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Neuroprotection of Insulin against Oxidative Stress-induced Apoptosis in Cultured Retinal Neurons: Involvement of Phosphoinositide 3-kinase/Akt Signal Pathway

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Abstract In order to investigate the neuroprotection of insulin in retinal neurons, we used retinal neuronal culture as a model system to study the protective effects of insulin against H_2O_2-induced cytotoxicity and apoptotic death. Primary retinal neuronal cultures were grown from retinas of 0–2-day old Sprague-Dawley rats. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. Apoptotic cell death was evaluated by the TdT-mediated digoxigenin-dUTP nick-end labeling assay, and by DNA laddering analysis. Phosphoinositide 3-kinase (PI3K) activity was measured using phosphoinositide 4,5-bisphophate and [γ-^{32}P]ATP as substrate. Western blot analysis with anti-phospho-Akt (pS473) antibody was performed to examine the level of phosphorylated Akt. We observed that treatment with 100 μM H_2O_2 for 24 h significantly decreased cell viability and induced apoptotic death of retinal neurons, and that pretreatment with 10 nM insulin significantly inhibited or attenuated H_2O_2-induced cytotoxicity and apoptosis. Pretreatment with LY294002, a specific PI3K inhibitor, abolished the cytoprotective effect of insulin. Insulin also strongly activated both PI3K and the downstream effector Akt. These results suggest that insulin protects retinal neurons from oxidative stress-induced apoptosis and that the PI3K/Akt signal pathway is involved in insulin-mediated retinal neuroprotection.

Key words apoptosis; insulin; phosphoinositide 3-kinase/Akt; oxidative stress; neuroprotection; retinal neuronal culture

Oxidative stress, the result of cellular production of reactive oxygen species, has been implicated in a number of diseases of the retina. There is substantial evidence that light injures photoreceptors by increasing the formation of reactive oxygen species [1] and that antioxidants can rescue light-damaged photoreceptors [2–4]. The role of oxidative stress as a mediator of apoptosis has been examined [5,6], and hydrogen peroxide (H_2O_2), a by-product of oxidative stress, has been implicated in triggering apoptosis in various cell types including cultured retinal neurons [7–9]. Apoptosis has been described in a wide variety of hereditary retinal degenerations [10,11], in light damaged retinas [12,13], and following retinal detachment [14]. Other types of retinal degeneration, such as retinal ischemia [15], glaucoma [16], and diabetic retinopathy [17] have also been associated with apoptosis.

Insulin is a metabolic hormone, but accumulating evidence suggests that it, as an important survival factor, has a neuroprotective role [18]. Insulin can protect against brain damage induced by stress conditions, such as oxidative stress or ischemia [19–21]. Insulin can also protect against apoptosis by regulating a number of factors associated with apoptosis in the nervous system [22–25]. It has been demonstrated that insulin can protect against apoptosis induced by serum deprivation in neuronal culture from different nervous areas including cortex [26], cerebellar granule cells [27], spinal motor neurons [28], and PC12 cells [29]. The retina is an integral part of the central nervous system and also expresses abundant insulin receptors [30]. However, the role of insulin as a survival factor in the protection of retinal neurons is not well understood.

In order to investigate the neuroprotection of insulin in retinal neurons, we used cultured retinal neurons as a model

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system to examine the protective effect of insulin on neurons against H_2O_2-induced cell death. Our data demonstrated that insulin increased cell viability and inhibited apoptosis in cultured retinal neurons and that activation of phosphoinositide 3-kinase (PI3K)/Akt was involved in insulin-mediated retinal neuroprotection.

Materials and Methods

Materials

Anti-p85α regulatory subunit of PI3K antibody was from Upstate Biotechnology (Lake Placid, USA). Anti-Akt and anti-phospho-Akt (pS473) antibodies were obtained from Cell Signaling Technology (Beverly, USA). [γ-^{32}P]ATP was from New England Nuclear (Boston, USA). Phosphoinositide 4,5-bisphophate (PI-4,5-P_2) was from Echelon Research Laboratories (Salt Lake, USA). Electrophoresis reagents, nitrocellulose sheets, and alkaline phosphatase-conjugated goat antirabbit immunoglobulin (Ig) G were from Bio-Rad (Richmond, USA). Horseradish peroxidase (HRP)-conjugated donkey antirabbit IgG and sheep antimouse IgG, enhanced chemiluminescence (ECL) reagents, and Hyperfilm ECL were from Amersham Biosciences (Piscataway, USA). Silica gel thin-layer chromatographic (TLC) plates were from Sigma (St. Louis, USA). Dulbecco's modified Eagle's medium with F-12 (DMEM/F12) was purchased from Gibco-BRL (Grand Island, USA). Apoptosis detection kits were purchased from Oncor (Gaithersburg, USA). All other reagents were of analytical grade from Sigma.

Primary culture of retinal neurons

The preparation of primary cultures of retinal neurons was as described previously [9,31]. Retinas of 10–15 Sprague-Dawley rat pups, 0–2 d 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 DMEM/F12 plus 10% fetal calf serum in a plastic bag and mechanically dissociated. The suspension was first filtered through a 230 μm sieve, which was then rinsed once more 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 1000 *g* in a clinical centrifuge for 5 min, the supernatant decanted, and the cell pellets resuspended in 25 ml of medium 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 1×10^5 cells per milliliter. The cells (1 ml per well) were plated in 24-well tissue culture plates on 12 mm coverslips that had been pre-treated overnight with poly-*D*-lysine (10 μg/ml). The cells were maintained in DMEM/F12 with 2% fetal calf serum for the first 3–4 d and then in synthetic serum-free medium. The cultures were used in experiments 10–14 d after plating.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

As described previously [32], MTT was dissolved at a concentration of 5 mg/ml in phosphate-buffered saline. Lysing buffer was prepared as follows: 20% (*W*/*V*) of sodium dodecylsulfate (SDS) was dissolved at 37 ºC in a solution of 50% of N,N-dimethyl formamide in deionized water. Twenty-five microliters of the 5 mg/ml stock solution of MTT was added to each well and, after 2 h of incubation at 37 ºC, 100 μl of the lysing buffer was added. After an overnight incubation at 37 ºC, absorbance of the samples was read at 562 nm using a microtiter plate enzyme-linked immunosorbent assay reader.

TdT-mediated digoxigenin-dUTP nick-end labeling (TUNEL) assay

Detection of apoptosis using the TUNEL method was carried out with a commercially available *in situ* apoptosis detection kit as described previously [9]. Staining for the TUNEL assay was performed according to the protocol provided by the manufacturer. Cells were stained with diaminobenzidine as the substrate for the HRP in the kit. TUNEL-positive cells were identified with a Nikon Eclipse 800 microscope (Yokohama, Japan), and images were captured by a digital camera and stored in a computer. The percentage of apoptotic cells was calculated by dividing TUNEL-positive cells by the total number of cells visualized by Nomarski optics in the same field. Three digitized images of similar total cell numbers were selected from each coverslip for counting and averaging, and were considered as one independent experiment. Three independent experiments were then averaged.

DNA fragmentation

DNA laddering was carried out as follows. The cells were homogenized in 1 ml of extraction buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% SDS, and 0.5 mg/ml of freshly prepared proteinase K) using a Tissue-Tearor (Biospec Products Inc., Bartlesville, USA). Each sample was placed on ice for 20 min then centrifuged at 15,000 *g* for 10 min. After centrifugation, the supernatant from each sample was extracted with phenol/chloroform until the

white precipitate was no longer visible in the aqueous fraction. This usually took 3–6 extractions. The genomic DNA was then precipitated overnight at –20 ºC with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol. The samples were then centrifuged at 5000 *g* for 20 min and the resulting pellets were resuspended in 100 μl of TE buffer (Tris-HCl and EDTA, pH 8.0). RNase A was then added to a final concentration of 20 μg/ml and the samples were incubated at 37 ºC for 2 h. Finally, 3–5 μl of each sample was run on a 2% agarose gel at 40 V for 2 h.

PI3K activity assay

Enzyme assays were carried out essentially as described previously [33]. Briefly, assays were performed directly on total cells in 50 μl of the reaction mixture containing 0.2 mg/ml PI-4,5-P_2, 50 μM ATP, 0.2 μCi [γ-^{32}P]ATP, 5 mM $MgCl_2$, and 10 mM HEPES buffer (pH 7.5). The reactions were performed for 15 min at room temperature and stopped by the addition of 100 μl of 1 M HCl followed by 200 μl of chloroform-methanol (1:1, *V*/*V*). Lipids were extracted and resolved on oxalate-coated TLC plates (silica gel 60) with a solvent system of 2-propanol/2 M acetic acid (65:35, *V*/*V*). The TLC plates were prepared by placing in 1% (*W*/*V*) potassium oxalate in 50% (*V*/*V*) methanol and baked in an oven at 100 ºC for 1 h before use. TLC plates were exposed to X-ray film overnight at –70 ºC and radioactive lipids were scraped and quantified by liquid scintillation counting.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Protein samples were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The blots were washed twice for 5 min with TTBS (100 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.1% Tween-20) and blocked with 10% non-fat dry milk in TTBS overnight at 4 ºC. The blots were then incubated with anti-p85α regulatory subunit of PI3K (1:1000), anti-Akt (1:500), and anti-phospho-Akt S473 (1:2000) antibodies for 2 h at room temperature. Following primary antibody incubations, the blots were incubated with HRP-linked secondary antibodies (antirabbit, antimouse, or antigoat IgG) and developed by ECL, according to the manufacturer's instructions.

Statistical analysis

Data were expressed as mean±SD and analyzed by means of analysis of variance, and further assessed by Dunnett's tests. Statistical differences reaching $P<0.05$ were accepted as significant.

Results

Insulin protects retinal neurons from H_2O_2-induced cytotoxicity

We tested the cytoprotective effects of insulin in a well-characterized retinal neuronal culture system, using 10 μM H_2O_2 to generate an oxidative stress [9]. We first examined the cytotoxicity of insulin on neurons by treating the cultures with different concentrations of insulin ranging from 0.1 to 100 nM. The results showed that these concentrations of insulin did not significantly affect cell viability of cultured retinal neurons compared with the untreated control cultures (data not shown). To evaluate the role of insulin in protecting retinal neurons from H_2O_2-induced cytotoxicity, we pretreated retinal neuron cultures with different concentrations of insulin (0.1, 1, 10, 100 nM) for 30 min prior to exposure to H_2O_2 (100 μM) for 24 h. Cell viability was determined by the MTT assay. As shown in **Fig. 1**, insulin attenuates cytotoxicity induced by H_2O_2 in a dose-dependent fashion. Insulin (0.1 and 1 nM) did not significantly increase cell viability, whereas 10 nM insulin significantly raised cell viability from 32% to 80%. A higher concentration of insulin (100 nM) did not further increase cell viability, indicating that the maximal effect was at 10 nM. These data suggest that insulin protects cultured retinal neurons from H_2O_2-induced cytotoxicity.

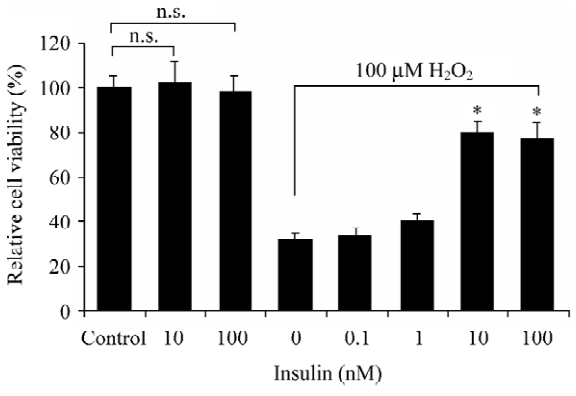

Fig. 1 Insulin attenuates H_2O_2-induced cytotoxicity in cultured retinal neurons

Cultured retinal neurons were pretreated with different concentrations of insulin for 30 min before exposure to 100 μM H_2O_2 for 24 h. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. *$P<0.05$ vs. the same dose of H_2O_2 exposure without insulin pretreatment (mean±SD, $n=9$). n.s., no significant difference.

Insulin inhibits H_2O_2 induced apoptosis

The TUNEL assay was performed to determine whether insulin could inhibit apoptotic cell death induced by H_2O_2. A few positive-staining cells were noted in control cultures [**Fig. 2(A)**], whereas cultures treated with 100 μM H_2O_2 for 24 h had a large number of cells undergoing apoptosis [**Fig. 2(B)**]. A few TUNEL-positive cells were noted in the group pretreated with insulin without exposure to H_2O_2 [**Fig. 2(C)**]. However, pretreatment of retinal neurons with 10 nM insulin for 30 min prior to H_2O_2 (100 μM) exposure led to a dramatic decrease in the number of apoptotic cells [**Fig. 2(D)**]. The percentage of TUNEL-positive cells [**Fig. 2(E)**] in control cultures or insulin-treated cultures without H_2O_2 exposure from three independent experiments was 3%–5%. H_2O_2-treated cultures without insulin pretreatment exhibited 48% positive cells, whereas, with insulin pretreatment, the number of TUNEL positive cells in H_2O_2-treated cultures was only 8%. This inhibition of apoptosis by insulin was also evidenced in a DNA fragmentation study showing a complete prevention of DNA fragmentation induced by H_2O_2 [**Fig. 2(F)**].

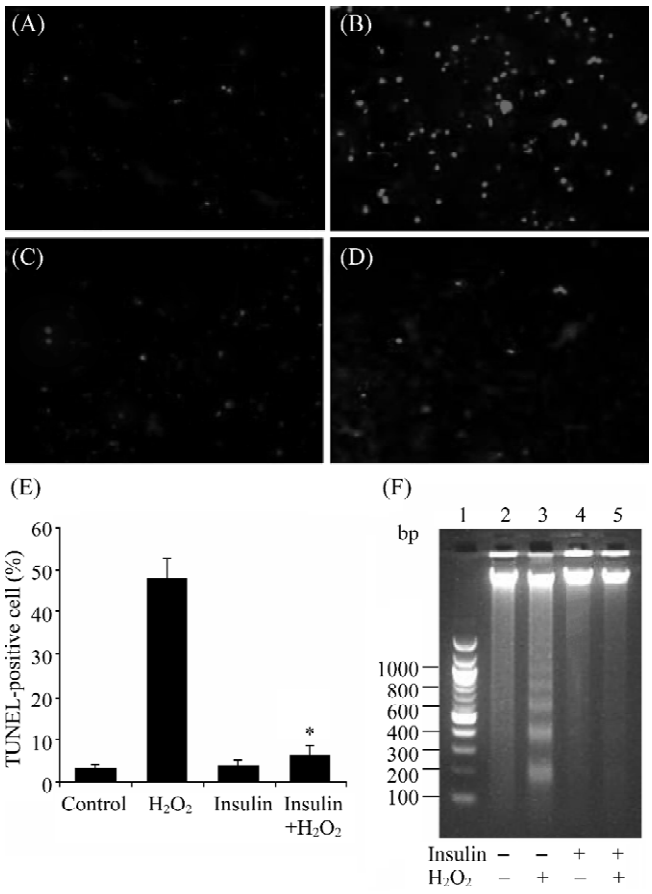

Fig. 2 TdT-mediated digoxigenin-dUTP nick-end labeling (TUNEL) assay of cultured retinal neurons showing insulin inhibits H_2O_2-induced apoptosis

(A) Untreated normal control. (B) Cells treated with 100 μM H_2O_2 for 24 h. (C) Cultured retinal neurons treated only with 10 nM insulin for 24 h. (D) Cultured retinal neurons pretreated with 10 nM insulin for 30 min before exposure to 100 μM H_2O_2 for 24 h. (E) Percentage inhibition of H_2O_2-induced apoptosis by insulin in cultured retinal neurons as determined by the TUNEL assay. *P<0.05 vs. the same H_2O_2 exposure without insulin pretreatment (mean±SD, n=9). (F) Insulin prevents DNA fragmentation induced by H_2O_2. 1, DNA molecular weight marker; 2, untreated cells; 3, cells treated with 100 μM H_2O_2 for 24 h; 4, cells treated with 10 nM insulin for 24 h; 5, cells pretreated with 10 nM insulin for 30 min before exposure to 100 μM H_2O_2 for 24 h.

Specific PI3K inhibitor, LY294002, abolishes protection of insulin

The PI3K cascade has been shown to provide neuroprotection to stressed neuronal cells [34]. To investigate whether the cytoprotective effect of insulin was mediated by activation of PI3K, we pretreated cultured retinal neurons with 10 μM LY294002, a PI3K-specific inhibitor, for 30 min prior to the addition of insulin, and then determined cell viability by MTT assay. As shown in **Fig. 3**, pretreatment with 10 μM LY294002 greatly inhibited the insulin-induced protective effect. Cell viability was significantly decreased (from 80% to 40%). These results

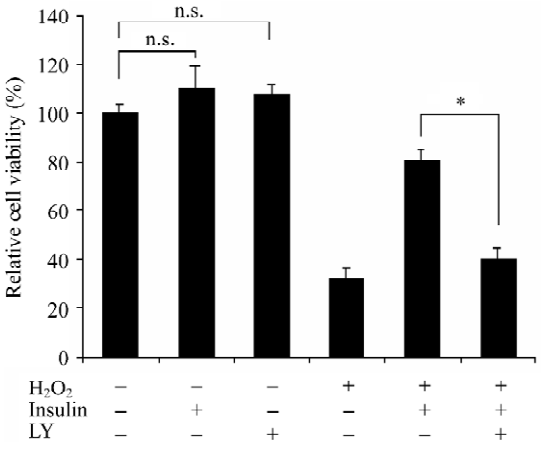

Fig. 3 Phosphoinositide 3-kinase inhibitor blocks neuroprotective effect provided by insulin in cultured retinal neurons

Cultured retinal neurons were pretreated with 10 μM LY for 30 min prior to 10 nM insulin treatment. After 30 min treatment with insulin, cultures were exposed to 100 μM H_2O_2 for 24 h. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. LY significantly blocked the neuroprotective effect of insulin. *P<0.05 vs. the same treatment of insulin and H_2O_2 exposure without LY pretreatment (mean±SD, n=9). n.s., no significant difference. LY, LY294002.

suggest that activation of PI3K may play a critical role in the insulin-induced neuroprotective effect in retinal neurons.

Insulin activates PI3K in cultured retinal neurons

We next examined whether insulin actually activates PI3K in cultured retinal neurons. When cultured retinal neurons were treated with 10 nM of insulin, PI3K activity was increased at 15 min. This increase reached a peak at 30 min [**Fig. 4(A)**], slowly declined after 90 min, and returned to control level after 240 min. The percentage increase in PI3K activity by insulin is shown in **Fig. 4(B)**. Moreover, we also detected that insulin activated PI3K in a dose-dependent manner. Ten nanomoles of insulin maximally activated PI3K, whereas a higher concentration of insulin (100 nM) had no further effect on PI3K activity [**Fig. 4(C)**].

To determine whether the increased activity of PI3K is due to upregulated PI3K expression by insulin in the retina, retinal neuron cultures were treated with 10 nM of insulin over the same time course, and extracts were subjected to Western blot analysis with anti-p85α regulatory subunit of PI3K (1:1000). Western blot showed that the expression of the p85α regulatory subunit of PI3K did not respond to insulin treatment [**Fig. 4(A)**], indicating that the increased enzymatic activity of PI3K was not due to increased p85 expression in the retina.

To further investigate the effect of H_2O_2 with or without insulin on PI3K activity, retinal neuron cultures were treated with either H_2O_2 alone or pretreated with 10 nM of insulin for 30 min prior to exposure to H_2O_2. As shown in **Fig. 5**, addition of H_2O_2 alone to culture medium for 2 h only slightly reduced PI3K activity compared to the basal level of PI3K activity (P>0.05). Similar to **Fig. 4**, PI3K activity was increased in the culture pretreated with 10 nM of insulin without exposure to H_2O_2. This increase, however, was still evident in the culture pretreated with 10 nM of insulin for 30 min before exposure to H_2O_2 for an additional 2 h. The difference between the insulin-treated group and the insulin+H_2O_2 group was statistically insignificant (P>0.05).

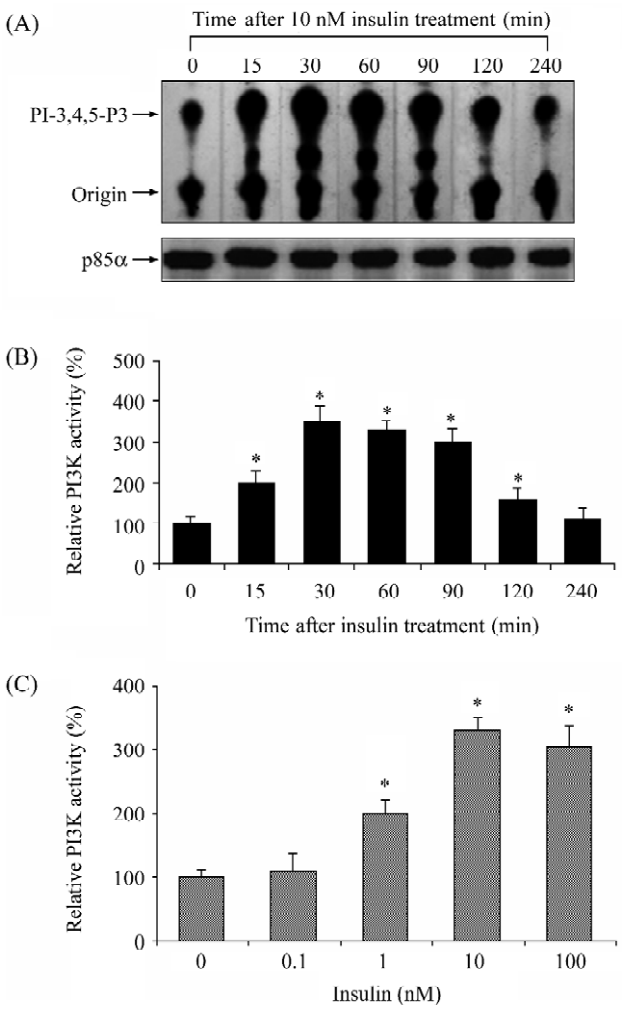

Fig. 4 Activation of phosphoinositide 3-kinase (PI3K) by insulin in cultured retinal neurons

(A) Cultures were treated with 10 nM insulin for indicated periods of time. Upper panel, PI3K activity was measured using phosphoinositide 4,5-bisphophate (PI-4, 5-P_2) and [γ-^{32}P] ATP as substrates. Thin-layer chromatographic autoradiogram of PI-3,4,5-P_3 showed a transient increase in PI3K activity by insulin in cultured retinal neurons. Lower panel, Western blots showed that the expression of the p85α regulatory subunit of PI3K did not respond to insulin treatment. (B) PI3K activity was quantified. (C) Cultures were treated with indicated concentrations of insulin for 30 min, and then PI3K activity was quantified. Data from six independent experiments were averaged and presented relative percentage to control (100%). *P<0.05 vs. control group (mean±SD, n=6).

Insulin activates Akt through PI3K

It is well known that the serine/threonine protein kinase Akt is one of the downstream effectors of the PI3K signaling pathway, and the activation of Akt by phosphorylation has been reported to play a critical role in protecting cells from apoptotic cell death [24,25,35]. Therefore, we examined whether Akt can be activated by insulin in cultured retinal neurons. Retinal neuron cultures were treated with 10 nM insulin for different time periods (0, 15, 30, 60, 120, 240 and 480 min) and extracts were subjected to Western blot analysis with anti-phospho-Akt S473 antibody (1:2000). As shown in **Fig. 6**, the phosphorylation of Akt was significantly enhanced from 15 min and peaked at 60 min after addition of insulin. Phosphorylated levels of Akt slowly declined after 120 min, and returned to control level after 480 min. Over the same time course, Western blot showed that the expression of the Akt protein did not respond to

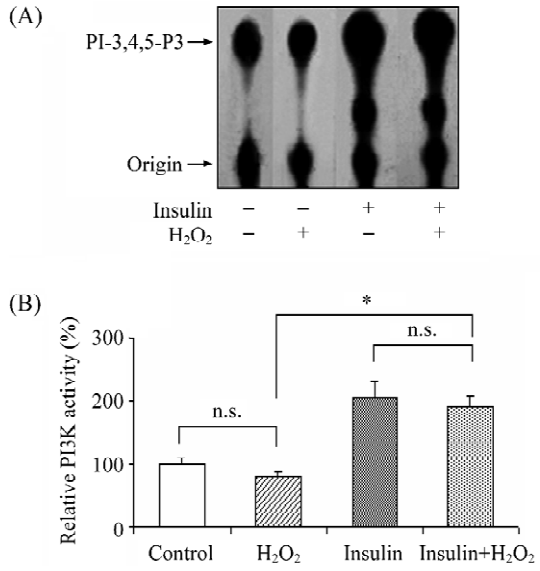

Fig. 5 Effect of H_2O_2 with or without insulin on phosphoinositide 3-kinase activity in cultured retinal neurons

(A) Cultured retinal neurons were pretreated with 10 nM of insulin for 30 min before 100 μM H_2O_2 or phosphate-buffered saline was added to the medium for an additional 2 h, or treated with 100 μM H_2O_2 alone for 2 h. Cultures without treatment served as controls. (B) Data from six independent experiments were averaged and presented relative percentage to control (100%). **P*<0.05 vs. the same dose of H_2O_2 exposure without insulin pretreatment (mean±SD, *n*=6). n.s., no significant difference.

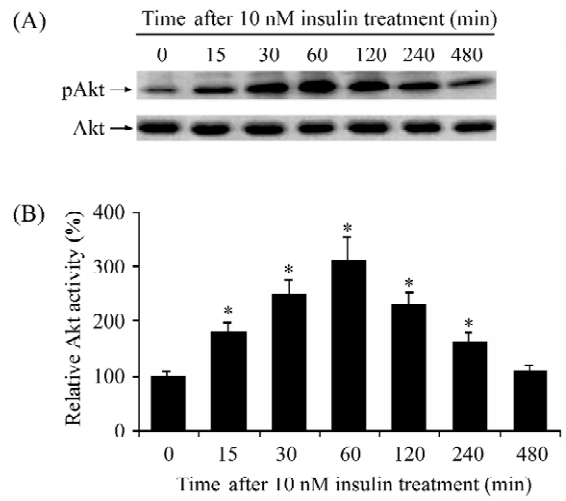

Fig. 6 Activation of Akt by insulin in cultured retinal neurons

(A) Cultures were treated with 10 nM of insulin for indicated periods of time and extracts were subjected to Western blot analysis with anti-phospho-Akt S473 antibody or anti-Akt antibody. Western blots showed that levels of phosphorylated Akt (pAkt) were increased by insulin (upper panel), but the expression of Akt protein did not respond to insulin treatment (lower panel). (B) Levels of phosphorylated Akt were quantified. Data from three independent experiments were averaged and presented relative percentage to control (100%). **P*<0.05 vs. zero-time control group (mean±SD, *n*=3).

insulin treatment [**Fig. 6(A)**], indicating that the increased phosphorylated Akt (pAkt) was not due to increased Akt expression in the retina. Insulin-induced activation of Akt was significantly attenuated by pretreatment with 10 μM LY294002 for 30 min (**Fig. 7**), suggesting that insulin activates Akt through the PI3K pathway in retinal neurons.

To investigate the effect of H_2O_2 on activation of Akt in retinal neuron cultures, retinal neuron cultures were treated with either H_2O_2 alone for 2 h or pretreated with 10 nM of insulin for 30 min prior to exposure to H_2O_2 for an additional 2 h. As shown in **Fig. 7**, when H_2O_2 alone was added to culture medium for 2 h, the level of pAkt was not significantly different to the basal level. Akt activity was still increased in the culture pretreated with 10 nM of insulin for 30 min and then exposure to H_2O_2 for an additional 2 h. The difference between the insulin-treated group and the

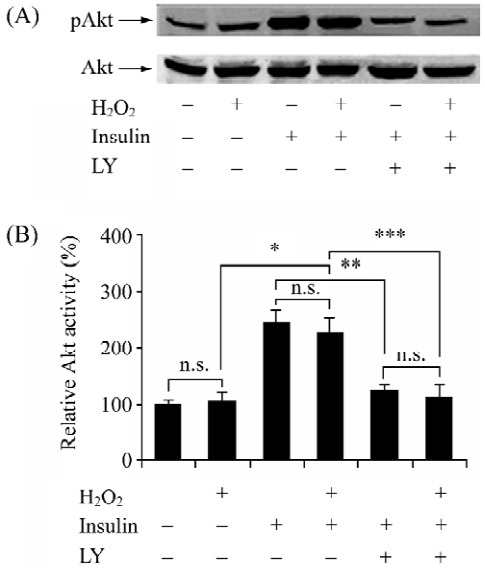

Fig. 7 Effect of H_2O_2 with or without insulin on Akt activity and inhibition of Akt activity by LY294002 (LY) in cultured retinal neurons

(A) Retinal neuron cultures were treated with 10 μM H_2O_2 alone for 2 h, or pretreated with 10 nM insulin for 30 min before 10 μM H_2O_2 or phosphate-buffered saline was added to the medium for an additional 2 h, or pretreated with 10 μM LY for 30 min prior to 10 nM insulin treatment for 60 min. Extracts were subjected to Western blot analysis with anti-phospho-Akt S473 antibody or anti-Akt antibody. Upper panel, levels of phosphorylated Akt (pAkt) in response to different treatments; lower panel, the expression of Akt protein did not respond to different treatments. (B) Levels of pAkt were quantified. Data from three independent experiments were averaged (mean±SD, *n*=3) and presented relative percentage to control (100%) **P*<0.05 vs. the same dose of H_2O_2 exposure without insulin pretreatment; ***P*<0.05 vs. the same dose of insulin treatment without LY pretreatment; ****P*<0.05 vs. the same treatment of insulin and H_2O_2 exposure without LY pretreatment. n.s., no significant difference.

insulin+H_2O_2 group was statistically insignificant (P>0.05). These data suggest that addition of H_2O_2 in cultures had not influenced activation of Akt by insulin.

Discussion

In the present study, we used H_2O_2 as a toxic stimulus in a retinal neuron culture model to examine the neuroprotective role of insulin. We chose H_2O_2 to induce retinal neuron death in our cell cultures because: (1) oxidative stress is believed to be an important mediator of neuronal cell death and has been postulated to contribute to the pathogenesis of retinal degeneration; (2) H_2O_2 is a precursor of highly oxidizing, tissue-damaging radicals such as hydroxyl radicals and is known to be toxic to many systems; (3) exogenous H_2O_2 can enter the cells and induce cytotoxicity due to its high membrane permeability [36]; and (4) H_2O_2 has been reported to trigger apoptosis in various cell types [7,8]. We found that exposure of cultured retinal neurons to H_2O_2 induces cell death. We have also noted that at a concentration of 100 μM H_2O_2, most TUNEL-positive cells exhibited apoptotic morphology. At present, neither the precise sequence of events nor the critical triggering mechanisms involved in H_2O_2-induced neuronal injury has been established. Inhibition of mitochondrial activity has been shown to result in apoptosis [37,38]. The MTT assay is a colorimetric assay for the non-radioactive quantification of cell viability and is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. The formation of formazan is thought to take place by way of intact mitochondria. A decrease in the number of living cells results in a decrease in the total metabolic activity in the sample. Our MTT assays demonstrated that the reduction of metabolic activity was significant, suggesting an inhibition of mitochondria function by H_2O_2 in this culture system.

Insulin has been reported to promote survival of neurons of the central nervous system [18–21]. The data presented here demonstrate that the pretreatment of cultured retinal neurons with 10 nM insulin inhibited H_2O_2-induced apoptosis and cytotoxicity. This is the first report demonstrating that insulin has a neuroprotective effect inhibiting H_2O_2-induced apoptosis in retinal neurons.

To further determine the mechanism of insulin against H_2O_2-induced apoptosis in cultured retinal neurons, we examined the role of PI3K because insulin has been reported to have anti-apoptotic effects through PI3K activation [24–26]. It is clear in the present study that insulin significantly increased PI3K activity in cultured retinal neurons, and the protective effect provided by insulin can be blocked by pretreatment with LY294002, a PI3K-specific inhibitor, for 30 min, suggesting a direct role of PI3K in insulin-mediated anti-apoptotic effects. PI3K has been reported to phosphorylate and activate many lipids and proteins, including the serine/threonine kinase Akt. Akt was initially described as an oncogene and is activated by a variety of growth factors through the PI3K-dependent pathway. Activation of Akt has been reported to inhibit apoptosis in many cell types [24,25,39,40]. Anti-Akt pS473 is a polyclonal antibody developed against the singly phosphorylated Akt. We used this antibody to clearly show that addition of insulin to retinal neuronal cultures significantly activates Akt, and this activation was strongly suppressed by a specific PI3K inhibitor, LY294002. These results suggest that PI3K may mediate an anti-apoptotic effect of insulin in retinal neuronal cultures through activating Akt.

In conclusion, H_2O_2, a free radical generator, significantly induces apoptotic cell death in cultured retinal neurons and pretreatment of the cells with insulin prevents apoptosis. The anti-apoptotic mechanism of insulin in cultured retinal neurons could be involved in activation of the PI3K/Akt signal pathway.

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