Estrogen Prevents Oxidative Stress–Induced Endothelial Cell Apoptosis in Rats

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Background—Estrogen replacement attenuates the increased risk of cardiovascular disease in postmenopausal women. Recent studies using an in vitro culture system have shown that estrogen inhibits endothelial cell (EC) apoptosis. The in vivo relevance of this finding, however, is not defined. To do so, we have developed a rat vascular injury model in which EC apoptosis induced by hydrogen peroxide plays a role.

Methods and Results—Intracarotid arterial administration of 0.01 mmol/L hydrogen peroxide for 5 minutes evoked EC apoptosis after 6 to 24 hours, determined by nuclear staining with Hoechst 33342, terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling, and electron microscopy. Apoptosis was associated with EC loss and was followed by EC regeneration at 72 hours and neointima formation at 1 to 2 weeks. Estradiol replacement in ovariectomized female Wistar rats decreased the rate of apoptotic ECs by ~50%, assayed by nuclear morphology of en face specimens, resulting in increased remaining ECs and decreased neointima formation. Progesterone did not influence the effects of estradiol on EC apoptosis.

Conclusions—These results provide new insight into the cardioprotective action of estrogen as well as a paradigm of the response-to-injury hypothesis. (Circulation. 2001;103:724-729.)

Key Words: hormones • apoptosis • hydrogen peroxide • atherosclerosis

It has been suggested that estrogens may protect postmenopausal women against cardiovascular disease. Previous epidemiological studies have shown beneficial effects of estrogens on incidence of ischemic heart disease,1–3 although this has not been confirmed in several recent trials in patients with ischemic heart disease.4 A number of animal studies have also shown that estrogen replacement inhibits the development of arteriosclerotic lesions.5–8 In addition to the effects on lipid metabolism,2,5,9 direct actions on vascular cells, including endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), appear to be involved in the mechanism.6–8 The existence of estrogen receptors in ECs10,11 and VSMCs12,13 supports the notion that these cells can be targets for estrogen.

Endothelial injury is considered to be an initial event in the development of atherosclerosis.14 Endothelial injury may lead to EC apoptosis, a morphologically distinct type of cell death. Importantly, regions in which atherosclerotic lesions develop are characterized by enhanced EC turnover,15 which may be due to the increased rate of preceding EC apoptosis. Indeed, EC apoptosis is detected in atherosclerotic lesions.16,17 The recent report by Tricot et al17 on evidence of EC apoptosis in atherosclerotic plaques in humans and its association with shear stress supports the importance of EC apoptosis in atherosclerosis. Moreover, factors that influence EC apoptosis are thought to play a role in atherogenesis,18 suggesting a contribution of EC apoptosis to atherogenesis and other vascular diseases. Recent reports have demonstrated that estrogen inhibits apoptosis in cultured ECs16,19 however, there is no report documenting protection against EC apoptosis by estrogen in humans or in animal experiments. Therefore, it is intriguing to study whether estrogen can modulate this process in vivo.

In the present study, we established a rat model in which oxidative stress produced by hydrogen peroxide (H$_2$O$_2$) induces EC apoptosis, followed by neointima formation. Then, using this model, we examined the effect of estrogen replacement on EC apoptosis and demonstrated that estrogen acted on ECs as a survival factor.

Methods

Animals

Eight-week-old female Wistar rats (Nippon Bio-Supply Center, Tokyo, Japan) were used in this study. They were housed in...
individual cages in a room in which lighting was controlled (12 hours on, 12 hours off) and room temperature was kept at \( \approx 22{\text{\degree}}C \). They were given a standard diet and water ad libitum. All the surgical procedures were performed under pentobarbital sodium anesthesia (50 mg/kg IP). All of the experimental protocols were approved by the Animal Research Committee of the University of Tokyo.

**Surgical Procedures**

Rats were bilaterally ovariectomized and randomly divided into 2 groups: an estradiol (E\(_2\)) replacement group and a vehicle group. At 10 weeks of age, rats received subcutaneous implants with a 3-week releasing pellet containing 0.5 mg E\(_2\) or placebo.\(^{20}\) In some experiments, pellets containing 10 mg progesterone or its placebo were added.\(^{20}\) When the experimental period was \( \geq 3 \) weeks, the original pellet was removed and a new pellet containing the same drug was implanted. Two weeks later, rats were treated with H\(_2\)O\(_2\) to induce endothelial injury in the carotid artery. Plasma E\(_2\) concentration at this point was 6.7±0.4 pg/mL in the vehicle group (\( n=6 \)) and 88.3±10.6 pg/mL in the E\(_2\) group (\( n=8 \)).

The right common carotid artery, including the bifurcation, was exposed, and a PE10 catheter (Becton Dickinson) was placed in the common carotid artery via the external carotid artery. The blood-stream at the site of surgical manipulation was temporarily interrupted by occlusion of the common, internal, and external carotid arteries with surgical ligatures. After the right common carotid lumen was flushed with saline, the catheter was pulled back to just above the bifurcation. Then the saline in the lumen of the carotid artery was replaced with H\(_2\)O\(_2\) (Wako Pure Chemical Industries) diluted in saline for 5 minutes. After the H\(_2\)O\(_2\) solution was completely removed by flushing of the lumen with blood, the external carotid artery was ligated, and the wound was closed. In the preliminary experiments to optimize the concentration of H\(_2\)O\(_2\) solution, 0.01, 0.1, 1.0, or 10 mmol/L H\(_2\)O\(_2\) was applied in the vehicle group. In other experiments, 0.01 mmol/L H\(_2\)O\(_2\) was used in the E\(_2\) and vehicle groups. Some rats in the vehicle group were subjected to balloon denudation of ECs as previously described\(^{21}\) to compare the EC and VSMC damage with that in H\(_2\)O\(_2\)-treated rats.

**Evaluation of EC Denudation and Apoptosis**

After the experimental period, rats were anesthetized with pentobarbital, and a PE50 catheter (Becton Dickinson) was placed in the jugular vein. Evans blue dye (60 mg/kg) (Sigma Chemical Co), which stains the deendothelialized area blue, was perfused for 30 minutes to identify the remaining ECs.\(^{22}\) After the rats had been exsanguinated through a cannula placed in the left cardiac ventricle, they were perfused with heparinized PBS at a pressure of 90 mm Hg for 5 minutes, followed by 5 minutes of fixation with 100% methanol. The carotid artery from the aortic arch to the bifurcation was dissected and incised longitudinally. The en face specimen was pinned to a plastic board, postfixed in 100% methanol, and microphotographed to examine the Evans blue staining. Then, the arterial specimen was washed with PBS for 2 hours at room temperature, followed by fluorescent staining with Hoechst 33342 (8 µg/mL in PBS) for 30 minutes at \( 37{\text{\degree}}C \).\(^{23}\) The specimen was viewed under high power \(( \times 125 )\) with a UV microscope, which was focused on the luminal endothelial side. Apoptotic cells were identified by their typical morphological appearance: chromatin condensation, nuclear fragmentation, or apoptotic bodies. The numbers of apoptotic cells and intact cells were counted in 6 high-power fields for each specimen by an observer blinded to the treatment group.

To confirm EC apoptosis, we performed terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) and dUTP, was performed according to the manufacturer’s protocol (Takara). Transmission electron microscopy was performed as previously described.\(^{24}\) Cross sections of carotid arteries 6 hours after H\(_2\)O\(_2\) treatment were fixed in situ with 2.5% glutaraldehyde and 1% paraformaldehyde. Sections were postfixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Ultrathin sections were stained with 1% uranyl acetate and examined with an electron microscope.

**Morphometric Analysis of Neo-intima Formation**

Two weeks after H\(_2\)O\(_2\) treatment, rats were anesthetized with pentobarbital and euthanized by perfusion with heparinized PBS. The rats were then perfusion-fixed with 10% neutral buffered formalin at 90 mm Hg. The carotid artery was dissected, postfixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. The middle segment of the artery was cut into 5 subserial cross sections 5 µm thick at intervals of 2 mm. The sections were stained by van Gieson elastica staining. The areas of the intima and of the media were measured by image analyzing software (NIH Image Version 1.61) by an observer blinded to the treatment group, and the ratio of the intimal area to the medial area (I/M ratio) was calculated. The average of 5 sections was taken as the value for each animal.

**Data Analysis**

The values are expressed as mean±SEM in the text and figures. The data were analyzed by 1-factor ANOVA followed by Newman-Keuls multiple comparison test. Differences with a value of \( P<0.05 \) were considered statistically significant.

**Results**

**EC Apoptosis Induced by H\(_2\)O\(_2\) Treatment**

We first examined the EC damage immediately after chemical injury induced by various concentrations of H\(_2\)O\(_2\) in the vehicle group. H\(_2\)O\(_2\) evoked immediate and patchy EC depARATION in a dose-dependent manner, presumably due to necrosis or cell lysis. According to the microscopic appearance of the Evans blue staining, \( \approx 90\% \) of ECs remained after treatment with 0.01 mmol/L H\(_2\)O\(_2\), and 50% remained with 0.1 mmol/L H\(_2\)O\(_2\). However, \( \approx 90\% \) of ECs disappeared with 1 mmol/L H\(_2\)O\(_2\) and 10 mmol/L H\(_2\)O\(_2\) resulted in complete denudation of ECs. Nuclear staining with Hoechst 33342 confirmed this finding (Figure 1a through 1c). Furthermore, VSMC damage was rarely seen in H\(_2\)O\(_2\)-treated artery, in contrast to the widespread VSMC damage in balloon-injured artery (Figure 1d). These results indicate that 0.01 mmol/L H\(_2\)O\(_2\) is appropriate to study EC apoptosis, which occurs later, as described below.

Figures 2 and 3 show the histological changes in carotid artery treated with 0.01 mmol/L H\(_2\)O\(_2\) in the vehicle group. Apoptotic ECs were found as early as 3 hours after injury, increasing at 6 hours, documented by en face Hoechst 33342 nuclear staining (Figure 2). Typical apoptotic ECs exhibiting apoptotic morphology, such as chromatin condensation, nuclear fragmentation, and apoptotic bodies, are shown in Figure 2a. EC apoptosis was confirmed by DNA fragmentation with TUNEL staining (Figure 2b). TUNEL-positive cells were identical to the apoptotic ECs determined by nuclear morphology. These apoptotic changes were not observed when the carotid artery was treated with saline without H\(_2\)O\(_2\). In contrast to the en face specimens, significant apoptotic morphology was hard to detect in cross-sectional segments stained with Hoechst 33342, although the same segments were positively stained for TUNEL (Figure 2c). This result indicates that en face specimens are better for evaluating EC apoptosis by nuclear morphology than cross-sectional segments. Transmission electron microscopy corroborated the morphological changes of apoptotic cells. As shown in Figure 2d, the nuclei of apoptotic ECs 6 hours after H\(_2\)O\(_2\) treatment...
were occupied by heterochromatin, in contrast to the control intact ECs. At 6 hours and 24 hours after injury, a substantial number of ECs had disappeared (Figure 3b and 3c), followed by EC regeneration at 72 hours (Figure 3d). Neointima formation appeared at 1 week and was increased at 2 weeks (Figure 3e and 3f). Neointima consisted predominantly of VSMCs, determined by immunostaining with an antibody against smooth muscle α-actin (data not shown).

**Effect of Estrogen Replacement on EC Apoptosis**

Using this injury model, we examined the effect of E₂ replacement on vascular remodeling, including EC apoptosis and neointima formation, in ovariectomized female rats. EC apoptosis was evaluated by en face Hoechst 33342 nuclear staining and quantified by counting of apoptotic cells and intact cells. The number and percentage of apoptotic ECs increased in a time-dependent manner, peaking at 6 hours, and then decreased until up to 2 weeks in both the vehicle and E₂ groups (Figure 4a and 4c). In contrast, the number of intact cells was decreased at 6 to 24 hours and had recovered at 72 hours in both the vehicle and E₂ groups (Figure 4b). There was no difference in the number of intact cells at 3 hours between the vehicle and E₂ groups, suggesting that estradiol did not inhibit necrosis of ECs. Interestingly, the number of

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**Figure 1.** Cell damage immediately after injury in rat carotid artery with saline (a), 0.01 (b) or 10 (c) mmol/L H₂O₂, or balloon injury (d). After each type of injury, en face specimens were stained with Hoechst 33342. Nuclear morphology of ECs (round) and VSMCs (spindle-shaped) can be observed. Compared with control saline treatment, 0.01 mmol/L H₂O₂ evoked patchy and slight EC denudation, whereas 10 mmol/L H₂O₂ or balloon injury resulted in complete EC denudation. Note that massive VSMC damage was induced by balloon injury.

**Figure 2.** Representative morphological changes of apoptotic ECs determined by en face Hoechst 33342 staining at 6 hours after H₂O₂ injury in rat carotid artery (a), comparisons between Hoechst 33342 and TUNEL staining (b, c), and representative morphological changes determined by transmission electron microscopy (d). a. Typical apoptotic morphology, such as chromatin condensation (large arrow), nuclear fragmentation (arrowhead), and an apoptotic body (small arrow) can be observed. b. Cells exhibiting apoptotic body by Hoechst 33342 staining were also positive for TUNEL staining (arrowheads) in en face specimens. c. Cells stained for TUNEL (arrowheads) did not show significant apoptotic nuclear morphology in cross-sectional segments. d. Nuclei of apoptotic ECs 6 hours after H₂O₂ injury were occupied by heterochromatin (bottom), in contrast to control intact cells 6 hours after saline treatment (top).
apoptotic cells was significantly lower in the E2 group at 6 hours and 24 hours than in the vehicle group, whereas the number of intact cells was greater in the E2 group at 6 hours and later than in the vehicle group. Consequently, the percentage of apoptotic cells was significantly lower in the E2 group at 6 hours and later than in the vehicle group (by ≈50%). The number of intact ECs determined in the contralateral carotid artery at 3 hours was comparable between vehicle and E2 groups, showing that estradiol does not influence the basal EC number. These results indicate that estrogen replacement inhibited EC apoptosis induced by H2 O2 and thus allowed more ECs to survive in the intima.

We also examined the effect of progesterone (alone or combined with estrogen), because the estrogen replacement regimen usually includes progesterone, and some previous studies have shown that progesterone antagonized the beneficial effects of estrogen.23 As shown in Figure 4d, progesterone alone did not exhibit any effects on the number of apoptotic cells. Furthermore, addition of progesterone did not influence the effects of estrogen.

Because estrogen is reported to accelerate endothelial recovery after balloon injury,22,26 we assessed EC regeneration as the increase in intact cell number from 24 hours to 72 hours after H2O2 treatment (Figure 4b). EC regeneration was comparable, however, between the vehicle and E2 groups.

To investigate the effect of estradiol on the resulting neointima formation, morphometric analysis was performed on the cross-sectional carotid arteries at 2 weeks. The I/M ratio in the E2 group was <50% of that in the vehicle group (Figure 5).

Discussion

In this study, we have developed a new vascular injury model in which H2O2 evokes EC apoptosis. Most ECs were denuded immediately by high doses of H2O2, presumably as a result of necrosis, whereas immediate EC loss was modest at low doses of H2O2 (10% at 0.01 mmol/L). We applied 0.01 mmol/L H2O2 to evaluate EC apoptosis, which occurred several hours later. Apoptosis, determined by the nuclear morphology (Hoechst 33342 staining), was also documented by DNA fragmentation (TUNEL staining) and transmission electron microscopy. Apoptosis led to cell loss, which peaked at 6 to 24 hours after injury. EC regeneration was followed by neointima formation, which consisted predominantly of VSMCs. Of interest, by gross morphological observations, acute VSMC injury was rare, in contrast to the balloon-denudation model, in which reendothelialization and neointima formation also occur but VSMCs undergo massive cell death.27 Because we did not examine VSMC injury precisely, the reaction of VSMCs in this model is not known, and the contribution of VSMC reactions to the observed results, such as neointima formation, is not determined by the present study. Thus, this H2O2-induced vascular injury model is appropriate to study EC apoptosis and its role in vascular remodeling.

A possible limit of this model is the use of a strong oxidative stimulus, H2O2, which may not have a clear correlation with human pathological conditions. Although cigarette smoke, oxidized lipoproteins, and polymorphonuclear leukocytes, which play important roles in atherogenesis, can generate H2O2 concentrations of 0.05 to 0.2 mmol/L in vitro,28 it is unknown how much H2O2 is actually produced in the lesions in vivo. But it may be possible that locally produced H2O2 provokes a stronger oxidative stress than short exposure (5 minutes) to the low concentrations (0.01 mmol/L) of H2O2 used in this study.

In the present study, we demonstrated that estrogen replacement attenuated the rate of EC apoptosis compared with that with vehicle treatment. Plasma estradiol concentrations in rats with estrogen replacement were <100 pg/mL, indicating that the dose was physiological.29 Although there is no previous report demonstrating the protective action of estrogen against EC apoptosis in vivo,22 two studies have shown that estrogen prevents EC apoptosis in culture. Spyrigopoulos et al19 reported that estradiol inhibited tumor necrosis factor-α–induced cell death in human umbilical vein ECs. Alvarez et al30 reported that estradiol inhibited apoptosis of bovine or human aortic ECs induced by estradiol withdrawal. Using bovine carotid ECs, we have also found that estradiol decreases the rate of H2O2-induced EC apoptosis (unpublished data). Conversely, it has been reported that estrogen accelerates reendothelialization after arterial balloon injury.22,26 Therefore, we attempted to evaluate this process after EC loss induced by H2O2 injury. Under our experimental conditions, however, we could not detect increased EC proliferation in the group with E2 replacement. A more precise time course or method, such as quantification of bromodeoxyuridine uptake, may be necessary to study EC proliferation in our model.

Previous studies have shown that H2O2 causes EC apoptosis in culture.30–32 Activation of p38 mitogen–activated protein kinase31 and tyrosine kinase–dependent upregulation of Fas32 may be involved in the mechanism. Conversely, activation of extracellular signal–regulated kinase by H2O2 con-
tributes to cell survival. We do not know whether the same mechanisms are involved in the in vivo model, nor could we show the mechanism responsible for the protective effect of estrogen against EC apoptosis. Increased endothelial release of nitric oxide, prostacyclin, or basic fibroblast growth factor by estrogen might be implicated, although no direct causal relationship has been shown between estrogen-stimulated production of these substances and endothelial apoptosis or proliferation. Another possibility is an antioxidant effect of estrogen. We examined whether estradiol directly inactivated H$_2$O$_2$; however, incubation with 1 nmol/L estradiol did not influence the activity of H$_2$O$_2$ assayed by the peroxidase-coupled oxidation of a donor substrate (data not shown). Further studies are necessary to elucidate the molecular mechanism involved in our in vivo model and the effect of estrogen.

We further examined the effect of progesterone (alone or in combination with estrogen), because, in most cases, hormone replacement therapy includes both estrogen and progestin, and some previous studies have shown that progesterone abrogated the beneficial effects of estrogen. In the present study, progesterone did not elicit any effects on EC apoptosis or influence the effects of estradiol. It is not easy to compare this result with those of previous studies, such as the study by Levine et al that showed the antagonizing effect of medroxyprogesterone against estradiol in the rat balloon-injury model. Levine et al used a higher dose of medroxyprogesterone (500-fold higher than estradiol) than the dose of native progesterone used in this study (20-fold higher than estradiol). Because the relative and absolute dose of progesterone used in this study is much higher than that used in humans (~10-fold higher than estradiol), our results may contribute to understanding the actions of progesterone and estrogen.

Figure 4. Time course and effect of estradiol (E$_2$) replacement on number of apoptotic ECs (a), number of intact ECs (b), and percentage of apoptotic ECs (c) after H$_2$O$_2$ injury in ovariectomized female rats. At indicated times, apoptotic and intact ECs were counted per high-power field (HPF, ×125) in en face specimens of carotid artery stained with Hoechst 33342. Contralateral side at 3 hours after injury served as control. n = 8 to 10 for each group. *P < 0.05, **P < 0.01 vs vehicle. d. Effects of progesterone on number of apoptotic ECs 24 hours after H$_2$O$_2$ injury were also examined. n = 8 to 10 for each group. **P < 0.01 vs vehicle.

Figure 5. Neointima formation of carotid artery after H$_2$O$_2$ injury in vehicle (n = 5) or estradiol (E$_2$)-supplemented (n = 4) ovariectomized rats. I/M ratio (a) and representative photomicrographs (b,c).
In the present study, we also demonstrated that neointima formation after H2O2 injury was decreased by estrogen replacement. This may be attributable to inhibition of EC apoptosis and exaggerated EC regeneration by estrogen. Furthermore, estrogen may have directly inhibited VSMC migration and proliferation, which follow EC regeneration, as previously reported. Accordingly, the effect of estrogen on neointima formation observed in this study might reflect synergistic effects on EC apoptosis and regeneration and VSMC migration and proliferation.

In summary, we have developed a rat vascular injury model in which EC apoptosis induced by H2O2 plays a role. Using this model, we demonstrated that estrogen replacement inhibited EC apoptosis, resulting in reduced neointima formation. This is the first report to address the in vivo role of estrogen in endothelial apoptosis, thus supporting the use of estrogen in postmenopausal women to prevent vascular diseases.

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