Effects of Oral Estrogen on Aortic ROS-Generating and -Scavenging Enzymes and Atherosclerosis in apoE-Deficient Mice

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The effect of hormone replacement therapy (HRT) on cardiovascular diseases remains controversial. Studies conducted on postmenopausal women indicate that oral HRT increases risk factors that may counteract the atheroprotective effect of estrogen. However, the effects of estrogen on atherosclerosis have been examined using subcutaneous estrogen in most animal studies, which points to the need for evaluating the effect of oral estrogen. Reactive oxygen species (ROS) have emerged as critical factors in the pathogenesis of atherosclerosis. This study examined the effect of long-term oral estrogen treatment on aortic oxidative stress and atherosclerosis in female apoE$^{-/-}$ mice to mimic HRT in humans. Ovariectomized apoE$^{-/-}$ mice were given 6 μg/day of oral 17β-estradiol (E_2) or control vehicle for 12 weeks. Estrogen treatment reduced atherosclerotic lesions by 38% (E_2: 0.20 ± 0.01 mm^2/section; control vehicle: 0.32 ± 0.02 mm^2/section) and intima by 32% (E_2: 0.44 ± 0.02 mm^2/section; control vehicle: 0.65 ± 0.04 mm^2/section) in the aortic root. Serum levels of total and low-density lipoprotein cholesterol were significantly decreased after estrogen treatment. Aortic superoxide anion levels and the expression of NAD(P)H oxidase subunit p22^{phox} markedly decreased, and two ROS scavenging enzymes, Cu/ZnSOD and MnSOD, were upregulated after estrogen treatment. Estrogen at physiological concentration inhibited tumor necrosis factor-α-stimulated NAD(P)H oxidase activity in both cultured smooth muscle cells and peritoneal macrophages. These results showed that long-term oral estrogen treatment reduces ROS levels and atherosclerosis progression in apoE$^{-/-}$ mice. Oral estrogen alters ROS-generating and -scavenging enzyme expression, suggesting that anti-oxidative actions in the vessel wall contribute to atheroprotective effects of estrogen. Exp Biol Med 234:1037–1046, 2009

Key words: oral estrogen; atherosclerosis; reactive oxygen species; NAD(P)H oxidase; ROS scavenging enzymes

Introduction

Atherosclerosis is a multifactorial disorder that underlies cardiovascular diseases, including coronary heart disease (CHD) and stroke (1). Overwhelming evidence has indicated that atherosclerosis is characterized by chronic inflammation involving interaction between infiltrating leukocytes and resident cells of the vascular wall. Emerging evidence indicates that oxidative stress is critical in the pathogenesis of atherosclerosis. The overproduction as well as the inadequate removal of reactive oxygen species (ROS) may result in oxidative stress. The electron transport chain of mitochondria, xanthine oxidase, and NAD(P)H oxidases generate ROS, and superoxide dismutases (SOD), catalase, and glutathione peroxidases remove ROS. NAD(P)H oxidases, a primary source of ROS in the vascular wall (2), were upregulated both in human atheroma (3) and in experimental atherosclerosis models (4). Overexpressed NAD(P)H oxidase subunit p22^{phox} increased neointima formation in a carotid ligation model, and a deficiency in NAD(P)H oxidase subunit p47^{phox} decreased atherosclerotic plaques in apoE$^{-/-}$ mice (5, 6). On the other hand, overexpressed MnSOD as well as co-overexpressed CuZnSOD and catalase both inhibited atherosclerosis formation (7, 8).

Cardiovascular diseases occur at a much lower rate in women of reproductive age compared with age-matched

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men, yet are the leading cause of death in postmenopausal women (9). Women undergoing early menopause also face higher risks of cardiovascular mortality (10). These data indicate that female sex hormones are vasoprotective, a notion supported by various animal studies (11–13). Recent studies on long-term hormone replacement therapy (HRT) in postmenopausal women have, however, obtained controversial results (14). The route of estrogen administration is likely a critical factor that contributes to the varied effects of estrogen on cardiovascular diseases. Accumulating evidence indicates that orally and transdermally administered estrogens have different effects on the risk factors for cardiovascular diseases in postmenopausal women. Oral estrogen has a significant effect on the serum lipid profile, but transdermal estrogen has a statistically and clinically non-significant effect. Furthermore, these two routes have opposite effects on triglyceride levels (15). Oral estrogen, but not transdermal estrogen, increased the risk of venous thromboembolism (16, 17), the concentration of circulating proinflammatory molecule C-reactive protein (CRP) (18, 19), and angiotensinogen (20). In addition, transdermal E_2, but not oral conjugated equine estrogen (CEE), decreased arterial stiffness (19). All of these differential results point to the need for evaluating the role of oral estrogen in the pathogenesis of cardiovascular diseases.

The vasoprotective effects of estrogen may in part be attributed to its modulation of ROS-generating and ROS-scavenging enzymes. In ovariectomized mice, subcutaneous estrogen treatment reduced NAD(P)H oxidase subunit expression and activity (21) and raised SOD expression in the aorta (22). While these findings suggest that the ROS-regulating system is involved in the vasoprotective effect of subcutaneous estrogen, whether oral estrogen acts in a similar manner remains unclear. Apolipoprotein E-deficient ($apoE^{-/-}$) mice spontaneously develop atherosclerotic lesions that cover the whole spectrum of human lesions, including fatty streaks, intermediate lesions, fibrous plaques, and vulnerable plaques exhibiting necrotic core and intraplaque hemorrhage (23–25). During the past decade, the $apoE^{-/-}$ mouse has become an invaluable model for studying the pathogenesis of atherosclerosis (26). However, the effects of estrogen on cardiovascular diseases have been examined by subcutaneous treatment in most animal models, including in the $apoE^{-/-}$ mouse (27). Furthermore, there are no published reports on the effects of oral estrogen on oxidative stress and enzymes that regulate oxidative stress in diseased blood vessels. Therefore, we examined the effects of estrogen treatment on the progression of atherosclerosis and on ROS-regulating enzymes as the underlying mechanisms in ovariectomized $apoE^{-/-}$ mice treated with oral estrogen to mimic HRT.

Materials and Methods

Materials. Rabbit polyclonal (pRb) antibodies for $p22^{phox}$ and Nox 1, goat polyclonal antibody for Nox 4, and mouse monoclonal antibody for $p47^{phox}$ were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); pRb antibodies for SOD isoforms were purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada); and pRb antibody for catalase was purchased from Chemicon International, Inc. (Temecula, CA, USA). Mouse monoclonal antibody for β-actin and HRP-conjugated rabbit anti-goat IgG were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA); HRP-conjugated goat anti-rabbit IgG was purchased from Chemicon; and HRP-conjugated horse anti-mouse IgG was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA).

Animals and Treatment. This study was approved by the institutional animal care and use committee and conformed to the procedures described in the *Guide for the Care and Use of Laboratory Animals* of the National Institute of Health (USA). Female $apoE^{-/-}$ mice provided by National Cheng Kung University Animal Center were ovariectomized at eleven weeks of age. One week later, the mice were fed an estrogen-free diet containing 0.15% cholesterol (TestDiet; Purina Mills, Richmond, IN, USA) and orally administered 17β-estradiol (E_2) (6 μg/day) or 5% ethanol (control vehicle) for 12 weeks. The E_2 dose was based on a previous study (28) that compared drug absorption in six mammalian species. Each treatment group contained ten or sixteen mice from two separate experiments.

Measuring Atherosclerotic Lesion Size. Mice were placed in a CO_2-filled chamber and intraperitoneally (i.p.) anesthetized with sodium pentobarbital (140 mg/kg). The aortic root with the ascending aorta was immersion-fixed in 4% buffered paraformaldehyde, embedded in O.C.T. compound, frozen in liquid nitrogen and stored at –80°C. Ten-micrometer thick sections of the aortic root were stained with oil red-O (3 mg/ml) and hematoxylin-and-eosin. The lesion and intimal areas were quantified using specially designed computer software (Yen-Ting Chen and Chih-Chao Liu, Institute of Electrical Engineering, Southern Taiwan University of Technology, Tainan, Taiwan). Ten sections taken 900 μm apart were stained per aorta and six aortas per treatment group were randomly picked for the analysis.

Analyzing Serum Lipid Profiles. Levels of total cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol in the sera of mice fasted overnight were determined by the Biochemical Division, Pathology Department, National Cheng Kung University Hospital.

Detecting Superoxide Production. Unfixed frozen sections of ascending aorta (20 μm thick) plus 2 μM of dihydroethidium (DHE) in modified Krebs' solution containing 20 mM of HEPES were incubated in a dark chamber for 30 min at 37°C. Ethidium produced from the DHE and superoxide reaction was detected using fluorescence microscopy (DMIRE2; Leica Mikrosysteme Vertrieb GmbH, Bensheim, Germany).

Immunoblotting Analysis. Frozen thoracic aortas were placed in a dry ice-acetone slurry containing 10% trichloroacetic acid (TCA) and 10 mmol/L dithiothreitol (DTT); they were then thawed and washed with acetone containing 10 mmol/L DTT to remove the TCA. Aortas were homogenized with homogenization buffer containing 20 mmol/L morpholinopropanesulfonate (MOPS), 2% SDS, 10% glycerol, 1 μmol/L aprotinin, 1 μmol/L leupeptin, and 1 mmol/L PMSF. The protein content in tissue lysate was analyzed using a kit (BCA protein assay kit; Pierce Biotechnology Inc., Rockford, IL). Aortic homogenate was separated with SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with Tris-buffered saline (TBS) containing 3% non-fat dry milk and then incubated overnight with anti-$p22^{phox}$, anti-$p47^{phox}$, anti-Nox 1, anti-Nox 4 (1:1000 each), anti-Cu/Zn SOD (1:5000), anti-Mn SOD (1:5000), anti-ecSOD (1:1000) or anti-catalase (1:5000), followed by incubation with HRP-conjugated secondary antibody (1:5000). Immunoreactive bands were detected with a chemiluminescence (ECL) kit (PerkinElmer, Fremont, CA, USA) and quantified with densitometry. To normalize the variation in protein loading, the membranes were stripped with stripping buffer (62.5 mmol/L Tris, 10 mmol/L DTT, 2% SDS [pH 6.7]) for 30 min at 50°C, and then reprobed with anti-β-actin (1:5000) as the internal control.

NADPH Oxidase Activity Assay. Vascular smooth muscle cells (VSMC) derived from an explant culture of aortas of male Sprague-Dawley rats were grown as previously described (29). Rat VSMC expressed both estrogen receptor-α and -β as detected with reverse transcription-polymerase chain reaction (30, data not shown). The cells were grown to 80% confluence, serum-starved for 24 h and treated with TNF-α (5 ng/ml), E_2 (10^{-8} M), TNF-α combined with E_2 or apocynin (250 μM), or with solvent vehicle (0.01% ethanol) for 24 h. The cells were then scraped in 5 ml cold phosphate buffered saline (PBS) and centrifuged at 750 *g* for 5 min at 4°C. The cell pellet was suspended in lysis buffer (20 mM KH_2PO_4, 0.34 M sucrose, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin), mixed well, and then centrifuged at 20,000 *g* for 20 min. The membrane fraction was collected by ultra-centrifuging the supernatant at 100,000 *g* for 1 h at 4°C and then resuspended in balanced salt solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4). Chemiluminescence was measured for 10 min in a mixture containing 4–6 μg of membrane protein, 5 μM of lucigenin, and 100 μM of NADPH in 1 ml balanced salt solution using a β liquid scintillation counter (LS 6500; Beckman Coulter, Inc., Fullerton, CA, USA) set at out-of-coincidence mode.

Female C57BL/6NCrj mice were ovariectomized at 10 weeks of age. Two weeks later, mouse peritoneal macrophages were collected using peritoneal lavage. The mice were asphyxiated using CO_2, and peritoneal fluid was collected by irrigating the abdominal cavity with 1 ml of cold PBS plus 0.02% EDTA. The cells were washed with ice cold PBS and allowed to adhere to plates for 30 min in RPMI 1640 medium containing 10% FBS; nonadherent cells were removed. Macrophages were treated with TNF-α (50 ng/ml), E_2 (10^{-8} M), or TNF-α plus E_2 for 20 h with or without solvent vehicle. Macrophages (4–5 $\times$ 10^6 cells) were lysed in 0.5 ml of cold lysis buffer and centrifuged at 750 *g* for 5 min at 4°C. Membrane fractions were collected by ultra-centrifuging the supernatant at 100,000 *g*, for 1 h at 4°C; the membrane fractions were then resuspended in balanced salt solution. Chemiluminescence was measured for 10 min in a mixture containing 10 μg of membrane protein, 5 μM of lucigenin, and 100 μM of NADPH in 1 ml balanced salt solution using the β liquid scintillation counter set at out-of-coincidence mode.

Statistical Analysis. Data are expressed as means ± SEM; *n* is the number of mice. The data were analyzed using unpaired Student's *t* tests. A one-way ANOVA and then Tukey's Honestly Significant Difference test were used for multiple comparisons. Statistical significance was set at $P < 0.05$.

Results

Oral Estrogen on Atherosclerotic Lesion Formation. Lipid deposition and the cross-sectional area of the intima were used to analyze the effect of oral E_2 on atherosclerosis formation of the aortic root. Oil red-O staining of lipid deposition was more extensive in the Control group (Fig. 1A) than in the oral-estrogen-treated (E_2) group (Fig. 1B). After 12 weeks of E_2 treatment, the mean lesion area of the aortic root (0.20 ± 0.01 mm^2) decreased by 38% compared with the control (0.32 ± 0.02 mm^2), a significant ($P < 0.05$) difference (Fig. 1C). Similarly, hematoxylin-and-eosin stained intimal thickening was greater in the Control group (Fig. 1D) compared with the E_2 group (Fig. 1E). The intimal area of the E_2 group (0.44 ± 0.02 mm^2) decreased by 32%, significantly ($P < 0.05$) smaller than that of the Control group (0.65 ± 0.04 mm^2) (Fig. 1F).

Oral Estrogen on Uterine Weight and Serum Lipid Profile. Table 1 shows the effect of E_2 on uterine weight and serum lipid levels. E_2 treatment markedly increased uterine weight while body weight remained similar. In addition, serum levels of total cholesterol and LDL cholesterol were decreased whereas no difference in HDL cholesterol or triglycerides was detected.

Oral Estrogen on Superoxide Production. To determine whether E_2 modulates superoxide production in the arterial wall, DHE-derived fluorescence in aortic sections was examined. As shown in Figure 2A, high intensity of DHE-derived fluorescence was detected in medial VSMCs and cells of the aortic intima of the control group. E_2 treatment significantly decreased the fluorescent signals in both intima and media (Fig. 2B).

Oral Estrogen on Aortic Expression of

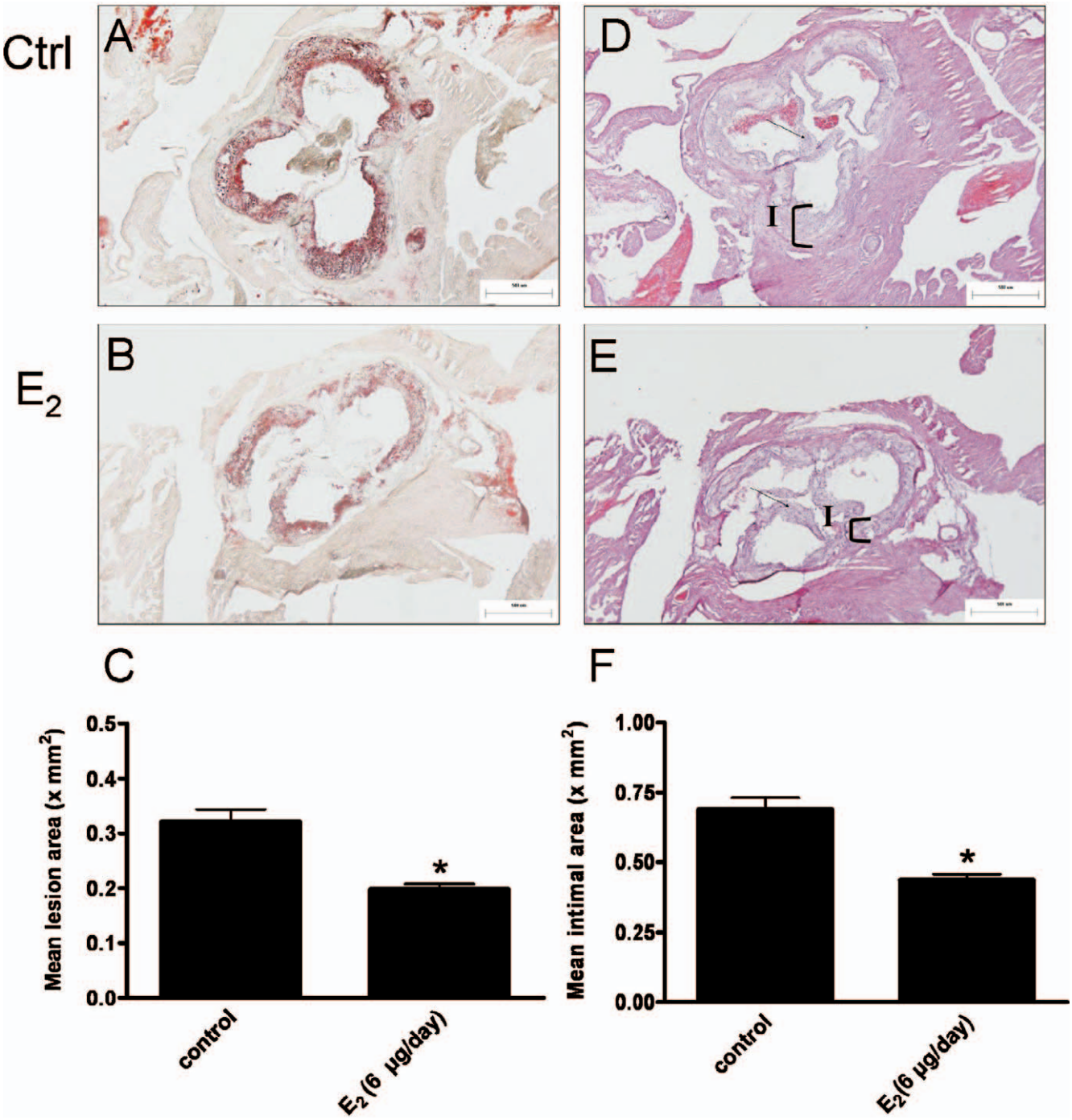

Figure 1. Estrogen treatment on lipid deposition and intimal area in the aortic root of apoE$^{-/-}$ mice. Ovariectomized apoE$^{-/-}$ mice were orally administered 6 μg/day E_2 (B, E) or vehicle control (A, D) for 12 weeks. A & B showed representative photomicrographs of oil red-O-stained sections (in red); D & E showed hematoxylin-and-eosin-stained sections. C & F summarize the lesion (C) and intimal area (F) in the aortic root quantified by computer-assisted image analysis and expressed as mean ± SEM ($n = 6$). I indicates intima; arrows indicate aortic valve. * $P < 0.05$ compared to the control. Scale bar = 500 μm. A color figure is available in the online journal.

NAD(P)H Oxidase Subunits, SODs, and Catalase. Since E_2 reduced aortic superoxide levels, which reflect the balance between ROS production and scavenging, the expression of NAD(P)H oxidase subunits, three SODs and catalase was examined following E_2 treatment. Figure 3A shows the representative immunoblots of p22^{phox}, Cu/ZnSOD, MnSOD, and catalase from aortae of each treatment group. E_2 significantly decreased aortic expression ratio of p22^{phox} (Fig. 3B, $P < 0.01$, $n = 9$ for control and $n = 10$ for E_2 group) without affecting the expression of p47^{phox}, Nox1 and Nox4 (Supplementary Fig. I, A–C). In addition, E_2 significantly increased aortic expression of Cu/ZnSOD

Table 1. Effects of Oral E_2 on Body Weight, Uterine Weight and Serum Lipids in Ovariectomized apoE-Deficient Mice[a]

Treatment groups	Body weight (g)	Uterine weight (mg)	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Control	22.3 ± 0.67 (6)	15.3 ± 0.79 (6)	584 ± 22.8 (19)	44.6 ± 2.30 (19)	5.0 ± 0.28 (19)	570 ± 22.5 (19)
E_2 (6 μg/d)	23.6 ± 0.49 (6)	61.5 ± 2.95* (6)	520 ± 18.7* (18)	39.6 ± 2.78 (18)	5.6 ± 0.30 (18)	507 ± 18.8* (18)

[a] Number in parentheses indicates sample size. Serum lipids were analyzed with samples from both experiments.
* $P < 0.05$ vs. control.

(Fig. 3C, $P < 0.05$, $n = 6$) and MnSOD (Fig. 3D, $P < 0.05$, $n = 14$) with the trend to upregulate catalase (Fig. 3E, $P = 0.071$, $n = 10$). By contrast, no difference in ecSOD expression was detected between the E_2-treated and control groups (Supplementary Fig. ID). In order to know whether oral E_2 treatment also modulated unitary enzymatic activity of ROS-scavenging enzymes, we examined the effect of E_2 on aortic Cu/ZnSOD and MnSOD activities. E_2 did not increase Cu/ZnSOD unit activity compared to the control group (1.13 ± 0.09 U/μg protein for E_2-treated; 1.31 ± 0.10

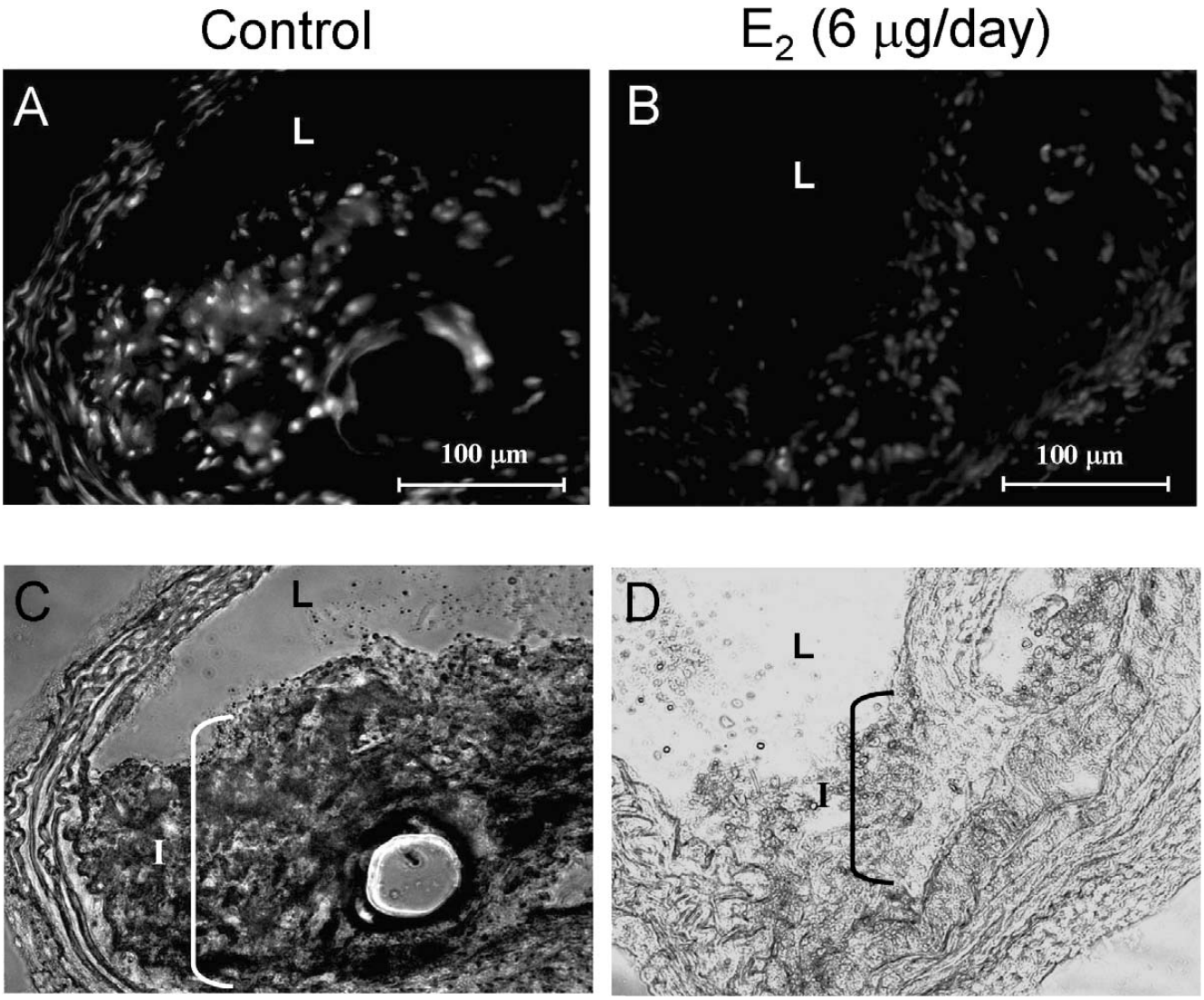

Figure 2. Estrogen treatment on aortic superoxide production in apoE$^{-/-}$ mice. Unfixed cryosections of ascending aorta from mice treated with E_2 (B, D) or vehicle control (A, C) were incubated with dihydroethidium (2 μM) and fluorescence detected at 585 nm. A & B showed representative fluorescent images detected in three animals, whereas C & D showed their corresponding phase contrast images. L indicates lumen, I indicates intima. Scale bar = 100 μm.

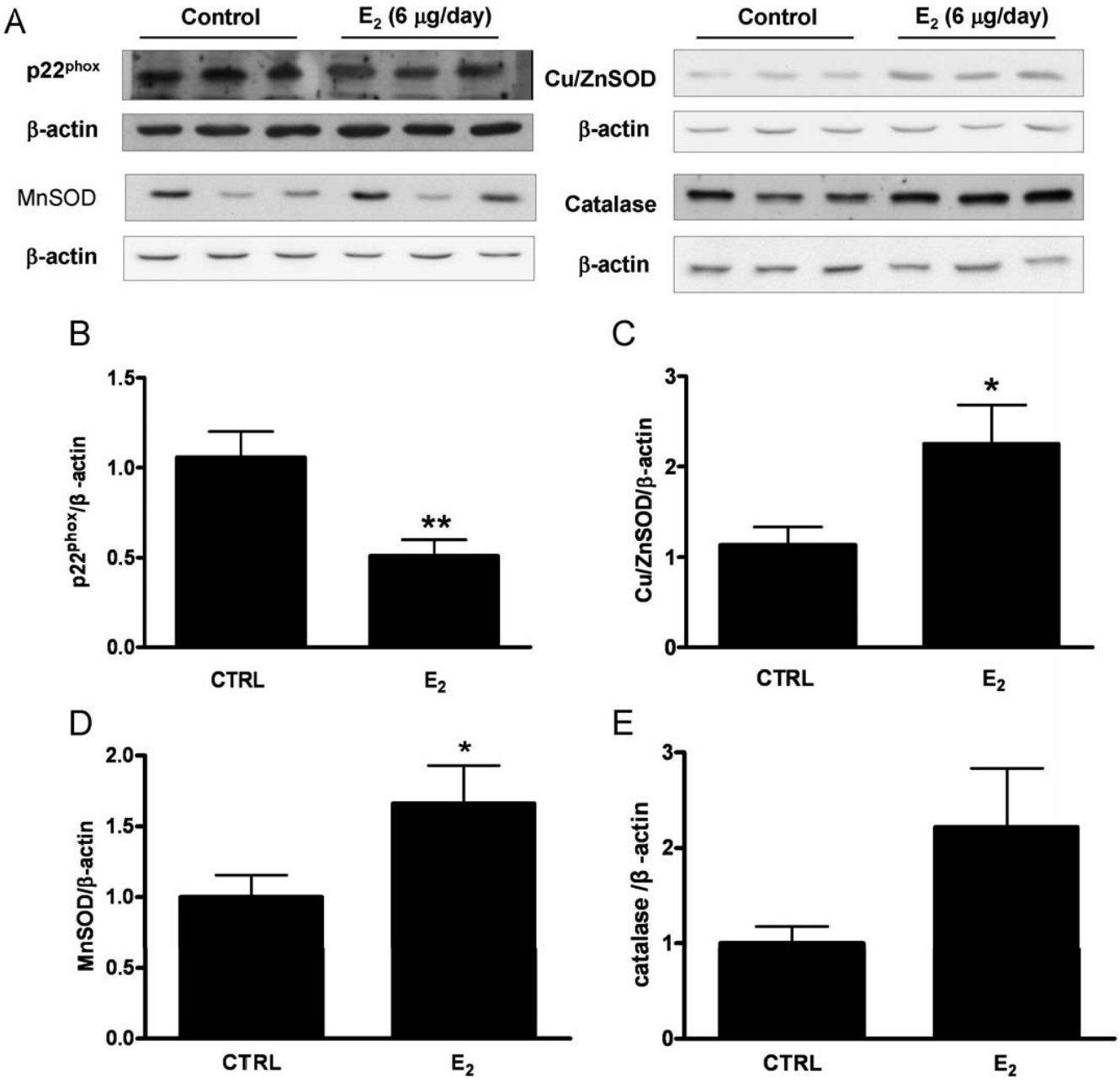

Figure 3. Estrogen treatment on aortic expression of NAD(P)H oxidase subunit $p22^{phox}$, Cu/ZnSOD, MnSOD, and catalase in $apoE^{-/-}$ mice. Aortic homogenate (30 μg for $p22^{phox}$; 10 μg for SODs and catalase) from the E_2-treated and control groups was analyzed by immunoblotting. A shows representative results from three aortae. B–E summarize the quantitative results of $p22^{phox}$ (B), Cu/ZnSOD (C), MnSOD (D), and catalase (E). Enzyme expression levels were normalized with β-actin as loading control. Values are mean ± SEM of 6 to 14 animals. Large variation was detected in the expression of MnSOD ($n = 14$). * $P < 0.05$ compared to the control; ** $P < 0.01$ compared to the control.

U/μg protein for control, $P > 0.05$, $n = 6$) whereas MnSOD activity was below the detection limit under our experimental conditions.

Estrogen on NAD(P)H Oxidase Activity in Cultured VSMC and Macrophages. To examine whether estrogen directly acts on cells that are present in atherosclerotic lesions, the effect of estrogen on NAD(P)H oxidase activity was examined in primary culture of VSMC and macrophages. Rat aortic VSMC and mouse peritoneal macrophages were used in order to obtain enough cells. As shown in Figure 4A, 24-h TNF-α treatment stimulated NAD(P)H oxidase activity in rat aortic VSMC approximately 3-fold compared to the untreated control (7705 ± 161.3 cpm/μg protein and 20742 ± 2170 cpm/μg protein for control and TNF-α, respectively, $n = 5$, $P < 0.001$). The cotreatment of E_2 (10^{-8} M) inhibited 70% of TNF-α-stimulated activity (11594 ± 757.1 cpm/μg protein, $n = 3$, $P < 0.01$ vs. TNF-α alone), the efficacy being compatible with that of 250 μM apocynin, an inhibitor for NAD(P)H oxidase activation (11218 ± 1189 cpm/μg protein, $n = 3$, P

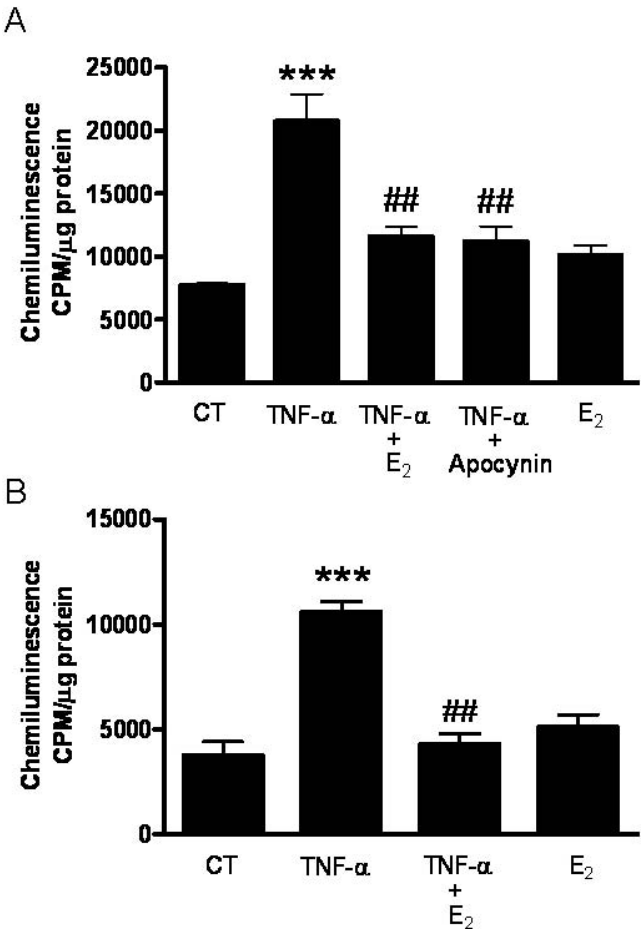

Figure 4. Estrogen treatment on NAD(P)H oxidase activity in cultured VSMC (A) and peritoneal macrophages (B). Rat aortic VSMC were treated with E_2, TNF-α, TNF-α combined with E_2 or apocynin for 24 h. Mouse peritoneal macrophages were treated with TNF-α, E_2, or E_2 plus TNF-α for 20 h in the presence of solvent vehicle. NAD(P)H oxidase activity was measured with membrane protein (VSMC, 4–5 μg; macrophages, 10 μg). Values are mean ± SEM (VSMC, $n = 5$; macrophage, $n = 3$). *** $P < 0.001$ compared to the control; ## $P < 0.01$ compared to TNF-α alone.

< 0.01 vs. TNF-α alone). By contrast, E_2 alone and vehicle (data not shown) had no significant effect.

As illustrated in Figure 4B, estrogen treatment resulted in similar inhibition of TNF-α-activated NAD(P)H oxidase activity in mouse peritoneal macrophages. TNF-α stimulated NAD(P)H oxidase activity nearly 3-fold compared to the vehicle control (3764 ± 643.4 cpm/μg protein and 10600 ± 480.8 cpm/μg protein for control and TNF-α treatment, respectively, ($n = 3$, $P < 0.001$). The E_2 cotreatment inhibited over 90% of TNF-α stimulation (4296 ± 495.7 cpm/μg protein, $n = 3$, $P < 0.001$ vs. TNF-α alone) whereas E_2 alone had no significant effect (5101 ± 583.4 cpm/μg protein, $n = 3$).

Discussion

Results of this study clearly showed that long-term oral administration of estrogen reduced atherosclerosis formation and intimal thickening in the aortic root of ovariectomized female apoE$^{-/-}$ mice. Oral E_2 reduced atherosclerotic lesions with concomitant decreases in aortic ROS production by both medial VSMC and intimal cells. In accordance with this effect, the expression of NAD(P)H oxidase subunit p22^{phox} was also markedly inhibited by oral E_2 treatment. Similar results were obtained in a previous study in which E_2 given by daily subcutaneous injection reduced aortic atherosclerotic lesion and NAD(P)H oxidase activity in apoE$^{-/-}$ mice (21). The subcutaneous E_2 also reduced aortic ROS production with a concomitant decrease in p22^{phox} mRNA in Dahl salt-sensitive hypertensive rats (31). P22^{phox} forms complex with various Nox members including Nox1, Nox2 and Nox4 and is required for NAD(P)H oxidase activity (32). Therefore, estrogen-induced decreases in p22^{phox} should decrease NAD(P)H oxidase activity and hence reduce ROS production. Our result that oral E_2 significantly decreased aortic superoxide anion production fully supports this notion.

Estrogen at physiological concentration inhibited NAD(P)H oxidase activity in both VSMC and macrophages stimulated by TNF-α, suggesting that E_2 can directly act on component cells of atherosclerotic vessels. Previous studies also showed that estrogen inhibited cytokines-stimulated NAD(P)H oxidase activity/expression in endothelial cells, VSMC, and macrophages (33–35). In VSMC, TNF-α activates NAD(P)H oxidase and upregulates the expression of various NAD(P)H oxidase subunits (36, 37). In THP-1 macrophages, co-stimulation of TNF-α and interferon-γ upregulates p47^{phox} expression mediated by NF-κB and is inhibited by E_2 (34). In our experiments measuring NAD(P)H oxidase activity, VSMC and macrophages were treated with TNF-α and inhibitors for 24 h, it is highly possible that E_2 inhibited TNF-α-stimulated NAD(P)H oxidase activity via inhibiting subunit up-regulation stimulated by the cytokine. It is also noteworthy that apocynin was recently shown to be an antioxidant that does not inhibit vascular NAD(P)H oxidases but is effective in inhibiting redox-sensitive kinases (38). Thus, it is likely that the inhibition of apocynin on TNF-α-stimulated NAD(P)H oxidase activity was an indirect effect. Given that oral and subcutaneous estrogen treatments exhibit differential effects on serum lipids and other risk factors, their consistent effect on inhibiting NAD(P)H oxidase activity and ROS production by component cells of atherosclerotic lesions is likely to present a critical mechanism for antiatherosclerotic effect of estrogen.

Oral estrogen-induced ROS decrease in the aorta of apoE$^{-/-}$ mice was accompanied by the upregulation of two ROS scavenging enzymes, Cu/ZnSOD and MnSOD, but not ecSOD in the current study. The upregulation of MnSOD by E_2 was also detected in two previous studies in which subcutaneous E_2 reversed ovariectomy-induced downregulation of MnSOD both in the aorta of B6 mice (22) and in cerebral blood vessels of rats (39). Mitochondrial dysfunction plays critical roles in atherogenesis and can be rescued by overexpressing MnSOD, a mitochondrial enzyme, in

apoE$^{-/-}$ mice (7). The upregulation of MnSOD by estrogen regardless of administration route could be crucial for atheroprotective effect of estrogen. This notion is supported by recent evidence implicating decreased oxidative stress as a major component for estrogen-enhanced mitochondrial functions in various tissues including the vasculature (39). In addition, upregulation of Cu/ZnSOD and possibly catalase may further enhance this effect (8).

Clinical studies reported that the effect of hormone therapy depends on hormone formula, dosage, and the route of administration. In the United States, the daily dosage for oral CEE has been decreased from 1.25 mg to 0.625 mg, even to 0.3125 mg to circumvent potential risk on breast cancer and thrombosis (14, 40). In Europe, E_2 at 2 or 1 mg/day is often used for hormone therapy (15). Recent studies further showed that the route of hormone replacement affects the protective effect of estrogen on cardiovascular system. While oral CEE/E_2 increased plasma levels of pro-inflammatory molecules and hemostatic factors which counteract the beneficial effects of estrogen, transdermal E_2 had less effect on those factors (16–19, 40). Unlike hormone therapy in human, E_2 was administered subcutaneously in most animal studies. In a previous study, 12 weeks of subcutaneous E_2 at 6 or 12 μg/day reduced approximately 40% of lesion area in the aortic root of apoE$^{-/-}$ mice (12). Interestingly, same period of oral E_2 at 6 μg/day led to similar reduction in atherosclerosis in the present study. The E_2 dose of 6 μg/day, which was selected based on a formula using dose/body surface area instead of dose/body weight for quantitative comparison of drug absorption between human and mice (28), is approximately equivalent to the HRT dose of 1.5 mg/day E_2 in human. Using an estradiol enzyme immunoassay (EIA) kit (Cayman Chemical, MI, USA), we measured serum E_2 levels in apoE$^{-/-}$ and B6 mice with or without oral E_2 treatment. We did not obtain reliable serum E_2 levels in apoE$^{-/-}$ mice resulting from severe interferences from serum components. In B6 mice, the serum E_2 levels were 12.5 ± 4.57 pg/ml ($n = 6$) for the control and 49.6 ± 5.77 pg/ml ($n = 7$) for 6 μg/day E_2-treated group. These values were lower than those measured with the same method in a previous study reporting 23 pg/ml and 168 pg/ml in ovariectomized and intact B6 mice (41) but were comparable with values reported in cycling B6 mice (20–80 pg/ml) using radioimmunoassay (42) and fluoroimmunoassay (43). These results, though circumstantial, support the notion that oral E_2 treatment at 6 μg/day provided E_2 levels within physiological range. Our results show that long-term oral E_2 at HRT dose indeed reduces atherosclerosis progression.

Oral E_2 significantly reduced serum levels of total and LDL cholesterol without changing HDL cholesterol or triglycerides in this study. By contrast, previous studies conducted in apoE$^{-/-}$ mice with subcutaneous E_2 treatment obtained conflicting results on plasma/serum lipid profile albeit exhibiting consistent reduction in atherosclerotic lesions (27). The differences between our results and those obtained by subcutaneous E_2 treatment in apoE$^{-/-}$ mice are compatible with human data showing oral E_2 to be more effective in lipid modulation. A review on postmenopausal HRT studies published between 1974 and 2000 showed that all estrogen regimens at HRT doses lowered LDL and total cholesterol and raised HDL cholesterol and triglycerides (15). The effects of oral E_2 on serum lipids detected in this study agree only partially with those observed in human studies, probably reflecting the fact that both lipid profiles and metabolism are different between apoE$^{-/-}$ mice and humans (24, 25). Nonetheless, lower serum total/LDL cholesterol may present additional mechanism for atheroprotective effects of oral estrogen in apoE$^{-/-}$ mice.

In this and previous studies conducted in apoE$^{-/-}$ mice, both oral and subcutaneous E_2 reduced atherosclerotic lesions. These results appear to contradict with those reported in postmenopausal women showing no beneficial effects on CHD of oral HRT (44). However, in addition to hormone application route, the effects of hormone replacement on cardiovascular diseases are modulated by various risk factors, such as aging, metabolic syndrome and changed hemodynamic functions. These factors appear to interact with each other and modify the outcome of HRT (45). Previous studies showed that timing of receiving hormone replacement affects the estrogenic effect on cardiovascular system. Data from two clinical trials, Women's Health Initiative and the Nurses' Health Study, both showed that estrogen treatment initiated between 50 and 59 years reduced the risk of CHD in women while no beneficial effect on CHD risk was observed with estrogen administered at 60 years or older (44, 46). In a monkey model, estrogen treatment was effective in preventing coronary artery atherosclerosis when administered shortly after menopause induction but completely lost its beneficial effects when treatment was delayed for two years (47). In apoE$^{-/-}$ mice, estrogen treatment inhibited the initiation of fatty streaks without reducing established atherosclerotic lesions (48). In the current study, E_2 was given to apoE$^{-/-}$ mice one week after ovariectomy at a relatively young age (12 weeks) when atherosclerotic lesions were at earlier stages (23). Taken together, time point of initiating estrogen treatment presents a critical factor that should not be overlooked.

In conclusion, long-term oral administration of E_2 at HRT dose reduced atherosclerosis formation with concomitant downregulation of ROS-generating enzyme and upregulation of ROS-scavenging enzymes in the aorta of apoE$^{-/-}$ mice. These results suggest that anti-oxidative actions in the vessel wall contribute to atheroprotective effects of estrogen.

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