Increase of Protein Tyrosine Phosphorylation in Rat Retina After Ischemia–Reperfusion Injury

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**Purpose.** This study was conducted to examine the effect of retinal ischemia–reperfusion injury on protein tyrosine phosphorylation, the production of angiogenic growth factors, and the activation of signal proteins in tyrosine kinase pathways.

**Methods.** Ischemia–reperfusion injury was induced in rats by compression of the optic nerve for 2 hours. The rats were killed, and the retinas were collected at 0, 1, 6, 24, 48, 96, or 168 hours of reperfusion. Tyrosine phosphorylation of proteins in the retina was examined by Western blot analysis and immunohistochemistry. Angiogenic growth factors and their receptors, such as basic fibroblast growth factor (bFGF) and Flk, vascular endothelial growth factor (VEGF) and Flk-1, platelet-derived growth factor (PDGF)-B chain and PDGF-β receptor, and five intracellular signal proteins (phosphatidylinositol 3-kinase [PI3K], phospholipase Cγ [PLCγ], C-Src, SHC, and mitogen-activated protein kinase [MAPK]) were examined by Western blot analysis.

**Results.** Protein tyrosine phosphorylation increased after ischemia–reperfusion injury, reaching a peak at 48 hours of reperfusion. Increased staining of tyrosine-phosphorylated proteins in the inner retina were evident on immunohistochemical examination. The amount of bFGF decreased after injury, but the amounts of VEGF and PDGF-B chain increased. Tyrosine phosphorylation of PLCγ, SHC, and MAPK was increased at 48 hours of reperfusion, and tyrosine phosphorylation of PDGF-β receptor and PI3K was increased at 168 hours of reperfusion.

**Conclusions.** Ischemia–reperfusion injury in the rat retina leads to activation of the tyrosine kinase pathway, increasing the amounts of angiogenic growth factors. The resultant activation of signal proteins PLCγ, SHC, MAPK, PI3K, and PDGF-β receptor may play an important role in ischemia-induced retinal changes such as cell proliferation. Invest Ophthalmol Vis Sci. 1996;37:2146–2156.
inositol phosphate turnover; PLCγ produces diacylglycerol, which activates protein kinase C (PKC); SHC participates in activation of the Ras signaling pathway, which activates mitogen-activated protein kinase (MAPK), and MAPK translocates from the cytosol to the nucleus and phosphorylates a number of transcription factors and other protein kinases such as S6 kinases; and C-Src interacts with PDGF-β receptor and participates in mitogenic signal transduction.

From these findings, it is apparent that protein tyrosine phosphorylation plays a crucial role in the signal transduction of various cellular responses, such as mitogenesis and differentiation.

Ischemia–reperfusion injury in the rat retina is one model of experimental retinal ischemia and has been shown to induce a mitogenic response in cells located in the inner nuclear layer and in the retinal pigment epithelium with retinal degeneration. Recently, others using this model have shown that the expression of cytokines, such as interleukin-1β and adult T-cell leukemia-derived factor, increase in the ischemia–reperfusion-injured retina. However, it is unknown whether these ischemia-induced changes are related to protein tyrosine phosphorylation, whether angiogenic growth factors are induced by this model of retinal ischemia, and, if angiogenic growth factors are induced, which signal proteins in the tyrosine kinase pathways are activated by the injury.

In the current study, we used a rat model of retinal ischemia–reperfusion injury and Western blot analysis and immunohistochemical techniques to examine overall protein tyrosine phosphorylation in the retina after injury; changes over time in the amounts of three angiogenic growth factors associated with ischemia (bFGF, VEGF, and PDGF-B chain); and, in tyrosine phosphorylation, of their receptors (Flk-1, and PDGF-β receptor); and changes in the total amount and tyrosine phosphorylation of five signal proteins that are important in the tyrosine kinase pathways (PI3K, PLCγ, SHC, C-Src, and MAPK).

METHODS

Induction of Ischemia–Reperfusion Injury

Eighty-four adult male Sprague–Dawley rats (each weighing 250 to 300 g) were used for this study of retinal ischemia–reperfusion injury. In rats that underwent surgery, only one eye was operated on. All procedures involving the animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The rats that were operated on were anesthetized with ketamine (40 mg/kg) and xylazine (4 mg/kg). The surgical procedures were modified from those reported by Stefansson et al. With the animal under full anesthesia, the conjunctiva of the eye in which ischemia–reperfusion injury was to be induced was dissected temporally. The lateral rectus muscle was removed, and the optic nerve was exposed with blunt forceps. A ligature of 4-0 silk was placed on the optic nerve, and the ligature was tightened while the surgeon watched, using an operating microscope, for blood flow in the retinal vessels to stop. When cessation of blood flow was confirmed, the eyelids were removed with 6-0 silk (to protect the cornea from drying during ischemia). After 2 hours, the sutures in the eyelids were removed, and retinal vessels were examined under the operating microscope to confirm non-perfusion. Then the ligature was removed, and the retinal vessels were allowed to reperfuse. Only those eyes in which reperfusion was confirmed within 10 minutes of ligature removal were used in these experiments.

Rats were killed at 0, 1, 6, 24, 48, 96, and 168 hours of reperfusion. Nonoperated rats were used as controls for Western blot analysis and immunohistochemistry. Two other rats underwent a sham operation that included all steps of the ischemia–reperfusion procedures except ligation of the optic nerve. These rats were killed at 48 hours after the sham operation.

Sample Preparation

After the eyes had been enucleated, the anterior segments and the vitreous were removed. The retinas were dissected carefully from the choroid and prepared as previously described, as follows. Isolated retinas were immersed in ice-cold lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, 100 µg/ml phenylmethylsulfonfonyl fluoride, 0.3 µg/ml pepstatin A, 0.5 µg/ml leupeptin, 1 mM orthovanadate, and 50 µM sodium fluoride) and homogenized. An equal amount of 2X SDS sample buffer (made up of 160 mM Tris, pH 6.8, 4% SDS, 30% glycerol, 5% β-mercaptoethanol, 10 mM dithiothreitol, and 0.01% bromophenol blue) was added to the lysate. The samples were boiled at 95°C for 5 minutes. They were then centrifuged at 13,000 rpm for 10 minutes, and the supernatants were collected. Samples were stored at −80°C. Protein concentrations were measured by the Pierce BCA method.

Antibodies Used for Analysis

Primary antibodies used for Western blot analysis were mouse monoclonal antiphosphotyrosine antibody (PY20) (1:500 dilution); rabbit anti-human vascular endothelial growth factor antibody (1:100 dilution); rabbit anti-human C-Src antibody (1:100 dilution); rabbit anti-rat phospholipase Cγ1 antibody (1:100 dilution); rabbit anti-mouse Flk-1 antibody (1:100 dilution).
Immunoprecipitation

Ten of the rats in which ischemia–reperfusion injury to the retina had been induced were killed at 48 hours of reperfusion, and another 10 rats were killed at 168 hours of reperfusion. Ten nonoperated rats served as controls.

After enucleation, the retinas in each group were collected, immersed in ice-cold lysis buffer (10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton-X 100, 0.5% NP40, 100 μg/ml phenylmethylsulfonyl fluoride), and homogenized. Then 160 μg of agarose-conjugated anti-phosphotyrosine antibody (PY20) (Santa Cruz Biotechnology) was added to each retinal homogenate, and the homogenate was incubated at 4°C overnight with shaking.

Pellets were collected by centrifugation and rinsed in the lysis buffer three times, resuspended in 150 μl of 1X SDS sample buffer, and boiled at 95°C for 5 minutes. Immunoprecipitated samples were stored at −80°C. Equal amounts of each of these three immunoprecipitated samples were used for gel electrophoresis and Western blot analysis to study tyrosine-phosphorylated forms of growth factor receptors and intracellular signal proteins.

Gel Electrophoresis

Procedures used for gel electrophoresis and Western blot analysis have been described previously.25,26 Equal amounts of protein in each of the samples were electrophoresed by SDS–polyacrylamide gel electrophoresis. The samples were run on 4% to 20% gradient mini-gels (Bio-Rad) on a Bio-Rad Protean II apparatus. Biotinylated and prestained molecular markers (Bio-Rad) were used for protein staining with Coomassie brilliant blue dye or for Western blot analysis.

Coomassie Blue Staining of Gels

After electrophoresis, the gels were fixed in 45% methanol and 10% acetic acid aqueous solution for 30 minutes. They were soaked in saturated picric acid solution briefly and stained with 0.25% aqueous Coomassie brilliant blue (R-250) for a minimum of 2 hours. Gels were destained in 10% acetic acid solution. This procedure was repeated three times.

Western Blot Procedures

For Western blot analysis, gels were electroblotted onto nitrocellulose membranes (Costar Scientific, Cambridge, MA) with the use of a transblot SD apparatus (Bio-Rad). The membranes for detection of tyrosine-phosphorylated proteins were incubated with 3% bovine serum albumin in Tris-buffered saline (TBS; 20 mM Tris and 150 mM NaCl, pH 7.5) for 1 hour at room temperature. The membranes for detection of other proteins were incubated with 3% nonfat dried milk (Bio-Rad) in TBS for 1 hour at room temperature. Then each membrane was incubated with a primary antibody solution at 4°C overnight.

The membranes were rinsed three times with TBS and incubated with a solution of hors eradish peroxidase-conjugated goat anti-mouse IgG antibody or anti-rabbit IgG antibody (Bio-Rad) (1:2000 dilution) and avidin–horseradish–peroxidase complex (Bio-Rad) (1:10000 dilution) for 2 hours at room temperature. After the membranes were rinsed three times with TBS containing 0.3% Tween 20 and twice with TBS, they were incubated with enhanced chemiluminescence reagents (Amersham Life Science, Arlington Heights, IL) and exposed to Kodak X-ray film (Eastman Kodak, Rochester, NY) for 15 seconds to 5 minutes, as described in Amersham’s enhanced chemiluminescence protocols. Each Western blot analysis was repeated at least three times.

Immunohistochemistry of Tyrosine-Phosphorylated Proteins

Four rats were used to investigate the immunohistochemistry of tyrosine-phosphorylated proteins in the retina. Two rats were not operated on, and the other two rats underwent surgery in one eye for the induction of retinal ischemia–reperfusion injury, as described above, 48 hours earlier.

Under deep anesthesia with ketamine and xylazine, the four rats were perfused transcardially with 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 1 mM orthovanadate, followed by 2% paraformaldehyde in 0.1 M PBS to fix tissues. The eyes of each rat were then removed and immersed in the same fixative solution for 30 minutes.

Anterior portions of the eyes were removed, and the posterior portions were transferred through graded solutions of sucrose (5%, 10%, and 15% for 30 minutes each) and incubated in 20% sucrose solution overnight at 4°C. After cryoprotection, the eyes were...
molded in OCT compound (Miles, Elkhart, IN) and frozen in isopentane with dry ice.

Retinas were sectioned at 8 μm thickness with a cryostat and thaw-mounted onto slides. Sections were treated with 3% peroxide to block intrinsic peroxidase and incubated with 2% normal horse serum for 20 minutes. After they were rinsed three times in 0.1 M PBS, the sections were incubated overnight at 4°C in a solution of 1:2500 anti-phosphotyrosine antibody (PY20):0.1 M PBS. The sections were then rinsed three times in 0.1 M PBS and incubated with biotinylated anti-mouse IgG antibody solution (Vector Laboratories, Burlingame, CA) (1:500 dilution) for 30 minutes at room temperature, followed by incubation with peroxidase-labeled streptavidin (Kirkegaard & Perry Laboratories, Gaithersburg, MD) (1:500 dilution).

Sections were treated with 3-amino-9-ethylcarbazole solution (Sigma) and were mounted with glycerin jelly (7% gelatin, 54% glycerin, 0.7% phenol). Photomicrographs were obtained, using a microscope with Nomarski optics (Carl Zeiss, Thornwood, NY), of three slides from each rat retina.

RESULTS

Changes in Total Protein Profile After Ischemia–Reperfusion Injury

Coomassie blue staining (to show protein profiles) of the gel containing retinas from eyes in which ischemia–reperfusion injury had been induced and from nonoperated eyes was shown (Fig. 1). At least three bands in retinas exposed to injury were stronger (arrowheads) than bands from the control retina (lane C). These three stronger bands represent molecular weights of approximately 85, 72, and 21 kDa. The increased 85 kDa band was first apparent in retinas reperfused for 6 hours and became more noticeable with increased reperfusion time up to 48 hours, but it was decreased at 96 hours and 168 hours of reperfusion. The 72 kDa band was increased after 1 hour of reperfusion and was markedly increased at 48 hours of reperfusion, but this band was decreased after 96 hours of reperfusion. The 21 kDa band became apparent after 24 hours of reperfusion, was less noticeable at 48 hours of reperfusion, and was more prominent again at 96 hours of reperfusion.

At least one protein, of approximately 35 kDa MWt, was less apparent in injured retinas than in the control retina; this band was weaker in retinas obtained at 24 or 48 hours of reperfusion after ischemia. However, the band was stronger after 96 hours of reperfusion. None of the abnormalities just described was seen in the protein profiles of retinas from eyes exposed to sham operation (data not shown).

Increase in Tyrosine-Phosphorylated Proteins After Ischemia–Reperfusion Injury

Western Blot Analysis. Western blot analysis with anti-phosphotyrosine antibody (PY20) of retinas reperfused for 0, 1, 6, 24, 48, 96, or 168 hours after ischemia showed an overall decrease in tyrosine-phosphorylated proteins for the first 6 hours of reperfusion, followed by a marked increase in tyrosine-phosphorylated proteins with molecular weights between 14 kDa and 140 kDa at 24 and 48 hours of reperfusion (Fig. 2). The amounts of these proteins decreased toward control levels in retinas obtained at 96 hours

FIGURE 1. Protein profiles of retinas from normal rat eyes and eyes obtained 0, 1, 6, 24, 48, 96, or 168 hours (indicated by the number at the top of each lane) of reperfusion after 2 hours of ischemia. The retinas from each group of rat eyes were processed by SDS-PAGE, and the gel was stained with Coomassie brilliant blue. Molecular weights represented by each band (in kilodaltons) are shown to the left. Compared to control (C) retinas, retinas exposed to ischemia–reperfusion showed increases in three proteins (indicated by arrowheads) and a decrease in one protein (arrow).
At least four bands of tyrosine-phosphorylated proteins were increased, representing approximate molecular weights of 88, 63, 35, and 33 kDa (arrowheads, Fig. 2). These bands were markedly increased at 24 and 48 hours of reperfusion. Tyrosine-phosphorylated proteins in the retinas from eyes that had undergone the sham operation were not different from those in the control retina (data not shown).

Immunohistochemistry. Immunohistochemical examination of the distribution of tyrosine-phosphorylated proteins showed a slight positive reaction in the nerve fiber layer of normal rat retinas (Fig. 3A) and a marked reaction in the retina at 48 hours of reperfusion after 2 hours of ischemia (Fig. 3B). As Figure 3B shows, the intense positive immunoreaction occurred mainly in the inner retina, especially in the inner plexiform layer and the ganglion cell layer, although the inner nuclear layer and the nerve fiber layer also stained positively, indicating immunoreactivity in these layers. Incubation of the adjacent retinal section with nonimmune mouse IgG instead of anti-phosphotyrosine antibody showed no reaction (Fig. 3C).

Changes in Growth Factors and Their Receptors After Ischemia–Reperfusion Injury

Basic Fibroblast Growth Factor. Three forms of bFGF, identified by molecular weights of 28, 25, and 24 kDa, were detected in the rat retina exposed to ischemia–reperfusion. The amounts of all three forms of this growth factor gradually decreased after reperfusion, starting at 24 hours (Fig. 4A). The decrease was marked for all three forms at 168 hours of reperfusion, and the proteins with higher molecular weights (28 kDa and 25 kDa) were almost undetectable (Fig. 4A).

Vascular Endothelial Growth Factor. In rat retinas exposed to ischemia–reperfusion, VEGF appeared as two bands, representing molecular weights of 29 kDa and 15 kDa. As Figure 4B shows, the 15 kDa band was increased markedly after 6 hours of reperfusion and then decreased gradually, although at 168 hours of reperfusion, the amount of VEGF in the injured retina was still higher than in the control. In contrast, the 29 kDa band decreased in amount after ischemia–reperfusion injury, with a marked difference, compared to the control, after 24 hours of reperfusion.

Platelet-Derived Growth Factor-B Chain. Figure 4C shows amounts of PDGF-B chain in the control retina and in retinas allowed to reperfuse for various times after ischemia. Two forms of PDGF-B chain, with molecular weights of 15 kDa and 12 kDa (arrowheads), are evident in the control retina in Figure 4C. Neither band was detectable at 6 hours of reperfusion after ischemia, but at 24 hours of reperfusion, the 12 kDa band was detectable, and by 168 hours of reperfusion, both bands were clearly present and were more prominent than in the control retina.

Changes in Tyrosine Phosphorylation of Signal Proteins in the Tyrosine Kinase Pathways

Because the amounts of tyrosine-phosphorylated proteins were increased markedly at 48 hours of reperfu-
had at least six tyrosine-phosphorylated Flg proteins, with approximate molecular weights of 145, 120, 85, 65, 60, and 37 kDa (arrowheads). The amounts of the 140 kDa and 85 kDa proteins were decreased after 48 and 168 hours of reperfusion, the amount of the 120 kDa protein was unchanged, and the amounts of the 65 kDa and 60 kDa proteins were slightly decreased at 48 hours of reperfusion and slightly increased after 168 hours compared to the control. Because of its low molecular weight, the tyrosine-phosphorylated 37 kDa protein could be a degraded protein of the intracellular part of Flg proteins.

Figure 5B shows the result of evaluating for tyrosine-phosphorylated Flk-1 in rat retinas. The molecular weight of Flk-1 is approximately 190 kDa, and the amount of this protein appeared to be approximately the same after the injury as before it.

Figure 5C shows the result of evaluating for tyrosine-phosphorylated form of PDGF-β receptor, which has an approximate molecular weight of 190 kDa (arrowhead).
This protein appears to be induced at 168 hours of reperfusion because it was almost undetectable in either the control or the 48-hour postreperfusion sample. The low molecular weight of the second band could be a degraded protein of PDGF-β receptor.

**Intracellular Signal Proteins.** Figures 6A and 6B show the levels of total P13K and the tyrosine-phosphorylated form of this protein in the control retina and retinas exposed to ischemia–reperfusion injury. The arrowhead points to this protein of 85 kDa MWt. Although the total amount of P13K decreased after ischemia–reperfusion injury in the retina (Fig. 6A), the amount of tyrosine-phosphorylated P13K was increased at 168 hours of reperfusion (Fig. 6B). The arrow shows a positive band of mouse IgG in the samples immunoprecipitated with mouse monoclonal anti-phosphotyrosine antibody (Fig. 6B).

Figures 6C and 6D show changes in the levels of total PLCγ1 and tyrosine-phosphorylated form of this 145 kDa molecular weight protein (arrowhead). The total amount of PLCγ1 did not change after ischemia–reperfusion injury. However, the amount of the tyrosine-phosphorylated form of PLCγ1 was increased at 48 hours of reperfusion, although it was decreased by 168 hours of reperfusion.

Figures 6E and 6F show the levels of total C-Src and tyrosine-phosphorylated C-Src in the control retina and retinas exposed to ischemia–reperfusion. The arrowhead points to this protein of 60 kDa MWt. The total amount of C-Src in the control retina was much larger than the amount in retinas exposed to ischemia–reperfusion, as shown by the much less stained bands in retinas obtained at 48 and 168 hours of reperfusion. The tyrosine-phosphorylated form of C-Src was notably decreased after injury, especially at 168 hours of reperfusion (Fig. 6F).
DISCUSSION

Neovascularization of the retina or iris has not been shown in the rat model of ischemia–reperfusion injury we used. However, the model is easily reproducible and has been used by several other research groups. One group showed that ischemia–reperfusion injury induced proliferation of glial cells, capillary endothelial cells, and vascular cells. We thus considered this model to be appropriate for use in our study of the tyrosine phosphorylation of proteins involved in retinal ischemia–reperfusion injury. Our study showed that the profile of total proteins in the retina changes after ischemia–reperfusion injury, and some of the differences may represent proteins involved in the process of retinal ischemia.

Increase in Tyrosine-Phosphorylated Proteins

Western blot analysis and immunohistochemical study showed that ischemia–reperfusion injury in the rat retina leads to an increase in a broad range of tyrosine-phosphorylated proteins. Studies of brain ischemia have shown that this type of injury is followed by an increase in tyrosine phosphorylation of proteins in general and of specific tyrosine-phosphorylated proteins, including MAPK. The results of our study cannot be compared directly to those of brain studies because the duration of retinal ischemia was 2 hours in our study, and the duration of brain ischemia was 5 to 10 minutes. Nevertheless, our results are consistent with these findings, although the molecular weights of the proteins that increased were different.

Angiogenic Growth Factors

We found that the levels of VEGF and PDGF-B chain increased after ischemic injury, but the amount of bFGF gradually decreased with increasing reperfusion time (Fig. 4). These results are consistent with previous studies showing that hypoxic conditions induce mRNA expression of VEGF but not bFGF in vascular smooth muscle cells and in retinal glial cells.

In a study of brain ischemia, increased bFGF was found in brain astrocytes after injury, whereas we found an overall decrease in bFGF by Western blot analysis of total proteins in retinas exposed to ischemia. We speculate that the decrease in bFGF in our study might have occurred because increasing numbers of retinal neurons, which contain bFGF, were lost with the progression of retinal degeneration.

The increase in VEGF that followed ischemia–reperfusion injury in our study was consistent with results of previous studies in which mRNA expression of VEGF was found to increase in hypoxic conditions. A previous immunohistochemical study found evidence that Muller glial cells contain VEGF but did not specify which of the four molecular forms of VEGF was involved. We found that the amount of the 15 kDa form of VEGF increased after ischemia–reperfusion injury, whereas the amount of the 29 kDa form of VEGF decreased with increasing reperfusion time after ischemia. Others have suggested that low-molecular-weight forms of VEGF might be derived from partial proteolysis of high-molecular-weight forms of VEGF. Although our study could not clarify this issue, our results suggest that the 15 kDa form of VEGF may play an important role in the proliferative responses of vascular endothelial cells after retinal ischemia.

The amount of PDGF-B chain decreased, compared to the control, after 6 hours of reperfusion but
then rose at 168 hours of reperfusion. PDGF-B chain has been shown to localize to retinal blood vessels, and PDGF-BB stimulates proliferation of endothelial cells by PDGF-β receptor, and in vitro angiogenesis. From these findings, we speculate that the initial decrease of PDGF-B chain that occurred after ischemia–reperfusion injury in our study might have been caused by severe damage to endothelial cells. The increase of PDGF-B chain that occurred in our study at 96 and 168 hours of reperfusion might have resulted from proliferation of retinal endothelial cells.

**Tyrosine Phosphorylation of Growth Factor Receptors**

Alternative splicing has resulted in synthesis of many isoforms of the Flg protein. By Western blot analysis, we detected tyrosine-phosphorylated Flg proteins of six molecular weights: 145, 120, 85, 65, 60, and 37 kDa. The Flg proteins of molecular weights 145, 120, 85, and 60 kDa corresponded, respectively, to FGF receptors 1α, 1β, 1β2, and 1β2. We also consider the 65 kDa protein to be an isoform of FGF receptor 1β. In our study, two tyrosine-phosphorylated Flg proteins (145 kDa and 85 kDa) were decreased after ischemia–reperfusion injury in the rat retina, and the time courses of these changes were consistent with the timing of changes in the amount of bFGF. Because Flg proteins have been shown to be distributed widely in the ganglion cell layer and the inner and outer nuclear layers of the retina, we speculate that the decrease in tyrosine-phosphorylated Flg proteins might have resulted from retinal degeneration after injury.

Flk-1 and PDGF-β receptor proteins have been shown to be expressed selectively in vascular endothelial cells and to mediate mitogenesis. In our study, the tyrosine-phosphorylated form of Flk-1 did not change after the injury in spite of the presence of excessive amounts of VEGF. As expected by the increase in the amount of PDGF-B chain that was evident at 96 and 168 hours of reperfusion, an increase in the amount of tyrosine-phosphorylated form of PDGF-β receptor was evident at 168 hours of reperfusion. However, examining tyrosine phosphorylation of growth factor receptors is complicated by the fact that this process also is regulated by autophosphorylation.

**Tyrosine Phosphorylation of Intracellular Signal Proteins**

Although the amount of total PI3K protein decreased after ischemia–reperfusion injury in the rat retina in our study, the amount of tyrosine-phosphorylated form of PI3K was increased at 168 hours of reperfusion. PI3K has been shown to bind to PDGF-β receptor; thus, increases in the tyrosine phosphorylation of PDGF-β receptor and PI3K at 168 hours of reperfusion in our study may indicate that these proteins may be involved in mitogenic responses in the retina.

The amount of total PLCγ protein did not change after ischemia–reperfusion injury in our study, but the amount of tyrosine-phosphorylated form of PLCγ was increased at 48 hours of reperfusion. Because PLCγ has been shown to activate PKC, which then induces cell proliferation, the increase we found in the tyrosine-phosphorylated form of PLCγ may indicate that PLCγ participates in mitogenesis in the injured retina.

In our study, decreases occurred in the amounts of total C-Src and tyrosine-phosphorylated form of C-Src in the rat retina after ischemia–reperfusion injury, suggesting that the pathways activated by C-Src do not play major roles in retinal changes after this type of injury.

Several receptor tyrosine kinases, including PDGF-β receptor, have been shown to activate SHC, which has been shown to be an important signal protein in mitogenic transductions. Increases in the amounts of total and tyrosine-phosphorylated forms of SHC shown in our study (Figs. 6G, 6H) confirm that SHC probably plays a role in mitogenesis in the retina after ischemia–reperfusion injury.

We found increases in the amount of tyrosine-phosphorylated form of MAPK, especially extracellular regulated protein kinase, after ischemia–reperfusion injury in the rat retina. In addition to its involvement in mitogenesis, MAPK is involved in neurotrophic signal transduction, as shown by the fact that several neurotrophic factors, such as nerve growth factor and brain-derived neurotrophic factor, activate MAPK. Although the mechanisms by which MAPK is activated after injury are complicated, the fact that the amounts of tyrosine-phosphorylated forms of MAPK increased after injury suggests that MAPK might be an important protein in mitogenesis and other retinal responses induced by ischemia–reperfusion injury.

Increases in protein tyrosine phosphorylation of PLCγ, SHC, and MAPK after ischemia–reperfusion injury reflect not only mitogenesis but neuronal degeneration as well. For this reason, further studies are needed to examine the roles of these signal proteins and to clarify which signaling pathways are activated after retinal ischemia. Our study did show that ischemic injury to the retina causes an increase in protein tyrosine phosphorylation and an increase in amounts of angiogenic growth factors, which suggests that inhibition of the abnormal levels of tyrosine phosphorylation and/or activation of specific signal proteins, especially PLCγ, SHC, and MAPK, might help to prevent pathologic changes in the retina, such as cell proliferation and retinal degeneration, after retinal ischemia.
Phosphotyrosine Induction in the Ischemic Retina

Key Words
growth factor, ischemia—reperfusion, phosphorylation, protein tyrosine
rat, retina

Acknowledgment
The authors thank Rhonda R. Grebe for assistance in immunohistochemical studies.

References


