In the present study, we tested the hypothesis that 17β-estradiol (E2) is a neuroprotectant in the retina, using two experimental approaches: 1) hydrogen peroxide (H2O2)-induced retinal neuron degeneration in vitro, and 2) light-induced photoreceptor degeneration in vivo. We demonstrated that both E2 and 17α-estradiol (E2) significantly protected against H2O2-induced retinal neuron degeneration; however, progesterone had no effect. E2 transiently increased the phosphoinositide 3-kinase (PI3K) activity, when phosphoinositide 4,5-bisphosphate and [32P]ATP were used as substrates. Phospho-Akt levels were also transiently increased by E2 treatment. Addition of the estrogen receptor antagonist tamoxifen did not reverse the protective effect of E2, whereas the P13K inhibitor LY294002 inhibited the protective effect of E2, suggesting that E2 mediates its effect through some PI3K-dependent pathway, independent of the estrogen receptor. Pull-down experiments with glutathione S-transferase fused to the N-Src homology 2 domain of p85, the regulatory subunit of PI3K, indicated that βE2 and αE2, but not progesterone, activated the insulin receptor β-subunit (IRβ) as a binding partner. Pretreatment with insulin receptor inhibitor, HNMPA, inhibited IRβ activation of PI3K. Systemic administration of E2 significantly protected the structure and function of rat retinas against light-induced photoreceptor cell degeneration and inhibited photoreceptor apoptosis. In addition, systemic administration of βE2 activated retinal IRβ, but not the insulin-like growth factor receptor-1, and produced a transient increase in PI3K activity and phosphorylation of Akt in rat retinas. The results show that estrogen has retinal neuroprotective properties in vivo and in vitro and suggest that the insulin receptor/PI3K/Akt signaling pathway is involved in estrogen-mediated retinal neuroprotection.

Oxidative stress-induced neuronal cell death has been implicated in different neurological disorders and neurodegenerative diseases (1, 2). There is substantial evidence that light injures photoreceptors by increasing the formation of reactive oxygen species (3) and that antioxidants can rescue light-damaged photoreceptors (4–6). The role of oxidant stress as a mediator of apoptosis has been examined (7, 8), and hydrogen peroxide (H2O2), a by-product of oxidative stress, has been implicated in triggering apoptosis in various cell types including cultured retinal neurons (9–11). Apoptosis has been described in a wide variety of hereditary retinal degenerations (12, 13), in light-damaged retinas (14, 15), and following retinal detachment (16). Other types of retinal degeneration, such as retinal ischemia (17) and glaucoma (18), have also been associated with apoptosis. During the past decade, several therapeutic approaches, including retinal transplantation (19), gene therapy (20), growth factors (21, 22), and antioxidants (23–25), have been used to treat retinal degeneration. However, no effective medical therapy is currently available in humans, although vitamin A supplementation has shown some beneficial effect by slowing down the progression of retinitis pigmentosa (23).

The female sex hormone estrogen has a variety of metabolic activities including numerous effects on neurons (24). Therefore, the notion that estrogen is only important for sex differentiation and maturation has changed, to include its function as a neuromodulator and neuroprotectant. It appears that estrogen specifically maintains verbal memory in women and may prevent the deterioration in short and long term memory which is associated with normal aging (25). In addition, estrogen is not restricted to females because the male sex hormone testosterone (and other steroids with a 19-carbon atom structure, so-called C-19 steroids) can be converted chemically to estradiol in various tissues, including the brain, by an aromatase P450 enzyme (26).

17β-Estradiol (E2) is a steroid hormone synthesized enzymatically mainly in the ovaries from acetate, cholesterol, progesterone, and testosterone, but also by the placenta during pregnancy and, to a lesser extent, in the adrenal cortices, testes, and peripheral tissues (26). Several lines of evidence suggest that estrogen has neurotrophic and neuroprotective properties (27, 28). For many years, it has been known that E2 promotes viability and survival of neurons in primary...
neuronal cultures. Addition of βE2 to defined culture media increased the viability, survival, and differentiation of primary neuronal cultures from different brain areas including amygdala (29), hypothalamus (30), and neocortex (31). However, the role of estrogen in the protection of retinal neurons is not well understood. We have previously used both H2O2-induced cell death of cultured retinal neurons and light-induced photoreceptor degeneration as model systems to study the role of neurotrophic factors, pigment epithelium-derived factor, or basic fibroblast growth factor on retinal neuronal cell protection (11, 22). In that previous study, we have used in vitro and in vivo approaches to examine the role of estrogen as a neuroprotectant in the retina.

EXPERIMENTAL PROCEDURES
Primary Culture of Retinal Neurons—Timed pregnant Sprague-Dawley rats were ordered each week and the retinas of 10–15 pups, 0–2 days old, were removed with the aid of a dissecting microscope under sterile conditions in a tissue culture hood. The retinas were suspended in 25 ml of Dulbecco’s modified Eagle’s medium with F-12 medium plus 10% fetal calf serum in a plastic bag and mechanically dissociated for 2 min using a Stomacher set on low power. The suspension was first filtered through a 230-μm sieve, which was then rinsed once with medium, and the combined filtrates were passed through a 140-μm sieve followed by a rinse with undiluted fetal calf serum. The filtered suspension was centrifuged at 800 rpm in a clinical centrifuge for 5 min, the supernatant decanted, and the cell pellets resuspended in 25 ml of media using a sterile 5-ml pipette. The concentration of cells was determined with a cell counter or hemocytometer and the suspension diluted with medium to 1 × 10^6 cells/ml. The cells (1 ml) were plated in 24-well tissue culture plates on 12-mm coverslips that had been pre-treated overnight with 10 μg/ml poly-L-lysine. The cells were maintained in either Dulbecco’s modified Eagle’s medium with F-12 medium or in synthetic serum-free media. The cultures were used in experiments 7–10 days after plating.

Immunocytochemistry—Cells grown in poly-L-lysine-coated coverslips were fixed for 30 min in 4% paraformaldehyde in 0.1 M Tris-buffered saline (TBS, pH 7.5) and then rinsed three times with 1.0 ml of 0.1 M Tris-HCl, pH 7.5, and maintained at 4 °C in that buffer until processed for immunocytochemistry as described in a previous report (33). Briefly, nonspecific binding sites were blocked by 2% normal rabbit serum in TBS containing 0.1% Triton X-100. Recoverin was detected using goat anti-rabbit IgG conjugated to fluorescein isothiocyanate. The control experiments were performed without nonspecific labeling by secondary antibody. Cells treated without primary antibody were unlabeled. The cells on coverslips were then scraped and quantified by liquid scintillation counting.

GST-p85 Fusion Proteins and Pull-down Experiments—Glutathione S-transferase (GST-p85-N-SH2) (314–446 amino acids) fusion proteins were generated by PCR amplification of the p85α cDNA and cloned into a GST vector (37). The sequence of each clone was verified by DNA sequencing. All inductions yielded proteins of the expected size, as judged by Coomassie Blue staining. Pull-down experiments were performed as described previously (38), using 5 μg of GST fusion proteins that had been absorbed onto GST-Sepharose 4B matrix. Cultured retinal neurons were incubated with GST/GST-p85 fusion proteins with continuous mixing at 4 °C for 1.5 h. The Sepharose beads were washed three times in 500 μl of HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10 mM glycerol) and centrifuged at 5,000 rpm for 1 min at 4 °C. Proteins bound to GST-p85 were eluted by boiling in 2 × SDS sample buffer and subjected to SDS-PAGE. The gels were transferred to nitrocellulose membranes followed by Western blot analysis with anti-IRβ antibody.

Light-induced Photoreceptor Degeneration—Female Sprague-Dawley adult rats were raised and maintained in a cyclic light environment (12 h on, 12 h off at an in-cage illumination of less than 10 lux). Ovariec-
tomy was performed 2 weeks before exposure to constant light for 24 h at age 3–4 months. Constant light at an illumination level of 1,700 lux was provided by two 40-W white fluorescent light bulbs that were suspended 50 cm above the floor of the cage. During light exposure, rats were maintained in transparent polycarbonate cages with stainless-steel wire bar covers. A water bottle was kept in the appropriate depression in the cage cover, but food was placed in the bottom of the cage on the bedding. Animals received intraperitoneal injection of βE2 (500 μg/kg of body weight) or vehicle 1 h before exposure to constant light for 24 h.

Functional Rescue of Photoreceptor Cells Evaluated by Electroretinogram—Electroretinogram (ERG) recordings were performed as described previously (22). Briefly, animals were kept in total darkness overnight before the ERG recording. Pupils were dilated with 1% atropine and 2.5% phenylephrine HCl. Animals were anesthetized intra-
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RESULTS

βE2 Attenuates H2O2-induced Cytotoxicity—We tested the cytoprotective effects of βE2 in our well-characterized retinal neuronal culture system, using H2O2 to generate an oxidant stress. We first examined the cytotoxicity of βE2 on neurons by treating the cultures with different concentrations of βE2 ranging from 0.0001 to 100 μM. A significant reduction in cell viability was observed only at the relatively high 100 μM concentration (Fig. 1B). To evaluate the role of βE2 in protecting retinal neurons from H2O2-mediated cytotoxicity, we pretreated retinal neuron cultures with different concentrations of βE2 (ranging from 0.001 to 10 μM) 30 min prior to H2O2 (100 μM) treatment; significant increases in cell viability were observed between 0.1 and 10 μM of βE2 (Fig. 1C). We used absolute ethanol to dissolve βE2. When culture medium contained 10 μM βE2, the concentration of ethanol was 0.01%. There was no significant cytotoxicity of 0.01% ethanol treatment for up to 24 h as measured by the MTT assay (Fig. 1A).

Effect of the Estrogen Receptor Inhibitor Tamoxifen—To investigate whether the cytotoxic effects of βE2 are mediated through the estrogen receptor, we repeated the experiments described above in the presence of tamoxifen, an estrogen receptor blocker. Fig. 2A shows that additions of tamoxifen at 5 μM for 24 h did not significantly affect cell viability, although decreases in cell viability were observed at 10 and 100 μM concentrations. Pretreatment with 5 μM tamoxifen for 30 min prior to the addition of βE2 did not significantly block the neuroprotective effect of βE2 in this study (Fig. 2B). Immunocytochemical analysis of cultured primary retinal neurons showed that a very small population of cells (less than 1%) was estrogen receptor-positive. These cells were recoverin-negative, indicating that they were not photoreceptor cells, and were usually double or triple the size of recoverin-positive cells (Fig. 3).

βE2 Inhibits Apoptosis Induced by H2O2—The TUNEL assay was performed to determine whether βE2 can inhibit apoptotic cell death induced by H2O2. A few positive staining cells were noted in control cultures, whereas cultures treated with 100 μM H2O2 for 24 h had large numbers of cells undergoing apoptosis. However, pretreatment of retinal neurons with 10 μM βE2 for 30 min prior to H2O2 (100 μM) exposure led to a dramatic decrease in the numbers of apoptotic cells. A few TUNEL-positive cells were noted in the group pretreated with βE2 without exposure to H2O2. The percentage of TUNEL-positive cells (Fig. 4A) in control cultures or βE2-treated cultures without H2O2 exposure from three independent experiments was 3–6%, whereas H2O2-treated cultures exhibited 53% positive cells. Pretreatment with βE2 significantly reduced the positive cells to 16%. This inhibition of apoptosis by βE2 was also evidenced in DNA fragmentation study showing a complete prevention of DNA fragmentation induced by H2O2 (Fig. 4B, lane 9).

Effects of 17α-Estradiol (αE2) and Progesterone—The specificity of the βE2 effect was determined by testing two other steroids, αE2, an isomer of βE2 that does not activate the estrogen receptor, and progesterone. Neither was cytotoxic to the cells at the concentration of 10 μM when added in the absence of H2O2. A few positive staining cells were noted in control cultures (Fig. 5A), whereas large numbers of cells undergoing apoptotic cell death (Fig. 5B) were present in cultures
Moxifen did not significantly block the neuroprotective effect of recoverin-positive cells; recoverin-negative cells. After a 30-min treatment with neurons with 10E2 for 30 min prior to H2O2 (100M) exposure without tamoxifen pretreatment (mean ± S.D., n = 6). Tamoxifen did not significantly block the neuroprotective effect of αE2.

**Fig. 2.** Tamoxifen does not block neuroprotective effect by βE2. A, effect of tamoxifen, an estrogen receptor antagonist, on the cell viability of cultured retinal neurons. Cultured retinal neurons were treated with different concentrations of tamoxifen for 24 h. The cytotoxic responses were measured by the MTT assay. B, cultures were pretreated with 5μM tamoxifen 30 min prior to 10μM βE2 treatment. After a 30-min treatment with βE2, cultures were exposed to 100μM H2O2 for 24 h. *, p < 0.05 versus the same treatment of βE2 and H2O2 exposure without tamoxifen pretreatment (mean ± S.D., n = 6). Tamoxifen did not significantly block the neuroprotective effect of αE2.

**Fig. 3.** Visualization of recoverin-positive and estrogen receptor-α-positive cells in culture. A, Nomarski image; B, fluorescein green-stained estrogen receptor-α-positive cells; C, Texas Red-stained recoverin-positive cells; D, superimposition of fluorescein isothiocyanate (green) image and Texas Red image on the Nomarski image. The white arrows indicate estrogen receptor-positive cells that are recoverin-negative.

treated with 100μM H2O2 for 24 h. Pretreatment of retinal neurons with 10μM αE2 for 30 min prior to H2O2 (100μM) exposure caused a significant decrease in the numbers of apoptotic cells (Fig. 5D). However, pretreatment with 10μM progesterone for 30 min prior to H2O2 (100μM) exposure did not appear to inhibit apoptosis induced by H2O2 significantly (Fig. 5F). Quantification of the number of apoptotic cells showed that cultures treated with either αE2 or progesterone had no more apoptotic cells than untreated cultures (4–7%). However, pretreatment with αE2 decreased the percentage of apoptotic cells in H2O2-treated cultures from 49 to 22%, whereas progesterone-pretreated cultures remained 42% TUNEL-positive (Fig. 5G). Thus, αE2 significantly reduced the number of apoptotic cells, although to a lesser extent than βE2 (Fig. 4A). Another indication of the effectiveness of αE2 was that it significantly reduced DNA fragmentation, whereas progesterone did not (Fig. 4B, lanes 5 and 7).

**Morphological and Functional Evaluations of Photoreceptor Cell Rescue in Vivo—Neuroprotection by systemic administration of βE2 was evaluated by quantitative histology, ERG, and TUNEL assay. 24 h of exposure to fluorescent light (1,700 lux) reduced the thickness of the ONL of photoreceptor cell nuclei from the normal 10–13 rows in control animals (Fig. 6, A and B) to 3 rows in the most degenerated region of the retinas in vehicle-injected animals (Fig. 6C). However, in βE2-treated animals, there was significant rescue of photoreceptors with the ONL having 7–8 rows of nuclei (Fig. 6D). Quantitative analysis of ONL thickness as a function of the retinal location showed a significant protection of photoreceptors by systemic injection of βE2 across the entire retina (Fig. 7A). βE2 also protected retinal function, as demonstrated in the ERG tracings in Fig. 6. Both A- and B-wave responses were greater in the light-stressed rats given βE2 than in controls given a placebo. Measurement of B-wave amplitudes at different flash intensities clearly demonstrates the functional protection provided by βE2 (Fig. 7B).

The TUNEL assay was performed to determine whether βE2-mediated photoreceptor protection is through inhibition of apoptosis. Fig. 8A showed intensive TUNEL-positive cells in the ONL in the superior region (the most degenerated area) of the retinas in vehicle-treated animals after exposure to constant light for 24 h at an illumination of 1,700 lux. Systemic injection of βE2 significantly reduced light-induced photoreceptor cell apoptosis in this region (Fig. 8B).

The serum level of βE2 was 71 ± 21 pg/ml (0.3 nM) in ovariectomized animals without βE2 treatment and 587 ± 184 pg/ml (2.1 nM) after intraperitoneal injection of 500μg βE2/kg of body weight. The serum level of βE2 in non-ovariectomized adult rats without βE2 treatment was 158 ± 35 pg/ml (0.6 nM).

**Involvement of Insulin/PI3K/Akt Signal Pathway**—The PI3K cascade has been shown to provide neuroprotection to stressed neuronal cells (39). PI3K activity was increased by a 30-min

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Recently it has been shown that IRβ is involved in the regulation of PI3K activity in the retina (37, 38). We examined whether IRα activation may be involved in the estrogen effect. We presented preliminary data on this at the Retinal Degeneration meeting held at Burginstock, Switzerland (40) that 1) IRβ is expressed in our cultured rat retinal neurons; 2) 5 µM βE2 and 5 µM αE2 activated IRβ; and 3) 5 µM progesterone was ineffective. In the present study, using GST (GST-p85-N-SH2) pull-down experiments to study IRβ phosphorylation (activation), we further demonstrated that both insulin and βE2 (Fig. 11A) activated IRβ. The data from in vivo experiments showed that both IRβ and insulin-like growth factor-I receptor (IGF-IR) were present in the retina (Fig. 11B). Systemic administration of βE2 activated IRβ but not IGF-IR (Fig. 11C) using GST (GST-p85-N-SH2) pull-down experiments. We also demonstrated by immunohistochemistry that insulin receptor is present in the inner and outer segments of photoreceptor cells (Fig. 12). In addition, we found that the insulin receptor blocker, HNMPA (200 µM), did not completely inhibit βE2-activated PI3K (Fig. 13A), and incubation of βE2 (10 µM) directly with a homogenate of cultured retinal neurons did not activate PI3K (Fig. 13B).

It is well known that Akt is the downstream target of PI3K in receptor-mediated signal cascades and is activated by phosphorylation (41). To determine whether Akt can be activated by βE2, retinal neuron cultures were treated with 10 µM βE2 for different times (0.5, 1, 3, 6, and 12 h), and extracts were subjected to Western blot analysis with anti-phospho-Akt (pS473) antibody (1:2,000) (preliminary data presented at the Retinal Degeneration meeting held at Burginstock, Switzerland (40)). As shown in Fig. 14A, addition of 10 µM βE2 to the culture for 30 min significantly increased phosphorylated Akt expression more than 2-fold, and this increase was maintained for at least 3 h before declining to 1.5-fold by 6 h. Systemic injection of βE2 at the concentration of 500 µg/kg also transiently increased pAkt level in vivo (Fig. 14B, upper panel). Over the same time course, Western blots showed that the expression of the pan-Akt protein did not respond to βE2 treatment (Fig. 14B, lower panel), indicating that the increased pAkt was not caused by increased Akt expression in the retina.

DISCUSSION

In the present study, we demonstrated that estrogen has a neuroprotective effect in the retina which is mediated via the insulin/PI3K/Akt signal transduction pathway. Although it has been known that βE2 promotes viability and survival of other primary neuronal culture systems, such as cortex (42), hippocampus (43), hypothalamus (30), this is the first observation in primary neuronal culture systems, such as cortex (42), hippocampus (43), hypothalamus (30). As shown in Fig. 14A, addition of 10 µM βE2 to the culture for 30 min significantly increased phosphorylated Akt expression more than 2-fold, and this increase was maintained for at least 3 h before declining to 1.5-fold by 6 h. Systemic injection of βE2 at the concentration of 500 µg/kg also transiently increased pAkt level in vivo (Fig. 14B, upper panel). Over the same time course, Western blots showed that the expression of the pan-Akt protein did not respond to βE2 treatment (Fig. 14B, lower panel), indicating that the increased pAkt was not caused by increased Akt expression in the retina.

In some tissues, neuroprotection of βE2 may be mediated by the estrogen receptor and protein synthesis (27, 44) because βE2 can bind intracellular specific estrogen receptors, and the complex binds to specific sites on genomic DNA and control its transcription. Indeed, βE2 was reported to provide neuroprotection mediated by estrogen receptors in cultured cortical neurons (45) and in hippocampus-derived cell line (46). We observed that a competitive estrogen receptor antagonist, tamoxifen, did not significantly attenuate the protection provided by βE2. Although the absence of any effects by tamoxifen did not completely exclude the possibility that the neuroprotection was mediated by estrogen receptors, αE2, a biologically inactive stereoisomer that

treatment of cultured retinal neurons with βE2 and reached a plateau at 1 and 3 h (Fig. 9A), then activity decreased after 6 h and returned to the control level after 12 h. A similar pattern of PI3K activation was observed in rat retina after systemic injection of βE2 (Fig. 9B, upper panel). Synthesis of phosphoinositide trisphosphate increased up to 12 h and returned to base line by 24 h. Over the same time course, Western blots showed that the expression of the p85α regulatory subunit of PI3K did not respond to βE2 treatment (Fig. 9B, lower panel), indicating that the increased enzymatic activity of PI3K was not caused by increased p85 expression in the retina.

Pretreatment with LY294002, a PI3K inhibitor, for 30 min prior to the addition of βE2 greatly inhibited the βE2-induced protective effect (Fig. 10C). Neither the carrier (dimethyl sulfoxide) nor 100 µM LY294002 drug was cytotoxic after 24 h (Fig. 10, A and B). These results suggest that the βE2 cytoprotective effect may be mediated through some downstream effector generated by activation of the PI3K pathway. The cytoprotective effect of the two estrogen isomers and the absence of a tamoxifen effect as well as the absence of estrogen receptor expression in the photoreceptor cells (Fig. 3) suggested that the estrogen receptor is not involved in the estrogen-mediated photoreceptor protection.

Fig. 5. αE2 but not progesterone inhibits H2O2-induced apoptosis. A, vehicle-treated control. B, H2O2 treated (100 µM, 24 h) cells. C, cultured retinal neurons treated only with 10 µM αE2 for 24 h. D, cultured retinal neurons pretreated with 10 µM αE2 30 min before exposure to 100 µM H2O2 for 24 h. E, cultured retinal neurons treated only with 10 µM progesterone for 24 h. F, cultured retinal neurons pretreated with 10 µM progesterone 30 min before exposure to 100 µM H2O2 for 24 h. G, comparison of TUNEL-positive cells as a function of treatment. *, p < 0.05 versus the same H2O2 exposure without αE2 pretreatment (mean ± S.D., n = 3).
has little effect as a female sex steroid hormone, also provides neuroprotection of retinal neurons against H2O2-induced apoptotic cell death. Furthermore, we could not detect estrogen receptors in photoreceptor cells in vitro. These results suggest that neuroprotective effects of E2 in our culture system do not involve activation of estrogen receptors. In addition to the activation of genome transcription mediated by estrogen receptors, estrogens have been reported to activate IGF-IR kinase, resulting in enhanced binding of p85, the regulatory subunit of PI3K, to insulin receptor substrate-1 and -2 in the mouse uterus (47). However, we found that E2 does not activate IGF-IR in the retina. Insulin receptors are present in neuronal retina (48), and the interaction of the IRβ

**Fig. 6. E2 protects retina from light damage.**

A, control: the black arrow indicates the optic nerve, and the white arrowhead indicates where the high magnification picture was taken. The inset shows a typical normal ERG waveform. B, E2 treatment without light damage did not affect retinal morphology and function. C, 24 h constant light (CL) damage with intraperitoneal injection of 1 ml of 1% ethanol. D, pretreatment of E2 at the concentration of 500 μg/kg of body weight for 1 h before exposure to 24 h of constant light. RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

**Fig. 7. Measurements of ONL thickness and ERG B-wave amplitude as function of protection.**

A, rat retinal outer nuclear layer (ONL) thickness along the vertical meridian, and the results are expressed as mean ONL thickness ± S.D. (n = 6 for each point). B, ERG B-wave sensitivity curves, and the results are expressed as mean microvolt ± S.D. (n = 6 for each point). ONH, optic nerve head.

**Fig. 8. E2 inhibits photoreceptor apoptosis in vivo.**

A, 24 h of constant light damage with intraperitoneal injection of 1 ml of 1% ethanol as vehicle control results in extensive TUNEL-positive cells in ONL. B, systemic administration of E2 at the concentration of 500 μg/kg of body weight for 1 h before exposure to constant light at the illumination of 1,700 lux for 24 h, shows a great reduction of TUNEL-positive cells in the outer nuclear layer (ONL).
Because the insulin receptor blocker did not completely inhibit E2-activated Akt, suggesting the possible involvement of Akt in the protection of cells from apoptosis (53). Anti-Akt pSer473 is a polyclonal antibody developed against the singly phosphorylated Akt. We used this antibody to show clearly that phosphorylation of IR serves to trigger the phosphorylation of Akt.

Both E2 and insulin activate the IR. Whether the estrogen effect on IRβ is direct or indirect is not known at present. However, insulin implants significantly reduced the number of apoptotic cells in retinas of streptozotocin diabetic rats (50), and insulin has been shown to prevent apoptotic cell death of proliferating neuroepithelial cells in the embryonic retina (51) and to rescue retinal neurons by inhibiting caspase-3 (50).

Studies examining the role of PI3K, mainly using inhibitors, suggest involvement of PI3K in numerous biological responses that encompass the regulation of cell growth (52). It is clear in the present study that βE2 significantly increased PI3K activity in cultured retinal neurons and in the retina in vivo. This neuroprotective effect provided by βE2 can be blocked by a PI3K inhibitor, suggesting a direct role of PI3K in estrogen-mediated neuroprotection. Akt, also known as protein kinase B, is a serine/threonine, mitogen-regulated protein kinase involved in the protection of cells from apoptosis (53). Anti-Akt pS473 is a polyclonal antibody developed against the singly phosphorylated Akt. We used this antibody to show clearly that addition of βE2 to retinal neuronal cultures significantly activates Akt, suggesting the possible involvement of Akt in βE2-mediated retinal neuronal protection. It is generally thought that insulin acts as a neurotrophic factor via the PI3K/Akt signaling pathway, which in turn inhibits many proapoptotic targets (49, 54). These observations led to the hypothesis that estrogen-mediated insulin receptor/PI3K/Akt signaling could be one of the mechanisms of βE2-mediated retinal neuron protection. Because the insulin receptor blocker did not completely inhibit βE2-activated PI3K, this suggests that additional mechanisms might be involved in this protection. It has been reported that the extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathway is involved in estrogen-mediated cell survival (55, 56). We have found that systemic injection of βE2 increased Ras activity in a time-dependent manner in mouse retina.

The effects of βE2 and αE2 on the insulin receptor are likely not through a nonspecific membrane effect, based on following evidence: 1) ethanol and acetic acid did not activate insulin receptor; 2) progesterone at the same concentration as βE2 did not activate the insulin receptor; 3) in vitro injection of βE2 activates insulin receptor but not the IGF-IR. The mechanism that triggers the phosphorylation of IRβ in response to βE2 and αE2 is not known. Recently light-induced tyrosine phosphorylation of IRβ independent of insulin in the retina has been reported (37). We speculate that there could be at least two possible mechanisms that lead to the phosphorylation of IRβ. The first could involve ligand(s) other than insulin, which are induced or released in response to estrogens. The second mechanism involves the activation of a nonreceptor tyrosine kinase(s) in response to estradiols. Nonreceptor tyrosine kinase Src phosphorylates insulin receptors and IGF-IRs on autophosphorylation sites, and Src kinase has been shown to substitute for the ligand-dependent receptor activation (59, 60). Consistent with this mechanism, we have also reported previously the in vitro phosphorylation of IRβ by c-Src in reactive oxygen species (38). It has been shown that αE2-induced vascular endothelial growth factor-A gene expression in rat pituitary tumor cells is mediated through an estrogen receptor-independent mechanism.

*W. Cao, unpublished observation.
dent but PI3K-Akt-dependent signaling pathway (61). Akt activation has also been shown by estrogen in estrogen receptor-negative breast cancer cells (32). In those studies, insulin receptor phosphorylation was not studied.

It is worth noting that in our in vitro primary rat retinal neuronal cultures were incubated in the presence of 100 nM insulin, 5 µM βE2, 0.01% acetic acid, and 0.01% ethanol was subjected to GST pull-down assay with GST-p85N-SH2 domain. The bound proteins were subjected to Western blot analysis with anti-IRβ antibody. Both βE2 and insulin, but not vehicles, activated insulin receptor in cultured retinal neurons. B, presence of IRβ and IGF-IR in rat retina. C, βE2 activates IRβ but not IGF-IR in the retina in vivo.

![Fig. 11. Activation of IRβ by βE2. A, primary rat retinal neuronal cultures were incubated in the presence of 100 nM insulin, 5 µM βE2, 0.01% acetic acid, and 0.01% ethanol was subjected to GST pull-down assay with GST-p85N-SH2 domain. The bound proteins were subjected to Western blot analysis with anti-IRβ antibody. Both βE2 and insulin, but not vehicles, activated insulin receptor in cultured retinal neurons. B, presence of IRβ and IGF-IR in rat retina. C, βE2 activates IRβ but not IGF-IR in the retina in vivo.](image1)

![Fig. 12. Presence of IRβ in outer and inner segments of photoreceptors. A, fluorescein green-stained photoreceptor protein, arrestin, in outer segments of photoreceptors. B, Texas Red-stained IRβ presented in outer and inner segments of photoreceptors. C, DAPI-stained nuclei. D, superimposition of fluorescein isothiocyanate (green) image and Texas Red image on DAPI blue image.](image2)

![Fig. 13. A, addition of insulin receptor inhibitor, HNMPA (200 µM), inhibited estrogen-mediated activation of PI3K. DMSO, dimethyl sulfoxide. B, direct incubation of βE2 with cell homogenate for 30 min did not activate PI3K.](image3)

![Fig. 14. Activation of Akt by βE2. A, primary retinal neuron cultures were treated with 10 µM βE2, and extracts were subjected to Western blot analysis with anti-phospho-Akt (pS473) antibody. βE2 significantly increase in the activation of Akt. B, upper panel, anti-phospho-Akt S473 antibody (1:2,000) detects a phospho-Akt (pAkt) enzyme band at ~60 kDa showing an increase in the pAkt level in the retina by systemic administration of βE2 at the concentration of 500 µg/kg of body weight; lower panel, Western blots showed that the expression of Akt protein did not respond to βE2 treatment.](image4)
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