17β-Estradiol Protects against Oxidative Stress-induced Cell Death through the Glutathione/Glutaredoxin-dependent Redox Regulation of Akt in Myocardiac H9c2 Cells*  

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The GSH/glutaredoxin (GRX) system is involved in the redox regulation of certain enzyme activities, and this system protects cells from H2O2-induced apoptosis by regulating the redox state of Akt (Murata, H., Ihara, Y., Nakamura, H., Yodoi, J., Sumikawa, K., and Kondo, T. (2003) J. Biol. Chem. 278, 50226–50233). Estrogens, such as 17β-estradiol (E2), play an important role in development, growth, and differentiation and appear to have protective effects on oxidative stress mediated by estrogen receptor α (ERα). However, the role of the ERβ-mediated pathway in this cytoprotection and the involvement of E2 in the redox regulation are not well understood. In the present study, we demonstrated that E2 protected cardiac H9c2 cells, expressing ERβ from H2O2-induced apoptosis concomitantly with an increase in the activity of Akt. E2 induced the expression of glutaredoxin (GRX) as well as γ-glutamylcysteine synthetase, a rate-limiting enzyme for the synthesis of GSH. Inhibitors for both γ-glutamylcysteine synthetase and GRX and ICI182,780, a specific inhibitor of ERs, abolished the protective effect of E2 on cell survival as well as the activity of Akt, suggesting that ERβ is involved in the cytoprotection and redox regulation by E2. Transcription of the GRX gene was enhanced by E2. The promoter activity of GRX was up-regulated by an ERβ-dependent element. These results suggest that the GRX/GSH system is involved in the cytoprotective and genomic effects of E2 on the redox state of Akt, a pathway that is mediated, at least in part, by ERβ. This mechanism may also play an antiapoptotic role in cancer cells during carcinogenesis or chemotherapy.

Oxidative stress is a principal cause of the development of aging and diseases such as inflammation, infection, cancer, and cardiovascular disorders (1, 2). Exogenous or endogenous sources of oxidative stress and weakened antioxidative defenses can damage macromolecules such as DNA, lipids, and proteins.

Estrogens play an important role in development, growth, and the differentiation of both female and male secondary sex characteristics (3). Protective effects of estrogen, such as 17β-estradiol (E2), on oxidative stress have been indicated (4). E2 regulates longevity signals to enhance resistance to oxidative stress in mice. Inhibitory effects of E2 on atherosclerosis are mediated by COX-2-derived prostacyclin (5). E2 induces production of antioxidative enzymes, such as superoxide dismutase (6), γ-glutamylcysteine synthetase (γ-GCS), and glutathione S-transferase (7). The effects of E2 are mediated mostly through ERα, which functions as a ligand-induced transcription factor and belongs to the nuclear receptor superfamily (8). ERα binds to a variety of ligands and displays tissue-specific effects through estrogen-response element (ERE). When estrogen-responsive genes do not contain EREs, ERα can up-regulate gene expression through AP-1 and Sp1 sites (9). Another ER, ERβ, is expressed in cells targeted by E2, including cardiomyocytes (10). However, the role of ERβ in protection against oxidative stress has not been well studied.

Protein thiols act as redox-sensitive switches and are believed to be a key element in maintaining the cellular redox balance. The redox state of protein thiols is regulated by oxidative stress and redox signaling and important to cellular functions. To maintain the cellular thiol-disulfide redox status, living cells possess two major systems, the thioredoxin (TRX)/TRX reductase system and the glutathione (GSH)/glutaredoxin (GRX) system (11). GSH is synthesized in two sequential enzymatic reactions that are catalyzed by a rate-limiting enzyme, γ-GCS, and GSH synthetase (12). GRX, also known as thioredoxin, was first discovered as a GSH-dependent hydrogen donor for ribonucleotide reductase in Escherichia coli mutants lacking TRX (13). Oxidized GRX is recycled to the reduced form by GSH with the formation of glutathione disulfide and regeneration of GSH by coupling with NADPH and glutathione disulfide reductase (14). GRX functions via a disulfide exchange reaction by utilizing the active site, Cys-Pro-Tyr-Cys, which specifically and efficiently catalyzes the reduction

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3 The abbreviations used are: E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen-response element; GRX, glutaredoxin; γ-GCS, γ-glutamylcysteine synthetase; PP2A, protein phosphatase 2A; MT2, 3-(4-(5-dimethyl-thiazole-2-yl)-2,5-diphenyltetrazo- 

lum bromide; HRP, horseradish peroxidase; PPT, propylpyrazoletriol; TBS, Tris-buff- ered saline; PKD1, 1,3-phosphoinositide-dependent protein kinase-1; TRX, thioredoxin; AMS, 4-acetamido-4′-maleimidylstilbene-2′-2-diol; PBS, phosphate-buff- ered saline; RT, reverse transcription; BSO, buthionine sulfoximine; EpRe, electrophoretic response element.
of protein-SSG mixed disulfide (15). GRX also partially shares its function as a redox sensor with TRX (16, 17). Recently, we have found that GRX protects against oxidative stress-induced cell death from apoptosis by regulating the redox state of Akt (18).

Akt/protein kinase B is a pleckstrin homology domain-containing serine/threonine kinase and a critical component of an intracellular signaling pathway that exerts effects on survival and apoptosis (19). Akt has been found to be responsive to extracellular signaling factors, oxidative and osmotic stress, irradiation, and ischemic stress (20). Akt can phosphorylate Bad, caspase-9, and forkhead-related transcription factors, leading to an inhibition of apoptosis (21). The unphosphorylated form of Akt is virtually inactive, and phosphorylation at Thr308 and Ser473 stimulates its activity. Inactivation of Akt also occurs via dephosphorylation of the two phosphorylation sites by protein phosphatase 2A (PP2A) (22, 23). The activation of Akt contributes to the survival of H$_2$O$_2$-treated cells (24).

It has been reported that the function of ER-mediated transcriptional activity is regulated by redox (25). However, the precise mechanisms of redox regulation in the E$_2$-mediated signal pathways have not been clarified. Here we describe a mechanism for the antiapoptotic effect of E$_2$ through the regulation of the redox state of Akt under oxidative stress. Treatment of cardiac H9c2 cells with E$_2$ for 18 h protected against H$_2$O$_2$-induced apoptosis. E$_2$ induced the expression of GRX and γ-GCS, at least in part, through ERβ-mediated regulation. Elevated GSH and GRX levels potentiated the redox of Akt on the exposure of cells to H$_2$O$_2$.

**MATERIALS AND METHODS**

**Reagents**—Anti-PP2A scaffolding A subunit (PR65) antibody was obtained from Santa Cruz Biotechnology. Antibodies against human ERα (clone ER88) and ERβ (polyclonal) were from Kyowa Medex (Tokyo, Japan). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG F was purchased from MBL (Nagoya, Japan). HRP-goat anti-mouse IgG F was from Chemicon International (Temecula, CA). Normal goat IgG, normal rabbit IgG, and normal mouse IgG were from Sigma. Anti-Akt and anti-phospho-(Ser473)-Akt antibodies were from Cell Signaling Technology. Anti-PP2A catalytic C subunit antibody was from BD Transduction Laboratories. 3-[4,5-dimethyl-thiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was from Sigma. 4-Acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) was purchased from Molecular Probes, Inc. (Eugene, OR). H$_2$O$_2$ and DcCl$_2$ were from Wako Pure Chemicals (Osaka, Japan). ICI182,780 and propylypyrazoletriol (PPT) were from Tocris (Ballwin, MO).

**Cell Culture**—H9c2 cells, a clonal line derived from embryonic rat heart, and human breast cancer SK-BR-3 (SKB3) cells, and MDA-MB-231 (MDA) cells, were obtained from the American Type Culture Collection (CRL-1446). Human breast cancer MCF7 cells were from the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). H9c2 cells were routinely maintained in Dulbecco’s modified Eagle’s medium, or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. MDA-MB-231 cells were maintained in a four-well Lab Tec Chamber (Nalgene Nunc International, Naperville, IL). After treatment with H$_2$O$_2$, cells were treated with 10 μM Hoechst 33342 for 30 min to estimate the extent of nuclear condensation. They were then washed again with PBS. Fluorescence intensity was examined using an Axioskop2 fluorescence microscope (Carl Zeiss, Jena, Germany), and the findings were analyzed using a charge-coupled device camera (Axio-Cam) and AxioVision software.

**Determination of Redox States**—The redox states of proteins were assessed by modifying free thiols with AMS (28). Briefly, after incubation with or without H$_2$O$_2$, cell lysates or proteins were treated with trichloroacetic acid at a final concentration of 7.5% to denature and precipitate...
the proteins as well as to avoid any subsequent redox reactions. The protein precipitates were collected by centrifugation at 12,000 × g for 10 min at 4 °C. The pellets were rinsed in acetone and centrifuged twice and then dissolved in a buffer containing 50 mM Tris-HCl (pH 7.4), 1% SDS, and 15 mM AMS. Proteins were then separated by 10% SDS-PAGE without using any reducing agents and blotted to nitrocellulose membranes. Proteins in the membranes were visualized by immunoblotting as described above.

**Northern Blot Analysis**—A 764-bp DNA fragment (bp 865–1628) of full-length γ-GCS heavy subunit cDNA was obtained by digestion with PstI (29). The probes were radiolabeled with 32P using a random primer labeling kit (Takara Biomedicals, Shiga, Japan). The isolation of cytoplasmic RNA and Northern blotting were essentially performed as described by Sambrook et al. (30). Isolated RNAs (30 μg) were electrophoresed on a 1% agarose gel containing 0.6 ionic formaldelyde, transfere to a nylon mem-

branes. Proteins in the membranes were visualized by immunoblotting

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**3-Phosphoinositide-dependent Protein Kinase-1 Activity**—3-Phosphoinositide-dependent protein kinase-1 (PDK1) activity was estimated using an assay kit according to the manufacturer’s instructions (Upstate Biotechnology). Briefly, recombinant human active PDK1 (Upstate Biotechnology) protein was incubated first with inhibitors for 30 min at 30 °C and then with inactive glucocorticoid-inducible kinase 1 (SGK1) for 30 min at 30 °C. Next, the PDK1-dependent SGK1 kinase activity was estimated by incubating the reaction mixture with glycosyn synthase kinase 3 (GSK3) peptide as a subst-

rate for 10 min at 30 °C in the presence of [γ-32P]ATP.

**Generation of Luciferase Reporter Constructs**—A 2.0-kb fragment of the human GRX gene promoter (−22 to −2) (31) was amplified by PCR using Pfu turbo DNA polymerase (Stratagene). The primers used were as follows: a forward primer (5′-GGT CTG ATG AGT GAG CAG ATA ATG GTC TCC-3′) and a reverse primer (5′-CGG GAA GAA TCC TCA GTT GCA GGT ATT GTT GCG-3′). The PCR product was subcloned into pUC18 to obtain pUC18-pro-GRX. PUC18-pro-GRX was digested with HindIII, and the resulting fragment containing the promoter region from −22 to −2 was inserted into the HindIII site of the reporter vector pGL3-Basic (Stratagene) to give pGL3-pro-GRX.

To generate a deleted version of the luciferase reporter construct (pGL3-pro-GRX-del), pGL3-pro-GRX was digested with KpnI and PvuII (Takara Biomedicals). Site-directed mutagenesis for luciferase vectors was performed with pGL3-pro-GRX (−22 to −2) as a tem-
plate by using a QuikChange site-directed mutagenesis kit (Stratagene).

The oligonucleotides used were as follows: electrophoretic response element (EpRE)-like 1 forward (5′-GCT CCC CCT CCG GGA GCA CTC AGA ATG GTC TCC-3′) and EpRE-like 1 reverse (5′-CCA GAT TCT GAG TCC CGG AGG GGG AGC-3′). The nucleotide sequence was confirmed by sequencing with an ALFExpress II system (Amersham Biosciences).

**Luciferase Activity Assay**—Each vector was introduced into H9c2 cells by using Lipofectamine 2000 (Invitrogen) according to the manu-
facturer’s instructions. After 12 h of transfection, cells were harvested for 24 h and then treated with E2 (100 nM) or left untreated for 18 h. Then luciferase assay was assayed with cellular extracts by using a dual luciferase reporter assay system (Promega).

**Electrophoretic Mobility Shift Assay**—The electrophoretic mobility shi	shift assay for the GC box and EpRE-like 1 element was performed as described (32). Briefly, oligonucleotides were annealed to double-stranded oligonucleotides and then labeled with [γ-32P]ATP using T4 polynucleotide kinase. Oligonucleotides specific for the GC box and EpRE-like 1 element were prepared according to the nucleotide sequence of the human GRX promoter region. Oligonucleotides used were as follows: EpRE-like 1 element, 5′-CCC TCC GTG ACT CAG AAT CTG GTC TCC-3′; mutated EpRE-like 1 element, 5′-CCC TCC GGG ACT GTA AGT TTA TGC TGC TCC-3′. Binding reactions were performed in 15 μl of reaction mixture (25 mM Tris, pH 7.0, 6.25 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM KCI, and 10% glycerol) containing 10 μg of nuclear extract and 25 ng of labeled oligo-
nucleotide. For the supershift assay, specific antibodies were added to the reaction mixture during the binding reaction for 30 min.

**Statistical Analysis**—Data were presented as means ± S.D. Differences were examined by using Student’s t test. A value of p < 0.05 was considered significant.

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**RESULTS**

**Expression of ERs**—The expression of ERs in H9c2 cells was estimated immunohistochemically and genetically. Fig. 1 shows the results of the immunohistochemical analysis. Unlike MCF7 cells, which are known to express both ERα (Fig. 1A) and ERβ (Fig. 1B), H9c2 cells expressed ERβ (Fig. 1F) but not ERα (Fig. 1E). Fig. 1F shows the results of the RT-PCR
analysis. ERβ mRNA but not ERα mRNA was detected in H9c2 cells. On the other hand, both ER mRNAs were detected in MCF7 cells.

Cytoprotective Effect of E2 on Oxidative Stress—We tested the cytoprotective effect of E2 on oxidative stress-induced apoptosis in H9c2 cells. Hydrogen peroxide induces apoptosis or early mitochondrial dys-

function in cardiac H9c2 cells (32, 33). Since 10% fetal calf serum, required for maintaining cultured cells, reduces oxidative stress modification of cells, in order to observe the effect of E2 on H2O2-induced oxidative stress, the concentration of fetal calf serum in the medium was changed from 10 to 0.5% in the experiments that followed. As shown in

FIGURE 1. Immunohistochemical analysis for ERs. The expression of ERs was examined by immunohistochemical analysis. A–D, MCF7 cells were treated with antibody against ERα (A) and ERβ (B). E–H, H9c2 cells were treated with antibody against ERα (E) and ERβ (F). As a negative control, normal mouse IgG (C and G) or normal rabbit IgG (D and H) was used. The gene expression of ERs was examined by RT-PCR analysis (I) using sense-primers for rat ERα and -β mRNAs in H9c2 cells and those for human mRNA in MCF7 cells.
Fig. 2A, the cell viability decreased by H$_2$O$_2$, as assessed photometrically with the MTT assay. The cell viability upon treatment with 100 nM E$_2$ for 18 h was ~27% of the control. Prior treatment of the cells with 100 and 200 nM E$_2$ for 18 h prevented the H$_2$O$_2$-induced cell damage by 1.4-fold and 1.8-fold of the control level, respectively (Fig. 2B). The increase in cell viability caused by E$_2$ observed in 18 h continued until 24 h and then declined until 36 h (Fig. 2C). Morphologically, H$_2$O$_2$-induced DNA condensation was observed (Fig. 3, A versus E). E$_2$ protected against DNA condensation (Fig. 3, B versus F). ICI182,780, an ER antagonist, abolished the protective effect of E$_2$ (Fig. 3, C versus G). PPT (0.5 mM), a specific inhibitor of ER, had no apparent influence on the protective effect of E$_2$ (Fig. 3, D versus H). These results suggest that the protective effect against oxidative stress observed on treatment of the cells with E$_2$ for 18 h involves transcriptional regulation mediated by ER through a genomic pathway in this cell line. Unless otherwise indicated, subsequent experiments on the effect of E$_2$ were done by incubating the cells with 100 nM E$_2$ for 18 h.

**E$_2$ Stimulated the Activity of Akt in Response to H$_2$O$_2$**—The Akt cascade is known to mediate the survival function. The Akt signal is involved in both the genomic (34) and the nongenomic pathway of E$_2$ (35). We tested the involvement of Akt in the cytoprotective effect of E$_2$ in ER-positive H9c2 cells. Phosphorylation of Akt (Ser473) was promoted by H$_2$O$_2$ in 10 min by 1.7-fold, and the control level was reached in 60 min (Fig. 4, A and B). Prior treatment with E$_2$ for 18 h resulted in a further increase in the H$_2$O$_2$-induced phosphorylation of Akt in 10 min by 4.1-fold, and the phosphorylation continued until 30 min (Fig. 4, A and B, lanes 6 versus lanes 2, respectively). ICI182,780 abolished the effect of E$_2$ (Fig. 4, C and D, lanes 6 versus lanes 4). The H$_2$O$_2$-induced enhancement of Akt activity estimated using GSK3 as a substrate was increased by E$_2$ (Fig. 4, E and F, lanes 6 versus lanes 2 and lanes 6 versus lanes 3, respectively). ICI182,780 abolished the effect of E$_2$ (Fig. 4, C and D, lanes 6 versus lanes 4). The H$_2$O$_2$-induced enhancement of Akt activity estimated using GSK3 as a substrate was increased by E$_2$ (Fig. 4, E and F, lanes 6 versus lanes 2 and lanes 6 versus lanes 3, respectively). The activity of PDK1, upstream of Akt, was stimulated by H$_2$O$_2$; however, E$_2$ had no apparent effect on the activity of PDK1 (Fig. 4G). The phosphorylation of Akt is regulated by PP2A (18). The activity of PP2A assayed spectrophotometrically using RKpTIRR and p-nitrophenylphosphate as substrates was not affected by H$_2$O$_2$ and E$_2$ (Fig. 4H). The data suggest that the change in the activity of PP2A...
is not involved in the up-regulation of the phosphorylation of Akt by E2.

It has been reported that inactive Akt develops a redox-sensitive intramolecular disulfide bond close to its activation loop (18), and recently we found that the redox state of Akt is modulated by H2O2 (19). Fig. 5A shows the redox state of Akt assessed by modifying free thiol with AMS. In control cells, Akt existed mostly in an oxidized form (lane 1). Treatment of cells with H2O2 resulted in a further increase in an oxidized form of Akt (lanes 2 and 3). In the cells treated with E2 for 18 h, Akt existed more in a reduced form (lane 4). The reduced form of Akt, once decreased by H2O2 for 30 min, was restored again in 60 min (lanes 5 and 6). The data suggested that E2 maintains Akt in a reduced form under oxidative stress. The redox state of Akt is regulated by the GSH/GRX system, and this system protects cells against H2O2-induced apoptosis by preventing the association of Akt with PP2A (19). Then we estimated the effect of E2 on the phosphorylation of Akt in the presence of buthionine sulfoximine (BSO), a specific inhibitor of H9253-GCS, or cadmium, an inhibitor of GRX. H9253 is a rate-limiting enzyme of GSH synthesis. The effect of E2 on the phosphorylation was abolished both by BSO (Fig. 5B) and by cadmium (Fig. 5C). These results suggest that E2 increases the levels of GSH/GRX to protect cells against oxidative stress.

E2 Induces the Expression of H9253-GCS and GRX—We tested if E2 increases the levels of GSH and GRX. E2 increased the levels of GSH.
Redox Regulation of Akt Signaling by Estradiol

A

Red

Gx

E2

H2O2

0

30

60

0

+ + +

+ + +

B

Anti-Akt-P

(Ser 473)

Anti-Akt

C

Relative intensity

H2O2 (10 min)

E2

2.5 µM CdCl2 (1 h)

BSO (2 h)

lane 1

2

3

4

5

6

7

8

E2 retained the redox state of Akt. A, the redox state of Akt was assessed based on mobility shifts of these proteins in an immunoblot analysis as described under “Materials and Methods.” The positions of reduced (red) and oxidized (Gx) proteins are indicated. The data are from a typical analysis. B, effect of modification of the redox on the phosphorylation of Akt was estimated, using 200 µM BSO, a specific inhibitor of γ-GCS (lanes 5 and 6), and 2.5 µM cadmium, an inhibitor of GRX (lanes 7 and 8). C, the activity of Akt phosphorylation is shown as relative intensity in the absence (open bar) and presence of H2O2 (closed bar). The data are the mean ± S.D. of three independent analyses. *, p < 0.05 compared with cells with H2O2 and E2 without inhibitors.

FIGURE 5. E2 retains the redox state of Akt. A, the redox state of Akt was assessed based on mobility shifts of these proteins in an immunoblot analysis as described under “Materials and Methods.” The positions of reduced (Red) and oxidized (Gx) proteins are indicated. The data are from a typical analysis. B, effect of modification of the redox on the phosphorylation of Akt was estimated, using 200 µM BSO, a specific inhibitor of γ-GCS (lanes 5 and 6), and 2.5 µM cadmium, an inhibitor of GRX (lanes 7 and 8). C, the activity of Akt phosphorylation is shown as relative intensity in the absence (open bar) and presence of H2O2 (closed bar). The data are the mean ± S.D. of three independent analyses. *, p < 0.05 compared with cells with H2O2 and E2 without inhibitors.

FIGURE 6. β-Actin → GRX → IC182,780

D

β-Actin

GRX

E2

ICI182,780

lane 1

2

3

E

GRX/β-Actin (% control)

FIGURE 6. GSH synthesis and GRX. Effects of E2 on levels of GSH, the γ-GCS heavy subunit, and GRX were estimated in the presence or absence of IC182,780, as described under “Materials and Methods.” A, cells were treated with 100 nM E2 for 0–24 h, and the levels of GSH in the cell lysates were estimated. Cells were incubated with 100 nM E2 for 6 h for the analysis of the expression of the γ-GCS heavy subunit by Northern blotting (B) and that of GRX by RT-PCR (D). The expression of γ-GCS was expressed as relative intensity (percentage of control) (C), and that of GRX was expressed as the intensity of GRX/β-actin (E). Each datum is a mean ± S.D. of three independent analyses. *, p < 0.05 compared with untreated cells.

FIGURE 6. GSH synthesis and GRX. Effects of E2 on levels of GSH, the γ-GCS heavy subunit, and GRX were estimated in the presence or absence of IC182,780, as described under “Materials and Methods.” A, cells were treated with 100 nM E2 for 0–24 h, and the levels of GSH in the cell lysates were estimated. Cells were incubated with 100 nM E2 for 6 h for the analysis of the expression of the γ-GCS heavy subunit by Northern blotting (B) and that of GRX by RT-PCR (D). The expression of γ-GCS was expressed as relative intensity (percentage of control) (C), and that of GRX was expressed as the intensity of GRX/β-actin (E). Each datum is a mean ± S.D. of three independent analyses. *, p < 0.05 compared with untreated cells.

(64A). The level of GSH was 24.8 ± 4.0 nmol/106 cells in control cells and 38.5 ± 5.2 nmol/106 cells in cells treated with 100 nM E2 for 18 h. The level of GSSG in the control cells was ~2 nmol/106 cells and was not changed by E2 treatment (data not shown). The expression of γ-GCS was up-regulated by 100 nM E2 by 1.6-fold in 18 h (Fig. 6, B and C). Similarly, 100 nM E2 increased the expression of GRX by 1.8-fold in 18 h (Fig. 6, D and E). IC182,780 abolished the E2-dependent up-regulation of GSH synthesis as well as GRX synthesis (Fig. 6, A–E). It is suggested that the redox state of Akt is regulated by an E2-dependent enhancement of the GRX/GSH system.

The Gene Promoter Activity of GRX Is Regulated by E2 via an EpRE-like Element—As reported by Montano et al. (7), the expression of the γ-GCS heavy (catalytic) subunit is up-regulated by E2 via an EpRE (5′-G(A)/TGACNNNGC(G/A)-3′), not by an ERE. To investigate the mechanism of the transcriptional regulation of GRX by E2, we used a 2.0-kb genomic fragment containing the promoter region of GRX inserted into a luciferase vector, pGL3 Basic. The promoter region contains no apparent ERE or EpRE. There were two EpRE-like sites (EpRE-like 1 (−1380 to −1370; GTGACTCAGAA) and EpRE-like 2 (−347 to −337; GTGAGTAAGCA)) and Sp1 (−1217 to −1208, GCCCCGC-CTC). The luciferase activity of the cells previously treated with E2 for 18 h was almost lost when the EpRE-like 1 site was deleted or mutated (Fig. 7). Deletion of EpRE-like 2 or Sp1 had no apparent effect on the E2-induced up-regulation of the luciferase activity.

E2 Up-regulated the ERβ-EpRE-like 1 Complex Formation—To investigate the importance of the EpRE-like elements in the E2-induced expression of GRX, an electrophoretic mobility shift assay was performed with nuclear extracts from the cells treated with E2 for 18 h using 32P-labeled oligonucleotides designed for EpRE-like 1. As shown in Fig. 8, a protein-DNA complex of EpRE-like 1 (lane 2) increased by E2 (lane 3) and appeared in the presence of an excess of unlabeled probe (lane 4), or 32P-labeled probe with the disabled mutant for EpRE-like 1 (lane 5). The addition of the anti-ERβ antibody caused the ERβ-DNA-binding complex to disappear (lane 12), indicating the involvement of ERβ as a transcription factor that bound to the EpRE-like 1 site. The EpRE-like 1 of GRX did not bind with Nrf2, Sp1, c-Jun, or c-Fos (lanes 9–11), different from the EpRE site of the γ-GCS heavy subunit (7). On the other hand, neither EpRE-like 2 site nor the Sp1 site was stimulated by E2 (data not shown).
Important Role of ERβ in Other Cells—To further confirm the role of ERβ in protection against oxidative stress through redox regulation of Akt, we employed human breast cancer cells, SK-BR-3 and MDA-MB-231 cells. As shown in Fig. 9A, an RT-PCR analysis revealed that these cells mainly expressed ERβ mRNA. A stimulatory effect of E2 on the activity of Akt was observed in these cells (Fig. 9, B and C). However, ICI182,780 abolished the protective effect of E2 (Fig. 9, B and C). E2 induced the expression of GRX (Fig. 9, D and E). The results suggested that the cytoprotective effect of E2 is mediated through redox regulation of Akt activity in ERβ-expressing cells.

DISCUSSION

ERβ-mediated Cytoprotection against Oxidative Stress—Estrogenic hormones are required for the growth and differentiation of female
Redox Regulation of Akt Signaling by Estradiol

FIGURE 9. Protective effect of E2 in other cell lines. The effect of E2 was studied using ERα-expressing human breast cancer SK-BR-3 cells and MDA-MB-231 cells. A, expression of ERs. The expression of ERs was estimated by RT-PCR analysis as described in the legend to Fig. 1. B, phosphorylation of Akt. The effect of E2 on the phosphorylation of Akt under oxidative stress was estimated by immunoblot analysis using specific antibodies as described under “Materials and Methods.” C, band intensity was estimated densitometrically and expressed as the relative intensity of phosphorylated Akt to total Akt (Aktp/Aktt). The data are the mean ± S.D. of three independent analyses. *, p < 0.05 compared with cells with E2 without ICI 182,780. D, expression of GRX. The effect of E2 on levels of the GRX was estimated as described under “Materials and Methods.” E, band intensity was estimated densitometrically and expressed as the intensity of GRX/β-actin. Each datum is a mean ± S.D. of three independent analyses. *, p < 0.05 compared with cells with H2O2 without E2; **, p < 0.05 compared with cells with E2 without ICI182,780.

reproductive tissues, contribute to male fertility, and play a role in maintaining cardiovascular, skeletal, and neural cell functions (9). Estrogen has been widely used to regulate fertility, relieve postmenopausal symptoms, and decrease the incidence and recurrence of mammary tumors. The ERs were the first members of the nuclear receptor family to be identified. ERα has been well characterized and plays a major role in E2-mediated genomic actions in both reproductive and nonreproductive tissues. ERα-mediated cytoprotection against oxidative stress-induced cell damage has been reported in neurological cells (33, 34) and breast cancer cells (35). On the other hand, the role of ERβ is not well understood. A report using microarray analyses showed that most of the genes regulated by ERβ are distinct from those regulated by ERα in response to E2 and selective estrogen receptor modulators (36). ERβ regulates plasminogen activator inhibitor-1 in endothelial cells, and a clinical evaluation of ERβ was suggested as a prognostic or predictive factor of drug resistance in breast cancer (37). These results suggest a significant role for ERβ in the regulation of cellular function, although the function of ERβ and its precise mechanism are still unclear (3).

Thus, this is the first report aimed at the significant role of ERβ-mediated signals of E2 in redox regulation in response to oxidative stress.

Involvement of Akt in the Cytoprotection of E2 Mediated by ERβ—The importance of Akt has been suggested in the cytoprotective effect of E2 against oxidative stress. This effect of E2 was rapid and nongenomic in neurological cells (38), vascular endothelial cells (39), and ovarian cancer cells (40). On the other hand, Stoica et al. (41) reported that ERα-mediated signals up-regulated the expression of Akt in ERα-positive MCF-7 breast cancer cells. They also demonstrated that Akt-mediated signals up-regulated the expression of ERα in these cells, suggesting that Akt plays a central role in the growth and survival of breast cancer cells; however, the mechanism by which Akt is activated by E2 was not fully characterized.

In the present study, we were interested in the possible involvement of Akt signals in the ERβ-mediated anti-apoptotic effect against oxidative stress. We employed H9c2 cells that apparently express only ERβ (Fig. 1). We found that 1) H2O2-induced apoptosis was prevented when the cells were incubated with E2 for over 18 h; 2) the anti-oxidative effect of E2 was mediated by a genomic pathway through ERβ; and 3) E2 retained the level of phosphorylated Akt in response to H2O2 via the GSH/GRX system.

Role of the GSH/GRX System in ERβ-mediated Akt Signals—We reported previously a role for the GRX/GSH system in the regulation of Akt phosphorylation (19). Akt is a Ser/Thr protein kinase with anti-apoptotic and oncogenic activities. Akt is activated through a growth factor receptor-mediated activation of the phosphatidylinositol 3-kinase pathway (21). The unphosphorylated form of Akt is virtually inactive, and phosphorylation at Thr308 and Ser473 stimulates its activity. Inactivation of Akt also occurs via dephosphorylation of the two phosphorylation sites by PP2A (23, 24). The activation of Akt contributes to the survival of H2O2-treated cells (25). H2O2 induces oxidation of Akt at Cys224, Cys297, and Cys311, and the oxidized form of Akt can be dephosphorylated by PP2A (26). PP2A is a major Ser/Thr phosphatase implicated in the regulation of many cellular processes, including the regulation of different signal transduction pathways, cell cycle progression, DNA replication, gene transcription, and protein translation (42). Yasukawa et al. (43) reported that Akt is also inactivated by S-nitrosylation at Cys473 in NO donor-treated cells (44). Furthermore, we recently reported that the phosphorylation of Akt is down-regulated by cytoplasmic calcium (32).
Calcium induced the expression of the PP2A catalytic subunit mediated by cAMP via the cAMP-response element. In the present study, the activity and the expression of the anti-PP2A catalytic C subunit did not change upon treatment with E2 in H9c2 cells (Fig. 4H), suggesting that the modulation of calcium levels may not be involved. Inactivation by ROS of protein phosphatases, such as protein-tyrosine phosphatase 1B (44), mitogen-activated protein kinase (MAPK) phosphatases (45), and PP2A (46), has been reported. In the present study, the activity of PP2A was not changed by H2O2 (Fig. 4H), suggesting that inactivation of PP2A by ROS is not involved. The redox state of Akt is regulated by GSH/GRX (19). Oxidation of Akt at Cys397 and Cys311 facilitates the association of PP2A, leading to the dephosphorylation of Akt. However, the activity of Akt is not affected by the oxidation. In the present study, oxidation of Akt was observed in the medium with 0.5% fetal calf serum in the absence of H2O2, and after the treatment with H2O2, the oxidation of Akt continued for 60 min. In such conditions, E2 maintained Akt in the reduced form (Fig. 5). This suggested that E2 potentiates the functions of the GSH/GRX system. The GSH/GRX system regulates many signals, such as ASK-1, NFI, PTP1B, protein kinase C, and protein kinase A (49). The present study indicates for the first time that ERβ-mediated signaling via E2 up-regulates the activity of the GSH/GRX system to stimulate Akt and protects cells against oxidative stress.

Up-Regulation of γ-GCS and GRX by E2—The ERα-mediated expression of antioxidant enzymes in response to oxidative stress has been reported. Genomic effects on the expression of antioxidant enzymes reported were Mn-SOD (4, 6), Cu,Zn-SOD (6), COX-1 (47), and COX-2 (5). The induction of antioxidants in response to oxidative stress has been reported. Up-regulation of γ-GCS and GRX by E2 was studied further. In the present study, we found that the induction of γ-GCS expression by E2 is mediated by an EpRE-like element (Figs. 7 and 8). The human GRX promoter employed here possessed no apparentERE or EpRE but had two EpRE-like sites. Interestingly, one of these sites, EpRE-like 1, bound to ERβ and promoted the transcriptional activity of GRX. Transcription of the GRX gene was increased by E2, but decreased by anti-ERβ antibody. However, EpRE-like 1 did not bind to Nrf-2 or AP-1. This element may be a novel kind ofERE. In summary, E2 has a cytoprotective effect against oxidative stress in H9c2 cells expressing ERβ. The genomic effect of E2 on the GSH/GRX redox system potentiates Akt activity, a mechanism that may also play an antiapoptotic role in cancer cells during carcinogenesis or chemotherapy.

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REFERENCES

1. Berlett, S. B., and Stadtman, E. R. (1997) J. Biol. Chem. 272, 20313–20316
2. Finkel, T., and Holbrook, N. J. (2000) Nature 408, 239–247
3. Yang, S. H., Liu, R., Perez, E. J., Wen, Y., Stevens, S. M., Jr., Valencia, T., Brun-Zinkernagel, A. M., Prokai, L., Will, Y., Dykems, J., Koulou, P., and Simpkins, J. W. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 4130–4135
4. Baba, T., Shimizu, T., Suzuki, Y., Ogawa, M., Sonno, K., Koseki, H., Kurosawa, H., and Shiraaka, T. (2005) J. Biol. Chem. 280, 16417–16426
5. Egan, K. M., Lawson, J. A., Fries, S., Koller, B., Rader, D. J., Smyth, E. M., and Fitzgerald, A. G. (2004) Science 306, 1954–1957
6. Streibow, K., Rotter, S., Wassmann, S., Adam, O., Grohe, C., Laufs, K., Bohm, M., and Nickenberg, G. (2005) Circ. Res. 93, 170–177
7. Montano, M. M., Deng, H., Liu, M., Sun, X., and Singal, R. (2004) Oncogene 23, 2442–2453
8. Beato, M., Herrlich, P., and Schütz, G. (1995) Cell 83, 851–857
9. Schulz, J. R., Petz, L. N., and Nardulli, A. M. (2005) J. Biol. Chem. 280, 347–354
10. Foster, C., Keitz, S., Hultenby, K., Warner, M., and Gustafsson, J. A. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 14232–14239
11. Holmgren, A. (1989) J. Biol. Chem. 264, 13963–13966
12. Meister, A. (1973) Science 180, 33–39
13. Holmgren, A. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2275–2279
14. Gan, Z.-R., and Wells, W. W. (1986) J. Biol. Chem. 261, 996–1001
15. Gravina, S. A., and Mieyal, J. J. (1993) Biochemistry 32, 3368–3376
16. Song, J. J., Rhee, J. G., Suntharalingam, M., Walsh, S. A., Spitz, D. R., and Lee, Y. J. (2002) J. Biol. Chem. 277, 46566–46575
17. Song, J. J., and Lee, Y. J. (2003) Biochem. J. 373, 845–853
18. Murata, H., Ibara, T., Nakamura, H., Yodoi, J., Sumikawa, K., and Kondo, T. (2003) J. Biol. Chem. 278, 50226–50233
19. Huang, X., Begley, M., Morgenstern, K. A., Gu, Y., Rose, P., Zhao, H., and Zhu, X. (2003) Structure (Camb.) 11, 21–30
20. Brazile, D. P., and Hemmings, B. A. (2001) Trends Biochem. Sci. 26, 657–664
21. Franke, T. F., Hornik, C. P., Segev, L., Shostak, G. A., and Sugimoto, C. (2003) Oncogene 22, 8983–8998
22. Luikenhuis, S., Perrone, G., Dawes, I. W., and Grant, C. M. (1998) Mol. Biol. Cell 9, 1081–1091
23. Andjelkovic, M., Jakowiczow, T., Cron, P., Ming, X. F., Han, J. W., and Hemmings, B. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5699–5704
24. Pham, F. H., Sugden, P. H., and Clark, A. (2000) J. Cell Sci. 113, 1252–1258
25. Hayashi, S., Hairo-Nakanishi, K., Makino, Y., Eguchi, H., Yodoi, J., and Tanaka, H. (1997) Nucleic Acids Res. 25, 4035–4040
26. Goto, S., Kamada, K., Soh, Y., Ibara, Y., and Kondo, T. (2002) Jpn. J. Cancer Res. 93, 1047–1056
27. Tamura, N., Hashikawa, Y., Ejima, K., Nagasue, N., Inoue, S., Muramatsu, M., Hayashi, T., and Koji, T. (2004) Lab. Invest. 84, 1460–1471
28. Kobayashi, T., Kishigami, S., Sone, M., Inokuchi, K., Mogi, T., and Ito, K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11857–11862
29. Iida, T., Kijima, H., Uraha, T., Goto, S., Ibara, Y., Oka, M., Kohno, S., Scanlon, K. J., and Kondo, T. (2001) Cancer Gene Ther. 8, 803–814
30. Sambrook, J., Frisch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Padilla, C. A., Bajalica, S., Lagercrantz, I., and Holmgren, A. (1996) Genomics 32, 455–457
32. Yasuoka, C., Ibara, Y., Ikeda, S., Miyahara, Y., Kondo, T., and Kohno, S. (2004) J. Biol. Chem. 279, 51182–51192
33. Prokai, L., Prokai-Tattari, K., Perjesi, P., Zbarkiova, A. D., Perez, E. J., Liu, R., and Simpkins, J. W. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 11741–11746
34. Kenschapp, R. S., Diwakar, L., Annepu, J., and Ravindranath, V. (2004) FASEB J. 18, 1102–1104
35. Fernando, R. I., and Wimalasena, J. (2004) Mol. Biol. Cell 15, 3266–3284
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36. Tee, M. K., Rogatsky, I., Tzagarakis-Foster, C., Cvoro, A., An, I., Christy, R. J., Yamamoto, K. R., and Leitman, D. C. (2004) Mol. Biol. Cell 15, 1562–1572
37. Smith, L. H., Coats, S. R., Coats, S. R., Qin, H., Petrie, M. S., Covington, J. W., Su, M., Eren, M., and Vaughan, D. E. (2004) Circ. Res. 95, 269–275
38. Yu, X., Rajala, R. V., McGinnis, J. F., Li, F., Anderson, R. E., Yan, X., Li, S., Elias, R. V., Knappe, R. R., Zhou, X., and Cao, W. (2004) J. Biol. Chem. 279, 13086–13094
39. Lu, Q., Pallas, D. C., Surks, H. K., Baur, W. E., Mendelsohn, M. E., and Karas, R. H. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 17126–17131
40. Mabuchi, S., Ohmichi, M., Kimura, A., Nishio, Y., Arimoto-Ishida, E., Yada-Hashimoto, N., Tasaka, K., and Murata, Y. (2004) Endocrinology 145, 49–58
41. Stoica, G. E., Franke, T. F., Moroni, M., Mueller, S., Morgan, E., Iann, M. C., Winder, A. D., Reiter, R., Wellstein, A., Martin, M. B., and Stoica, A. (2003) Oncogene 22, 7998–8011
42. Janssens, V., Goris, J., and Van Hoof, C. (2005) Curr. Opin. Genet. Dev. 15, 34–41
43. Yasukawa, T., Tokunaga, E., Ota, H., Sugita, H., Martyn, J. A., and Kaneki, M. (2005) J. Biol. Chem. 280, 7511–7518
44. Salmeen, A., Andersen, J. N., Myers, M. P., Meng, T. C., Hinks, J. A., Tonks, N. K., and Barford, D. (2003) Nature 423, 769–773
45. Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., and Karin, M. (2005) Cell 120, 649–661
46. Rao, R. K., and Clayton, L. W. (2002) Biochem. Biophys. Res. Commun. 293, 610–616
47. Gilson, L. L., Hahner, L., Osborne-Lawrence, S., Germain, Z., Wu, K. K., Chambliss, K. L., and Shaul, P. W. (2005) Circ. Res. 96, 518–525
48. Ejima, K., Nanri, H., Araki, M., Uchida, K., Kashimura, M., and Ikeda, M. (1999) Endocrinology 140, 608–613
49. Shelton, M. D., Chock, P. B., and Mieyal, J. J. (2005) Antioxid. Redox Signal. 7, 348–366
50. Helguero, L. A., Fauld, M. H., Guslafsson, J. A., and Haldosen, L. A. (2005) Oncogene 24, 6605–6616
51. Connor, E. E., Wood, D. L., Sonstegard, T. S., da Mota, A. F., Bennet, G. L., and Williams, J. L. (2005) J. Endocrinol. 185, 593–603