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Estrogen Stimulates Dimethylarginine Dimethylaminohydrolase Activity and the Metabolism of Asymmetric Dimethylarginine

Desmond P. Holden, MRCOG, PhD; Judith E. Cartwright, PhD;
Stephen S. Nussey, FRCP, DPhil; Guy St J. Whitley, PhD

Background—Experimental evidence suggests that estrogens stimulate the production of nitric oxide (NO) by vascular endothelial cells. This effect has been attributed to increased expression and enzymatic activity of both the constitutive and inducible isoforms of NO synthase. In this study, we have investigated whether estrogens regulate the metabolism or release of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase.

Methods and Results—The concentration of ADMA in the plasma of 15 postmenopausal women was 0.722±0.04 μmol/L (mean±SEM). Two weeks after subcutaneous implantation with estradiol, there was an increase in plasma estradiol concentration from 0.693±0.075 to 0.81±87 nmol/L, which was accompanied by a significant fall in plasma ADMA concentration to 0.588±0.03 μmol/L (P=0.006). Human and murine endothelial cell lines previously cultured in estrogen-free medium and then exposed to 17β-estradiol showed a dose-dependent decrease in the release of ADMA. This reached statistical significance at 10^{-14} mol/L 17β-estradiol and was accompanied by a corresponding increase in the activity of dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that catalyzes the metabolism of ADMA.

Conclusions—We have demonstrated that estrogens can alter the catabolism and release of ADMA in vitro and reduce the circulating concentration in vivo. We therefore propose that increased DDAH activity and the subsequent fall in ADMA could contribute to the positive effect of estrogen on NO synthesis. **(*Circulation*. 2003;108:1575-1580.)**

Key Words: endothelium ■ nitric oxide ■ cardiovascular diseases ■ asymmetric dimethylarginine

Significant differences in cardiovascular function and disease have been reported between men and women. The incidence of coronary heart disease (CHD) in men exceeds that in women of similar age, whereas CHD among women rises sharply after the onset of natural or surgically induced menopause. Many of these effects have been attributed at least in part to gonadal steroids, in particular estrogen. Additional support comes from numerous studies using experimental models.[1] Although the concept that estrogen replacement therapy (ERT) is cardioprotective has been challenged recently by the negative results of randomized clinical trials in coronary heart disease,[2,3] several retrospective and cross-sectional studies indicate that women treated with ERT have improved vascular function and a lower incidence of CHD.[4]

Mechanistically, the effects of estrogen on cardiovascular function have been attributed to favorable changes in plasma lipid profile, which may account for 25% to 50% of the protective effects. Direct effects of estrogens on endothelial function and vascular reactivity as a result of enhanced production or activity of several vasoactive compounds including nitric oxide (NO) have also been reported.[5] Estradiol stimulates the expression of both endothelial NO synthase (eNOS) and inducible NOS (iNOS) in vascular cells[5,6] and stimulates NO-dependent vasodilatation in vivo.[7] There is also evidence that estrogen stimulates eNOS activity directly via the activation of membrane-associated steroid hormone receptors.[8] The mechanisms involved have not been fully elucidated, but there is evidence that activation of the phosphatidylinositol 3-kinase/Akt pathway is involved.[9,10]

Endogenous competitive inhibitors of NO synthesis have been identified; N^g-monomethylarginine (L-NMMA) and N^g-N^g-dimethylarginine (ADMA) and their contribution to the maintenance of basal vascular tone have been demonstrated.[11] Both L-NMMA and ADMA are found in the plasma and urine of healthy individuals and are altered in those with several diseases, including renal failure, hypercholesterolemia, and atherosclerosis.[12–14] We[15] and others[16] have shown that the plasma concentration of ADMA drops early in normal pregnancy (hyperestrogenic state) but is elevated in preeclampsia. L-NMMA and

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From the Department of Obstetrics and Gynaecology (D.P.H.), Royal Sussex County Hospital, Eastern Road, Brighton, and Departments of Biochemistry and Immunology (J.E.C., G.S.J.W.) and Oncology, Gastroenterology, Endocrinology, and Medicine (S.S.N.), St George's Hospital Medical School, London, UK.

Dr Whitley is an inventor on patent No. WO0044888 relating to screening methods for inhibitors of DDAH. The patent is held by University College London, which was not involved in this study.

Correspondence to Guy Whitley, Department of Biochemistry and Immunology, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK. E-mail g.whitley@sghms.ac.uk

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ADMA, but not symmetric dimethylarginine (SDMA), are metabolized to citrulline by dimethylarginine dimethylaminohydrolase (DDAH), which is present in several tissues and cells that synthesize NO, including endothelial cells.[17,18]

The following study was performed to test the hypothesis that estrogens can regulate the concentration of ADMA through changes in DDAH enzymatic activity. We propose that this may be one mechanism by which estrogens exert their cardiovascular effects.

Methods

Effect of Subcutaneous Ethynylestradiol Implant on Plasma Methylarginine Concentration

Fifteen postmenopausal women attending outpatient clinics at St George's Hospital were chosen at random and gave informed, verbal consent for blood sampling. None had received ERT before the study. All were healthy and were not taking any medication for blood pressure. At the time of recruitment, their average age, blood pressure, and plasma cholesterol was 55.3±1.5 years, 123±4.0/75.8±2.3 mm Hg, and 5.58±0.334 mmol/L, respectively. Their diet was unrestricted, and samples were taken immediately before and 2 weeks after the insertion of a 100-mg ethynylestradiol implant into subcutaneous fat of the abdominal wall. Blood was taken by venipuncture for the subsequent determination of methylarginines. Serum estradiol concentration was determined by the Department of Clinical Biochemistry using an enzyme-linked immunosorbent assay.

Isolation and Measurement of Dimethylarginines

Quantitation of dimethylarginines was achieved by HPLC using the method described previously.[15] Concentrations of ADMA and SDMA in the samples were determined by comparison with authentic standards. Extraction efficiency was determined by the addition of 10 μg L-NMMA to each sample before extraction.

Cell Culture

In this study, we used the human umbilical vein endothelial cell line SGHEC-7 and the murine endothelial cell line sEnd-1. Both exhibit an endothelial cell phenotype and have been characterized and used in several studies of ADMA and DDAH biology.[17,19–21] The cells were cultured as previously described.[19,22] For experimental purposes, estrogen was removed from the serum by overnight incubation with 0.2% (wt/vol) charcoal and 0.02% (wt/vol) dextran, stirring constantly, at 4°C. This was then added to phenol red-free medium, which was otherwise identical in composition to the normal growth medium.

Effect of 17β-Estradiol on the Release of Dimethylarginines by Endothelial Cells in Culture

SGHEC-7 or sEnd-1 cells were seeded at a density of 2.5×10^5 cells/mL, 10 mL per 90-mm culture dish, and maintained for 24 hours under standard incubation conditions. After washing with PBS, the cells were incubated in estrogen-depleted, phenol red–free medium. After 72 hours, the medium was replaced with identical medium containing varying concentrations of 17β-estradiol or ethanol as a vehicle control. In selected experiments, the effect of the estrogen receptor antagonists tamoxifen and ICI 182780 or their vehicle methanol on dimethylarginine release was investigated. At the end of the incubation period, the culture supernatant was removed and the methylarginines were determined as above. The cells were washed with PBS, and the protein content was determined by the Bradford assay (Bio-Rad).

Effect of 17β-Estradiol on DDAH Activity

SGHEC-7 cells were seeded into 24-well plates (10^5 cells/mL, 0.5 mL/well) and cultured as above. The cells were washed, and 0.4 mL medium containing 17β-estradiol (final concentration, 10^{-14} to 10^{-8} mol/L) was added to each well. The medium was removed after 24 hours, and cells were incubated with 0.25 mL Krebs solution containing ^{14}C-L-NMMA (0.04 μCi/mL) alone or with either ADMA (3 mmol/L) or SDMA (3 mmol/L) for 1 hour at 37°C. Cells were then washed twice with ice-cold PBS and lysed with sodium dodecyl sulfate (SDS; 0.1% [wt/vol]; 0.4 mL). The lysate was added to 1 mL of Dowex 50X8-400 resin, and ^{14}C-citrulline was determined by liquid scintillation counting.

Determination of DDAH, Estrogen Receptor-α, and Estrogen Receptor-β Protein Expression

For the detection of DDAH, sEnd-1 cells were seeded into 90-mm plates in standard medium and cultured as above. After 24 hours, cells were washed with PBS and incubated in estrogen-depleted, phenol red–free medium for 72 hours. The medium was replaced with identical medium containing 17β-estradiol (final concentration, 10^{-14} to 10^{-8} mol/L), which was added for 24 hours. After this time, protein isolation, electrophoresis, and western blot analysis were carried out. The protein concentration was determined before loading. Detection of membrane-bound antibodies was carried out using enhanced chemiluminescence (Boehringer Mannheim). For the detection of the estrogen receptors, confluent plates of SGHEC-7, sEND-1, and human umbilical vein endothelial cells were used.[19,22] Antibodies to estrogen receptor-α (ER-α) and ER-β were obtained from Santa Cruz. DDAH-I monoclonal antibody was a gift from Dr Kimoto.[23]

Data Analysis

Data obtained from cell culture experiments were analyzed using the nonparametric Mann-Whitney *U* test. The Student's *t* test was used for statistical analysis of clinical data. Statistical significance was assumed at $P<0.05$.

Results

Effect of 17β-Estradiol Implants on Plasma Dimethylarginine Concentration In Vivo

Fifteen postmenopausal women (mean age, 55.3 years; range, 46 to 63) allowed venipuncture immediately before and 2 weeks after the insertion of a 100-mg ethynylestradiol implant. Before implant, the mean±SEM serum estradiol concentration was 0.693±0.075 nmol/L, whereas after implant there was a small but significant increase to 0.810±0.087 nmol/L (Figure 1, $P<0.05$ as determined by Student's *t* test). The mean plasma ADMA concentration decreased from a mean±SEM of 0.722±0.04 μmol/L to 0.588±0.03 μmol/L ($P<0.05$). Although there was an apparent drop in the mean plasma SDMA concentration after treatment with 17β-estradiol, this did not reach statistical significance (0.486±0.07 μmol/L SEM compared with 0.395±0.14 μmol/L; $P=0.54$).

Endothelial Estrogen Receptor Expression

Western blot analysis showed the expression of both ER-α and ER-β by SGHEC-7 and sEnd-1 cells. Primary human umbilical endothelial cells, which were used as a positive control for the expression of the receptors (Figure 2).

Effect of 17β-Estradiol on Dimethylarginine Accumulation in Endothelial Cell Cultures

Stimulation of SGHEC-7 cells with 17β-estradiol (10^{-14} to 10^{-8} mol/L) significantly reduced the concentration of ADMA in the medium by a maximum of 65.7±23.6%. At 10^{-10}mol/L, compared with control cultures ($P<0.001$; Figure 3A), the concentration of SDMA was unaffected (data not

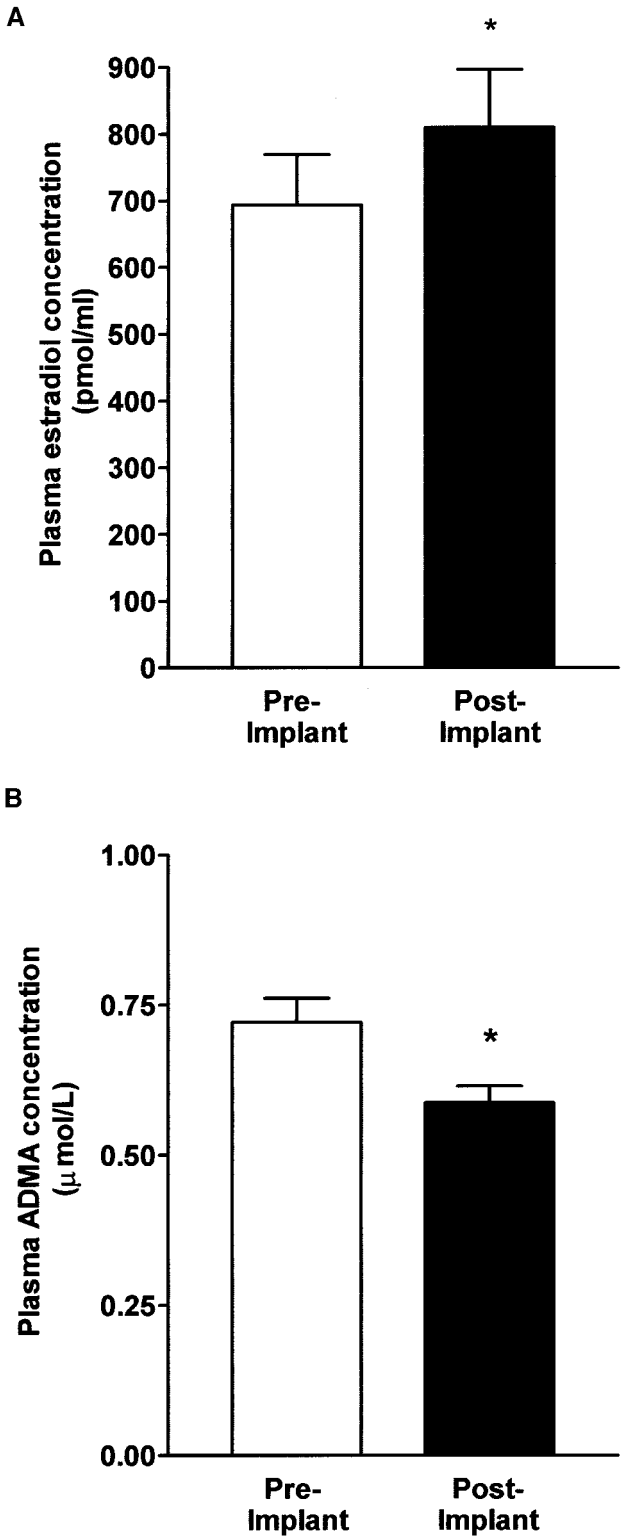

Figure 1. Plasma concentration of 17β-estradiol and ADMA. Blood was taken from 15 postmenopausal women just before and 2 weeks after the subcutaneous insertion of a 100-mg estradiol implant. The circulating concentrations of 17β-estradiol (A) and ADMA (B) are expressed as mean+SEM before and after implant as measured by ELISA and HPLC, respectively. Data analysis was by 2-tailed *t* test. *$P<0.05$.

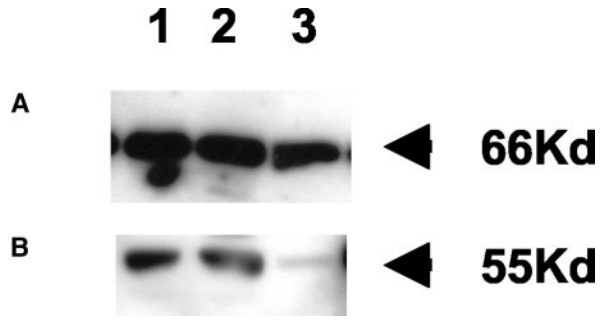

Figure 2. Estrogen receptor expression. Untreated cell lysates were subjected to Western blot analysis. Lane 1, SGHEC-7 cells; lane 2, sEnd-1 cells; lane 3, primary human umbilical vein endothelial cells. Separate blots were probed for ER-α (panel A; 49.5 μg/well) and ER-β (panel B; 34.5 μg/well).

shown). Using sEnd-1 cells, a similar dose-dependent reduction in the concentration of ADMA was seen in the culture supernatant (Figure 3B). Changes in the release of ADMA were not detected at earlier time points.

Effect of Tamoxifen on the Accumulation of Dimethylarginines in Endothelial Cell Cultures

In the presence of 10^{-14} mol/L 17β-estradiol, there was a significant reduction in the ADMA concentration of the culture supernatant from SGHEC-7 cells, as seen previously (Figure 4). Under these conditions, tamoxifen at 10^{-8} mol/L inhibited the reduction in ADMA concentration associated with 10^{-14} mol/L 17β-estradiol (83±16%, $P<0.01$); the methanol control had no effect. In the presence of tamoxifen, the effect of 17β-estradiol on ADMA was not significantly different from control. A similar inhibitory effect could also be demonstrated after the incubation of sEnd-1 cells with tamoxifen (data not shown).

Neither tamoxifen nor its vehicle methanol had any effect on the concentration of SDMA in the culture supernatant (data not shown). Neither ethanol nor methanol had any effect on dimethylarginine accumulation in SGHEC-7 cell culture supernatants at concentrations up to 10-fold higher than those used in these experiments. The specific high-affinity estrogen receptor antagonist ICI 182780 was also able to partially inhibit the effects of 17β-estradiol (17β-estradiol ADMA as a percent of control, 42.89±12, n=5; compared with 17β-estradiol plus ICI 182780, 87.4±13%, n=5; $P<0.05$).

Effect of 17β-Estradiol on DDAH Activity

DDAH activity in SGHEC-7 cells was stimulated by 17β-estradiol (10^{-14} and 10^{-8} mol/L) as determined by the production of ^{14}C-citrulline by 23±6.3% ($P<0.01$) and 35±5.3% ($P<0.001$), respectively (Figure 5A). This activity was inhibited by tamoxifen. Cells incubated with tamoxifen in the absence of 17β-estradiol showed a small increase in DDAH activity, although this did not reach statistical significance (data not shown). The DDAH-specific conversion of ^{14}C-L-NMMA to ^{14}C-citrulline was investigated, and the results from a representative assay are shown in Figure 5B. The production of ^{14}C-citrulline was inhibited by the addition of 3 mol/L ADMA to the assay from 13.8±2.4 to 0.89±0.7 pmol citrulline per min per mg cell protein ($P<0.001$). The

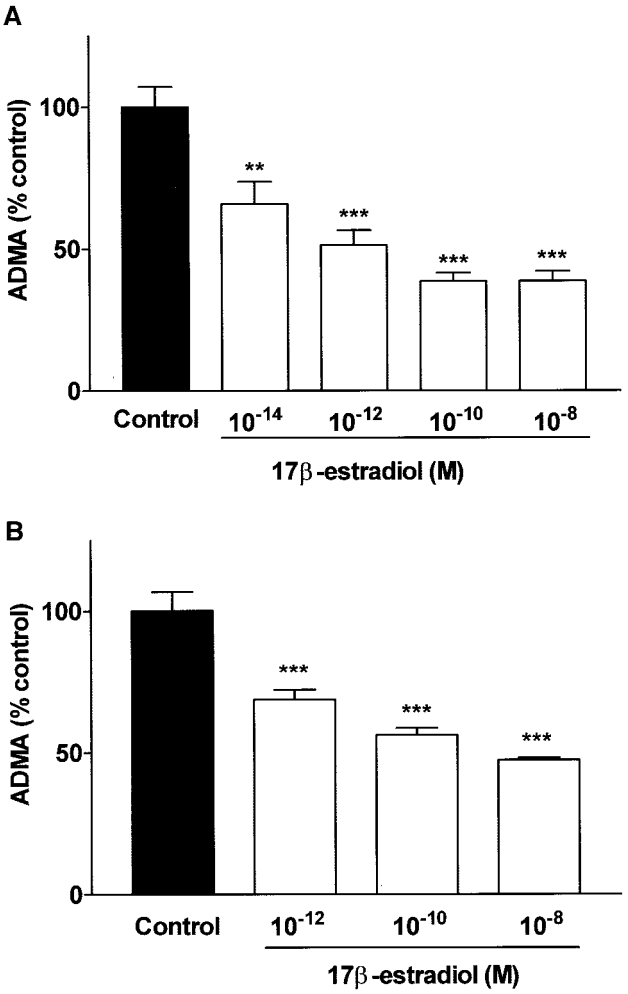

Figure 3. Effect of 17β-estradiol on the release of dimethylarginine by endothelial cells in culture. 17β-estradiol was added to SGHEC-7 (A) and sEnd-1 (B) cells for 24 hours. The concentration of ADMA and SDMA was determined by HPLC. Results were normalized in each experiment by taking the mean ADMA concentration for cells in phenol red–free, estrogen-deplete medium as 100%. Results are presented as mean+SEM of 3 independent experiments carried out in triplicate. Comparisons were made with ethanol vehicle control using Mann-Whitney *U* analysis; $^{**}P<0.01$, $^{***}P<0.001$.

addition of 3 mmol/L SDMA had no effect on ^{14}C-citrulline production.

Effect of 17β-Estradiol on DDAH-I Protein Expression

Having established that 17β-estradiol increased DDAH activity in both SGHEC-7 and sEnd-1 cells, we used sEnd-1 cells to determine whether DDAH-I protein expression was altered after stimulation. The use of murine cells in this experiment overcame poor species cross-reactivity previously reported when using the monoclonal antibody raised against rat DDAH-I.[17,23] Stimulation with 17β-estradiol up to 10^{-8} mol/L had no effect on DDAH-I expression in these cells (Figure 6).

Discussion

The significant findings of this investigation are the following: (1) circulating ADMA is reduced in women after ERT;

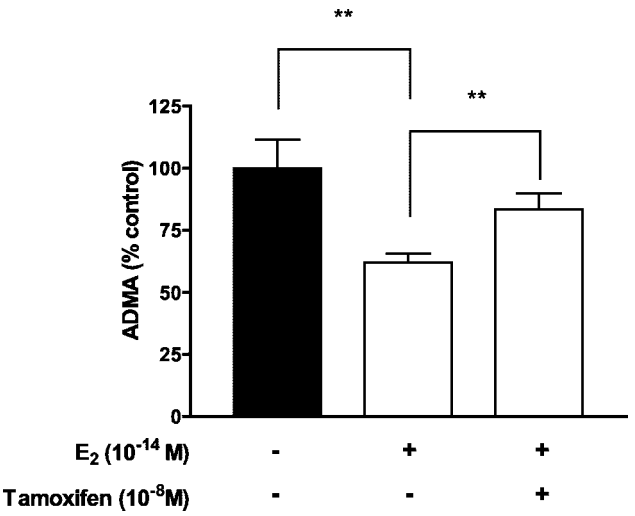

Figure 4. Effect of estrogen receptor antagonists on ADMA accumulation in the culture supernatant of SGHEC-7 cells. Cells were incubated with either vehicle alone or tamoxifen±17β-estradiol. The results were normalized in each experiment by taking the mean ADMA concentration in untreated cells as 100%. The results presented are mean+SEM from 2 independent experiments carried out in triplicate; $^{**}P<0.01$.

(2) in vitro, estrogen reduces the release of ADMA by endothelial cells; (3) estrogen stimulates endothelial cell DDAH enzyme activity; (4) both the inhibition of the release of ADMA and the increased DDAH activity are inhibited by estrogen receptor antagonists; and (5) increased DDAH activity was not attributable to increased expression of DDAH I.

There has been considerable interest in the regulation of cardiovascular function by estrogen and in particular how estrogens may regulate the production of NO. Mechanistic studies demonstrate the ability of estrogens to increase the bioavailability of NO by directly stimulating changes in the expression of eNOS or through indirect regulation of genes encoding essential cofactors. In addition, estrogen may alter NO synthesis via its direct antioxidant effects.[24] We examined whether estrogen could also regulate the concentration of ADMA, an endogenous inhibitor of NO synthesis.

Previous studies have shown that ADMA is synthesized by and released from endothelial cells in culture.[19,25] It competes with arginine for both the active site of NOS and the Y^+ transporter.[21,26] Significantly, the intracellular concentration of ADMA can reach 5 times that of the extracellular or circulating concentration. At this concentration, inhibition of NOS is a possibility.[27] Therefore, manipulation of intracellular or the circulating concentration of ADMA could regulate both basal and stimulated NO synthesis. In support of this hypothesis, inhibiting ADMA metabolism in isolated vascular rings induced contraction, which could be reversed by L-arginine.[17] More recently, a role for ADMA as a marker of cardiovascular disease has been proposed,[28] and significant correlation between the circulating concentration of ADMA and intima-media thickening in the carotid artery and impaired endothelium-dependent relaxation has been found.[29] We have shown during normotensive pregnancies (a hyperestrogenic state) that an early drop in

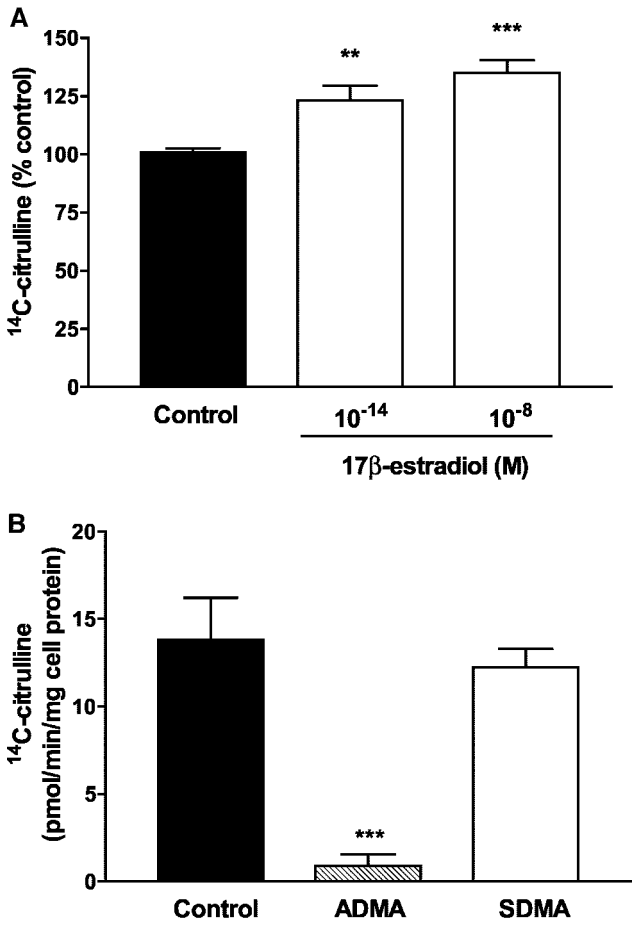

Figure 5. Effect of 17β-estradiol on DDAH enzyme activity. A, SGHEC-7 cells were stimulated with 17β-estradiol for 24 hours. DDAH activity was determined by the conversion of ^{14}C-L-NMMA to citrulline. The production of ^{14}C-citrulline is normalized to that of unstimulated control cells. Results presented are mean+SEM of n=12 determinations from 3 independent experiments. Significance was determined using Mann-Whitney *U* test; $^{**}P<0.01$, $^{***}P<0.001$. B, DDAH-specific conversion of ^{14}C-L-NMMA to ^{14}C-citrulline was investigated, and the results from a representative assay are shown. Cells were treated as above but were incubated in the presence and absence of either 3 mmol/L ADMA or 3 mmol/L SDMA. Results are mean+SEM of n=8 determinations from a representative experiment. Significance was determined using Mann-Whitney *U* test; $^{***}P<0.001$.

blood pressure correlated with a significant fall in plasma ADMA concentration.[15]

Increased NO synthesis after the administration of estradiol has been demonstrated.[7] In premenopausal women, plasma NO peaks midcycle, when estrogen reaches its maximum circulating concentration.[30] Administration of estradiol to postmenopausal women induced a sustained increase in NO production,[31] whereas increased expression of eNOS mRNA and enhanced calcium-dependent NOS activity has been reported in animals.[32]

Contrary to expectations, the serum ADMA concentration of our sample group before treatment was not significantly different from our previous report, in which 20 healthy young nonpregnant women were studied.[15] However, there was a significant reduction in the plasma concentration of ADMA after estradiol implantation. Our failure to detect any change in the concentration of SDMA would be predicted after the specific

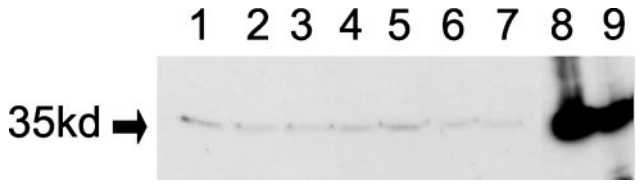

Figure 6. Effect of 17β-estradiol on DDAH-I expression. sEnd-1 cells were stimulated with 17β-estradiol (final concentration, 10^{-14} to 10^{-8} mol/L) and subjected to western blot analysis. Lanes 1 and 2 show control, unstimulated cells; lanes 3 and 4 show 10^{-8} mol/L; lanes 5 and 6 show 10^{-11} mol/L; lane 7 shows 10^{-14} mol/L 17β-estradiol; and lanes 8 and 9 show a positive control of rat glioma cells transfected to overexpress DDAH I.

activation of DDAH by estrogen. Although the limited nature of the study group restricts the extent of the interpretation, this study is in agreement with a recent study indicating a reduction in circulating ADMA in postmenopausal women undergoing ERT.[33] The magnitude of the fall in ADMA in response to a modest rise in estradiol probably reflects the sensitivity of endothelial cells to 17β-estradiol, which is supported by our in vitro studies.

To examine the mechanism at a cellular level, experiments were performed using 2 endothelial cell models, which express both ER-α and ER-β. In both cell types, stimulation with physiological concentration of 17β-estradiol significantly reduced the concentration of ADMA. The involvement of the estrogen receptor was investigated using receptor antagonists. Tamoxifen, which inhibits estrogen-induced NO synthesis by endothelial cells,[34] significantly increased endothelial cell ADMA concentrations. Consistent with mixed agonist/antagonist activity, 10^{-11} mol/L tamoxifen alone reduced the accumulation of ADMA but not SDMA (data not shown). In this regard, it is interesting to speculate that a reduction in the circulating concentration of ADMA may contribute to the cardioprotective effects observed in some groups undergoing cancer treatment with tamoxifen.[35] We were able to confirm the involvement of estrogen receptors by the use of ICI 182780, which, like tamoxifen, was able to partially inhibit the fall in ADMA observed with 17β-estradiol treatment.

The active metabolism of ADMA and L-NMMA, but not SDMA or L-arginine, to citrulline and methylamines by DDAH has been described in vascular endothelial cells.[17] The differential effect of estradiol on ADMA but not SDMA observed in vivo in this study would indicate a specific change in ADMA metabolism, perhaps by alterations in DDAH activity. This involvement could be confirmed, because treatment with 17β-estradiol significantly increased DDAH activity in vitro. However, the exact mechanism for this is unknown. Two isoforms of DDAH have been identified.[18] Whereas a change in DDAH-I expression was not detected, in this study, undetected changes in DDAH-II protein expression may in part be responsible for the increased DDAH activity observed. The expression of DDAH-II was examined using recently available commercial antibodies; however, these proved unsatisfactory both in the detection of DDAH-II from test samples as well as recombinant DDAH II. Alternatively, 17β-estradiol may act as an antioxidant, because oxidative stress can inhibit DDAH without affecting DDAH expression.[36]

Two large randomized studies, one of healthy post menopausal women and the other of postmenopausal women with preexisting cardiovascular disease, found significant adverse cardiovascular effects after the combined use of estrogen and progesterone therapy[2,3] despite significant advantageous changes in the lipid profiles compared with placebo group.[3] However, because neither study was designed to determine the effects of estrogen alone, direct comparison with the present study is not possible. It is interesting to speculate how administration of progesterone might interact with estradiol to regulate DDAH activity and, therefore, ADMA accumulation.

In conclusion, we have demonstrated that 17β-estradiol decreases the circulating concentration of ADMA in vivo and release from endothelial cells in vitro, the latter because of increased DDAH activity. These results may in part explain increased NO synthesis observed in women undergoing hormonal replacement therapy and therefore could contribute to some of the cardiovascular effects attributed to estrogens.

Acknowledgments

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