Estradiol counteracts oxidized LDL-induced asymmetric dimethylarginine production by cultured human endothelial cells

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Abstract

Objective: Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide (NO) synthase, is a novel cardiovascular risk factor produced by endothelial cells. ADMA levels are mainly regulated by the activity of dimethylarginine dimethylaminohydrolases (DDAH). Endothelial release of ADMA is increased in the presence of oxidized LDL cholesterol (oxLDL), whereas estrogens stimulate NO production by endothelial cells by increasing both expression and activity of NO synthase and by reducing ADMA levels. Thus, the aim of the present study was to evaluate the estradiol effects on the DDAH/ADMA/NO pathway in cultured human umbilical vein endothelial cells (HUVEC) exposed to LDL.

Methods: After 24 h of exposure to various treatments, culture medium was collected to measure NO production by using an amperometric sensor specific for NO, and to measure dimethylarginines by high-performance liquid chromatography (HPLC). DDAH-I and II mRNA expression and protein content were quantified by real-time PCR assay and immunoblotting, respectively.

Results: Exposure of HUVEC to 100 μg/mL oxLDL, but not to 100 μg/mL of native LDL (nLDL), reduced DDAH-II expression at both the mRNA as well as the protein levels, which in turn increased ADMA levels and reduced NO production. Estradiol (1 nM) alone increased DDAH-II mRNA and protein expression, which reduced ADMA levels and increased NO production. In cells exposed to estradiol in combination with either nLDL or oxLDL, levels of DDAH-II, ADMA, and NO were the same as those for estradiol alone.

Conclusion: Estradiol completely reverses the effects induced by oxLDL on the DDAH/ADMA/NO pathway.

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1. Introduction

The incidence of coronary heart disease (CHD) in men is greater than it is in premenopausal women of the same age, but CHD increases in frequency after menopause, an effect that has been attributed, at least in part, to estrogens [1]. Estrogens have been used as contraceptive agents or as principal constituents of hormonal therapy formulations in postmenopausal women. The protective effect detected in a considerable number of observational clinical studies [2] has not been confirmed by more recent randomised placebo-controlled trials designed to study the effects of hormonal therapy in either secondary [3,4] or primary [5] prevention. Nevertheless, clinical and experimental data support the consideration of endothelium as a target for estrogens [1,6], and a number of studies have demonstrated a favorable profile for estrogens in both experimental animal as well as in in vitro models [6,7].

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Among its vascular actions, estradiol regulates the production of such vasoactive compounds as prostacyclin, thromboxanes and nitric oxide (NO) [7]. Experimental evidences suggest that estrogens stimulate NO production by endothelial cells through both increased expression and activity of NO synthase (NOS) [8,9]. Endothelial cells are capable of synthesizing the l-arginine analogue asymmetric dimethylarginine (ADMA), a major endogenous inhibitor of NOS, and its biologically inactive isomer symmetric dimethylarginine (SDMA). ADMA reduces NO production and decreases vasodilator responses in vitro and in vivo [10]. There is growing evidence that an elevation of the circulating ADMA level is involved in endothelial dysfunction in some cardiovascular abnormalities [11]. ADMA is nowadays defined as an independent risk factor for coronary artery disease [11]. Moreover, ADMA endothelial release increases in the presence of oxidized LDL cholesterol for coronary artery disease [11]. Malondialdehyde levels (mean ± SEM of 6 values corresponding to 3 experiments) were 0.8 ± 0.1 nmol/mg protein for nLDL and 3.3 ± 0.7 for oxLDL, p < 0.05.

This investigation conforms to the principles outlined in the Declaration of Helsinki, was approved by the institutional review board at our center, and written informed consent was obtained from all donors.

2.2. NO production

After 24 h of incubation with the desired treatments, cells were washed and incubated with HEPES buffer (5 mM HEPES containing (in mM) 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2 and 10 glucose, pH adjusted to 7.4), for 120 min. Then, incubation medium was collected and stored at −20 °C until NO measurement. Culture wells were then washed with PBS, and adherent cells were collected in 0.5 N NaOH solution for protein determination [20].

Endothelial NO production was determined in culture medium using the ISONOP nitric oxide sensor (World Precision Instruments, Sarasota, FL, USA), an amperometric sensor specific for NO [21]. A chemical titration calibration was performed with use of an acidic iodide solution (0.1 mol/l H2SO4, 0.14 mol/l K2SO4, 0.1 mol/l KI) against varied volumes of KNO2. NO was formed stoichiometrically and measured directly. A standard curve was constructed from the preceding with a plot of picocampers vs. NO in nmol. The quantity of NO was converted to nitrite and expressed as nmol/mg protein.

2.3. Isolation and measurement of dimethylarginines

After 24 h of incubation with the desired treatments, medium was collected and stored at −20 °C until dimethylarginines quantification. Culture wells were then washed with PBS and adherent cells were collected in 0.5 N NaOH solution for protein determination [20]. Measurement of ADMA and SDMA was accomplished by high-performance liquid chromatography (HPLC) as described earlier [22]. In brief, dimethylarginines from 1 mL of culture medium were purified with Bond Elut SCX columns (Varian Inc., Palo Alto, CA, USA) and eluted with 4 mL of methanol containing 30% distilled water and 2% triethylamine. The eluent was then evaporated to dryness at 60 °C, and the dried extract was redissolved in running buffer. HPLC was carried out on a Shimadzu chromatography system (Shimadzu Corporation, Kyoto, Japan). Separation of dimethylarginines was achieved with a 250 × 4.6-mm (inner diameter), 5-μm “Kromasil C18” analytical column (Scharlau,
2.4. Immunoblotting

HUVEC were treated in 25 cm² flasks for 24 h with the desired products. Then, flasks were washed twice with pre-warmed Medium 199. A volume of 150 μL of lysis buffer (0.1% triton X-100, 0.5% sodium deoxicolic acid, 0.1% SDS, in 100 mL of phosphate saline buffer containing protease inhibitors: 1 μg/mL leupeptin, 0.5 μg/mL pepstatin and 1 μg/mL bestatin) was added, and incubation was maintained at 4 °C for 30 min. Then, cells were collected using a cell scraper, boiled for 5 min and sonicated for 10 s. Protein content was measured [20], and samples were frozen at −20 °C until assay.

Equal amounts of protein (ranging 60–130 μg) were then separated by 12% of SDS-Polyacrylamide gel electrophoresis. Protein was then transferred to PVDF sheets (PVDF Transfer Membrane, Bio-Rad, Madrid, Spain). Immunostaining was achieved using specific antibodies anti-DDAH-I (PC716; Calbiochem, San Diego, CA, USA) or anti-DDAH-II (PC717; Calbiochem). Development was performed with alkaline-phosphatase-linked anti-goat antibody (Sigma), followed with nitroblue tetrazolium (NBT)/5-Bromo-4-Chloro-3-Indolyl Phosphate, p-toluidine salt (BCIP) color development reaction. Blots were digitalized using a Gelprinter PLUS (TDI, Madrid, Spain), and the densities of spots were analyzed with the program 1-D Manager. Equivalent protein loading and transfer efficiency were verified by staining for β-actin (Sigma).

2.5. RNA isolation and quantitative real time PCR (QRT-PCR) assay

Total cellular RNA was extracted using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. Reverse transcription (RT) was carried out using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) with a personal Mastercycler Eppendorf Thermocycler (Eppendorf, Hamburg, Germany).

Primers for QRT-PCR were designed using the Primers Express Software (Applied Biosystems, Fosters City, CA, USA) and synthesized by Custom Primers (Life Technologies, Barcelona, Spain). The sequence of the GAPDH sense primer was 5′-CTGCTCCTCCTGGTCAGCAGT-3′ and that of the antisense primer was 5′-CCGTTGACTCCGACCTCACC-3′ (NCBI#: NM_001736) giving rise to an expected PCR product of 100 bp. The DDAH-I primers, 5′-GGACAAATCAAC GAGGTGCT-3′ for the sense primer and 5′-TACGCGGTGT CACTCATCTG-3′ for the antisense one (NCBI#: BC_033680), were designed to amplify a 193 bp PCR product. For DDAH-II, the primers used were 5′-GATCTGGC CAAAAGTCAAAG-3′ for the sense primer and 5′-CAAACC GAGGACAGAAAAGA-3′ for the antisense one, and a 573 bp product was expected (NCBI#: NM_013974).

A QRT-PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with a heated lid (105 °C), an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To amplify cDNA, the RT samples were diluted 1/10. In each reaction, a total of 1 μL from each RT tube was mixed with 12.5 μL of SYBR Green PCR master mix (Applied Biosystems) containing nucleotides, Taq DNA polymerase, MgCl2 and reaction buffer with SYBR green; 1.5 μL of either 2.5 μM DDAH-I primers or 10 μM DDAH-II primers and double distilled water were added to a final volume of 25 μL. Each sample was amplified in duplicate for DDAH-I, DDAH-II and GAPDH. In parallel, 5 fold serial dilutions of well-known cDNA concentrations were run as calibration curves. After the amplification process was ended, the melting curves program was used to assure that all the amplicons were obtained at the same temperature, and to assure there was no amplification of other products. Data were analysed with the ABI PRISM Sequence Detection v. 1.7 analysis software (Perkin Elmer, Nieuwkerk, The Netherlands). Duplicates showing more than a 5% variation were discarded. To validate a QRT-PCR, standard curves with r²>0.95 and slope values between −3.1 and −3.4 were required.

The amount of DDAH-I and DDAH-II was relative quantified based on the work of Pfaffl MW [23]. In some samples, PCR bands were purified using a MiniElute PCR Purification Kit (Qiagen, Valencia, CA, USA) and then sequenced to prove that the amplified products corresponded to previously published DDAH-I, DDAH-II and GAPDH sequences. Agarose gel electrophoreses were also performed to demonstrate that QRT-PCR yielded a unique band.

Fig. 1. Estradiol reverses oxLDL-decreased NO production. Endothelial cells were exposed to 100 μg/mL of either nLDL or oxLDL in the absence/presence of 1 nM estradiol for 24 h. Culture medium was then collected and NO concentration was measured as described in Materials and methods. Data are expressed as percentage of control values and are mean±SEM of 6–8 values corresponding to 3 experiments. Average control values for all experiments were 7.3±0.2 nmol/mg protein (range: 6.9–7.7 nmol/mg protein). *p<0.05 vs. control, †p<0.01 vs. control, and ‡p<0.05 vs oxLDL alone.
2.6. Statistical analysis

Values shown in the text and figures are mean ± SEM from data obtained in 3 experiments. ANOVA test was applied for comparisons of mean, and then Bonferroni’s test was performed. *P < 0.001 vs. control, †P < 0.05 vs. control, ‡P < 0.001 vs. nLDL, and §P < 0.001 vs oxLDL alone.

3. Results

We first tested the effect of nLDL and oxLDL, with and without estradiol, on NO production by endothelial cells. As expected, oxLDL (100 μg/mL) reduced NO production by 32% (p < 0.01 vs. control), whereas production remained unchanged when cells were exposed to the same concentration of nLDL (Fig. 1). Estradiol alone (1 nM) enhanced NO production (52%, p < 0.05 vs. control). Estradiol also increased NO production in cells exposed to nLDL (p < 0.05 vs. control) and oxLDL (p < 0.05 vs. oxLDL alone). These results suggested that oxLDL reduced NOS activity and estradiol increased it.

Consequently, the levels of the endogenous inhibitor of NOS, ADMA, and its inactive isomer SDMA were studied. The effects of nLDL, oxLDL and estradiol, on ADMA production are presented in Fig. 2. After 24 h of exposure, oxLDL significantly increased ADMA production by HUVEC (159% of control values, p < 0.001), whereas nLDL was not able to modify it. Estradiol decreased ADMA production, not only by itself, but also when used in combination with nLDL or oxLDL (p < 0.05 vs. control values). In fact, estradiol was able to completely prevent ADMA production induced by oxLDL (p < 0.001 vs. oxLDL alone).

Fig. 2. Estradiol recovers oxLDL-increased ADMA production. Endothelial cells were exposed to 100 μg/mL of either nLDL or oxLDL in the absence/presence of 1 nM estradiol for 24 h. Culture medium was then collected and ADMA concentration was measured as described in Materials and methods. Data are expressed as percentage of control values and are mean ± SEM of 6–9 values corresponding to 3 experiments performed in cells from different cultures. Average control values for all experiments were 1.7 ± 0.2 ng/mg protein (range: 1.2–2.3 ng/mg protein). *p < 0.001 vs. control, †p < 0.05 vs. control, ‡p < 0.001 vs. nLDL, and §p < 0.001 vs oxLDL alone.

Fig. 3. Neither nLDL nor oxLDL modifies SDMA production. Endothelial cells were exposed to 100 μg/mL of either nLDL or oxLDL in the absence/presence of 1 nM estradiol for 24 h. Culture medium was then collected and SDMA concentration was measured as described in Materials and methods. Data are expressed as percentage of control values and are mean ± SEM of 6–9 values corresponding to 3 experiments. Average control values for all experiments were 1.6 ± 0.3 ng/mg protein (range: 0.9–2.2 ng/mg protein).

Fig. 4. LDL does not modify DDAH-I protein or mRNA expression. Endothelial cells were exposed to 100 μg/mL of either nLDL or oxLDL in the absence/presence of 1 nM estradiol for 24 h. For immunoblotting assays, cells were collected in lysis buffer, and equal amounts of protein (range: 60–130 μg) were subjected to 12% acrylamide gel electrophoresis and immunoblotted with specific antibody anti-DDAH-I, as described in Materials and methods. A typical immunoblotting image (A) and relative levels assessed by densitometry of bands of 40-kDa (B) are presented. For mRNA expression (C), total RNA was extracted, and the relative expression of DDAH-I was quantified by QRT-PCR, as described in Materials and methods. Data are expressed as a percent of control values and are mean ± SEM of 4–7 values corresponding to 3 experiments.
selective, since SDMA production was unaffected when cells were exposed to the above mentioned treatments (Fig. 3).

ADMA levels in cultured cells are mainly controlled through the activity of DDAH. The relative implication of DDAH-I and DDAH-II on ADMA production was examined by studying the changes in their mRNA expression and on their protein content (by QRT-PCR and by immunoblotting, respectively) in HUVEC exposed to desired treatments for 24 h. DDAH-I protein content and mRNA expression remained unchanged when cells were exposed to nLDL, oxLDL, estradiol, and their combinations (Fig. 4). In contrast, DDAH-II protein content and mRNA expression were significantly reduced when cells were exposed to oxLDL (Fig. 5). Estradiol alone increased both DDAH-II protein content, as well as mRNA expression ($p<0.05$ vs. control). Estradiol increased DDAH-II, when used in combination with nLDL and oxLDL, to the same levels as estradiol alone did ($p<0.05$ vs. control). In the case of oxLDL, DDAH-II protein content and mRNA expression were also significantly increased when compared to the values for oxLDL alone ($p<0.05$). Therefore, data suggest that estradiol decreases oxLDL-increased ADMA levels in endothelial cells by increasing the expression and protein content of DDAH-II.

4. Discussion

The major finding of our investigation is that estradiol is able to completely reverse the changes induced by oxLDL on the DDAH/ADMA/NO pathway. Mainly, estradiol reverses (1) decreased NO release, (2) increased ADMA production, and (3) decreased DDAH-II mRNA and protein content. Estradiol exerts a number of cardiovascular actions through regulation of NO production. Estradiol increases NO production acutely [24], as well as by genomic, estrogen receptor dependent mechanisms [25]. In the present study, physiological concentrations of estradiol increase NO production and decrease ADMA levels (Figs. 1 and 3). These effects are mediated through estrogen receptor activation, since a blockade by antagonist ICI 182780 (Fulvestrant, a competitive antagonist of both estrogen receptor subtypes α and β) completely prevents it (Figs. 1, 3, 4 and 5, on-line supplementary data). Data are in accordance with previous studies performed in HUVEC line SGHEC-7 [16]. Moreover, the effect is specific for ADMA, since estradiol has no effect on HUVEC production of the inactive isomer, SDMA (Fig. 2, on-line supplementary data).

Additionally, estradiol exerts diverse biological effects on the cardiovascular system, including not only antiatherogenic changes in the lipoprotein pattern, but also such direct effects on the vascular wall as decreased LDL plasma levels [26] and decreased LDL oxidation [27]. As stated before [12], cell exposure to oxLDL results in decreased NO release as a consequence of increased ADMA production, whereas the same concentration of nLDL has no effect (Figs. 1 and 2). Furthermore, SDMA remains unaltered when cells are exposed to the different treatments, which is in complete agreement with previous studies performed in human umbilical artery endothelial cells [12].

The combination of these effects, however, has not been studied. In the present work, our results clearly indicate that estradiol completely reverses the effects induced by oxLDL on NO (Fig. 1) and ADMA production (Fig. 2). Therefore, the antiatherogenic activity of estrogens could be mediated not only by reducing LDL oxidation, for instance, but also by decreasing its consequences on the vascular tone regulation mediated by NO. Furthermore, ADMA reduction could contribute to the antiatherogenic effect of estradiol since ADMA has been found to have proatherogenic activities [28,29], and it is thought of as an independent risk factor for cardiovascular diseases [11].

ADMA and SDMA are derived from the catabolism of proteins containing methylated arginine residues by protein
arginine methyltransferases and are degraded by DDAH, which hydrolyzes ADMA to L-citrulline and dimethylamine. The key role of DDAH in the regulation of ADMA levels has been demonstrated through overexpressing the DDAH gene [30,31]. It has also been established that the elevation of ADMA levels is related to the decrease of DDAH activity in cultured endothelial cells [13,14]. To date, two isoforms of DDAH have been characterized: DDAH-I and DDAH-II. DDAH-I is typically found in tissues expressing neuronal NOS, whereas DDAH-II is highly expressed in cardiovascular tissues and has a tissue distribution with similarities to that of eNOS [32].

Results of the present study revealed that both enzymes are expressed in HUVEC, but only DDAH-II is modified by exposure to oxLDL or estradiol (Figs. 4 and 5). The absence of estradiol stimulation on DDAH-I protein expression is in accordance with previous studies performed in murine endothelial cells, in which DDAH-II expression was not studied [16]. In fact, the expression of DDAH-II, but not DDAH-I, protein has been shown to be transcriptionally regulated [15,33,34]. In the current study, a decrease in mRNA expression of DDAH-II was observed in endothelial cells exposed to oxLDL, associated with an increase of 57% in ADMA levels. Estradiol, on the other hand, increased both mRNA and protein expression of DDAH-II by ~50%, both alone and in combination with nLDL or oxLDL. Taken together, results suggest that estradiol counteracts the oxLDL-induced down-regulation of DDAH-II at the transcriptional level, thus increasing DDAH-II protein expression, which further decreases the accumulation of ADMA and restores NO production.

The exact mechanism by which oxLDL decreases DDAH-II and, therefore increases ADMA levels, is unknown. One possibility arises from the fact that oxLDL is not only a product of oxidative stress but also a stimulus for oxygen radical formation [35]. Reactive oxygen radicals have been reported to decrease DDAH activity [36] and mRNA expression [37], thus suggesting that oxLDL-induced DDAH decrease and ADMA increased production in HUVEC could be due to oxidative stress. Estradiol has been reported to exert antioxidant actions, including the reduction of LDL oxidation [18]. Therefore, estradiol could counteract oxLDL-induced changes in the DDAH/ADMA/NO pathway by reducing oxidative stress. Nevertheless, care should be taken, since the required estradiol concentrations to exert antioxidant actions are usually at least 1000-times higher than used in the present study, within the micromolar range [18].

Actions of oxLDL in endothelial cells are mainly mediated through LOX-1, a lecitin-like receptor for oxLDL. LOX-1 facilitates uptake and mediates several biological effects of oxLDL [38]. LOX-1 increases the generation of oxygen radicals that can, in turn, up-regulate LOX-1 expression, therefore contributing to further oxidative stress [39]. LOX-1 up-regulation by oxLDL is also associated with decreased eNOS expression and NO production [39]. Interestingly, ADMA increases LOX-1 expression in endothelial cells, resulting in augmented uptake of oxLDL and increasing oxidative stress and LOX-1 expression, causing a vicious circle [40]. Taken together, it can be hypothesized that estradiol could antagonize oxLDL-induced changes in the DDAH/ADMA/NO pathway by modulating the expression of LOX-1.

In different clinical trials performed in healthy postmenopausal women, hormone replacement therapy with estradiol, either unopposed or combined with progestogens, decreased plasma ADMA levels, whereas SDMA plasma levels remained unchanged or slightly reduced [41,42]. In women with coronary heart disease, endogenous estradiol plasma concentrations were inversely associated with ADMA plasma levels [43]. Our results support these clinical observations, and provide a role for DDAH-II in the observed effects. Moreover, although oxLDL was not measured in the above mentioned clinical studies, total cholesterol remained unchanged after different treatments, supporting an independent role for estradiol in ADMA reduction.

In conclusion, clinical and experimental studies support a role for estradiol in ADMA regulation through DDAH. In our study, estradiol was able to completely reverse the effects induced by oxLDL on the DDAH/ADMA/NO pathway.

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Appendix A. Supplementary data


References


