoxyde diminue probablement la biodisponibilité du NO par la réaction chimique produisant des peroxynitrites, qui peuvent à leur tour favoriser le découplage de la eNOS ce qui augmente encore le stress oxydant vasculaire (Förstermann 2010; Rochette, Zeller et al. 2014). L'augmentation du stress oxydant vasculaire va aussi altérer le fonctionnement des canaux potassiques dépendants du calcium SK_{Ca} and IK_{Ca} , résultant dans une altération de la conductivité électrique et des voies de signalisation électriques, conduisant à la réduction de la composante EDH de la relaxation (Behringer, Shaw et al. 2013; Ellinsworth, Sandow et al. 2016).

Ces résultats soulignent le rôle majeur du stress oxydant dans la dysfonction endothéliale induite par l'Ang II via la diminution des composantes NO et EDH de la relaxation qui suivent la surexpression des sous-unités de la NADPH oxydase ($p47^{phox}$ and $p22^{phox}$). La consommation chronique de la formulation EPA:DHA 6:1 a significativement diminué le niveau du stress oxydant vasculaire, du moins en partie, en prévenant la surexpression de la NADPH oxydase et de AT1R. Ces résultats sont en accord avec ceux d'étude cliniques qui montrent une diminution du stress oxydant vasculaire chez des patients athérosclérotiques recevant des omega-3 (Eftekhari, Aliasghari et al. 2013).

Dans le système cardiovasculaire, l'Ang II active la NADPH oxydase via l'activation de AT1R, ce qui augmente le stress oxydant vasculaire qui est fortement associé au développement des maladies cardiovasculaires (Manrique, Lastra et al. 2009). Plusieurs voies de signalisation en réponse à l'Ang II impliquent ainsi des espèces réactives de l'oxygène (ROS) (Mehta and Griendling 2007). Diverses enzymes ont été impliquées dans l'augmentation du stress oxydant vasculaire tels que

AT1R, la NADPH oxydase, la xanthine oxydase, les COX-1 et COX-2, le cytochrome P450, les enzymes de la chaîne respiratoire mitochondriale, et la eNOS découplée. Les ROS ($O_2^{\bullet-}$) peuvent réagir chimiquement avec le NO pour former des peroxynitrites (Koppenol, Moreno et al. 1992), ce qui conduit à l'oxydation du cofacteur de la eNOS, la tétrahydrobioptérine (BH_4), et subséquemment au découplage de la eNOS augmentant à son tour le stress oxydant (Cai and Harrison 2000).

La consommation chronique de la formulation EPA:DHA 6:1 est associée à une augmentation significative des proportions plasmatiques en acides gras omega-3, principalement sous forme d'EPA, de DHA et de l'intermédiaire métabolique allongé de l'EPA, l'acide docosapenténoïque (DPA), conduisant à la diminution significative du ratio omega-6/omega-3. Des quantités excessives d'acides gras polyinsaturés omega-6 ainsi qu'un ratio omega-6/omega-3 élevé promeuvent la genèse de nombreuses pathologies, dont les maladies cardiovasculaires, les cancers, les maladies inflammatoires et auto-immunes, alors qu'à l'inverse des niveaux importants en omega-3 (et un faible ratio omega-6/omega-3) ont des effets protecteurs. La réduction de ce rapport omega-6/omega-3 a été associée à un basculement vers les effets bénéfiques pour la santé des omega-3 (Simopoulos 2008). Les êtres humains ont évolué avec un régime alimentaire ayant un rapport entre acides gras omega-6 et omega-3 approchant de 1/1, alors que l'alimentation occidentale a un rapport de l'ordre de 10/1-22.5/1 (Simopoulos 2011). De ce fait, l'alimentation occidentale est trop riche en omega-6 et présente un déficit en omega-3 (Simopoulos 2008).

Dans la présente étude, la consommation chronique de la formulation EPA:DHA 6:1 a significativement diminué la surexpression induite par l'Ang II de AT1R, de COX-1 et de COX-2. Les acides gras omega-3 ont des effets antiinflammatoires reconnus chez l'homme qui pourrait contribuer à leur effets bénéfiques vis-à-vis du système cardiovasculaire (Wall, Ross et al. 2010). Les acides gras omega-6 sont convertis en acide arachidonique qui est lui-même métabolisé en eicosanoïdes de la série omega-6. La consommation des omega-3 augmente le taux d'EPA dans les membranes cellulaires, où il va entrer en compétition avec l'acide arachidonique pour la conversion en ses propres métabolites, les eicosanoïdes de la série omega-3 (Wall, Ross et al. 2010).

L'augmentation de la concentration et de l'activité de l'Ang II est fortement associée à l'inflammation. L'Ang II exerce un effet pro-inflammatoire sur le système cardiovasculaire qui va

stimuler les dommages vasculaires, induire l'expression de molécules d'adhésion, recruter des cellules inflammatoires, et augmenter l'expression de cytokines (Brasier, Recinos et al. 2002). Dans un modèle expérimental de rat, l'infusion d'Ang II induit une hypertension associée à une forte infiltration monocyttaire et une expression de VCAM-1 et MCP-1 pro-inflammatoire dans la paroi des microvaisseaux ou dans l'espace périvasculaire (Cheng, Vapaatalo et al. 2005). Dans les cellules endothéliales, les cellules musculaires lisses vasculaires et les mononucléaires, l'Ang II induit une augmentation de l'expression de MCP-1, la principal chimiokine recrutant les monocytes/macrophages, et d'interleukine-8, un puissant chimioattractant et activateur des neutrophiles (Yadav, Saini et al. 2010). L'Ang II augmente aussi la production d'interleukine-6 dans les macrophages et les cellules vasculaires, et régule positivement l'expression des gènes du TNF- α et de l'interleukine-6 (Libby, Ridker et al. 2002). Les inhibiteurs de l'enzyme de conversion (IEC) et les antagonistes du récepteur AT1R (ARAs) réduisent l'expression des marqueurs inflammatoires, des molécules d'adhésion et des cytokines (Mezzano, Ruiz-Ortega et al. 2001). L'Ang II active les cellules vasculaires et inflammatoires pour induire la sécrétion des médiateurs pro-inflammatoires qui vont recruter de nouveau mononucléaire, ce qui va encore augmenter la réponse inflammatoire contribuant à la progression de la lésion vasculaire. Les huiles de poisson peuvent limiter les cytokines pro-inflammatoires et réduire l'expression des molécules d'adhésion cellulaires chez l'homme de façon indépendante de leurs effets sur le métabolisme des eicosanoïdes (Calder 2006).

Conclusions et perspective

En conclusion, la présente étude a évalué le potentiel d'une formulation optimisée EPA:DHA 6:1 à protéger la fonction endothéliale *in vivo*. Les résultats obtenus montrent que l'infusion d'Ang II est associée avec le développement dans les branches secondaires de l'artère mésentérique d'une dysfonction endothéliale affectant fortement la composante EDH de la relaxation ainsi que plus légèrement la composante NO. La dysfonction endothéliale induite par l'Ang II passe par un mécanisme redox-sensible impliquant la NADPH oxydase, COX-1 et COX-2. La consommation chronique de la formulation EPA:DHA 6:1 a prévenu la dysfonction endothéliale et l'hypertension induite par l'Ang II fort probablement en diminuant le stress oxydant conduisant à l'atteinte des

composantes NO et EDH de la relaxation et à l'augmentation des réponses contractile dépendantes de l'endothélium.

De nombreuses études supportent le rôle clé des acides gras omega-3 dans la prévention de la dysfonction endothéliale, de l'hypertension et des maladies cardiovasculaires. De plus amples études sont nécessaires pour déterminer les métabolites actifs des acides gras omega-3 en sus des resolvines, protectines, thromboxane A3, leucotriène 5 and lipoxines. Il serait aussi nécessaire de déterminer le degré d'imprégnation des tissus vasculaires en omega-3.

De même, des études seront nécessaires pour déterminer le potentiel curative de la formulation EPA:DHA 6:1 vis-à-vis de la dysfonction endothéliale et de l'hypertension dans des modèles d'hypertension induite par l'Ang II chez le rat (Figure 24).

Enfin, sur la base des effets vasoprotecteurs mis en évidence par la présente étude, il serait intéressant de proposer une étude clinique visant à démontrer le potentiel de la formulation EPA:DHA 6:1 à réduire la dysfonction endothéliale et les facteurs de risques cardiovasculaires chez les patients hypertendus (Figure 25).

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Annexes



The Endocrine Pancreas, an Early Sensor of Senescence in Middle-aged Rats with Still Normal Vascular Function

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Keywords:	Pancreatic islet, microparticles, tissue factor, ROS, extracellular vesicles
Abstract:	Objectives: Aging is a determinant factor in the progressive deterioration of all organs in adulthood. We compared the senescence features and organ function of pancreatic and vascular tissues in young and middle-aged rats. Methods: Islet morphology and the area of cells secreting insulin or glucagon was investigated using immunohistology in young rats (12weeks) and middle-aged (52weeks) (n=8). Senescence markers, oxidative stress

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	(ROS) and tissue factor (TF) were measured in tissue lysates or fresh frozen tissues sections. Circulating microparticles (MPs) were measured as surrogates of vascular cell injury. Vascular function was studied in mesenteric arterial rings. Results: Larger islets were twice as frequent in Young vs. Middle-aged rats. In middle-aged rats, there was a significant decrease of the β-cells/ islet area ratio ($P<0.05$). Western blot analysis showed an increased expression of p53, p21 and p16 senescence markers (2, 7 and 3-folds respectively), with no modification in caspase-3-activation. A 30% decrease of eNOS was observed together with a 4-fold increase in TF expression. ROS formation increased significantly (2-fold) in middle-aged rats and their main source determined by pharmacological inhibition were the NADPH oxidase and uncoupled Nitric oxide (NO) synthase. No sign of vascular injury (Microparticles) or dysfunction was evidenced. Conclusions: Morphological and functional pancreatic islet alterations were detected in middle-aged rats before any measurement of macro-vascular alteration. Altogether, the data indicate an early driven pancreatic senescence in the process of aging associated with uncontrolled accumulation of oxidative species.

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The Endocrine Pancreas, an Early Sensor of Senescence in Middle-aged Rats with Still Normal Vascular Function

Running title: Endocrine Pancreas: Sensor of Senescence

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27 **Abstract**

28 **Objectives:** Aging is a determinant factor in the progressive deterioration of all organs in adulthood. We
29 compared the senescence features and organ function of pancreatic and vascular tissues in young and middle-
30 aged rats.

31 **Methods:** Islet morphology and the area of cells secreting insulin or glucagon was investigated using
32 immunohistology in young rats (12weeks) and middle-aged (52weeks) (n=8). Senescence markers, oxidative
33 stress (ROS) and tissue factor (TF) were measured in tissue lysates or fresh frozen tissues sections. Circulating
34 microparticles (MPs) were measured as surrogates of vascular cell injury. Vascular function was studied in
35 mesenteric arterial rings.

36 **Results:** Larger islets were twice as frequent in Young vs. Middle-aged rats. In middle-aged rats, there was a
37 significant decrease of the β -cells/ islet area ratio ($P<0.05$). Western blot analysis showed an increased
38 expression of p53, p21 and p16 senescence markers (2, 7 and 3-folds respectively), with no modification in
39 caspase-3-activation. A 30% decrease of eNOS was observed together with a 4-fold increase in TF expression.
40 ROS formation increased significantly (2-fold) in middle-aged rats and their main source determined by
41 pharmacological inhibition were the NADPH oxidase and uncoupled Nitric oxide (NO) synthase. No sign of
42 vascular injury (Microparticles) or dysfunction was evidenced.

43 **Conclusions:** Morphological and functional pancreatic islet alterations were detected in middle-aged rats before
44 any measurement of macro-vascular alteration. Altogether, the data indicate an early driven pancreatic
45 senescence in the process of aging associated with uncontrolled accumulation of oxidative species.

46 **Keywords:** Pancreatic islet, microparticles, tissue factor, ROS, extracellular vesicles.

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58 Introduction

59 Aging is a determinant factor for the progressive post- maturational deterioration of all organs, leading to
60 chronic and metabolic diseases including obesity, impaired glucose tolerance, and type 2 diabetes (T2D).[1,2] In
61 organ transplantation and particularly in pancreatic islet transplantation, donor age is determinant for the
62 efficacy of graft function and survival.[3]

63 The increased incidence of type 2 diabetes (T2D) in the elderly is a health problem worldwide,[4,5] where one
64 third of the elderly have diabetes and three quarters have diabetes or pre-diabetes.[6] During the pathogenesis of
65 T2D, beta cell (β -cell) proliferation increases to compensate the increased insulin demand caused by insulin
66 resistance, and the generation of ROS is induced by hyperglycaemia. It would seem that β -cell proliferation
67 and/or ROS generation accelerate cellular senescence and lead to diabetes.[7,8] Pancreatic β -cell senescence has
68 been suggested a contributor to type 2 diabetes, at least in a model of high-fat feeding.[9] and could be a direct
69 mechanism through which senescence contributes to diabetes, as the decline of β -cell function and mass is a
70 hallmark of type 2 diabetes progression.

71 In turn, features of diabetes may cause an increase in senescent cell number in many organs and play a pivotal
72 role in tissue damage thereby amplifying diabetes complications. Indeed, cellular senescence is a feed-forward
73 mechanism that promotes the accumulation of an increasing number of senescent cells in nearby and distant
74 tissues.[10]

75 Senescence arises from age or from organ premature dysfunction as a consequence of altered cell function in
76 response to stress. Cellular senescence is characterized by an irreversible arrest of cell division that occurs in
77 response to various cellular stressors, such as telomere erosion, DNA damage, oxidative stress, or oncogenic
78 activation and by shifted optimum pH of lysosomal β -galactosidase (Senescence-Associated β -galactosidase
79 [SA- β gal] activity).[11,12] The proteins p53, p21, and p16 are cell cycle inhibitors contributing to the
80 senescence process.

81 Pancreatic islet undergo morphological changes and functional decline during normal aging,[13] and diabetes
82 in the elderly is a cardiovascular risk factor, thereby pointing at a possible interaction between islets cells and
83 the vascular endothelium. Indeed, intra-islet endothelial cells play a role in the revascularization of transplanted
84 islet.[14]

85 Endothelial cells contribute to many vital physiological functions such as the control of hemostasis and blood
86 pressure.[15,16] Endothelium-mediated vasodilatory function progressively declines with age.[17] It is

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87 associated with decreases in eNOS expression and in NO formation, a major vasoprotector, by aging endothelial
88 cells, leading
89 to a progressive deterioration of cell function owing to cell cycle arrest.[18,19]
90 Oxidative stress causes endothelial aging and dysfunction through uncontrolled production of reactive oxygen
91 species.[20] Inflammation, which increases with age, is another major cause of cell dysfunction. At the surface
92 of the inflamed endothelium, the expression of tissue factor (TF), the membrane initiator of coagulation and an
93 early responsive gene, is upregulated as well as pro-adhesive membrane proteins involved in leucocyte
94 recruitment.[21]
95 Microparticles (MPs) are submicron plasma membrane vesicles shed by stressed or apoptotic cells. They are
96 circulating markers of vascular cell damage and behave as cellular effectors. In blood flow, MPs are
97 procoagulant and convey the active form of TF. MP cell origin can be determined by detection of specific
98 membrane proteins. We showed that an inflammation-driven β -cell membrane remodeling leads to insulin
99 impairment and the release of TF-bearing MPs able to induce β -cell apoptosis.[22,23] Furthermore, MPs are
100 circulating surrogate markers of endothelial damage and associated with cardiometabolic risk factors.[24]
101 The aim of the present study was to (i) investigate whether islets morphological or functional changes, and
102 alteration of pancreatic endocrine cell distribution within the process of aging could sense pancreatic
103 senescence-driven dysfunction, (ii) compare the variations of markers of senescence and oxidative stress with
104 age in pancreatic islets and vessels using plasma MPs as surrogate of vascular tissue damage.

105 **Materials and Methods**

106 **Ethics statement**

107 Male Wistar rats (Janvier-Labs, Le Genest-St-Isle, France) were housed in a temperature-controlled (22°C)
108 room and maintained on a standard 12-h light/dark cycle (lights on at 07:00 am) with free access to food and
109 water. Experiments conform to the Guide of Care and the Use of Laboratory Animals published by the US
110 National Institutes of Health (NIH publication No. 85-23, revised 1996) and were authorized by the French
111 Ministry of Higher Education and Research and by a local ethic committee (Comite d'éthique en Matière
112 d'expérimentation animale de Strasbourg, authorization 03799.01). All animal experiments were done in a
113 registered animal yard within Faculty of Pharmacy (Authorization number E-67-218-26).

114 **Animal model, blood and tissue sampling**

115 Young, 12-week-old male Wistar rats (n=8) with average body weight (463.6 ± 9.49 g) and 52-week-old
116 middle-aged male Wistar rats (n=8) with average body weight (739.4 ± 46.29 g) were used. Rats were

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3 117 anaesthetized by sub-lethal IP injection of 50 mg/kg sodium pentobarbital (Ceva Animal Health, Libourne,
4 118 France), and euthanized by exsanguination. The pancreas, aorta, and mesenteric artery were removed and
5
6 119 weighed. The pancreas was cut into 3 parts: one was fixed in freshly depolymerized paraformaldehyde 4 %
7
8 120 (Electron microscopy sciences 15710,
9
10 121 Hatfield) and subsequently embedded in paraffin for histological analysis, the second embedded in Tissue-Tek
11
12 122 O.C.T. Compound (Sakura 4583, Leiden, Netherlands) and snap-frozen for immunofluorescence studies and the
13
14 123 determination of the formation of ROS. The third part was frozen in liquid nitrogen for Western blot analysis.
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16 124 Blood samples were withdrawn by heart puncture and harvested on 13 mM sodium citrate or 1.8 mg/ml EDTA.
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18 125 Plasma was obtained by centrifugation and stored at -80°C for subsequent analysis.

126 **Histological and morphological analyses**

127 Four micrometer (μm) thick paraffin sections of pancreas samples were stained with haematoxylin-eosin and
128 Gomori's trichrome stain. Four sections were taken from each sample every 100 μm . For each section, the
129 surface of the islets was measured using ImageJ software. The mean islet surface area was calculated by adding
130 the values measured for each of the 4 sections to cover the whole islets area.

131 **Immunocytochemistry and immunofluorescence determinations**

132 Paraffin-embedded sections of pancreas were subjected to microwave antigen retrieval in 10 mM sodium citrate
133 buffer (pH 6.0). After, incubation with the primary antibody, mouse monoclonal anti-PSA-NCAM (1/500)
134 (Millipore, clone 2-2B, MAB5324, Darmstadt, Germany), overnight labelling was revealed by anti-mouse (2 h)
135 and ABC complex formation (30 min) (VECTASTAIN Elite ABC Kit (Standard), California, USA); VIP
136 (VECTOR VIP Peroxidase (HRP) Substrate Kit, California, USA) as a chromogen.

137 Double labeling of insulin and glucagon, was performed on paraffin-embedded pancreas sections by overnight
138 incubation with rabbit polyclonal anti-insulin (1/200, 4590S Cell Signaling, Danvers, USA) or mouse
139 monoclonal anti-glucagon primary antibodies (1/5000, G2654 Sigma, Missouri, USA), followed by 2 h
140 incubation at room temperature with an Alexa Fluor 488 FITC-goat anti-mouse IgG (H+L) and Alexa Fluor 633
141 goat anti-rabbit IgG (H+L), Life technologies, USA antibodies. Nuclei were stained with DAPI (Roche
142 Diagnostics, Meylan, France), and sections were cover-slipped with fluorescence-mounting medium (Dako
143 S3023, Carpinteria, USA). For negative controls, primary antibodies were omitted.

144 **Intra peritoneal glucose tolerance test (IPGTT)**

145 Rats were fasted 8h before intraperitoneal injection of glucose (2 g/Kg). Glucose plasma concentration was
146 measured in the blood of the tail vein using a glucometer (BG STAR, Agamatrix USA) at different times.

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147 **Biological markers**

148 Glucose was measured in plasma collected at the moment of sacrifice by the Hexokinase Spectrophotometry
149 method, insulin by ELISA kit (Millipore). Cholesterol and triglycerides were measured by enzymatic methods.
150 HDL and LDL levels were established using the Elimination method (catalase + reaction trinder).

151 **Western blot analysis**

152 Protein lysates from frozen pancreatic tissue were incubated with extraction buffer (Tris/HCl 20 mM (pH 7.5;
153 Q- Biogene, California, USA), NaCl 150 mM, Na₃VO₄ 1 mM, sodium pyrophosphate 10 mM, NaF 20 mM,
154 okadaic acid 0.01 mM (Sigma-Aldrich, Missouri, USA) containing a tablet of protease inhibitor (Complete
155 Roche, Basel, Switzerland) and 1% Triton X-100 (Euromedex). Total proteins (30 µg) were separated on 10%
156 SDS-polyacrylamide gels at 100 V for 2 h and transferred electrophoretically onto polyvinylidene difluoride
157 membranes (Amersham, Life Sciences, Germany) at 100 V for 120 min. Aspecific binding was blocked by
158 incubation in TBS buffer (Biorad) containing 5% bovine serum albumin (BSA), and 0.1% Tween 20 (Sigma)
159 (TBS-T) for 2 h.
160 Membranes were then incubated overnight at 4⁰C in TBS-T containing 5 % BSA and polyclonal antibody raised
161 against human p53 (1/1000) (Santa Cruz Biotechnology FL-393 SC-6243, Dallas, Texas, USA), human p21
162 (1/1000) (Santa Cruz Biotechnology C-19 SC-397, Dallas, Texas, USA), human p16 (1/1000 Santa Cruz
163 Biotechnology, Dallas, Texas, USA), rat eNOS (1/1000) (BD Transdaction Laboratory cat: 610297), human TF
164 (1/1000 Sekisui diagnostics, Sekisui Wiretech Gbmh, Russelsheim, Germany), and rat cleaved Caspase-3 (Cell
165 Signaling Technology, Danvers, USA, 1/1000). After 3 washings with TBS-T, membranes were incubated with
166 an appropriate horseradish peroxidase-conjugated secondary antibody and density signals of each band detected
167 using ECL Substrate (170-0561, BIO-RAD, United States). Membranes were incubated with a mouse
168 polyclonal anti-glyceraldehyde-3-phosphate deshydrogenase (GAPDH) antibody (Millipore MAB 374,
169 Darmstadt, Germany), or monoclonal anti-β-tubulin I mouse antibody (sigma) for normalization purposes.

170 **Detection of senescence associated β-galactosidase activity in aorta**

171 After sacrifice, SA-βgal activity was revealed by blue staining using the chromogenic substrate 5-bromo-4-
172 chloro-3-indolyl-β-D-galactopyranoside (X-gal) at pH 6.0. Analysis of images was performed by comparing
173 color intensities after correction by background adjustment in unstained areas.

174 **Determination of mitochondrial ROS formation and sources**

175 *In situ* formation of ROS was assessed as previously described.[25] The redox-sensitive red fluorescent dye
176 dihydroethidium (DHE, 2.5 µM) was applied to 25 µm unfixed cryosections of the pancreas for 30 min at 37⁰C

in a light-protected humidified chamber. Sections were washed three times with PBS, mounted in DAKO, and cover-slipped. The sections were examined under a confocal microscope (Confocal Leica TSC SPE-Mannheim, Germany). To determine source of ROS, sections were incubated 30 min at 37°C before DHE staining with the specific inhibitors VAS-2870 (VAS, NADPH oxidase 10 μ M), N-nitro-L-arginine (L-NA, NO synthase inhibitors 300 μ M), Indomethacin (Indo, cyclooxygenases COX 10 μ M) and MRK (inhibitors of the mitochondrial respiration chain, myxothiazol 0.5 μ M + rotenone 1 μ M + potassium cyanide (KCN) 1 μ M).

Vascular reactivity

Vascular reactivity studies of secondary mesenteric artery rings were performed as described [26]. Briefly, the secondary mesenteric artery was cleaned of connective tissue, cut into rings (2–3 mm in length) and suspended in organ baths containing oxygenated (95% O₂, 5% CO₂) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.18, CaCl₂ 1.25, NaHCO₃ 25 and D-glucose 11, pH 7.4, 37°C) for the determination of changes in isometric tension. After equilibration and functional tests, rings were pre-contracted with phenylephrine (PE, 1 μ M) before construction of concentration-response curves to acetylcholine (ACh). In some experiments, rings were exposed to an inhibitor for 30 min before contraction with PE. Relaxations were expressed as percentage of the contraction induced by PE.[27]

Microparticles measurement and characterization

Platelet-free rat plasma (PFP) was obtained from blood by sequential centrifugation steps (15 min) at 500g, and supernatant further submitted to 13,000g for 5 min. The PFP was kept at -80°C. MPs measurement was performed using prothrombinase assay after capture onto annexin-5 as previously described. The assay allows extensive washing of insolubilized MPs, taking advantage of the high affinity of annexin-5 for the phosphatidylserine (Phtdser) exposed at MPs surface. MPs concentration was referred to as Phtdser equivalent, by reference to a standard curve made with synthetic vesicles of known amounts of PhtdSer.[28]

In addition, MPs cell origin was determined by replacing annexin-5 with specific biotinilated monoclonal antibodies, each directed against one typical CD: mouse IgG against rat CD61 for platelet (Biolegend, San Diego, CA, USA), anti-rat CD45 for leucocytes (Biolegend, San Diego, CA, USA), anti-rat CD54 for endothelial cells (Biolegend, San Diego, CA, USA), anti-rat CD245 for erythroid cells (BD Pharmingen, New Jersey, USA).

Image analysis

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206 For immunohistochemistry and immunofluorescence analysis, four sections from each pancreas sample were
207 taken every 100 μm , and the mean was calculated to cover all the islets's area. Images were analyzed by the
208 NIH Image J software.

209 **Statistical analyses**

210 Data are expressed as mean $\pm$ S.E.M. Mean values were compared using unpaired Student's t test was performed
211 for the comparisons of groups using GraphPad Prism version 6.01 for Windows™ (GraphPad Software, San
212 Diego, USA). $P < 0.05$ was considered statistically significant.

213 **Results**

214 **Size distribution and morphology of islets is altered in middle-aged rats**

215 Histological examination of pancreas sections from the young rats showed a normal islet morphology with a
216 regular, spherical shape surrounded by a thin collagen capsule, whereas the middle-aged rats islets showed an
217 altered shape with high fragmentation of the biggest islets into small irregular units surrounded by dense fibrous
218 tissue (Fig 1 A, B). Regardless of age, the size distributions ranged from 300 μm^2 to $>80000 \mu\text{m}^2$, though small
219 islets (300 μm^2 to 20000 μm^2) were significantly more abundant in middle-aged $92.7\% \pm 1.9$ compared to
220 young rats $86.8\% \pm 1.4$ ($P < 0.05$). The larger islets (20000 μm^2 to 40000 μm^2) appeared characteristic features of
221 young rats $8.6\% \pm 0.9$ vs. middle-aged $4.3\% \pm 1$ ($P < 0.01$) (Fig 1 C).

222 **Islet cell populations, plasticity, and activity**

223 Because PSA-NCAM was shown by Karaca et al. [29] as a specific marker of β -cell function and activity, we
224 investigated its expression in pancreatic tissue of both rat subsets by measuring the PSA-NCAM surface area.
225 PSA-NCAM staining remained restricted to the islet endocrine tissue in both young and aged pancreas, and the
226 labeling area was identical between the young 0.42 ± 0.01 and middle-aged rats 0.41 ± 0.01 . Double
227 insulin/glucagon immunolabelling showed a similar distribution of both β and α cells in the islets of young and
228 middle-aged rats (Fig 1 D). However, in middle-aged rats, the glucagon/islets surface ratio increased
229 significantly from $6.04 \pm 0.01 \%$ to $10.40 \pm 0.01 \%$ ($P < 0.05$) (Fig 1 E), while the insulin area/islets surface ratio
230 significantly decreased from $93.96 \pm 0.01 \%$ to $89.60 \pm 0.01 \%$ ($P < 0.05$) (Fig 1 F).

231 **IPGTT, lipid profile, and insulin levels are modified in middle-aged rats**

232 Compared with young rats, aged rats experienced a shift in glucose response, shown by IPGTT tests at 15-30
233 min (250 mg/dl shifting to 267 ± 16.7 vs 211 ± 6.7 mg/dl in young rats after 30 min) (Fig 2 A). In addition, values
234 of glycaemia remained elevated when compared to young rats (211 ± 6.7 to 267.3 ± 16.7 ($P < 0.01$)) after 30 min
235 and from 156 ± 5.4 to 199.8 ± 16.7 ($P < 0.05$) after 120 min intraperitoneal injection of glucose in young and

middle-aged rats respectively. Plasma glucose (Fig 2 B) and HDL levels (Table 1) remained unchanged. Conversely, insulin levels significantly increased in middle aged rats from 5.69 ± 0.47 to 7.05 ± 0.35 ng/ml ($P < 0.05$) (Fig 2 B), cholesterol from 1.59 ± 0.03 to 2.02 ± 0.19 mM ($P < 0.05$), and LDL-CHOL from 0.15 ± 0.01 to 0.21 ± 0.02 mM ($P < 0.05$). Triglycerides trended towards an increase in middle-aged rats (Table 1).

Senescence markers are over-expressed and oxidative stress increases with aging in rat pancreatic tissue

Because ROS production was reported to promote senescence, we measured ROS in pancreas cryosections and detected a 2-fold increase in middle-aged compared to young rats (Fig 3 A). ROS staining was homogenous and detected in the whole pancreatic tissue. Pharmacological inhibitors applied on pancreatic sections revealed that the major sources of ROS in middle-aged rat were the NADPH oxidase and uncoupled NO synthase (Fig 3A). Furthermore, a 30% decrease in eNOS expression was demonstrated by western-blot in the pancreas lysates from middle-aged compared to young rats (Fig 3 B). Interestingly, no modification in activated caspase-3 levels could be established between young and middle-aged samples, thereby excluding apoptosis induction in the older pancreas (Fig 3 C). In addition, a significant 4-fold up regulation of TF expression was evidenced, suggesting enhanced thrombogenicity and inflammation (Fig 3 D).

Senescence markers were significantly up regulated among aged individuals with a doubled p53 expression, along with a seven-fold increase in cyclin-dependent kinase inhibitor protein p21, and a three-fold rise in its downstream p16 effector, as previously demonstrated [30] (Fig 3 E). Interestingly, at sites of branched aorta vessels, where blood flow disturbance favors reduced endothelial NO formation, premature senescence was only observed in middle-aged rats, using the specific SA- β gal activity probe after 24 h incubation with the chromogenic blue X-gal substrate (Fig 3 F).

Endothelial-dependent vascular reactivity and circulating MPs level were unmodified in middle-aged rats

Acetylcholine caused similar concentration-dependent relaxations in mesenteric arterial rings from young and middle-aged rats (Fig 4 A). The NO component of the relaxation was assessed in the presence of indomethacin and charybdotoxin plus apamin to prevent the formation of vasoactive prostanoids and Endothelium-Derived Hyperpolarizing (EDH) (Fig 4 B); the EDH-mediated component of the relaxation was assessed in the presence of indomethacin plus L-NA (an inhibitor of eNOS) (Fig 4 C). In both cases, no variation between the 2 groups could be established. In addition, the sodium nitroprusside, an NO donor, induced similar endothelium-independent relaxation in both groups (Fig 4 D). Phenylephrine-induced contraction reached identical ranges in mesenteric artery rings from young and middle-aged rats (Fig 4 E). Because premature senescence is not a feature of vascular tissues under condition of protective laminar flow, we assessed circulating MPs as plasma

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surrogates of an eventual vascular damage. In young and middle-aged rats, total MPs levels remained similar 5.08 ± 0.23 vs. 4.53 ± 0.43 nM PhtdSer EQ. respectively and no variation in the concentration of each MPs subpopulation could be observed for platelet, leucocyte, erythrocyte, or endothelial cell origin (Table 2).

Discussion

In this study, we report the first experiment aiming to simultaneously compare the development of senescence in pancreas and vascular tissues. We have found by studying islet morphology and function that larger islets were twice as frequent in young vs. middle-aged rats and that β -cells/ islets area ratio was decreased significantly with age. A shift in glucose response was shown by IPGTT at 15-30 min with age, together with a significant increase in plasma insulin, cholesterol and LDL concentrations. Altogether, our morphological and histological data were in accordance with other reports showing the impact of age on the ratio of α -cells to β -cell area.[31] Interestingly, our data demonstrate that in pancreatic tissue p53, p21 and p16 senescence markers were overexpressed, oxidative stress increased by ROS production and eNOS down-expression, in middle-aged rats. Although a significant overexpression of TF was measured in pancreas, no sign of vascular injury by MPs measurement, or of vascular dysfunction by contraction or relaxation studies in mesenteric artery rings, could be detected.

Our findings showed no difference in PSA-NCAM expression by β -cells between young and middle-aged rats, in accordance with the report of Gu et al. who could only detect a significant 50 % decrease in β -cell proliferation by PCNA labeling in 2 years old rats.[31]

Oxidative stress as an early inducer of senescence and endocrine dysfunction in the pancreas of middle-aged rats

Although our data showing early senescence in the pancreas of middle-aged rats were obtained by global assessment in the pancreas lysates, it is tempting to speculate that islet dysfunction is at least in part driven by senescence. Our data indeed confirm and extend the previous observation by Liu et al.[30] showing an up regulation of p16 in the isolated islets from adult rats and the presence of SA- β gal activity only in the endocrine pancreatic tissue. Since no apoptosis could be detected by active caspase-3 labeling in the pancreas lysates, senescence appears a mechanism specifically prompted in middle-aged rat pancreas.

Our data are in favor of an excessive production of ROS in middle-aged rat pancreas driven by NADPH oxidase that is not counter-balanced due to the reduced expression of functional eNOS. Therefore, excessive oxidative stress seems to trigger early senescence in the pancreas while no evidence of oxidative stress-driven endothelial dysfunction could be evidenced in macro-vessels such as mesenteric arteries.

The Pancreas serves as an early sensor of oxidative stress and senescence, before any sign of vascular injury in middle-aged rats

Our data on pancreatic function correlate with the abnormal islets morphology occurring in middle-aged rats. In accordance with the previous report by Liu et al.,[30] IPGTT assays showed an early alteration in glucose control, which we found concomitant with the occurrence of smaller, collagen-surrounded islets characterized by lower insulin and higher glucagon labeling, yet occurring to a small but significant extent. Furthermore, variations of glucose and insulin blood concentrations were highly suggestive of an early insulin resistance in middle-aged rats. Indeed, cholesterol and LDL levels were also significantly increased.

Most interestingly, while pancreas function was initially altered, vascular damage could not be evidenced in middle-aged rats when assessed in mesenteric arteries. In particular, accumulation of oxidative stress and the higher expression of thrombogenic and senescence markers, all widely recognized as a pattern of vascular dysfunction, were solely detected in the pancreatic tissue. Because early endothelial alteration could have remained limited and undetectable by vascular reactivity assays or Western blot, we also investigated circulating MPs as surrogates of vascular damage or thrombogenicity. Again, no significant variation in MPs levels from endothelial platelets or leucocyte origin could be shown between young and middle-aged rats.

However, despite an absence of vascular dysfunction in mesenteric arteries, we found evidences of senescence in the branching areas of middle-aged rat aortas suggesting that at that stage, the damage remained limited to vascular areas with flow disturbances.

This observation is of prime importance for pancreas transplantation and of particular impact in the context of islet transplantation, owing to the limited availability of organs. Indeed, in the latter case, allocated islets are generally isolated from elder grafts with presumed higher tissue damage and oxidative stress with possible impact on the 3 years post-transplantation insulin independence.[32]

Our data suggest that oxidative stress and consecutive senescence may become a therapeutic target in islet graft pre-conditioning, where multiple cellular lineages, like endothelial progenitor cells, contribute to islet protection [33] or constitute a local immunoprivileged site, like mesenchymal stem cells.[34] Further studies on the underlying mechanisms specifically favoring the early development of senescence in the pancreas of middle-aged individuals and to link cellular senescence to the pathogenesis of diabetes are needed, where many studies have showed that senescent cell burden is increased in tissues.[35]

In islet and pancreas transplantation, senescence would favor the progression of age-related dysfunction while in diabetes a senescent cell burden was shown.[10,36] Therefore senescent cell clearance could have major

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benefits to human healthspan and lifespan,[37] by halting their systemic effect and the progression of diabetes-induced tissue damage, and by improving insulin sensitivity.[38] Indeed, shared mechanisms between the development of insulin resistance and cellular senescence indicate that perhaps the therapeutic clearance of senescent cells, could ameliorate the complications of diabetes by protecting the microenvironment of aging tissues and their regenerative potential. The development of new protective strategies during islets transplantation and at the onset of diabetes will further rely on the identification of senescence pathways and on the characterizations of reliable sensors of islets senescence.

In summary, our data indicate that at least in the rat model, pancreas is an early sensor of oxidative stress before any sign of vascular injury, and that middle-aged healthy individuals present abnormal islets morphology and early signs of endocrine dysfunction and senescence.

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Figures legends:

Figure 1: Islets morphology, Size distribution and glucagon and insulin-secreting cells area are altered in middle-aged rats: (A) Morphological analysis of islets structure was determined on formaldehyde-fixed 4 μm thick paraffin sections after haematoxylin-eosin staining (scale bar=500 μm). (B) Accumulation of collagen was evidenced using Gomori's trichrome (scale bar=500 μm) at 10 x. (C) Size range distribution was determined by measurement of total islet area every 20000 μm^2 . Four sections for each sample were taken (section every 100 μm). (D) The distribution of glucagon and insulin-secreting cells was analyzed on 4 μm paraffin sections following double labeling with fluorescent anti-insulin and anti-glucagon antibodies; cell nuclei were revealed with blue DAPI staining (magnification 40 x). The ratio of glucagon (E) and insulin (F) areas per islet area and quantification was performed using Image J software, results expressed as mean $\pm$ SEM of 8 rats per group, * $p<0.05$, ** $p<0.01$.

Figure 2: IPGTT and Plasma glucose and insulin concentrations: (A) Intraperitoneal glucose tolerance test IPGTT: glucose was measured in blood samples obtained from the tail vein using a glucometer in fasting rats. (B) Plasma glucose concentration was assessed by Hexokinase Spectrophotometry method. Insulin concentration was established by ELISA kit (Millipore cat# EZRMI-13K) using samples drawn by heart puncture at the time of sacrifice. Results expressed as mean $\pm$ SEM of 5-8 rats per group, * $p<0.05$, ** $p<0.01$.

Figure 3: Expression of oxidative stress and senescence in pancreatic and aorta tissues: (A) In situ ROS accumulation measured by fluorescent immunohistochemistry using the redox-sensitive probe Dihydroethidium and sources of ROS investigated by Pharmacological inhibition for 30 min using VAS-2078 (NADPH oxidase), L-NA (NO synthase inhibitor), Indomethacyn (Cyclooxygenases COX) and MRK (inhibitors of Mitochondrial respiration chain) incubated on section by confocal laser-scanning microscope (Confocal Leica TSC SPE) magnification 20 x, (B) Markers of oxidative stress: eNOS, (C) of apoptosis: cleaved caspase-3, (D) of inflammation: TF, (E) of senescence: p53, p21, and p16 were measured by Western blot (lower panel

representative blot, upper panel: cumulative data). (F) SA- β gal enzymatic activity measured by staining aortas with the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) at pH 6.0. Quantification of red fluorescence as well as that of Chemiluminescence as a ratio of protein of interest / housekeeping protein levels (Gapdh or β -Tubulin) performed by Image J software, Results expressed as means $\pm$ SEM of 5-8 rats per group, * $p < 0.05$, ** $p < 0.01$.

Figure 4: No variation in vascular reactivity studies on the secondary mesenteric artery: Concentration-relaxation curves to acetylcholine in mesenteric artery rings with endothelium in young and middle-aged rats. (A) The global relaxation in presence of acetylcholine, (B) The NO component of the relaxation was assessed in the presence of indomethacin (10 μ M) and apamin plus charybdotoxin (100 nM each), (C) the EDH component in the presence of indomethacin and L-NA (300 μ M), (D) Relaxations resulted from sodium nitroprusside (an exogenous donor of NO) and (E), contraction occurred in presence of phenylphrine. Results are shown as mean $\pm$ SEM of 5-8 different rats.

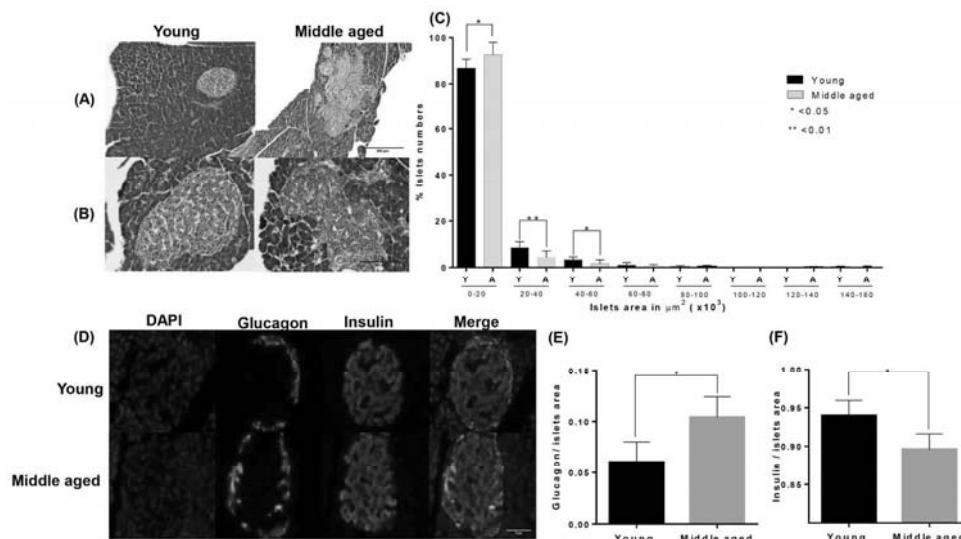
Tables legends:

Table 1: Metabolic markers in rats at time of sacrifice

HDL: High-density lipoprotein, **LDL:** Low-density lipoprotein. Results expressed as mean $\pm$ SEM of 5-8 rats per group, * $p < 0.05$

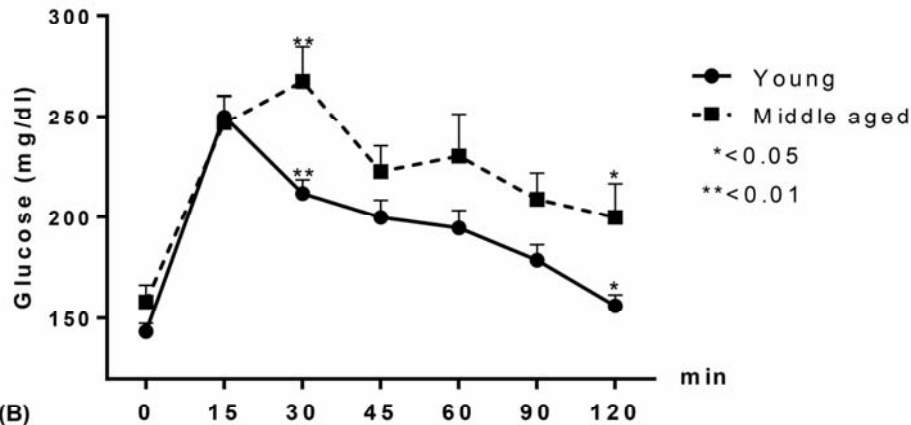
Table 2: Characterization of circulating MPs in young and middle-aged rats

CD61 (Platelet), CD 45 (Leucocyte), and CD 54 (Leucocyte and endothelial), CD Erythrocyte. Results expressed as means $\pm$ SEM of 8 rats per group

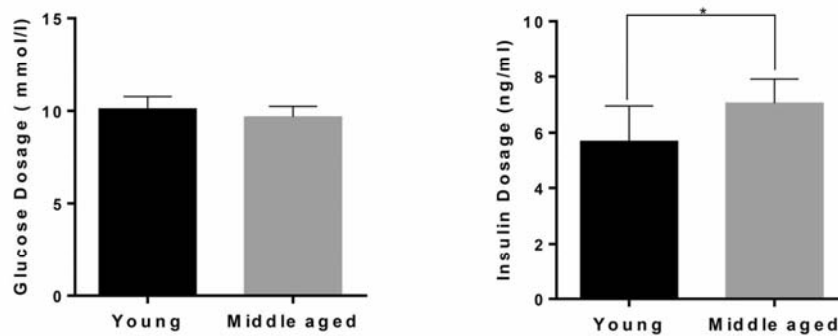


Islets morphology, Size distribution and glucagon and insulin-secreting cells area are altered in middle-aged rats: (A) Morphological analysis of islets structure was determined on formaldehyde-fixed 4 μm thick paraffin sections after haematoxylin-eosin staining (scale bar=500 μm). (B) Accumulation of collagen was evidenced using Gomori's trichrome (scale bar=500 μm) at 10 x. (C) Size range distribution was determined by measurement of total islet area every 20000 μm^2 . Four sections for each sample were taken (section every 100 μm). (D) The distribution of glucagon and insulin-secreting cells was analyzed on 4 μm paraffin sections following double labeling with fluorescent anti-insulin and anti-glucagon antibodies; cell nuclei were revealed with blue DAPI staining (magnification 40 x). The ratio of glucagon (E) and insulin (F) areas per islet area and quantification was performed using Image J software, results expressed as mean $\pm$ SEM of 8 rats per group, * $p<0.05$, ** $p<0.01$. 268x155mm (300 x 300 DPI)

(A) Intraperitoneal Glucose Tolerance Test (IPGTT)

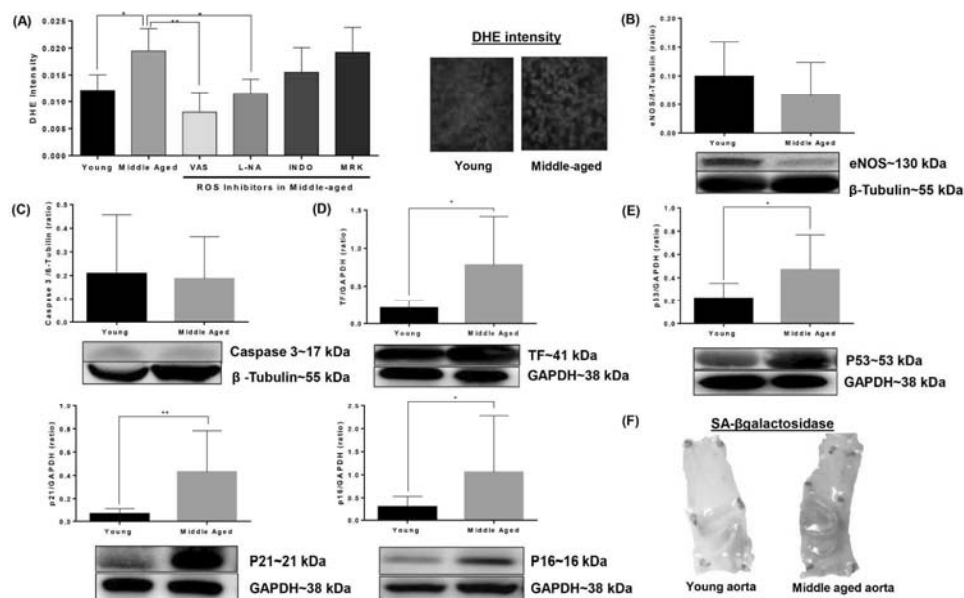


(B)

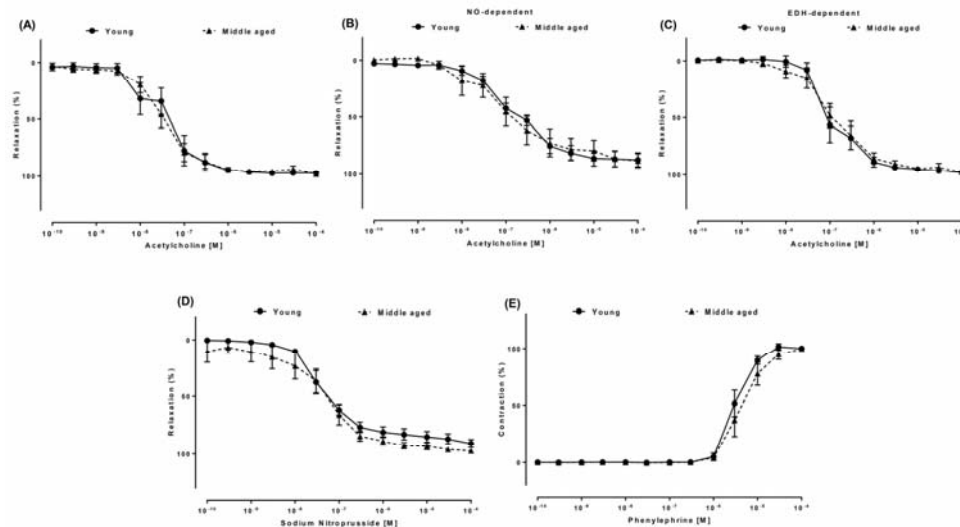


IPGTT and Plasma glucose and insulin concentrations: (A) Intraperitoneal glucose tolerance test IPGTT: glucose was measured in blood samples obtained from the tail vein using a glucometer in fasting rats. (B) Plasma glucose concentration was assessed by Hexokinase Spectrophotometry method. Insulin concentration was established by ELISA kit (Millipore cat# EZRMI-13K) using samples drawn by heart puncture at the time of sacrifice. Results expressed as mean $\pm$ SEM of 5-8 rats per group, * $p < 0.05$, ** $p < 0.01$.

157x139mm (300 x 300 DPI)



Expression of oxidative stress and senescence in pancreatic and aorta tissues: (A) In situ ROS accumulation measured by fluorescent immunohistochemistry using the redox-sensitive probe Dihydroethidium and sources of ROS investigated by Pharmacological inhibition for 30 min using VAS-2078 (NADPH oxidase), L-NA (NO synthase inhibitor), Indomethacyn (Cyclooxygenases COX) and MRK (inhibitors of Mitochondrial respiration chain) incubated on section by confocal laser-scanning microscope (Confocal Leica TSC SPE) magnification 20 x, (B) Markers of oxidative stress: eNOS, (C) of apoptosis: cleaved caspase-3, (D) of inflammation: TF, (E) of senescence: p53, p21, and p16 were measured by Western blot (lower panel representative blot, upper panel: cumulative data). (F) SA-βgal enzymatic activity measured by staining aortas with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) at pH 6.0. Quantification of red fluorescence as well as that of Chemiluminescence as a ratio of protein of interest / housekeeping protein levels (Gapdh or β-Tubilin) performed by Image J software, Results expressed as means ± SEM of 5-8 rats per group, * p<0.05, **p<0.01. 299x190mm (300 x 300 DPI)



No variation in vascular reactivity studies on the secondary mesenteric artery: Concentration-relaxation curves to acetylcholine in mesenteric artery rings with endothelium in young and middle-aged rats. (A) The global relaxation in presence of acetylcholine, (B) The NO component of the relaxation was assessed in the presence of indomethacin ($10 \mu\text{M}$) and apamin plus charybdotoxin (100 nM each), (C) the EDH component in the presence of indomethacin and L-NA ($300 \mu\text{M}$), (D) Relaxations resulted from sodium nitroprusside (an exogenous donor of NO) and (E), contraction occurred in presence of phenylphrine. Results are shown as mean $\pm$ SEM of 5-8 different rats.
190x107mm (300 x 300 DPI)

Table 1: Metabolic markers in rats at time of sacrifice

Plasma analysis (mmol/l)	Young	Middle-aged
Cholesterol	1.59 ± 0.03	2.02 ± 0.19*
HDL	0.40 ± 0.01	0.41 ± 0.04
LDL	0.15 ± 0.01	0.21 ± 0.02*
Triglycerides	1.00 ± 0.14	1.26 ± 0.13

Table 2: Characterization of circulating MPs in young and middle-aged rats

Plasma MPs Concentration (nM Phtdser EQ.)	Young	Middle-Aged
CD 61 +	1.23 ± 0.11	1.03 ± 0.18
CD 45 +	0.68 ± 0.09	0.61 ± 0.13
CD 54 +	0.43 ± 0.04	0.36 ± 0.06
CD Erythrocyte	0.36 ± 0.05	0.26 ± 0.08
Total MPs	5.08 ± 0.23	4.53 ± 0.43

The omega-3 EPA:DHA 6:1 formulation prevents the monocrotaline-induced pulmonary hypertension, endothelial dysfunction and vascular remodeling in rats

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Short title: The EPA:DHA 6:1 prevents the monocrotaline-induced pulmonary hypertension in rat

ABBREVIATIONS : Ach, acetylcholine; CO, cardiac output; COX, cyclooxygenase; DHA, docosahexaenoic acid; DHE, dihydroethidium; eNOS, endothelial nitric oxide synthase; EPA, eicosapentaenoic acid; ET-1, endothelin-1; ET_{A/B}, ET receptor A and B; MCT, monocrotaline; mPAP, mean pulmonary arterial pressure; PAAT, pulmonary artery acceleration time; PAH, pulmonary arterial hypertension; PHE, phenylephrine; PVR, pulmonary vascular resistance; ROS, reactive oxygen species; RVSP, right ventricular systolic pressure and VTI, velocity time integral.

ABSTRACT

Background: Pulmonary arterial hypertension (PAH) is characterized elevated pulmonary arterial resistance leading to right heart failure. Proliferation of pulmonary arterial smooth muscle cells, endothelial dysfunction, oxidative stress and inflammation promote the development of pulmonary hypertension. Omega-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acids (DHA) have been shown to protect the cardiovascular system and reduce inflammation and oxidative stress. The possibility that EPA:DHA 6:1 a superior omega-3 formulation prevents pulmonary arterial and right ventricular remodeling and dysfunction was evaluated an experimental model PAH.

Methods: Male Wistar rats received 500 mg/kg/day of either EPA:DHA 6:1 or corn oil by daily gavage. After one week, PAH was induced by a single subcutaneous injection of monocrotaline (MCT, 60 mg/kg). After three weeks, cardiac function and morphology were assessed by echocardiography, pulmonary artery reactivity using organ chambers, vascular morphometry by histology, proteins level by immunofluorescence and western blot, and oxidative stress using dihydroethidium.

Results: MCT treatment was associated in the pulmonary artery with a significant increased mean pulmonary arterial pressure (mPAP), vascular resistance, and blunted endothelium-dependent relaxations to acetylcholine, in pulmonary arterioles with increased wall thickness and oxidative stress, and in the heart with increased RV systolic pressure (RVSP), RV hypertrophy and a reduced cardiac output (CO). Compared to the MCT group, the EPA:DHA 6:1 treatment prevented the MCT-induced changes in the morphology and pressure in the pulmonary artery and the RV, and also prevented the decreased CO. EPA:DHA 6:1 treatment also reduced the MCT-induced pulmonary artery endothelial dysfunction, and the level of oxidative stress in pulmonary arterioles. The MCT-induced vascular oxidative stress was significantly reduced by N-acetylcysteine, VAS-2870, N^G-nitro-L-arginine and indomethacin. The protective effect of EPA:DHA 6:1 was associated with the prevention of the MCT-induced upregulation of eNOS, angiotensin type 1 receptors, endothelin A and B receptors, COX-1 and COX-2, and the NADPH oxidase subunits (p22phox and p47phox) in pulmonary arterioles, and a reduced pulmonary infiltration of macrophages and lymphocytes.

Conclusion: The present findings indicate that the EPA:DHA 6:1 formulation has a vascular effect in PAH by preventing RV failure, pulmonary arterioles remodeling and dysfunction, and inflammation in lungs, most likely by preventing the NADPH oxidase-, COX- and uncoupled eNOS-mediated vascular oxidative stress.

KEY WORDS: Omega-3 . pulmonary vascular remodeling . right ventricular hypertrophy . inflammation . oxidative stress

Introduction

Pulmonary arterial hypertension (PAH) is defined by a mean pulmonary arterial pressure (mPAP) ≥ 25 mmHg at rest (1). It is a chronic and progressive lung disease characterized by pronounced small pulmonary artery remodeling leading to chronic elevation of pulmonary vascular resistance and subsequent right ventricular failure (1-3). It is also characterized by an endothelial dysfunction involving decreased NO and thrombosis (4). Moreover, oxidative stress and inflammatory responses have been shown to play a critical role in both human and experimental PAH (5-7). Indeed, pro-inflammatory cytokines including interleukin (IL)-1 β and IL-6 and intense lung perivascular infiltrates of macrophages and lymphocytes are observed in human idiopathic PAH (8, 9) and monocrotaline (MCT)-induced PAH (5, 10). High levels of oxidative stress are observed in pulmonary vascular lesions of patients with severe PAH as a consequence of tissue hypoxia (11), ischemia (12) and possibly also involving an inflammatory response (13, 14).

Several studies have reported that long chain polyunsaturated omega-3 fatty acids including eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acids (DHA, 22:6n-3) have a beneficial effect on the cardiovascular system (15-17). Their potential protective effects include antioxidant, anti-inflammatory, anti-proliferative and the endothelial formation of NO, a vasoprotective factor (18-22). Indeed EPA:DHA 6:1 a superior omega-3 formulation caused pronounced endothelium-dependent relaxations of porcine coronary artery rings, and increased the endothelial formation of NO subsequent to the redox-sensitive activation of the PI3-kinase/Akt pathway leading to the phosphorylation of eNOS at Ser 1177 (15, 22).

Therefore, the aim of the present study was to determine the ability of EPA:DHA 6:1 to prevent the development of PAH using MCT-induced PAH in rats. To test this hypothesis, we investigated the chronic efficacy of oral EPA:DHA 6:1 treatment in MCT-treated rats, and, if so, to characterize the underlying mechanism. The MCT-induced PAH is a well-established experimental model of PAH causing similar morphologic damage as that observed in humans with idiopathic PAH (23, 24). The MCT-induced PAH is associated with endothelial cell injury followed by an inflammatory response and vascular oxidative stress (25, 26).

Materials and methods

Animals

Experiments were conducted according to the European Union regulations (Directive 86/609 EEC) for animal experiments, and complied with our institution's guidelines for animal care and handling. This study was approved by the Strasbourg Regional Committee of Ethics in Animal Experimentation and with the French law for the protection of animals.

The monocrotaline (MCT) of pulmonary arterial hypertension

Adult male Wistar rats (200 to 220 g) were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and maintained in a temperature-controlled room with a 12:12 light-dark cycle. Rats were randomly divided into 4 groups: control group (n = 7), EPA:DHA 6:1 group (n = 7), MCT group (n = 13) and MCT + EPA:DHA 6:1 group (n = 13). Rats received daily by oral intake gavage 500 mg/kg/day of either EPA:DHA 6:1 (Pivotal Therapeutics, Inc Woodbridge, ON, Canada) or corn oil as control. One week after the beginning of gavage, rats received a single subcutaneous injection of either an isotonic saline solution (control groups) or MCT (60 mg.kg⁻¹). The pyrrolizidine toxic alkaloid MCT (Sigma Aldrich, Saint-Quentin Fallavier, France) was dissolved in 1 N HCl and neutralized to pH 7.4 with 1 N NaOH. Thereafter, the oral intake of either corn-oil or EPA:DHA 6:1 was continued for 3 weeks. Body weight was measured weekly to adjust the dose accordingly.

Echocardiographic and hemodynamic studies

Three weeks after MCT-injection, transthoracic two-dimensional, M-mode, and Doppler pulse wave images were obtained in rats anesthetized with sodium pentobarbital (50 mg/kg ip) to evaluate the progression of PAH. Both long- and short-axis views at the papillary muscle level and apical-4 chamber views were done with a Sonos 5500 (Philips, USA) equipped with 12-MHz sectorial transducer.

M-mode measurements

Pulmonary artery diameter is obtained in the parasternal long-axis. The left ventricular (LV) and right ventricular (RV) end-diastolic and end-systolic diameters were measured in the parasternal short axis view. The fractional area change (FAC) for the RV was measured at the apical 4-chamber view and calculated as [(end-diastolic - end-systolic area)/end-diastolic

area] (27). Cardiac output and stroke volume were obtained from the B-mode long axis according to Simpson's method (28).

Doppler imaging

Pulse-wave Doppler of pulmonary outflow was recorded in the parasternal long-axis view at pulmonary valve leaflets. In addition, to characterize the pulmonary outflow Doppler envelope, the pulmonary artery acceleration time (PAAT) and the velocity time integral (VTI) were measured. The VTI was obtained by tracing the outer edge of the pulmonary outflow Doppler profile. PAAT was measured from the time of onset of systolic flow to peak pulmonary outflow velocity. The tricuspid valve was used to determine the tricuspid regurgitation velocity (TR) with color flow and pulsed-wave Doppler in the apical 4-chamber view so that the tricuspid and mitral valves could be clearly visualized. If TR was observed, the transducer was aligned to achieve the maximal peak velocity. The RV systolic pressure was calculated using the peak TR velocity ($V_{\max}$) in the modified Bernoulli ($RVSP = 4 \times V_{\max}^2$) (29, 30). The pulmonary vascular resistance was calculated as $[PVR = V_{\max} \text{ (m/s)}/VTI \text{ (cm)}]$ of flow wave of pulmonary artery. Right atrial area from the 4-chamber apical view and the inferior vena cava diameter and collapsibility were measured. All measurements and calculated indexes are presented as the average of three cardiac cycles.

Hemodynamic Measurements and Tissue Preparation

Following echocardiography, the mean pulmonary arterial pressure (mPAP) was monitored with a heparinized saline catheter inserted into the RV through the jugular vein and placed in the lumen of the pulmonary artery. The systemic arterial blood pressure was monitored with a pressure catheter inserted into the femoral artery, and steady-state hemodynamic was recorded using a blood pressure transducer (EMKA Technologie, Paris, France).

Subsequent to hemodynamic measurements, rats were euthanized and the hearts and lungs were isolated, the left lungs lobe and some group of hearts were in fixated in 4% paraformaldehyde for 48 h for morphology study. The RV and right lungs were embedded in Tissue-Tek[®] O.C.T. and snap-frozen on liquid nitrogen and quantitative as immunofluorescence and Western blot analysis.

Assessment of right ventricular hypertrophy and remodeling

Subsequent to hemodynamic measurements, rats were euthanized and the hearts were isolated to separate the right ventricle (RV) wall from the left ventricle wall and the septum (LV + S) and then weighed. The RV hypertrophy was assessed by the weight ratio of RV to LV plus the septum [RV/LV + S] as a Fulton's index (Mam et al. 2010). Cardiomyocytes cross-sectional area and interstitial collagen content were determined in the RV and LV to determine cardiac ventricle tissue remodeling. Four μm sections of heart were stained with hematoxylin and eosin (H-E) or Gomori's trichrome to assess fibrosis. 50 cardiomyocytes in each ventricle per rat were determined on transversely cut myocardial.

Assessment of pulmonary arteriolar wall thickness

The left lung lobe from each rat was isolated, harvested and perfused via the trachea with a 4% paraformaldehyde solution, and then immersed in the fixative solution for 48 h. Following dehydration, lungs were embedded in paraffin blocks and cut into 4 μm thick sections. Lungs sections were stained with H-E for morphology analysis (Olympus camera and microscope). The images of terminal arterioles were captured with magnification 20X and measured using ImageJ Software. Pulmonary vascular remodeling was evaluated by determining the percentage wall thickness (%WT) in H-E stained sections and wall areas were measured in smooth muscle α -actin-stained sections (%WA). A minimum of 16 arterioles of comparable size ($< 50 \mu\text{m}$) per lung sections were examined for each group. The percent WT of pulmonary arterioles was calculated as follows: $\% \text{ WT} = [(2 \times \text{medial thickness}/\text{external diameter}) \times 100]$ and the percent wall area, $\% \text{ WA} = [(\text{vessel wall area}/\text{vessel lumen ratio}) \times 100]$.

Immunohistochemistry

The antibodies against smooth muscle α -actin (mouse monoclonal, 1:20000; Santa Cruz) to assess the degree of muscularization of small peripheral pulmonary arterioles, CD68 (mouse monoclonal [ED1], 1:1000; Abcam) for macrophages and CD3 (rabbit monoclonal [SP7], 1:100; Abcam) for lymphocytes staining were used for this study. Microwave antigen retrieval (10 mM citrate buffer, pH 6.0) of 4 μm paraffin sections was followed by incubation in blocking buffer. Endogenous peroxidases were blocked (3% H_2O_2 for 10 min) before incubation with a primary antibody overnight at 4°C. Thereafter, sections were incubated with

a secondary biotinylated antibody for 2h, and then streptavidin-biotin-peroxidase complex linked to HRP (Vectastain Elite ABC kit, Vector Laboratories, AbCys, Paris, France) for 30 min. VIP peroxidase substrate kit (Vector Laboratories) was used as chromogen. Sections were counterstained with methyl green, air-dried and cover slipped with Eukitt (Labonord, Templemars, France). Ten photographs were taken from each lung sample, and the fraction area occupied by the macrophages and the number of lymphocytes was evaluated from each photograph using the ImageJ software (National Institutes of Health, <http://rsweb.nih.gov/ij/>).

Immunofluorescence studies

Right lung lobes were embedded in Tissue-Tek[®] O.C.T. Compound (Sakura Finetek, Villeneuve d'Ascq, France), frozen in liquid nitrogen bath and cryosectioned at 14 μ m. Lung sections were first fixed with 4% paraformaldehyde, washed and treated with 5% bovine serum albumin in PBS containing 0.1% Triton X-100 for 1 h at room temperature to block non-specific binding. Lung sections were then incubated overnight at 4°C with an antibody directed against either eNOS (mouse monoclonal, 1/1000, Santa Cruz), angiotensin II type 1 receptor (rabbit polyclonal AT-1, 1/500; Santa Cruz), endothelin-1 type A and B receptors (rabbit polyclonal ET_A, ET_B, 1/1000; Abcam), cyclooxygenase (rabbit monoclonal COX-1 and COX-2, 1/1000, Abcam) or the NADPH oxidase subunits (p22^{phox} and p47^{phox}, 1/500, Santa Cruz). Sections were then washed with PBS, incubated with the secondary antibody (1/400, immunoglobulin G coupled to Alexa 488- or 633) for 2h at room temperature in the dark before being washed with PBS and mounted in Dako fluorescence mounting medium (Dako France SAS, Les Ulis, France) and cover-slipped. All samples for immunofluorescence studies were observed using a confocal laser-scanning microscope (Leica SP2 UV DM IRBE; Leica, Heidelberg, Germany) with a 20X magnification lens. Quantification of fluorescence levels was performed using the ImageJ software.

Determination of vascular oxidative stress

The redox-sensitive fluorescent dye dihydroethidium (DHE, 2.5 μ M) was applied onto 25 μ m unfixed lung cryosections for 30 min at 37°C in a light protected humidified chamber to determine the *in situ* formation of ROS. To characterize the source of ROS, sections were incubated with either N-acetylcysteine (NAC, antioxidant 1 mM), VAS-2870 (VAS, NADPH oxidase inhibitor, 10 μ M), N-nitro-L-arginine (L-NA, NO synthase inhibitor, 300 μ M), indomethacin (Indo, cyclooxygenase inhibitor, 10 μ M), or MRK (inhibitors of the

mitochondrial respiration chain (myxothiazol, 0.5 μ M + rotenone, 1 μ M + potassium cyanide (KCN), 1 μ M) for 30 min at 37°C before DHE staining. Sections were then washed three times, mounted in DAKO and cover-slipped. The level of fluorescence in each section was examined under a confocal laser-scanning microscope (Leica SP2 UV DM IRBE; Leica, Heidelberg, Germany) with a 20X magnification lens. Quantification of fluorescence levels was performed using the ImageJ software.

Evaluation of O_2^- and NO levels

The levels of NO and O_2^- in lungs was determined by electron paramagnetic resonance (EPR) in frozen tissues at liquid nitrogen temperature.

Nitric oxide (NO) determination

Lung samples were incubated for 30 min in Krebs-Hepes buffer containing bovine serum albumin (20.5 g/L), $CaCl_2$ (3 mM), and L-arginine (0.8 mM). NaDETC (3.6 mg) (DETC: diethyldithiocarbamate) and $FeSO_4 \cdot 7H_2O$ (2.55 mg) were separately dissolved under nitrogen gas bubbling in 10-mL volumes of ice-cold Krebs-Hepes buffer. These compounds were rapidly mixed to obtain a pale yellow-brown opalescent colloid $Fe(DETC)_2$ solution (0.4 mM), which was used immediately. The colloid $Fe(DETC)_2$ solution was added to the incubation solution of lungs for 45 min at 37°C. The level of NO was determined using a table-top x-band spectrometer Miniscope (Magnettech, MS200, Berlin, Germany). The quantification of the signal is based on the mean of the height (amplitude) of the three signals. Values are expressed in signal amplitude (amplitude, arbitrary units).

Superoxide anion (O_2^-)

Lung samples were allowed to equilibrate in deferoxamine-chelated Krebs-Hepes solution containing 1 hydroxy-3 methoxycarbonyl 2,2,5,5-tetramethylpyrrolidin (CMH, Noxygen, Germany, 500 μ M), deferoxamine (25 μ M), and DETC (5 μ M) under constant temperature (37°C) for 1 h. The reaction was stopped by freezing the samples in liquid nitrogen before EPR spectroscopy analysis. Values were expressed as arbitrary units per milligram weight of dried tissue (A/Wd).

Vascular reactivity studies

Vascular reactivity studies are performed in main pulmonary artery and secondary mesenteric artery rings. Briefly, the arteries were excised, carefully cleaned of connective tissue in Krebs bicarbonate solution and cut into rings (3 mm length). Rings were suspended in organ chambers containing oxygenated (95% O₂; 5% CO₂) Krebs bicarbonate solution (mM: NaCl 119, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.18, CaCl₂ 1.25, NaHCO₃ 25, and D-glucose 11, pH 7.4 at 37°C) for the determination of changes in isometric tension. After an equilibration period, rings were subjected to functional test before construction of either a concentration-contraction curve to phenylephrine, or a concentration-relaxation curve to acetylcholine on rings precontracted with phenylephrine (10 µM). The concentration-response curves were constructed both in absence or presence of an eNOS inhibitor (L-NA, 300 µM) to assess the role of the basal endothelial NO formation.

Statistical analysis

All values are expressed as the mean $\pm$ SEM of n different rats. Statistical analysis was performed using either a one-way or a two-way analysis of variance test (ANOVA), followed by Bonferroni's post-hoc test as appropriate using GraphPad Prism software (version 5.04 for Windows, GraphPad software, Inc., San Diego, CA, USA). A *P* value < 0.05 was considered to be statistically significant.

RESULTS

EPA:DHA 6:1 prevents the MCT-induced increase in mPAP and RVSP

Three weeks after MCT injection, hemodynamic parameters were monitored by echocardiography and right ventricular catheterization. MCT-treated rats consistently developed pulmonary hypertension associated with a significant increase in mPAP from 16 ± 0.3 to 35.2 ± 0.7 mmHg and RVSP from 17.4 ± 0.5 to 40.5 ± 1.9 mmHg (Fig. 1A, B). In addition, CO was significantly decreased in the MCT group compared with the control group (124 ± 7.4 to 67.7 ± 3.7 ml/min) (Figure 1C). Oral intake of EPA:DHA 6:1 significantly reduced mPAP to 29.6 ± 0.8 mmHg and RVSP to 31.9 ± 0.8 mmHg and also improved CO to 90.2 ± 4.4 mL/min (Fig. 1A, C).

The heart rate was slightly but significantly lowered from 392.7 ± 8.1 bpm to 333.2 ± 4.9 bpm by the MCT treatment and the mean systemic arterial pressure is not significantly different in the four groups (Fig. 1D, E).

EPA:DHA 6:1 prevents the MCT-induced RV remodeling and hypertrophy

RV morphology and function were evaluated by echocardiography. The MCT group presented a significant dilatation of the RV indicated by the increased RV area, end-diastolic and systolic diameters compared to the control group. The dilatation of the RV was associated with a decreased of the RV fractional area change (RVFAC %) (control vs MCT: 42.4 ± 1.6 vs 27.6 ± 3.1). Oral intake of EPA:DHA 6:1 prevented RV remodeling by improving RV morphology and function (Fig. 2A-D).

Right ventricular hypertrophy was assessed by the weight ratio of RV/(LV+S). The MCT group developed severe RV hypertrophy manifested by an RV/(LV+S) ratio of 0.54 ± 0.03 compared with 0.22 ± 0.01 in the control group (Fig. 2E). Similarly, the ratio of RV weight to body weight was significantly higher in the MCT-treated group compared to the control group. The marked MCT-induced RV hypertrophy was reduced significantly to 0.33 ± 0.01 by the EPA:DHA 6:1 treatment indicating that chronic oral intake of EPA:DHA 6:1 was able to prevent PAH-induced RV hypertrophy.

EPA:DHA 6:1 prevents the MCT-induced RV cardiomyocytes hypertrophy and macrophages infiltration

In the RV, representative H-E staining showed that the MCT treatment induced a significant cardiomyocytes hypertrophy as compared to the control group (Figure 3A). The RV cardiomyocytes hypertrophy was associated with enhanced perivascular infiltration of macrophages detected by anti-CD68 staining (Figure 3B). The EPA:DHA 6:1 treatment prevented the MCT-induced RV cardiomyocytes hypertrophy by restored RV area and reduced infiltration of macrophages (Fig. 3A, B). In contrast, in the left ventricle, cardiomyocytes size was similar in all group studied, and only a low level of infiltration of macrophages was observed.

EPA:DHA 6:1 prevents MCT-induced pulmonary arterial remodeling

Pulmonary vascular remodeling was assessed by pulse-wave Doppler of pulmonary outflow. MCT significantly increased the pulmonary artery diameter and pulmonary vascular resistance (control vs. MCT: 0.37 ± 0.03 vs. 0.84 ± 0.05 unite wood), indicating hypertrophy and stiffness of the pulmonary artery, and reduced the velocity-time integral (VTI) and pulmonary artery acceleration time (43.05 ± 2.39 vs. 26.06 ± 0.91 ms), indicating an increased pulmonary arterial pressure. EPA:DHA 6:1 treatment significantly prevented the MCT-induced changes on pulmonary artery diameter, PVR, PAAT, and pulsatility (Fig. 4). Indeed, to examine the level of pulmonary vascular remodeling, morphometric analysis was performed on lung tissue sections stained with H-E or α -actin. Quantitative morphometric analyses showed pulmonary arterioles remodeling in MCT group (Figure 5). The medial thickness of pulmonary arterial was markedly increased in arterioles with external diameter $< 50 \mu\text{m}$ in the MCT group (control vs. MCT: 25.9 ± 0.6 vs. 73.7 ± 0.5). The medial wall hypertrophy was accompanied with muscularization of small pulmonary arteries evidenced by enhanced α -actin staining (Fig. 5). The EPA:DHA 6:1 treatment significantly attenuated the MCT-induced increase in wall thickness and pulmonary arterioles muscularization. In addition, an increased collagen deposition in MCT group as assessed by Gomori's Trichrome, which was prevented by the EPA:DHA 6:1 treatment (Fig. 5).

To determine the perivascular inflammatory cell infiltration, immunohistochemical staining was performed in lung sections with CD68 for macrophages and CD3 for T lymphocytes. The MCT-treatment was associated with a significantly macrophages and lymphocytes infiltration

surrounding pulmonary remodeled arterioles. The EPA:DHA 6:1 treatment significantly prevented the MCT induced infiltration of macrophages and lymphocytes (Fig. 5B).

EPA:DHA 6:1 treatment prevents MCT-induced vascular oxidative stress involving several sources in pulmonary arterioles

Reactive oxygen species (ROS) have been proposed as a pathogenic mechanism underlying the vascular remodeling observed in MCT-induced PAH (5). The pulmonary vascular level of oxidative stress was assessed using the redox-sensitive fluorescent probe DHE. The MCT treatment increased the formation of ROS as indicated by the markedly increased the DHE fluorescence signal throughout the medial wall in comparison to the control group. These effects were significantly prevented by EPA:DHA 6:1 treatment (Figure 6A). In order to determine the source of ROS, lung sections were treated with different inhibitors major vascular sources of ROS. The MCT-induced formation of ROS in the pulmonary arterioles wall was significantly inhibited by N-acetylcysteine (NAC, antioxidant), VAS-2870 (an NADPH oxidase inhibitor and antioxidant), L-NA (an eNOS inhibitor), indomethacin (a cyclooxygenase inhibitor) and by a combination of inhibitors of the mitochondrial respiration chain (MRK: myxothiazol, rotenone and KCN) suggesting the involvement of NADPH oxidase, COXs, uncoupled eNOS and the mitochondrial respiration chain (Fig. 6B). The levels of NO^- and O_2^- in lungs was determined by electron paramagnetic resonance (EPR) in frozen tissues at liquid nitrogen temperature. The MCT treatment increased NO^- and O_2^- production in lungs. EPA:DHA treatment significantly prevented the increased of NO^- and O_2^- production (Fig. 6 C, D).

To obtain further evidence for a role of eNOS, NADPH oxidase and COXs. Their expression level was determined by immunofluorescence staining in the pulmonary arterioles. A significantly increased immunofluorescence signal of the NADPH oxidase subunits p22^{phox}, p47^{phox}, and of COX-1 and COX-2 was observed in pulmonary arterioles of the MCT group compared to the control group (Fig. 7). The EPA:DHA 6:1 treatment significantly reduced the MCT-induced stimulatory effect for p22^{phox}, p47^{phox}, COX-2 and COX-1 (Figure 7A). In addition, an upregulation of eNOS was observed in the MCT group which was prevented by the EPA:DHA 6:1 treatment (Fig. 7).

EPA :DHA 6 :1 prevents the expression of endothelin-1 receptors (ET_A, ET_B) and angiotensin II receptor on pulmonary arterioles wall.

It has been suggested that PAH is associated with the increased expression of endothelin-1 (31) and angiotensin II (32) may contribute to the arterioles remodeling. An increased immunofluorescence level of endothelin-1 receptors (ET_A, ET_B) and angiotensin II receptor (AT1) throughout the pulmonary arterioles wall was observed in the MCT group (Fig 7). The EPA:DHA 6:1 treatment prevents MCT-induced changes of ET_A, ET_B and AT1 expression in pulmonary arterioles (Fig. 7).

EPA:DHA 6:1 prevents MCT-induced endothelial dysfunction in the pulmonary artery

Vascular reactivity was performed in primary pulmonary artery rings to evaluate the endothelial function using organ chambers. The MCT treatment significantly reduced the contractile responses to phenylephrine, and also the acetylcholine-induced endothelium-dependent relaxation of pulmonary artery rings pre-contracted with phenylephrine. The concentration-response curves were constructed both in absence or presence of an eNOS inhibitor (L-NA) to assess the role of the basal endothelial NO formation. The MCT treatment increased basal nitric oxide (NO) production. The EPA:DHA 6:1 prevented MCT-induced endothelial dysfunction as indicated by the increased relaxation to Ach (Fig. 8).

DISCUSSION

The present study demonstrated that the optimized EPA:DHA 6:1 formulation prevents the development of PAH in MCT-treated rat. Since, rats were treated with EPA:DHA 6:1 one week before the induction of PAH, the findings indicate the capacity of the omega-3 products to prevent the disease and not to cure it. The MCT-induced PAH rat model has been widely used as an experimental model of PAH (33) and it is tough to the most similar animal model to the human form of the disease (34). It is characterized by pulmonary endothelial cell damage, associated with an inflammatory response and vascular oxidative stress. It also leads to small pulmonary arterial and RV remodeling and dysfunction (35, 36). Previous studies focused on the effects of omega-3 on the systemic circulation but provided little information on its pulmonary effects.

The presents findings indicate that daily oral treatment with EPA:DHA 6:1 significantly prevented an elevation of mean pulmonary arterial pressure, RV systolic pressure and improved cardiac output in rats model of MCT-induced PAH. EPA:DHA 6:1 treatment also prevented pulmonary arterioles remodeling and dysfunction, RV hypertrophy and dilation, attenuated oxidative stress and inhibited inflammation. Our studies show that EPA:DHA 6:1 exerts anti-inflammatory, antioxidant and antiproliferative effects in the pulmonary arteries, which may contribute to prevent pulmonary hypertension. Recent study showed that DHA therapy reduced mPAP in a rat model of hypoxia-induced PAH and this effect was linked with inhibition of pulmonary vascular remodeling (37).

Echocardiography is widely used in the evaluation of PAH in the rat MCT model (38). The echocardiography analysis of control and MCT groups indicates that oral treatment of EPA:DHA 6:1 prevented in the pulmonary arteries of the MCT-treated rats the elevation of vascular resistance and the reduction of pulmonary acceleration time compared to control rats, suggesting that this formulation has the beneficial effect of reducing the stiffness of the pulmonary artery. In addition, oral treatment with EPA:DHA 6:1 significantly prevented the MCT-induced RV dilatation as indicated by increased RV area and decreased RV fractional area change (RVFAC), suggesting a beneficial effect of EPA:DHA 6:1 to reduce RVSP. The FAC is an ideal echocardiography parameter reflecting RV dysfunction and disease severity in PAH (39). Oral treatment with EPA:DHA 6:1 prevent RV hypertrophy characterized by increased RV/(LV + S) ratio, cardiomyocytes hypertrophy and extracellular matrix changes with fibrosis.

Previous studies have been revealed that inflammation plays a key role in human PAH as well as in experimental models including MCT-induced PAH (7, 40). In response to injury and stress, a pronounced lung vascular inflammatory response is observed including macrophages, monocytes, lymphocytes and mast cells (41, 42) and they have been involved in the initiation of pulmonary vascular remodeling by matrix remodeling, collagen deposition, and vascular cell proliferation and migration in PAH (43, 44). These processes lead to increased pulmonary resistance and right heart failure. Consistent with these previous findings, a substantial increased number of CD68 (ED-1) and CD3⁺ positive cells were observed in the lungs of MCT-treated rats. In addition, an increased pulmonary arterial medial thickness and RV cardiomyocytes hypertrophy were observed and associated with an increased number of macrophages in perivascular intra-alveolar spaces, and of lymphocytes around the pulmonary arteries in the MCT-treated rats relative to the control group. Importantly, oral intake of EPA:DHA 6:1 significantly prevented the stimulatory effect of MCT on macrophages and T lymphocytes infiltration in the lung and right ventricle. Thus, these findings suggest that the EPA:DHA 6:1 formulation prevented MCT-induced PHA by reducing the inflammatory responses.

In addition to the inflammatory response, oxidative stress has been implicated in the pathogenic mechanism underlying the vascular remodeling and heart failure observed in pulmonary hypertension (14, 32, 45). Dysregulation of the pro-oxidant/antioxidant balance contributes to impair vascular tone and the pathological activation of anti-apoptotic and mitogenic pathways, leading to cell proliferation and obliteration of the vasculature (46). There is solid evidence in MCT-treated rats, that oxidative injury to the pulmonary vascular endothelium precedes pulmonary arterial smooth muscle cells proliferation and medial hypertrophy in the distal pulmonary vascular bed and the rise in pulmonary artery pressure. Small pulmonary arterioles and RV remodeling is associated with an increased inflammatory infiltrate and oxidative stress as indicated by high levels of ROS formation throughout the pulmonary arterioles wall of MCT-treated rats. The characterization of the cellular sources of ROS in the small pulmonary arterioles indicated the involvement of several sources including NADPH oxidase, uncoupled eNOS, cyclooxygenase (COXs) and the mitochondrial respiration chain. Moreover, an up-regulation of several pro-oxidant enzymes including NADPH oxidase subunits p22^{phox} and p47^{phox}, COX-1, and COX-2 is observed in the small pulmonary arteries of MCT-treated rats. Oral intake treatment of EPA:DHA 6:1 substantially attenuated the level of vascular oxidative stress and remodeling by reducing in arterioles as

well as the upregulation of the NADPH oxidase subunits p22^{phox} and p47^{phox}, COX-1 and 2 and eNOS expression in MCT-treated rat. Both *in vitro* and *in vivo* studies have shown that ROS promote cardiomyocyte hypertrophy as well as fibrosis (47) and also right ventricular failure in the MCT-induced PAH, (48). EPA:DHA 6:1 due to its antioxidant, anti-inflammatory and cardioprotective properties, improved right ventricular function as indicated by an increased CO and with inhibition of cardiomyocyte hypertrophy and fibrosis.

Endothelial dysfunction has been shown to play a key role in the development of PAH and results in a decrease of vasodilator and antiproliferative factors (prostacyclin, nitric oxide) and in an increase in vasoconstrictor and proliferative factors (endothelin [ET]-1) (49). Moreover, inflammation and oxidative stress have been shown to contribute to the MCT-induced endothelial dysfunction most likely by reducing the bioavailability of NO and oxidizing tetrahydrobiopterin, an essential cofactor for eNOS (5, 10). Although decreased endothelium-dependent relaxation was observed in the pulmonary artery (50). The present findings indicated that MCT-induced pulmonary hypertension is associated with an endothelial dysfunction in main pulmonary artery without any effect on systemic vascular functions. The MCT treatment significantly reduced the contractile response of pulmonary artery rings to α -adrenergic agonist PHE. Blockade of NO production by L-NA enhanced PHE potency in MCT-treated rats. In addition, ACh-induced endothelium-dependent relaxation was reduced in pulmonary arteries of MCT treated-rats. Although the reduced ACh relaxation in the PH rats could be due to increased oxidative stress and decreased NO bioavailability in MCT treated-rats. The decreased bioavailability of NO reduces the antiproliferative effects of NO and thus contributes to the increased of pulmonary vascular remodeling and resistance. Previous studies have shown in isolated pulmonary arteries a reduced responsiveness to endothelium-dependent vasodilators including ACh and A23187, whereas others have suggested an increased basal NO production in the MCT-treated rats from (51, 52). We have showed that inflammation and oxidative stress of the lung in PAH stimulates the eNOS uncoupled. Oral intake of EPA:DHA 6:1 prevented the MCT-induced the development of PAH, reversed vascular remodeling, reduced vascular inflammation and improved the endothelial function, as indicated by an improvement ACh-induced relaxation. Thus, it could be hypothesized that EPA:DHA 6:1-induced elevation of cGMP. It is possible that up-regulation of eNOS contributes to the therapeutic action of omega-3. Furthermore, EPA:DHA 6:1 has been shown to stimulate the endothelial release of NO and to improve

endothelial function subsequent to phosphorylation of PI3K/Akt/eNOS signaling pathway (22).

The endothelin (ET) system is activated in human pulmonary hypertension (PH) of various pathogenesises (53, 54). ET-1 could contribute to the development of human PH through its strong vasoconstrictive and promitogenic properties (55). Previous studies have shown enhanced ET-1-induced pulmonary vasoconstriction and vascular resistance in the MCT-PAH (56) and increased vasomotor tone in hypoxic PAH (5, 32). The present findings support a role for the ET-1 in the MCT-induced endothelial dysfunction. Indeed, an increased expression of ET_A and ET_B receptors was observed throughout the arterial wall of the MCT treatment group. The ET_A receptors are located on smooth muscle cells, where they mediate vasoconstrictive and proliferative effects (57). The ET_B receptor is the only subtype found predominantly on the vascular endothelium, where it promotes vasodilation through the release of nitric oxide and prostacyclin (31). There is also evidence that the ET_B receptor indirectly modulates ET-1 synthesis through negative feedback under the action of nitric oxide (53). They are also consistent with the fact that chronic bosentan, a non-selective ET-1 receptor antagonist reduced pulmonary hypertension in MCT-treated rats and prevented the MCT-induced endothelial dysfunction and oxidative stress (31, 58). Our data show that EPA:DHA 6:1 prevented the MCT-induced upregulation of the expression ET_A and ET_B receptors in MCT-induced PAH rats.

However, the mechanism explaining best the beneficial effect of omega-3 treatment to prevent pulmonary hypertension most likely includes its antioxidant, anti-inflammatory and endothelial protective properties. Two previous studies have demonstrated potential benefits of oral administration of MAG-DPA to reduce inflammation (decreased NF- κ B and p38 MAPK activation) and proliferation (reduction in MMP-2, MMP-9 and VEGF expression levels in lung tissue homogenates) of pulmonary artery smooth muscle cells in MCT-treated rats (59) and DHA to inhibit the development of hypoxic pulmonary hypertension *in vitro* and *in vivo* studies (60). The effects of EPA and DHA have not been investigated in PAH; however, these two fatty acids affect cell function differently (61, 62).

Moreover, the beneficial role of omega-3 in cardiovascular health is supported by its metabolism. Indeed, omega-3, unlike omega-6, decreases the production of pro-inflammatory cytokines and leukocyte reactive oxygen species, increase the synthesis of anti-inflammatory

cytokines, lead to the release of resolvins and modulate the activation of genes involved in the inflammatory process (63, 64). Furthermore, when administrated per os, omega-3 represents a stable compound that could serve as a precursor to generate proresolving (resolvins and protectins) molecules, which are known for their anti-inflammatory properties (65, 66).

Study limitation

This study indicates the ability of a formulation omega-3 to prevent MCT-induced PAH, in which inflammatory mechanisms related to pulmonary vascular endothelial dysfunction and remodeling may contribute to the development and progression of the pathology. A subsequent study will be necessary to determine the efficacy of omega-3 in the treatment of an established PAH.

CONCLUSION

The present findings indicate that EPA:DHA 6:1 exerts a significant anti-oxidant, anti-inflammatory and endothelial protective effect in the pulmonary circulation and prevents the progression of pulmonary hypertension in MCT-treated rats by reducing pulmonary vascular remodeling, endothelial dysfunction, right ventricular hypertrophy and failure. The beneficial effects were associated with a reduced vascular inflammatory responses and oxidative stress mostly by preventing overexpression of NADPH oxidases, COXs and also of uncoupled eNOS. Further investigations are necessary to better assess the biological effects, elucidate the underlying mechanisms and to evaluate the political of this omega-3 formulation in the clinical setting.

Conflict interests

The authors declare that they have no conflict of interest.

Authors' contributions

AS performed most of the experiments and analyzed data and wrote the manuscript. NZ and MB performed some experiments. AS, CA and VSK performed contributed to the discussion and wrote. RK, LM, NB and VSK designed the experiments and edited the manuscript.

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Table 1. Characteristics of the different omega-3 fatty acid products

Fatty acids	Purity (<i>in</i> %)	Ratio EPA:DHA	Content of Omega-3 EPA-DHA as EE		Sum Omega-3 as EE (mg/g)
			EPA (mg/g)	DHA (mg/g)	
EPA:DHA	93.4	01:01	476	386	934
EPA:DHA	91.3	06:01	694	121	913
EPA:DHA	46.0	06:01	352	65	460
EPA	99.5		991		995
DHA	98.6			945	986

Table 2. Echocardiography parameters of the inferior vena cava, the right atrium and the left ventricular.

	Control	Omega	MCT	MCT+OM
RA diameter (mm)	1.6 ± 0.1	1.8 ± 0.1	2.9 ± 0.2*	2.2 ± 0.1 [#]
RA area (mm ²)	9 ± 0.2	9.1 ± 0.1	13.3 ± 0.1*	10.2 ± 0.1 [#]
IVC diameter (mm)	1.6 ± 0.2	1.8 ± 0.2	2.7 ± 0.1*	2.4 ± 0.1
IVC systolic area (mm ²)	3.6 ± 0.7	3.6 ± 0.4	4.5 ± 0.3*	3.9 ± 0.2
LV Stroke volume (ml)	0.32 ± 0.02	0.31 ± 0.01	0.20 ± 0.01*	0.24 ± 0.01
LV End-diastolic (mm)	7.9 ± 0.1	7.8 ± 0.1	6 ± 0.1*	6.4 ± 0.1
LV End-systolic (mm)	4.3 ± 0.1	4.4 ± 0.2	3.6 ± 0.1	3.7 ± 0.2
Septal wall thickness (mm)	1.5 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	1.7 ± 0.1
LVP wall thickness (mm)	1.7 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	1.8 ± 0.1
LV area (mm ²)	53.3 ± 1.1	50.2 ± 3.4	35.1 ± 0.7	39.6 ± 2.0
LV fractional shortening (%)	45.3 ± 1.5	42.9 ± 2.2	39.4 ± 1.4	42.5 ± 2.4
LV ejection fraction (%)	70 ± 1.7	67.2 ± 2.4	62.1 ± 1.6	66.4 ± 2.9

*All values are mean ± SEM, n = 6-11/group. IVC, inferior vena cave, MCT, monocrotaline, LV, left ventricle; RV, right ventricle. *P < 0.05 versus control; [#]P < 0.05 versus MCT.*

Figure 1. Oral intake of EPA:DHA 6:1 prevents MCT-induced pulmonary hypertension and right heart dysfunction. (A) Mean pulmonary artery pressure (mPAP) was monitored by catheter inserted into the jugular vein and advanced to the pulmonary artery. (B) Right ventricular systolic pressure (RVSP) was evaluated by echocardiography from tricuspid regurgitation velocity ($RVSP = 4 \times V_{max}^2$). (C) Cardiac output (CO) and (D) heart rate (HR) were monitored by pulsed wave Doppler. (E) Systemic arterial pressure (SAP). All measurements are presented as the average of three cardiac cycles. Values are given as mean $\pm$ SEM. * $P < 0.05$ versus control, # $P < 0.05$ versus MCT.

Figure 2. The EPA:DHA 6:1 treatment prevents MCT-induced right ventricular remodeling and hypertrophy. (A) Echocardiographic views of the right ventricle. (B) RV end-diastolic (RVEDD), (C) end-systolic diameter (RVESD), (D) RV fractional area changes (RVFAC). Images were obtained by two-dimensional and M-mode echocardiography from a parasternal short-axis view. (E) RV hypertrophy was assessed by Fulton's index, calculated as RV to LV weight ratio $[RV/(LV+S)]$, (F) RV weight/body weight and (G) LV+S weight/body weight. Values are given as mean $\pm$ SEM. * $P < 0.05$ versus control, # $P < 0.05$ versus MCT.

Figure 3. The EPA:DHA 6:1 treatment prevents the MCT-induced RV cardiomyocytes hypertrophy and macrophages infiltration. (A) Quantitative morphometric analyses of cardiomyocytes section area were done using hematoxylin and eosin and gomori's blue trichrome staining of right and left ventricle walls. (B) Evaluation of macrophage infiltration was performed by quantitative analysis of the macrophages counted in 10 fields of immunohistochemical staining with CD68 antibody (20X, objective). Results are presented as representative micrographies (left) and corresponding cumulative data (right). Values are given as mean $\pm$ SEM. * $P < 0.05$ versus control, # $P < 0.05$ versus MCT.

Figure 4. The EPA:DHA 6:1 treatment prevents MCT-induced pulmonary artery remodeling. (A) Notching of the pulse wave Doppler profile in the pulmonary artery outflow, (B) pulmonary artery diameter, (C) pulmonary artery acceleration time (PAAT), (D) pulmonary vascular resistance (PVR) and (E) pulsatility. Pulse-wave Doppler of pulmonary outflow was recorded in the parasternal view at the level of the aortic valve. PAAT was measured from the time of onset of systolic flow to peak pulmonary outflow velocity. Values are given as mean $\pm$ SEM. * $P < 0.05$ versus control, # $P < 0.05$ versus MCT.

Figure 5. The EPA:DHA 6:1 treatment prevents pulmonary vascular remodeling, pulmonary macrophages and lymphocytes infiltration in MCT-treated rats. (A) Representative images of hematoxylin-eosin, gomori's blue trichrom and immunohistochemical (α -actin as a measure of the dedree of muscularization) staining of lung section in rats for determination of the wall thickness and wall area of small pulmonary arteries sized $< 20 \mu\text{m}$. Images are shown on the left and quantification analysis on the right. Sixteen pulmonary arteries from 6 rats/experimental group were analyzed ($n= 8$). (B) Immunofluorescent (*green*) and immunohistochemical (*purple arrows*) staining with antibodies against CD68 [ED-1] for detection of macrophages and CD3 for lymphocytes T, respectively. Quantification of positive cells was analyzed of the fraction area occupied macrophages and lymphocytes T were counted in 10 different fields. Scale bar, $20 \mu\text{m}$ (20X objective). Values are given as mean $\pm$ SEM. $*P < 0.05$ versus control, $^{\#}P < 0.05$ versus MCT.

Figure 6. The EPA:DHA 6:1 treatment prevents MCT-induced vascular oxidative stress in pulmonary arterioles. (A) The determination of the vascular formation of ROS formation was done in unfixed cryosections of the right lung using the redox-sensitive probe dihydroethidium (DHE). (B) The characterization of the source of ROS formation was performed in presence of either N-acetylcysteine (NAC, antioxydant 1 mM), VAS-2870 (NADPH oxidase inhibitor, $10 \mu\text{M}$), N-nitro-L-arginine (LNA, NO synthase inhibitor, $300 \mu\text{M}$), indomethacin (Indo, cyclooxygenase inhibitor, $10 \mu\text{M}$), or MRK (inhibitors of the mitochondrial respiration chain, myxothiazol, $0.5 \mu\text{M}$ + rotenone, $1 \mu\text{M}$ + potassium cyanide (KCN), $1 \mu\text{M}$) for 30 min before DHE staining. Thereafter, ethidium fluorescence was determined by confocal laser-scanning microscope (Leica SP2 UV DM IRBE). (C and D) Nitric oxide ($\text{NO}\cdot$) and superoxide anion ($\text{O}_2\cdot^-$) measured by electron paramagnetic resonance in small pulmonary arterioles. Values are given as mean $\pm$ SEM. $*P < 0.05$ versus control, $^{\#}P < 0.05$ versus MCT.

Figure 7. The EPA:DHA 6:1 treatment prevents MCT-induced changes of protein expression in pulmonary arterioles. Protein expression levels were determined in unfixed cryosections of right lung, the determination of the expression level of endothelial NO synthase (eNOS), NADPH oxidase subunits $\text{p}22^{\text{phox}}$ and $\text{p}47^{\text{phox}}$, cyclooxygenase (COX-1 and COX-2), ET-1 receptors (ET_A , and ET_B) and the AT1R was done by immunofluorescence. All samples for immunofluorescence studies were observed using a confocal laser-scanning microscope (Leica SP2 UV DM IRBE).

Figure 8. The EPA:DHA 6:1 treatment prevents MCT-induced endothelial dysfunction in the main pulmonary artery as assessed in organ chamber. The concentration-contraction curves in response to phenylephrine (A and B) and the concentration-relaxation curves to acetylcholine in pulmonary artery rings pre-contracted by phenylephrine (10^{-6} mol/L) (C and D) were constructed in the absence (A and C) and the presence (B and D) of eNOS inhibitor (L-NA, 300 μ M) to assess the role of the basal formation of endothelial NO. (n = 7 to 11). Values are given as mean $\pm$ SEM. * P < 0.05 versus control, # P < 0.05 versus MCT.

Figure 1.

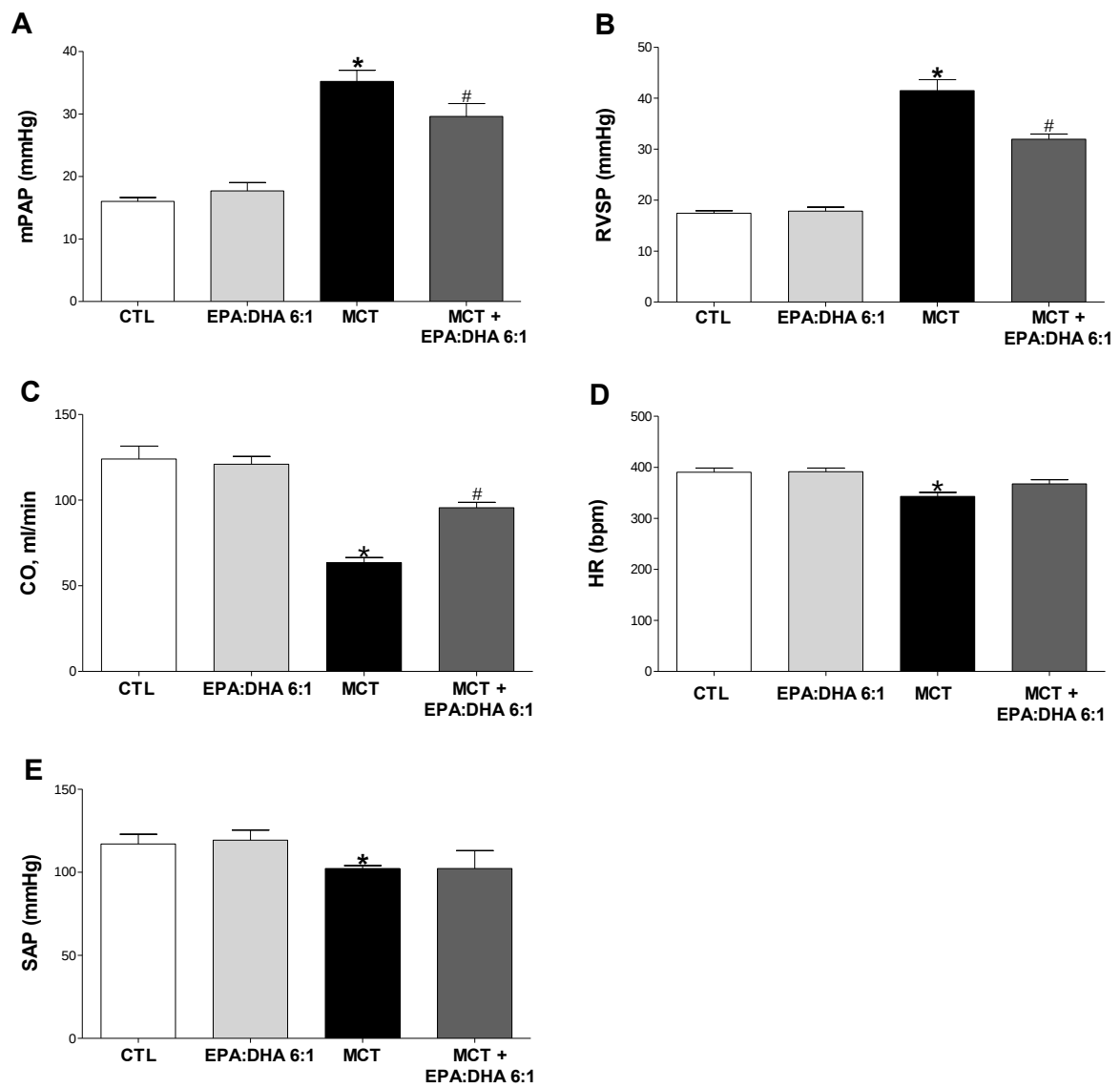


Figure 2.

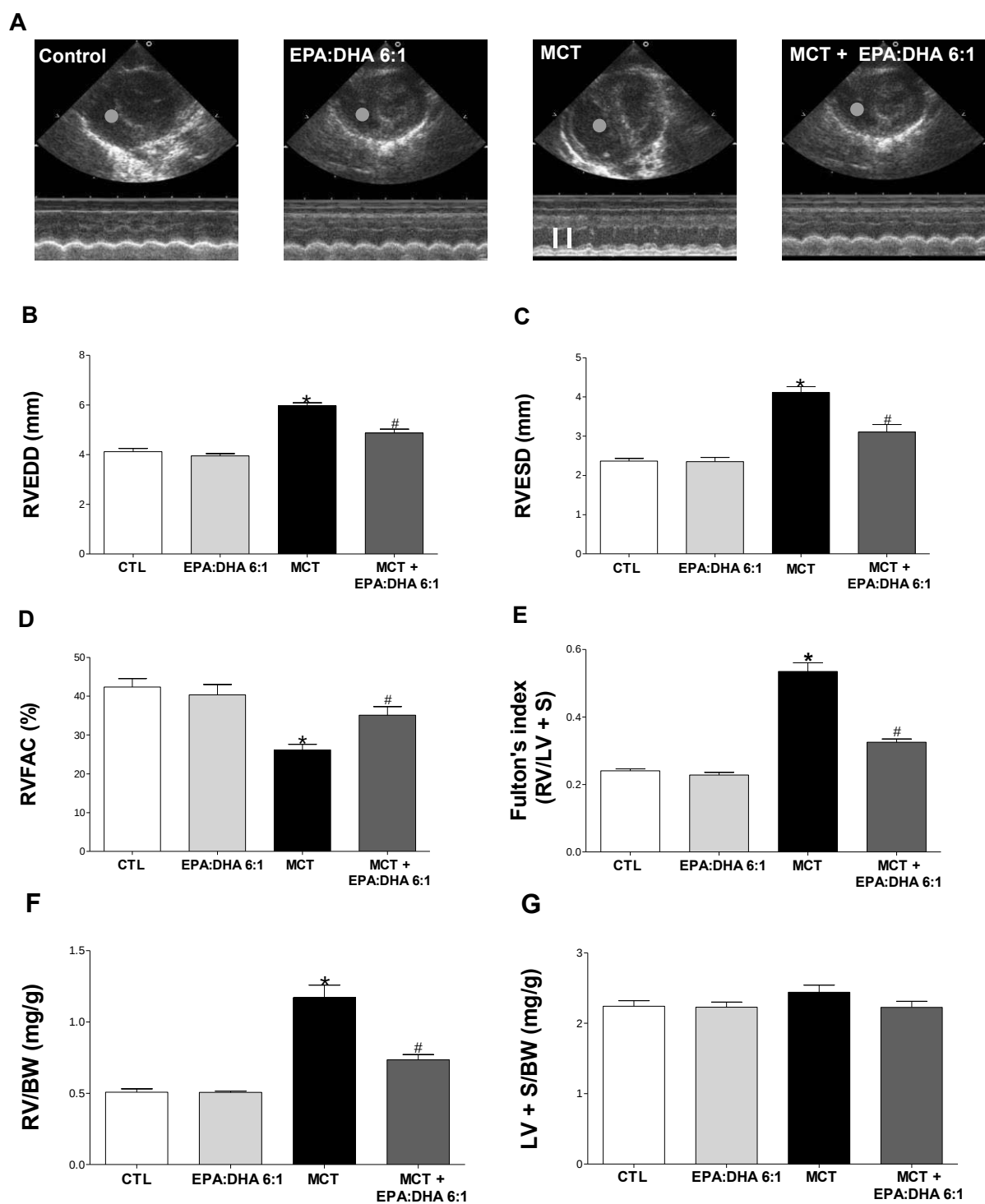


Figure 3.

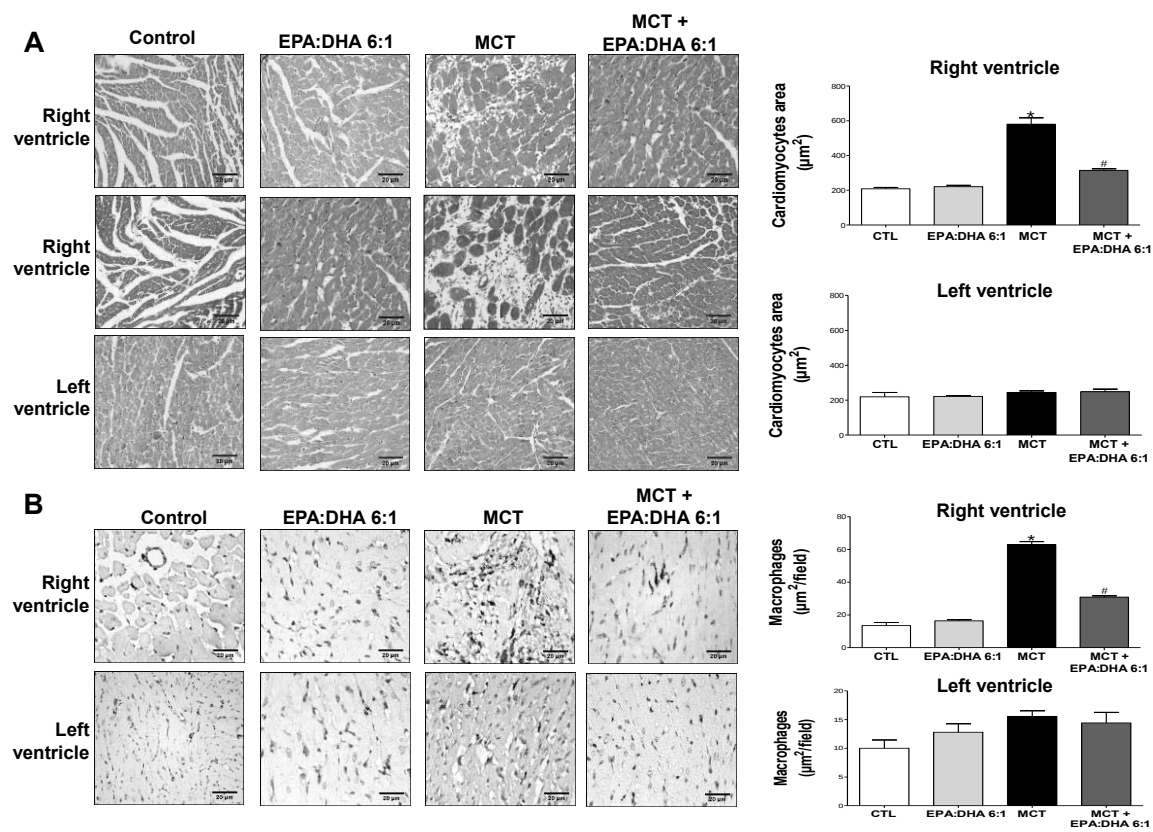


Figure 4.

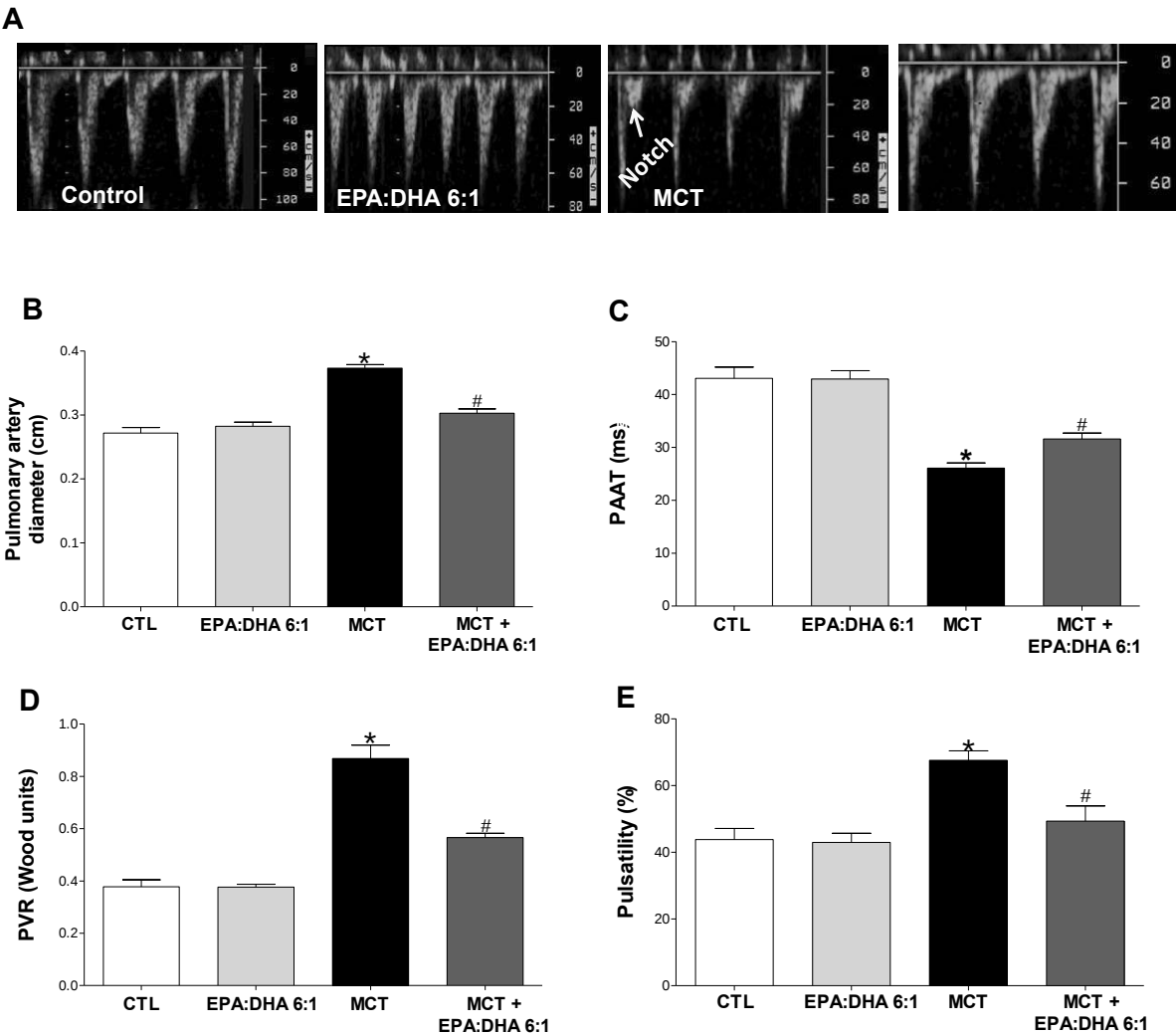


Figure 5.

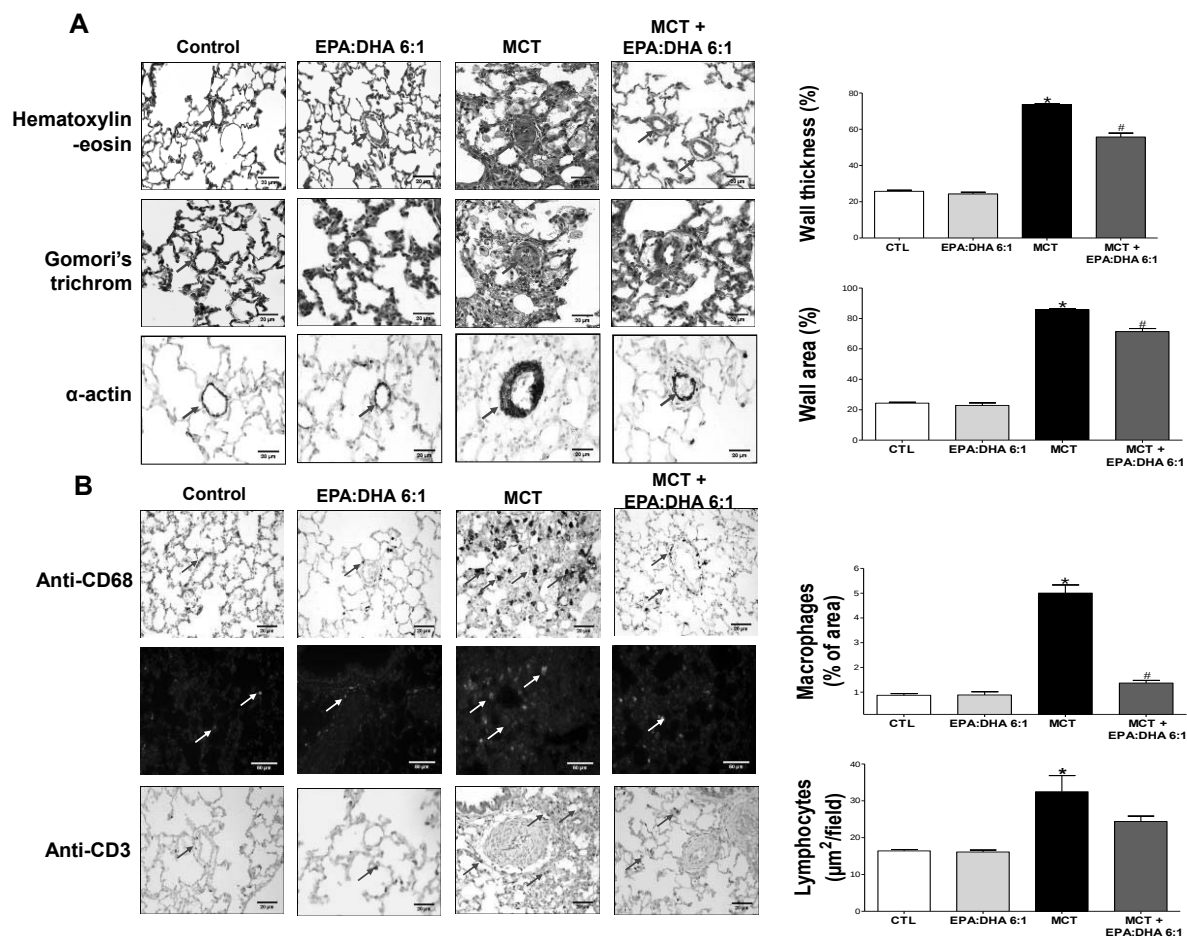


Figure 6.

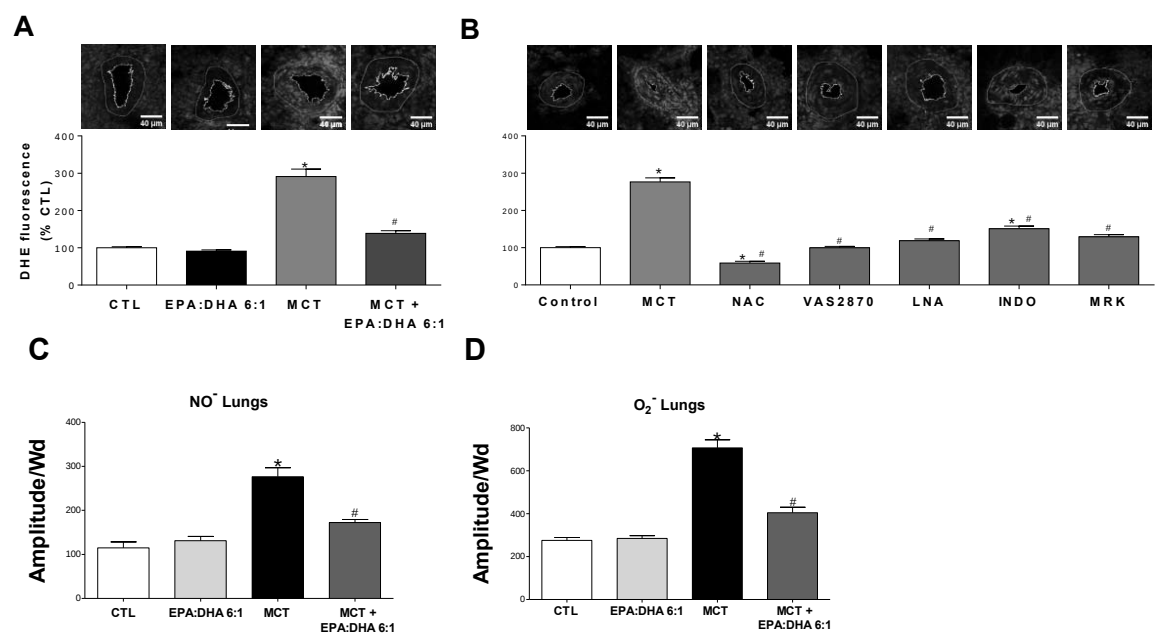


Figure 7.

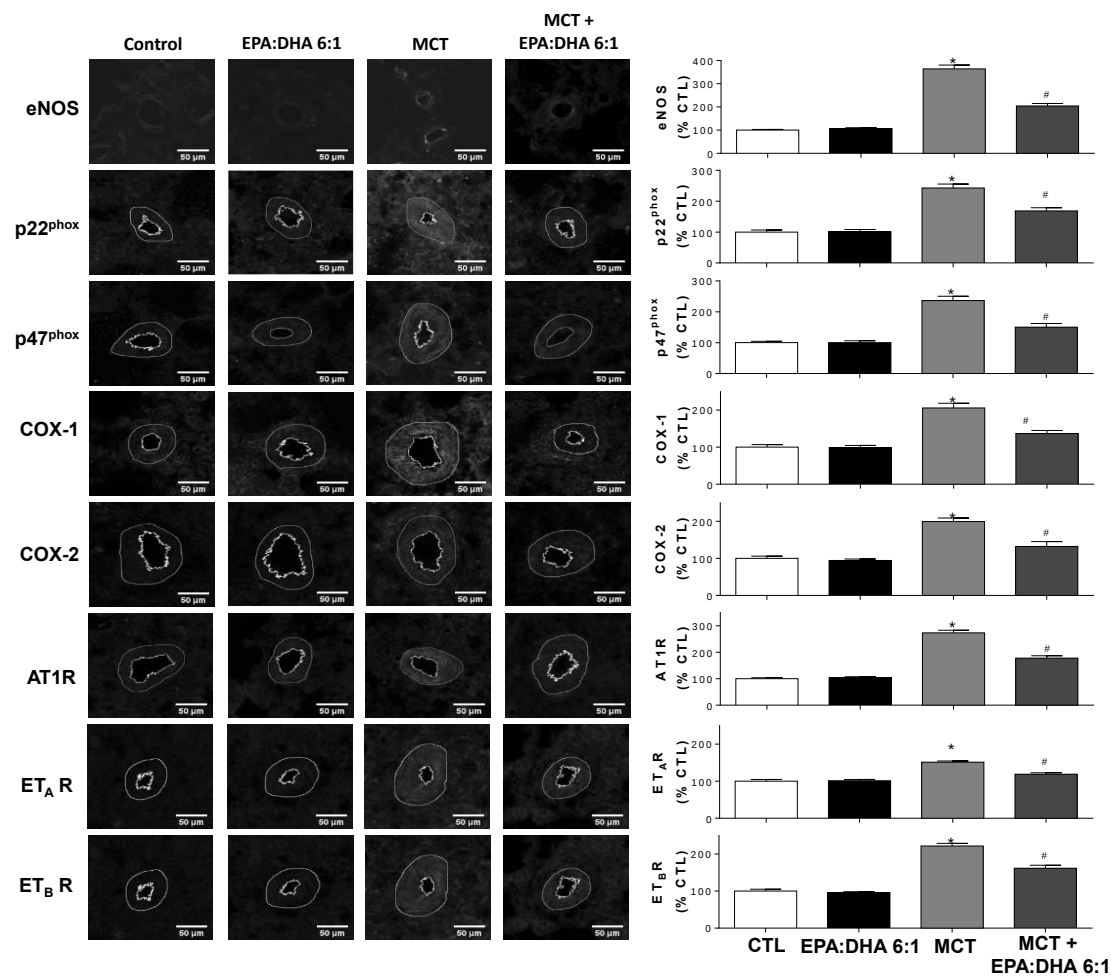
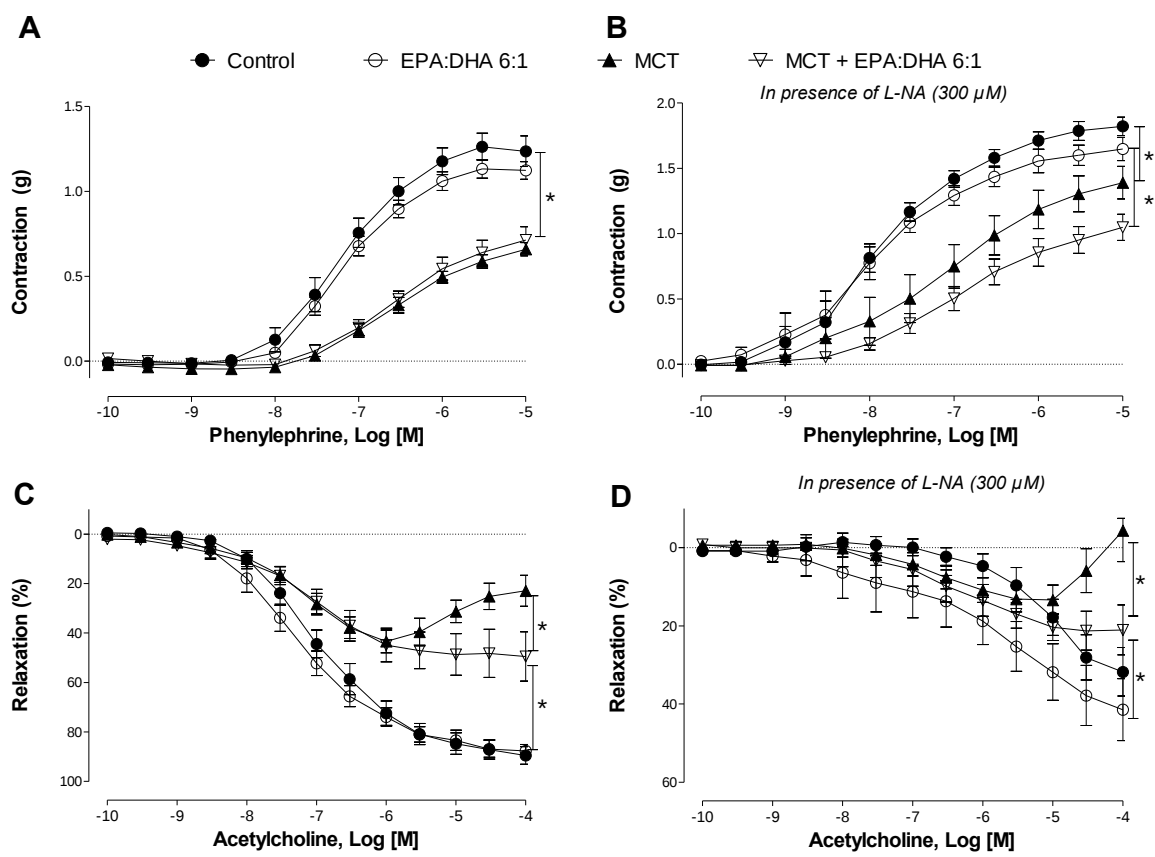


Figure 8.



Chronic treatment with an aqueous extract of *Phyllanthus amarus* prevents hypertension and endothelial dysfunction in DOCA-salt rats

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Abstract

Ethnopharmacology relevance: *Phyllanthusamarus*(Euphorbiaceae family) has been reported in traditional medicine to possess beneficial effects in the management of hypertension. In different animal models of hypertension, an impairment of the vascular function has been linked to an endothelial dysfunction. Therefore, the aim of the present study was to determine if an aqueous extract of *Phyllanthus amarus* (AEPA)obtained by decoction was able to prevent hypertension and endothelial dysfunction in DOCA-salt rats, and, if so, to clarify the underlying mechanism.

*Materials and methods:*Male Wistar rats were randomly assigned into the control group, the AEPA group (100 mg/kg/day, by gavage), the DOCA-salt group (50 mg/kg, s.c, per week), and the DOCA-salt + AEPA group (100 or 300 mg/kg/day, by gavage). DOCA-salt-treated rats were allowed free access to water containing 1% NaCl. Systolic blood pressure (SBP) was determined by tail-cuff plethysmography twice a week, in the morning during 5 weeks. Vascular reactivity using main mesenteric artery rings was assessed in organ chambers. Dihydroethidine (DHE) and immunofluorescence methods were used for the determination of the vascular formation of reactive oxygen species (ROS) and the expression level of proteins, respectively.

Results: After 5 weeks, SBP (mmHg) increased significantly in DOCA-salt hypertensive rats. It was significantly lowered by the treatment with AEPA (100 or 300 mg/kg/day) by 24 and 21mmHgrespectively. In mesenteric artery rings, the phenylephrine induced contractile response was increased significantly in the DOCA-salt group in comparison to the control group. After treatment by AEPA, the contractile response was shifted to the right. Both the NO-mediated (assessed in the presence of indomethacin and TRAM-34 plus apamin) and the endothelium-dependent hyperpolarization (EDH)-mediated (assessed in the presence of

indomethacin and N^ω-nitro-L-arginine) relaxation to acetylcholine were significantly reduced in the DOCA-salt group compared to the control group. Fluorescence study showed that the endothelial dysfunction was associated with a reduced expression level of Cx37, an increased expression of eNOS and the formation of ROS in main mesenteric artery. The antihypertensive effect of AEPA was related to an improvement of the blunted NO- and EDH- mediated relaxation, and an increased vascular oxidative stress and modulation of the expression levels of target proteins in DOCA- salt rats.

Conclusion: Altogether, our study shows that AEPA is able to act as an antihypertensive agent, and to prevent endothelial dysfunction in DOCA-salt hypertensive rats, in part, by preventing vascular oxidative stress.

Keywords: *Phyllanthusamarus*, DOCA-salt, hypertension, endothelial dysfunction,

Introduction

Hypertension is a major public health problem (WHO, 2013). In chronic situation, hypertension increases the risk factor for cardiovascular diseases including atherosclerosis, coronary disease, congestive heart failure and stroke (Kannel, 2000; Tsoetetsi et al., 2001; D'Agostino et al., 2008). For a better understanding of hypertensive disease physiopathology, several animal models such as spontaneously hypertensive rats (SHR), angiotensin II (Ang II)-induced hypertension, Dahl salt-sensitive hypertensive rats, DOCA-salt rats and other models are used as a mimetic of human essential hypertension (Galisteo et al., 2004; Wilcox and Pearlman, 2008). A DOCA-salt model is a characteristic of human volume-overload induced hypertension with sodium retention (Galisteo et al., 2004; Iyer et al., 2010), obtained through several weeks administration of a synthetic mineralocorticoid deoxycorticosterone acetate (DOCA) to rat allowed free access to water containing NaCl. This model is also associated to a low rennin and potassium-depleted (Galisteo et al., 2004; Liu et al., 2014).

Almost deleterious effects found in human hypertension are observed in DOCA-salt hypertensive rat as a model of endothelial dysfunction, cardiac hypertrophy and renal damage, and inflammation (Fenning et al., 2005). These phenomena are related to an excessive production of reactive oxygen species (ROS) by NADPH oxidase (O'Brien et al., 2010). Left ventricular hypertrophy is found in most animal models of hypertension (Perez-Vizcaino et al., 2009). Numerous studies have indicated that endothelial dysfunction in pathological models was linked to an impairment of endothelium-derived relaxing factors mediated-relaxations, such as NO and EDH component (Li et al., 2013; Rashid et al., 2014).

Hypertension management required the use of classic antihypertensive drug agents such as diuretics, beta-blockers, angiotensin converting enzyme inhibitors or angiotensin II type 1 receptor (AT1R) blockers, and calcium channel blockers. Since efficacy of antihypertensive

drugs was relative, in most patients, two or more drugs combined are required in antihypertensive therapy (Chobanian et al., 2003). Moreover, chemical drugs cause a risk of side effects. During the last few decades, besides chemical drugs, other therapeutic approaches were privileged. Within this framework, the modifications of lifestyle or the use of natural products derived from plant extracts gained much attention (Perez-Vizcaino et al., 2009; Zapata-Sudo et al., 2014). Besides, many researches are focusing on herbal preparations for their cardioprotective properties. For instance, *Moringa oleifera* (Moringaceae) seeds show a beneficial effect on cardiac structure and function in SHR associated with an upregulation of PPAR α and δ signaling (Randriamboavonjy et al., 2016). *Euterpe oleracea* (Arecaceae) prevented cardiac dysfunction, hypertrophy and fibrosis on rats subjected to myocardial infarction (Zapata-Sudo et al., 2014).

In Ivorian folk medicine, *Phyllanthus amarus* Schum. & Thonn. a plant belonging to the Euphorbiaceae family is used by local population as decoction of whole/leave of plant to treat hypertension and cardiovascular disorders (N'guessan et al., 2009). *P. amarus* is an erect annual found in all the tropical regions of the world for instance, Africa, India or South America (Kuttan and Harikumar, 2012).

P. amarus extracts are rich in several secondary metabolites such as lignans, hydrolysable tannins, flavonoids, alkaloids, triterpenes, sterols and volatile oils (Kuttan and Harikumar, 2012; Patel et al., 2011). Preliminary phytochemical analysis of aqueous extract of *phyllanthus amarus* revealed the presence of alkaloids, polyphenols, terpenes and sterols (Amonkan et al., 2013).

Numerous studies have indicated that *P. amarus* extract exhibited different pharmacological activities such as, anti-inflammatory (Kierner et al., 2003), antioxidant (Roengrit et al., 2014) or vasodilatation and hypotensive effects (Srividya and Periwal, 1995; Amaechina et al., 2007; Inchoo et al., 2011). *P. amarus* induced a potent anti-inflammatory effect resulting by

inhibition of iNOS, COX-2, and cytokines via the NF- κ B pathway (Kiemer et al., 2003). The antihypertensive activity has been shown to be related to stimulation of muscarinic receptor and involving endothelium-derived nitric oxide (NO) and also to blocking of calcium channel (Amaechina et al., 2007; Amonkan et al., 2013). Little data are available about cardioprotective and antihypertensive effects of *P. amarus* in chronic hypertension. Therefore, the aim of the present study was to determine if an aqueous extract of *P. amarus* (AEPA) obtained by decoction is able to prevent hypertension, cardiac hypertrophy and endothelial dysfunction in DOCA-salt hypertensive rats, and, if so, to clarify the underlying mechanism.

Materials and Methods

Plant material and extraction procedure

The whole plant of *Phyllanthus amarus* (Euphorbiaceae) was collected in the district of Cocody (Abidjan, Côte d'Ivoire) in April 2014. The plant is registered under No. 3, 141 and 248 at the Centre National de Floristique (CNF). Extraction procedure was similar to a method previously described (Amonkan et al., 2013). Briefly, the whole plant was harvested, washed, and extracted in boiling distilled water for 30 min at ratio of 500 g of plant for 1 liter. The decoction was filtered and then lyophilized to obtain a powder of aqueous extract of *Phyllanthus amarus* (11.72 g from 1 kg of plant).

Animals and experiments groups

Experiments were performed in accordance to the Guidelines for experiments involving animals (McGrath et al., 2010). Male Wistar rats (Janvier, Le Genest-Saint-Isle, France) weighing between 180 and 200 g were maintained under standard laboratory conditions (21-22 °C) with dark and light cycle (12/12 h) and had free access to a standard dry pellet diet

from Scientific Animal Food and Engineering (SAFE, France) and water *ad libitum*. Animals were randomly assigned 7-8 rats per group, into the control group, the AEPA group (100 mg/kg/day, by gavage), the DOCA-salt group (50 mg/kg, s.c, per week), and the DOCA-salt + AEPA groups (100 or 300 mg/kg/day, by gavage). DOCA-salt-treated rats were allowed free access to water containing 1% NaCl.

Blood pressure measurements

Prior to start the blood pressure monitoring, rats were subjected to a period (one week) of adaptation to the system. Systolic blood pressure (SBP) was determined by tail-cuff sphingomanometry twice weekly during 5 weeks using blood pressure analysis system (Bioseb, Vitrolles, France). Blood pressure was monitored at 9 a.m., and at least 12 determinations were made for each session.

Echocardiographic studies

After the 5 weeks treatment, the rats were anaesthetized by intraperitoneal injections of pentobarbital (50 mg/kg). Cardiac structure and function were determined by echocardiography transthoracic using the Phillips Sonos 5500 machine equipped with a probe 12 MHz, transducer. Two-dimensional short axis views of the left ventricle and M-mode tracings were recorded through anterior and posterior LV walls at the papillary muscle level. Morphological characterization of the cardiac left ventricle (LV) was assayed following the determination of these parameters: LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), posterior and septum diastolic wall thickness (PWT and SWT, respectively). Left ventricular mass (LVM) and LV ejection fraction (% LVEF) were subsequently derived from these parameters. The pulsed Doppler was used to assess the

isovolumetric relaxation time (IVRT), measured as the interval between aortic closure and the start of the mitral flow.

Assessment of cardiac and kidney weight indices

At the end of study, rats were prior weighed, anaesthetized by intraperitoneal injections of pentobarbital (50 mg/kg) and sacrificed. Blood was taken directly by cardiac puncture. The heart and the left kidney, were carefully taken, cleaned, and then weighed. The heart was after separation into left and right ventricles respectively, also weighed. Thereafter, cardiac and renal weight ratios were determined.

Biochemical analysis

After taken blood pressure, it was put into heparinized tubes and, thereafter the plasma was obtained by centrifugation 1500 g for 15 min. Plasma aliquots were stored at -80°C for subsequent determination of the electrolyte content, urea and uric acid.

Vascular studies

Vascular studies were performed using main mesenteric artery rings according to a similar method described previously in our team (Lee et al., 2013). Briefly, rings were contracted with 1 μ M of phenylephrine (PE) before the application of increasing concentrations of acetylcholine (ACh) ranging from 0.1 nM - 10 μ M to construct concentration-response curves. In some experiments, rings were exposed to an inhibitor for 30 min before contraction with PE. The NO-mediated component of relaxation was determined in the presence of indomethacin (10 μ M) and TRAM-34 (1 μ M) plus apamin (100 nM) to inhibit the formation of prostanoids and EDH-mediated relaxation, respectively. The EDH-mediated component of the relaxation was determined in the presence of indomethacin (10 μ M) and N^ω-nitro-L-

arginine (L-NA, 300 μ M) to inhibit the formation of prostanoids and NO, respectively. In endothelial-intact rings, rings were contracted with an increasing concentration of PE (0.1 nM-10 μ M) in order to obtain PE induced contractile responses.

Immunofluorescence studies

Main mesenteric arteries ring were removed, embedded in OCT compound (Tissue-Tek®, Sakura Finetek, Villeneuve d'Ascq, France) and snap-frozen in liquid nitrogen. Frozen arteries rings were cryosectioned at 14 μ m. Sections were air-dried for 15 min and stored at -80 °C until use. The slides were fixed with paraformaldehyde (4%), washed and treated with 10% milk containing 0.1% Triton X-100 for 1 h at room temperature to block non-specific binding. Next, overnight at 4 °C, mesentery artery sections were incubated with an antibody directed against either eNOS(1:50), NADPH oxidase subunits p22phox (1:50), cyclooxygenase-1(COX-1, 1:250), cyclooxygenase-2 (COX-2, 1:200), or connexin 37 (Cx, 1: 100). Sections were then washed with PBS, incubated with the secondary antibody (1/400, 633-conjugated goat anti-rabbit or anti mouse IgG) for 2 h at room temperature in the dark before being washed with PBS and mounted in Dako fluorescence mounting medium (Dako, Carinteria, USA) and cover-slipped. For negative controls, primary antibodies were omitted. The samples were kept in the dark until, observation using a confocal laser-scanning microscope (Leica TSC SPE-Mannheim, Germany). Analyse for quantification of protein levels expression was performed using Image J software (NIH, Bethesda, Maryland, USA).

Determination of vascular reactive oxygen species formation

Determination of in situ formation of reactive oxygen species (ROS) was performed into the main mesenteric artery rings using the oxidative fluorescent dye dihydroethidium (DHE) method. Mesenteric arterial rings (3-4 mm length) were embedded in OCT compound and snap-frozen in liquid nitrogen. Frozen arteries were cryosectioned at 25 μm . Sections were air-dried for 15 min and stored at -80 °C until use. Dihydroethidium (2.5 μM , Sigma) was applied onto unfixed cryosections of mesenteric arteries for 30 min at 37 °C in a light-protected humidified chamber, before being mounted in Dako fluorescent mounting medium and cover-slipped. The sample were kept in the dark until fluorescence was determined using a confocal laser-scanning microscope. Analyse for quantification of the fluorescence intensity was performed using ImageJ software.

Drugs

DOCA was obtained from Sigma

Antibodies

Antibodies were purchased as indicated: mouse anti-eNOS (BD Transduction Laboratories, East Rutherford, New Jersey, United States), COX-1 monoclonal antibody (Abcam, Paris France), COX-2 polyclonal antibody (Abcam, France), rabbit anti-p22phox (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Alexa 633-conjugated goat anti-rabbit or anti mouse IgG, (Life technologies, USA).

Statistical analysis

Data are expressed as means $\pm$ standard error of mean (SEM) of n experiments. Mean values were compared by ANOVA followed by the Bonferroni post-hoc test to identify significant difference between treatments, using GraphPad Prism (version 5 for Microsoft windows. GraphPad software, Inc, San Diego, CA, USA). The difference was considered to be significant when the $P < 0.05$.

Results

Intake of AEPA improves the DOCA-salt-induced hypertension

After 5 weeks, the SBP (mmHg) increased significantly in DOCA-salthypertensive rats, compared to the control group ($P < 0.05$; Fig. 1). Daily oral administration with AEPA (100 or 300 mg/kg/day) significantly prevented the increasing of SBP observed in DOCA-saltrats by 24 and 21 mmHg, respectively at the end of treatment. The SBP of animal receiving only AEPA (100 mg/kg/day) remained unaffected compared to the control groups ($P > 0.05$; Fig. 1).

Effect of AEPA treatment in cardiac structure and function

Transthoracic echocardiography revealed that after 5 weeks, DOCA-salthypertensive rats developed an important modification of cardiac structure, marked by left ventricular hypertrophy, compared to the control group. We observed a significant increase of PWT (1.58 ± 0.02 mm versus 2.24 ± 0.05 mm in the control and DOCA-salthypertensive group respectively; Fig. 2A) and SWT (1.54 ± 0.03 mm versus 1.77 ± 0.05 mm in the control and DOCA-salt hypertensive group respectively; Fig. 2B). The LVM was also significantly

greater in DOCA-salt hypertensive group than control (1103.61 ± 40.13 mg versus 755.29 ± 24.66 mg; Fig. 2C). No significant modification of LVEDD was observed ($P > 0.05$; Fig. 2D). The AEPA treatment (100 or 300 mg/kg/day) reduced significantly the increase of PWT by 0.36 mm and 0.30 mm respectively, SWT by 0.18 mm and 0.17 mm, LVM by 268.55mg and 251.61mg respectively. Parameters for AEPA (100 mg/kg/day) remained unaffected compared to the control groups ($P > 0.05$).

Concerning cardiac function, our results show that, in DOCA-salt rats, IVRT was significantly increased compared to the control (35.5 ± 2.83 ms versus 45.71 ± 1.96 ms, respectively; Fig. 3A). AEPA treatment (100 or 300 mg/kg/day) improves these parameters at a value close to normal, 36.67 ± 3.47 and 36.88 ± 2.37 ms respectively (Fig. 3A). No significant difference was observed between rats treated only with AEPA (100 mg/kg/day) and control group. The ejection fraction was affected slightly but not significantly in DOCA-salt rats compared to the control ($P > 0.05$; Fig. 3B).

Analyse of morphological parameters

Post mortem morphometric analysis of organ shows that except right ventricular DOCA-salt rats increase significantly the cardiac (3.4 ± 0.23 versus 2.73 ± 0.08), left ventricular (1.34 ± 0.11 versus 1.04 ± 0.05) and , left kidney (3.71 ± 0.18 versus 3.18 ± 0.12) weight indices, as compared respectively to the control group (Table 1). The cardiac and kidney weight indices were reduced in the DOCA-salt + AEPA groups (100 or 300 mg/kg/day), compared to the DOCA-salt rats ($P < 0.05$; Table 1). These parameters remained similar between AEPA (100 mg/kg/day) group and control rats ($P > 0.05$; Table 1).

Effect of DOCA-salt on plasma electrolyte levels

DOCA-salt treatment increases the plasma uric acid level (11.25 ± 0.48 in DOCA-salt rats versus 7.00 ± 1.00 in control; Table 2) and decrease the plasma potassium level (4.18 ± 0.08 versus 5.54 ± 0.2 in the control; Table 2). AEPA treatments (100 or 300 mg/kg/d) normalize the plasma levels of potassium (5.26 ± 0.3 and 5.14 ± 0.3 respectively; Table 2) and uric acid level (6.67 ± 0.52 and 8.0 ± 0.50 respectively; Table 2).

At the end of experiment, the plasma sodium level in DOCA-salt rat increase slightly, but not significantly compare to the control group (147.50 ± 0.65 in DOCA-rats versus 143.20 ± 1.11 in control; Table 2). The plasma chlorine and urea levels remained also unaffected (Table 2).

AEPA improves the DOCA-salt-induced endothelial dysfunction in the mesenteric artery

In mesenteric artery rings with endothelium, DOCA-salt treatment increase significantly phenylephrine-induced vasoconstriction compared to the control rats ($P < 0.05$; Fig. 4A). In the DOCA-salt + AEPA groups (100 or 300 mg/kg/day), the phenylephrine induced-contraction response was shifted to the right (Fig. 4A). The significant effect was obtained with AEPA at dose of 300 mg/kg/day ($P < 0.05$; Fig. 4A).

DOCA-salt group, present an endothelial dysfunction, marked by a significant reduction of acetylcholine induced endothelium-dependent vasorelaxation, compared to the control group ($P < 0.05$; Fig. 4B). Treatment with AEPA (100 or 300 mg/kg/day) improve these endothelial dysfunction ($P < 0.05$; Fig. 4B).

AEPA improves the DOCA-salt-blunted NO- and EDH-mediated relaxations in the mesenteric artery

In mesenteric artery rings with endothelium, both the NO-mediated (assessed in the presence of indomethacin and Tram-34 plus Apamin) and the endothelium-dependent hyperpolarization (EDH)-mediated (assessed in the presence of indomethacin and N^ω-nitro-L-arginine) relaxation to acetylcholine were significantly reduced in the DOCA-salt group compared to the control group ($P < 0.05$; Fig. 5A and Fig. 5B). Treatment with AEPA improves the DOCA-salt-blunted NO- and EDH-mediated relaxations in the mesenteric artery (Fig. 5A and Fig. 5B).

AEPA prevents the DOCA-salt-induced increased vascular oxidative stress and up-regulation of NADPH oxidase

Immunofluorescence studies show that DOCA-salt treatment induced an important increase of vascular oxidative stress, attested by the significant increase of DHE fluorescence signal amounted to $124.17 \pm 4.89 \%$, compared to control ($100.00 \pm 3.97 \%$; Fig. 6A). This oxidative stress was accompanied by a significant increase of NADPH oxidase subunits p22phox ($198.82 \pm 17.17\%$ in DOCA-salt rat versus $100.00 \pm 17.34 \%$; Fig. 6B). AEPA treatment prevents the DOCA-salt-induced increased vascular oxidative stress and up-regulation of NADPH oxidase ($P < 0.05$; Fig. 6A and Fig. 6B).

AEPA prevents the DOCA-salt-induced increased expression of eNOS and decreased expression of Cx37

Immunofluorescence staining indicated a significant increased expression of eNOS ($188.22 \pm 12.91 \%$; Fig. 7A) and a decreased expression of Cx37 ($32.39 \pm 3.23 \%$; Fig. 7B) in DOCA-salt rats compared to control rats. ($P < 0.05$). AEPA treatment normalized these deleterious effects by preventing the DOCA-salt-induced increased expression of eNOS and decreased expression of Cx37 ($P < 0.05$; Fig. 7A and Fig. 7B).

DOCA-salt-induced increased expression of COX-2, but not expression of COX-1

The expression of COX-2 signal was significantly increased in DOCA-salt rats compared to the control (153.72 ± 12.44 %, versus 100.00 ± 9.07 %; Fig. 8A). After treatment, a preventing effect was observed by a significant reduction of COX-2 levels expression in DOCA + AEPA (100 or 300 mg/kg/day) group ($P < 0.05$; Fig. 8A). In contrast to COX-2 signal, the expression COX-1 levels expression remained unaffected ($P < 0.05$; Fig. 8B).

Discussion

The present findings show that after 5 weeks of injection of deoxycorticosterone acetate (DOCA) followed by 1% NaCl diet, rats become hypertensive. This hypertension is associated to left cardiac hypertrophy and endothelial dysfunction.

Concerning cardiac structure, our result is in agreement with previous study indicated that DOCA-salt induces cardiac hypertrophy, especially in left ventricular in rat (Fenning et al., 2005). Transthoracic echocardiography result show an increase of PWT and SWT associated with an increase of LVM without any significant dilation of the left ventricular chamber, indicating that hypertrophy observed in rat was concentric type (Chan et al., 2006). Analyses of cardiac function show an impairment of diastolic function in DOCA-salt rats, attest by an increase of IVRT. Ejection fraction (LVEF) was slightly reduced, in DOCA-salt group. However, this parameter remained in the normal range i.e. upper than 50 % (Kawaguchi et al., 2003). This result is in agreement with previous study indicating that in hypertension, at early stages, cardiac diastolic function was prior affected before systolic function (Ayme-Dietrich et al., 2015).

Moreover, the beneficial cardioprotective effect of AEPA in DOCA-salt rats was related to its ability to exhibit an antihypertrophic effect by reducing near to the normal PWT, SWT and

LVM, together with an improvement of cardiac function. Similar effect was reported with oral extract of *Moringaoleiferaseeds* which decreases the LVM and improves diastolic function in SHR (Randriamboavonjy et al., 2016).

The experimental models of salt-insensitive hypertension induce also a renal damage (Manning et al., 2005). In this study, we found an increase of kidney weight indices, an increase of uric acid and a depletion of potassium plasma levels in untreated pathological rats. Chronic hypokalemia has been involved in cardiac hypertrophy, thus, potassium supplementations seem to be a strategy to prevent the development of cardiac hypertrophy (Wang et al., 2005; Liu et al., 2014). Our result shows that in hypertensive rats, EAPA treatment was able to restore near to the normal, the potassium plasma level. The same result was obtained with sesame oil and quercetin in DOCA-salt hypertensive rats (Galisteo et al., 2004; Liu et al., 2014).

Endothelial dysfunction is a common vascular deleterious effect found in hypertension (Perez-Vizcaino et al., 2009). In the present study, we demonstrated that AEPA treatment prevent endothelial dysfunction by reducing the maximal contractile response to phenylephrine and an improvement of vascular effect of acetylcholine in DOCA-salt hypertensive rat. Moreover, endothelial dysfunction was corroborating by an impairment of both NO- and EDH-mediated relaxations in the mesenteric artery in DOCA hypertensive rats. Previous reports indicate that in animal artery, endothelium-dependent relaxations to acetylcholine most likely involve a NO component (Furchgott and Zawadzki 1980) and an EDH component (Pannirselvam et al., 2006). Thus EAPA treatment prevent endothelial dysfunction by improvement of the blunted of NO- and EDH-mediated relaxations in the mesenteric artery. Other natural product derived from plant extract such as red wine polyphenols (RWPs) or the sesame lignan, sesamin have been reported to restore endothelial function in DOCA-salt hypertension mainly by preventing vascular oxidative stress

(Jiménez et al., 2007; Nakano et al., 2008). We therefore examine the possibility that AEPA exert a beneficial effect on cardiovascular system by such mechanism, since the DOCA-salt hypertensive rat is described as a model of cardiovascular oxidative and inflammatory stress (Iyer et al., 2010). Interestingly, in the present study, in mesenteric artery of DOCA-salt hypertensive rat, we observed an increase of reactive oxygen species production and an overexpression of NADPH oxidase subunits p22phox. It is well established that NADPH oxidase activation induces a release of ROS production, contributing to vascular endothelial dysfunction (Nediani et al., 2007).

eNOS expression was up-regulated in the mesenteric artery of DOCA-salt hypertensive. We can explain this phenomenon by the fact that, in presence of vascular oxidative stress, tetrahydrobiopterin (BH4) can promote oxidation, and a compensatory mechanism take place and lead consequently to eNOS uncoupled (Landmesser et al., 2003). Thus, the effect of AEPA was similar in part to resveratrol which attenuates hypertension spontaneously in hypertensive rats by preventing endothelial nitric oxide synthase uncoupling (Bhatt et al., 2011). Connexin (Cx) protein subunits present in the gap junctions play a crucial role in EDH component of endothelium-dependent relaxation (de Wit et al., 2010). In accordance with our results, previous results have indicated that vascular oxidative stress also reduces connexin subunits expression such as Cx37, and thus leading to an impairment of EDH-mediated relaxation (Rashid et al., 2014).

DOCA-salt hypertension promotes an overexpression of COX-2 in artery (Callera et al., 2006). Such effect was most likely restored and the value observed was close to the normal level after AEPA treatment. These results are in agreement with previous *in vivo* and *in vitro* studies, which have been showed that *Phyllanthus* species such as *P. amarus* or *P. acidus* are able to reduce inflammatory stress by inhibiting COX-2 overexpression (Kiemer et al., 2003; Hossen et al., 2015).

In most human and experimental salt sensitive hypertension, antioxidant level is down-regulated, thus antioxidant therapy can be helpful (Manning et al., 2005). Taken together, the antioxidant properties of *P. amarus* previously described (Roengrit et al., 2014), might contribute to explain the ability of AEPA to counteract the deleterious effects described in DOCA-salt rats.

Altogether, our study indicates that AEPA has antihypertensive properties, and prevents endothelial dysfunction and cardiac hypertrophy in DOCA-salt hypertensive rats, in part, by preventing vascular oxidative stress. The antihypertensive effect of AEPA was associated with an improvement of the blunted NO- and EDH- mediated relaxation, and a reduced vascular oxidative stress level and improvement of target proteins (eNOS, Cx37, COX-2 and p22phox) and cardiac hypertrophy in DOCA-salt rats.

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Conflicts of Interests

The authors declare that they have no conflicts of interest

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**Optimized EPA: DHA 6:1
formulation prevents
Angiotensin-II induced
hypertension and endothelial
dysfunction in rats**

Résumé

La présente étude évalue la capacité de EPA:DHA 6:1, une formulation d'omega-3 capable d'induire la formation continue de monoxyde d'azote par la NO synthase endothéliale, à prévenir l'hypertension et la dysfonction endothéliale induites par l'angiotensine II (Ang II) chez le rat. L'hypertension induite par l'Ang II est associée à une dysfonction endothéliale caractérisée par une altération des composantes de la relaxation et une augmentation des réponses contractiles dépendantes de l'endothélium. L'Ang II augmente le stress oxydant vasculaire et l'expression de NADPH oxydase, COXs, eNOS, et AT1R, alors que SK_{Ca} et connexin 37 sont sous-exprimés. EPA:DHA 6:1 prévient l'hypertension, la dysfonction endothéliale et la surexpression des protéines cibles. En conclusion, la consommation chronique de EPA:DHA 6:1 prévient l'hypertension et la dysfonction endothéliale induites par l'Ang II chez le rat, probablement en prévenant le stress oxydant dû à la NADPH oxydase et aux cyclooxygénases.

Résumé en anglais

EPA:DHA 6:1 has been shown to be a superior omega-3 formulation inducing a sustained endothelial NO synthase-derived formation of nitric oxide. This study examined whether chronic intake of EPA:DHA 6:1 prevents hypertension and endothelial dysfunction induced by angiotensin II (Ang II) in rats. Ang II-induced hypertension was associated with endothelial dysfunction characterized by blunted components of relaxation and increased endothelium-dependent contractile responses. Ang II increased the vascular oxidative stress, and the expression of NADPH oxidase subunits, COXs, eNOS, and AT1R whereas SK_{Ca} and connexin 37 were down-regulated. Intake of EPA:DHA 6:1 prevented the Ang II-induced hypertension and endothelial dysfunction, and improved expression of target proteins. In conclusion, chronic intake of EPA:DHA 6:1 prevented the Ang II-induced hypertension and endothelial dysfunction in rats, most likely by preventing NADPH oxidase- and cyclooxygenase-derived oxidative stress.