Endocrine Journal 2007, **54** (6), 1027–1031

RAPID COMMUNICATION

2-Methoxyestradiol Reduces Monocyte Adhesion to Aortic Endothelial Cells in Ovariectomized Rats

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Abstract. 2-Methoxyestradiol (2-ME) is an endogenous metabolite of estradiol with no affinity for estrogen receptors. It inhibits cell proliferation, thus is a potentially useful drug to block the progression of atherosclerosis. As a first step to examining the anti-atherosclerotic effects of 2-ME, we investigated monocyte adhesion to aortic endothelial cells, which is considered a prerequisite to atherosclerosis *in vivo*. Eight-week-old Sprague-Dawley rats were ovariectomized then treated by slow-release pellets with placebo, 17-β-estradiol (5 μg/day), low-dose 2-ME (10 μg/day), or high-dose 2-ME (100 μg/day). After 6 weeks, *enface* analysis showed an increased number of monocytes adhering to endothelial cells of the thoracic aorta in ovariectomized rats compared with sham-operated controls. This increase was predominantly inhibited by treatment with 17β-estradiol, and low-dose or high-dose 2-ME. The observed effects were unrelated to changes in serum lipids, blood glucose, or blood pressure. Our data suggested that 2-ME mediates the anti-atherosclerotic actions of estradiol at least in part by preventing monocyte adhesion to the aortic endothelium.

Key words: Menopause, Estrogen, Hormone replacement therapy, Cardiovascular disease
(*Endocrine Journal* **54**: 1027–1031, 2007)

CARDIOVASCULAR disease is a major cause of morbidity and mortality in modern societies. In women, menopause increases the risk of cardiovascular disease [1, 2], probably due to reduced estrogen levels [3–5]. Hormone replacement therapy is reported to delay the onset of cardiovascular disease in postmenopausal women [6, 7]. In contrast, two major randomized prospective clinical trials, the Heart and Estrogen/progestin Replacement Study (HERS) [8] and the Women's Health Initiative Study (WHI) [9], found that hormone replacement therapy increased the risk of cardiovascular disease following menopause. This discrepancy might reflect differences in study conditions. The efficacy of hormone replacement therapy to protect against atherosclerosis seems dependent on the atherosclerotic state, type of estrogen used, and the co-administration of progestin [10]. Research into the anti-atherosclerotic effect of estrogen is thus needed to establish a better method to deliver estrogen to postmenopausal women.

The endogenous estrogen 17β-estradiol (E_2) binds to both estrogen receptor (ER) α and β. Activation of ERα and β has been linked with some, but not all, anti-atherosclerotic effects of estrogen [11–13]. 2-methoxyestradiol (2ME), a metabolite of estradiol with no affinity for estrogen receptors, is a potent inhibitor of cell proliferation, tumor growth, and angiogenesis, and might, at least in part, mediate anti-atherosclerotic effects of estrogen [14–17].

Received: August 27, 2007
Accepted: September 4, 2007
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Abbreviations: E_2: 17β-estradiol, ER: estrogen receptor, FFA: free fatty acid, 2-ME: 2-methoxyestradiol, NEMOes: New *enface* method for optimal observation of endothelial surface, TC: total cholesterol, TG: triglyceride.

Atherosclerosis is a complex disease associated with functional changes in the vascular endothelial layer. An increasing body of evidence points to a critical role for monocyte-endothelial cell interactions in atherosclerotic plaque formation. The adhesion of circulating monocytes to the intimal endothelial cells is one of the earliest events in naturally occurring experimental animal models of atherosclerosis [18]. We recently established a new *en face* method for optimal observation of endothelial surfaces (NEMOes) to quantify adhesion of monocytes to the endothelium *in vivo*. This method allows us to image the entire endothelial surface in clear focus, and thus quantify the number of monocytes adhering to every part of the rat thoracic aorta after immunostaining for the monocyte/macrophage-specific protein, CD68 [19].

The present study examined whether 2-ME modulates monocyte adhesion to endothelial cells in ovariectomized female rat, a model of the human postmenopausal state.

Materials & Methods

Animals

The Animal Care and Use Committee of Juntendo University reviewed and approved the study protocol. Six- to seven-week-old female Sprague-Dawley rats ($n = 25$) obtained from Sankyo Laboratory Services (Tokyo, Japan) were housed in polycarbonate cages with wooden-chip mat flooring. Water was available *ad libitum* for all rats, which were fed standard CRF-1 chow (22.6% protein, 53.8% carbohydrate, 5.6% fat, 6.6% mineral and vitamin mixture, and 3.3% fiber; total: 356 kcal/100 g; Charles River Japan, Yokohama, Japan). The animal room was kept on a 12-hour light/dark cycle (7:00 am to 7:00 pm/dark, 7:00 pm to 7:00 am/light), at constant temperature (22 ± 1°C) and relative humidity (55 ± 5%) throughout the experimental period.

Study protocol

At 8 weeks of age, rats were anesthetized with pentobarbital and then subjected to a sham operation or bilateral ovariectomy. The animals were then implanted subcutaneously with small pellets (Innovative Research of America, Sarasota, FL) that released E_2 or 2-ME gradually over 6 weeks. The animals were divided randomly into five groups of 5 rats each: sham-operated; ovariectomized and supplied with placebo; ovariectomized and supplied with E_2 (5 μg/day); ovariectomized and supplied with low-dose 2-ME (10 μg/day); and, ovariectomized and supplied with high-dose 2-ME (100 μg/day).

Oral glucose tolerance test (OGTT)

Rats in all groups underwent an OGTT at the age of 14 weeks. Briefly, following fasting for 12 hours, 1 g/kg of glucose was administered by oral gavage. Blood samples were taken from tail veins at 0, 30, 60, and 120 minutes for measurement of blood glucose.

Blood pressure measurement and laboratory data

Blood pressure was measured by the tail-cuff method (BP-98A; Softron, Tokyo). Blood samples were taken from the tail veins at 14 weeks of age 17 h after fasting. Total cholesterol (TC), HDL cholesterol, triglycerides (TG), and free fatty acid (FFA) estimations were outsourced to a private laboratory (SRL, Tachikawa, Japan). The plasma glucose level was measured by the glucose oxidase method (Glutest sensor; Sanwa Kagaku, Nagoya, Japan).

NEMOes

Monocyte adhesion to the wall of the thoracic aorta was quantitated by NEMOes, as described previously [19], in 14-week-old rats. Briefly, rats were perfused with normal saline followed by 10% buffered formalin. After fixation, the aorta was divided into segments of 8–12 mm, and incubated in 0.05% hydrogen peroxide in methanol for 20 min at room temperature. The segments were incubated with mouse anti-rat CD68 antibody (Serotec, Raleigh, NC), diluted 1:100 in PBS for 60 min at 37°C, followed by biotinylated anti-mouse IgG for 30 min at room temperature, and finally with horseradish peroxidase-conjugated streptavidin using an LSAB2 kit (Dako, Glostrup, Denmark). Immunoreactivity was visualized by incubation with a substrate-chromogen solution. The segments were then cut open longitudinally along the ventral side with scissors. Specimens were placed on glass slides with the intimal side facing up, and a coverslip applied by surface tension. Specimens were viewed under a

Table 1. Effect of ovariectomy, 17β-estradiol and 2-methoxyestradiol on body weight, serum parameters and mean arterial blood pressure.

	Sham	P	E	ME low	ME high
Body weight (g)	250 ± 5	299 ± 5[a]	241 ± 2[b]	301 ± 6[a,c]	278 ± 9[a,b,c,d]
Total cholesterol (mg/dl)	102 ± 7	101 ± 6	165 ± 7[b]	100 ± 5	129 ± 27
HDL cholesterol (mg/dl)	29.8 ± 1.0	29.4 ± 1.4	45.2 ± 1.3[a,b]	32.2 ± 1.4[c]	35.4 ± 4.3[c]
Triglyceride (mmol/l)	21.8 ± 2.1	23.9 ± 3.7	83.1 ± 7.4[a,b]	42.0 ± 7.3	49.6 ± 14.6
Free fatty acid (μEq/l)	551 ± 31	609 ± 31	762 ± 97[a,b]	638 ± 77	596 ± 35
Blood sugar (mg/dl)	64 ± 3	68 ± 1	61 ± 5	59 ± 6	73 ± 5
AUC of blood sugar during OGTT (× 100 mg/dl·min)	152 ± 17	159 ± 20	137 ± 41	121 ± 5	161 ± 24
Mean blood pressure	NA	95.3 ± 4.3	102.3 ± 6.7	107.0 ± 9.0	97.3 ± 12.0

Sham, sham operated group; P, placebo-treated group; E, E_2-treated group; ME low, mice treated with 10 μg/day 2-ME; ME high, mice treated with 100 μg/day 2-ME. Data are expressed as mean ± SEM (n = 5). [a]p<0.05% compared with Sham group; [b]p<0.05% compared with P group; [c]p<0.05% compared with E group; [d].
AUC of BS during OGTT; Area under the curve of blood sugar during oral glucose tolerance test; BP, blood pressure.

microscope (E800; Nikon, Tokyo) connected to an XYZ controller and a digital camera (Media Cybernetics Inc, Silver Spring, MD). Pictures were captured at various focal lengths with an automatically regulated Z-stepper and the clearest images were selected automatically to produce a composite image of the whole thoracic aorta by Image-Pro4.5J (Planetron Co, Tokyo). For precise counting of adherent monocytes, we counted separately the number of CD68-immunopositive cells around the intercostal-artery opening in each aorta (1400 μm × 1000 μm). The cell density in each area was calculated as the cell count divided by the total area by examiners blinded to the treatment regimen.

Statistical analysis

All data were expressed as mean ± SEM. All statistical analyses were performed with SPSS Version 11 (SPSS Inc, Chicago, IL). One-way ANOVA and Post-Hoc tests were used to compare groups. A *P* value less than 0.05 was considered significant.

Results

Effect of 2-ME on body weight, serum parameters, and blood pressure

Six weeks after each treatment, we measured body weight, fasting serum parameters, and blood pressure. As shown in Table 1, ovariectomy caused significant body weight gain without affecting other parameters. E_2 treatment inhibited the weight gain, as well as increased lipid parameters compared to control rats. In contrast, low-dose 2-ME treatment had no effect, and high-dose 2-ME treatment significantly reduced only the weight gain induced by ovariectomy without affecting other parameters.

2-ME reduces monocyte adhesion to endothelial cells

We counted the number of monocytes attached to the aortic endothelium after immunohistochemical staining with anti-rat CD68 antibody. Compared with the sham-operated group, the mean densities of monocytes attached to the endothelium were significantly increased in the ovariectomized rats treated with placebo (Fig. 1). However, the increased adhesion of monocytes following ovariectomy was suppressed by treatment with E_2. Low- and high-dose treatments with 2-ME also reduced monocyte adhesion to endothelial cells to a level comparable with the sham-operated rats.

Discussion

E_2 exerts anti-atherosclerotic effects in ERα-knockout mice [20]. In addition, E_2 is fully protective against atherosclerosis in the absence of ERβ [21]. Thus, it is possible that the anti-atherosclerotic effect of E_2 in ERα-knockout mice is mediated by E_2 metabolite, 2-ME. Previous studies demonstrated that 2-

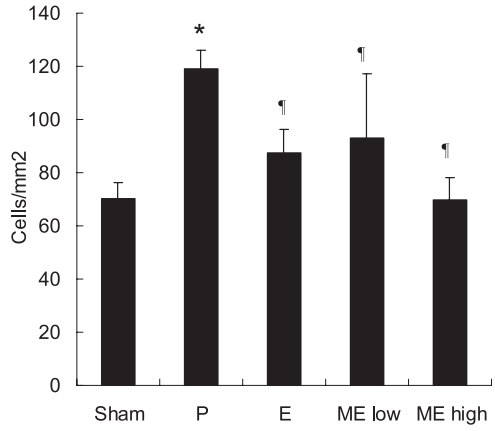

Fig. 1. Monocyte adhesion to the endothelium assessed by NEMOes. (a) Density of adherent CD68-positive cells on endothelial cells in each group. Data are mean ± SEM. **P*<0.05 compared with sham-operated group, ¶*P*<0.05, compared with placebo-treated group. Sham, sham-operated group; P, placebo-treated group; E, E_2-treated group; ME low, 10 μg/day 2-ME-treated group; ME high, 100 μg/day 2-ME-treated group.

ME reduces atherosclerotic lesion formation in female ApoE-deficient mice [17]. In this context, 2-ME inhibited neointima formation by inhibiting smooth-muscle cell growth [14], and exerted cardiovascular-protective effects in a model of severe cardiovascular and renal injury [15, 16]. In addition, 2-ME reduced oxidative stress [14, 22] in smooth muscle cells, and increased prostacyclin in endothelial cells [23]. Our study showed for the first time that 2-ME reduces monocyte adhesion to endothelial cells *in vivo* without affecting body weight, serum parameters, and blood pressure. Certainly, our data adds new insight into the mechanism underlying the anti-atherosclerotic effect of 2-ME.

In this study, we investigated the effect of several treatments on body weight, serum markers, and blood pressure in ovariectomized and control rats. Our data confirmed previous observations that estrogen deficiency significantly increases the weight gain associated with ovary removal, and that the effect can be ameliorated by treatment with E_2 [24]. In contrast to the previous data, our E_2 treatments increased TC, HDL-cholesterol, TG, and FFA levels in serum. We do not know the exact reason of these discrepant findings. Since the different method and/or timing of administration of estrogen results in the different effects, these discrepancy might be derived from different study condition [25]. However, low-dose 2-ME treatment did not affect body weight, serum lipid parameters, or blood pressure, and high-dose 2-ME modestly reduced body-weight gain only. Both doses of 2-ME suppressed the increased number of monocytes adhering to endothelial cells following ovariectomy. These results suggest that 2-ME inhibits monocyte adhesion to the endothelium via a direct action on one or both cell types or via an unknown serum factor. Further studies are therefore needed to clarify this mechanism.

Taken together with recent studies, our results support the notion that 2-ME might be beneficial for the progression of atherosclerosis through multiple mechanisms. 2-ME is currently undergoing evaluation in Phase II clinical trials for cancer, and evaluation of its clinical efficacy on cardiovascular disease or atherosclerosis is of great interest.

Acknowledgments

We thank Mrs. Naoko Daimaru, Eriko Magoshi and Shouzou Ichinose for the excellent technical assistance.

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