Induction of a 55-kDa PKN Cleavage Product by Ischemia/Reperfusion Model in the Rat Retina

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PURPOSE. To investigate the physiological role of a protein kinase, PKN, and its relation to apoptosis in vivo.

METHODS. An ischemia/reperfusion model of the rat retina was created by elevating the intraocular pressure. Retinal samples were obtained after ischemic insult (15–45 minutes) followed by reperfusion (1–7 days). The effect of ischemia on the fragmentation of PKN was examined by immunoblotting and immunocytochemical procedures using the antibody against PKN. N-methyl-D-aspartate (NMDA) or a caspase-3 inhibitor (DEVD-CHO) was administered intravitreally to investigate its effect on the induction of PKN fragmentation. The retinal cell loss in each sample was evaluated by toluidine blue staining.

RESULTS. Ischemia induced a 55-kDa PKN cleavage fragment corresponding to the molecular size of the constitutively active fragment of PKN. The appearance of the cleavage fragment depended on the duration of reperfusion and correlated with the occurrence of retinal cell loss. Immunocytochemical analysis revealed that ischemia increased PKN immunoreactivity in the inner layers of the retina. DEVD-CHO significantly inhibited the appearance of the 55-kDa fragment and protected against retinal cell loss. The administration of NMDA also induced cleavage of PKN.

CONCLUSIONS. PKN is specifically cleaved by caspase-3 or a related protease during apoptosis in vivo, and PKN cleavage is at least partially initiated by activation of the NMDA receptor. (Invest Ophthalmol Vis Sci. 2000;41:29–35)

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KN is a serine/threonine kinase with a molecular mass of 120 kDa, which has a catalytic domain highly homologous to that of protein kinase C in the carboxyl terminus and a unique regulatory region in the amino terminus. Its kinase activity is enhanced by unsaturated fatty acids such as arachidonic acid.1–3 The amino-terminal region of PKN contains three repeats of a leucine zipperlike motif presumed to be critical for regulating biological activity1 and has the basic region adjacent to the first leucine zipperlike motif. The amino-terminal region also contains the binding site for a small GTP-binding protein Rho that activates PKN in a GTP-dependent manner.4–6 Recent studies have demonstrated that PKN is proteolytically cleaved by caspase-3 or a related protease during apoptosis in the cultured cells, which generates a constitutively active 55-kDa kinase fragment.7 However, the physiological role of PKN and its relation to apoptosis remain to be clarified.

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Apoptosis is a fundamental cellular process in the development and homeostasis of all multicellular organisms. Although it is initiated by various physiological and pathologic stimuli, all apoptotic cells undergo a similar series of morphologic and biochemical events, such as blebbing of plasma membranes, actin cytoskeletal disruption, nuclear condensation, decreases in adhesion and intercellular contacts, and DNA fragmentation.9–10 These changes during apoptosis include the limited proteolysis of cellular proteins by caspases, a family of aspartate-specific cysteine proteases.11–13 Caspases are normally present in cells as catalytically inactive proenzymes, and an apoptotic signal causes the cleavage of the proenzymes into active proteases, which subsequently cleave other caspases that are downstream in the cascade.14–16 Among the 12 mammalian caspases identified, caspase-3, -6, and -7 are suggested to be involved in the execution of apoptosis,12 and caspase-3 is reported to be activated by caspase-1, -8, and -9.16–19 Caspases activated by apoptotic signals cleave various cellular substrates, such as lamin,20 focal adhesion kinase,21 actin,22 and poly (ADP-ribose) polymerase,23 which may be responsible for the morphologic changes that occur during apoptosis.

In the present study, we investigated PKN fragmentation in the rat retina using a pressure-induced ischemia/reperfusion model, a well-established model system of apoptosis.24–26 We also examined the involvement of caspase in the cleavage of PKN to elucidate the physiological role of PKN during apoptosis in vivo.
MATERIALS AND METHODS

Materials

The constitutively active fragment of PKN (AF3; residues 561–942) and the antibody (αC6) against the carboxyterminal fragment (residues 863–946) of rat PKN27 were provided by Y. Ono. Caspase-3 inhibitor DEVD-CHO was purchased from Peptide Institute Inc. (Osaka, Japan). The compounds were dissolved in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) containing 0.6% dimethylsulfoxide (DMSO). N-methyl-D-aspartate (NMDA) was obtained from Sigma (St. Louis, MO) and dissolved in 0.1 M PBS. The temperature control unit and the heating pad were obtained from Neuroscience Inc. (Tokyo, Japan). All other agents were obtained from Nacalai tesque (Kyoto, Japan).

Ischemia Models

All investigations involving animals adhered to the ARVO Statement for the use of Animals in Ophthalmic and Vision Research. Adult male Wistar rats weighing 150 to 250 g were used in the present study. Retinal ischemia was induced by elevating the intraocular pressure according to the modified method of Hughes.24 Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg; Abbott Laboratories, North Chicago, IL), and pupils were dilated with topical instillation of phenylephrine hydrochloride and tropicamide. The anterior chamber of the right eye was cannulated with a 27-gauge infusion needle and connected to a normal saline bag. The intraocular pressure of the cannulated eye was raised to 130 mm Hg by elevating the saline chamber. Retinal ischemia was confirmed by fundoscopic examination with a microscope to verify the absence of blood flow in all retinal vessels. After a specific time period of ischemia, the needle was removed to allow resumption of normal ocular blood flow. Reperfusion was verified again by fundoscopic examination. The eyes from untreated animals were used as controls. A sham operation was performed in the eye of an untreated animal without increasing the intraocular pressure. The core temperature of the rats was maintained at 37 ± 1°C using a temperature control unit and a heating pad from the start of the procedure until 1 hour after reperfusion. Animals were kept at room temperature during the reperfusion period. Animals in which severe cataract or corneal opacity developed were excluded from the study.

Intravitreal Injection of Drugs

Intravitreal injections were carried out according to the method of Morizane28 using a 33-gauge needle connected to a Teflon tube with a 25-μl Hamilton syringe, after pupil dilation with phenylephrine hydrochloride and tropicamide. Briefly, the 5-mm tip of the needle was inserted through the dorsal limbus of the eye under a stereoscopic microscope. Injections were completed over a period of 1 minute. A single 5 μl intravitreal injection of 40 mM NMDA (corresponding to 200 nmol/injection) was administered into the right eye. Considering that the vitreous humor volume is approximately 60 μl in rat eyes, the actual concentration of NMDA delivered to the retina was approximately 3.3 mM. A sham operation was performed in the eye of an untreated animal with a corresponding volume of 0.1 M PBS, which was used as a control. A single 5-μl intravitreal injection of 3.9 mM DEVD-CHO in 0.6% DMSO was administered into the right eye in a similar fashion immediately after ischemic insult. The actual concentration of DEVD-CHO and DMSO delivered to the retina was approximately 300 μM and 0.05%, respectively. A sham operation was performed using a corresponding volume of vehicle in the eye of an untreated animal, which was then used as the control. Animals in which severe cataract or vitreous hemorrhage developed were excluded from the study.

Immunoblotting

After a specific time period of reperfusion, animals were killed and the ocular bulbs were enucleated. The cornea was excised from the bulbs, and the lens, iris, and vitreous were carefully dissected. The posterior globe of the eye was then homogenized in a Teflon-glass homogenizer with a 300 μl ice-cold homogenizing buffer (20 mM Tris/HCl, pH 7.4, containing 250 mM sucrose, 2 mM EDTA, 10 mM EGTA, 1 mM PMSF, and 200 μg/ml leupeptin) and sonicated (UD-210 Tomy, Japan; output 3, duty 50%, 10 times) at 4°C, because our immunocytochemical study showed little PKN in the choroid and sclera (data not shown). After centrifugation at 100g for 3 minutes, the supernatant was collected. A portion of the supernatant was used as the total fraction of samples, and the remainder was further centrifuged at 16,000g for 20 minutes at 4°C. The supernatant and the pellet were then collected. Protein concentrations of the samples were determined and then the samples were mixed with 2× sample buffer.29 Equal amounts of the samples (30 μg/lane) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli.29 The separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) filter (Millipore, Bedford, MA). Nonspecific binding sites on the PVDF filter were blocked by incubation with 5% skim milk in 0.01 M PBS containing 0.03% Triton X-100 (PBS-T) for 12 hours. The filter was then incubated with the antibody (αC6, diluted 1:1000) for 60 minutes at 20°C. After washing with PBS-T, the filters were incubated with goat anti-rabbit IgG for 30 minutes and then with rabbit peroxidase anti-peroxidase complex for 15 minutes. After three rinses, the immunoreactive bands were made visible with a chemiluminescence detection kit (Amersham, Buckinghamshire, England) according to the manufacturer’s standard procedure. The density of the immunoreactive band was quantified with a microcomputer imaging device (NIH image).

Immunocytochemistry

In preparing the sections, enucleated eyes were immediately immersed in 0.1 M phosphate buffer (PB) (pH 7.4) containing 4% paraformaldehyde (PFA) and 1% glutaraldehyde for 70 minutes at 4°C. After dissection of the cornea, lens, iris, and vitreous, the eyes were further fixed in 0.1 M PB (pH 7.4) containing 4% PFA overnight at 4°C and then in 30% sucrose in 0.1 M PB for 4 days. Sagittal sections of retinas through the optic disc (10 μm-thick, within 1–2 mm of the optic disc) were created by a cryostat and dipped in 0.01 M PBS containing 0.3% Triton-X and 0.02% NaN3 for 4 days. The cryostat sections were used as follows: For immunostaining, the cryostat sections, enucleated eyes were immediately immersed in 0.1 M phosphate buffer (PB) (pH 7.4) containing 4% paraformaldehyde (PFA) and 1% glutaraldehyde for 70 minutes at 4°C. After dissection of the cornea, lens, iris, and vitreous, the eyes were further fixed in 0.1 M PB (pH 7.4) containing 4% PFA overnight at 4°C and then in 30% sucrose in 0.1 M PB for 4 days. Sagittal sections of retinas through the optic disc (10 μm-thick, within 1–2 mm of the optic disc) were created by a cryostat and dipped in 0.01 M PBS containing 0.3% Triton-X and 0.02% NaN3 for 4 days. The cryostat sections were used as follows: For immunostaining, the cryostat sections were incubated with the antibody (αC6, diluted 1:4000) for 7 days. After washing with PBS-T, sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) for 12 hours and then with avidinbiotinylated rabbit peroxidase complex for 90 minutes. After three rinses, the
A very faint 55-kDa band was detected in the total and molecular size of the constitutively active fragment of PKN but not in particulate fractions, which corresponded to the supernatant fractions of retinas, which was detected in the total and supernatant fractions of retinas. After 45 minutes of ischemia and 7 days of reperfusion, the total, supernatant, and particulate fractions of the control retina. PKN was detected as a single band of 120 kDa in the retina. PKN was detected as a single band of 120 kDa in all fractions of the ischemic and control retinas. An additional band of 55 kDa, corresponding to the molecular size of AF3 was detected in the total and supernatant fractions of the ischemic retina. Molecular sizes (kDa) of the standard proteins are indicated on the right of the blot.

immunoreaction was made visible with 0.05 M Tris-HCl containing 3,3-diaminobenzidine-tetrahydrochloride and 1% H2O2 and observed under a light microscope (Zeiss, Oberkochen, Germany). For confocal analysis, the cryostat sections were incubated with the antibody (αC6, diluted 1: 4000) for 7 days. After washing with PBS-T, the sections were incubated with fluorescein isothiocyanate–labeled goat anti-rabbit IgG (MBL, Nagoya, Japan) for 30 minutes and observed under a confocal scanning laser fluorescence microscope (Zeiss). The remaining cryostat sections were stained with toluidine blue and observed under a light microscope.

**Statistical Analysis**

Data are presented as means ± SD. Statistical comparisons were made by one-way analysis of variance and by the Student’s t-test. P < 0.05 was considered statistically significant.

**RESULTS**

**Cleavage of PKN by Ischemia and Reperfusion in the Retina**

We first checked for the existence of PKN in the rat retina by immunoblotting using the antibody (αC6) against PKN. As shown in Figure 1, a considerable amount of PKN was present in the retina. PKN was detected as a single band of 120 kDa in the total, supernatant, and particulate fractions of the control retinas. After 45 minutes of ischemia and 7 days of reperfusion, an additional immunoreactive band of approximately 55 kDa was detected in the total and supernatant fractions of the control retinas (data not shown). There was no significant difference in the amount of the 120-kDa protein between the ischemic and control retinas. We therefore used the supernatant sample fractions for the after immunoblot analysis. Both the 120- and 55-kDa bands were not detected by normal rabbit serum (Fig. 1) or preabsorbed serum (data not shown).

The cleavage of PKN was examined at various reperfusion periods after 45 minutes of ischemia. As shown in Figure 2, the 55-kDa PKN cleavage product was detectable at 5 days of reperfusion and further increased at 7 days of reperfusion. Toluidine blue staining revealed that the thickness of the retina was slightly reduced at 5 days of reperfusion (data not shown). At 7 days of reperfusion, the histologic changes became more obvious, and the marked reduction in thickness was seen in the inner nuclear layer (INL), with the ganglion cell layer (GCL) and the inner nuclear layer (INL) showing mild reduction.

We further examined the induction of the PKN cleavage product and the morphologic changes in retinal tissues after various durations of ischemia (15, 30, and 45 minutes) and 7 days of reperfusion. As shown in Figure 3, the PKN cleavage product was evident after 15 minutes of ischemia with no significant increase in the amount of the 55-kDa PKN fragment after longer periods of ischemia (30 and 45 minutes). In contrast, no obvious retinal cell loss was observed after 15 and 30 minutes of ischemia. Significant retinal cell loss was observed after 45 minutes of ischemia (data not shown).

**Immunocytochemistry**

The distribution of PKN immunoreactivity in the rat retina with or without ischemia/reperfusion also was examined. No immunoreaction was detected in the retina by normal rabbit serum, retinas.
Although a slight nonspecific immunoreaction was found in the GCL (Fig. 4A). In the control retina, intense PKN immunoreactivity was observed in the GCL, with moderate reactivity in the IPL (Fig. 4B). Weak varicose-fiber—like immunoreactivity was seen in the outer plexiform layer (OPL). Faint immunoreactivity was seen in the proximal half of the INL and the photoreceptor layer (PRL). No immunoreaction was found in the outer nuclear layer (ONL) and the distal half of the INL. The immunoreactivity of the ischemic retina was increased mainly in the GCL and IPL with the INL and OPL showing slightly higher reactivity compared with the control retina (Fig. 4C). No immunoreaction was found in the ONL.

Under confocal laser scanning fluorescent microscopy, PKN immunoreactivity was present in the perikarya of ganglion cells of both control (Fig. 4D) and ischemic retinas (data not shown). The moderate dotlike immunoreactivity encircled the cells in the INL of the ischemic retina (Fig. 4E).

**Effect of a Caspase Inhibitor on the Ischemia-Induced Cleavage of PKN**

We investigated the effects of a caspase inhibitor on the ischemia-induced cleavage of PKN in the retina, because the cleavage of PKN recently has been reported to be mediated by caspase-3 or a related protease in vitro.7 As shown in Figure 5, the administration of a caspase-3-directed inhibitor DEVD-CHO (final concentration of 300 μM in the eye) significantly inhibited the appearance of the 55-kDa PKN cleavage product, whereas the PKN cleavage product was evident in the vehicle-injected retina. The ischemia-induced retinal cell loss was also blocked by treatment with DEVD-CHO (Fig. 6). These results indicate that caspase-3 or a related protease plays an important role in the ischemia-induced cleavage of PKN and cell death in the retina.

**Induction of the 55-kDa PKN Cleavage Product by NMDA**

Because the NMDA receptor recently has been reported to be involved in ischemia-induced retinal cell death,30,31 we examined the effect of NMDA on the appearance of the 55-kDa PKN cleavage product. The PKN cleavage product of 55 kDa, corresponding to the molecular size of AF3, also was found in the NMDA-injected retina. A very faint 55-kDa band was detected in the sham-operated retina (Fig. 7A). The 55-kDa PKN cleavage product was significantly detected at 7 days after NMDA injection but not at 1 and 3 days postinjection (Fig. 7B). Toluidine blue staining revealed that the retinal cell loss was not recognized until 7 days after NMDA injection (data not shown). These results indicate that activation of the NMDA receptor leads to cleavage of PKN.

**DISCUSSION**

It has been reported recently that PKN expressed in cultured cells is specifically cleaved to generate the constitutively active kinase fragment of 55 kDa (AF3) by caspase-3 or related protease during apoptosis in vitro.7 In the present study, we demonstrated for the first time that PKN was present in the retina, and that the 55-kDa PKN fragment was induced in vivo, using a pressure-induced ischemia/reperfusion model in the rat retina. We also showed that cleavage of PKN occurred in...
accordance to the occurrence of ischemia-induced retinal cell loss and that the fragmentation of PKN was inhibited by a caspase-3-directed inhibitor DEVD-CHO. These results suggest that PKN is specifically cleaved by caspase-3 or related protease during the execution phase of apoptosis in the retina and that the cleaved PKN might play specific roles in the ischemia-induced retinal cell death. Recently, we found that a similar 55-kDa PKN-immunoreactive protein appeared in the hippocampus extract after ischemic insult (Hashimoto and Tanaka, personal communication 1999) using a ischemia/reperfusion model in the gerbil brain.32,33 These results suggest that the generation of a 55-kDa PKN fragment representing the constitutively active kinase activity may be a common event in apoptotic cells.

Immunohistochemical studies revealed that PKN immunoreactivity was abundant and increased after ischemia/reperfusion in the inner layers of the retina, where the apoptotic process is suggested to be active after ischemia/reperfusion.26 Furthermore, under our experimental conditions, ischemia-induced retinal cell loss in the inner layers of the retina, which was consistent with recent findings.54,55 These facts suggest that PKN may be involved in apoptosis and ischemia-induced cell death in the inner layers of the retina. It is noteworthy that PKN is expressed ubiquitously in human tissues7 and is relatively higher in the thymus, spleen, and testis,1 where the apoptotic process is suggested to be active.56–58 This supports the idea that PKN is involved in apoptosis.

The ischemia-induced increase in PKN immunoreactivity in the inner layers of the retina may be due to the production of the 55-kDa PKN fragment after ischemic insult, although
there is a possibility that the increase in the relative intensity of PKN in the inner layers of the retina was caused by the decrease of the thickness of the inner retina after ischemic insult.

Recently, it has been shown that the NMDA receptor is involved in ischemia-induced cell death in the retina.50,51 and that a caspase acts downstream in NMDA-induced apoptosis in cerebral neurons.59,60 In the present study, the 55-kDa PKN cleavage product was shown to occur in the NMDA-injected retina. Furthermore, PKN immunoreactivity was increased by ischemic insult, as well as by NMDA injection (data not shown), in the inner layers of the retina where NMDA receptors were abundantly expressed.41–44 These results suggest that the fragmentation of PKN after ischemia/reperfusion is at least partly initiated by the activation of NMDA receptors in the retina, although how NMDA stimulation leads to the activation of caspase in the brain and retina remains to be clarified.

In this study, the appearance of the PKN cleavage product temporarily seemed to correspond with retinal cell loss after ischemia/reperfusion or NMDA injection. It is possible that the degrading process of the ischemic retina may cause PKN fragmentation. However, the 55-kDa PKN cleavage product was detected even at 7 days after 15 minutes of ischemia (Fig. 3), when no obvious retinal cell loss was even observed (data not shown). This suggests that the cleavage of PKN may not result from the retinal cell loss, because the cleavage occurred before the retinal cell loss. Recently, Kuroiwa et al.26 demonstrated that DNA fragmentation, a hallmark of apoptosis, was observed in the pressure-induced ischemia/reperfusion model in the rat retina reaching its peak at 9 to 48 hours after ischemic insult. We also confirmed that DNA fragmentation occurred at 24 hours after 45 minutes of ischemia by agarose gel electrophoresis and terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining (data not shown). Accordingly, apoptotic signals may cause PKN cleavage after DNA fragmentation but before degradation of the retinal cells, although there is a possibility that the two phenomena are not related to each other.

The present results indicate that PKN is specifically cleaved by caspase-3 or a related protease during apoptosis in the retina, which generates the PKN fragment of 55 kDa, corresponding to the molecular size of AF3. It is known that PKN is activated by limited tryptic proteolysis,45 presumably by removal of an inhibitory N-terminal domain.5 These facts suggest that PKN is activated through the caspase-mediated proteolysis during apoptosis as well as MEKK-1,46 p21 activated kinase 2,47 and PKCδ.48 Based on recent findings that PKN binds to and phosphorylates intermediate filaments such as glial fibrillary acidic protein, vimentin,49 and subunits of neurofilament,50 and that the phosphorylation results in inhibition of the filament assembly in vitro,49,50 phosphorylation of such proteins by the 55-kDa PKN cleavage product might contribute to the morphologic manifestations of the cells undergoing apoptosis, together with the caspase-mediated proteolysis of cytoskeletal components such as Gas,51 fodrin,52 and gelsolin.53 Identification of the substrates for the 55-kDa PKN cleavage product as well as PKN itself will provide further understanding of the signal transduction pathways involved in apoptosis.

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