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Intake of omega-3 formulation EPA:DHA 6:1 by old rats for 2 weeks improved endothelium-dependent relaxations and normalized the expression level of ACE/AT1R/NADPH oxidase and the formation of ROS in the mesenteric artery

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Abstract

Omega-3 polyunsaturated fatty acids (PUFAs) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to protect the cardiovascular system, in part, by stimulating the endothelial formation of nitric oxide (NO). EPA:DHA 6:1 has been identified as a potent omega 3 PUFA formulation to induce endothelium-dependent vasorelaxation and activation of endothelial NO synthase (eNOS). This study examined whether intake of EPA:DHA 6:1 (500 mg/kg/day) for 2 weeks improves an established endothelial dysfunction in old rats (20 months old), and, if so, the underlying mechanism was subsequently determined.

In the main mesenteric artery rings, an endothelial dysfunction characterized by a blunted NO component, an abolished endothelium-dependent hyperpolarization component, and increased endothelium-dependent contractile responses (EDCFs) are observed in old rats compared to young rats. Age-related endothelial dysfunction was associated with increased vascular formation of reactive oxygen species (ROS) and expression of eNOS, components of the local angiotensin system, senescence markers, and cyclooxygenase-2 (COX-2), and the downregulation of COX-1. The EPA:DHA 6:1 treatment improved the NO-mediated relaxation, reduced the EDCF-dependent contractile response and the vascular formation of ROS, and normalized the expression level of all target proteins in the old arterial wall. Thus, the present findings indicate that a 2-week intake of EPA:DHA 6:1 by old rats restored endothelium-dependent NO-mediated relaxations, most likely, by preventing the upregulation of the local angiotensin system and the subsequent formation of ROS.

Keywords (6 max): Aging; Omega-3 polyunsaturated fatty acids; Artery; Endothelium; Nitric oxide; Angiotensin system

1. Introduction

Cardiovascular diseases such as ischemic heart disease and stroke are a major cause of death responsible for 32% of deaths worldwide in 2013 [1]. Age has been identified as a main risk factor for the development of cardiovascular diseases. The impact of aging on the vascular system is characterized predominantly by increased thickness and stiffness of the arterial wall and impaired regulation of vascular tone as indicated by the appearance of endothelial dysfunction [2,3]. Under physiological conditions, the vascular endothelium has a pivotal protective role in the control of vascular homeostasis mainly by inducing potent vasoprotective mechanisms including the formation of endothelium-derived nitric oxide (NO) and endothelium-dependent hyperpolarization (EDH). Both these responses are blunted with increasing age in experimental animals and humans and often are associated with the appearance of endothelium-dependent contractile responses (EDCFs) [4–7]. Moreover, old arteries are characterized by increased formation of reactive oxygen species (ROS) throughout the arterial wall involving several cellular sources such as NADPH oxidase, cyclooxygenases (COXs), the mitochondrial respiratory chain, and uncoupled endothelial NOS (eNOS)[4,6]. Previous studies have shown a pivotal role of both circulating and local angiotensin systems in the induction of endothelial dysfunction in several experimental models of hypertension, atherosclerosis, diabetes, and aging [4,6,8–10] and also in humans [11]. Angiotensin II (Ang II)-induced endothelial dysfunction is characterized by the vascular formation of ROS subsequent to the upregulation of the expression level of NADPH oxidase [12,13], thereby promoting eNOS uncoupling to further generate ROS [14], alteration of calcium-dependent K^+ channels involved in EDH responses [15], and increased expression of COXs, promoting EDCF responses [16,17].

Recently, premature endothelial senescence has been suggested to be an early event contributing to age-related endothelial dysfunction and the development of cardiovascular

diseases [18]. Premature endothelial senescence is observed in response to several pro-oxidant stimuli such as H₂O₂, Ang II, and elevated glucose concentration [19,20]. These pro-senescence inducers promote the downregulation of eNOS and the induction of a pro-inflammatory and pro-atherothrombotic phenotype in endothelial cells [21–23].

Several, but not all clinical studies, have indicated that omega-3 polyunsaturated fatty acids (PUFAs), in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), improve the endothelial function and reduce cardiovascular events in patients with cardiovascular risk factors [24–26]. Recently, the REDUCE-IT clinical trial has reported a decreased risk of cardiovascular events after supplementation with 2 g of an EPA ethyl ester (icosapent ethyl) twice daily in patients with established cardiovascular disease or with diabetes and other risk factors, who were on statin therapy and had a fasting triglyceride level of 135 to 499 mg/dL [27]. The beneficial effect was stronger in patients with elevated baseline triglycerides despite moderate-to-high statin therapy intensity [27] and observed across a wide range of baseline triglycerides [28]. A beneficial effect on the cardiovascular system is also supported by the fact that both purified EPA and DHA alone induced endothelium-dependent relaxations of isolated rat conductance and resistance arteries mainly through stimulation of both NO and EDH pathways [29]. Our previous study has indicated that both the ratio and the purity of the EPA:DHA formulations are of major importance for the biological activity, as highly purified EPA:DHA 6:1 and 9:1 formulations demonstrated greater endothelium-dependent NO-mediated relaxation of porcine coronary artery rings than other ratios [30] and acted through a redox-sensitive activation of Src/PI3-kinase/Akt and MAPK pathways, leading to eNOS phosphorylation at Ser1177 and subsequent formation of NO [30]. EPA:DHA 6:1 also induced endothelium-dependent NO-mediated relaxations and inhibited serotonin-induced contractile responses in human internal thoracic artery rings [31].

Moreover, oral intake of EPA:DHA 6:1 significantly prevented Ang II-induced hypertension, endothelial dysfunction, and vascular pro-oxidant responses in rats [32].

The aim of the study was to evaluate the potential of a 2-week oral intake of EPA:DHA 6:1 to improve an established blunted endothelium-dependent relaxation in old rats and, if so, to determine the underlying mechanism and, in particular, the role of the pro-oxidant and the pro-senescence angiotensin system.

2. Methods and materials

2.1. Ethics statement

This study was performed in accordance with the guidelines on animal care published by the US Institute of Health (Bethesda, MD, USA; NIH publication number 85–23, revised 1996) and the French Legislation. The protocol for this study was approved by the local Ethics Committee (Comité Régional d’Ethique en Matière d’Expérimentation Animale de Strasbourg) and authorized by the French Ministry of Higher Education, Research, and Innovation (authorization no. #7626-201611171554293).

2.2 Chemicals

All chemicals were purchased from Sigma-Aldrich (Sigma Aldrich Chimie S.a.r.l, St.-Quentin-Fallavier, France) except TRAM-34, UCL-1684, levcromakalim, NS-398, and SC-560, which were bought from Tocris (Bio-Techne, Abingdon, UK), VAS-2870 from Enzo Life Science (Villeurbanne, France), and PBS and Triton X100 from Euromedex (Souffelweyersheim, France).

2.3. Preparation of Omega-3 PUFAs

Highly purified EPA and DHA products were provided by Pivotal Therapeutics, Inc. (Woodbridge, ON, Canada). EPA:DHA 6:1 (w/w) was prepared by mixing the respective purified products in the ratio. The final solution was aliquoted in amber-colored vials. All the processes were done under nitrogen flux to avoid oxidation of omega-3 PUFAs.

2.4. *In vivo* treatment of rats

Thirty male Wistar rats were kept in the animal facility and given free access to standard diet and tap water from the age of 12 weeks until they were 20 months old. They were then divided randomly into 3 groups and administered daily with 500 mg/kg/day of either EPA:DHA 6:1, corn oil (isocaloric control without omega-3 PUFAs, Mazola 100% corn oil), or untreated (control old rats) for 14 days. A group of untreated 12-week-old rats was used as young controls. After 14 days of treatment, the rats were euthanized by an intraperitoneal injection of a lethal dose of pentobarbital (150 mg/kg) before collection of organs.

2.5. Vascular reactivity study

The main mesenteric artery was dissected, cleaned of connective tissue, and cut into rings (2-3 mm) before suspension 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. The rings were allowed to stabilize for 30-45 min at a tension of 1 g before the reactivity of the vascular smooth muscle was assessed in response to Krebs buffer containing 80 mM potassium. The integrity of the endothelium was checked with acetylcholine (ACh 1 μM) after sub-maximal contraction (approximately 70%) with phenylephrine (PE 1 μM). To assess the endothelium-dependent relaxations, the rings were contracted with PE (1 μM) before the construction of concentration-relaxation curves in

response to ACh. To assess the contractile responses, concentration-contraction curves were constructed in response to PE. In some experiments, the rings were exposed to a pharmacological agent for 30 min before contraction. To study the role of cyclooxygenase-derived prostanoids, the rings were incubated with indomethacin (10 μ M, a nonselective COX inhibitor). To study the role of NO-mediated relaxation, the rings were incubated in the presence of indomethacin and TRAM-34 plus UCL-1684 (1 μ M each, inhibitors of IK_{Ca} and SK_{Ca} , respectively) to prevent the formation of vasoactive prostanoids and EDH-mediated relaxation, respectively. The role of EDH-mediated relaxation was studied in rings incubated with indomethacin and N^{ω} -nitro-L-arginine (L-NA, 300 μ M, an eNOS inhibitor) to prevent the formation of vasoactive prostanoids and NO, respectively. To study endothelium-dependent vasoconstrictor factors (EDCFs), the rings were exposed to L-NA and TRAM-34 plus UCL-1684 to prevent NO and EDH, respectively, in the absence or presence of indomethacin, before an approximate 30% pre-contraction with PE and the subsequent construction of a concentration-contraction curve in response to ACh. To assess the vascular smooth muscle function, the rings were contracted with PE (1 μ M) before the construction of concentration-relaxation curves in response to either sodium nitroprusside (SNP, a NO donor) or levcromakalim (Lev, an ATP-sensitive potassium channel opener) in the presence of indomethacin, L-NA, and TRAM-34 plus UCL-1684.

2.6. Immunofluorescence studies

Rings of the main mesenteric artery and thoracic aorta were embedded in histomolds containing Tissue-Tek optimum cutting temperature (OCT) compound (Sakura 4583, Leiden, The Netherlands) and snap-frozen in liquid nitrogen. Rings were cryosectioned at 14 μ m and stored at -80 $^{\circ}$ C until use. The sections were defrosted with phosphate-buffered saline (PBS) and fixed for 1 h with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA,

USA) before blocking for 2 h at room temperature with 10% non-fat powdered milk in PBS containing 0.1% Triton X-100 to prevent nonspecific binding. All sections, excluding negative controls, were then incubated overnight at 4 °C with a solution of blocking buffer containing a primary antibody against either COX-1 (1/250, Cat. ab109025, Abcam, Paris, France), COX-2 (1/200, Cat. Ab15191, Abcam), eNOS (1/100, Cat. 610297, BD Transduction Laboratories, Le Pont de Claix, France), p16 (1/200, Cat. db018, Delta Biolabs, California, USA), p21 (1/100, Cat. Sc817, Santa Cruz Biotechnology, CliniSciences, Nanterre, France), p53 (1/100, Cat. Sc6243, Santa Cruz Biotechnology), p22^{phox} (1/100, Cat. Sc11712, Santa Cruz Biotechnology), p47^{phox} (1/100, Cat. Sc7660, Santa Cruz Biotechnology), AT1R (1/200, Cat. Sc1173, Santa Cruz Biotechnology), and ACE (1/200, Cat.250450, Abbiotec, San Diego, USA). The next day, all sections were washed with PBS before incubation with a solution of blocking buffer containing a fluorescent secondary antibody (Alexa Fluor 633 antirabbit or antimouse, Invitrogen, Fisher Scientific France, Illkirch, France) for 2 h at room temperature in the dark, followed by washing with PBS and air drying for 15-20 min. The slides were then cover-slipped with Dako fluorescence mounting solution (Ref. S3023, Dako, California, USA) and dried for 20 min at room temperature. The slides were then analyzed using a confocal laser scanning microscope (Leica TSC SPE, Mannheim, Germany). Signals were collected in the red wavelength between 645 and 719 nm following excitation of Alexa 633 using the 635 nm red laser. Autofluorescence was measured in the green wavelength between 500 and 600 nm following excitation with the 488 nm cyan laser. Quantitative analysis of fluorescence was performed using ImageJ software (version 1.49 for Windows, NIH). The mean fluorescence intensity of the elastic lamina was measured in both green and red channels to calculate the signal ratio between the two channels. The autofluorescence signal (green channel) is then subtracted for the signal of the red channel using the signal ratio. The mean fluorescence signal in the resulting image is then determined in the area of interest

delimited manually. The mean fluorescence signal was measured in the endothelial layer for eNOS and in the endothelium plus media for the other proteins.

2.7. Determination of the *in situ* level of ROS

The vascular level of ROS was determined using the redox-sensitive fluorescent probe dihydroethidium (DHE, Calbiochem, Sigma-Aldrich). Cryosections of the mesenteric artery or the aorta (25 μm) were defrosted with PBS and incubated with DHE (2.5 μM) for 30 min at 37 °C in a black box to protect them from light. To determine the sources of ROS, aorta sections were pretreated with inhibitors such as N-acetylcysteine (antioxidant), indomethacin (inhibitor of COX), NS-398 (COX-2 inhibitor), SC-560 (COX-1 inhibitor), L-NA (inhibitor of eNOS), VAS-2870 (inhibitor of NADPH oxidase), and inhibitors of mitochondrial respiratory chain (KCN, myxothiazol, potassium cyanide, and rotenone) for 30 min at 37 °C before incubation with DHE. The sections were then washed with PBS before mounting under a cover slip in DAKO fluorescence solution. The slides were allowed to dry in the dark for 20 min before being analyzed by confocal laser scanning microscopy. The images were processed as in section 2.6 except that signals were collected between 580 and 650 nm following the excitation of the ethidium with the 561 nm yellow-green laser, and autofluorescence between 495 and 581 nm following excitation with the 488 nm cyan laser.

2.8. Statistical analysis

Values were expressed as mean \pm S.E.M. Statistical analysis was performed by two-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc test for vascular reactivity and by two-tailed unpaired *t*-test for other data. The normality of the data was verified by Shapiro-Wilk test. Values were considered to show statistically significant difference for

$P < 0.05$. All statistical analyses were performed using GraphPad Prism software (version 5 for Windows, GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Intake of EPA:DHA 6:1 by old rats for 2 weeks improves vascular reactivity of intact mesenteric artery rings

To assess the vascular effect of a 2-week oral intake of the omega-3 EPA:DHA 6:1 formulation by old rats, the reactivity of the main mesenteric artery with endothelium was studied using organ chambers. After precontraction with phenylephrine (PE, 1 μ M), the rings from old rats showed significantly decreased relaxations in response to acetylcholine (ACh, 0.1-10 μ M) when compared with the rings from young rats (Figure 1A). Similarly, the rings from old rats showed significantly increased contractile responses to increasing concentrations of PE (1-10 μ M) when compared with those from young rats (Figure 1B). Treatment with EPA:DHA 6:1, but not with corn oil, significantly improved ACh-induced relaxations and reduced PE-induced contractions in the mesenteric artery rings of old rats (Figures 1A, B).

To assess EDCF-mediated responses, the rings were preincubated with N^o-nitro-L-arginine (L-NA, an eNOS inhibitor) and TRAM-34 plus UCL-1684 (inhibitors of IK_{Ca} and SK_{Ca}, respectively) to prevent NO- and EDH-mediated relaxation, respectively. Rings from untreated old rats showed significantly greater contractions in response to ACh than those of young rats (Figure 1C), such responses were abolished in the presence of indomethacin (a nonselective COX inhibitor, Figure 1D), indicating the involvement of an increased formation of COX-derived EDCFs. Treatment of old rats with EPA:DHA 6:1 partially, but significantly, reduced EDCF-mediated contractile responses in the mesenteric artery, whereas corn oil was without effect (Figure 1C).

In addition, the endothelium-independent relaxations to either SNP (an NO donor) or levcromakalim (Lev, an ATP-sensitive potassium channel opener) were similar in old and young rats, indicating that the function of the vascular smooth muscle, in contrast to endothelial function, is preserved in the old mesenteric artery (Figures 1E, F).

3.2. EPA:DHA 6:1 treatment improves the NO-mediated relaxation and decreases the PE-induced contractile response in the old mesenteric artery

In the mesenteric artery rings of young rats, the ACh-induced relaxation was significantly reduced in the presence of L-NA and slightly but significantly in the presence of TRAM-34 plus UCL-1684 (from $50.4 \pm 5.3\%$ to $25.6 \pm 7.1\%$ at 3.10^{-8} M of ACh for control and in the presence of TRAM-34 plus UCL-1684, respectively), indicating the involvement predominantly of NO and, to some extent, of EDH (Figure 2A, Table 1). In contrast to young rats, the ACh-induced relaxation in the rings from control old rats was not affected by TRAM-34 plus UCL-1684 but abolished in the presence of L-NA, indicating that the relaxation is exclusively mediated by NO (Figure 2B, Table 1). In addition, indomethacin slightly improved the relaxation to ACh at concentrations greater than $1 \mu\text{M}$ in old rats, but this effect did not reach statistical significance (Table 1).

Similarly, in old rats treated with either EPA:DHA 6:1 or corn oil, the relaxation to ACh was not affected by TRAM-34 plus UCL-1684 and by indomethacin but abolished in the presence of L-NA, indicating that they are exclusively mediated by NO (Figures 2 C, D, Table 1).

In the mesenteric artery rings of old but not young rats, the PE-induced contractile response was significantly reduced in the presence of indomethacin, suggesting the involvement of COX-derived vasoconstrictor prostanoids (Figure 2F, Table 1). The inhibitory effect of indomethacin was also observed in the corn oil-treated group but not in the EPA:DHA 6:1-treated group of old rats. In addition, the contractile response to PE was markedly increased in

the presence of L-NA in the EPA:DHA-treated group but not in the control and corn oil-treated groups of old rats, suggesting an increased basal formation on NO (Figure 2 G, H, Table 1).

3.3. EPA:DHA 6:1 treatment normalizes the expression level of eNOS and COXs in the old mesenteric artery and aorta

As the endothelial dysfunction of the old mesenteric artery is characterized by a reduced NO-mediated relaxation and increased COX-dependent EDHF-mediated contractile response, the expression level of proteins involved in these pathways, eNOS and COXs, respectively, was assessed by immunofluorescence in both the mesenteric artery and the aorta. The eNOS fluorescent signal was observed selectively in the endothelium, whereas that of COX-1 and COX-2 was observed throughout the arterial wall in the mesenteric artery and the aorta in all groups (Figure 3). Compared to young rats, sections of old arteries showed higher eNOS and COX-2 signals but lower COX-1 signals (Figure 3). The EPA:DHA 6:1 but not the corn oil treatment normalized eNOS, COX-1, and COX-2 signals in old arteries to a similar level as those observed in young arteries (Figure 3).

3.4. EPA:DHA 6:1 treatment normalizes the formation of ROS in the old mesenteric artery and aorta

As age-related endothelial dysfunction is associated with an increased level of oxidative stress [4–6], the formation of ROS was assessed in the mesenteric artery and aorta using the redox-sensitive probe DHE. Sections of the mesenteric artery and aorta of control and corn oil-treated old rats showed similar higher levels of ethidium fluorescence as those of young rats, demonstrating an increased formation of ROS (Figures 4 A, B). The EPA:DHA 6:1 but not

the corn oil treatment normalized the level of ROS in both the old mesenteric artery and aorta to a similar level as that observed in young arteries (Figures 4 A, B).

In addition, the formation of ROS in the aortic section of control old rats was significantly lower in the presence of N-acetylcysteine (NAC, an antioxidant), indomethacin (nonselective COX inhibitor), SC-560 (a selective COX-1 inhibitor), NS-398 (a selective COX-2 inhibitor), VAS-2870 (a NADPH oxidase inhibitor), L-NA (eNOS inhibitor) or by the combination of potassium cyanide, rotenone, and myxothiazol (inhibitors of the mitochondrial respiratory chain, Figure 4C), indicating the involvement of several sources of ROS including COX-1 and COX-2, uncoupled eNOS, NADPH oxidase, and mitochondrial respiratory chain (Figure 4C).

3.5. EPA:DHA 6:1 treatment improves the expression level of p47^{phox} and p22^{phox}

NADPH oxidase subunits in the old aorta

As the age-related increased ROS formation involves NADPH oxidase, a major cellular source of ROS in cardiovascular diseases [33], the expression level of the NADPH oxidase subunits p47^{phox} and p22^{phox} was assessed in the aorta by immunofluorescence. Compared to young rats, the fluorescent signals of both p47^{phox} and p22^{phox} NADPH oxidase subunits were significantly higher in the old aorta (Figure 5). The EPA:DHA 6:1 but not the corn oil treatment normalized the signals of both NADPH oxidase subunits in the old aorta (Figure 5).

3.6. EPA:DHA 6:1 treatment improves the expression level of ACE and AT1R in the old aorta

As an angiotensin-converting enzyme (ACE) inhibitor and an AT1R antagonist improved age-related endothelial dysfunction and excessive vascular ROS formation [6,34], the expression level of two major components of the local angiotensin system, namely, ACE and angiotensin

II type 1 receptor (AT1R), was assessed by immunofluorescence. Aortic sections of control old rats showed higher fluorescence signals of both ACE and AT1R than those of young rats (Figure 6). The EPA:DHA 6:1 but not corn oil treatment normalized signals of both ACE and AT1R in the old aorta to a similar level as those observed in the young aorta (Figure 6).

3.7. EPA:DHA 6:1 treatment improves the expression level of the senescence markers p53, p21, and p16 in the old aorta

As endothelial senescence has been identified as an early event leading to endothelial dysfunction [35], the expression level of senescence markers was assessed by immunofluorescence. Aortic sections of old rats showed a higher fluorescence signal of p53, p21, and p16 throughout the arterial wall than those of young rats (Figure 7). EPA:DHA 6:1 but not corn oil treatment normalized signals of p53, p21, and p16 in the old aorta to a similar level as those observed in the young aorta (Figure 7).

4. Discussion

Both preclinical and clinical studies have indicated that the progressive increase in age is associated with the development of endothelial dysfunction, as observed in the present study in the mesenteric artery of old rats. The characterization of age-related endothelial dysfunction has indicated the involvement of a reduced NO-mediated relaxation, induction of EDCF-mediated contractile responses, and induction of vascular senescence as pointed out by an upregulation of the senescence markers p53, p21, and p16. Old mesenteric arteries are also characterized by an increased ROS formation involving several sources and, in particular, NADPH oxidase. As old arteries showed upregulation of the expression level of ACE and ATR1, whose activation promotes NADPH oxidase-derived formation of ROS, the local angiotensin system seems to be involved. Moreover, the present findings also show that intake of EPA:DHA 6:1 by old rats for 2 weeks improved the age-related endothelial dysfunction, normalized the vascular ROS formation, and normalized the expression of ACE and ATR1 and senescence markers.

Circulating levels of omega-3 PUFAs have been inversely associated with the risk of cardiovascular diseases. Indeed, a low content of omega-3 PUFAs in erythrocyte membranes has been associated with an increased risk of cardiovascular and all-cause mortality in the Framingham Heart Study Offspring cohort [36]. Several clinical studies and meta-analyses have evaluated the effects of various omega-3 PUFAs-rich products (fish, fish oil, marine oil, krill oil, etc.) or purified omega-3 PUFAs such as EPA and DHA in primary and secondary cardiovascular outcome trials [24,25,27,28,37,38]. Although some studies reported a beneficial effect of omega-3 PUFA supplementation on major cardiovascular endpoints [24,26,39], no such effect was observed in other studies and meta-analyses [38,40]. Such differences in outcomes might possibly relate to differences in study design, heterogeneity in the patient population and in the endpoints reported, the fact that patients in the more recent

trials were optimally treated with statins, and most likely the use of different doses, omega-3 PUFA sources, the degree of purity, and the formulation of the omega-3 PUFA products [41]. The dose of 500 mg/kg/day of EPA:DHA 6:1 used in the present study is equivalent to 5.67 g/day of omega-3 PUFAs in a 70 kg human [42], which is within the range of doses reported in different clinical studies, ranging from 0.18 to up to 10 g/day [24,27,40,43,44]. In a previous preclinical study with hypertensive rats, we have shown that oral intake of 500 mg/kg/day of the EPA:DHA 6:1 formulation was associated with increased circulating levels of omega-3 PUFAs and improved Omega Score and n-6/n-3 ratio [32]. The beneficial effects of omega-3 PUFAs on the cardiovascular system have been attributable to several effects such as reduction of the chronic inflammatory response, inhibition of the thrombogenesis, reduction of hypertension, and improvement of the myocardial function [45].

Previous studies have indicated that the age-related endothelial dysfunction is characterized by blunted NO- and often also EDH-components of the endothelium-dependent relaxation as observed in the mesenteric artery [4,6, present findings], femoral arteries [46], aorta [47], and coronary arterioles [48] of rats. The present study indicates that in the old mesenteric artery, the EDH component is abolished, whereas the NO component is partially but significantly decreased. Moreover, a 2-week oral intake of EPA:DHA 6:1 by old rats significantly improved NO-mediated relaxations but not the EDH component. The fact that eNOS inhibition by L-NA increased to a greater extent the PE-induced contractions in the EPA:DHA 6:1-treated group than in the control group of old rats suggests an increased basal release of NO. This is in agreement with our previous investigations showing that EPA:DHA 6:1 is a potent stimulator of the endothelial formation of NO in isolated porcine coronary arteries [30] and human internal thoracic artery [31].

The local vascular angiotensin system appears to have a major role in the induction of endothelial dysfunction and the associated vascular oxidative stress in *in vitro* and preclinical

models of aging and hypertension [8,12,49]. Indeed, ACE inhibitors and/or AT1R antagonists restored, at least partially, the endothelial function in old rats and normalized the level of vascular oxidative stress [34,50,51], indicating the involvement of the AT1R-mediated activation of NADPH oxidase [12,49]. In the present study, the increased expression level of ACE and AT1R points to an increased activation of the local angiotensin system as observed previously in old rats [52]. This is also consistent with the observation that the NADPH oxidase inhibitor VAS-2870 reduced the level of ethidium fluorescence and that an upregulation of the expression of the NADPH oxidase subunits p22^{phox} and p47^{phox} was observed in the old artery. The EPA:DHA 6:1 treatment of old rats normalized the activation of the local angiotensin system including ACE, AT1R, NADPH oxidase subunits p22^{phox} and p47^{phox}, and formation of ROS to a level similar to that observed in young rats. Similar observations to the EPA:DHA 6:1 treatment have been observed in Ang II-induced hypertensive rats [32].

Vascular oxidative stress has also been shown to contribute to age-related vascular alterations [53] and endothelial dysfunction in the mesenteric artery of middle-aged rats [4,6]. The age-related increased formation of ROS involving several vascular sources including COXs, uncoupled eNOS, NADPH oxidase, and the mitochondrial respiratory chain can cause direct inactivation of NO by chemically converting it to peroxynitrite anion (ONOO⁻) and a reduced formation of NO subsequent to the oxidation of BH₄, the most important cofactor of eNOS, thus leading to uncoupled eNOS to further promote the formation of ROS [54]. Uncoupling of eNOS is strongly suggested in the present study by the fact that the increased formation of ROS in the old artery is reduced in the presence of the eNOS inhibitor L-NA. Moreover, the increased expression level of eNOS in the old artery is mostly likely part of a compensatory mechanism subsequent to the reduced bioavailability of NO.

Previous studies have reported that premature endothelial senescence leads to endothelial dysfunction [18,55]. Several cellular stresses including oxidative stress, angiotensin II, high glucose, and reduced availability of NO are associated with the induction of premature endothelial senescence, which causes the upregulation of the cyclin-dependent kinase inhibitors p53, p21, and p16, leading to cell cycle arrest [20]. Moreover, replicative endothelial senescence is associated with an upregulation of the angiotensin system (ACE, AT1R) and NADPH oxidase and increased oxidative stress [19,22]. The present study shows an upregulation of p53, p21, and p16 in the old aorta, suggesting the induction of vascular senescence, which is normalized by the EPA:DHA 6:1 treatment. A previously published study has also shown that omega-3 PUFAs prevented hydrogen peroxide-induced senescence in endothelial cells through upregulation of NRF2-mediated antioxidant responses [56]. Taken together, the present findings indicate that the old mesenteric arteries are characterized by endothelial dysfunction with blunted NO-mediated relaxation, no more EDH-mediated relaxation, and increased COX-derived EDCF responses. Furthermore, the endothelial dysfunction is associated with increased vascular ROS formation, activation of the local angiotensin system, and premature senescence in the arterial wall. Intake of the omega-3 PUFA EPA:DHA 6:1 formulation for 14 days was able to significantly improve the endothelial function by restoring NO-mediated relaxation and decreasing EDCF-mediated contractile responses. The beneficial effect of EPA:DHA 6:1 on the endothelial function is associated with the normalization of the expression level of the local angiotensin system and, subsequently, of the vascular formation of ROS and premature senescence.

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Conflict of interest:

The authors report no conflict of interest.

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Figure legends

Figure 1: EPA:DHA 6:1 improves the age-related endothelial dysfunction in the main mesenteric artery.

Main mesenteric artery rings with endothelium were suspended in organ baths to determine changes in isometric tension. (A) To study the endothelium-dependent relaxation, the rings were contracted with 1 μ M phenylephrine (PE) before the addition of increasing concentrations of acetylcholine (ACh). (B) To assess the contractile responses, concentration-contraction curves were constructed in response to phenylephrine. (C, D) To study endothelium-dependent vasoconstrictor factors (EDCFs), the rings were exposed to L-NA (N^{ω} -nitro-L-arginine, 300 μ M, an eNOS inhibitor) and TRAM-34 plus UCL-1684 (1 μ m each, inhibitors of IK_{Ca} and SK_{Ca} , respectively) to prevent NO and EDH, respectively, with (C) or without (D) indomethacin (10 μ M, a nonselective COX inhibitor) before precontraction by approximately 30% with PE and the subsequent construction of a concentration-contraction curve in response to ACh. (E, F) The function of the vascular smooth muscle was assessed in rings with endothelium contracted with 1 μ M PE before the construction of a concentration-relaxation curve either to sodium nitroprusside (SNP, a NO donor) or to levcromakalim (Lev, an ATP-sensitive potassium channel opener) in the presence of L-NA and TRAM-34 plus UCL-1684 and indomethacin to prevent the contribution of NO, EDH, and vasoactive prostanoids, respectively. Results are expressed in % relaxation (A, D-F) or in grams of contraction (B) as means \pm SEM of 10 rats per group. * $P < 0.05$ vs. Young rats, # $P < 0.05$ vs. Old rats.

Figure 2: EPA:DHA 6:1 improves the age-related decreased NO component of the relaxation and blunted the EDCF component.

Rings with endothelium were prepared from the main mesenteric artery and suspended in organ baths for the determination of changes in isometric tension. (A-D) To study the

endothelium-dependent relaxation, the rings were contracted with 1 μ M phenylephrine (PE) before the addition of increasing concentrations of acetylcholine (ACh). (E-H) To assess the contractile responses, concentration-contraction curves were constructed in response to PE. In some baths, the rings were exposed to a pharmacological agent for 30 min before contraction. To study the role of cyclooxygenase-derived prostanoids, the rings were incubated with indomethacin (10 μ M, a nonselective COX inhibitor). To study the NO-mediated component, the rings were incubated with N^o-nitro-L-arginine (L-NA, 300 μ M, an eNOS inhibitor). The EDH-mediated component was studied in the rings incubated with TRAM-34 plus UCL-1684 (1 μ M each, inhibitors of IK_{Ca} and SK_{Ca}, respectively). Results are expressed in % relaxations (A-D) or in grams of contraction (E-H) as means \pm SEM of 10 rats per group. * P <0.05 vs. control without inhibitors.

Figure 3: EPA:DHA 6:1 improves the age-related upregulation of eNOS and COX-2 and the downregulation of COX-1 in the mesenteric artery and aorta.

Protein immunoreactive signals were determined in fixed cryosections of the main mesenteric artery and thoracic aorta. The determination of the expression level of eNOS, COX-1, and COX-2 was done by immunofluorescence and analyzed using a confocal microscope. Representative confocal images are shown in the upper panel and the corresponding cumulative data in the lower panel. The scale bar corresponds to 50 μ m. Results are expressed as means \pm SEM of 4-5 rats per group. * P <0.05 vs. Young rats, # P <0.05 vs. Old rats.

Figure 4: EPA:DHA 6:1 improves the age-related increased vascular formation of ROS in the mesenteric artery and aorta.

ROS formation was determined in unfixed cryosections of the main mesenteric artery (A) and the thoracic aorta (B, C) by fluorescence histochemistry using the redox-sensitive probe

dihydroethidium and analyzed using a confocal microscope. (C) To determine the sources of the age-related increased ROS formation, the aortic sections of old rats were pretreated with inhibitors including N-acetylcysteine (antioxidant, 1 mM), indomethacin (inhibitor of COX, 10 μ M), NS-398 (COX-2 inhibitor 3 μ M), SC-560 (COX-1 inhibitor, 0.3 μ M), L-NA (inhibitor of eNOS, 100 μ M), VAS-2870 (inhibitor of NADPH oxidase, 10 μ M), and inhibitors of mitochondrial respiratory chain (myxothiazol 0.5 μ M, potassium cyanide 1 μ M, and rotenone 1 μ M) for 30 min at 37 °C before incubation with DHE. Representative confocal images are shown in the upper panel and the corresponding cumulative data in the lower panel. The scale bar corresponds to 50 μ m. Results are expressed as means \pm SEM of 4-5 rats per group. * P <0.05 vs. Young rats, # P <0.05 vs. Old rats control.

Figure 5: EPA:DHA 6:1 improves the age-related upregulation of the NADPH oxidase subunits p22^{phox} and p47^{phox} in the thoracic aorta.

Protein immunoreactive signals were determined in fixed cryosections of the thoracic aorta. The expression level of p22^{phox} and p47^{phox} was determined by immunofluorescence and analyzed using a confocal microscope. Representative confocal images are shown in the upper panel and the corresponding cumulative data in the lower panel. The scale bar corresponds to 50 μ m. Results are expressed as means \pm SEM of 4-5 rats per group. * P <0.05 vs. Young rats, # P <0.05 vs. Old rats.

Figure 6: EPA:DHA 6:1 improves the age-related activation of the local angiotensin system in the thoracic aorta.

Protein immunoreactive signals were determined in fixed cryosections of the thoracic aorta. The expression level of the component of the angiotensin system ACE and AT1R was determined by immunofluorescence and analyzed using a confocal microscope.

Representative confocal images are shown in the upper panel and the corresponding cumulative data in the lower panel. The scale bar corresponds to 50 μm . Results are expressed as means \pm SEM of 4-5 rats per group. * $P < 0.05$ vs. Young rats, # $P < 0.05$ vs. Old rats.

Figure 7: EPA:DHA 6:1 improves the age-related upregulation of senescence markers in the thoracic aorta.

Protein immunoreactive signals were determined in fixed cryosections of the thoracic aorta. The expression level of the senescence markers p53, p16, and p21 was determined by immunofluorescence and analyzed using a confocal microscope. Representative confocal images are shown in the upper panel and the corresponding cumulative data in the lower panel. The scale bar corresponds to 50 μm . Results are expressed as means \pm SEM of 4-5 rats per group. * $P < 0.05$ vs. Young rats, # $P < 0.05$ vs. Old rats.

Figure 1

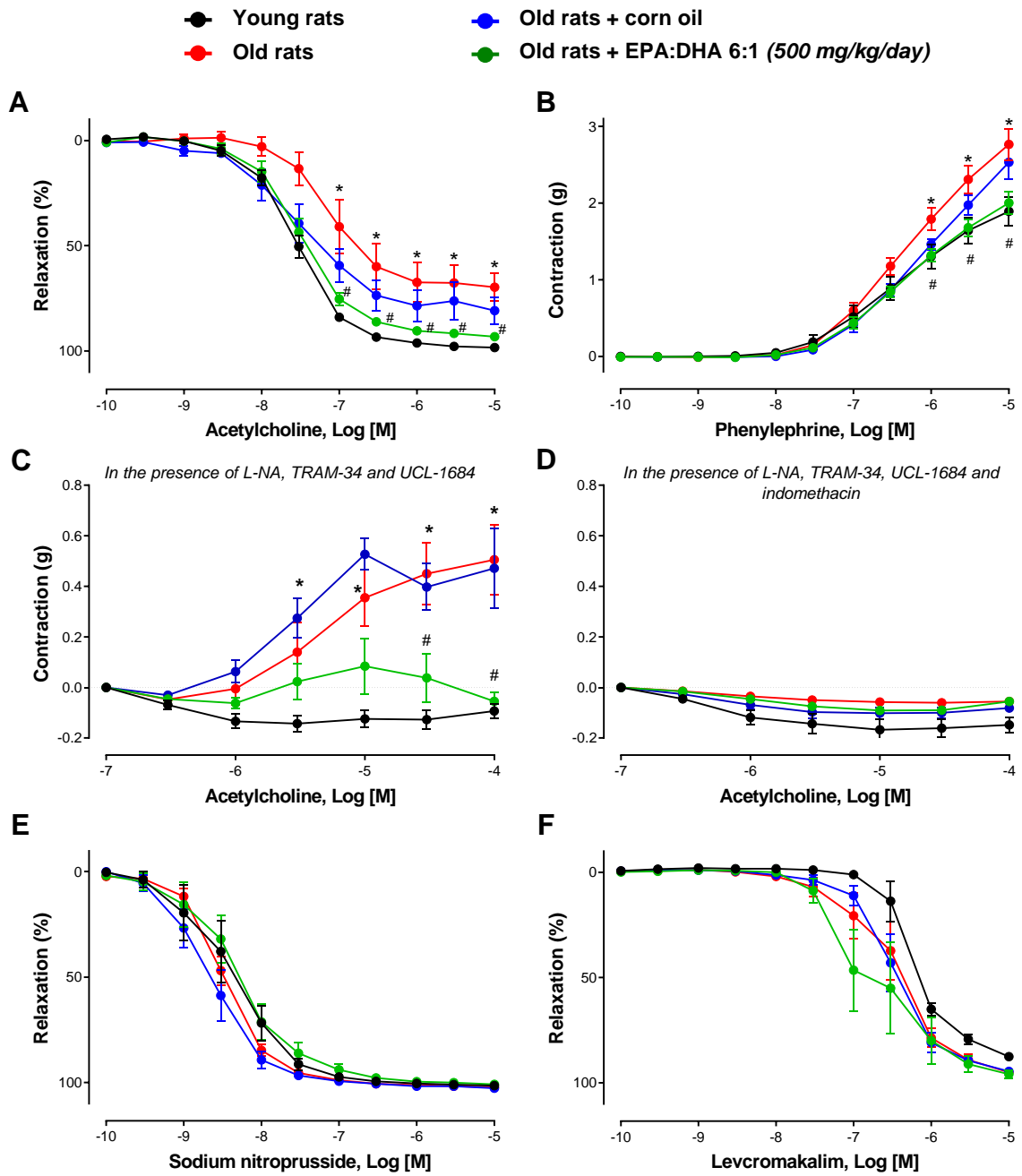


Figure 2

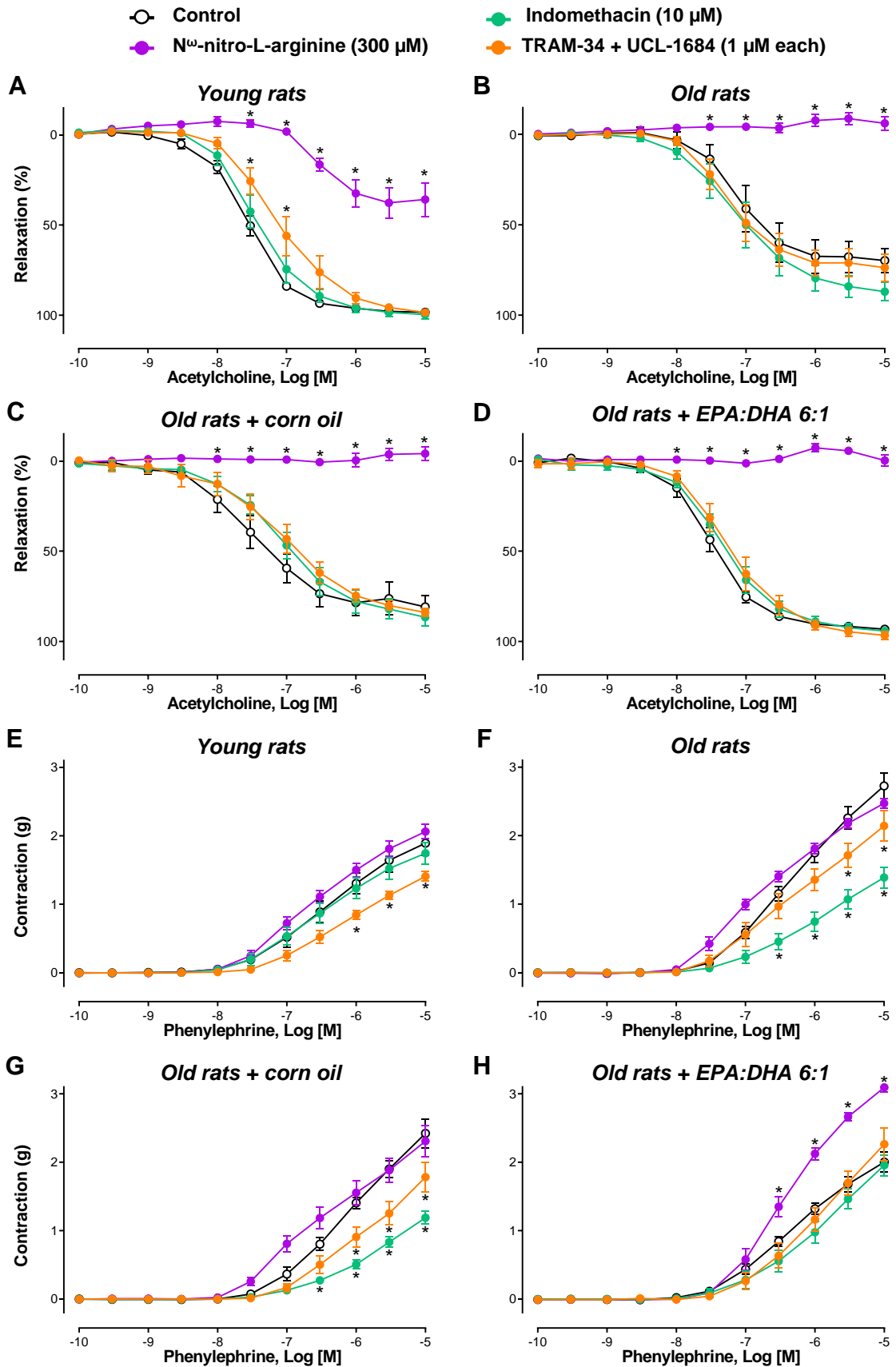


Figure 3

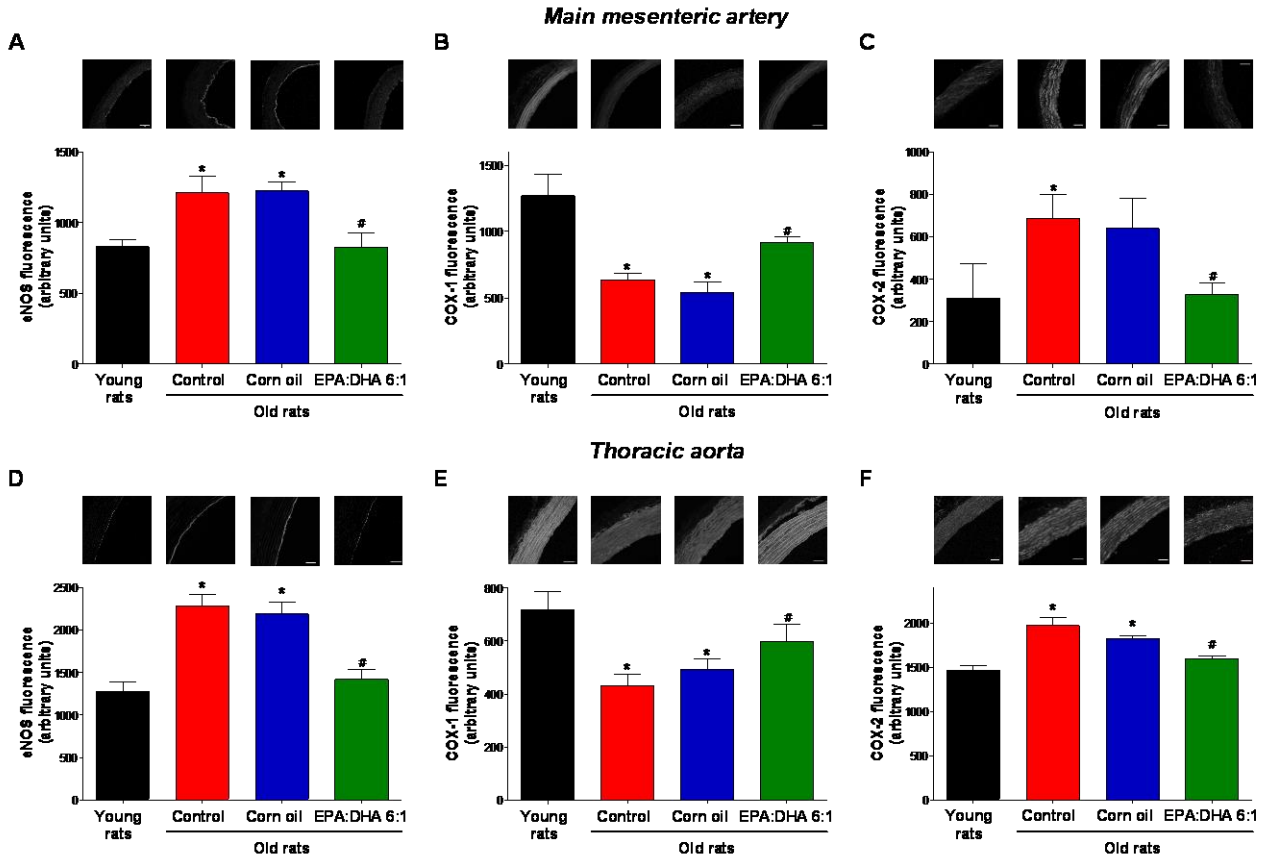


Figure 4

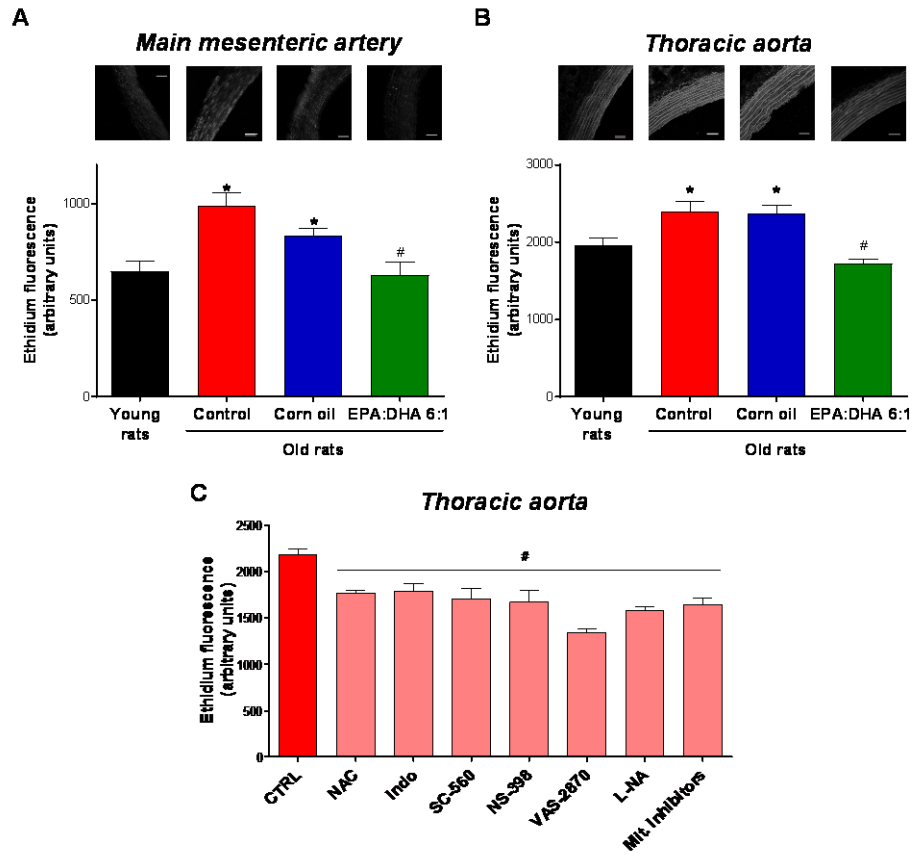


Figure 5

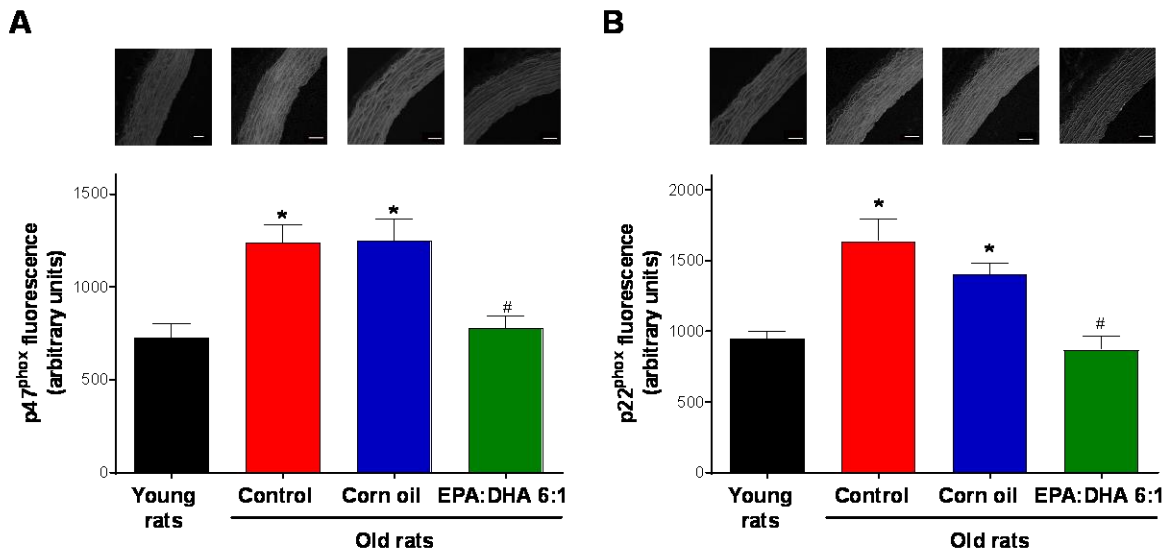


Figure 6

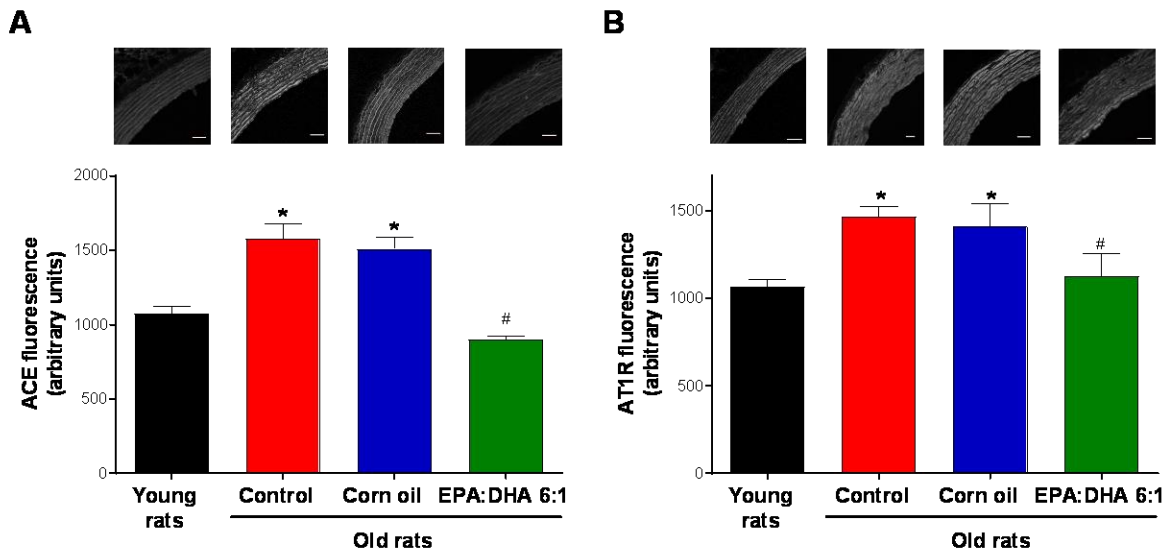


Figure 7

