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17β-Estradiol Increases Expression of the Oxidative Stress Response and DNA Repair Protein Apurinic Endonuclease (Ape1) in the Cerebral Cortex of Female Mice Following Hypoxia

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Abstract

While it is well established that 17β-estradiol (E_2) protects the rodent brain from ischemia-induced damage, it has been unclear how this neuroprotective effect is mediated. Interestingly, convincing evidence has also demonstrated that maintaining or increasing the expression of the oxidative stress response and DNA repair protein apurinic endonuclease 1 (Ape1) is instrumental in reducing ischemiainduced damage in the brain. Since E_2 increases expression of the oxidative stress response proteins Cu/Zn superoxide dismutase and thioredoxin in the brain, we hypothesized that E_2 may also increase Ape1 expression and that this E_2-induced expression of Ape1 may help to mediate the neuroprotective effects of E_2 in the brain. To test this hypothesis, we utilized three model systems including primary cortical neurons, brain slice cultures, and whole animals. Although estrogen receptor α and Ape1 were expressed in primary cortical neurons, E_2 did not alter Ape1 expression in these cells. However, immunofluorescent staining and quantitative Western blot analysis demonstrated that estrogen receptor α and Ape1 were expressed in the nuclei of cortical neurons in brain slice cultures and that E_2 increased Ape1 expression in the cerebral cortex of these cultures. Furthermore, Ape1 expression was increased and oxidative DNA damage was decreased in the cerebral cortices of ovariectomized female C57Bl/6J mice that had been treated with E_2 and exposed to hypoxia. Taken together, our studies demonstrate that the neuronal microenvironment may be required for increased Ape1 expression and that E_2 enhances expression of Ape1 and reduces oxidative DNA damage, which may in turn help to reduce ischemia-induced damage in the cerebral cortex and mediate the neuroprotective effects of E_2.

Keywords

Apurinic endonuclease 1; estrogen; estrogen receptor; reactive oxygen species; neuroprotection; hypoxia

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1. Introduction

The human brain utilizes 20% of the oxygen consumed, but accounts for only 2% of total body mass, making it the most metabolically active organ in the human body [1]. Because reactive oxygen species (ROS) are produced as byproducts of normal cellular metabolism, the massive consumption of oxygen by the brain can lead to substantial ROS production. ROS play a role in cellular signaling such as MAP kinase activation and tyrosine phosphorylation [2–5]. However, if not effectively dissipated, ROS can accumulate and the ability of the cell to maintain a reduced intracellular environment is compromised, which can result in oxidative stress and damage to resident proteins, lipids, and DNA.

Cells rely on a variety of proteins to dissipate ROS, reduce oxidative stress, and, if damage does occur, to repair ROS-induced damage to cellular macromolecules. The oxidative stress response protein apurinic endonuclease (Ape1) is a multifunctional protein involved in DNA repair and redox regulation. Ape1 is the primary mammalian endonuclease and plays an essential role in repairing the most common DNA lesions, apurinic and 8-hydroxydeoxyguanosine (8-OHG) sites [6–11]. In addition to its role in DNA repair, Ape1 is required for the reduction of oxidized cellular proteins and is especially important in maintaining numerous transcription factors in a reduced, active state [12–15]. Thus, Ape1 is required to maintain DNA integrity and protein structure and function in the brain.

Ape1 is also instrumental in repairing cellular damage caused after blood vessel occlusion as might occur during a stroke [16–18]. While the hypoxia resulting from blood vessel occlusion is deleterious, reoxygenation can be even more damaging as the oxygen supply is reestablished and ROS production rapidly escalates [19–21]. Studies in rodents have demonstrated that Ape1 levels decline following middle cerebral artery (MCA) occlusion leading to DNA damage and cell death in the infarct region [16,22,23]. However, by simply maintaining Ape1 levels, cell death and DNA damage can be reduced [16,17]. Furthermore, if Ape1 is overexpressed, DNA damage and cerebral infarct volume resulting from MCA occlusion is significantly reduced [18].

While best known for its role in female fertility, the steroid hormone 17β-estradiol (E_2) also alters the expression of proteins involved in oxidative stress response, anti-inflammatory processes, and programmed cell death in the brain [24–35]. A number of laboratories have demonstrated that E_2 diminishes neuronal injury associated with cerebral ischemia and brain trauma in rodents [36–40]. This E_2-induced neuroprotection is most apparent in the cerebral cortex, which is particularly vulnerable to ischemia-induced injury [17,18,24–27,41]. An elegant series of studies by Wise and coworkers demonstrated that exposing ovariectomized female rodents to physiological levels of E_2 reduces the infarct volume and cell death that occurs following MCA occlusion [24–27], but that this neuroprotective effect was only observed when the E_2 was administered prior to artery occlusion [28]. Furthermore, the fact that E_2 pretreatment decreases infarct volume in estrogen receptor β (ERβ), but not in estrogen receptor α (ERα) null mice that have been subjected to MCA occlusion, demonstrates that ERα, not ERβ, is involved in this E_2-mediated neuroprotection [29,30].

We previously demonstrated that E_2 increases expression of the oxidative stress response protein Cu/Zn superoxide dismutase in brain slice cultures and that this E_2-induced expression helps to limit protein and DNA damage of cerebral cortical neurons [42]. From these studies we hypothesized that E_2 might also increase Ape1 expression in the brain and that this E_2-induced expression of Ape1 could help to protect the cerebral cortex from hypoxia-induced cell damage.

2. Materials and Methods

2.1. Mice

C57BL/6J breeding pairs for the generation of mouse pups for isolation of primary cortical neurons and brain slice cultures and female mice (12–15 weeks) were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a 12 h light/dark schedule with access to water and food ad libitum. Ovariectomized mice were maintained on phytoestrogen-free chow. Primary cortical neurons were prepared from P0 female mouse pups, non-neuronal cultures for the preparation of conditioned media were prepared from P0–P3 female mouse pups, and brain slice cultures were prepared from P7 to P9 day old female pups. Female pups were identified by the smaller anogenital distance and the absence of an adjacent pigmented region. All procedures were performed in accordance with guidelines of the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee and Division of Animal Resources.

2.2. Primary cortical neurons

The isolation of primary cortical neurons was performed as described [43] with the following modifications. Conditioned plating medium (Neurobasal-A with 0.5 mM GlutaMAX, B27 supplement without antioxidants [Life Technologies, Grand Island, NY], and antibiotics) and conditioned maintenance medium (Neurobasal-A with 0.5 mM GlutaMAX, custom-formulated media supplement [Table 1], and antibiotics) were prepared by incubating non-neuronal cultures with media for 24 h. Cortical pieces were triturated in conditioned plating media, cells were counted and seeded on poly-D-lysine coated 60 mm petri dishes at 115,000 cells/cm_2 or on poly-D-lysine and fibronectin pre-coated German glass coverslips (EMS, Hatfield, PA) at 50,000 cells/cm_2. Neurons were incubated overnight and the conditioned plating media was removed and replaced with conditioned maintenance media. Primary cortical neurons were treated with 1 μM cytosine ß-D-arabinofuranoside once a week to reduce glial cell proliferation. Media was removed from the primary cortical neurons and replaced with conditioned maintenance media twice a week. Ethanol or 20 nM E_2 was added 24 h prior to cell harvest. All cells were maintained in a 5% CO_2 incubator at 36°C.

2.3. Brain slice cultures

Brain slice cultures were prepared essentially as described [44] with modifications. Pups were decapitated and whole brains were quickly removed, mounted, and sectioned with a Leica VT1200 vibratome (Leica Microsystems, Nussloch, Germany). 300 μm coronal sections were sliced into chilled slicing solution (1.25 mM NaH_2PO_4, 2.5 mM KCl, 10 mM $MgSO_4$, 0.5 mM $CaCl_2$, 234 mM sucrose, 11 mM glucose and 26 mM $NaHCO_3$). Slices were immediately placed on a sterile Millicell culture plate insert (Millipore, Billerica, MA) in each well of a chilled 12-well plate. Excess slicing solution was removed following sectioning and replaced with 1.2 ml of Neurobasal-A medium (Gibco, Carlsbad, CA) containing 0.5 mM GlutaMAX (Gibco), antibiotics (penicillin, streptomycin, and gentamycin) and 10% charcoal dextran-treated fetal bovine serum with ethanol or 20 nM E_2. Slices were maintained in a 5% CO_2 incubator at 36 °C for 24 h.

2.4. Ovariectomy

12–15 week old female mice were anesthetized by inhalation of 4% isoflurane, bilaterally ovariectomized, and implanted subcutaneously with silastic tubing (0.062 in/0.125 in, inner/outer diameter, 1 in length; Dow Corning, Midland, MI) plugged at both ends with medical adhesive (Dow Corning). Tubing contained 35 μl cottonseed oil (vehicle) or 35 μl of 180 μg/

ml E_2 in cottonseed oil, which produces a low physiological level of circulating E_2 (~25 pg/ml) [29] that is equivalent to estrous levels of E_2 in mice [45–47].

2.5. Hypoxia treatment

7 days after ovariectomy and implantation of silastic tubing, oil- and E_2-treated mice were placed in cages inside a hypoxia chamber (BioSpherix, Lacona, NY) that was equilibrated to 7% O_2 and 93% N_2 for 3 h, conditions which have been shown to induce substantial changes in mRNA expression [48]. Oxygen concentration was monitored continuously throughout the experiments. Oil- and E_2-treated mice were also maintained at normoxic conditions for 3 h. After hypoxia, animals were allowed to reoxygenate for 1 h (RT-PCR) or 3 h (protein expression and DNA damage). Following hypoxia and re-oxygenation, all animals were anesthetized with isoflurane, decapitated, and cerebral cortices were harvested for quantitative real time-PCR, immunofluorescent staining, Western blot, or DNA damage analyses.

2.6. Western blot analysis

Primary neurons, brain slice cultures, or cerebral cortical hemispheres were combined with 400, 250, or 800 μl RIPA buffer (Thermo Scientific, Rockford, IL), respectively, with 1× Protease Inhibitor Cocktail (Sigma) and homogenized for 10 seconds at high speed with a Pro Homogenizer (ProScientific Inc., Oxford, CT). The buffer was adjusted to 400 mM NaCl with 5 M NaCl, placed on ice, and vortexed every 5 min for 15 min. The extract was spun at 20,800×g in a 4°C microfuge, the supernatant was removed, and protein assays were performed with bicinchoninic acid using BSA as the protein standard (Thermo Scientific). Whole cell lysates (25μg) were loaded onto each lane of a denaturing gel and fractionated. Proteins were transferred to a nitrocellulose membrane and blots were probed with an Ape1- (1:2000, ab194, Abcam Inc., Cambridge, MA), β-tubulin-(1:5000, sc9104, Santa Cruz Biotechnologies, Santa Cruz, CA) or α-tubulin- (1:100,000, T6199, Sigma) specific antibody followed by a secondary antibody covalently linked to infrared fluorophores (IRDye 800CW donkey anti-mouse IgG [1:5,0000, LI-COR Biosciences, Lincoln, NE] or IRDye 800CW donkey anti-rabbit IgG [1:5,0000, LI-COR Biosciences]). The membranes were scanned with an Odyssey infrared imager (LI-COR Biosciences) to quantitate the level of protein present. The integrated intensity function with the automated median background correction method was used since this system provides a wide dynamic range and reduces error to improve quantitative accuracy [49,50].

2.7. Immunofluorescence imaging

Brain slices from cultures and whole animals were rinsed 2× with PBS, fixed in PBS with 4% formaldehyde for 1 h, washed 3× with PBS, permeabilized with PBS containing 1% Triton X-100 for 30 min, and incubated in blocking solution (PBS with 0.05% Tween-20 and 5% normal donkey serum) for 1–2 h. Slices were then incubated in blocking solution with an ERα-, (1:600, ab31312, Abcam Inc.), Ape1- (1:100, sc9919, Santa Cruz Biotechnologies), or NeuN- (1:500, MAB377, Millipore, Temecula, CA) specific antibody for 2 h at room temperature. Slices were washed 3× with PBS containing 0.1% Tween-20 (PBS-T) and incubated with DyLight 549-, 649- or 488-conjugated anti-rabbit, anti-goat or anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), respectively, for 1 h in the dark at room temperature, washed 3× with PBS-T, incubated with DAPI nucleic acid stain for 30 min at room temperature, washed 3× with PBS, and mounted with Pro-Long Gold antifade reagent (Life Technologies). 4′,6-diamidino-2-phenylindole (DAPI) co-staining was included with each treatment to identify nuclei and ensure that similar numbers of cells were present. Control slices, which had not been exposed to primary antibody, were processed in parallel.

Primary cortical neurons were stained as described for the brain slices with the following modifications. After primary neurons were rinsed 2× with PBS, they were fixed in PBS with 4% formaldehyde for 15 min, washed 3× with PBS, permeabilized with PBS containing 0.5% Triton X-100 for 10 min, and incubated in blocking solution for 30 min. DAPI staining time was reduced to 10 min.

2.8. Image collection and quantitation

All images were obtained with a 40× oil-immersion objective using the Leica DM 4000 B confocal microscope and Leica TCS SPE system and Application Suite Advanced Fluorescence software (Leica Microsystems, Inc., Bannockburn, IL). Detector gain and offset, laser power, and bandwidth of emission collection were kept constant for all treatments in each experiment and adjusted so that images had a full range of pixel intensities (0–255) and saturation was minimized. Images from 8 independent experiments were collected from primary cortical neurons to examine effect of E_2 on Ape1 expression.

Image Pro Plus software (Media Cybernetics, Bethesda, MD) was used for quantitative immunofluorescent analysis of Ape1 staining in brain slice cultures. Analysis included 477 (ethanol) or 742 (E_2) z-stack images from 6 (ethanol) or 7 (E_2) individual brain slice cultures. The perimeter and area were adjusted so that only specifically stained cells were detected and recorded in a script designed to analyze the individual images in an entire z-stack. Data were exported into Excel and the mean density/intensity of specifically stained cells in each z-stack image was quantitated for each treatment.

2.9. RT-PCR

Total RNA was isolated from mouse cortices using RNAqueous reagents (Ambion, Life Technologies, Austin, TX) according to the manufacturer's instructions. RNA concentrations were measured and cDNA was synthesized using the iScript kit (Bio-Rad, Hercules, CA) as described by the manufacturer. 1μl of cDNA was combined with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), forward and reverse primers for HIF3α (5'-GGACTCAGACTCAGGCTACAG-3' and 5'- TCAGGAAGTGGACGCAGATG-3') and VefgA (5'- GGCTGCTGTAACGATGAAG-3' and 5'- TCTGCTGTGCTGTAGGAAG-3') and real-time PCR was carried out using a Bio-Rad iQ5 multicolor Real-Time PCR Detection System. Standard curves were created using cDNA equivalents of 0.125, 1.25 and 12.5 ng of RNA and were run in triplicate with each primer set for each experiment.

2.10. 8-hydroxydeoxyguanosine quantitation

Genomic DNA was isolated by digesting each cerebral cortex in 500 μl of DNA isolation buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, pH 8.0, 0.5% SDS, 1 mg/ml proteinase K [Sigma], 200 μg/ml RNase A [Sigma]) overnight at 50°C in a rotating incubator. After tissue digestion, 500 μL of phenol-chloroform-isoamyl alcohol (25:24:1) was added and samples were vortexed and centrifuged at 15,000×g for 5 min at room temperature. The top, aqueous phase was transferred to a fresh tube. DNA was precipitated, resuspended in Tris-EDTA and 8-hydroxydeoxyguanosine (8-OHG) oxidative DNA damage was quantitated using the OxiSelect Oxidative DNA Damage ELISA kit (Cell Biolabs, San Diego, CA). A 96 well plate was coated with 8-OHG conjugate overnight. Sample DNA was denatured, digested, and treated with alkaline phosphatase. Individual samples or 8-OHG standards were added to the coated plate and 8-OHG antibody was added to each well for 1 hr. Wells were rinsed with wash buffer 3× and secondary antibody was added. After 1 hr, wells were washed 3× and the substrate solution was added. Stop solution was added to each well after the color had developed and the absorbance was read at 450 nm using a SpectraMax Plus 384 plate reader.

2.11. Statistics

Combined data are expressed as the mean ± SEM. SAS version 9.2 (SAS Institute Inc., Cary, NC) was used for statistical analysis. Ape1 protein expression in brain slice cultures was analyzed using Student's *t*-test. Statistical analyses of HIF3α and VegfA mRNA expression, Ape1 protein expression, and 8-OHG levels among all groups of animals were compared using a two-way analysis of variance (ANOVA). A p value of <0.05 was considered statistically significant (95% confidence interval).

3. Results

3.1. Ape1 expression in primary cortical neurons

Since neuronal ERα is essential for E_2-mediated neuroprotection [29,30,36] and the cerebral cortex is particularly susceptible to ischemia-induced damage [17,18,24–27,41], we examined the expression of ERα and Ape1 in primary cultures of cortical neurons. Primary neurons were isolated from the cerebral cortices of newborn C57Bl/6J female mouse pups and cultured for 9 or 10 days in vitro. When an ERα antibody was preincubated with purified ERα and used in immunofluorescence assays, no staining was detected (Data not shown). In contrast, antibody that had not been preincubated with receptor detected robust ERα staining, thus confirming the specificity of the antibody (Fig. 1A). Quantitative real-time PCR demonstrated that ERα transcripts were present in primary neurons that had been cultured for 9 days (data not shown). Furthermore, immunofluorescent staining demonstrated that the expression of Ape1 was quite robust in these cells as well suggesting that they might be an appropriate model system to study the potential effects of E_2 on Ape1 expression.

When primary cerebral cortical neurons were treated with ethanol vehicle or 20 nM E_2 for 24 h, no changes in Ape1 expression were detected using Western blot (Fig. 1B) or immunofluorescence (Fig. 1C) analyses. Ape1 staining was confined to the nuclear compartment of the primary neurons (Fig. 1C, compare Ape1 and NeuN staining). Varying the concentration (10–100 nM) or time (3–48 h) of E_2 treatment did not alter Ape1 expression (data not shown). Even after eliminating the superoxide dismutase, catalase, corticosterone, and progesterone from the B27 media supplement, which are typically used for primary neuronal cultures (Table 1), we were still unable to detect an E_2-induced expression of Ape1. Thus, in spite of the fact that ERα was present and would presumably have been able to respond to E_2 treatment, no changes in Ape1 expression were observed suggesting that the primary neurons may require other cell types and/or the organizational features and structural architecture present in the intact brain.

3.2. Ape1 expression in brain slice cultures

To more closely recapitulate the neuronal environment present in the brain, we used brain slice cultures, which maintain much of the spatial architecture and many of the organizational features and local synaptic connections present in the brain [44]. Since the cellular environment can be carefully defined and manipulated, brain slice cultures have been used extensively to study electrophysiological properties, angiogenesis, dendritic growth, neural cell migration, and the responsiveness of neural cells to various drugs and treatments including E_2 [42,51–55].

Brain slice cultures were prepared from 7–9 day old C57Bl/6J mouse pups. Immunofluorescent staining was used to characterize the expression of Ape1 and ERα in the cerebral cortex. In order to decrease variation and limit bias, the same regions of the cerebral cortex (Fig. 2A, red boxed regions) were examined in each of the experiments described herein. Ape1 was expressed in the nuclei of cerebral cortical neurons as shown by co-

staining with DAPI and the neuronal marker NeuN (Fig. 2B and 2C). Similarly, ERα was expressed in the nuclei of cerebral cortical neurons (Fig. 2D). In contrast, no staining was observed when the Ape1- or ERα-specific antibody was omitted (data not shown). The co-expression of ERα and Ape1 in the nuclei of the same cerebral cortical neurons was evident when the ERα and Ape1 channels were merged (Fig. 2E).

When brain slice cultures were treated with ethanol or 20 nM E_2 for 24 h and stained with an Ape1-specific antibody, Ape1-staining was observed in the absence of E_2, but when brain slice cultures were treated with E_2, Ape1 staining was increased (Fig. 3A). Immunofluorescence image analysis of ethanol- or E_2-treated brain slice cultures demonstrated that Ape1 expression was significantly increased in the cerebral cortex when cultures were treated with E_2 compared to cultures that were treated with ethanol vehicle (Fig. 3B).

Whole cell extracts were prepared from brain slice cultures that had been treated with ethanol or 20 nM E_2 for 24 h and Western blot analysis was performed to examine the effect of E_2 on Ape1 expression using another independent method (Fig. 3C). Combined data from 8 individual brain slice cultures demonstrated that E_2 significantly increased Ape1 expression (Fig. 3D). These findings confirmed that E_2 increased Ape1 expression in cortical neurons and demonstrated that brain slice cultures provide a valuable model system to study estrogen action in the brain.

3.3. Effect of hypoxia on Ape1 expression and DNA damage in the cerebral cortex of mice

Previous studies have demonstrated that significantly less neural damage is observed when ovariectomized female rodents are treated with E_2 and subjected to MCA occlusion than when animals have been treated with oil [24–27]. Thus, MCA occlusion experiments have been extremely informative in demonstrating the neuroprotective effects of E_2. However, because the infarct region contains dead or dying cells using this experimental paradigm, the amount of information that can be gathered from these cells is limited. Thus, rather than use an artery occlusion method, we examined the effect of hypoxia, a hallmark of ischemia, in ovariectomized female mice that had or had not been treated with E_2.

C57Bl/6J female mice were ovariectomized and silastic tubing containing oil or E_2 was implanted. After 7 days, a time when E_2-mediated neuroprotection is observed in rodents [25,28–30,56,57], mice were placed in a hypoxia chamber that was equilibrated to 7% oxygen to mimic the decrease in oxygen that can occur with ischemia. The animals were allowed to recover in normoxic conditions and then sacrificed. Control mice that had not been exposed to hypoxia were processed in parallel.

Previous studies have shown that hypoxia induces the expression of several genes in the brain including HIF3α and VegfA [58–62]. Quantitative real-time PCR demonstrated that when animals were exposed to hypoxia for 3 h and allowed to recover for 1 h, HIF3α and VegfA transcript levels were significantly increased (Figs. 4A and 4B, respectively).

Immunofluorescent staining of the cerebral cortex from ovariectomized female mice that had been treated with oil or E_2 for 7 days, exposed to hypoxia for 3 h, and allowed to recover to normoxic conditions for 3 h indicated that Ape1 (Fig. 5A) and ERα (Fig. 5B) were present in the nuclei of the cerebral cortical neurons. Whole cell extracts were prepared from cerebral cortices to determine whether E_2 altered Ape1 expression during hypoxia using Western blot analysis (Fig. 6A). As seen in Fig. 6B, a modest but significant increase in Ape1 expression was observed when mice were treated with E_2 and exposed to hypoxia. Thus, E_2 treatment and hypoxia together increased Ape1 expression in the cerebral cortex of ovariectomized female mice.

Guanine is particularly susceptible to oxidation and is the most common target of oxidative DNA damage. 8-hydroxydeoxyguanosine (8-OHG) mispairs with adenine and results in a guanine to thymine transversion and perturbation in DNA conformation [63]. To determine whether E_2 altered DNA damage during hypoxia, the level of 8-OHG was examined. Ovariectomized female mice were treated with oil or E_2 for 7 days, exposed to hypoxia for 3 h, recovered at normoxic conditions for 3 h, and then sacrificed. Control mice that had not been exposed to hypoxia were processed in parallel. Genomic DNA was isolated from cerebral cortices and 8-OHG levels was measured and reported as the normalized mean ± SEM (Fig. 6C). E_2 significantly reduced 8-OHG levels in the cerebral cortex of mice that had been exposed to hypoxia.

4. Discussion

Although it has been known for some time that E_2 decreases ischemia-induced damage in the rodent brain, the mechanisms mediating this neuroprotective effect have remained unclear [24–27]. We now provide evidence for a link between E_2 treatment, Ape1 expression, and neuroprotection. Our studies demonstrate that E_2 increased Ape1 expression in the cerebral cortex of brain slice cultures and that combined hypoxia and E_2 treatment increased Ape1 expression and decreased 8-OHG oxidative DNA damage in the mouse cerebral cortex.

4.1. Ape1-induced neuroprotection

Previous studies have convincingly demonstrated that Ape1 protects the brain from ischemia-induced injury by decreasing DNA damage and cell death [16–18,22,23]. One way Ape1 might protect the cerebral cortex from ischemia-induced damage is by repairing DNA. In fact, results from our studies demonstrate that when E_2 was administered prior to a hypoxic event, the levels of the oxidative DNA damage marker 8-OHG were significantly decreased.

Ape1 is an essential enzyme in the base excision repair pathway. Ape1 recognizes and repairs apurinic sites and also enhances the activity of 8-oxoguanine-DNA glycosylase, the enzyme responsible for removing 8- OHG lesions [6,9–11]. Furthermore, Ape1 activity in base excision repair is especially vital in post-mitotic cells such as neurons that rely predominantly on this pathway to maintain DNA integrity [64–66]. When Ape1 expression declines after MCA occlusion, extensive DNA and cellular damage is observed [16,17]. However, if Ape1 expression is maintained or elevated, the number of DNA lesions significantly decline [17,18,67,68]. Our work demonstrates that even the modest elevation of Ape1 protein expression observed in mice that had been treated with E_2 and then exposed to hypoxia is sufficient to decrease oxidative DNA damage. Thus, the E_2-induced increase in Ape1 expression may help to protect the cerebral cortex from ischemia-induced DNA damage.

Another mechanism by which Ape1 can reduce ischemic injury is by maintaining numerous proteins in an active, reduced state. When ROS accumulate, cellular proteins can become oxidized and are no longer functional. Ape1 reduces a number of transcription factors including p53, NFκB, Fos, Jun, and HIF1α [12–15]. The ability of these transcription factors to modulate expression of numerous genes could have widespread effects on gene expression, cellular function, and neuronal survival [7,69]. Thus, Ape1 is required to maintain protein structure and function and preserve cellular homeostasis that is critical in the central nervous system. The overall importance of Ape1 is evident in the embryonic death of Ape1 null mice [70,71].

4.2. Combined data from the three models

At first glance the findings from our cell-based, brain slice, and whole animal studies may seem inconsistent. However, a more careful inspection highlights the fact that each model system provides clues about the requirements for Ape1 expression in the cerebral cortex. The fact that no increase in Ape1 expression was observed in primary cortical neurons seems contradictory to the brain slice and whole animal studies where E_2 increased Ape1 expression. However, it seems plausible that the failure of E_2 to alter Ape1 expression in primary neurons might be attributed to the lack of architectural and organizational features present in the brain microenvironment and/or the absence of other cells such as astroglia, microglia, or oligodendrocytes. Recently, E_2 has been shown to increase glial cell release of growth factors that promote neuronal survival [72]. The presence of these other cells and/or organizational features in the brain slice cultures may be required for E_2-induced expression of Ape1 as observed in the brain slice cultures. When the neuronal environment was more closely recapitulated in brain slice cultures, E_2 treatment was sufficient to enhance Ape1 expression. However, both hypoxia and E_2 treatment were required for increased Ape1 expression in the whole animal. If one considers that brain slice cultures are exposed to significant stress during harvesting and slicing, this stress combined with E_2 treatment may be sufficient to increase Ape1 expression in brain slice cultures and supports the idea that E_2 alone may not be sufficient to increase Ape1 expression.

4.3. Role of E_2-induced proteins in neuroprotection

Although our studies focused on the E_2-induced expression of a single oxidative stress response protein, Ape1, E_2 enhances the expression of other oxidative stress response proteins including Cu/Zn superoxide dismutase and thioredoxin, which are known to reduce ischemia-induced damage [42,73–75]. Superoxide dismutase reduces superoxide levels and decreases protein and DNA damage in neurons [42]. Thioredoxin, like Ape1, reduces oxidized cellular proteins and enhances ischemia-induced neuronal survival [76,77]. Overexpression of Ape1, Cu/Zn superoxide dismutase, or thioredoxin confers a neuroprotective effect following MCA occlusion [18,77,78]. Furthermore, intravenous administration of thioredoxin in mice following ischemia reduces infarct volume, protein damage, and cell death [79]. Together these studies emphasize the important role that oxidative stress response proteins have, as a whole, in sustaining cell viability after an ischemic event [16,17,23,77].

Although an E_2-mediated increase in Bcl-2 expression has also been implicated in inhibiting apoptosis after an ischemic event [34,35], it seems unlikely that simply limiting apoptosis in neurons with extensive DNA damage would be beneficial. However, if the E_2-mediated increase in Bcl-2 expression was coupled with increased expression of oxidative stress response and DNA repair proteins, together these proteins could reduce protein and DNA damage and enhance neuronal cell survival.

In addition to reducing oxidative stress and apoptosis by mediating the induction of oxidative stress response proteins and Bcl-2 in the brain, E_2 attenuates inflammation by reducing pro-inflammatory molecules, decreases cytokine and chemokine release following administration of a neuroinflammatory endotoxin, and reduces microglial activation and peripheral monocyte recruitment [31–33].

While it is clear that ERα plays a role in E_2-mediated neuroprotection [29,30], other studies have implicated a role for ERβ in neuroprotection [80]. Both ERα and ERβ are involved in E_2-mediated neurogenesis in the subventricular zone, which generates neural stem cells that migrate to the site of injury. [81]. In addition, estrogen binding sites in the mitochondria and

plasma membranes have also been implicated in conferring estrogen responsiveness. [82,83] [82,83].

4.4. Biological relevance of E_2-induced Ape1 expression

The protective effects of E_2 have been documented in numerous animal studies but have also been reported in humans as well [24–30,84,85]. It has been suggested that the decline in production of ovarian hormones contributes to the increased incidence of stroke in postmenopausal women [24,86]. In fact, a number of observational and epidemiological studies have reported that E_2 replacement therapy in postmenopausal women reduces stroke as well as coronary heart disease and neurodegenerative diseases [87–93]. Importantly, recent analysis of data from the Women's Health Initiative indicates that women who initiate E_2 replacement therapy before 60 years of age or within ten years of menopause have reduced rates of coronary heart disease and mortality [84,85].

5. Conclusions

Taken together, our studies demonstrate that E_2 treatment increases Ape1 expression in the cerebral cortex and suggest that E_2 mediates its neuroprotective effects in the brain in part by increasing expression of oxidative stress response proteins and reducing oxidative DNA damage. Because the maintenance or overexpression of Ape1 results in decreased ischemia-induced damage [16–18] and the decline of Ape1 expression leads to an increase in ischemic damage [16,22,23], we believe that the E_2-mediated increase in Ape1 expression enables neurons to repair DNA lesions, reduce oxidized cellular proteins, and more effectively regulate transcription.

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Abbreviations

DAPI	4′,6-diamidino-2-phenylindole
8-OHG	8-hydroxydeoxyguanosine
Ape1	Apurinic endonuclease 1
E_2	17β-estradiol
ERα	estrogen receptor α
ERβ	estrogen receptor β
ROS	reactive oxygen species
RPL7	ribosomal protein L7

Highlights

- Apurinic endonuclease (Ape1) is required to maintain protein and DNA integrity.
- 17β-estradiol (E_2) increases Ape1 protein expression in cerebral cortical neurons.
- E_2 reduces hypoxia-induced oxidative DNA damage in cortical neurons.
- The E_2-induced expression of Ape1 helps to reduce hypoxia-induced neuronal damage.
- These studies identify a mechanism by which E_2 neuroprotection may be conferred.

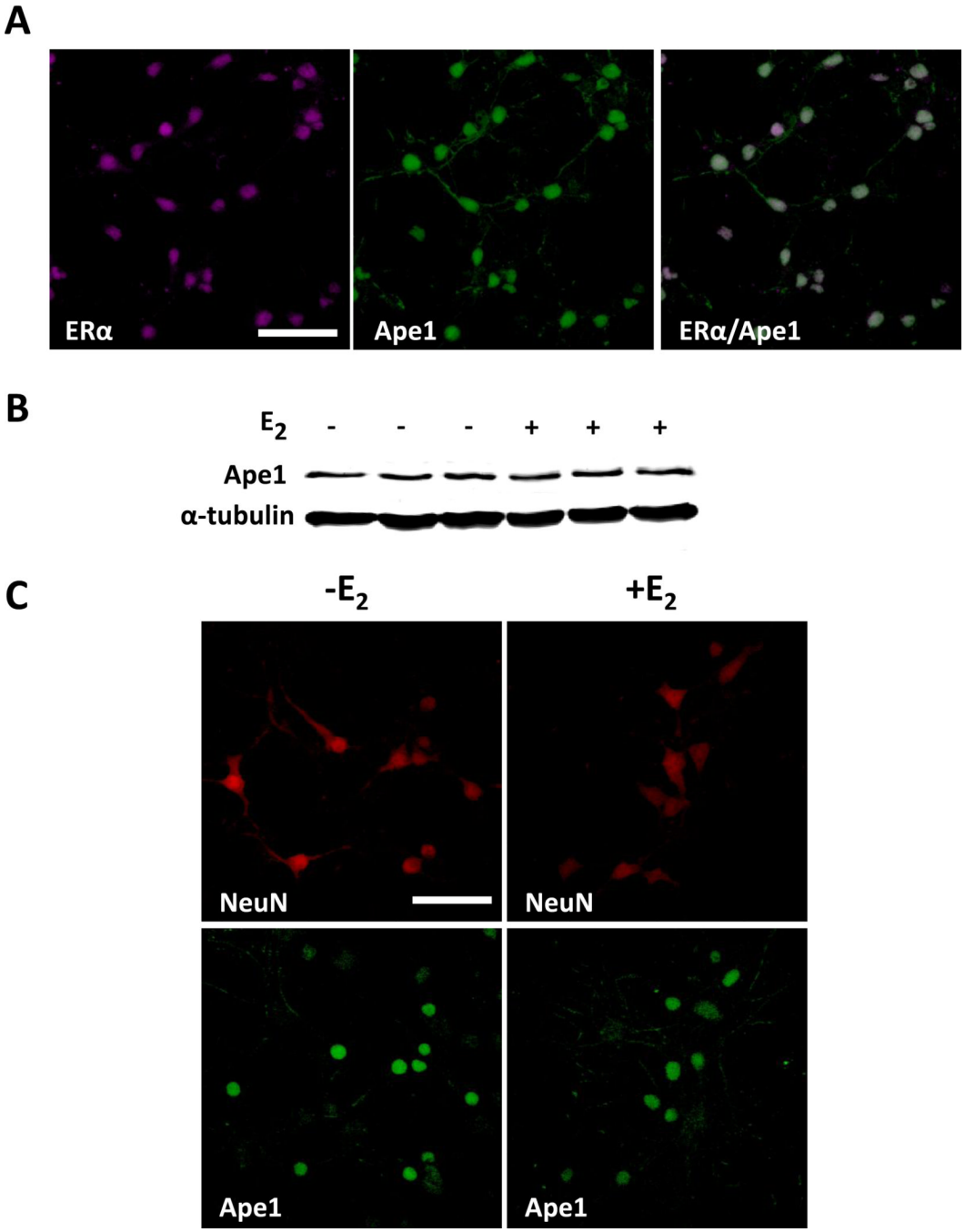

Fig. 1. Ape1 and ERα are expressed in primary cortical neurons

Cerebral cortical neurons were isolated from P0 mouse pups and cultured in vitro for 10 days. (A) Immunofluorescent staining was performed using ERα- and Ape1-specific antibodies. Neurons were treated with ethanol or 20 nM E_2 for 24 h and whole cell extracts were prepared for (B) Western blot analysis using Ape1- and α-tubulin-specific antibodies or (C) immunofluorescent staining using Ape1- and NeuN-specific antibodies. Scale bars indicate 25 μm.

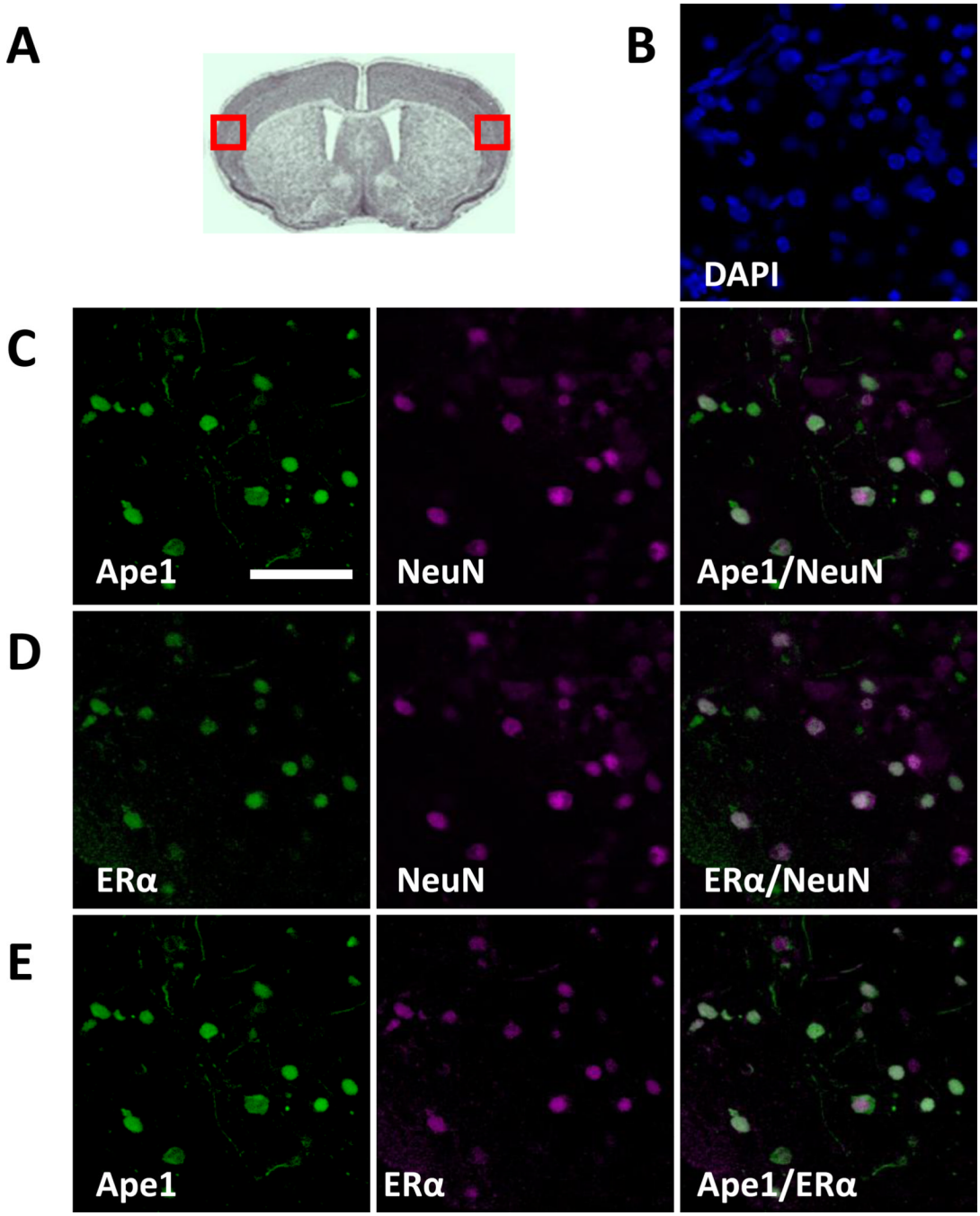

Fig. 2. Ape1 and ERα are expressed in cortical neurons in brain slice cultures

(A) Red boxes indicate the regions of the cerebral cortex examined by immunofluorescence. Immunofluorescent staining was performed with brain slice cultures using (B) DAPI to identify cortical cell nuclei, (C) Ape1- and NeuN-, (D) ERα- and NeuN-, or (E) Ape1- and ERα-specific antibodies. Scale bar indicates 25 μm.

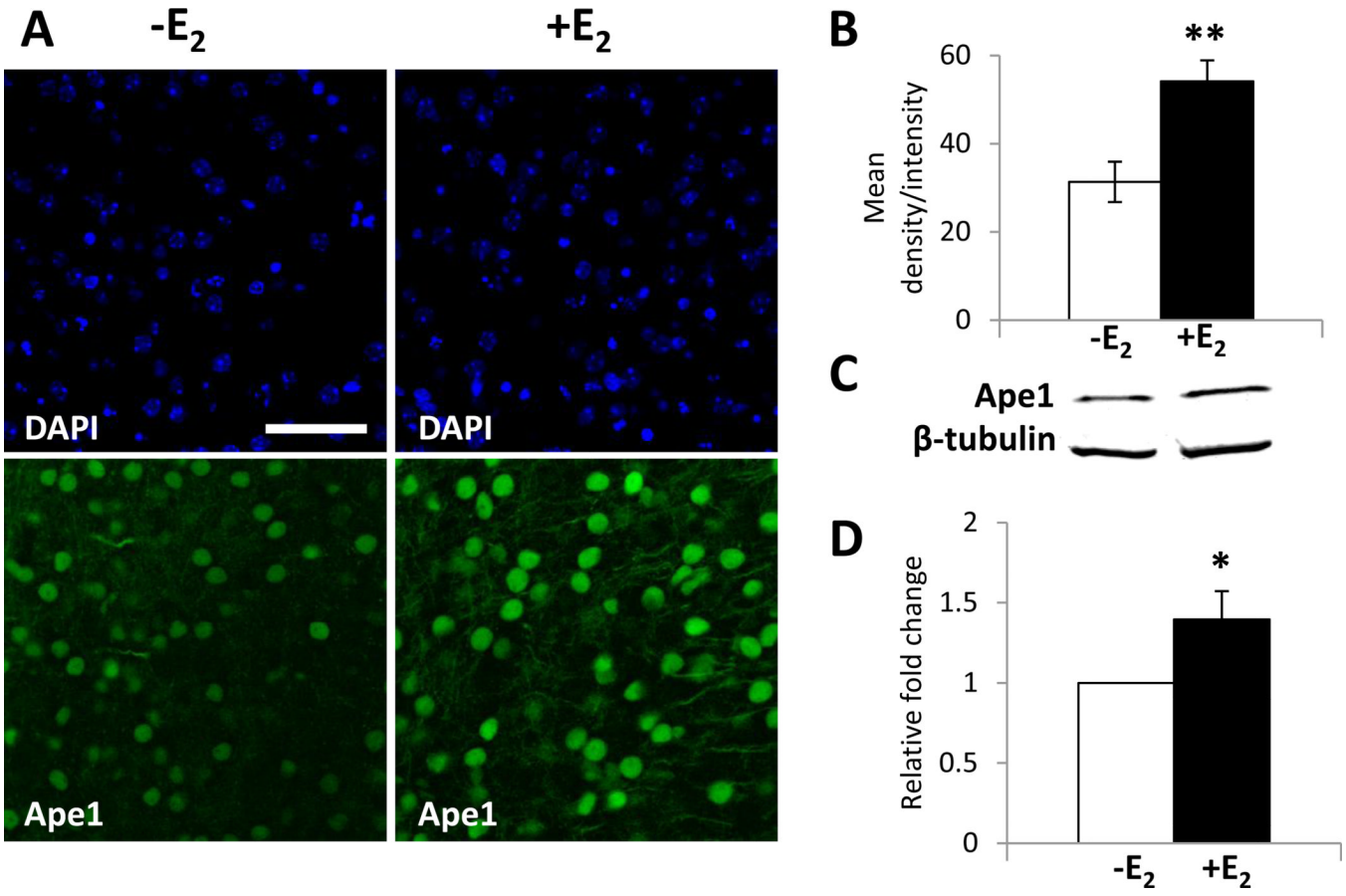

Fig. 3. E_2 increases Ape1 expression in brain slice cultures

Brain slice cultures were treated with ethanol or 20nM E_2 for 24 h. (A) Immunofluorescent staining was performed using an Ape1-specific antibody. DAPI staining was included to identify cortical cell nuclei. Scale bar indicates 25 μm. (B) Image analysis of Ape1 expression from 6 individual ethanol- or 7 individual E_2-treated brain slice cultures were combined and are expressed as the mean density/intensity ± SEM. (C) Whole cell extracts from brain slice cultures were prepared and quantitative Western blot analysis was performed using Ape1- and β-tubulin-specific antibodies. Ape1 expression was normalized to β-tubulin expression. (D) Data from 8 individual brain slice cultures were combined and are expressed as the normalized expression ± SEM. Ape1 expression from ethanol- or E_2-treated brain slice cultures were compared using Student's *t* test to determine statistical significance (* $p < 0.05$, ** $p < 0.001$).

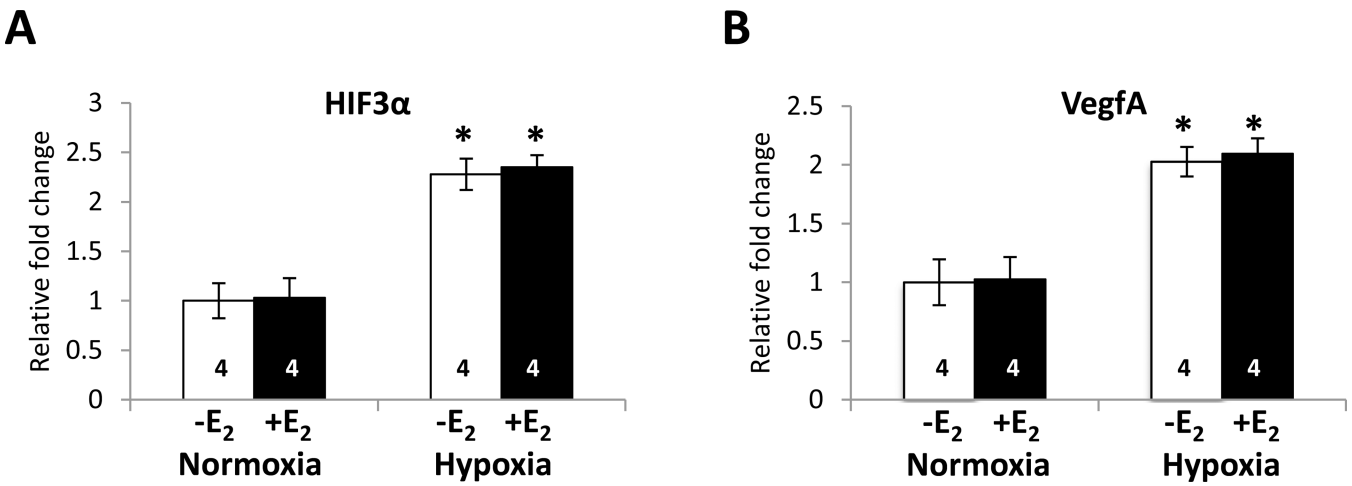

Fig. 4. Hypoxia significantly increases HIF3α and VegfA transcript levels

Ovariectomized female mice were treated with oil or E_2 for 7 days and subjected to normoxic or hypoxic conditions. Cortices were dissected and total RNA was isolated. cDNA was synthesized and quantitiative real-time PCR was carried out with (A) HIF3α- or (B) VegfA-specific primers. The relative fold change was calculated using the delta-delta Ct method with ribosomal protein L7 (RPL7) as a control. The mean relative fold change in each group is shown ± SEM. Two-way analysis of variance (ANOVA) was used to detect a significant difference in mRNA levels from mice that had been exposed to hypoxia compared with mice that had been exposed to normoxia (* $p < 0.0001$). The number of animals in each treatment group is indicated at the base of each bar.

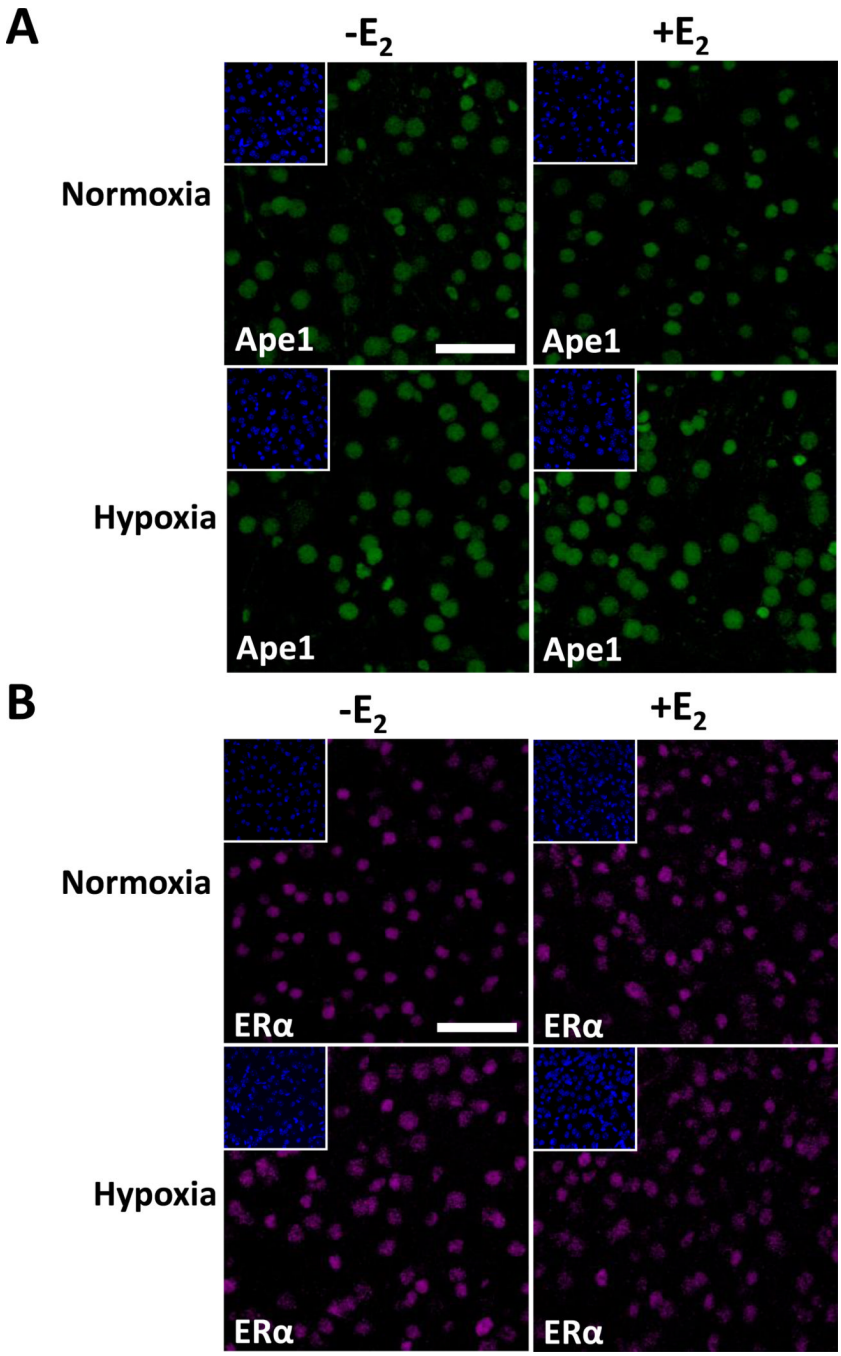

Fig. 5. E_2 increases Ape1 expression after hypoxia in the mouse cerebral cortex

Ovariectomized female mice were treated with oil or E_2 for 7 days, subjected to normoxic or hypoxic conditions, and allowed to recover for 3h. Immunofluorescent staining was performed using an (A) Ape1- or (B) ERα-specific antibody. DAPI staining is shown in the inserts. Scale bars indicate 25 μm.

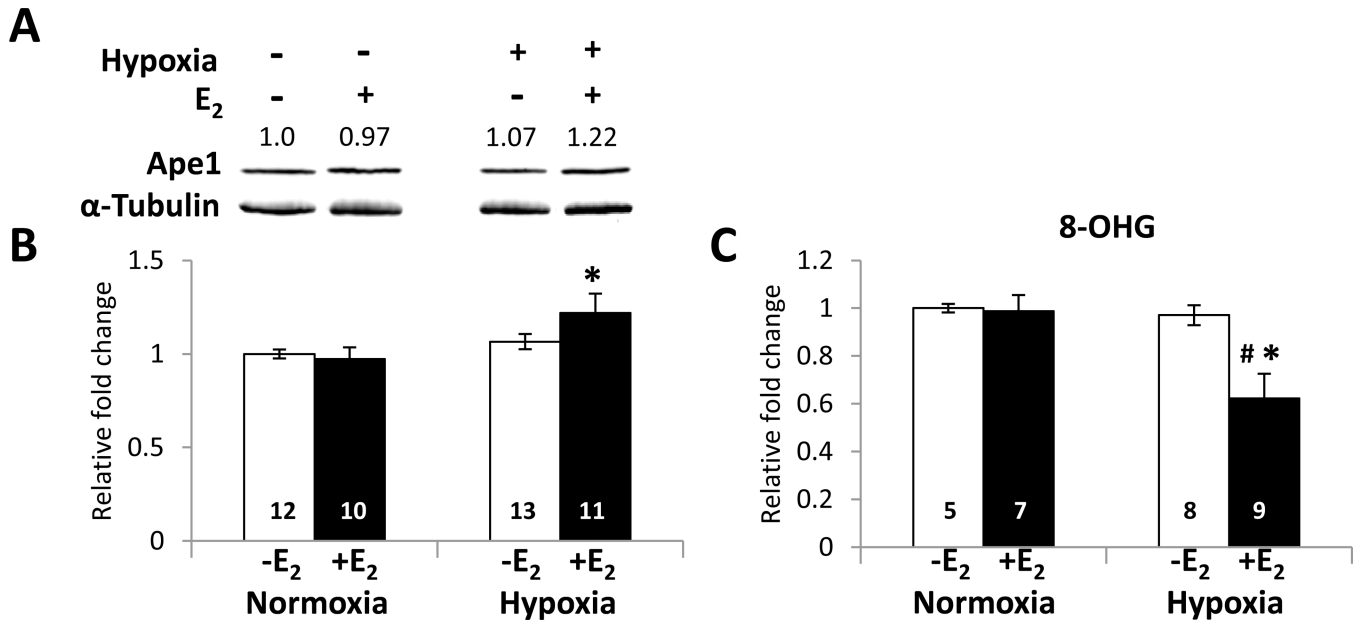

Fig. 6. E_2 and hypoxia increase Ape1 protein expression and decrease 8-OHG damage in the mouse cerebral cortex

Ovariectomized female mice were treated with oil or E_2 for 7 days and subjected to hypoxic conditions. (A) Quantitative Western blot analysis using whole cell extracts from cerebral cortices was performed using Ape1- and α-tubulin-specific antibodies. (B) Ape1 expression was normalized to α-tubulin expression and data are expressed as the relative fold change ± SEM. The value above each band indicates the relative fold change for each condition. (C) Genomic DNA was isolated from each cerebral cortex and 8-OHG concentrations were measured. Data represent the normalized mean ± SEM. Two-way analysis of variance (ANOVA) was used to detect significant differences in Ape1 protein expression or 8-OHG level in response to hypoxia (* $p < 0.05$) or E_2 (# $p < 0.05$). Relative fold change on the Y-axis indicates the normalized mean value for each treatment with the value of oil-treated mice maintained at normoxic conditions set at 1. The number of animals in each treatment group is indicated at the base of each bar.

Table 1

Final concentrations of our custom-formulated, serum-free media supplement. Ingredients and concentrations are based on Price and Brewer (74) and Roth et al (75) with some modifications.

H_2O soluble		**Ethanol soluble**	
D-galactose	15 μg/ml	Linolenic acid	0.1 μg/ml
Putrescine dihydrochloride	16.1 μg/ml	Linolenic acid	0.1 μg/ml
Sodium Selenite (Na_2SeO_3)	0.016 μg/ml	Retinyl acetate	0.1 μg/ml
Albumin, bovine	2.5 mg/ml	D,L-a-Lipoic acid	0.047 μg/ml
Holo-Transferrin	5 μg/ml		
L-carnitine hydrochloride	2 μg/ml	**Antioxidant, ethanol soluble**	
Ethanolamine	1 μg/ml	D,L-a-Tocopherol	1 μg/ml
$CuSO_4$	5.2 nM	D,L-a-Tocopherol acetate	1 μg/ml
$ZnSO_4$	1.4 mM		
$MnSO_4$	0.3 nM	**HCl soluble**	
		Insulin (bovine)	4 μg/ml
Antioxidant, H_2O soluble			
L-Glutathione reduced	1 ug/ml	**NaOH soluble**	
		3,3`,5-Triiodo-L-thryronine	2 ng/ml (3.1nM)
NH_4OH soluble			
Biotin	0.1 μg/ml		

*Superoxide dismutase (2.5 μg/ml), catalase (2.5 μg/ml), progesterone (20 nM), and corticosterone (20 ng/ml), which are included in Complete B27 media supplement, were omitted in our custom-formulated media supplement.