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Treatment with n-3 polyunsaturated fatty acids reverses endothelial dysfunction and oxidative stress in experimental menopause☆

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Abstract

Menopause is associated with endothelial dysfunction and oxidative stress. In this condition, reduced n-3 polyunsaturated fatty acids (n-3 PUFAs) contribute to cardiovascular disease. We investigated whether treatment with n-3 PUFA reverses endothelial dysfunction and oxidative stress in experimental menopause. Thirty female rats underwent either sham-surgery or bilateral ovariectomy or bilateral ovariectomy+oral n-3 PUFA (0.8 g kg^{-1} day^{-1} for 2 months).

Ovariectomy caused endothelial dysfunction to acetylcholine, which was reversed by superoxide scavenger Tiron. Erythrocyte membrane lipid composition was characterized by reduced n-3 PUFA total content and omega-3 index, and by concomitant increase in n-6:n-3 PUFA ratio. Ovariectomy-related oxidative stress, demonstrated by both enhanced superoxide production and 3-nitrotyrosine expression in aorta, was associated with increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit NOX-4 protein expression. Endothelial nitric oxide synthase (eNOS) functional inhibition by L-NG-nitroarginine methyl ester, protein expression and activity did not change.

In ovariectomized rats, treatment with n-3 PUFA increased n-3 PUFA total content and omega-3 index and decreased n-6:n-3 PUFA ratio in erythrocyte membrane, reversed vascular oxidative stress, endothelial dysfunction, aortic 3-nitrotyrosine and markedly lowered NOX-4 protein expression; eNOS protein expression also increased, paralleled by reversal of inhibitory binding to Caveolin-1, while ex-vivo functional inhibition and NOS synthesis were unchanged.

These findings demonstrate in vivo a therapeutic benefit of n-3 PUFA on menopause-associated endothelial dysfunction by reversal of alterations in membrane lipid composition induced by ovariectomy and by reduction of vascular oxidative stress. In this setting they also identify NOX-4 as a potential target to reduce oxidative stress-mediated vascular complications.

Keywords: Menopause; Endothelial dysfunction; n-3 polyunsatured fatty acids; Oxidative stress; Endothelial nitric oxide synthase

1. Introduction

Menopause is strictly associated with endothelial dysfunction and atherosclerosis [1]. Estrogen deficiency increases cardiovascular risk, as a result of atherogenic modifications of plasma lipid profile, activation of the renin-angiotensin system and overproduction of reactive oxygen species (ROS) which quench nitric oxide (NO) [2,3]. Also, menopause-related decrease in estrogen receptors correlates with lower endothelial nitric oxide synthase (eNOS) expression and activation [4] as eNOS activity is notably regulated by estrogens through the modulation of the eNOS/caveolin-1 (Cav-1) complex formation [5]. Endothelial dysfunction determined by reduced NO bioavailability promotes the development of atherosclerosis and its complications. Impaired vascular function due to ROS overoproduction which occurs during estrogen deficiency is normalized by hormone replacement therapy in human and experimental menopause [6,7].

Compelling evidence demonstrates the cardiovascular benefits of n-3 polyunsaturated fatty acids (n-3 PUFA). Consumption of fish oil significantly reduces cardiovascular mortality in both primary and secondary prevention trials of ischemic heart disease [8]. In observational studies, the relative risk of cardiac events is inversely correlated with baseline blood and tissue levels of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids [9,10]. Potential mechanisms underlying beneficial effects of n-3 PUFA on endothelial dysfunction and atherosclerosis include enhanced vasodilation by correction of the imbalance between vasoconstrictor and vasodilator endothelium-derived factors [11], inhibition of nuclear factor kappa B (NF-kB) activation and of cyclooxygenase-2 (COX-2) expression [11], reduction of endothelial expression of adhesion molecules [12,13], suppression of leukocyte adhesion to endothelial cells [14] and modulation of oxidative stress [15–17]. Several studies suggest a link

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between n-3 PUFA-mediated cardioprotective effects and the involvement of caveolae [18], a subset of membrane lipid rafts which plays a key role in endothelial function [19]. By modulating caveolar lipid composition, dietary fatty acids ultimately regulate caveolar signal transduction pathway responses [20]. In endothelial cells eNOS localizes in caveolae [21] where its binding with Cav-1 causes direct inhibition of its activity [22]. In vitro studies have shown that both EPA [23] and DHA [24] can be incorporated in endothelial caveolar membranes, altering the liquid ordered state of caveolae, displacing both Cav-1 [20] and eNOS, this way activating the enzyme [23,24]. Also nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex co-localizes with Cav-1 in both endothelial [21] and vascular smooth muscle cells [25]. Caveolae-associated NADPH oxidase isoform 1 (NOX-1) is reportedly the major source of intracellular superoxide in coronaric smooth muscle cells in diabetic mice [26]. In addition, n-3 PUFA are direct modulators of superoxide anion production from the non caveolar NADPH oxidase isoform 4 (NOX-4) [27], highly expressed in the vasculature and localized in focal adhesion sites [25,28], and one of the primary sources of superoxide production together with its regulatory subunit $p22^{phox}$ [29,30]. Finally, n-3 PUFA improve lipid profile by reducing free fatty acids and triglyceride levels [31]. In patients prone to atherosclerosis, which is characterized by multiple cardiovascular risk factors, administration of n-3 PUFA reverses endothelial dysfunction [32].

Despite reported therapeutic benefits of n-3 PUFA in coronary artery disease, hypertension and dyslipidemia, their precise role in the prevention of endothelial dysfunction and oxidative stress in the estrogen-deficient state remains unclear. Since low tissue and plasma levels of n-3 PUFA define a population of menopausal women at increased risk of cardiovascular disease [10], restoration of normal levels of n-3 PUFA may represent an effective therapeutic approach to prevent atherosclerosis and its complications during menopause.

Therefore, the present study investigates the hypothesis that chronic treatment with n-3 PUFA can restore endothelial dysfunction in estrogen-deficient ovariectomized (OVX) rats, an animal model widely employed to mimic menopause.

2. Methods

2.1. Drugs and reagents

A commercially available pharmaceutical preparation from refined fish oil was used for n-3 PUFA treatment (actual content of EPA and DHA is 450±50 mg/g and 395±55 mg/g respectively, Eskim, SigmaTau, Italy). The manufacturing process fulfils the good manufacturing practice standards, as certified by the manufacturer. The production and encapsulation steps were optimized to reduce the risk of lipid peroxidation and to improve stability of the compounds. The amount of lipid peroxides in the drug, tested both at release and at the end of validity period, was below the limit set by the European Pharmacopoeia (NMT 10 mEq/kg). Alpha tocopherol (0.29 mg/g) is also added to the preparation in order to prevent lipid peroxidation. 0.9% saline was manufactured by Diaco (Trieste, Italy). All other drugs and chemicals for ex-vivo experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA) and of the highest purity.

2.2. Animals and experimental protocol

Adult (n=30) female Wistar rats (2 months old weighing 170 g) were supplied by Harlan (San Pietro al Natisone, Italy) and fed a standard rat chow (Harlan 2018, 3.4 cal/g, Harlan, San Pietro al Natisone, Italy) for at least 2 weeks. Animals were then randomly assigned to three experimental groups (n=10 rats each): (1) sham-operated rats (CTR); (2) OVX rats; (3) OVX animals receiving daily oral administration of n-3 PUFA (0.8 g kg^{-1} day^{-1}) by esophageal gavage (OVX+PUFA). Bilateral ovariectomy and sham surgery were performed via a mid-abdominal route after rats were sedated and anesthetized using Xylazine and Ketamine [10 mg kg^{-1}+100 mg kg^{-1}, intraperitoneal injection (IP)]. Anesthesia was evaluated by complete absence of limb retraction upon painful stimulation. To standardize study protocol CTR and OVX rats received an equal volume of normal saline solution by gavage per day. Throughout the study animals were individually housed in wire-bottom cages in a controlled environment (12 h light–dark cycle, 20–22°C, 50–60% relative humidity), having free access to food and water. Body weight and food consumption were recorded regularly. Two months after surgery rats were anesthetized as previously described. Blood was collected through cardiac puncture and aortas were subsequently harvested with careful surgical dissection. In each animal, thoracic aorta was divided into seven rings: three were immediately snap frozen in liquid nitrogen and stored at −80°C for Western blot and 3-nitrotyrosine determination, while the rest were immediately used for ex-vivo vascular reactivity studies and superoxide anion synthesis assays. The same segment was used for each assay in all animals. Blood samples were centrifuged at 1000×g, 4°C for 15 minutes and plasma stored at −80°C until analysis. Animal care and experimental procedures were approved by the local Institutional Review Committee for animal studies.

2.3. Determination of blood glucose, plasma lipids and estrogen

Blood glucose and lipids were measured by standard commercial colorimetric enzymatic assays (BioMerieux, Marcy l'Etoile, France; Roche Diagnostics, Basel, Switzerland). Plasma high-density lipoprotein (HDL) cholesterol was measured using the same method as for total cholesterol, prior precipitation of non-HDL cholesterol by reaction with 2.5 volumes of a commercial reagent containing fosfotungstic acid (Roche, Basel, Switzerland). Non-HDL cholesterol was calculated by subtracting from total cholesterol the HDL fraction. The concentration of plasma estrogen was measured using a commercial immunosorbent assay kit (Estradiol sensitive ELISA, DRG Instruments, Marburg, Germany) according to manufacturer's recommendations.

2.4. Analyses of vascular reactivity

Rings (3 mm long) from each thoracic aorta were used for assessing vascular reactivity immediately after dissection. Rings were suspended in organ chambers filled with 25 ml of gassed (95% O_2 and 5% CO_2) modified Krebs-Ringer bicarbonate solution (composition in mmol L^{-1}: 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$,1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 0.026 EDTA, 11.1 dextrose, pH 7.4). The rings were allowed to equilibrate for 1 h at 37°C and then stretched to the optimal point as determined by repeated exposure to 20 mmol L^{-1} KCl. The maximal contraction of each ring was determined by phenylephrine (PHE) 10^{-5} mol L^{-1}. After washing and re-equilibration, submaximal contraction was then obtained using a 10^{-6} mol L^{-1} concentration of PHE. Dose-relaxation curves were finally performed by subsequent cumulative addition of acetylcholine (10^{-9} to 10^{-5} mol L^{-1}) or DEA-NONOate (2-(N,N-diethylamino)-diazenolate-2-oxide; 10^{-10} to 10^{-5} mol L^{-1}) to the precontracted rings. In single dose experiments, the effects of an addition of acetylcholine (10^{-6} mol L^{-1}) and DEA-NONOate (10^{-6} mol L^{-1}) on aortic rings in submaximal precontraction were tested in basal conditions, after 20 min incubation with the superoxide scavenger Tiron (200 μmol L^{-1}) and with the NOS inhibitor N_ω-nitro-L-arginine methyl ester (L-NAME, 100 μmol L^{-1}). Each cycle was performed after extensive washing of the bath chamber and a subsequent 20 min re-equilibration of the rings, as shown in Fig. 2A.

2.5. Total NOS activity in the aorta

Total NOS activity was assessed in rat aorta by measuring the conversion of oxyhemoglobin (HbO_2) to methemoglobin ($MetO_2$) by NO, as previously described [33]. Briefly, 12 μg of total protein extract from each frozen aortic sample were added in a 96-well microplate well and incubated at room temperature for 10 minutes in phosphate-buffered saline (PBS) 0.01 mol L^{-1} pH 7.2, with the addition of $CaCl_2$ (250 μmol L^{-1}). NOS dependance of the HbO_2 to MetHb conversion was ensured by addition, in some experiments, of L-NAME (100 μmol L^{-1}), a non-selective NOS inhibitor. An assay buffer (final concentrations in μmol L^{-1}: 0.15 HbO_2, 20 L-Arginine, 100 NADPH) was then added and HbO_2 to $MetO_2$ conversion was monitored for 15 min by measuring the difference in absorbance between 421 and 401 nm.

2.6. Superoxide anion production

Superoxide anion generation in intact aortic rings was measured by the SOD-inhibitable cytochrome c reduction assay as described by Landmesser et al. [34] with slight modifications. Before the assay, rings were equilibrated at room temperature in oxygenated Krebs-HEPES buffer (composition in mmol L^{-1}: 99 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1 KH_2PO_4, 1.9 $CaCl_2$, 25 $NaHCO_3$, 11 Glucose, 20 HEPES, pH 7.4) and then transferred to a 96-well transparent microplate, followed by immediate addition to each well of 150 μl of the appropriate freshly made assay buffer (Krebs-HEPES pH 7.4 containing 100 μmol L^{-1} cytochrome c, 125 U ml^{-1} catalase±100 U ml^{-1} SOD). Two rings from each aorta were assayed: one in the presence and one in the absence of SOD. Blanks were made for both assay buffers. Cytochrome c reduction was monitored kinetically for 40 minutes during incubation of the rings at 37° in a microplate spectrophotometer (SpectraMax Plus 384; Molecular Devices, Sunnyvale, CA, USA) reading absorbance at 540, 550 and 560 nm. Cytochrome c peak at 550 nm was corrected by background readings at the isobestic points of 540 and 560 nm with the formula: A_{550}-(A_{540}+A_{560})/2, and its variation over time was calculated. Area of the aortic wall surface was measured by appropriate software (ImageJ; NIH, Bethesda MD, USA) after image scanning and used to normalize results for each ring. Superoxide production was quantified in nmol min^{-1} mm^{-2} of aortic wall using the difference between calculated cytochrome c reduction rate with or without SOD for each rat, extinction coefficient for reduced cytochrome c being 21.1 mmol L^{-1} cm^{-1}.

2.7. 3-Nitrotyrosine

Nitration of protein tyrosine in aortic tissue samples was measured by chemiluminescence enhanced indirect ELISA, as previously published [33]. In brief, samples were coated on a white 96-well plate (Luminunc, Nunc, Roskilde, Denmark) for 2 h at 37°C. Nonspecific binding sites were then blocked with blocking buffer (Ovalbumin 0.5% (w/v), Tween-20 0.05% (v/v), PBS 0.01 mol L^{-1} (pH 7.4) for 2h at 37°C. Anti-nitrotyrosine monoclonal antibody (Anti-NT mAb clone 1A6, Millipore, Billerica, MA, USA; 1:2000) in blocking buffer was added and incubated at 37°C. Following extensive washing of unbound antibody, horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000) in blocking buffer was added for 1 h at room temperature. After washing and addition of enhanced chemiluminescence substrate (LumiGLO; Cell Signalling), at 10 min luminescence was read in a microplate luminometer (Synergy2, BioTek, Winooski, VT, USA). Measurements were obtained by interpolation from a standard curve of serial dilutions of nitrosylated bovine serum albumin (BSA^{NT}) and expressed in μg BSA^{NT} equivalents mg $protein^{-1}$.

2.8. Determination of fatty acid composition and Omega-3 index of erythrocyte membranes

Fatty acid (FA) composition of erythrocyte membranes was analyzed as referenced [35,36]. Briefly, erythrocytes (200 μl) were washed five times with decreasing concentrations (10 mmol L^{-1}, 2.5 mmol L^{-1}, 1.25 mmol L^{-1}, 0.625 mmol L^{-1}, 0.312 mmol L^{-1}) of PBS and resuspended in 500 μL of isotonic PBS. Total lipid extraction was performed in 5 ml of chloroform-methanol (2:1) solution, containing 50 mg l^{-1} of butylhydroxytoluene (Sigma-Aldrich) as antioxidant, and 1 ml of 1 M NaCl solution. After centrifugation the lower lipid phase was collected and dried under nitrogen flux at 40°C. Pellets were redissolved in toluene (500 μl) and, after the addition of 1 ml methanol solution containing 2% of H_2SO_4, samples were heated at 50°C for 2 hours. A neutralizing solution (1.0 ml, 0.25 M $KHCO_3$ and 0.5 M K_2CO_3) and hexane (1 ml) were added and, after centrifugation the hexane layer, containing fatty acid methyl esters (FAMEs) were collected; organic solvents were removed by nitrogen flux at 40°C. Extracted lipids were resuspended in hexane (150 μl) and analyzed by gas-chromatography-flame ionization detection (GC-FID; GC 6850 Agilent Technologies, Santa Clara, CA, USA). Helium was used as carrier gas. Detector temperature as well as injector temperature were set at 300°C. Column oven temperature started at 115°C (constant for 2 min) and increased afterwards by a gradient ramping of 10°C min^{-1} until 200°C. Temperature remained constant at 200°C for 11.5 min, and increased to 245°C by a gradient ramping of 60°C min^{-1}. Area-under-the-curve of each selected peak was determined by highly standardized hand integration performed using commercial software (HP Chem station; Agilent Technologies, Santa Clara, CA, USA). Individual FAMEs were identified comparing retention times of specific FA standards. Omega-3 index was calculated as the ratio between the sum of EPA and DHA peaks and the sum of all identified FA peaks; n-6 n-3 PUFA ratio as the ratio between the sum of n-6 and n-3 FA peaks expressed as percentage of the sum of all identified FA peaks.

2.9. Aortic protein expression and interaction

Protein levels of eNOS, NOX-1, NOX-4, $p22^{phox}$ and Cav-1 were measured by Western blot analysis. Briefly, aortic segments were isolated and immediately stored in liquid nitrogen. The frozen segments were then homogenized on ice in lysis buffer (composition in mmol L^{-1}: 50 Tris–HCl, 0.1 EDTA, 0.1 EGTA, 1 PMSF, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.1% (w/v) deoxycholate, 1%(w/v) nonidet NP-40, 2 μg ml^{-1}) supplemented with protease inhibitors (Sigma). After centrifugation at 16 000×*g* for 10 min, protein concentration was determined by the BCA method. After separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the resolved proteins were transferred to 0.2 μm nitrocellulose membrane by semi-dry electroblotting (Bio-Rad, Berkeley, CA, UK). Equal protein load was confirmed by Ponceau-S staining. Blots were blocked and incubated with anti-eNOS (610296; BD Transduction Laboratories, Franklin Lakes, NJ, USA; 1:1000), anti-NOX-1 (ab55831, Abcam; 1:300), anti-NOX-4 (ab61248, Abcam, Cambridge, UK; 1:400), anti-$p22^{phox}$ (ab75941, Abcam; 1:500) or anti-Cav-1 (610060, BD Transduction Laboratories; 1:2000) antibodies overnight at 4°C. After extensive washing, appropriate HRP-conjugated secondary antibodies were added. Chemiluminescence detection was performed by exposure of x-ray films to membranes after incubation with ECL reagents. Membranes were then extensively washed, re-blocked, and reprobed for β-actin using a HRP conjugated antibody (A3854, Sigma; 1:20000, 1h, 22°C). Densitometric scans (GS-700; BioRad) of films were analyzed by appropriate image analysis software (Molecular Analyst, BioRad; ImageJ; NIH, Bethesda, MD, USA). Data are expressed in arbitrary units (a.u.) after normalization by β-actin.

Protein-to-protein interaction was evalued by co-immunoprecipitation. In brief 500 μg of tissue homogenate were pre-cleared from antibodies using washed protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After addition of 4 μg/ml anti-Cav-1 antibody and overnight rocking incubation at +4°C, immunocomplexes were bound to A/G agarose beads during a subsequent 4 h incubation, washed in PBS and collected by centrifugation (13 000×*g*, 4°C). The pulled down proteins were released by addition of 2× Laemmli buffer and heating (10 min, 95°C). The unbound fraction, co-immunoprecipitated proteins and the cleared lysate were then resolved by SDS-PAGE and analyzed by Western Blot as described.

Table 1
Characteristics of CTR, OVX and OVX+n-3 PUFA rats

Variable	CTR	OVX	OVX+PUFA
Body weight (g)	211.0±3.9 [a]	246.4±11.9 [b]	263.3±7.3 [c]
Plasma 17β-estradiol (pmol L^{-1})	65.34±23.13[a]	5.87±1.84 [b]	7.34±3.30 [b]
Total cholesterol (mmol L^{-1})	1.07±0.11[a]	1.50±0.17 [b]	1.07±0.07 [a]
Triglyceride (mmol L^{-1})	0.71±0.08 [a]	0.58±0.08 [a]	0.58±0.07[a]
HDL (mmol L^{-1})	0.63±0.16 [a]	0.61±0.19 [a]	0.49±0.08 [a]
Non-HDL (mmol L^{-1})	0.41±0.09 [a]	1.00±0.10 [b]	0.61±0.10 [a]

Results are means±S.D. (n=10 animals/group). Means in the same row that share a common superscript letter do not differ, while those that do not share a letter differ at least by P<.05.

2.10. Statistical analysis

Data are presented as mean±S.E. Relaxation responses in ex-vivo vasodilation experiments are given as a percentage of precontraction induced by phenylephrine (PHE). Statistical analysis was performed by one-way analysis of variance to detect significant differences in multiple comparisons. Post hoc analysis was performed, where appropriate, by using a *t* test with Bonferroni's adjustment. Paired Student's

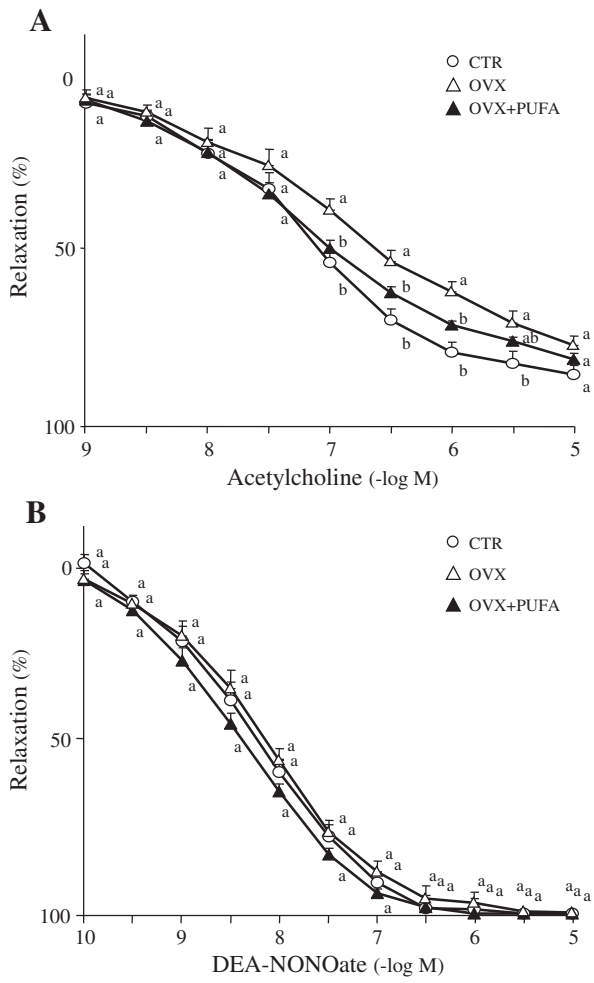

Fig. 1. Effects of OVX and n-3 PUFA treatment (OVX+PUFA) on endothelial function in thoracic aorta. (A) Dose-relaxation curves to acetylcholine (Ach) and (B) DEA-NONOate, an NO donor. Half-maximal contraction to phenylephrine was similar in the three groups. Values are expressed as means±S.E. Groups not sharing a letter differ at least by P<.05. n=10 animals/treatment group.

t test was used for repeated measures analysis in single dose vasodilation experiments. A value of $P<.05$ was considered to be statistically significant.

3. Results

3.1. Body weight, hemodynamic parameters and plasma biomarkers

Compared with sham-operated animals, 60 days after surgery body weight was higher ($P<.05$) in OVX rats, and it further increased ($P<.05$) in animals treated with n-3 PUFA (Table 1). The reduced serum estradiol levels in OVX rats were unaffected by n-3 PUFA treatment (Table 1). Ovariectomy increased plasma levels of total cholesterol and non-HDL cholesterol, while n-3 PUFA normalized plasma lipids (Table 1).

3.2. Ovariectomy inhibits endothelium-dependent but not –independent relaxation

Acetylcholine-induced endothelium-dependent relaxations were markedly ($P<.05$) impaired following OVX (Fig. 1A). By contrast, DEA-NONOate induced endothelium-independent relaxations were similar in control and OVX rats (Fig. 1B), indicating that the sensitivity of aortic smooth muscle cell in response to NO was unaltered in OVX animals.

3.3. ROS scavengers acutely ameliorate OVX-related endothelial dysfunction

The impaired endothelial acetylcholine-mediated relaxations in OVX rats were acutely reversed by 20-min treatment with Tiron (Fig. 2B and C). This finding supports a role for ROS in the ovariectomy-induced endothelial dysfunction. The acetylcholine-induced vasorelaxations were similarly abolished in the three experimental groups by preincubation with the NOS inhibitor L-NAME (Fig. 2B and C).

3.4. Chronic n-3 PUFA treatment prevents endothelial dysfunction

Chronic treatment of OVX rats with n-3 PUFA reversed the impaired acetylcholine-induced vasorelaxations (Fig. 1A). Endothelium-independent vasorelaxations were unaffected by n-3 PUFA supplementation (Fig. 1B).

3.5. n-3 PUFA increase aortic total eNOS protein expression but do not alter NOS activity

eNOS protein expression and activity were similar in control and OVX rats; following n-3 PUFA treatment eNOS expression increased significantly (Fig. 3A), while NOS activity did not change (Fig. 3B).

3.6. n-3 PUFA reverse OVX-mediated selective increase in NOX-4 protein expression, superoxide production and oxidative stress in rat aorta

Ovariectomy increased ($P<.05$) protein expression of NOX-4 in rat aortas (Fig. 4B) and there was a trend towards the increase of NADPH oxidase subunit $p22^{phox}$ (Fig. 4C). Overexpression of both NOX-4 and $p22^{phox}$ was inhibited by treatment with n-3 PUFA (Fig. 4B and C). Total NOX-1 protein expression was similar in all groups (Fig. 4A).

Superoxide anion production was markedly ($P<.05$) increased in OVX rat aorta; n-3 PUFA treatment prevented this increase (Fig. 4D). Likewise increased protein tyrosine nitration, a marker of peroxynitrite formation, was prevented by chronic n-3 PUFA treatment in OVX rat aorta (Fig. 4E).

3.7. Effect of ovariectomy and n-3 PUFA treatment on membrane lipid composition, Caveolin-1 protein expression and interactions

OVX caused remodeling of erythrocyte membrane lipid composition as showed in Table 2. Saturated FAs increased ($P<.02$) while monounsaturated as well as n-6 polyunsaturated FAs total content was unaffected by OVX. On the contrary, n-3 polyunsaturated FAs

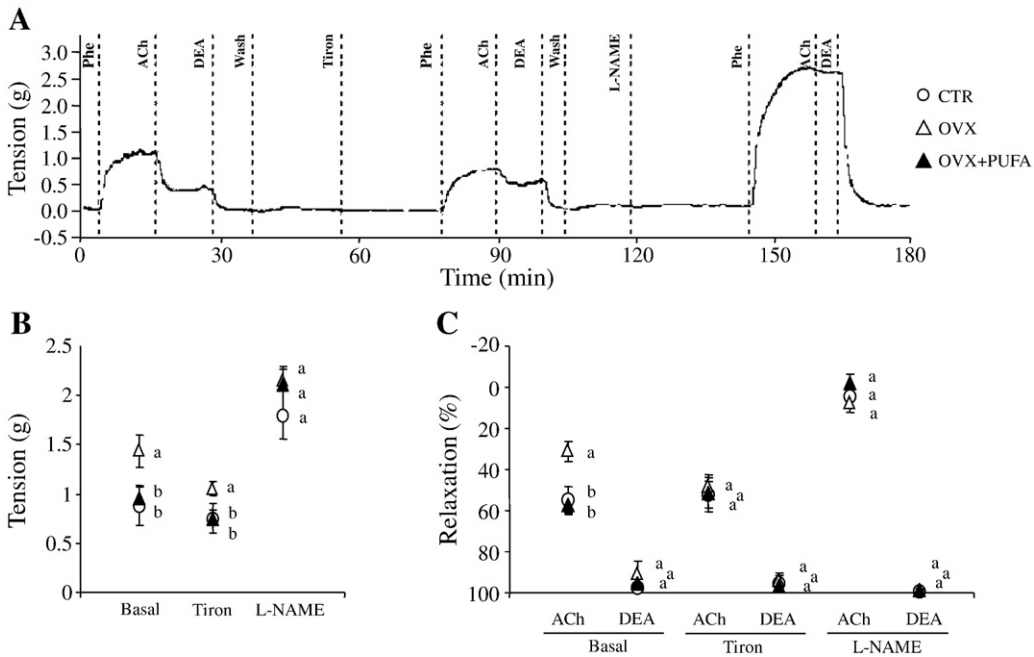

Fig. 2. Impact of superoxide anion and NOS on endothelial function. (A) Representative tension/time chart of the sequence of single dose experiments performed on thoracic aortic rings. (B) Vessel contraction response to 10^{-6} mol L^{-1} PHE and (C) subsequent Ach- or DEA-NONOate-mediated vasodilation in basal conditions or after 20 min incubation with 200 μmol L^{-1} Tiron or 100 μmol L^{-1} L-NAME. Values are expressed as means±S.E. Groups not sharing a letter differ at least by $P<.05$. $n=10$ animals/treatment group.

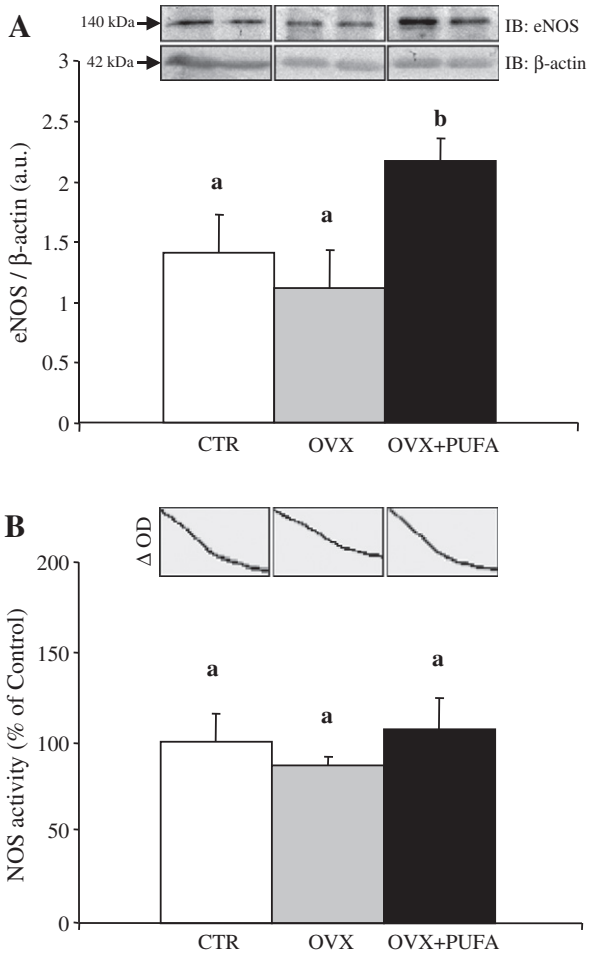

Fig. 3. eNOS protein expression and total NOS activity in thoracic aortas from experimental animals. (A) Western blot analysis of eNOS protein expression. (Top) Representative blots showing eNOS and β-actin bands. (Bottom) Densitometric analysis. (B) NO synthesis by NOS. (Top) Representative charts of time-related NO-dependent conversion of HbO_2 to $MetO_2$, by measuring the difference in absorbance between 421 and 401 nm (ΔOD), as described in Materials and Methods. a.u., arbitrary units. Values are expressed as means±S.E. Groups not sharing a letter differ at least by $P<.05$. $n=8$ animals/treatment group.

were reduced ($P<.02$). In particular, relative erythrocyte membrane content of EPA and DHA (omega-3 index) was diminished ($P<.05$) in OVX rats compared with the control group, while n-6:n-3 PUFA ratio increased ($P<.05$) (Fig. 5A and B). In this setting, Caveolin-1 protein expression did not change (Fig. 6A).

Treatment with n-3 PUFA markedly enhanced ($P<.0001$) total membrane content of n-3 unsaturated FAs and reduced ($P<.0001$) that of n-6 FAs. Omega-3 index increased ($P<.0001$) in OVX rats treated with n-3 PUFA (Fig. 5A). This change was paralleled by a concomitant reduction ($P<.0001$) in n-6:n-3 PUFA ratio in the same study group (Fig. 5B).

Total Caveolin-1 protein expression in rat aorta was also decreased by n-3 PUFA (Fig. 6A). OVX increased eNOS inhibitory interactions with Cav-1 and this finding was markedly reversed by n-3 PUFA (Fig. 6B). NOX-1 co-immunoprecipitation with Cav-1 was not altered in the three groups (Fig. 6B).

4. Discussion

n-3 PUFA reduces the risk of cardiovascular events in several conditions associated with atherosclerosis. Nevertheless, little is known about their effects to correct menopause-associated endothelial dysfunction and the underlying molecular mechanisms are still poorly characterized.

In this study, menopause-associated endothelial dysfunction resulted from increased oxidative stress, as demonstrated by enhanced vascular-derived superoxide production and aortic protein nitrosylation in OVX rats. ROS have been previously implicated in endothelial dysfunction and hypertension during estrogen deficiency [3]. In this condition, activation of angiotensin type 1 receptor-associated NADPH oxidase results in the overproduction of superoxide anion, which is responsible for vascular dysfunction. In our study, the causal role of oxidative stress in menopause-associated endothelial dysfunction was demonstrated by the finding that acute treatment with Tiron (a scavenger of superoxide anion) completely prevented endothelial dysfunction in OVX animals. Also, increased oxidative stress was paralleled by a selective increase in the expression of NAD(P)H oxidase isoform NOX-4 in aorta. Chronic treatment with n-3 PUFA blunted cellular oxidative stress and markedly down-regulated NOX-4 expression ultimately reversing endothelial dysfunction.

Experimental menopause led to a significant decrease in anti-inflammatory n-3 PUFA content of erythrocyte membrane, as shown by the low omega-3 index in OVX rats. This was paralleled by an increase of pro-inflammatory n-6:n-3 PUFA ratio. Changes in the fatty acid composition of erythrocyte membranes, specifically of omega-3 index, represent an early marker of increased cardiovascular risk [37]. In postmenopausal women, decreased plasma concentrations of n-3 PUFA are strictly associated with vascular inflammation and endothelial activation and with increased incidence of coronary heart disease [10,38]. Oral treatment with n-3 PUFA reversed n-3 PUFA content of erythrocyte membrane, omega-3 index, normalized n-6:n-3 PUFA ratio and reduced oxidative stress in thoracic aortas by down-regulating NOX-4 protein expression. NADPH oxidase is the major source of ROS in the vasculature [39] and NOX-4, which is distinctively associated with the cell membrane, is highly expressed in endothelial cells, where it represents the primary source of superoxide production together with the regulatory NOX subunit $p22^{phox}$ [29,30]. Previous in vitro studies using NADPH oxidase inhibitors and mRNA silencing, demonstrated that in fibroblasts NOX-4 is a specific target of DHA modulation, independently from intracellular antioxidant defenses [27]. Exposure of endothelial cells to DHA results in its incorporation into the cell membrane and in the inhibition of NOX-4 expression and activity [40,41]. Experimental evidence also shows that this effect is mediated through endothelial secreted phospholipase A2, ERK and PKC signaling pathways [40,41]. In this study we extend these findings from an in vitro to an in vivo setting, demonstrating that oral treatment with n-3 PUFA in menopause results in their efficient incorporation into plasma membrane, where they blunt oxidative stress by down-regulating NADPH oxidase activity through selective modulation of NOX-4.

The impact of n-3 PUFA on endothelial function in humans is currently unclear. Previous studies failed to demonstrate any effect of EPA and DHA on healthy individuals at dosages up to 3.4 g/day, despite a triglyceride-lowering effect at the highest dose [31,42]. In contrast, studies in patients with cardiovascular risk factors or overt coronary artery disease suggest that n-3 PUFA may improve endothelial function although the duration and dose of treatment are still unknown [32,43]. Vascular inflammation and endothelial dysfunction are closely linked in the natural history of atherosclerosis and its complications. n-3 PUFA restore endothelial dysfunction by decreasing NF-kB activation and reducing COX-2 expression [11]. The anti-inflammatory effects of n-3 PUFA are seen at relatively high doses of EPA and DHA [37,38]. In this study an oral dose of 0.8 g/kg/day of study supplement, corresponding to 0.65 g/kg per day of purified active compounds (EPA+DHA) was chosen to unveil their

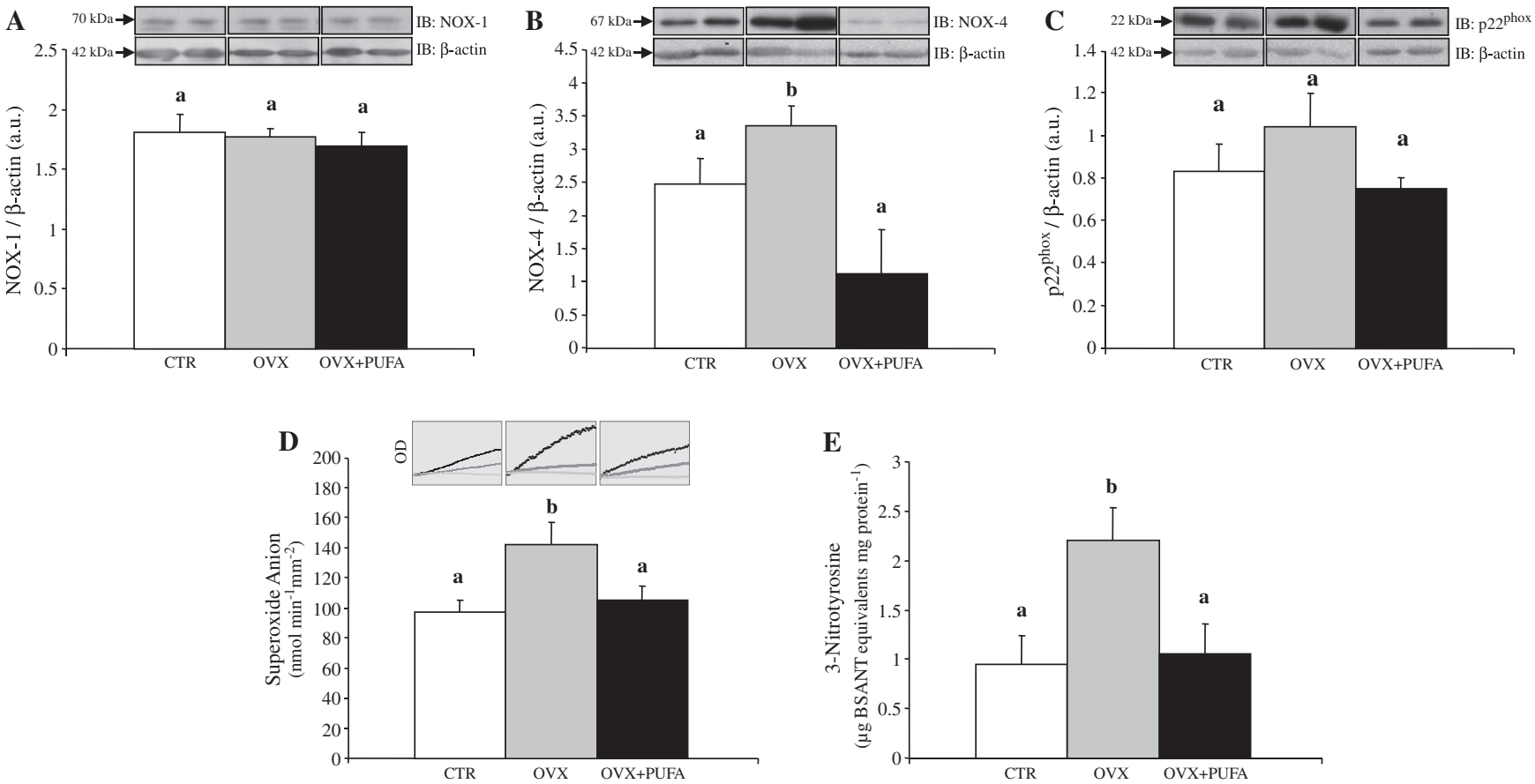

Fig. 4. Effects of OVX and n-3 PUFA supplementation (OVX+PUFA) on protein expression of NADPH oxidase subunits, superoxide anion production rate and 3-nitrotyrosine concentration in thoracic aortas. (A–C) Western blot analysis of NADPH oxidase isoforms NOX-1 (A), NOX-4 (B) and subunit $p22^{phox}$ (C) protein expression. (Top) representative blots showing target proteins and β-actin bands. Bottom: densitometric analysis. $n=8$ animals/experimental group. (D) Aortic superoxide anion production. (Top) Representative charts showing corrected cytochrome *c* absorbance (OD)/time, reflecting superoxide production from samples with (dark gray) or without (half gray) addition of SOD or the assay buffer (light gray). Bottom: Superoxide anion synthesis rate calculated as described in Materials and Methods. $n=10$ animals/experimental group. (E) Concentration of 3-nitrotyrosine in aortic proteins. $n=8$ animals/experimental group. a.u., arbitrary units. Values are expressed as means±S.E. Groups not sharing a letter differ at least by $P<.05$.

Table 2
Effects of ovariectomy and treatment with n-3 PUFA on major fatty acids (%) in erythrocyte membrane

	CTR	OVX	n-3 PUFA
Saturated FAs			
Myristic 14:0	0.34±0.03[a]	0.44±0.07[ab]	0.46±0.03[b]
Palmitic 16:0	25.27±0.21[a]	26.80±0.34[b]	27.89±0.97 [b]
Stearic 18:0	21.58±0.47 [a]	21.87±0.89 [a]	21.77±0.57 [a]
Sum	47.23±0.36 [a]	49.22±0.76 [b]	50.28±1.07 [b]
Monounsaturated FAs			
Palmitoleic 16:1 n-7	0.05±0.02 [a]	0.07±0.02 [a]	0.06±0.03 [a]
Oleic 18:1 n-9	7.29±0.21 [a]	7.23±0.27 [a]	6.76±0.44 [a]
Eicosaenoic 20:1 n-9	0.04±0.03 [a]	0.02±0.01 [a]	0.01±0.01 [a]
Sum	7.38±0.14 [a]	7.32±0.20 [a]	6.83±0.37 [a]
n-3 Polyunsaturated FAs			
α-Linolenic acid 18:3 n-3	0.12±0.03 [a]	0.23±0.10 [a]	0.15±0.03 [a]
Eicosapentaenoic acid 20:5 n-3	0.09±0.02 [a]	0.04±0.02 [a]	1.54±0.16 [b]
Docosapentaenoic acid 22:5 n-3	1.49±0.09 [a]	1.35±0.06 [a]	2.46±0.06 [b]
Docosahexaenoic acid 22:6 n-3	3.10±0.12 [a]	2.49±0.08 [b]	3.64±0.13 [c]
Sum	4.70±0.18 [a]	4.08±0.11 [b]	6.24±0.18 [c]
n-6 Polyunsaturated FAs			
Linoleic acid 18:2 n-6	11.13±0.22 [a]	10.89±0.28 [a]	11.7±0.46 [a]
Eicosadienoic acid 20:2 n-6	0.36±0.08 [a]	0.23±0.05 [a]	0.44±0.13 [a]
Dihomo-γ-linolenic 20:3 n-6	0.37±0.03 [a]	0.37±0.05 [a]	0.47±0.06 [a]
Arachidonic acid 20:4 n-6	25.60±0.23 [a]	24.90±0.50 [a]	21.09±0.36 [b]
Adrenic 22:4 n-6	2.51±0.09 [a]	2.50±0.14 [a]	1.28±0.08 [b]
Docosapentaenoic 22:5 n-6	0.70±0.02 [a]	0.50±0.09 [b]	0.18±0.04 [c]
Sum	40.66±0.35 [a]	39.43±0.68 [a]	35.16±0.73 [b]

Results are means±S.D. (n=10 animals/group). Means in the same row that share a common superscript letter do not differ, while those that do not share a letter differ at least by P<.05.

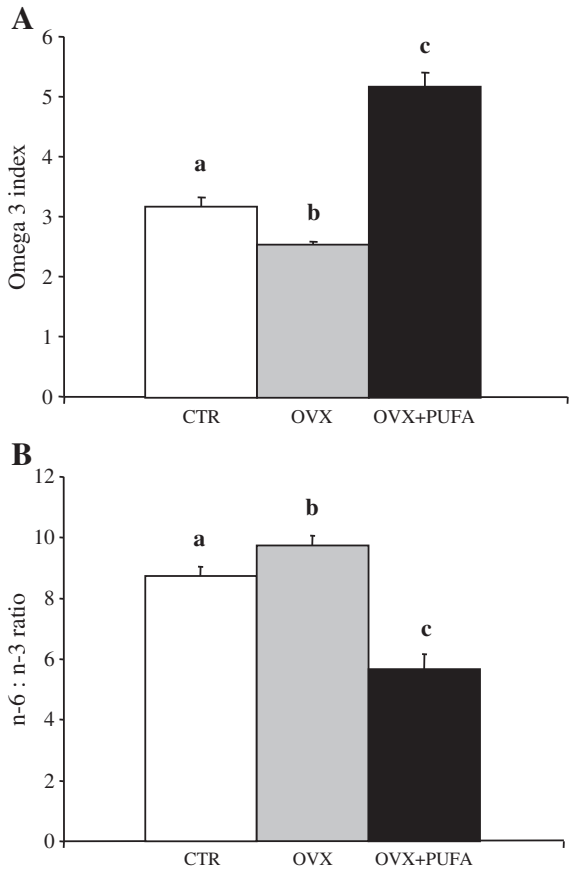

Fig. 5. n-3 PUFA incorporation in erythrocyte membrane. (A) Omega-3 index and (B) n-6:n-3 PUFA ratio in CTR, OVX and OVX+PUFA. Groups not sharing a letter differ at least by P<.05. n=10 animals/experimental group.

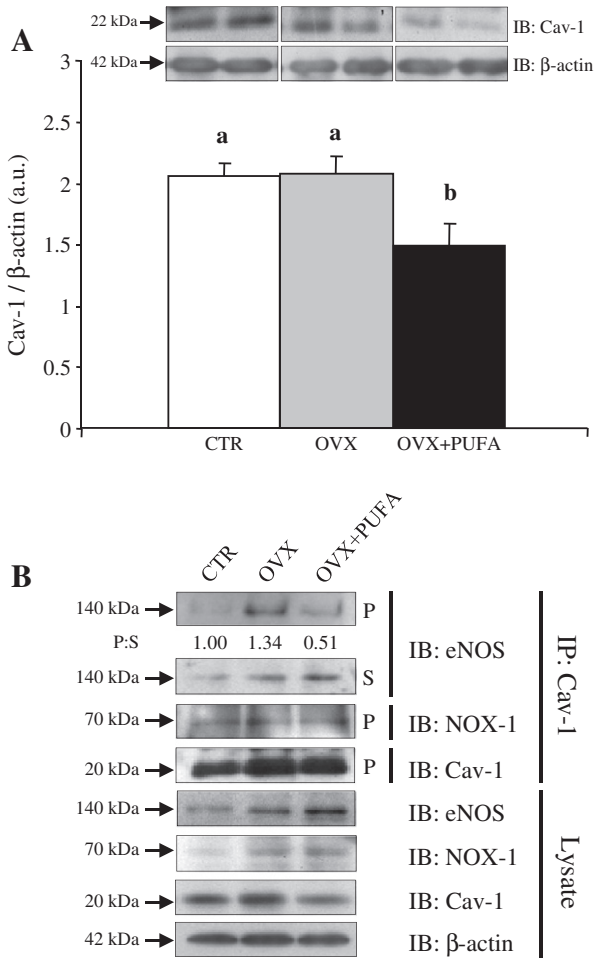

Fig. 6. Caveolin-1 protein expression and interactions. (A) Cav-1 protein expression. (Top) representative blots showing Cav-1 and β-actin bands. (Bottom) Densitometric analysis. Groups not sharing a letter differ at least by P<.05. n=8 animals/treatment group. (B) Blots showing co-immunoprecipitation experiments for Cav-1 interactions with eNOS and NOX-1. Immunoblots (IB) with anti-eNOS, anti-NOX-1, anti-Cav-1 and anti-β-actin antibodies are shown in Cav-1 IP or on tissue homogenate. The same samples were used in all blots. P: precipitated fraction; S: supernatant. IP:S ratio was calculated after band densitometry and set to 1.00 for CTR. a.u., arbitrary units. Values are expressed as means±S.E.

potential effects on vascular function. Since concern exists about the pro-oxidant potential of n-3 PUFA at increasing doses, our results provide evidence that n-3 PUFA at the study dose (corresponding to ~7 g/d extrapolated to a human equivalent dose) for 2 months do not increase oxidative stress in experimental menopause but, on the contrary, function as antioxidants via down-regulation of NADPH oxidase. These findings are in keeping with the observation that rat supplementation with EPA+DHA 1 g/kg body weight, corresponding for adult humans at an average intake of 10 g/d, does not increase the unsaturation index, a marker of peroxidability of plasma membranes [44].

Few data are available on vascular nitric oxide activity during menopause-associated endothelial dysfunction. Previous studies demonstrated normal or reduced eNOS expression and NO production in animal and human models of menopause [3,7]. Also incubation of endothelial cells in vitro with n-3 PUFA produces conflicting effects on eNOS enzyme [45,46]. In this study we did not find any difference in eNOS expression and NOS-related in vitro and ex vivo functional activity between control and OVX animals. n-3 PUFA treated animals showed an increase in membrane omega-3 index, and this was

associated with modifications at caveolar level, namely, lower caveolin-1 expression and inhibitory interaction with eNOS. Also eNOS protein expression increased following treatment. These data show an activation of pro NO-synthetic pathways in the aorta mediated by n-3 PUFA in accordance with previous reports [47]. However, these results, in an experimental menopause setting, do not cause an effective impact on endothelial function or total NO synthesis in the aorta, as shown both by ex vivo functional inhibition experiments and by isolated enzyme activity assays. This finding is consistent with recent evidence in patients with myocardial infarction suggesting that n-3 PUFA related improvement of endothelial function might not be directly related to NO production modifications [43]. Our data suggest that improved vasodilation in the n-3 PUFA OVX rats is not mainly mediated by mechanisms involving NO but is more strictly related to a decrease in oxidative stress.

n-3 PUFA improve vascular tone by endothelial-dependent and endothelial-independent mechanisms. The latter include a direct effect on arterial blood pressure which is achieved with relatively large doses of EPA+DHA [48]. In this study the effect on endothelial-dependent mechanisms was prominent, as demonstrated by similar endothelial-independent vasodilation to DEA-NONOate in the three groups in spite of different responses to acetylcholine in OVX and n-3 PUFA treated OVX rats.

Some of the cardioprotective effects of n-3 PUFA could result from their favorable effects on the lipid profile. As expected, treatment with EPA+DHA reduced by 30% total cholesterol and by 40% non-HDL cholesterol in OVX rats. Although improved lipid pattern could have contributed to reversal of endothelial dysfunction in OVX animals, the present data suggest that n-3 PUFA normalize endothelial function by an action that is beyond their lipid-lowering effect. This is supported by the evidence that n-3 PUFA suppress superoxide production and markedly reduce NOX-4 expression in OVX rat aorta. Moreover, n-3 PUFA induced alterations at caveolar level appear to be directly related to EPA and DHA incorporation into membranes, showing that n-3 PUFA treatment might modulate endothelial function regulatory pathways by direct action.

In this study n-3 PUFA treatment was administered in addition to the standard chow diet. This determined a significant increase in body weight in OVX compared with control and ovariectomized groups. Although the experimental design does not allow to distinguish the impact of the extra calories from fat versus the specific fats administered in the study (n-3 PUFA), nonetheless, the cardiovascular benefits overcame the detrimental effects of weight gain on endothelial function described in obesity [49]. Also, from the available data it can also be excluded that reversal of ovariectomy-associated endothelial dysfunction and oxidative stress following n-3 PUFA administration was attributable to any change in insulin sensitivity as no difference either in blood glucose or insulin levels were detected among groups both under basal conditions and at the end at the study period (data not shown).

In conclusion, these data indicate that chronic treatment with n-3 PUFA can reverse endothelial dysfunction and oxidative stress due to estrogen deficiency. The underlying mechanisms include restoration of altered FA pattern in membrane lipid composition, increased NO bioavailability as a result of reduced oxidative stress and improved lipid profile. Based on these data, n-3 PUFA treatment may represent a therapeutic option in menopausal women who are at higher risk for cardiovascular disease.

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