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Gender Difference in Cytoprotection Induced by Estrogen on Female and Male Bovine Aortic Endothelial Cells

Min-Liang Si,[1] Butheinah Al-Sharafi,[2] Chen-Ching Lai,[2]
Romesh Khardori,[2] Chawnshang Chang,[2] and Ching-Yuan Su[2]

Departments of [1]Pharmacology and Internal Medicine, Southern Illinois University School of Medicine, Springfield, IL

Before menopause, women have a lower risk of cardiovascular diseases than men. Studies attribute this gender difference to estrogenic protection in the female cardiovascular system. We have demonstrated that 17β-estradiol (E_2) protects female bovine aortic endothelial cells against oxidative injury, probably through the induction of antioxidant enzyme activities. In this study, we examined whether E_2 confers a differential protection on male and female cells. Bovine aortic endothelial cells from both genders were preconditioned for 24 h with E_2 (1 n*M* to 10 μ*M*), and their resistance to paraquat (1 m*M*, 3 h), a superoxide generator, was measured using an MTT assay. In contrast to the protection observed in female bovine aortic endothelial cells, there was no protective effect by E_2 on male bovine aortic endothelial cells at physiologic concentrations. However, E_2 at 1–10 μ*M* attenuated paraquat's toxicity in both male and female cells, probably through its direct antioxidant activity. E_2 at 1 n*M* increased in female, but not in male, cells the activities of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, which was associated with decreased levels of reactive oxygen species during subsequent paraquat exposure. This suggests that antioxidant enzyme induction plays some role in E_2-augmented oxidative resistance in female endothelial cells.

Key Words: 17β-estradiol; bovine aortic endothelial cells; cellular antioxidant defenses; heat-shock response; antioxidant enzymes.

Introduction

Cardiovascular disease is one of the leading causes of death in both men and women *(1)*. Before the age of 55, the incidence of cardiovascular disease in women is one-third that in men, but by age 75 the incidence is almost the same. Hormone replacement therapy (estrogen alone or a combination of estrogen and progestin) has been shown to reduce the risk of cardiovascular disease in postmenopausal women by up to 50%. Many theories exist regarding the beneficial effects of estrogen. The mechanism of action of estrogen may be owing to its ability to alter the plasma lipoprotein levels (increasing high-density lipoprotein and reducing low-density lipoprotein [LDL]), its protective effect on vascular wall following injury, and the preservation of endothelial functions *(1)*. In addition, estrogen at a supraphysiologic concentration has been found to exert a direct antioxidant effect on cells, such as in the clonal mouse hippocampal cell line HT22, against the toxicity of hydrogen peroxide *(2)*. However, the effectiveness of estrogen as a direct antioxidant at physiologic concentrations remains questionable. We have previously observed that 17β-estradiol (E_2) at 1–10 n*M* effectively augments the oxidative resistance in female bovine aortic endothelial cells (bAEC), probably through the induction of antioxidant enzyme (AOE) activities *(3)*. This may provide an alternative mechanism by which E_2 affords cardioprotection in females. It would be interesting to investigate whether similar oxidative protection can be actively induced by E_2 in male bAEC.

Since the estrogen level in premenopausal females (0.4–4.0 nmol/L or n*M*) is higher than in males (<180 pmol/L or p*M*), one may simply suggest that the cardioprotective effect of estrogen found in females may be owing to the higher concentration of this hormone compared with that of males. Nevertheless, studies have shown that E_2 at 10^{-11}–10^{-8} *M* inhibits the proliferation of female porcine coronary vascular smooth muscle cells but not male cells *(4)*. A similar gender difference in cellular responses to 10^{-9}–10^{-5} *M* E_2 was also observed in aortic smooth muscle cells isolated from both male and female rats *(5)*, suggesting the existence of intrinsic differences between male and female vascular cells regarding their responsiveness to E_2. In the present study, we compared the effect of E_2 at either physiologic or supraphysiologic concentrations on the endothelial oxidative resistance, and the induction of AOEs in both male and female bAEC.

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Author to whom all correspondence and reprint requests should be addressed: Ching-Yuan Su, PhD, Division of Endocrinology, Metabolism and Molecular Medicine, Department of Internal Medicine, Southern Illinois University School of Medicine, PO Box 19230, Springfield, IL 62794-9230. E-mail: csu@siumed.edu

Results

Effect of Estrogen on Female and Male bAEC Exposed to Oxidative Injury

To explore the difference between male and female cells in their response to E_2, bAEC originating from both genders were first treated with different concentrations of E_2 for 24 h and then challenged with paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride, methyl viologen). Paraquat was used because of its ability to generate superoxide anion O_2^- via intracellular metabolic conversion by living cells. Preliminary experiments showed that treatment with E_2 (1 n*M* to 10 μ*M*) did not significantly affect growth in both male and female bAEC, indicating that E_2 for 24 h has no effect on normal endothelial proliferation within the tested concentration. However, pretreatment with E_2 at concentrations equal to or higher than 100 μ*M* for 24 h significantly suppressed the growth of female and male bAEC (data not shown). The E_2 concentrations ranging from 1 n*M* to 10 μ*M* were subsequently selected for the following studies.

Interestingly, a marked augmentation of oxidative resistance as assessed by an MTT survival assay was observed in female bAEC pretreated with 1–10 n*M* E_2 for 24 h and then challenged with paraquat. The magnitude of this protection gradually decreased as the concentrations of E_2 were elevated to 0.1–10 μ*M* ($p < 0.05$ vs 1 n*M*, analysis of variance ANOVA and Tukey test; Fig. 1A). By contrast, no protection against paraquat challenge was observed in male bAEC pretreated with E_2 at any of the tested concentrations (p = not significant, ANOVA; Fig. 1A). These results were confirmed by cell count after the challenge (Fig. 1C). It appears that E_2 at physiologic concentrations induces or activates in the female, but not in the male, bAEC intrinsic protective mechanisms capable of defending against the intracellularly formed superoxide anion.

Direct Antioxidant Activity of E_2

Previous studies have indicated a direct free-radical scavenging activity of E_2 *(6,7)*. We postulated that this chemical protection should be concentration dependent and is readily available to both male and female bAEC, if E_2 is present during the oxidant challenge. To differentiate between such a chemical protection and the cell-mediated antioxidative activities, E_2 was added directly to both male and female cells during the 3-h paraquat exposure. As predicted, the presence of E_2 at the elevated concentration (≥1–10 m*M*) in the culture media provided a mild but notable oxidant protection in both male and female bAEC against paraquat killing (Fig. 1B). While the free radical scavenging by E_2 could account for these observed protections at

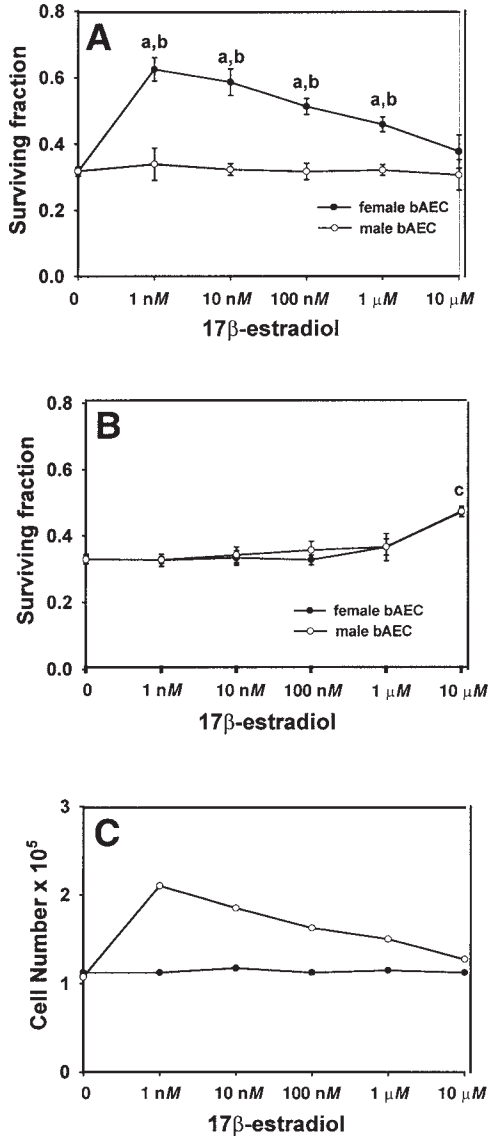

Fig. 1. (**A**) Physiologic levels of E_2 provide protection in female, but not male, bAEC against paraquat injury. Both male and female bAEC were treated with E_2 at concentrations from 1 n*M* to 10 μ*M* for 24 h. Following an extensive wash to remove E_2, the pretreated cells were challenged with paraquat at 1 m*M* for 3 h, and allowed to recover for 48 h. The relative cell survival (–○–, male; –●–, female), was measured by an MTT assay and is expressed as surviving fraction compared to cells without paraquat insult. (**B**) Direct antioxidant effect of E_2 on male and female bAEC. The cells were incubated without E_2 for 24 h. Subsequently, E_2 was added at the indicated concentrations, and the cells were challenged with 1 m*M* paraquat in the presence of E_2 for 3 h. Surviving fraction was measured using the MTT assay. Five female and four male animals were included. Cells from each animal were plated in four wells. Means ± SEM were derived from the averages of the tested wells. a: $p < 0.05$ vs male cells at the tested E_2 concentration, *t*-test; b: $p < 0.05$ vs 0 n*M* control, ANOVA, and Tukey test; c: $p < 0.05$ vs 0 n*M* control for both female and male cells, ANOVA and Tukey test. (**C**) E_2-mediated oxidative protection was confirmed by cell count. Experiment design was as mentionedin (A). After the 48-h recovery from paraquat exposure, cells in each well were harvested by trypsinization, resuspended in a small volume of medium, and counted with a hematocytometer. The averages derived from cells of two female and two male donors are shown. Cell counts among groups pretreated with various doses of E_2 without subsequent paraquat exposure were comparable, averaging 3.1×10^5 per well.

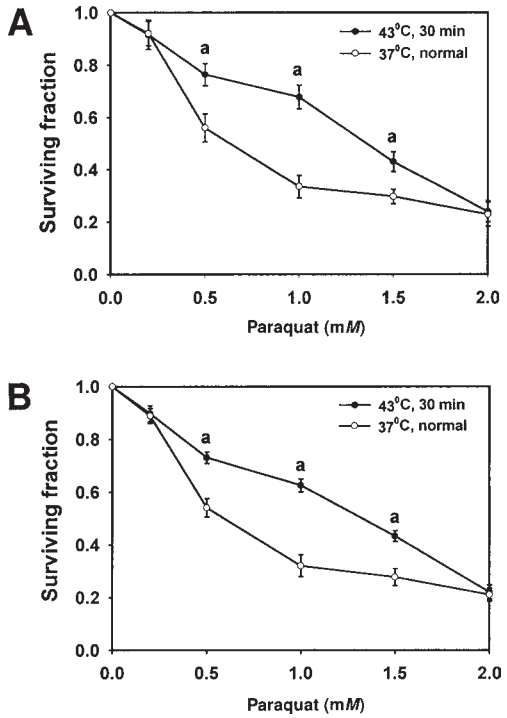

Fig. 2. Thermal preconditioning provides protection against paraquat injury in both (**A**) male and (**B**) female bAEC. Male and female bAEC were heat preconditioned at 43°C for 30 min (–●–) and then left to recover for 24 h. Control cells were grown at 37°C (–○–). The cells were then challenged with 0.2–2 m*M* paraquat, and the surviving fraction was measured after a 48-h recovery. Four female and three male animals were included. Cells from each animal were plated in four wells. Means ± SEM were derived from the averages of the tested wells. a: $p < 0.05$ vs 37°C control, *t*-test.

micromolar levels (Fig. 1B), the added E_2 could also hastily trigger some "low-sensitivity" cellular defense systems in both male and female bAEC. To eliminate the second possibility that E_2 may act through a fast, nongenomic signal pathway to enhance cellular defense, another experiment was conducted in which E_2 was added to bAEC at corresponding concentrations for only 30 min. Following the removal of E_2 by extensive washing, these short-term E_2-treated cells were similarly exposed to paraquat and assayed for their viability. No protection by E_2 was observed in both male and female endothelial cells receiving such a brief estrogenic exposure (data not shown). Taken together, our data suggest that E_2 can exert an effective free-radical scavenging activity against intracellular superoxide only at levels far exceeding the physiologic concentrations. It is thus conceivable that the observed difference between male and female bAEC in response to E_2 is owing to the induction of cellular antioxidant defense systems.

Heat Preconditioning Protects Both Male and Female Cells Against Oxidative Injury

Previous studies indicate that a universal adaptation can be induced by raised temperature *(8)*. Induction of this beneficial heat-shock response may provide protection to cardiovascular cells against oxidative insults, such as exposure to H_2O_2 or ischemic/reperfusion injury *(9–11)*. We hypothesize that this adaptive process exists in both male and female cells. To test whether male and female bAEC are equally responsive to a nonselective inducer, such as heat preconditioning, cells originating from both genders were preheated at 43°C for 30 min as previously described *(9–11)*. Following a 24-h recovery at 37°C, the preheated cells were challenged by different doses of paraquat (0.2–2 m*M*). Compared with the unheated control, the preheated male and female cells displayed a significant elevation of resistance to paraquat (Fig. 2A,B), indicating that both sexes contain similar defense systems inducible by heat shock. This finding suggests that the cultured male bAEC is still capable of adapting to environmental stresses.

Differential Induction of AOEs in Male and Female bAEC by E_2

Further experiments were conducted to explore the possible protective mechanisms that are differentially activated by physiologic concentrations of E_2 in male and female bAEC. We have previously observed *(3)* that the activities of AOEs, including superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase, are readily induced by E_2. The optimal E_2 concentration for AOE induction is about 1–10 n*M*, and the magnitude of AOE induction in female bAEC gradually decreased on increasing concentrations of E_2 *(3)*. This reverse dose response curve caused by E_2 coincides with the observed female cell survival following paraquat challenge (Fig. 1A), suggesting that AOEs play important roles in the E_2-induced oxidative protection of female bAEC. We further tested whether gender differences exist in the induction of these AOE activities by E_2. Following a 24-h 1 n*M* E_2 treatment, female bAEC showed a significant elevation of all four AOE activities tested. The SOD, catalase, glutathione peroxidase, and glutathione reductase activities were increased by ~four-, ~two-, ~three-, and ~twofold, respectively, in female bAEC (Fig. 3A–D). On the contrary, there was no induction of these AOE activities in male bAEC incubated with 1 n*M* E_2.

Reduced Levels of Reactive Oxygen Species in E_2-Pretreated Cells

SOD can dismutate the superoxide radical to hydrogen peroxide, which is subsequently detoxified by catalase or glutathione peroxidase. In theory, an induction of SOD, catalase, and glutathione peroxidase by E_2 as observed in female bAEC may enhance the catabolism of reactive oxygen species (ROS). We tested this theory by comparing the level of ROS in female and male bAEC during subsequent oxidant exposure. Cells after E_2 pretreatment for 24 h were loaded with dichlorofluorescein diacetate, which penetrates the plasma membranes and is converted to 2',7'-dichloro-

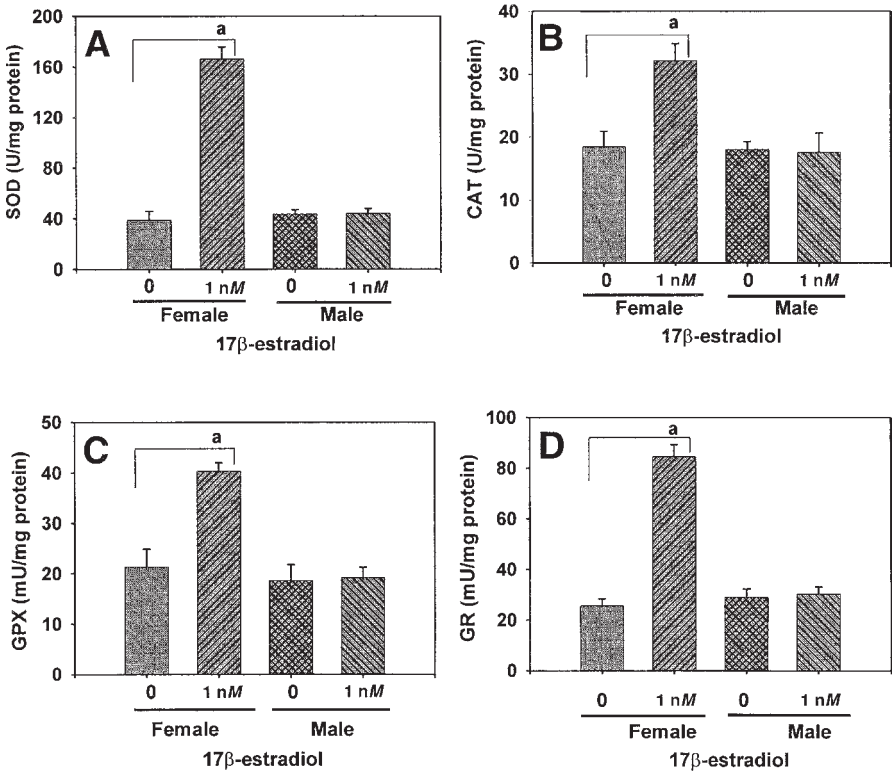

Fig. 3. Differential effects of E_2 on the induction of antioxidant enzyme activities in male and female bAEC. (**A**) SOD, (**B**) catalase, (**C**) glutathione peroxidase, and (**D**) glutathione reductase activities were measured following E_2 treatment. Both male and female bAEC were incubated with 1 n*M* E_2 at 37°C for 24 h and then harvested for the assays of different AOE activities as described in Materials and Methods. Three females and three males were included. Cells from each animal were assayed twice. The averages were used to calculate means ± SEM. a: $p < 0.05$ vs 0 n*M* control, *t*-test. GPX, glutathione peroxidase; CAT, catalase, GR, glutathione reductase.

fluorescein (DCFH) by intracellular esterases. This nonfluorescent compound, once oxidized by ROS such as hydrogen peroxide, turns to a highly fluorescent derivative. Figure 4 indicates that the basal level of DCFH oxidation in E_2-pretreated female and male bAEC was similar. On exposure to 1 m*M* of paraquat, DCFH oxidation increased. The fluorescence emitted by oxidized DCFH was significantly augmented in cells after a prolonged exposure to paraquat for 45 min. Interestingly, DCFH oxidation was lower in female (the mean fluorescence intensity was 795) than in male cells (the mean fluorescence intensity was 1323). This gender difference in DCFH oxidation was confirmed using bAEC from other donors (the mean DCFH fluorescence after a 45-min paraquat exposure was 1002 and 1581 for a separate set of female and male cells, respectively). In the absence of E_2 pretreatment, DCFH oxidation by paraquat, however, was comparable between female and male bAEC.

Discussion

The present data show that E_2 at physiologic concentrations induced AOE activities in the cultured female, but not male, bAEC (Fig. 3A–D), which was associated with a lower level of ROS in the female than in the male cells during subsequent oxidative insult (Fig. 4). The differential induction of AOE activities by E_2 between male and female bAEC correlated positively to the E_2-induced endothelial resistance to oxidative injury (Fig. 1A), suggesting that these AOEs may contribute significantly to the E_2-induced endothelial protection. This specific gender difference in the E_2-induced oxidative protection may provide some explanations for the lower risks of cardiovascular diseases in women.

On the other hand, our data also indicate that E_2 at micromolar levels could provide a direct oxidative protection in both male and female bAEC (Fig. 1B). Nevertheless, this common protection by E_2 depended on its existence at a sufficient level during the oxidant insult. Previous studies have shown that high concentrations of estrogens, such E_2, estrone, and estriol, can inhibit LDL oxidation mediated by copper ions in monocytes or endothelial cells, whereas testosterone had no such effect *(6)*. Other studies indicated that E_2 at micromolar levels inhibits oxidized LDL-induced cell injury *(7,12)*. However, the concentrations of E_2 used in these studies are several orders of magnitude higher than plasma E_2 levels (asymptotically nanomolar) found in women even during their reproductive years.

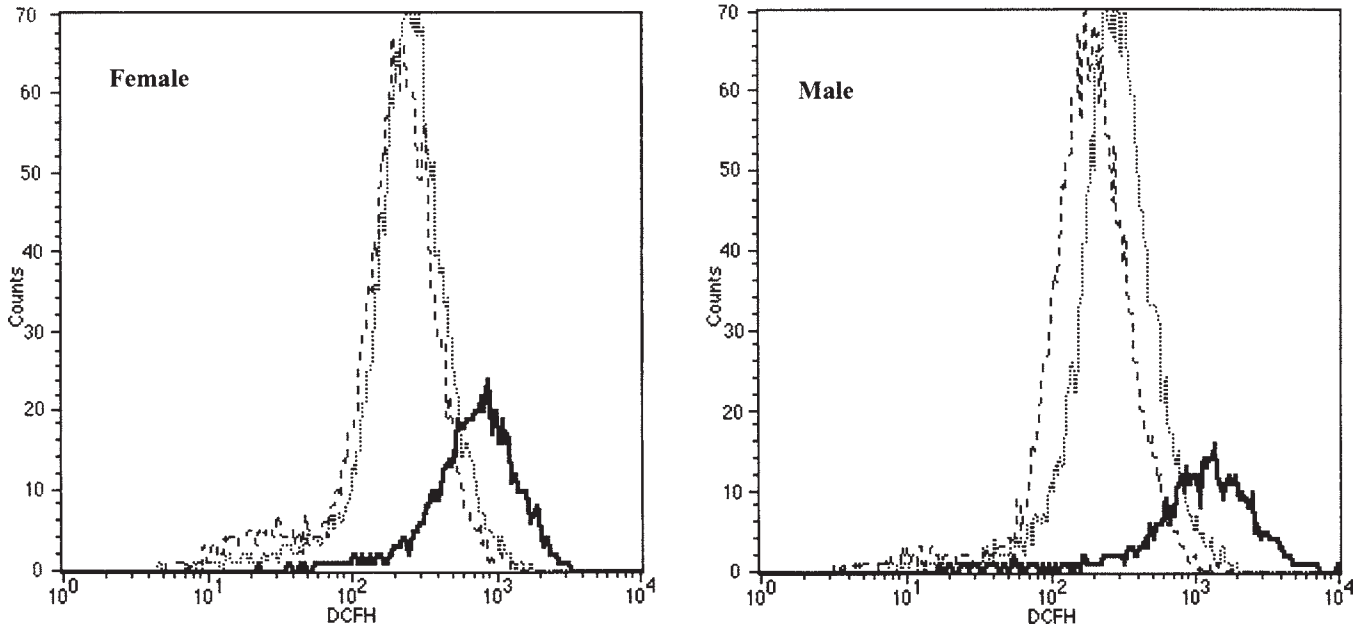

Fig. 4. Distribution of DCFH fluorescence in female and male bAEC on paraquat exposure. Cells were pretreated with 1 n*M* E_2 for 24 h and then harvested and incubated with dichlorofluorescein diacetate as described in Materials and Methods. Subsequently, the cells were exposed to 1 m*M* paraquat for 15 min (dotted line) or 45 min (solid line), and the fluorescence of oxidized DCFH was detected by flow cytometry. Compared with untreated controls (dashed line), paraquat exposure shifted DCFH fluorescence to the right.

Some studies did suggest that E_2 at nanomolar levels has an antioxidative activity both in vitro and in vivo *(13,14)*. This protection depends on the presence of plasma acyltransferase activity and may involve the incorporation of E_2 into LDL or cell membranes through esterification *(14)*. Such an accumulation of E_2 in cellular lipid compartments could have effectively increased the local concentration of E_2 to provide a direct chemical protection. One may argue that the observed oxidant protection in female bAEC following a 24-h E_2 preincubation (Fig. 1A) results from the local accumulation of E_2 through the acyltransferase activity potentially available in the serum. Nevertheless, the lack of protection in the male cells and a reverse dose-dependent protection in the female cells (Fig. 1A) argue against this acyltransferase-dependent protective mechanism. In the present study, we also examined the direct protection afforded by E_2 in the absence of serum during oxidant insults. We demonstrated in bAEC from both genders a notable endothelial protection by E_2 only at micromolar levels (Fig. 1B). It is thus conceivable that the observed selective protection in female bAEC by physiologic levels of E_2 (Fig. 1A) results from the induction of certain intrinsic defense systems, and their induction may be feedback suppressed by increasing concentrations of E_2.

Our observations that AOE induction by E_2 optimized at 1–10 n*M* and that the levels of induction decreased on the increasing concentration of E_2 *(3)* correlate well with the observed E_2-afforded oxidative protection in female bAEC (Fig. 1A), suggesting that AOEs play some roles in the E_2-mediated oxidative protection. More significantly, our data indicate that there exists a difference between male and female bAEC in their responses to the E_2-mediated oxidative protection (Fig. 1A) and the induction of AOE activities (Fig. 3A–D). Our finding is compatible with the observation that expression of glutathione reductase in response to oxidant challenge is higher in human endothelial cells derived from female umbilical veins than in those derived from male subjects, and the female cells are more resistant to the oxidant toxicity than the male cells *(15)*. Other studies have also shown that the female cardiovascular system is more tolerant to pathologic conditions, such as circulatory shock *(16)*, isoproterenol-induced myocardial necrosis *(17)*, and myocardial ischemia and infarction *(18)*. It has been shown that there is a sex difference in myointimal proliferation after vascular injury, and E_2 may modulate vascular smooth muscle cell proliferation via suppression of *c-myc* expression in response to injury *(19)*. A differential endothelium-dependent relaxation of male and female rat aortae in response to E_2 *(20)* and different levels of relaxation/constriction factors in the coronary circulation between female and male pigs *(21)* have also been reported. Recently, Kawano et al. *(22)* found that E_2 augments the flow-mediated vasodilation and level of serum nitric oxide (NO) metabolites in women but not in men, indicating that E_2 selectively improves the endothelium-dependent vasodilation in women, probably through the augmentation of NO production or release *(23)*. Our finding that E_2 mediated the induction of SOD suggests that E_2 may indirectly

facilitate the stabilization of NO through an enhanced SOD activity. This suggestion is strengthened by the detection of a reduced level of ROS in female than in male bAEC during paraquat insult in response to E_2 pretreatment (Fig. 4).

On the other hand, induction of nitric oxide synthase (NOS) activities by E_2 may also play a role in the E_2-mediated cardioprotection in female subjects *(24–29)*. Interestingly, the induction of NOS by E_2 in endothelial cells also optimizes at 1–10 n*M* and declines on increasing concentrations of E_2 *(26–28)*. The similarity on the reverse dose dependencies among the E_2-induced oxidative resistance, AOE, and NOS induction further suggests that E_2 can facilitate the preservation of female endothelial functions during oxidative injury through the induction of cellular defense systems.

The lack of cytoprotection and AOE induction by E_2 in male bAEC may be owing to a difference in the density of estrogen receptor (ER) between the male and female cells. Although ER density is generally higher in female than in male reproductive systems, contradictory data exist. It has been shown that the male mouse aortic tissue has more high-affinity cytosolic/nuclear estrogen-binding sites than the female aorta *(30)*. Nevertheless, this may not be so for bAEC owing to differences among species. We should point out that in the present study, bAEC of both genders were pregrown in steroid-free medium prior to E_2 stimulation, and ER expression may be dramatically suppressed under such conditions. Following E_2 stimulation, female bAEC might express ER more efficiently than male bAEC, thus responding to E_2 better and acquiring greater oxidative protection. A recent study suggests that methylation of ER genes could affect ER expression *(31)*. If ER genes are selectively methylated in male bAEC, ER reexpression could be less efficient in the male cells. It is also possible that the signaling pathways downstream from the ER may not be exactly the same between male and female bAEC. Under the latter circumstance, even though the density of ER is comparable, the cellular response evoked by E_2 would vary between these two genders. More studies are needed to differentiate these possibilities as well as to understand what type of ER is involved in the observed oxidative protection by E_2 in female bAEC.

In summary, our results indicated that the induction of cellular defense mechanisms, such as the AOEs, by E_2 might participate in oxidative protection of the female endothelial cells. The optimal protection of female bAEC by E_2 was within the physiologic levels, whereas E_2 conferred no survival advantage in male bAEC. While endothelial damage and dysfunction are the primary steps in atherogenesis, the differential endothelial protection between genders by physiologic levels of E_2 may have its biologic significance. Further understanding of this gender difference may facilitate the future development of preventive measurements against atherosclerosis.

Materials and Methods

Chemicals

All agents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Primary bAEC Culture

Batches of bAEC were independently established from five female and four male beef cattle (age ranged of 1 to 2 yr), respectively, based on the method described by Fenselau and Mello *(32)*. The cells were maintained under humidified air–5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). To confirm their endothelial characteristics, cells were stained with anti–factor VIII (von Willebrand factor) antibody (A0082; DAKO, Carpinteria, CA) as reported by Diglio et al. *(33)*. Only passages two to five bAEC cells were used in the following experiments to ensure their endothelial characteristics. Within these passage numbers, >95% cells were stained positively by factor VIII antibody.

E_2 Treatment and Oxidant Challenge of Female bAEC

Before E_2 treatment, bAEC were grown in phenol red–free DMEM supplemented with dextran charcoal-stripped FBS (10% final concentration) for at least one passage (3 to 4 d). The deprivation of serum steroids was conducted according to Yeh et al.'s *(34)* method. After deprivation, the E_2 level in the stripped serum was assayed using a [^{125}I] E_2 radioimmunoassay kit (TKE21; Diagnostic Products, Los Angeles, CA). The E_2 levels in the different batches of deprived sera were consistently undetectable (<0.1 n*M*; data not shown). In addition, phenol red–free DMEM was used to avoid potential estrogenic interference. After reaching confluence, the cells grown in E_2-free environment were plated into Falcon 24-well Primaria plates at a density of 5×10^4 cells/well. E_2 in dimethyl sulfoxide (DMSO) was added to each well with final concentrations from 1 n*M* to 10 μ*M* 3 h after plating. DMSO was added in a corresponding amount to the control group in a concentration of 0.05%. The cells were incubated with or without E_2 for 24 h at 37°C before receiving oxidant insult. Following treatment, the E_2-containing media was removed, and the cells were washed with phosphate-buffered saline (PBS) twice and refed with fresh medium without serum. The cells were subsequently treated with paraquat dichloride for 3 h at 37°C. The LD_{50} of paraquat in both male and female bAEC was about 0.5–1 m*M*. Two experimental groups were included in this study. In the first group, E_2 was added only during the 24-h pretreatment period. In the second group, E_2 was added only during the 3-h paraquat exposure. After paraquat exposure, both groups of cells were washed twice with PBS and allowed to recover for 48 h in fresh medium plus 10% regular FBS to exclude the possibility that different concentrations of E_2 interfere with the cellular recovery processes.

MTT Assay

Cell survival was estimated by the MTT assay *(35)*, which is based on the ability of mitochondrial dehydrogenase activity in viable cells to reduce 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (thiazolyl blue) to a blue formazan product. Briefly, cells were treated with 1/10 vol of MTT solution (5 mg/mL in PBS) and incubated at 37ºC for 2 h. The supernatant was then removed, and 600 μL of lysis buffer (50% dimethyl formamide, 5% sodium dodecyl sulfate, 0.35 *M* acetic acid, 50 m*M* HCl) was added to each well. Following cell lysis, the absorbency was measured at 570 nm and used as an indicator of relative cell survival. The surviving fraction was calculated as compared to the cells without paraquat challenge.

Heat Preconditioning of Male and Female bAEC

To determine whether intrinsic inducible defense mechanisms exist in both male and female bAEC, thermal preconditioning was applied to induce the beneficial heat-shock response *(8)*. Briefly, cells originating from both genders were heated at 43ºC for 30 min and allowed to recover at 37ºC for 24 h to induce this beneficial adaptation, which has been shown by other researchers and us to protect cultured cells against oxidative damages *(8–11)*. The control cells were maintained at 37ºC for this period. The cells were then challenged with different doses of paraquat (0.2–2 m*M*) and left to recover for 48 h before being subjected to the MTT assay.

Preparation of Cell Lysate for AOE Assays

Following E_2 treatment for 24 h, cells were harvested in a solution containing 50 m*M* Tris-HCl (pH 7. 4), 1 m*M* EDTA, 2 μg/mL of pepstatin A, 10 μg/mL of benzamidine, and 10 μg/mL of trypsin inhibitor. The cells were then lysed on ice by sonication at a setting of 50 mW, 15 s for three times using a Cole-Parmer Ultrasonic Homogenizer 4710 series (Chicago, IL). Protein concentration was determined by the Bradford *(36)* method. Bovine serum albumin (BSA) was used as a standard. The protein concentration of cell lysate was then adjusted to ~100 μg/100 μL.

SOD Activity

SOD activity was determined by a method based on the ability of SOD to suppress the autooxidation of epinephrine *(37)*. Briefly, 100 μL of 10X-diluted cell homogenate (~10 μg of protein) was added to 900 μL of carbonate buffer (0.1 *M* $NaHCO_3$, 0.1 *M* Na_2CO_3, 0.1 m*M* EDTA, pH 10.2) containing 0.7 m*M* epinephrine. The absorbency of the mixture was measured for 4 min at 480 nm using a Hitachi U-2000 spectrophotometer (Hitachi, San Jose, CA). One unit of SOD activity is defined as the amount of proteins that inhibits the oxidation of epinephrine by 50%.

Catalase Activity

CAT activity was determined by the method of Aebi *(38)*. Initially, 10 μL of absolute ethanol was mixed with 100 μL of cell homogenates and preincubated at 4ºC for 30 min. Following the addition of 10 μL of Triton X-100 (reduced form) a 100-μL aliquot (~100 μg of protein) of the resultant mixture was added to 500 μL of hydrogen peroxide (H_2O_2) (66 m*M*) and 400 μL of phosphate buffer (0.1 *M* NaH_2PO_4; 0.1 *M* Na_2HPO_4; 0.1 m*M* EDTA, pH 7.0). The absorbency of the final mixture was monitored for 1 min at 240 nm spectrophotometrically. The molar extinction coefficient of 43.6 *M*/cm was used to determine the catalase activity. One unit of catalase activity is defined as the amount of proteins that degrades 1 mmol of H_2O_2/min.

Glutathione Peroxidase Activity

Glutathione peroxidase activity was determined based on a modified method of Flohe and Gunzler *(39)*. In this assay, 100 μL of cell homogenate (~100 μg of protein) was added to 800 μL of phosphate buffer (0.1 *M* NaH_2PO_4; 0.1 *M* Na_2HPO_4; 0.1 m*M* EDTA, pH 7.0) containing 12.5 m*M* glutathione (reduced form, GSH), 0.2 m*M* β-nicotinamide adenine dinucleotide phosphate (reduced form, β-NADPH), and 0.34 U of glutathione reductase. This mixture was preincubated at 37ºC for 10 min and then 100 μL of 12 m*M* *t*-butyl hydroperoxide was added. The absorbency of the mixture was determined at 340 nm for 3 min. The molar extinction coefficient of 6.22 m*M*/cm was used to determine the activity of glutathione peroxidase. One unit of activity is defined as the amount of protein that oxidizes 1 nmol of β-NADPH/min.

Glutathione Reductase Activity

Glutathione reductase activity was determined by a slightly modified method of Carlberg and Mannervik *(40)*. Briefly, 50 μL of 2 m*M* β-NADPH in 10 m*M* Tris buffer (pH 7.0) was added to a cuvet containing 50 μL of 20 m*M* glutathione (oxidized form, GSSG) in phosphate buffer (0.1 *M* NaH_2PO_4; 0.1 *M* Na_2HPO_4; 0.1 m*M* EDTA, pH 7.0) and 800 μL of phosphate buffer. Following the addition of cell homogenate (~100 μg of protein/100 μL), the final mixture was measured at 340 nm for 3 min. The molar extinction coefficient of 6.22 m*M*/cm was used to determine the activity of glutathione reductase. One unit of activity is defined as the amount of proteins that oxidizes 1 nmol of β-NADPH/min.

Flow Cytometric Analysis

Female and male bAEC after E_2 pretreatment were trypsinized, rinsed, and resuspended in PBS containing 2% BSA at a density of 5×10^5 cells/mL. The cells were loaded with dichlorofluorescin diacetate (Molecular Probes, Eugene, OR) (dissolved in DMSO) at 5 mg/mL for 15 min followed with paraquat insult (1 m*M*) at room temperature in the dark. At the designated times, aliquots were withdrawn and

placed on ice, and then the fluorescence of oxidized DCFH *(41)* was analyzed by a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) equipped with an argon laser. A collection filter for the green emission of oxidized DCFH (530/30 nm) was used. Fluorescence signal was collected in log mode and analyzed using Cellquest software (Becton-Dickinson).

Data Analysis

All data of the cell surviving fractions and the enzyme activities were reported as mean ± SEM. Statistical differences between female and male bAEC were analyzed using student's *t*-test and those among cellular response to graded concentrations of E_2 were analyzed by ANOVA followed by Tukey test.

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