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17 β -Estradiol Reverses Shear Stress-Mediated LDL Modifications

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Abstract

Within arterial bifurcations or branching points, oscillatory shear stress (OSS) induces oxidative stress mainly via the NADPH oxidase system. It is unknown whether 17 β -estradiol (E₂) can regulate OSS-mediated low density lipoprotein (LDL) modifications. Bovine aortic endothelial cells (BAECs) were pre-treated with E₂ at 5 nmol/L, followed by exposure to OSS (0 ± 3.0 dynes/cm²sec and 60 cycles/min) in a flow system. E₂ decreased OSS-mediated NADPH oxidase mRNA expression, and E₂-mediated $\cdot$ NO production was mitigated by the $\cdot$ NO synthase inhibitor L-NAME. The rates of O₂^{•−} production in response to OSS increased steadily as determined by superoxide dismutase-inhibited ferricytochrome c reduction; however, pre-treatment with E₂ decreased OSS-mediated O₂^{•−} production (n=4, $P<0.05$). In the presence of native LDL (50 μ g/mL), E₂ also significantly reversed OSS-mediated LDL oxidation as determined by high performance liquid chromatography. In the presence of O₂^{•−} donor, xanthine oxidase (XO), E₂ further reversed XO-induced LDL lipid peroxidation (n=3, $P<0.001$). Mass spectra were acquired in the m/z 400–1800 range, revealing XO-mediated LDL protein nitration involving tyrosine 2535 in the α -2 domains, whereas pre-treatment with E₂ reversed this observation, consistent with the changes in nitrotyrosine intensities by the dot blots. E₂ plays an indirect antioxidative role. In addition to up-regulation of eNOS and down-regulation of Nox4 expression, E₂ influences LDL modifications via lipid peroxidation and protein nitration.

Keywords

17 β -Estradiol; Shear stress; LDL lipid oxidation and nitration; NADPH oxidase; Superoxide anion; Nitric oxide

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Introduction

The cardiovascular protective effects of estrogen in the regulation of endothelial function has become important in pathological states in which hemodynamics, specifically shear stress, influences the bioavailability of $\cdot\text{NO}$ [1]. Shear stress is a tangential force that exerts on the vascular endothelial cells by virtue of blood viscosity and velocity gradients [2]. Estrogen relaxes arteries, increases blood flow, and minimizes thickening of the carotid artery where oscillatory shear stress (OSS) develops in both humans and animals [3]. OSS is characterized as bidirectional net zero forward flow that commonly occurs within vascular bifurcations and branching points. OSS is a potent stimulus for proatherogenic expression of adhesion molecules and chemokines [4, 5] and enhancing vascular NADPH oxidase activity [6, 7]. Our previous studies demonstrated that OSS increased production of superoxide anion ($\text{O}_2^{\cdot-}$) [7, 8] and oxidative modification of low density lipoprotein (LDL) [5].

It has long been suspected that the level of circulating 17β -estradiol (E_2) contributes to gender-specific cardio-protection [9–11]. E_2 up-regulates endothelial $\cdot\text{NO}$ synthase (eNOS) gene expression leading to an increase in $\cdot\text{NO}$ production [12] and restores the regulation of wall shear stress in arterioles of spontaneously hypertensive male rats [13]. Further, estrogen has antioxidant effects that oppose oxidation of LDL [14, 15]. Vascular endothelial cells (EC) become hyperpermeable in response to oxidized LDL [16] and hyperlipoproteinemia [17] that favor intimal uptake of LDL and local oxidative degradation of trapped LDL [18–20]. Thus, oxidized LDL plays an important role in inflammatory responses and oxidative stress at the atherosclerosis prone regions [21, 22]. However, the mechanism(s) by which 17β -estradiol (E_2) mediates the effects of pro-atherogenic OSS within arterial bifurcations or curvatures remain unclear.

In the present study, we used a dynamic device generating precise flow patterns to investigate the antioxidant role of E_2 that occurs in human carotid arteries [2, 23, 24]. OSS-induced oxidative modification of LDL was accompanied by increased $\text{O}_2^{\cdot-}$ and decreased nitric oxide ($\cdot\text{NO}$) production. In contrast, E_2 reversed OSS-mediated LDL oxidation that was accompanied by decreased $\text{O}_2^{\cdot-}$ and increased NO formation. Pre-treatment of E_2 further attenuated xanthine oxidase-mediated LDL lipid peroxidation and protein nitration. Mass spectra analyses in the m/z 400–2000 range revealed LDL apo B 100 lipoprotein modifications involving tyrosine 2535 in the α -2 domains, consistent with XO-mediated nitrotyrosine by dot blots. These findings suggest that E_2 at a physiological concentration (5 nmol/L) suppresses OSS-mediated $\text{O}_2^{\cdot-}$ production and reverses LDL post-translational protein modifications.

Methods and Materials

Isolation of LDL from Human Plasma

Venous blood was obtained from fasting adult human volunteers under institutional review board approval at the University of Southern California Atherosclerosis Research Unit. Plasma was pooled and immediately separated by centrifugation at 1500 g for 10 minutes at 4°C . LDL isolation was performed by density gradient centrifugation using a SW-41 rotor (Beckman, Fullerton, CA) utilizing a Beckman L-80 XP type centrifuge as described by Hodis et al [25]. LDL ($d=1.019\text{--}1.063$) was then collected and washed with phosphate buffered saline (PBS) several times using a Millipore (Bedford, MA) centrifugal filtering device with a 30kDa cutoff. Concentrated LDL was then sterilized, stored at 4°C and used within 2–4 weeks. Protein concentrations were measured using Bio-Rad DC assay (Bio-Rad, Richmond, CA) with BSA used as a standard.

Experimental Protocol

Confluent bovine aortic endothelial cell (BAEC) monolayers were exposed to shear stress in a dynamic flow system (Fig. 1). The production of $O_2^{\cdot -}$ was measured by cytochrome c reduction rates and the expression of Nox4 and eNOS by quantitative real-time RT-PCR (described below). Native LDL at 50 $\mu\text{g}/\text{mL}$ was added to the culture media maintained under the four conditions described below: (Control, E_2 , OSS and $E_2 + \text{OSS}$) and aliquots of LDL isolated from the culture medium at 4 hours (and transferred to PBS) were subjected to anion exchange high performance liquid chromatography (HPLC) to analyze oxidized LDL, measured as $LDL^{\cdot -}$ [5, 26]. In some experiments, 100 μM N(G)-nitro-L-arginine methyl ester (L-NAME) (Sigma, St. Louis, MO), an inhibitor of NOS, was added at 20 min prior to loading with native LDL.

Endothelial Cell Culture

Between passages 3 and 6, BAECs were seeded on Cell-Tak cell adhesive (Becton Dickinson Labware, Bedford, MA) and Vitrogen (Cohesion, Palo Alto, RC 0701) coated glass slides (5 cm^2) at 3×10^6 cells per slide. Cells were grown to confluent monolayers in high glucose (4.5 g/L) Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% heat inactivated fetal bovine serum (Hyclone), 100 U/mL penicillin-streptomycin (Irvine Scientific) and 0.05% amphotericin B (Gibco) for 48 hours in 5% CO_2 at 37°C.

Dynamic Flow System

The physiological flow system was designed to generate well-defined temporal shear stress gradients and was mounted on an inverted microscope (Olympus FV-IX701) for real-time visualization of BAECs [27, 28] (Fig. 1). The system was capable of simulating both pulsatile and oscillatory flow conditions reported in the human arterial system [2, 23]. Micro Electro Mechanical Systems (MEMS) sensors were embedded in the channel to monitor real-time shear stress [29]. The circulating DMEM culture medium was maintained at 37 °C and pH 7.4 (Accumet AP) using 5% CO_2 .

Shear Stress Protocols

Confluent BAEC monolayers between passages 3 and 6 were plated on glass slides and incubated in phenol red free DMEM, 10% male human serum solution, 1% penicillin-streptomycin, and 0.05% fungizone. Cells were subjected to four conditions: (1) static condition, (2) pre-treatment with E_2 at 5 nmol/L for 12 hours at static condition, (3) OSS at 60 cycles/min and 0 ± 3.0 dynes/ cm^2sec (time-averaged shear stress (τ_{ave}) = 0 dynes/ cm^2) for 4 hours, and (4) pre-treatment with E_2 for 12 hours, followed by OSS for 4 hours. After 4 hours, BAECs were collected for quantification of Nox4 and eNOS mRNA expression. Effluents were collected for cytochrome c reduction assay and quantitative measurements of NO_2^- and NO_3^- . Cells were also exposed to the above four conditions in the presence of 50 $\mu\text{g}/\text{mL}$ of native LDL. After 4 hours, the medium was aliquoted to assess oxidative modification of LDL as described below.

Separation of Native and Oxidatively Modified LDL Subspecies by HPLC

The technique used for separating LDL was similar to that described previously [8]. Analysis of oxidatively modified LDL or electronegative LDL ($LDL^{\cdot -}$) was used as a measure of LDL modification according to methods previously described [25]. The total LDL and LDL subfractions were eluted through an anion exchange column (UNO-Q1, BioRad, Hercules, CA) at 1.0 mL/min and the effluent monitored at 280 nm. Native LDL (nLDL) and oxidatively modified LDL (or $LDL^{\cdot -}$ due to its electronegativity) [30] were isolated by a stepwise sodium chloride gradient. Area under the curve was measured and

oxidatively modified LDL (LDL⁻) percentage was determined. Accordingly, the ratio of LDL⁻ relative to total LDL was compared as a measure of LDL modification [5].

Quantitative Real-Time RT-PCR

After BAECs were exposed to the flow conditions, total RNA was isolated using the RNeasy kit (Qiagen). Real-time RT-PCR was performed according to the recommendations of the PE Applied Biosystems TaqMan PCR Core Reagent Kit [31]. RT and PCR were performed beginning with a manual ramp rate at 50°C for 2 minutes, 60°C for 30 minutes, and then 95°C for 5 minutes, followed by PCR for 50 cycles at 94°C for 20 seconds and annealing from 58 °C to 65 °C for 1 minute (MJ Research Opticon® System). C_T is the threshold cycle number at which the initial amplification becomes detectable by fluorescence [32]. ΔR_n normalizes fluorescence. TaqMan probes [33] were used for further specificity and sensitivity. The difference in C_T values for various flow conditions vs. control was used to mathematically determine the relative difference in the level of Nox4 and eNOS mRNA expression [31]. For quantification of relative gene expression, the target sequence was normalized to the expressed housekeeping gene GAPDH.

Measurement of Nitrate/Nitrite (NO₃⁻/NO₂⁻)

Quantitative measurements of NO₂⁻ and NO₃⁻ were performed as an index of global nitric oxide (·NO) production essentially following methods described previously [34, 35]. The level of ·NO produced by BAEC subjected to static (no flow) or oscillatory shear stress was measured by the Griess reaction [36]. Briefly, aliquots of the cell culture medium were collected from different treatment conditions for NO₂⁻ and NO₃⁻ using the Griess reaction (Cayman Nitrite/Nitrate Colorimetric Assay Kit, MI, USA). Briefly, collected cell culture medium was incubated with nitrate reductase for 2 hours to convert NO₃⁻ to NO₂⁻ [37]. After incubation, Griess reagents were added to the medium and NO₂⁻ levels quantified spectrophotometrically at 540 nm. The amount of ·NO produced by E₂ was verified by addition of N(G)-nitro-L-arginine methyl ester (L-NAME) [38] at 100μM, a dose at which optimal inhibition of ·NO production was demonstrated. The results were cross-checked with an analytical procedure based on acidic reduction of NO₂⁻ and NO₃⁻ to ·NO by vanadium (III) and purging of ·NO with helium into a stream of ozone and detected by an Antek 7020 chemiluminescence ·NO detector (Antek Instruments, Houston, TX). At room temperature, vanadium (III) only reduced NO₂⁻, whereas NO₃⁻ and other redox forms of ·NO (such as S-nitrosothiols) were reduced only if the solution was heated to 90–100 °C, so that the total ·NO could be quantified. This allowed for determination of the relative levels of NO₂⁻ and NO₃⁻, which might be indicative of differential oxidative metabolic routes of ·NO. For most analyses, 25–50 μl of extracellular fluids were utilized for this assay, and determinations made in duplicate. Quantification was performed by comparison with standard solutions of NO₂⁻ and NO₃⁻.

Measurement of Superoxide (O₂⁻) Formation

The production of O₂⁻ was measured by cytochrome c reduction rates as described previously [39, 40]. In each case, the medium contained 100μM acetylated-ferricytochrome c (Sigma Aldrich). Control samples were placed in a tissue culture dish in complete media with cytochrome c (100μM) and incubated at 37°C. Aliquots of culture medium (300μL) were collected at 0, 1, 2, 3, and 4 hours for absorbance measurements at 550nm (Beckman DU 640 Spectrophotometer). For the OSS conditions, BAEC monolayers were placed in the flow system. At 0, 1, 2, 3, and 4 hours, aliquots of medium bathing the BAECs in the flow apparatus were aspirated and mixed with acetylated-ferricytochrome c (1mM) and monitored for the absorbance at 550nm. The specificity for reduction by O₂⁻ was established by parallel measurements in the presence of PEG-SOD (60 μg/mL). The corrected rates for SOD-inhibited cytochrome c reduction were plotted after computing O₂⁻

formation using an extinction coefficient: $E_{550}=2.1 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ at an absorbance wavelength of 550 nm.

Xanthine Oxidase-Mediated LDL Modifications

BAECs at passage 5 were grown to confluent monolayers in high glucose (4.5 g/L) phenol red free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% male human serum (Omega Scientific, CA), 1% L-glutamine-penicillin-streptomycin solution (Sigma Aldrich) in 5% CO₂ at 37°C. BAECs were treated with E₂ at 5nM/L for 24 hours, followed by exposure to LDL (50µg/mL) in the presence and absence of xanthine oxidase (20mU/mL) for 4 hours. The culture media were collected for the determination of modified LDL (LDL⁻) by HPLC and LDL protein nitration by dot blot analysis for nitrotyrosine, respectively. LDL lipid peroxides were measured using the leucomethylene blue assay using tert-butyl hydroperoxide as a standard. Measurements were made at 650 nm.

Dot Blot Analysis

Dot blots were performed by spotting 12 µg of LDL protein which were exposed to BAEC for 4 hours. Membranes were soaked in methanol and washed with TBS-T. Samples were then blocked in milk and incubated with a 1:3000 dilution of monoclonal nitro-tyrosine antibody (Upstate, Lake Placid, NY) overnight followed by incubation with HRP-conjugated goat anti-mouse secondary antibody at a dilution of 1:10000. Blots were detected using ECL chemiluminescence kit (Pierce, Rockford, IL).

Liquid Chromatography and Tandem Mass Spectrometry (LC/MS/MS) Analyses

- a. *In-solution tryptic digest* was carried in 50 mM ammonium carbonate buffer with 5mM CaCl₂ using reductively methylated trypsin (Promega) to reduce self-hydrolysis. A 20:1 protein to enzyme ratio was used for the digestion reaction which was carried out at 37°C for at least three hours. Digestion products were prepared for LC/MS/MS analysis by drying down the reaction sample using an APD SpeedVac (ThermoSavant), resuspending evaporate in 5% acetonitrile/0.1 formic acid, and subjecting this sample to ZipTip purification using an elution buffer of 70% acetonitrile/0.1% formic acid. The ZipTip eluent was evaporated using the APD SpeedVac.
- b. *Analysis of tryptic peptide sequence tags* was performed by tandem MS. The digest samples were suspended in 10 µl 60% formic acid. Chromatographic separation was achieved using a ThermoFinnigan Surveyor MS-Pump with a BioBasic-18 100 mm C 0.18 mm reverse phase capillary column. Mass analysis was done with a ThermoFinnigan LQ Deca XP Plus ion trap mass spectrometer equipped with a nanospray ion source employing a 4.5 cm long metal needle, in the data-dependent acquisition mode. Electrical contact and voltage application to the probe tip took place via the nanoprobe assembly. Spray voltage was set to 2.9 kV and heated capillary temperature at 190°C. The column was equilibrated for 5 min with 95% solution A, 5% solution B (A, 0.1% formic acid in H₂O; B, 0.1 % formic acid in acetonitrile) prior to sample injection. A linear gradient was initiated 5 min after sample injection ramping to 35% A, 65% B after 50 min and 20% A, 80% B after 60 min. Mass spectra were acquired in the *m/z* 400–800 range.
- c. *Protein identifications* were carried out with the MS/MS search software Mascot 1.9 (Matrix Science) with confirmatory or complementary analyses with TurboSequest as implemented in the Bioworks Browsers 3.2, build 41 (ThermoFinnigan). NCBI Sus scrofa protein sequences were used as the primary search database; searches were complemented with the NCBI non-redundant

protein database. The presence of nitrotyrosine rendered an additional mass of 45 daltons to the peptides.

Statistical Analysis

Data are expressed as mean $\pm$ SD and compared among separate experiments. For comparisons between two groups, statistical analysis was performed using the two sample independent-groups t-test. Comparisons of multiple mean values were made by one-way analysis of variance (ANOVA), and statistical significance among multiple groups determined using the Tukey procedure (for pairwise comparisons of means between static-like and pulsatile flow conditions). *P*-values of < 0.05 are considered statistically significant.

Results

17- β Estradiol Down-Regulated Oscillatory Shear Stress-Mediated NADPH Oxidase Subunit (Nox4) Expression and $O_2^{\cdot-}$ Production

Under basal static cell culture conditions, Nox4 mRNA expression was markedly down-regulated in BAEC by E_2 compared with that of control cells (Fig. 2). Nox4 mRNA expression was further up-regulated in response to OSS; however, the presence of E_2 (5nmol/L) inhibited OSS-mediated Nox4 mRNA expression by $35.0 \pm 4.2\%$ ($P < 0.05$, $n=4$). Similarly, the rates of $O_2^{\cdot-}$ production in response to OSS increased steadily; however, the presence of E_2 decreased OSS-mediated $O_2^{\cdot-}$ production ($n=4$, $P < 0.05$) (Fig. 3), consistent with decreased Nox4 expression. The rates of $O_2^{\cdot-}$ production remained relatively steady under static conditions whereas the rates in the presence of E_2 were lower than that of controls ($n=4$, $P < 0.05$). The apparent negative rates for $O_2^{\cdot-}$ production were due to the lower measured values for optical densities (OD at 550 nm) after incubation as compared to initial values at time 0. Rates of $O_2^{\cdot-}$ production were correlated with Nox4 mRNA expression in the presence and absence of E_2 .

17- β Estradiol Up-regulated eNOS Expression and $\cdot NO$ Production in the Presence of Oscillatory Shear Stress

The presence of E_2 noticeably increased eNOS mRNA expression by 40-fold ± 5 under static conditions (Fig. 4). While eNOS mRNA was down-regulated in response to OSS, the presence of E_2 suppressed the inhibitory effect of OSS by up-regulating eNOS mRNA expression by 20-fold ± 1 ($n=4$, $P < 0.001$). The changes in eNOS mRNA were also correlated with an increase in $\cdot NO$ production in the presence of E_2 as compared with the control conditions ($3.98 \pm 0.14 \mu M$ versus $7.37 \pm 0.89 \mu M$, respectively $P < 0.05$, $n=4$) (Fig. 6). While OSS decreased $\cdot NO$ formation, the presence of E_2 restored $\cdot NO$ production to levels intermediate between controls and E_2 pre-treatment (OSS = $3.66 \pm 0.64 \mu M$ versus $E_2 + OSS = 5.65 \pm 0.26 \mu M$, $P < 0.05$, $n=4$) (Fig. 6a). The relative difference in NO_2^- production was statistically insignificant between OSS and $E_2 + OSS$ conditions (Control = $0.82 \pm 0.02 \mu M$, $E_2 = 1.21 \pm 0.06 \mu M$, OSS = $0.55 \pm 0.03 \mu M$, $E_2 + OSS = 0.93 \pm 0.032 \mu M$) (Fig. 6b). The reversing effect of E_2 on total $\cdot NO$ formation was reflected largely by the increased formation of NO_3^- (Control = $3.16 \pm 0.13 \mu M$, $E_2 = 6.16 \pm 0.68 \mu M$, OSS = $3.11 \pm 0.49 \mu M$, $E_2 + OSS = 4.73 \pm 0.23 \mu M$) (Fig. 6c). Overall, the levels of $\cdot NO$ production correlated with the general trend of eNOS mRNA expression. The amount of $\cdot NO$ produced as a result of E_2 treatment was verified by addition of L-NAME (100 μM) which mitigated E_2 -mediated $\cdot NO$ production (Fig. 7).

17- β Estradiol Reverses OSS-Mediated LDL Oxidation

The presence of E_2 decreased the extent of LDL modifications (LDL $^{\cdot-}$) under both static conditions and OSS conditions as demonstrated by the % LDL $^{\cdot-}$ formation (Fig. 8). The

chromatogram revealed two distinct sets of peaks: native LDL and LDL⁻ under static and OSS conditions in the presence and absence of E₂ (Fig. 8a). The relative levels of oxidized LDL as expressed in % LDL⁻ formation were compared in response to OSS in the presence and absence of E₂ pre-treatment (Fig. 8b). OSS induced the highest level of LDL modifications as measured by optical density (OD) of LDL⁻, followed by control, E₂+OSS, and E₂. While OSS significantly increased LDL modifications, the presence of E₂ significantly attenuated OSS-induced LDL⁻ formation (LDL⁻ to LDL ratios: control=1.05±0.34, E₂=0.58±0.18, OSS=2.28±0.41, OSS+E₂=0.80±0.25, *P*<0.01, n=4). The extent of % LDL⁻ formation correlated with the regulation of Nox4 mRNA expression and the relative rates of O₂⁻ and ·NO production in the presence of E₂ (Figs. 2,3 and 6).

17-β Estradiol Reverses Xanthine Oxidase-Mediated LDL Modification via Reduction in Lipid Peroxides and Protein Nitration

To elucidate a potential mechanism by which E₂ influenced LDL post-translational modifications in connection with the regulation of O₂⁻ and ·NO production by endothelial cells, we studied xanthine oxidase-mediated LDL lipid oxidation and protein nitration. Addition of XO significantly increased LDL⁻ formation (Fig. 9) and LDL lipid peroxidation (Fig. 10) compared with control, whereas pre-treatment with E₂ reversed XO-induced LDL modifications (*, *P*<0.01, n=3). LC/MS/MS analyses of XO-mediated LDL apo B 100 nitration revealed a tyrosine residue 2535 modification in a tryptic peptide (Y*LSLGQVYISDWTLAAK) from α-2 helix domains (Fig. 11), whereas pre-treatment with E₂ reversed this observation.

Sequence coverage for LDL protein modifications in control and XO- and/or E₂-treated samples was approximately 40% in samples subjected to LC/MS/MS. The tyrosine modification was observed over the course of analysis of three separate samples preparations. The tryptic peptide containing the identified tyrosine modification was observed by Mascot software analysis and LC/MS/MS analyses (Table 1). Nitration of tyrosine 2535 was observed in the same doubly charged 18 amino acid tryptic peptide in three separate LC/MS/MS analyses. Representative MS/MS spectrum for the tryptic peptide in the m/z 400–2000 range carrying a nitrative modification is presented (Fig. 11). The precursor ion was doubly charged with a mass of 1353.18 Da corresponding to an 18-residue tryptic peptide, comprising amino acids 2535 – 2559, with one nitration modification. Fragment b and y ions were observed for nearly all-observable single-charged fragments. LDL protein nitration was further corroborated by the dot blot analyses for nitrotyrosine (Fig. 12). While XO significantly increased nitrotyrosine intensity, E₂ significantly decreased its intensity (**, *P*<0.01, n=3). Collectively, these findings suggest that E₂ reverses LDL⁻ formation via lipid peroxidation and post-translation apo B100 protein nitration.

Discussion

The main finding of this study was that at a physiological concentration (5 nmol/L), E₂ reversed OSS-mediated LDL oxidation. In the presence of E₂, expression of the NADPH oxidase (Nox4) was down-regulated, whereas expression of eNOS was up-regulated. These changes in Nox4 and eNOS expression coincided with a decrease in O₂⁻ formation and an increase in ·NO production, respectively, suggesting that the effect of E₂ was mediated, in part, via concomitant down-regulation of NADPH oxidase (Nox4) and up-regulation of eNOS. This relative expression of Nox4 and eNOS indicates that E₂ may act at the transcriptional level to suppress oxidant production.

Vascular cells use reactive oxygen (ROS) and reactive nitrogen species (RNS) to modify LDL. ECs produce ROS and RNS from the enzymes and ·NO synthase and by specific

homologues of NADPH oxidase (gp91^{phox}, Nox1, and Nox4) [41]. We previously demonstrated that NADPH oxidases were capable of producing high levels of ROS in blood vessels in response to specific stimuli arising from flow conditions. Consequently, increasing quantities of modified LDL species were formed under oscillatory flow conditions that favor ROS production, whereas lower levels of LDL modification occur in response to pulsatile flow (unidirectional forward flow) conditions that produce lower levels of ROS [5]. Here, we further showed that estrogen attenuated the levels of LDL modification, suggesting linked effects between pulsatile shear stress and estrogen. The increase in NAD(P)H oxidase-derived O₂^{•-} level can account for the cross-talks with endothelium-derived ·NO; thus, the balance between O₂^{•-} and ·NO production may influence the ·NO signaling function, antioxidant action and generation of ONOO⁻ [42, 43]. The exposure of LDL to ONOO⁻ results in the nitration of apo B-100 tyrosine residues [44], the initiation of lipid peroxidation [45, 46], accompanied by the depletion of lipid-soluble antioxidants [47, 48].

Tyrosine nitration, a post-translational modification of proteins through the addition of a nitro (NO₂) group in the *ortho* position of tyrosine residues [49], has been detected under physiological settings and in a number of pathological states including inflammatory and septic conditions [46, 50, 51]. Peroxynitrite reacts rather specifically with tyrosine residues in proteins leading to the formation of nitrotyrosine, which in itself could be toxic, e.g., by undergoing redox cycling, by interfering with signal transduction, or by becoming incorporated into the microtubules protein tubulin and distorting the cytoskeleton [52].

Misfolding of apoB may account for one of the mechanisms for LDL⁻ formation. The α-2 and α-3 domains of apoB-100 contain the highest number of tyrosine residues (4.8% and 6.8% tyrosine respectively), and nitrotyrosine may play an important role in α helical integrity of apoB-100 [42]. Nitration of tyrosine residues could lend to a more hydrophilic residue carrying a dipole moment, altering the intact structure of α helices and other domains of the protein on the surface of the LDL particles. Mass spectra analysis (Fig. 11) demonstrated that pre-treatment with E₂ suppressed xanthine oxidase-mediated LDL protein nitration at tyrosine 2535 in the α-2 domain. These findings are consistent with dot blots for nitrotyrosine as a finger print for peroxynitrite-mediated nitration that reacts rather specifically with tyrosine residues (Fig. 12). Thus, E₂ plays an indirect antioxidant role in LDL⁻ formation by reversing xanthine oxidase-mediated LDL lipid peroxidation and LDL apo B 100 nitration.

A subclass of LDL described on the basis of its greater electronegativity and oxidative status has been previously characterized [25, 26, 53]. These particles, which were referred to as LDL⁻, are enriched with lipid hydroperoxides and other peroxidation products as compared to the bulk of the unmodified, normal LDL (nLDL) recovered from human plasma. We have demonstrated that LDL⁻ is a major carrier of lipid hydroperoxides and cholesterol oxides associated with plasma LDL and that LDL⁻ may arise from oxidative events in the vasculature [54]. Decreased rates of O₂^{•-} production in glucose free medium containing 2-Deoxyglucose further implicate NADPH as an important cofactor for NADPH oxidase activity and the necessity of this activity to produce O₂^{•-} and modify LDL [5]. The variation in LDL⁻ levels formed by BAECs under OSS conditions points to a role for NADPH oxidase activation in cell-mediated LDL modification.

The atheroprotective effect of estrogen favors women until menopause [55]. Several reports suggest that the atheroprotective effect of E₂ may be mediated by increased local concentrations of ·NO [9, 56] through up-regulation of eNOS expression [57] and/or an estrogen receptor-mediated activation of eNOS via the phosphatidylinositol-3-(PI3) kinase/Akt kinase pathway [58, 59]. E₂ transcriptionally up-regulates cytochrome P450 (CYP)

activity leading to the enhanced arteriolar vasodilation response to shear stress in $\cdot\text{NO}$ -deficient female rats and mice [1]. Interaction of cell membrane-bound estrogen receptors with PI3 kinase phosphorylates Akt induces phosphorylation of eNOS and release of $\cdot\text{NO}$ [48, 60]. By inhibiting NADPH oxidase activity, E_2 can exert an indirect 'antioxidant' effect through the reduced formation and interaction between $\text{O}_2^{\cdot-}$ and $\cdot\text{NO}$. Thus, the presence of estrogen exerts salutary effects on vascular endothelium largely via an increase in $\cdot\text{NO}$ formation and a decrease in oxidant production.

In figure 3, the effect of E_2 on the oxidative arm appears to be modest. There was a 35% change in Nox4 mRNA (Fig. 2); however, the effect of E_2 on $\text{O}_2^{\cdot-}$ production appears not to be a reduction in NADPH oxidase but activities. One possibility was an increased loss of already formed $\text{O}_2^{\cdot-}$. Another possibility was the effects of E_2 on other sources of $\text{O}_2^{\cdot-}$ production, including xanthine oxidase and mitochondria [61, 62].

Furthermore, $\cdot\text{NO}$ was metabolized or decomposed via various reactions to the major products NO_2^- and NO_3^- *in vivo*. They served as useful measurement of overall $\cdot\text{NO}$ production and metabolism, both under basal physiological conditions, as well as during exposure to xenobiotics and/or inflammatory response. Although NO_2^- could also react with heme proteins and participate in further reactions (including nitration), NO_3^- was an ultimate end product [63]. However, in Figure 6a, $\cdot\text{NO}$ production was comparable between control and OSS groups. OSS-mediated Nox4 expression and consequently, $\text{O}_2^{\cdot-}$ production (Figs. 2 and 3), was likely to react with $\cdot\text{NO}$ to form peroxynitrite (ONOO^-). ONOO^- would further decompose upon protonation through rapid isomerization to NO_3^- at physiologic pH [64]. Thus, the formation of ONOO^- could account for the paradoxical increase in NO_3^- between the control and OSS groups (Fig. 6c). In this context, measuring total NO_3^- and NO_2^- would not precisely reflect an impaired $\cdot\text{NO}$ bioavailability in the presence of increased $\text{O}_2^{\cdot-}$ formation.

The increase in eNOS mRNA by E_2 was impressive (Fig. 4); however, there was only a 2-fold change in $\cdot\text{NO}$ under control conditions and a smaller change under OSS. Two reasons may have accounted for the disparity between eNOS mRNA expression and $\cdot\text{NO}$ production. First of all, statistic analysis of real-time RT-PCR could have accentuated the differences between relative eNOS mRNA expressions. We used the delta delta C_t method as follows: C_T is the threshold cycle number at which the initial amplification becomes detectable by fluorescence [65]. The difference in C_T values for various flow conditions vs. control was used to mathematically determine the relative difference in the level of eNOS mRNA expression [65]. For quantification of relative eNOS mRNA expression, the difference in C_T values was normalized to the expressed housekeeping gene GAPDH. Secondly, a loss of already formed $\cdot\text{NO}$ could have attributed to the relatively small change in $\cdot\text{NO}$. Quantitative measurements of NO_2^- and NO_3^- were performed as an index of global nitric oxide ($\cdot\text{NO}$) production [33,34]. Collected cell culture medium was incubated with nitrate reductase for 2 hours to convert NO_3^- to NO_2^- [3], followed by addition of Griess reagents. Quantification of NO_2^- levels were performed spectrophotometrically.

We demonstrated that E_2 attenuated the extent of LDL oxidation in response to OSS. The hemodynamic forces, to which endothelial cells are exposed *in vivo*, especially at sites of arterial bifurcation, can predispose these regions to atherogenic processes that include up-regulation of $\text{O}_2^{\cdot-}$ formation [8] and subsequent oxidative reactions. Clinically known risk factors for coronary heart disease have been associated with endothelial dysfunction [66]. Enhanced oxidative stress may be another factor contributing to this dysfunction, although controversy remains [66]. A recent study in normotensive women showed that endothelial dysfunction secondary to acute endogenous estrogen deprivation is strongly linked to a reduction in the bioavailability of $\cdot\text{NO}$ [67]. Moreover, E_2 can attenuate $\text{O}_2^{\cdot-}$ formation in

phagocytes [68] and endothelial cells [6]. In summary, the reduction in $O_2^{\cdot-}$ formation combined with the salutary effects of $\cdot NO$ may account for decreased LDL oxidative modification by endothelial cells. Furthermore, E_2 suppresses xanthine oxidase-induced LDL post-translational modifications via reductions in both lipid peroxidation and protein nitration. This indirect antioxidant activity may provide a new insight into a potential mechanism whereby E_2 plays an atheroprotective role.

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Fig. 1.

(a) A dynamic flow system: Confluent BAEC monolayers were seeded on the bottom of the dynamic flow channel which will be mounted on the inverted microscope while an array of MEMS sensors will be embedded on the upper wall. (b) Monocyte and or BAEC were captured and digitized. (c) A magnification of shear stress sensor array. (d) Real-time recording of pulsatile (PSS) vs. oscillatory shear stress (OSS). This newly designed physiologic flow model enabled us to implement the specific shear stress values with temporal variations. The effluents was used for biochemical analyses, specifically, analysis of $\text{NO}_2^-/\text{NO}_3^-$, cytochrome c reduction assay for superoxide ($\text{O}_2^{\cdot-}$) and high performance liquid chromatography (HPLC) for LDL oxidation. The recovered BAEC were used for gene and protein expression.

Fig. 2. Relative NADPH oxidase subunit (Nox4) mRNA expression normalized to GAPDH at 4 hours. In the presence of E₂, Nox4 mRNA decreased significantly compared with control (*, $P<0.05$, $n=4$). Pre-treatment with E₂ under oscillatory shear stress (OSS) significantly down-regulated Nox4 expression by $35.0\pm4.2\%$ compared with OSS ($P<0.05$, $n=4$).

Fig. 3.

17 β -Estradiol (E2) inhibits OSS-induced superoxide ($O_2^{\cdot-}$) production. The rate of $O_2^{\cdot-}$ production in BAEC in response to OSS increased steadily whereas treatment with E2 decreased the rate of $O_2^{\cdot-}$ formation ($n=4$, $P<0.05$). The rate of $O_2^{\cdot-}$ production remained relatively steady under static conditions whereas the rate of $O_2^{\cdot-}$ production in the presence of E₂ was relatively lower than that of controls ($n=4$, $P<0.05$). The negative rates for $O_2^{\cdot-}$ production were due to the lower measured values for optical densities (OD at 550 nm) after incubation with E2 as compared to initial values at time 0.

Fig. 4. Relative eNOS mRNA expression normalized by GAPDH. E₂ pre-treated BAEC was associated with a significant increase in eNOS mRNA expression (*, $P < 0.001$, $n=4$). OSS decreased eNOS mRNA expression while pre-treatment with E₂ restored eNOS expression (**, $P < 0.001$, $n=4$).

Fig. 5. Nox4 protein expression normalized with β -actin at 4 hours. E₂ decreased Nox4 protein by $61 \pm 3\%$ compared with control (*, $P < 0.05$, $n = 4$). E₂ attenuated OSS-induced Nox4 protein expression by $23.9 \pm 5.4\%$ compared with OSS alone (**, $P < 0.05$, $n = 4$).

Fig. 6. Total NO, NO₂⁻ and NO₃⁻ production. (a) Total NO: Pretreatment of BAEC with E₂ significantly increased total NO production compared to control (static) conditions. OSS decreased NO production compared to E₂. Pretreatment with E₂ increased NO production compared to OSS ($P < 0.01$, $n = 4$). (b) NO₂⁻ production: the relative difference in NO₂⁻ production in response to OSS vs. E₂+OSS was statistically insignificant ($P < 0.05$, $n = 4$). (c) NO₃⁻ formation (total NO-NO₂⁻): the reversing effect of E₂ of total NO was reflected largely by the increased formation of NO₃⁻ ($P < 0.01$, $n = 4$).

Fig. 7. Addition of N(G)-nitro-l-arginine methyl ester (L-NAME) at 100 μM mitigated E₂-mediated NO production (control: 4.04 $\pm$ 0.37 μM L-NAME: 2.03 $\pm$ 0.68 μM E₂ : 7.7 $\pm$ 0.58 μM L-NAME+E₂ : 4.09 $\pm$ 0.47 μM).

Fig. 8.

(a) Chromatogram. The first set of peaks reflects nLDL and the second LDL-. OSS induced the highest optical density (OD) of LDL-, followed by control, E₂+OSS, and E₂. (b) LDL- to total LDL ratios as a measure of LDL oxidative modification. E₂ decreased LDL oxidation compared with control (*, $P < 0.05$, $n = 5$). While OSS increased oxidative modification of LDL, pre-treatment with E₂ significantly decreased OSS-mediated LDL oxidation (**, $P < 0.01$, $n = 5$).

E₂ reverses xanthine oxidase-induced LDL oxidation

Fig. 9.

E₂ reverses xanthine oxidase (XO)-mediated LDL oxidation. While addition of XO significantly increased LDL oxidation compared with control, pre-treatment with E₂ reversed XO-induced LDL oxidation (*, $P < 0.05$, $n = 3$).

Fig. 10.

(a) E_2 reverses xanthine oxidase (XO)-mediated lipid peroxidation (LP). XO increased LP to total protein ratios; pre-treatment with E_2 significantly decreased XO-mediated lipid peroxidation (**, $P < 0.01$, $n = 3$).

Fig. 11.

MS/MS spectrum for tryptic peptide carrying a nitrative modification at tyrosine 2535.

Above the spectrum is the peptide sequence with observed b and y ions indicated by their fragmentation number above (y ions) or below (b ions) the sequences. ++ indicates doubly charged ions. Single-charged ions are not designated with a +. The ion annotation is based on results presented in Sequest's "display ions view" window that were corroborated by the data file. Y* indicates a nitrotyrosine.

1. Positive control
2. LDL
3. LDL+ XO
4. LDL + E2
5. LDL + E2 + XO
6. Negative control

Fig. 12.

(a) Dot plot for nitrotyrosine as a finger print for LDL protein nitration. (b) Densitometry of nitrotyrosine. E2 attenuates XO-induced LDL protein nitration as measured by the presence of nitrotyrosine. While XO increased LDL protein nitration, pre-treatment with E2 significantly decreased XO-mediated LDL nitration.

LDL apo B 100 modifications in the m/z 400 – 2000 range. Nitrated tyrosines in the amino acid sequence of the tryptic fragments are denoted as asterisks. “M” is the Mascot score. Nitrotyrosine is absent in the samples treated with LDL and E2 (n=3).

Table 1

Treatments	Structure	Numerical sequence	Protein Sequence	Nitrotyrosine	Mascot score
LDL control	α-2	2535–2559	y*Islvgqvystlvtvysdwwtlaak	1	35
LDL + XO	α-2	2535–2559	y*Islvgqvystlvtvysdwwtlaak	1	25
LDL + E2	α-2	2535–2559	ylslvgqvystlvtvysdwwtlaak	0	NA
LDL + E2 + XO	α-2	2535–2559	y*Islvgqvystlvtvysdwwtlaak	1	10