Oestrogen replacement therapy lowers plasma levels of asymmetrical dimethylarginine in healthy postmenopausal women

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ABSTRACT

Oestrogen replacement therapy (ERT) has been shown to lead to favourable changes in the cardiovascular risk profile of postmenopausal women. Part of this effect is ascribed to increased production or bioavailability of nitric oxide (NO). We have tested the hypothesis that ERT lowers plasma levels of asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of NO synthase (NOS). In a randomized double-blind study design, 40 hysterectomized postmenopausal women received conjugated equine oestrogen (CEE; 0.625 mg/day; n = 14), the selective oestrogen receptor modulator raloxifene (150 mg/day; n = 13) or placebo (n = 13). At baseline and after 6, 12 and 24 months of treatment, plasma was analysed for levels of arginine, ADMA, and symmetrical dimethylarginine (SDMA), a stereoisomer of ADMA that does not inhibit NOS. An overall treatment effect on ADMA levels was observed in the CEE group (P = 0.004 compared with placebo), but not in the raloxifene group (P = 0.50). The decrease of ADMA levels by CEE treatment was consistent over the 2-year study period, without significant differences between the effects at 6, 12 and 24 months. The average post-baseline change in ADMA in the CEE group compared with placebo was −7.8% (95% confidence interval −12.8% to −2.9%; P = 0.003). Arginine or SDMA levels did not change during treatment in any of the groups. Thus ERT with oral conjugated oestrogen, but not with raloxifene, significantly reduced plasma concentrations of the cardiovascular risk factor ADMA in healthy postmenopausal women.

INTRODUCTION

Although randomized trials of hormone replacement therapy in women have not demonstrated a decrease in the rate of cardiovascular events [1,2], there is abundant evidence that the cardiovascular risk profile of postmenopausal women is changed in a favourable direction [3–5]. Oestrogen replacement therapy (ERT) has a beneficial effect on the lipoprotein profile by lowering levels of low-density lipoprotein (LDL) and lipoprotein(a), and by increasing levels of high-density lipoprotein (HDL). In addition, oestrogen may protect LDL against oxidation [6,7]. Plasma homocysteine, an independent risk factor for cardiovascular disease, is
also lowered by ERT [8,9]. ERT has been shown to improve vascular function as measured by endothelium-dependent vasodilation [10,11]. This improvement may be mediated by increased bioavailability of nitric oxide (NO), resulting from either increased NO synthesis [12] or reduced scavenging of NO by reactive oxygen species. Increased basal NO synthesis during ERT has been reported [12,13].

Asymmetrical dimethylarginine (ADMA), identified by Vallance et al. [14] as an endogenous inhibitor of NO synthase (NOS), has been shown to be inversely correlated with flow-mediated vasodilation [15] and to be an important determinant of carotid artery intima-media thickness [16]. Consequently, ADMA has been proposed as a risk factor for endothelial dysfunction and cardiovascular disease [15–17]. Recently it has been shown that even slightly elevated serum concentrations of ADMA are associated with an increased risk of acute coronary events [18].

Pregnancy, a state in which oestrogen levels are increased, is associated with decreased ADMA levels [19]. The aim of the present study was to test the hypothesis that ERT reduces ADMA levels. To gain insight into the mechanism of possible changes of ADMA levels induced by ERT, we also measured levels of symmetrical dimethylarginine (SDMA), a stereoisomer of ADMA that does not inhibit NOS. In addition, the effect of oestrogen was compared with that of raloxifene, a selective oestrogen receptor modulator that has a tissue-specific oestrogenic or anti-oestrogenic effect. The study design was a randomized double-blind placebo-controlled trial of 2 years’ duration, comparing the effects of conjugated equine oestrogen (CEE) and raloxifene with those of placebo.

METHODS

Subjects and study design
In the original study, 60 postmenopausal, hysterectomized women were included and randomly assigned to one of four treatment groups (n=15 each): CEE, 0.625 mg/day; raloxifene hydrochloride, 60 mg/day; raloxifene, 150 mg/day; and placebo. At baseline and after 6, 12, and 24 months of treatment, blood samples were collected and plasma was stored at −70 °C. The present substudy is based on analysis of available samples from the CEE (n=14), high-dose (150 mg/day) raloxifene (n=13) and placebo (n=13) groups. Informed consent was obtained from all subjects, and the hospital ethics committee approved the study. Details of the original study design have been described previously [4].

Biochemical measurements
At baseline, and after 6, 12, and 24 months of treatment, plasma levels of arginine, ADMA and SDMA were measured by HPLC with fluorescence detection [20]. Briefly, 0.2 ml of plasma was mixed with 0.1 ml of a 40 µmol/l solution of the internal standard monomethylarginine and 0.7 ml of PBS. This mixture was applied to Oasis MCX solid-phase extraction columns (Waters). The columns were washed consecutively with 1.0 ml of 0.1 mol/l HCl and 1.0 ml of methanol. Basic amino acids were eluted with 1.0 ml of conc. ammonia/water/methanol (10:40:50, by vol.). After evaporation of the solvent under nitrogen, the amino acids were derivatized with o-phthaldialdehyde reagent containing 3-mercaptopropionic acid. The derivatives were separated by isocratic reverse-phase chromatography on a Symmetry C18 column (3.9 mm×150 mm; 5 µm particle size; Waters) at a column temperature of 30 °C. Potassium phosphate buffer (50 mmol/l, pH 6.5), containing 8.7 % acetonitrile, was used as mobile phase at a flow rate of 1.1 ml/min. After elution of the last analyte, strongly retained compounds were quickly eluted by a strong solvent flush with acetonitrile. Fluorescence detection was performed at excitation and emission wavelengths of 340 and 455 nm respectively. All samples from individual patients were analysed in the same analytical series. The intra-assay coefficients of variation were 2.9 % for arginine, 2.0 % for ADMA and 1.2 % and 0.8 % respectively. Inter-assay coefficients of variation were 2.9 % for arginine, 2.0 % for ADMA and 2.6 % for SDMA.

At baseline, we measured serum levels of oestradiol by RIA (DPC) and of follicle-stimulating hormone by microparticle enzyme immunoassay (IMX; Abbott Laboratories, Chicago, IL, U.S.A.). Serum total cholesterol and HDL-cholesterol were measured by enzymic methods (Boehringer Mannheim). LDL-cholesterol was calculated using the Friedewald formula.

Statistical analyses
Variables with right-skewed distributions were logarithmically transformed and are presented as geometric means with 95 % confidence intervals (CIs). Analysis of treatment effects was performed on changes from baseline values. The overall treatment effect was tested by ANOVA for repeated measurements. After demonstration of significant differences among treatments, post hoc comparisons between active treatment groups and the placebo group were made using Dunnett’s two-sided t test. Differences between treatments at individual time points were analysed by one-way ANOVA. Pearson correlations were used to assess associations between variables. The significance level was set at P < 0.05.

RESULTS

Baseline characteristics of the subjects are shown in Table 1. There were no significant differences among the
The main novel finding of the present study is that treatment of postmenopausal women with conjugated oestrogen lowered plasma levels of the endogenous NOS inhibitor ADMA. In contrast with this effect on ADMA levels, the concentrations of SDMA and arginine did not change during treatment. The observed 7–10% reduction of ADMA levels by ERT was very consistent over the 2-year study period. The magnitude of this ADMA-lowering effect was smaller than ERT-induced changes in LDL and HDL [4], but similar to reported decreases in homocysteine levels [8,9]. It should be noted that we used a recently developed HPLC method for the analysis of ADMA, characterized by very high precision (intra-assay coefficient of variation of 1.2%), to allow reliable detection of small concentration differences [20]. The fact that healthy women were studied, with baseline ADMA levels within the normal range, may explain the observation that the decrease in ADMA was moderate. A more pronounced effect might be expected in women with cardiovascular disease that have elevated ADMA levels.

The trend towards increasing ADMA levels in the placebo group during the 2-year study period fits well with the fact that age has been shown by multiple regression analysis to be an independent determinant of plasma ADMA [16], and thus probably reflects the natural increase in ADMA with aging.

**DISCUSSION**

groups, except for levels of follicle-stimulating hormone, which were slightly lower in the raloxifene group. As ADMA levels were slightly higher in the placebo group, we analysed differences from baseline to assess treatment effects. An overall treatment effect on plasma levels of ADMA was observed in the CEE group (P = 0.004 compared with placebo), but not in the raloxifene group (P = 0.50 compared with placebo). Changes from baseline in the placebo and active treatment groups are shown in Figure 1. The decrease in plasma ADMA levels in the CEE group compared with the change in the placebo group was significant at all time points (P < 0.01). In the CEE group, the relative changes from baseline compared with placebo after 6, 12 and 24 months of treatment were −7.0% (95% CI −12.0% to −2.0%; P = 0.007), −6.8% (95% CI −12.1% to −1.5%; P = 0.013) and −9.7% (95% CI −17.1% to −2.3%; P = 0.012) respectively, without significant differences between these time points. The average post-baseline change was −7.8% (95% CI −12.8% to −2.9%; P = 0.003 compared with placebo).

As described previously in detail [4], both CEE and raloxifene decreased LDL-cholesterol to a similar extent, whereas CEE, but not raloxifene, increased HDL-cholesterol. ADMA levels at baseline were not associated with either LDL- or HDL-cholesterol. In addition, there were no associations between the treatment-induced changes in these lipoproteins and changes in ADMA levels in the CEE group (results not shown).

In contrast with the effects on plasma ADMA concentrations, changes in the levels of arginine and SDMA were not significant in either treatment group.
noted that effects of raloxifene on LDL-cholesterol and other cardiovascular risk factors are apparent at a dose of 60 mg/day [4], whereas in the present study women in the raloxifene group received a dose of 150 mg/day. The apparent lack of effect of raloxifene therefore cannot be ascribed to a suboptimal dose. We cannot, however, exclude the possibility that there was a small treatment effect of raloxifene that was not detected because of the limited power of our study.

Regarding the mechanism of the decrease in ADMA, some inferences can be made from the disparate effects of ERT on ADMA and SDMA levels and from the fact that ADMA levels were not lowered by raloxifene. Several tissues contain the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which hydrolyses ADMA, but not SDMA, into citrulline and dimethylamine [21]. Hydrolysis by DDAH is a major pathway for the elimination of ADMA, whereas renal clearance is the main mechanism for the elimination of SDMA. This is corroborated by the observation that plasma SDMA levels are closely correlated with creatinine clearance [22]. We can thus exclude increased renal clearance as an explanation for the effect of ERT, as this would affect levels of SDMA more than those of ADMA. Increased DDAH activity seems a more plausible mechanism. The active site of DDAH contains a reactive cysteine residue, raising the possibility that DDAH activity may be decreased in situations of oxidative stress [23]. In support of this notion is the observation that the activity of DDAH in endothelial cells is inhibited by incubation with oxidized LDL [24]. There are also indications that LDL plays a regulatory role in the synthesis of ADMA. ADMA is formed by transfer of two methyl groups from S-adenosylmethionine in a reaction catalysed by protein arginine N-methyltransferases. It has been shown that protein arginine N-methyltransferase activity in endothelial cells is up-regulated by native and oxidized LDL [25]. Oxidized LDL may thus both increase the synthesis of ADMA and decrease its metabolism by inhibition of DDAH. Oestrogen has been reported to increase the resistance of LDL to oxidation [6,7]. This effect has been shown to be mediated by HDL [26,27], levels of which are increased by ERT, but not by raloxifene [4,28]. A decrease in LDL levels and/or LDL oxidation thus seems a plausible mechanism for the decrease in ADMA levels due to ERT. The absence of significant associations between the changes in ADMA levels and changes in LDL and HDL in the CEE group does not lend support to this mechanism, but this may be due to lack of statistical power.

Another possible mechanism that could explain the effect of ERT on ADMA levels is based on the observation that homocysteine inhibits DDAH activity in endothelial cells and aortic segments [29]. Increased plasma concentrations of ADMA that accompany acute hyperhomocysteinaemia induced by oral methionine loading may be a reflection of this mechanism [30]. The homocysteine-lowering effect of ERT could thus be the cause of its effect on ADMA. However, in the present study, baseline plasma homocysteine concentrations were all within the normal reference range, and the modest homocysteine-lowering effect was more pronounced in the raloxifene group than in the CEE group [8], arguing against a substantial contribution of this mechanism to the ADMA-lowering effect of CEE.

In conclusion, our data show that conjugated oestrogen, but not the selective oestrogen modulator raloxifene, lowers plasma levels of the endogenous NOS inhibitor ADMA in healthy postmenopausal women. Further research is needed to clarify the mechanism by which oestrogen treatment lowers circulating ADMA levels, and to assess whether a decrease in oxidative stress is involved.

REFERENCES

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