17 β -Estradiol induces protein thiol/disulfide oxidoreductases and protects cultured bovine aortic endothelial cells from oxidative stress

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Abstract

Objective: To examine whether or not estrogens induced the expression of protein thiol/disulfide oxidoreductases such as protein disulfide isomerase (PDI), thioredoxin (Trx), Trx reductase, and glutaredoxin (Grx) in vascular endothelial cells.

Methods: The regenerative effects of the protein thiol/disulfide oxidoreductases, PDI, Trx and Grx, on oxidatively damaged proteins were assayed using H₂O₂-inactivated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reporter enzyme. The induction of protein thiol/disulfide oxidoreductases and the accumulation of protein adducts generated by lipid peroxidation were examined by Western blotting in estrogen-treated bovine aortic endothelial cells (BAECs).

Results: Reduced PDI, Trx and Grx regenerated the H₂O₂-inactivated GAPDH *in vitro*. The levels of these protein disulfide oxidoreductases in BAECs were increased by pretreatment with 0.01–10 μ mol/l 17 β -estradiol, the largest increase (about fourfold of the control) being found for PDI. Other sex hormones such as progesterone and testosterone did not affect the contents of these oxidoreductases in BAECs. 4-Hydroxy-2-nonenal (HNE)-protein adducts, which are generated by lipid peroxidation, were accumulated in BAECs exposed to paraquat, whereas the pretreatment of BAECs with 17 β -estradiol suppressed their accumulation.

Conclusions: The estrogen-mediated induction of the protein thiol/disulfide oxidoreductases such as PDI, Trx, Trx reductase and Grx suggested a possible involvement of these oxidoreductases in the antioxidant protection of estrogen observed in the vascular system.

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Introduction

Epidemiological studies have indicated a close relationship between menopause and an increase in the incidence of cardiovascular disease in women, suggesting that female hormones have strong effects in maintaining the integrity of vascular walls (1–3). Indeed, a number of studies have documented that estrogen has various beneficial effects on the vascular system. Some of the effects of estrogen have been shown to be associated with protection against oxidative injury to blood vessels (4), but the detailed mechanism of the cardiovascular protection mediated by estrogen still remains unknown.

We have reported that thioredoxin (Trx) and glutaredoxin (Grx) serve as a repair system for oxidatively damaged proteins via reduction of oxidized thiols in vascular endothelial cells (5–7). Furthermore, recent studies have shown that Trx and Trx reductase can also scavenge reactive oxygen species (8–11), indicating

that these proteins constitute a novel cellular defense system against oxidative injury in addition to the conventional antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase.

Growing evidence shows that estrogens have direct antioxidant activities to protect various tissues from oxidative damage (4), but the effects of estrogens on the antioxidant enzymes, particularly on the protein thiol/disulfide oxidoreductases, have not been well investigated. In the present study, we examined whether or not the effects of estrogens on the cardiovascular system were associated with the induction of these protein thiol/disulfide oxidoreductases. The results obtained from vascular endothelial cells as target tissues demonstrated that estrogen increased the protein levels of protein disulfide isomerase (PDI), Trx, Trx reductase and Grx, suggesting a possible involvement of these protein thiol/disulfide oxidoreductases in estrogen-mediated antioxidant defense in the cardiovascular system.

Materials and methods

Materials

Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Boehringer-Mannheim (Mannheim, Germany). Progesterone, 17 β -estradiol and testosterone were purchased from Sigma (St. Louis, MO, USA). Hormone stock solutions of 15 mg/ml were made with 100% ethanol as solvent. A final ethanol concentration of 0.04% did not affect cell morphology or viability of the cells in our experiments. Antibody against 4-hydroxy-2-nonenal (HNE)-protein adducts was prepared as described elsewhere (12). Antisera raised against PDI, Trx, Grx, and Trx reductase were prepared by immunizing albino rabbits with each of the purified proteins (5–7).

Cell culture

Bovine aortic endothelial cells (BAECs) were harvested from bovine thoracic aorta and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum as described by Kitazono *et al.* (13). Endothelial cells at up to passage 20 were used for the experiments. The number of living BAECs was estimated from cell associated lactate dehydrogenase activity. The protein content of the cell lysate was determined using the Bio-Rad (Hercules, CA, USA) protein assay kit, with bovine serum albumin as standard.

Regeneration of H₂O₂-inactivated GAPDH by purified PDI, Trx, and Grx

Trx, Trx reductase, Grx and PDI were purified from bovine liver or heart as described elsewhere (5, 6, 14). Reduced Trx, PDI and Grx were freshly prepared by reduction with 2 mmol/l dithiothreitol (5). We used GAPDH, which has critical cysteines susceptible to oxidative damage, as a reporter enzyme for the regeneration reaction. GAPDH (3.4 μ mol/l) was treated with 0.3 mM H₂O₂ for 5 min at 25 °C and 30 μ g/ml catalase was then added to eliminate the H₂O₂. Regeneration of the H₂O₂-inactivated GAPDH (1.7 μ mol/l) by reduced or oxidized forms of PDI (5 μ mol/l), Trx (4 μ mol/l), and Grx (4 μ mol/l) was carried out at 37 °C in 100 mmol/l triethanolamine (pH 7.6) as described previously (5, 6). After the indicated periods of incubation, GAPDH activity was assayed and expressed as a percentage of that of the untreated enzyme.

Western blotting analysis of protein thiol/oxidoreductases

BAECs were pretreated in the presence or absence of sex hormones for 24 h at 37 °C in a 5% CO₂ atmosphere. The final concentration of the hormones (17 β -estradiol, progesterone, testosterone) and tamoxifen (an estrogen

Figure 1 Regeneration of H₂O₂-inactivated GAPDH (1.7 μ mol/l) by reduced or oxidized PDI, Trx, and Grx was carried out at 37 °C in 100 μ mol/l triethanolamine (pH 7.6) as described in Methods. After the indicated periods of incubation, GAPDH activity was assayed and expressed as the percentage of that of the untreated enzyme. ○, Reduced Trx; □, reduced PDI; △, reduced Grx; ●, oxidized Trx; ■, oxidized PDI; ▲, oxidized Grx. Each point represents the average of the two single determinations from two independent experiments.

receptor inhibitor) ranged from 0.01 to 10 μ mol/l. After exposure to the hormones, the cells were lysed with buffer A containing 5 mmol/l Hepes (pH 7.4), 1 mmol/l EDTA, 0.2% Triton X-100, and protease inhibitors (chymostatin 5 μ g/ml, leupeptin 10 μ g/ml, antipain 10 μ g/ml, pepstatin 10 μ g/ml, and *p*-amidino phenylmethylsulfonyl fluoride 5 μ g/ml), and centrifuged at 10 000 r.p.m. for 10 min. An equal amount of protein in the supernatant was separated by SDS-PAGE, transferred onto Immobilon-P membrane (Millipore, Bedford, MA, USA) and probed with polyclonal rabbit antibodies raised to PDI, Trx, Grx, and Trx reductase. An enhanced chemiluminescence detection kit (Amersham, Little Chalfont, UK) was used to visualize the protein bands. Densitometric analysis of the immunoblots was performed using a public-domain computer program NIH Image (Wayne Rasband, NIH, Bethesda, MD, USA).

Effect of 17 β -estradiol on accumulation of HNE-modified proteins

We examined the effect of 17 β -estradiol on paraquat-mediated lipid peroxidation (15) in BAECs. After pretreatment with or without 17 β -estradiol (10 μ mol/l) for 24 h, the monolayers of BAEC were thoroughly washed and further incubated with paraquat (100–500 μ mol/l) for 24 h. The accumulation of HNE-modified proteins in BAECs was determined by Western blot analysis of the cell lysate using anti-HNE antibody.

Statistical analysis

Data for the expression of protein thiol/disulfide oxidoreductases were expressed as means $\pm$ S.D. from

at least three independent experiments. Statistical comparison was performed by Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

Results

GAPDH, which has critical cysteines susceptible to oxidative damage, was used as a reporter enzyme for the regeneration reaction. A brief exposure of GAPDH to 0.3 mmol/l H_2O_2 caused an approximately 70% decrease in its activity. Incubation of the inactivated GAPDH with reduced Trx, PDI, and Grx restored the GAPDH activity to 65–90% of the control, whereas

incubation with the oxidized enzymes failed to regenerate the inactivated GAPDH (Fig. 1), indicating that only the reduced forms of the protein disulfide oxidoreductases exerted regenerative effects on the H_2O_2 -inactivated enzymes.

We examined the effect of 17β -estradiol on the levels of the protein thiol/disulfide oxidoreductases in BAECs by Western blotting analysis. The pretreatment of BAEC with 17β -estradiol increased the levels of all these protein thiol/disulfide oxidoreductases in a concentration-dependent manner, with the maximal increases in PDI, Trx, Grx and Trx reductase being about 4.0-, 2.2-, 1.7- and 1.5-fold of the control, respectively (Figs. 2 and 3). Tamoxifen, a specific

Figure 2 Enhancement of the protein level of PDI in BAECs upon treatment with 17β -estradiol. Cells were incubated for 24 h in the absence (control) or presence of 0.01–10 mmol/l 17β -estradiol, progesterone or testosterone. The protein level of PDI was determined by Western blotting analysis as described in Methods. The results of densitometric analysis from three independent experiments were expressed as ratios relative to control and presented as means $\pm$ s.d. *Significant in comparison with control ($P < 0.05$). Representative Western blotting data are shown below the histogram.

Figure 3 Enhancement of the protein levels of (A) Trx, (B) Trx reductase, and (C) Grx in BAECs upon treatment with 17β -estradiol, determined by Western blotting analysis. Cells were incubated for 24 h in the absence (control) or presence of 0.01–10 μ mol/l 17β -estradiol. The results of densitometric analysis from three independent experiments were expressed as ratios relative to control and presented as means $\pm$ s.d. *Significant in comparison with control ($P < 0.05$).

antagonist of estrogen receptor (16), inhibited the induction of Trx, Grx and Trx reductase, but not that of PDI (data not shown), suggesting that the regulatory pathway of the estrogen-mediated induction of PDI may be different from that of the other members of the protein thiol/disulfide oxidoreductases. Pretreatment of BAECs with progesterone and testosterone did not affect the protein levels of PDI (Fig. 2) or the other protein thiol/disulfide oxidoreductases (data not shown).

Estrogen has been reported to have direct anti-oxidant effects against oxidative stress in various tissues, either *in vitro* or *in vivo* (17–20). We therefore examined whether or not estrogens protected BAECs from oxidative stress, using an experimental model of paraquat-mediated lipid peroxidation (15). As shown in Fig. 4, the accumulation of HNE-modified proteins in BAECs exposed to 100–500 μ mol/l paraquat was increased in a dose-dependent manner, whereas pretreatment of BAECs with 17β -estradiol, which caused several-fold increases in the levels of the protein thiol/disulfide oxidoreductases, markedly inhibited the accumulation of HNE-modified proteins.

Discussion

We have shown that Trx and Grx have a thiol-dependent regeneration action on oxidatively damaged proteins (5, 6). In the present study, we found that PDI also exhibited a similar regeneration activity, with a potency comparable to that of these enzymes. This finding suggests that the members of the protein thiol/disulfide oxidoreductases that have different subcellular localizations and different electron donors may serve as redundant repair systems for oxidatively damaged proteins.

The evidence demonstrating the effect of estrogens on the expression of antioxidant enzymes is very limited. Oberley *et al.* (21) have shown that exposure to diethylstilbestrol, a synthetic estrogen, increases manganese superoxide dismutase activity in normal hamster kidney proximal tubules, but the effects of estrogens on the expression of other antioxidant enzymes such as catalase and glutathione peroxidase have not been demonstrated experimentally. In the present study, we showed that the pretreatment of

Figure 4 Accumulation of HNE-modified proteins in BAECs exposed to 100–500 $\mu\text{mol/l}$ paraquat, and the protective effect of 17 β -estradiol. Cells were incubated for 24 h in the absence (–, control) or presence (+) of 17 β -estradiol, thoroughly washed and exposed to 100–500 $\mu\text{mol/l}$ paraquat for 24 h. HNE accumulation was determined by Western blotting analysis. Arrows at the left indicate the enhanced HNE-modified proteins.

BAECs with 17 β -estradiol induced the expression of the protein thiol/disulfide oxidoreductases, a novel antioxidant system recognized recently. As the protein thiol/disulfide oxidoreductases repair oxidatively damaged proteins (5–7) and eliminate reactive oxygen species (8–11), the present results suggested a potential mechanism of the estrogen-mediated antioxidant functions that occur in the cardiovascular system.

The estradiol concentrations (0.01–10 $\mu\text{mol/l}$) used in these experiments were higher than those observed in non-pregnant females (0.01–1.0 nmol/l). In certain conditions, particularly during pregnancy, however, comparable concentrations (0.01–0.1 $\mu\text{mol/l}$) of 17 β -estradiol are observed (22). As shown in Figs 2 and 3, the effect of estradiol was evident even at 0.01 $\mu\text{mol/l}$, suggesting that the estrogen-mediated induction of the protein thiol/disulfide oxidoreductases may occur *in vivo*. In fact, the expression of Trx mRNA has been reported to be enhanced in late pregnancy (23).

The expression of Trx and PDI is known to be induced by various stimuli including oxidative stresses (24, 25), cytokines (26), lipopolysaccharide (27, 28) and hormones (29, 30). In contrast, there are only a few reports documenting the effect of estrogens on the expression of the protein thiol/disulfide oxidoreductases. The content of Trx mRNA in the cervix of non-pregnant women has been reported to correlate positively with serum estradiol concentration and to increase in late pregnancy (23). HIP-70/PLC- α , which is a member of PDI family and possesses similar redox-active cysteines, has also been shown to be induced by estradiol in rat uterus and hypothalamus (31). The present results are the first to document the estrogen-mediated induction of the protein thiol/disulfide oxidoreductases in the vascular system.

The 5'-flanking regions of the genes for these protein thiol/disulfide oxidoreductases (32–34) do not

appear to contain an estrogen-responsive element. The published sequence of the promoter for the PDI gene (32), however, has a consensus sequence for the antioxidant responsive element (ARE), which is activated by planar aromatic compounds like flavonoids (35). The structural similarity of estrogen and tamoxifen to flavonoids (4) may suggest the involvement of AREs in the estrogen-mediated induction of PDI, which could also explain the lack of tamoxifen inhibition in PDI induction because tamoxifen could act as an agonist for AREs of the PDI gene. Estrogen has been reported to upregulate the expression of endothelial nitric oxide synthetase (36). Increased production of NO or peroxynitrite, a metabolite of NO reacted with O_2^- , may serve as an oxidative stress that induces the expression of the protein thiol/disulfide oxidoreductases.

In the present study, we showed that paraquat-mediated lipid peroxidation in BAECs was suppressed by pretreatment with estrogen under conditions in which the expressions of all these protein thiol/disulfide oxidoreductases were enhanced. Trx and Trx reductase are known to eliminate lipid peroxides (8–11). Furthermore, Grx and PDI are reported to have dehydroascorbate reducing activity (37), which enables the recycling use of the oxidized ascorbate as an antioxidant. The antioxidant properties of these protein thiol/disulfide oxidoreductases thus suggest that the induction of these protein oxidoreductases can, at least in part, contribute to the cardioprotective antioxidant activities exhibited by estrogens.

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