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Neuroprotective Effects of 17β-Estradiol and Nonfeminizing Estrogens against H_2O_2 Toxicity in Human Neuroblastoma SK-N-SH Cells

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ABSTRACT

Neuroprotective effects of estrogens have been shown in various in vitro and in vivo models, but the mechanisms underlying protection by estrogen are not clear. Mounting evidence suggests antioxidant effects contribute to the neuroprotective effects of estrogens. In the present study, we assessed the protective effects of estrogens against H_2O_2-induced toxicity in human neuroblastoma cells and the potential mechanisms involved in this protection. We demonstrate that 17β-estradiol (17β-E_2) increases cell survival against H_2O_2 toxicity in human neuroblastoma cells. 17β-E_2 effectively reduced lipid peroxidation induced by 5-min H_2O_2 exposure. Furthermore, 17β-E_2 exerts the protective effects by maintaining intracellular Ca^{2+} homeostasis, attenuating ATP depletion, ablating mitochondrial calcium overloading, and preserving mitochondrial membrane potential. Two nonfeminizing estrogens, 17α- and *ent*-estradiol, were as effective as 17β-E_2 in increasing cell survival, alleviating lipid peroxidation, preserving mitochondrial function, and maintaining intracellular glutathione levels and Ca^{2+} homeostasis against H_2O_2 insult. Moreover, the estrogen receptor antagonist fulvestrant (ICI 182,780) did not block effects of 17β-E_2, but increased cell survival and blunted intracellular Ca^{2+} increases. However, these estrogens failed to reduce cytosolic reactive oxygen species, even at concentrations as high as 10 μM. In conclusion, estrogens exert protective effects against oxidative stress by inhibiting lipid peroxidation and subsequently preserving Ca^{2+} homeostasis, mitochondrial membrane potential, and ATP levels.

In addition to their well established role as female sex hormones, estrogens have been shown to serve as neurotrophic and neuroprotective agents. Epidemiological studies show that early estrogen therapy can reduce the risk of neurodegenerative diseases such as Alzheimer's disease and improve cognition and memory in AD patients (Henderson et al., 1994; Birge, 1996; Sherwin, 1996; Tang et al., 1996; Yaffe et al., 1998; Costa et al., 1999; Bagger et al., 2005). In addition, estrogen therapy is associated with decreased incidence and enhanced recovery from ischemic stroke. In in vitro studies, protective effects of estrogen have been widely reported in different types of neuronal cells against a variety of insults, including H_2O_2 (Behl et al., 1995, 1997; Sawada et al., 1998; Singer et al., 1998; Moosmann and Behl, 1999; Green et al., 2000), serum deprivation (Bishop and Simpkins, 1994; Green et al., 1997a,b; Bae et al., 2000), oxygen-glucose deprivation (Regan and Guo, 1997; Wilson et al., 2000), iron (Goodman et al., 1996; Blum-Degen et al., 1998), amyloid β peptide-induced toxicity (Behl et al., 1995, 1997; Green et al., 1996; Gridley et al., 1997; Mattson et al., 1997; Pike, 1999), excitotoxicity (Goodman et al., 1996; Singer et al., 1996, 1999; Regan and Guo, 1997; Zaulyanov et al., 1999; Green and Simpkins, 2000), and mitochondrial toxins such as 3-nitropropionic acid (Wang et al., 2001a), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (De Girolamo et al., 2001), and sodium azide (Regan and Guo, 1997).

In in vivo studies, the neuroprotective effects of estrogens

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ABBREVIATIONS: E_2, estrogen; ROS, reactive oxygen species; ER, estrogen receptor; AM, acetoxymethyl ester; ICI 182,780, fulvestrant; DCFH-DA, 2,7-dichlorofluorescin diacetate; NAO, nonyl acridine orange; TMR, tetramethylrhodamine; rhod, rhodamine; TBA, 2-thiobarbituric acid; PBS, phosphate-buffered saline; HBSS, HEPES-buffered salt solution; $\Delta\Psi_m$, mitochondrial membrane potential; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive substances; GSH, reduced glutathione; ANOVA, analysis of variance.

have been demonstrated in a variety of models of acute cerebral ischemia. These include transient and permanent middle cerebral artery occlusion models (Simpkins et al., 1997; Alkayed et al., 1998; Dubal et al., 1998), global forebrain ischemia models (Sudo et al., 1997), photothrombotic focal ischemia models (Fukuda et al., 2000), and glutamate-induced focal cerebral ischemia models (Mendelowitsch et al., 2001). The protective effects of estrogens have been described in rats, mice, and gerbils (Simpkins et al., 1997; Culmsee et al., 1999; Chen et al., 2001). Estrogen-induced neuroprotection has been demonstrated in adult female rats, middle-aged female rats, and reproductively senescent female rats (Wise et al., 2001). Furthermore, nonfeminizing estrogens, including 17α-estradiol (17α-E_2), a weak natural estrogen, and *ent*-E_2, the enantiomer of 17β-estradiol (17β-E_2), showed neuroprotective effects (Green et al., 2001; Liu et al., 2002; Perez et al., 2005). As shown in Fig. 1, 17α-, 17β-, and *ent*-E_2 have phenolic A-ring, which is a key structure in neuroprotection by estrogen.

Oxidative stress, bioenergetic impairment, and mitochondrial failure have all been implicated in the etiology of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and stroke. Mitochondria are unique organelles in their involvement in the consumption of oxygen, production of ATP and oxygen radicals, and mobilization of calcium (Gunter and Pfeiffer, 1990; Melov, 2000). As the major source of intracellular ATP and free radicals, mitochondria sit at a strategic position of life–death decision of a cell. H_2O_2 is a major reactive oxygen species (ROS) and a by-product of normal cellular function produced by superoxide dismutase and monoamine oxidase.

The aim of the present study was to investigate the protective effects of estrogens on mitochondrial function against a major ROS, H_2O_2. To illuminate the role of estrogen receptors in mitochondrial protection, we evaluated the effects of two nonfeminizing estrogens, 17α-E_2 and *ent*-E_2, and assessed estrogen effects in the presence of an estrogen receptor (ER) antagonist, fulvestrant (ICI 182,780).

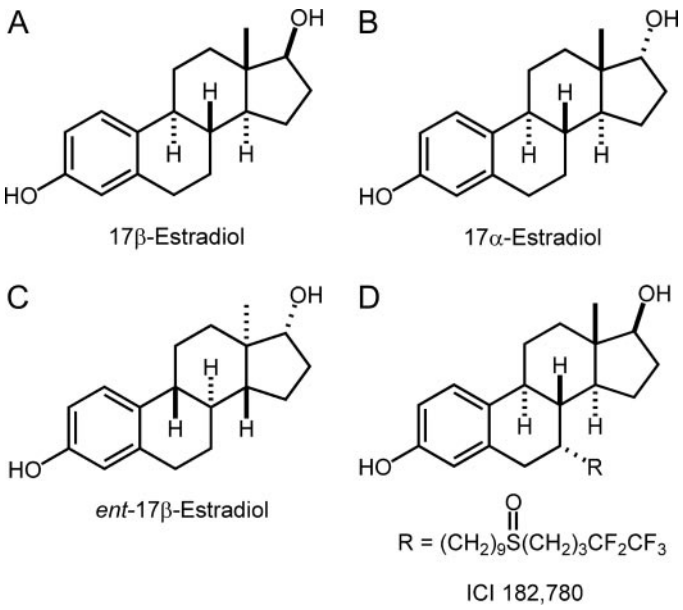

Fig. 1. Structures of estrogens and ER antagonist ICI 182,780. A, 17β-E_2. B, 17α-E_2. C, *ent*-E_2. D, ICI 182,780.

Materials and Methods

Chemicals. 17β-E_2 and 17α-E_2 were purchased from Steraloids (Newport, RI). ICI 182,780 was purchased from Tocris Cookson Inc. (Ellisville, MO). The enantiomer of 17β-estradiol, *ent*-E_2, was synthesized using methods that we have described previously (Green et al., 2001). H_2O_2 was purchased from Mallinckrodt Baker Inc. (Paris, KY). Calcein AM, 2,7-dichlorofluorescin diacetate (DCFH-DA), nonyl acridine orange (NAO), tetramethylrhodamine (TMR), Fura-2 AM, rhod-2 AM, and ATP determination kits were purchased from Molecular Probes (Eugene, OR). Trichloroacetic acid, 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, and HCl were purchased from Sigma-Aldrich (St. Louis, MO).

All steroids and ICI 182,780 were dissolved in ethanol at a final concentration of 10 mM and diluted to appropriate concentration in culture media as required. Unless otherwise stated, steroid treatments of cell cultures involved a 2-h preincubation followed by coadministration of the steroid with H_2O_2. Hereafter, this treatment is referred to as pretreatment. Those cells receiving vehicle (in place of estradiol) pretreatment were maintained in fresh culture medium at the same final ethanol concentration. Control cells were maintained in culture medium with appropriate changes of fresh medium. In experiments involving the estrogen receptor antagonist, ICI 182,780 was added 30 min before addition of 17β-E_2. H_2O_2 was diluted with culture media to final concentration before use.

Cell Culturing. SK-N-SH human neuroblastoma cells were obtained from American Type Culture Collection (Manassas, VA) at passage 38 and were grown to confluence in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT/Tissue Culture Biologicals, Tulare, CA), 20 μg/ml gentamicin (Sigma-Aldrich) in monolayers in plastic Nunc 75-cm^2 flasks (Fisher Scientific Co., Orlando, FL) at 37°C and under 5% CO_2, 95% air. Medium was changed three times weekly. Cells were observed with a phase-contrast microscope (Nikon Diaphot-300). SK-N-SH cells were back-cultured every 5 to 7 days using standard trypsinization procedures to maintain the cell line. SK-N-SH cells were used in passages 39 to 48.

Cell Viability Assay. Cell viability was determined using Calcein AM assay. SK-N-SH cells were plated at a density of 20,000 cells/well in 96-well plates 72 h before initiation of experiments. Cells were exposed to H_2O_2 (100 or 150 μM) for 18 h. Then, cells were rinsed with PBS, pH 7.4, and viability was assessed by calcein AM assay as described previously (Green et al., 2001). Percentage of viability was calculated by normalization of all values to the H_2O_2-free control group (=100%).

Intracellular Calcium Measurements. Cytosolic Ca^{2+} concentrations were measured using the ratiometric fluorescent indicator dye Fura-2 AM (Molecular Probes). SK-N-SH cells were plated on 25-mm coverslips in 35-mm dishes at the density of 6.0~7.0 $\times$ 10^5 cells/ml/dish 24 h before experimentation. Confluent SK-N-SH cell monolayers in the presence or absence of E_2 pretreatment grown on coverslips were incubated at 37°C in RPMI 1640 medium containing 3 μM Fura-2 AM for 30 min. Then, coverslips were washed with HEPES-buffered salt solution (HBSS, containing 145 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose; pH adjusted to 7.4 with NaOH) three times to remove excess Fura-2 AM. Each coverslip then was inserted into the microincubator chamber of a MetaFluor system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Three milliliters of HBSS containing vehicle (0.01% ethanol, estrogens, or ICI 182,780) was added into the chamber. Treatment with H_2O_2 was carried out by adding the appropriate concentrations into the chamber. The excitation wavelength was alternated between 340 and 380 nm, and emission fluorescence was recorded at 510 nm. The fluorescence ratio was calculated as F_{340}/F_{380}. The system was calibrated using solutions containing either no Ca^{2+} or a saturating level of Ca^{2+}. The equivalent in Ca^{2+} concentration was calculated using the formula $[Ca^{2+}]_i = K_d [(R - R_{min})/$

$(R_{max} - R)]$ (F_{min}/F_{max}). Data were analyzed using Prism 3.0 (GraphPad Software Inc., San Diego, CA).

Mitochondrial Calcium Measurement. Mitochondrial Ca^{2+} concentrations were measured using the mitochondrial-specific fluorescent indicator dye rhod-2 AM (Molecular Probes). SK-N-SH cells were plated on coverslips and cultured to reach 50% confluence. The cells were incubated with 1 mM rhod-2 AM for 45 min at 37°C and then washed five to six times with HBSS. The coverslips were placed into a 25-mm cell chamber (ALA Scientific Instruments Inc., Westbury, NY) followed by loading 3 ml of HBSS containing estrogens or vehicle. After stabilization for several minutes, H_2O_2 was added to reach a final concentration of 150 μM. The calcium variations were monitored by fluorescence confocal microscopy (Carl Zeiss GmbH, Jena, Germany) with excitation/emission of 568/590 nm. A time series of seven confocal images at 5-min intervals were recorded in each experiment. The time series were analyzed using the software system LSM 410 invert laser scan microscope. Fluorescence intensity was analyzed using CImaging software (Compix Inc., Imaging System, Cranberry Township, PA).

Measurement of ATP Levels. Experiments were initiated by plating SK-N-SH cells at a density of 1.0×10^6 cells/well in 12-well plates. Forty-eight hours later, cells were exposed to 150 μM H_2O_2 for 1 h in the presence or absence of estrogens (17β-E_2, 17α-E_2, and *ent*-E_2). Cellular ATP levels were quantified using a luciferin and luciferase-based assay (Garewal et al., 1986). Cells were rinsed with PBS and lysed with ATP-releasing buffer containing 100 mM potassium phosphate buffer at pH 7.8, 2 mM EDTA, 1 mM dithiothreitol, and 1% Triton X-100; 10 μl of the lysate was taken for protein determination. Another 10 μl of the lysate was added to a Nunc 96-well plate. ATP concentrations in lysates were quantified using an ATP determination kit (Molecular Probes) according to the manufacturer's instruction. The 96-well plates were read using a SpectraMax GeminiXS plate reader (Molecular Devices, Sunnyvale, CA). A standard curve was generated using solutions of known ATP concentrations. ATP levels were calculated as nanomolar ATP per milligram of protein and normalized to levels in untreated control cultures.

Monitoring Mitochondrial Membrane Potential. For $\Delta\Psi_m$ determination, 24 h before assay, cells were plated in clear-bottom, black-walled, 96-well plates (Costar 3606; Corning Glassworks, Corning, NY). Cells were plated at 60,000/well for use in high-throughput screening protocols as described previously (Green et al., 2000, 2001).

Mitochondrial membrane potential was measured in intact cells using an assay based on a fluorescence quenching assay between two dyes: NAO (Molecular Probes) that stains cardiolipin, a lipid found exclusively in the mitochondrial inner membrane; and TMR (Molecular Probes), a potentiometric dye taken up by mitochondria in accordance with Nernstian dictates of potential and concentration. The presence of TMR quenches NAO emission in proportion to $\Delta\Psi_m$, whereas loss of $\Delta\Psi_m$ with consequent efflux of TMR reduces the quenching of NAO fluorescence. The high specificity of NAO staining; selective monitoring of the fluorescence emitted by NAO, not TMR; and the stringent requirement for colocalization of both dyes within the mitochondrion all act in concert to allow the fluorescence quenching assay to report $\Delta\Psi_m$ unconfounded by background signal arising from potentiometric dye responding to plasma membrane potential.

Lipid Peroxidation Measurement. Lipid peroxidation was monitored by measuring malondiadehyde (MDA), a stable end product of lipid peroxidation cascades using the thiobarbituric acid reactive substances (TBARS) assay. As one of the main compounds among TBARS, MDA reacts with TBA under acid conditions and high heat, and the product of this reaction can be detected spectrometrically or fluorometrically. SK-N-SH cells were plated in 60-mm dishes at the density of 2.0×10^6 cells/dish 48 h before experiments. After 2-h preincubation of estrogens or vehicle, cells were exposed to 150 μM H_2O_2 for 5 min with the presence of estrogens or vehicle. Cells were washed twice with ice-cold PBS and harvested with 0.6 ml/dish ice-cold PBS using rubber policeman. Then, cells were homogenized by sonication. To prevent sample oxidation during homogenization, 0.5 M BHT (10 μl/ml cell suspension) was added before sonication. Cell homogenates were centrifuged at 3000*g* at 4°C for 10 min. The clear supernatant was used for TBARS assay and protein determination. For MDA measurement, 100 μl of sample was added into 48-well plate followed by addition of a solution containing 1% TBA, 12.5% trichloroacetic acid, and 0.8 N HCl. Reaction mixtures were incubated at 50°C for 60 min, and then precipitated proteins were removed by centrifuging at 12,000 rpm for 2 min. Supernatants were transferred to 96-well plates, and relative fluorescence values were determined using a BioTek FL600 plate reader (BioTek, Highland Park, VT) at an excitation wavelength of 530 ± 25 nm, emission wavelength of 590 ± 20 nm, and sensitivity of 100. External standards used in the TBARS assay were made from 1,1,3,3-tetramethoxypropane in reagent grade ethanol and diluted in 0.9% normal saline to give concentrations ranging from 0 to 20 μM.

Measurement of Cytosolic ROS. The extent of cytosolic cellular oxidative stress was estimated by monitoring the amount of ROS by the fluorescent dye DCFH-DA. Cells were plated 24 h before initiation of the experiment at a density of 15,000 cells/well in 96-well plates. Cells were loaded with DCFH-DA at a final concentration of 50 μM for 45 min. After incubation, DCFH-DA was removed, and cells were washed twice with PBS, pH 7.4, and incubated with minimum Eagle's medium containing 20% fetal bovine serum with a bolus dose of 50 or 100 μM H_2O_2 for 10 to 60 min. DCF2,7-dichlorofluorescin fluorescence was determined at an excitation of 485 nm and an emission of 538 nm using an FL600 microplate-reader (BioTek, Highland Park, VT). Values were normalized to percentage of untreated control groups.

Reduced Glutathione Measurement. Cellular GSH levels were determined using Northwest Life Science Specialties Glutathione Assay kit (Northwest Life Science Specialties, LLC, Vancouver, WA). Cells were seeded in 100-mm dishes at the density of 5.0×10^6 cells/dish. Forty-eight hours later, cells were exposed to various treatments. After treatments, cells were washed with PBS twice, and samples were collected as manufacturer's direction. GSH levels were normalized to the protein concentrations.

Protein Assays. Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin at concentrations ranging from 0 to 1 mg/ml as a standard curve.

Data Analysis. All data are presented as mean ± S.E.M. Comparisons between estrogen-treated groups and vehicle (H_2O_2 + vehicle) groups were performed using one-way ANOVA with Tukey's multiple comparisons test. For all tests, $p < 0.05$ was considered significant.

Results

Effects of Estrogens on H_2O_2-Induced Cell Death in SK-N-SH Cells. As shown in Fig. 2A, 18-h exposure to 150 μM H_2O_2 induced a 50% decline in cell viability. With 2-h pretreatment, 17β-E_2 dose dependently increased cell survival. At the concentration of 100 nM, 17β-E_2 increased cell survival from 49 ± 2 to 64 ± 3%. One μM 17β-E_2 increased cell viability from 45 ± 7 to 74 ± 5%.

Two nonfeminizing estrogens, 17α-E_2 and *ent*-E_2, also showed protective effects against H_2O_2 toxicity. At the concentration of 1 μM, 17α-E_2 and *ent*-E_2 enhanced cell viability from 57 ± 1% to 72 ± 2 and 91 ± 2%, respectively (Fig. 2B). ICI 182,780 alone (300 nM) significantly protected from H_2O_2 toxicity and did not block the protection from 100 nM 17β-E_2 (Fig. 2C).

Effects of Estrogens on H_2O_2-Induced $[Ca^{2+}]_i$ Increase. After 30-min incubation, 150 μM H_2O_2 increased

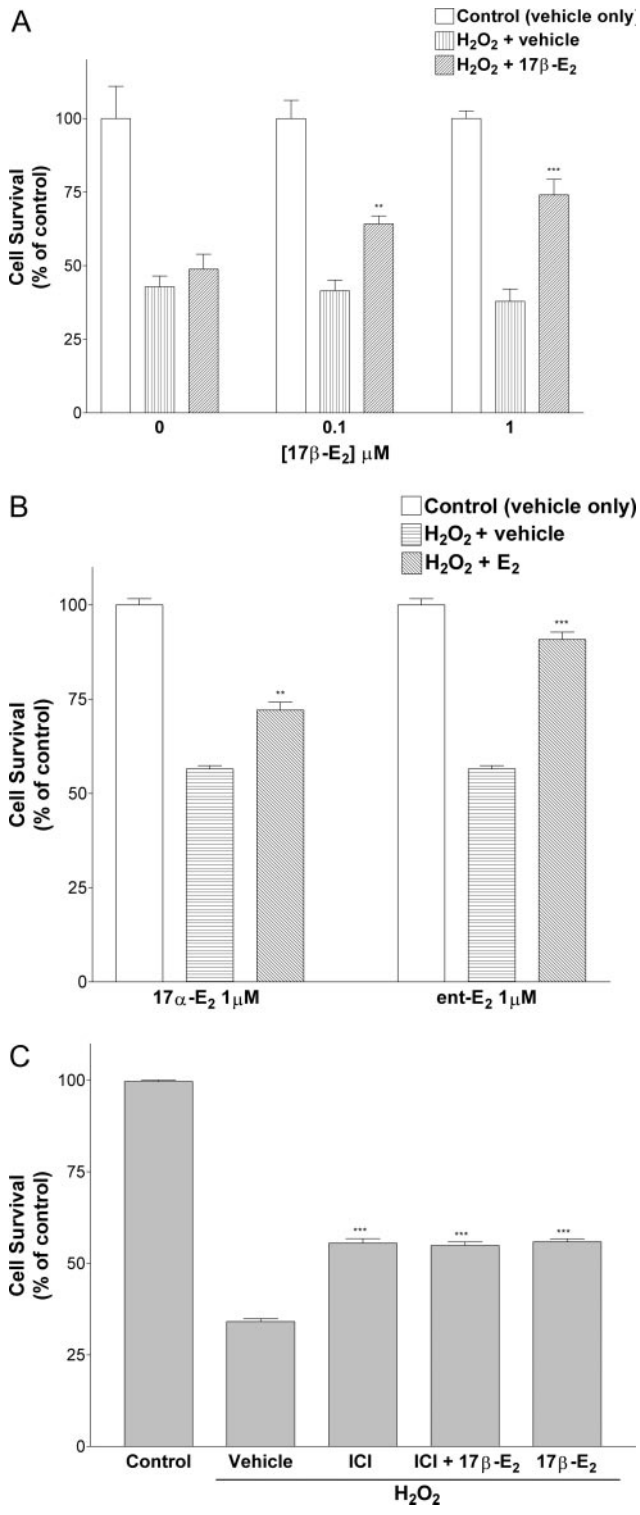

Fig. 2. A, effects of 2-h pretreatment with 17β-E_2 on 150 μM H_2O_2-induced cell death. Data are expressed as mean ± S.E.M. **, $p < 0.01$ versus vehicle (vehicle + H_2O_2) groups; ***, $p < 0.001$ versus vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test. B, effects of nonfeminizing estrogens 17α- and *ent*-E_2 on 150 μM H_2O_2-induced cell loss. Data are expressed as mean ± S.E.M. **, $p < 0.01$ versus vehicle (vehicle + H_2O_2) groups; ***, $p < 0.001$ versus vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test. C, effects of ER antagonist ICI 182,780 on protective effects of 17β-E_2 against H_2O_2 (150 μM) toxicity in SK-N-SH cells. Data are expressed as mean ± S.E.M. If S.E.M. values are not shown, the error was too small to be depicted. ***, $p < 0.001$ versus vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

$[Ca^{2+}]_i$ to 460 ± 35 nM, whereas $[Ca^{2+}]_i$ in vehicle only treated groups was approximately 80 ± 7 nM. Two-hour pretreatment with 17β-E_2 dose dependently reduced the increase in $[Ca^{2+}]_i$ induced by H_2O_2. One hundred nanomolar 17β-E_2 reduced $[Ca^{2+}]_i$ levels to 247 ± 11 nM, and 1 μM 17β-E_2 lowered $[Ca^{2+}]_i$ levels to 162 ± 9 nM (Fig. 3A).

With 2-h pretreatment, both 17α-E_2 and *ent*-E_2 significantly inhibited H_2O_2-induced $[Ca^{2+}]_i$ increase (Fig. 3B). At 1 μM concentration, 17α-E_2 and *ent*-E_2 reduced the H_2O_2 effects on $[Ca^{2+}]_i$ by 64 and 56%, respectively. The protective effects of 17β-E_2 on H_2O_2-induced $[Ca^{2+}]_i$ increase were consistently not blocked by coadministration of 300 nM ICI 182,780. On the other hand, ICI 182,780 alone caused a 57% decrease in $[Ca^{2+}]_i$ in cells exposed to H_2O_2 (Fig. 3C).

Effects of Estrogens on H_2O_2-Induced Mitochondrial Calcium Loading. Administration of 150 μM H_2O_2 increased mitochondrial calcium level to 147 ± 6% of baseline (Fig. 4), which was completely blocked by 2-h pretreatment of 1 μM 17β-E_2. Furthermore, 17α-E_2 and *ent*-E_2 at 1 μM had similar effect on mitochondrial calcium protection against H_2O_2 insult (Fig. 4).

Effects of Estrogens on H_2O_2-Induced ATP Depletion. Exposure to either 100 or 150 μM H_2O_2 triggered a rapid decrease in intracellular ATP levels. Within 1 h, 100 and 150 μM H_2O_2 reduced ATP levels to 44 ± 5 and 43 ± 3% of controls, respectively. ATP levels recovered after 2 h of exposure to H_2O_2, and then declined again; a temporal response that we previously reported in human lens epithelial cells (Wang et al., 2003). After 8 to 12 h, ATP levels were approximately 40% of control (data not shown).

As shown in Fig. 5A, 17β-E_2 protected SK-N-SH cells against H_2O_2-induced ATP depletion. Two-hour pretreatment with 17β-E_2 dose dependently reversed the decline of intracellular ATP induced by 100 μM H_2O_2. The ATP levels were completely restored to normal by 1 μM 17β-E_2 (from 58 ± 5 to 98 ± 12% of control). Similar effects were also demonstrated in 17α-E_2 and *ent*-E_2 (Fig. 5, B and C). At the concentration of 100 nM and 1 μM, 17α-E_2 restored intracellular ATP levels from 56 ± 6% to 68 ± 2 and 89 ± 7% of control, respectively. One micromolar *ent*-E_2 inhibited H_2O_2-induced ATP declines from 37 ± 4 to 59 ± 5%.

Effects of Estrogens on Mitochondrial Membrane Potential Collapse Caused by H_2O_2. As an acute cytotoxic stimulus, 30-min exposure of 3.0 mM H_2O_2 resulted in $\Delta\Psi_m$ collapse in SK-N-SH cells. As might be expected, the concentration of H_2O_2 required to cause acute collapse of $\Delta\Psi_m$ was substantially more than the concentration required for long-term cytotoxicity studies. 17β-E_2 significantly reduced the magnitude of $\Delta\Psi_m$ collapse induced by H_2O_2 (Fig. 6A). Treatments with 17α-E_2 and *ent*-E_2 at 1 μM reduced the magnitude of $\Delta\Psi_m$ collapse but not as effectively as 17β-E_2 (Fig. 6B).

Effects of Estrogens against H_2O_2-Induced Lipid Peroxidation. Lipid peroxidation levels were monitored by measuring a stable end product of lipid peroxidation cascades, MDA using TBARS assay. As shown in Fig. 7, under normal conditions, cellular MDA levels are approximately 1.52 ± 0.06 nmol/mg of protein. Exposure of 150 μM H_2O_2 for 5 min increased MDA levels to 2.51 ± 0.11 nmol/mg of protein. At concentrations ranging from 1 nM to 1 μM, 17β-, 17α-, and *ent*-E_2 all significantly and dose dependently attenuated lipid peroxidation. Among three compounds, 17α-E_2

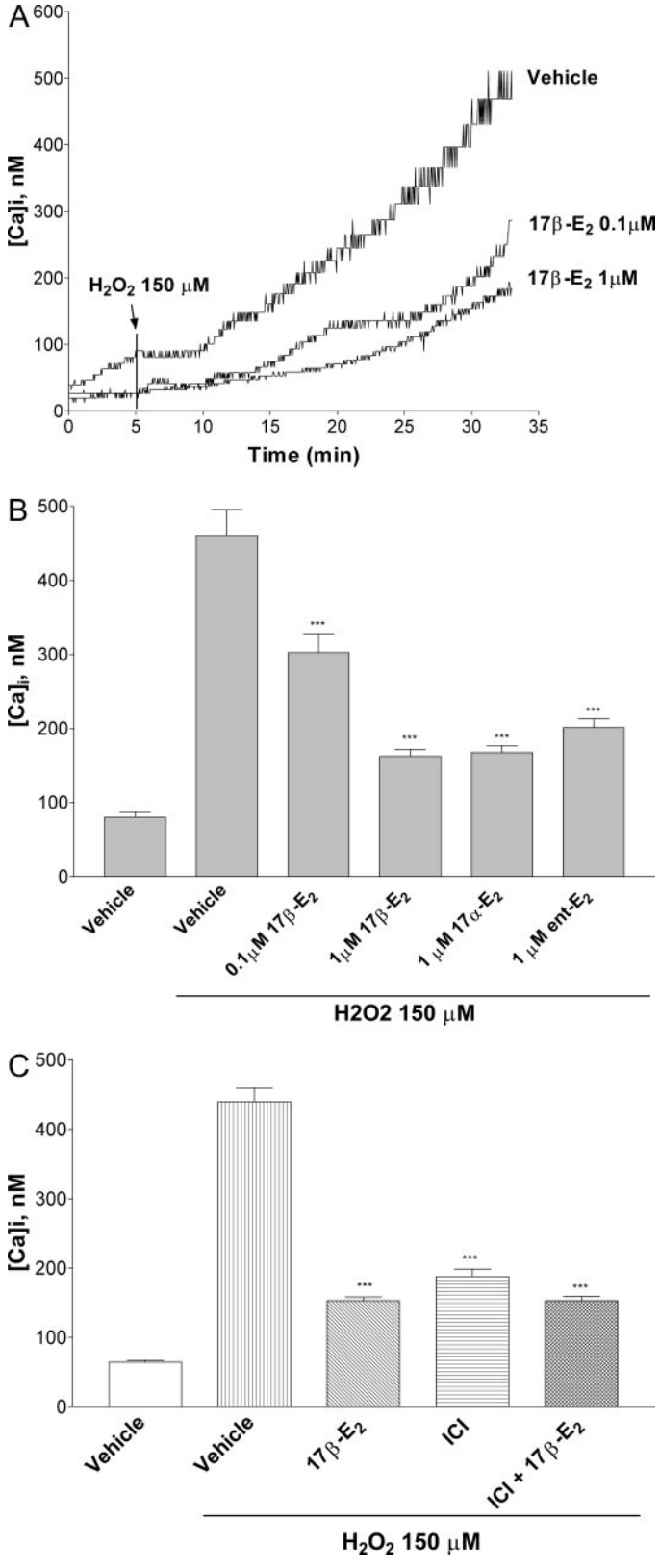

Fig. 3. A, dose-response effects of 17β-E_2 on 150 μM H_2O_2 (30-min)-induced $[Ca^{2+}]_i$ increase. B, effects of estrogens on 150 μM-H_2O_2 (30-min)-induced $[Ca^{2+}]_i$ increase. Data are expressed as mean ± S.E.M. ***, $p < 0.001$ versus vehicle (vehicle + 150 μM H_2O_2) groups as determined by one-way ANOVA by Tukey's test. C, effects of ER antagonist ICI 182,780 on protection by 17β-E_2 against 150 μM H_2O_2 (30-min)-induced $[Ca^{2+}]_i$ dyshomeostasis. Data are expressed as mean ± S.E.M. ***, $p < 0.001$ versus vehicle (vehicle + 150 μM H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

showed highest efficacy. At 1 μM, 17β-, 17α-, and *ent*-E_2 reduced MDA levels to from 2.51 ± 1.1 nmol/mg of protein to 1.51 ± 0.10, 1.27 ± 0.05, and 1.39 ± 0.16 nmol/mg of protein, respectively.

Effects of Estrogens against H_2O_2-Induced GSH Depletion. To further investigate estrogens antioxidant properties, we examined their effects on intracellular antioxidant-GSH levels. Six-hour treatment of H_2O_2 reduced intracellular total GSH levels from 18.5 ± 0.4 to 10.7 ± 0.7 nmol/mg of protein in SK-N-SH cells. Three estrogens, 17β-, 17α-, and *ent*-E_2 all significantly alleviated GSH depletion at concentrations ranging from 1 nM to 1 μM. At 1 μM, estrogens enhanced GSH concentrations to 87% or higher of normal levels (Fig. 8).

Concentrations of 17β-E_2 ranging from 1 nM to 10 μM failed to reduce cytosolic ROS, as determined by DCFH-DA fluorescence (data not shown).

Discussion

In the present study, we demonstrated that in human neuroblastoma SK-N-SH cells cultures, short-term 17β-, 17α-, and *ent*-E_2 protected against H_2O_2-incuded toxicity. The protective effects of the these three estrogens includes a potent attenuation of lipid peroxidation, enhanced cell survival, attenuated ATP depletion, alleviated intracellular calcium elevation, and ablated mitochondrial calcium loading and subsequent mitochondrial membrane potential maintenance. Furthermore, the ER antagonist ICI 182,780 did not block effects of 17β-E_2 but increased cell survival and blunted intracellular calcium increase induced by H_2O_2. These data suggest that the protective effects of estrogens in SK-N-SH cells are independent of ER-mediated genomic effects and are likely to involve a potent protection from lipid peroxidation.

ROS are implicated in neuronal damage and neurodegenerative diseases such as stroke and Alzheimer's disease (Brunelle and Rauk, 2002). Exposure to H_2O_2 induces a robust increase in ROS in cells; followed by oxidation of lipids, proteins, and DNA; increase in intracellular calcium; glutathione depletion; mitochondria dysfunction; caspase-3 activa-

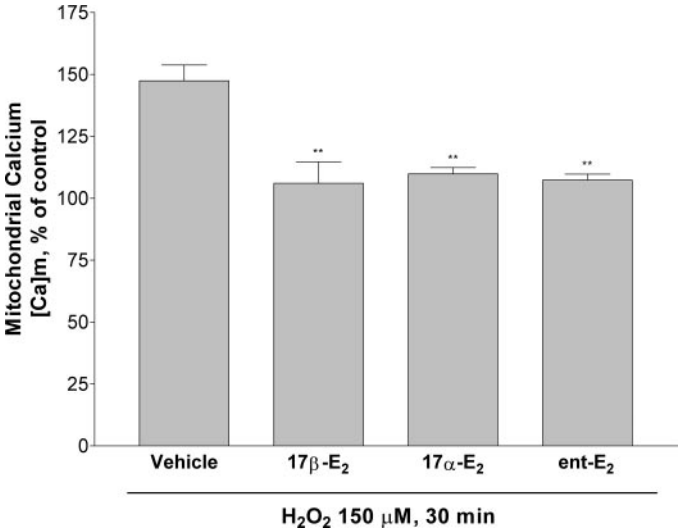

Fig. 4. Effects of estrogens 17β-, 17α-, and *ent*-E_2 on 150 μM H_2O_2 (30-min)-induced mitochondrial calcium loading. Data are expressed as mean ± S.E.M. **, $p < 0.01$ versus vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

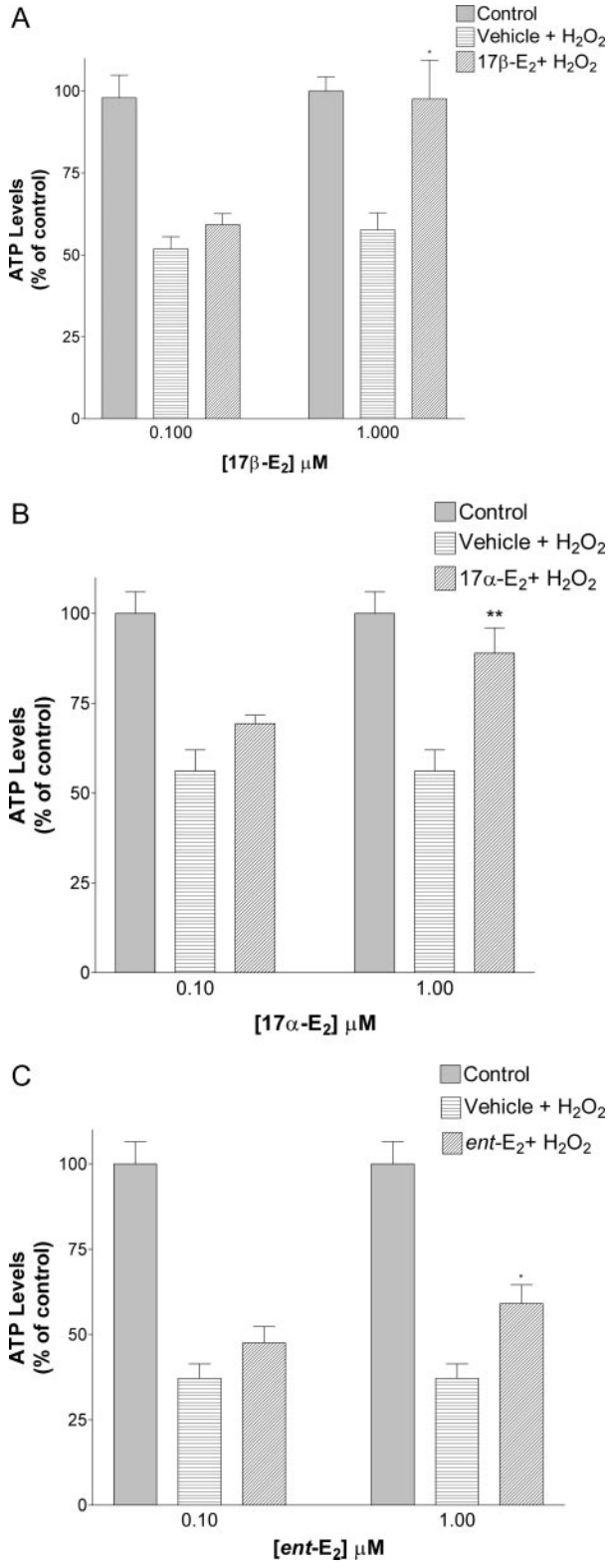

Fig. 5. A, dose-response effects of 2-h pretreatment of 17β-E_2 against 150 μM H_2O_2 (1-h)-induced ATP reduction. Data are expressed as mean ± S.E.M. *, $p < 0.05$ versus vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test. B, effects of 17α-E_2 against 150 μM H_2O_2 (1-h)-induced ATP depletion. Data are expressed as mean ± S.E.M. **, $p < 0.01$ versus vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test. C, effects of *ent*-E_2 against 150 μM H_2O_2 (1-h)-induced ATP depletion. Data are expressed as mean ± S.E.M. *, $p < 0.05$ versus vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

tion; and subsequent necrotic and apoptotic cell death. ROS have been shown to allow the influx of calcium, and this effect of oxidation is implicated in many neurodegenerative diseases (Gibson et al., 2002; Mattson, 2003; Zheng et al., 2003) Likewise, rapid disruption in cellular calcium leads to apoptosis (Simpkins et al., 2005). In our study, exposure to 150 μM H_2O_2 induces a rapid 4- to 5-fold elevation of the cellular free calcium levels within 30 min. After 18 h treatment, approximately 50% of cells died, which was accompanied by a dramatic increase in caspase-3 activation (data not shown). These findings agree with other studies showing that an early increases in intracellular calcium results in apoptosis (Yu et al., 2001). All three estrogens ablated H_2O_2-induced $[Ca^{2+}]_i$ increases. Furthermore, ER antagonist ICI 182,780 not only did not affect the protection by 17β-E_2 but also significantly attenuated H_2O_2-induced $[Ca^{2+}]_i$ increase. This agonist activity of ICI 182,780 has also been observed by other laboratories (O'Neill and Brinton, 2004).

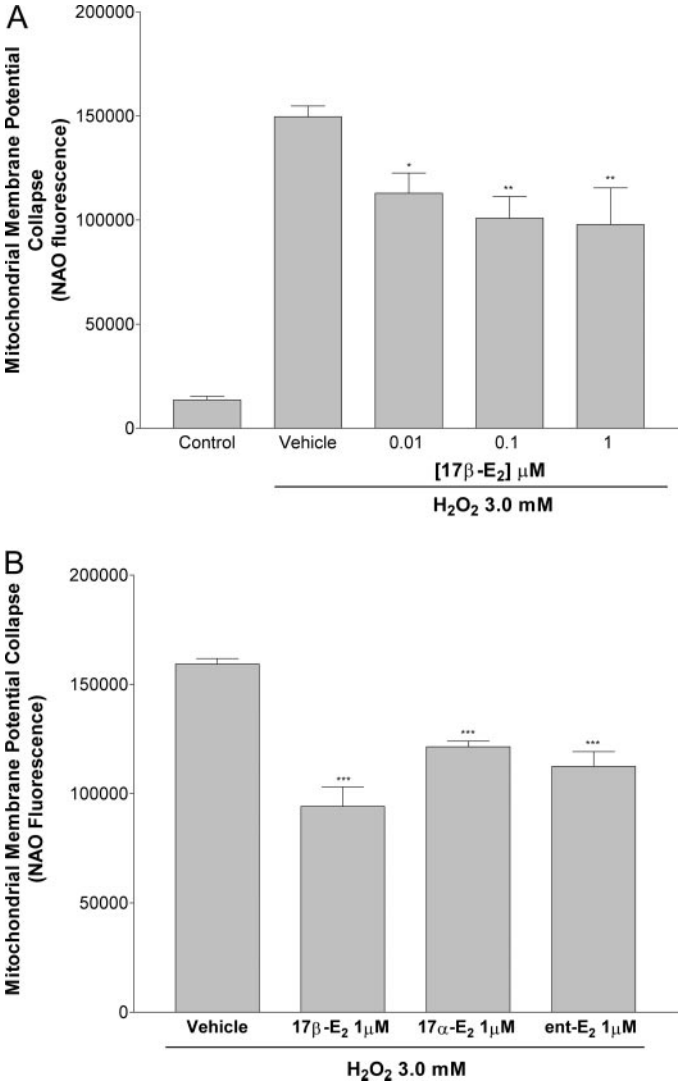

Fig. 6. A, dose-response effects of 17β-E_2 against 3.0 mM H_2O_2 (30-min)-induced mitochondrial collapse. *, $p < 0.05$ versus vehicle (vehicle + H_2O_2) groups; **, $p < 0.01$ versus vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test. B, effects of nonfeminizing estrogens 17α- and *ent*-E_2, against 3.0 mM H_2O_2 (30-min)-induced mitochondrial potential collapse. Data are expressed as mean ± S.E.M. ***, $p < 0.001$ versus vehicle (vehicle + H_2O_2) groups as determined by one-way ANOVA by Tukey's test.

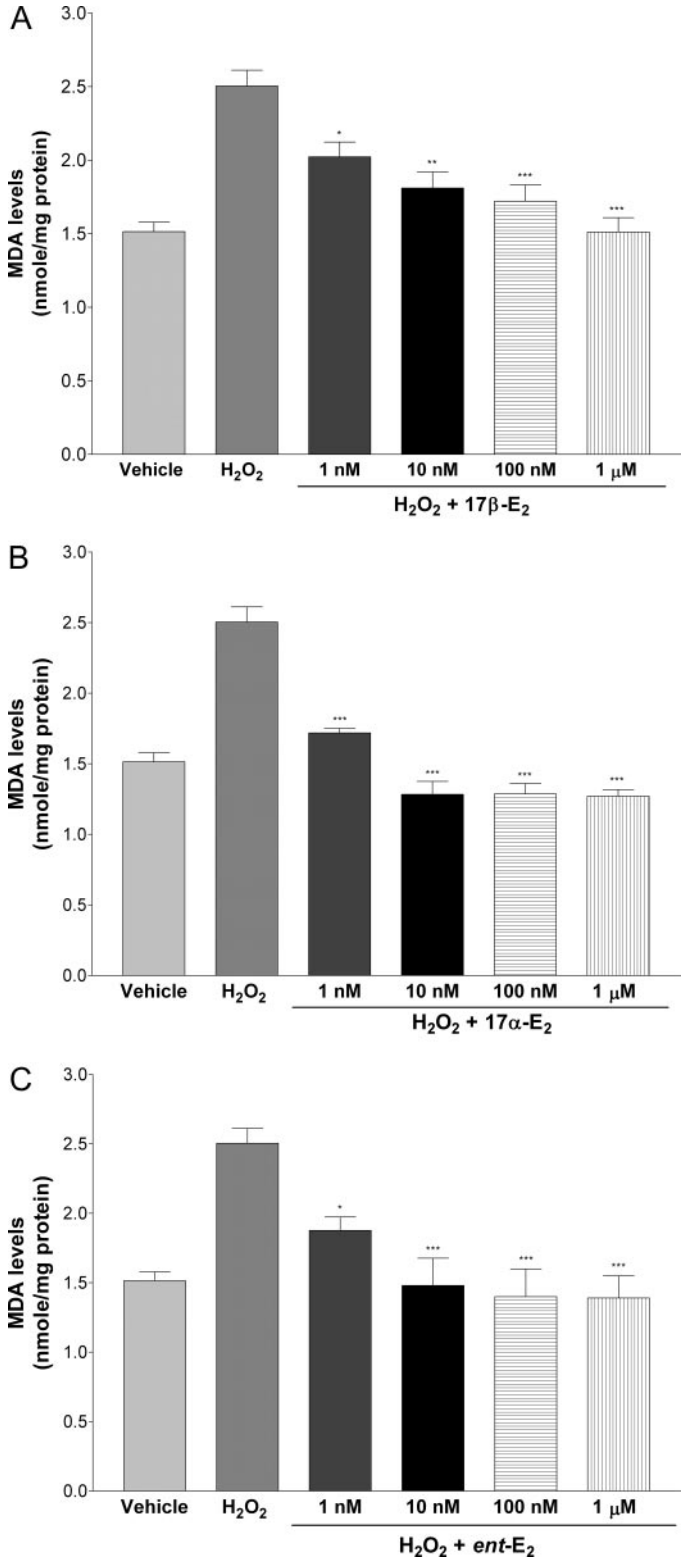

Fig. 7. Effects of estrogens on 150 μM H_2O_2-induced lipid peroxidation in SK-N-SH cell culture. Lipid peroxidation was determined by the method of TBARs and expressed as amount of MDA. A, 17β-E_2. B, 17α-E_2. C, *ent*-E_2. Data are expressed as mean ± S.E.M. If S.E.M. values are not shown, the error was too small to be depicted. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. Comparisons are performed between estrogen-treated groups (E_2 + H_2O_2) and corresponding vehicle groups (vehicle + H_2O_2) using one-way ANOVA by Tukey's tests.

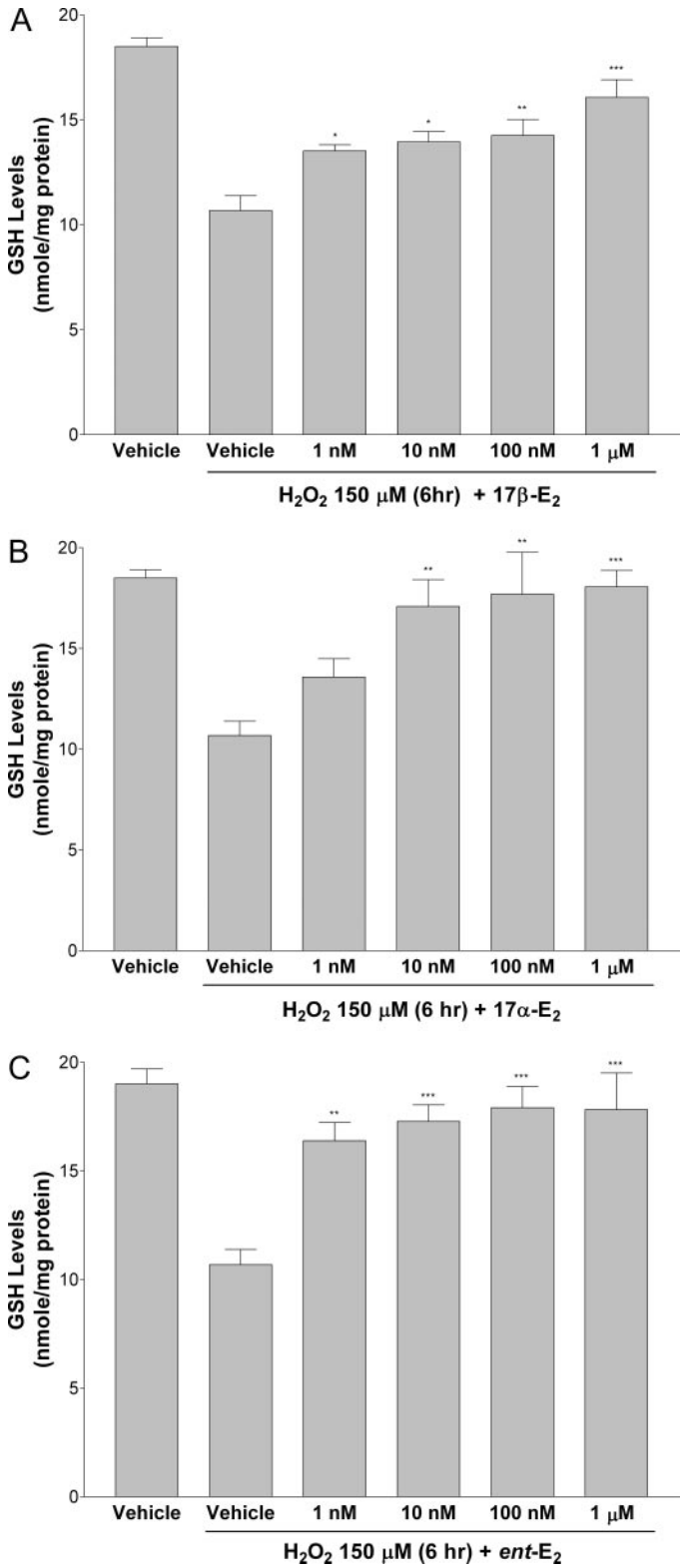

Fig. 8. Effects of estrogens on 150 μM H_2O_2-induced GSH depletion in SK-N-SH cells. A, 17β-E_2. B, 17α-E_2. C, *ent*-E_2. Data are expressed as mean ± S.E.M. **, $p < 0.01$; ***, $p < 0.001$. Comparisons are performed between estrogen treated groups (E_2 + H_2O_2) and corresponding vehicle groups (vehicle + H_2O_2) using one-way ANOVA by Tukey's tests.

The mechanisms involved in attenuation of $[Ca^{2+}]_i$ elevation by estrogen is not known. 17β-E_2 may exert this protective effect through both preventing extracellular calcium influx and inhibiting calcium release from intracellular calcium stores. Studies suggest that estradiol affects the Na^+-Ca^{2+} exchanger (Cross et al., 1998; Sugishita et al., 2001). Estrogen has been shown to modulate L-type Ca^{2+} channels in neuronal cells (Kim et al., 2000). In glial cells, estrogen has been demonstrated to inhibit both *N*-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, thereby, reducing Ca^{2+} influx (Lopez et al., 1997). The attenuation of $[Ca^{2+}]_i$ elevation in the early phase of H_2O_2 insult may contribute to the overall protective effects of estrogens.

Mitochondria are a major intracellular calcium store and mitochondrial calcium concentrations are critical in maintaining mitochondrial membrane potential and oxidative phosphorylation. Mitochondrial calcium overloading leads to mitochondrial membrane potential collapse and initiates cell death. Exposure of 150 μM H_2O_2 for 30 min induced a 50% increase in mitochondrial calcium levels. All three estrogens blunted H_2O_2-induced mitochondrial calcium influx. Studies from our laboratory and others also have shown that 17β-E_2 attenuates mitochondrial calcium overloading against oxidative stress (Wang et al., 2001b; Nilsen and Diaz Brinton, 2003). Mitochondrial calcium loading depends on uptake through the uniporter and efflux by Na^+-Ca^{2+} exchanger on mitochondrial membrane (Crompton et al., 1978). It has been shown that 17β-E_2 increases Na^+-dependent calcium efflux exponentially at concentrations above 10 nM in synaptosomal mitochondria (Horvat et al., 2000). The ability of estrogens to maintain mitochondrial calcium levels may be closely related to their modulatory effect on intracellular calcium homeostasis and mitochondrial sequestration of calcium under oxidative stress.

In the present study, we demonstrate that three estrogens restored cellular ATP levels against H_2O_2 toxicity. These results are consistent with previous studies on the effect of estrogen on ATP levels against various stressors. 17β-E_2 has shown to protect against ATP depletion, mitochondrial membrane potential decline, and the generation of reactive oxygen species induced by mitochondrial toxin 3-nitropropionic acid (Wang et al., 2001a). 17β-, 17α-, and *ent*-E_2 can protect human lens epithelial cells against H_2O_2-induced ATP depletion and mitochondrial potential collapse (Wang et al., 2003). E_2 stabilizes mitochondrial function against actions of mutant presenilin-1 (Mattson et al., 1997) and inhibits mitochondrial F0F1-ATP synthase/ATPase by binding to one of its subunits (Zheng and Ramirez, 1999). Estrogen can attenuate oxidative impairment of synaptic Na^+/K^+-ATPase activity, glucose transport, and glutamate transport induced by amyloid β-peptide and iron (Keller et al., 1997). In cerebral blood vessels, E_2 enhanced expression of mitochondrial specific proteins such as cytochrome *c* and subunit IV of complex IV (Stirone et al., 2005). Besides mitochondrial stabilization, estrogens may blunt ATP loss by enhancing ATP production from glycolysis. Estradiol has been reported to increase glyceraldehydes-3-phosphate dehydrogenase activity in the central nervous system (Ramirez et al., 2001). Furthermore, ERβ has been found located in the mitochondria of variety of tissues (Monje and Boland, 2001; Yang et al., 2004). The mitochondrial localization of ERβ suggests that ERβ could play a role in the effects of estrogens on mitochondria function.

Mitochondrial calcium sequestration and ATP production are closely correlated with mitochondrial membrane potential. 17β-, 17α-, and *ent*-E_2 effectively protected mitochondria from H_2O_2-induced membrane potential collapse. These data are consistent with other studies that show that 17β-E_2 treatment stabilizes mitochondrial potential against oxidative stress such as 3-nitropropionic acid (Wang et al., 2001a) and mutant presenilin-1 (Mattson et al., 1997). Mounting evidence suggests that estrogens, acting as mitochondrial energizers by targeting mitochondrial sites to inhibit opening of permeability transition pores, inhibit the mitochondrial calcium uniport, increase mitochondrial-specific proteins expression, cause recovery of ATP production, and up-regulate the antiapoptotic protein Bcl-2 (Nilsen and Diaz Brinton, 2003; Burris and Krishnan, 2005; Stirone et al., 2005).

To investigate the mechanisms underlying the antioxidant effects of estrogen, we evaluated the ability of 17β-, 17α-, and *ent*-estradiol to scavenge ROS and to prevent lipid peroxidation. We demonstrated that 150 μM H_2O_2 exposure significantly increased MDA levels within 5 min in SK-N-SH cells. In addition, all three estrogens effectively inhibited H_2O_2-induced lipid peroxidation at concentrations ranging from 1 nM to 1 μM. The quick action and high potency of estrogens indicate their effect on attenuating lipid peroxidation may serve as the primary role in neuroprotection. In contrast, 17β-E_2 did not reduce H_2O_2 exposure-induced cytosolic ROS increase with concentrations ranging from 1 nM to 10 μM (data not shown). Based on these observations, we propose that cellular membranes are one of the primary targets of antioxidant effects of estrogen. The antioxidant actions of estrogen on cell membranes are ER-independent, and the phenolic A-ring structure may play an important role in this effect. Mounting evidence shows that estradiol inhibits lipid peroxidation (Behl et al., 1995; Goodman et al., 1996; Gridley et al., 1997). Sugioka et al. (1987) first postulated that the phenolic A-ring is closely related with the effect of estrogen on inhibiting lipid peroxidation. Studies from Jellnick and Bradlow (1990) have shown that estrogens can inhibit oxidative cascades by donating hydrogen radicals on the A-ring of estrogens.

To further address the antioxidant properties of estrogen, we examined estrogens action on cellular GSH levels. Glutathione is an important cellular antioxidant and exerts its antioxidant activity through several mechanisms, including scavenging free radicals (Meister and Anderson, 1983). Previous studies from our laboratory showed the synergistic interaction between estrogens and glutathione in protecting neuronal cells against oxidative stress (Green et al., 1998; Gridley et al., 1998). In the presence of 3.25 μM GSH, the ED_{50} value of 17β-E_2 decreased from 3.27 μM to 5 nM against β-amyloid toxicity in HT-22 cells. In our current study, all three estrogens significantly increased intracellular GSH levels with dosages ranging from 1 nM to 1 μM. This evidence suggests the involvement of GSH in neuroprotection by estrogen.

We compared the potency of estrogens against various cytotoxic responses to H_2O_2 in SK-N-SH cells in an attempt to determine the primary mechanism of neuroprotection. In most cases, the effective concentrations of these estrogens were from 100 nM to 1 μM, consistent with effective antiox-

idant dosages from other studies. However, at the concentration of 1 nM, these estrogens significantly inhibit lipid peroxidation after 5-min H_2O_2 exposure. Based upon this time and potency relationship, we propose that inhibition of lipid peroxidation is the primary mechanism of estrogen protection in SK-N-SH cells followed by attenuation of intracellular calcium elevations, stabilization of mitochondrial Ca^{2+} and $\Delta\psi$ m, preservation of ATP and GSH levels, and subsequent inhibition of cell death.

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