Plasminogen activators promote excitotoxicity-induced retinal damage

Raghuveer S. Mali, Mei Cheng, and Shravan K. Chintala
Eye Research Institute of Oakland University, 409 Dodge Hall, Rochester, Michigan, USA

ABSTRACT  Increased levels of extracellular L-glutamate have been suggested to play a role in retinal damage in a number of blinding diseases such as glaucoma and diabetic retinopathy. Although glutamate can cause retinal damage in part by hyperstimulating its receptors (“excitotoxicity”), the downstream events that lead to retinal damage are poorly understood. In this study, we injected kainic acid (KA), a glutamate receptor agonist that specifically hyperstimulates non-NMDA-type receptors, into the vitreous humor of CD-1 mice and have investigated the role of plasminogen activators (PAs) [tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA)] in excitotoxicity-induced retinal damage. Injection of KA into the vitreous humor led to an up-regulation in tPA and an induction in uPA activity in the retina and this was associated with activation of zymogen plasminogen to active plasmin. Immunocytochemical analysis indicated that retinal ganglion cells (RGCs), constitutively express tPA and release it into the extracellular space upon KA injection. Immunocytochemical analysis also indicated an increase in uPA in the nerve fiber layer after KA injection that was absent in the control retinas. These events were associated with apoptotic death of cells initially in the ganglion cell layer and subsequently in the inner and outer nuclear layer, associated with loss of RGCs and amacrine cells. These phenomena were inhibited when recombinant plasminogen activator inhibitor (rPAI-1) or tPA-STOP were injected into the vitreous humor with KA, whereas a plamin inhibitor, α-2-antiplasmin, failed to attenuate KA-induced retinal damage. Taken together, these results suggest that inhibition of plasminogen activators might attenuate retinal damage in blinding retinal diseases in which hyperstimulation of glutamate receptors is implicated as a causative factor to retinal damage. — Mali, R. S., Cheng, M., Chintala, S. K. Plasminogen activators promote excitotoxicity-induced retinal damage. FASEB J. 19, 1280–1289 (2005)

Key Words: retina  •  ganglion cells  •  excitotoxicity  •  kainic acid  •  tissue plasminogen activator  •  urokinase plasminogen activator and non-NMDA receptors

Retinal damage is a major concern in blinding retinal diseases, including diabetic retinopathy and glaucoma (1–5). Although the mechanisms that underlie retinal damage in these diseases are poorly understood, previous studies have suggested that excitatory amino acid (EAA) such as L-glutamate might play a role (6–11). Accumulation of glutamate into the extracellular space activates two major classes of receptors: the ionotropic (N-methyl-D-aspartate [NMDA], α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA]/kainic acid [KA]-type) and the G-protein-coupled metabotropic receptors (12–14). A number of cells including ganglion cells and amacrine cells in the retina express these receptors (15, 16). Although activation of these receptors constitutes a physiological phenomenon, hyperstimulation of NMDA and non-NMDA type ionotropic receptors can lead to cell death. Indeed, previous studies have reported that glutamate and its receptor agonists such as NMDA and KA hyperstimulate glutamate receptors (“excitotoxicity”) and contribute to retinal damage (10, 17–25). Elevated levels of glutamate and glutamate-mediated excitotoxicity have been implicated in ganglion cell loss in animals models for retinal ischemia (7, 26, 27), axonal injury (28), and glaucoma (6, 29). Furthermore, previous studies have reported the presence of elevated levels of glutamate in human glaucoma (6). In spite of these observations, the role of glutamate in glaucoma is controversial; the mechanisms that underlie glutamate-mediated excitotoxic-retinal damage are poorly understood and remain open to debate.

Due to the lack of a clear understanding of the mechanisms involved in excitotoxic retinal damage, it is worthwhile to investigate and possibly draw some parallels with other neurodegenerative diseases in which the role of excitotoxicity has been investigated and continues to be investigated. Recent investigations into the mechanisms of excitotoxicity in the central nervous system (CNS) have identified tissue plasminogen activator (tPA) as one of the contributing factors to neuronal damage (30–32). Plasminogen activators (PAs), urokinase plasminogen activator (uPA), and tPA are serine proteases that convert plasminogen, a physiological zymogen, into active plasmin, a trypsin-like endopeptidase of broad substrate specificity (33). Endogenous plasminogen activator inhibitors (PAs) control plasminogen activation by regulating the activity of
plasminogen activators. In the CNS tPA plays a role in neuronal plasticity (34, 35) and neuronal regeneration (36–38), whereas uPA plays a role in astrocyte proliferation and tissue remodeling (39, 40). Previous studies have demonstrated the presence of tPA and uPA in various eye tissues, including retinal neovascular membranes (41, 42), and in normal (43–46) and diabetic retinas (47). In a clinical setting, tPA has been used to treat stroke patients (48, 49) as well as patients with submacular hemorrhage (50–54). Although the functional roles of these proteases in the retina are unclear, mounting evidence indicates that tPA and uPA might play a degenerative role in the CNS (30–32, 55–60) and retina (42, 61–64).

Previous studies from our laboratory have suggested that hyperstimulation of non-NMDA receptors (excitotoxicity) in the retina by kainic acid leads to an up-regulation of extracellular modulating proteases such as matrix metalloproteinase-9 (MMP-9); this in turn plays a role in retinal damage (65). However, inhibition of MMP activity by a synthetic inhibitor failed to offer complete protection against KA-induced cell loss, indicating the possible role of other proteases such as plasminogen activators uPA and tPA in retinal damage. Therefore, we injected kainic acid (KA) into the vitreous humor of normal CD-1 mice (to hyperstimulate non-NMDA-type glutamate receptors in the retina) and investigated the role of tPA and uPA in retinal damage. We provide some novel findings that hyperstimulation of non-NMDA-type receptors leads to an increase in tPA and uPA activity and protein levels in the retina. We show that the degenerative events associated with hyperstimulation of non-NMDA type glutamate receptors can be attenuated by injection of recombinant plasminogen activator inhibitor-1 (rPAI-1) or tPA-STOP into the vitreous humor along with KA. These results for the first time, to our knowledge, indicate that up-regulation of plasminogen activators (both tPA and uPA) play a causative role in retinal damage mediated by hyperstimulation of glutamate receptors.

MATERIALS AND METHODS

Intravitreal injections

All the experiments on animals were performed under general anesthesia according to institutional protocol guidelines and the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of Animals in Ophthalmology and Vision Research. Normal adult CD-1 mice (6–8 wk old; Charles River Breeding Labs, Wilmington, MA, USA) were anesthetized with an overdose of avertin, and their eyes were enucleated. Retinas were carefully removed with a sharp knife and placed in 0.1M glycine buffer (pH 8.0; for detection of tPA) and uPA) or 0.1M Tris-buffer (pH 8.0; for detection of plasmin). After electrophoresis, gels were washed twice with 2.5% Triton-X 100 (15 min each time), three times with 2.5% Triton-X 100 (15 min each time), and placed in 0.1M glycine buffer (pH 8.0; for detection of tPA and uPA) or 0.1M Tris-buffer (pH 8.0; for detection of plasmin), and incubated overnight at 37°C to allow proteolysis of the substrates in the gels. The gels were stained with 0.1% Coomassie Brilliant Blue-R250, then destained with a solution containing 25% methanol and 10% acetic acid. Samples containing standard recombinant tPA or plasmin were coelectrophoresed for comparison. tPA activity in zymograms was confirmed by incubating the gels with rPAI-1 or tPA-STOP (data not shown) and by Western blot analysis (as described below). A reduced molecular weight size standard was included on all gels (data not shown; Life Technologies, Gaithersburg, MD, USA). The area cleared by tPA and uPA in the zymograms was scanned by a flat-bed scanner, relative protease activity level was determined using image analysis software (Scion Corporation, Frederick, MD, USA), and the results from four independent experiments were represented as mean arbitrary densitometric units ±SE. Statistical significance was analyzed by using a nonparametric Newman-Keuls analog procedure (GB-Stat Software, Dynamic Microsystems, Silver Spring, MD, USA) and expressed as the mean ±SE.
Immunohistochemistry

Eyes enucleated after KA injection were fixed with 4% paraformaldehyde for 1 h at room temperature and embedded in OCT compound (Sakura Finetek USA, Torrance, CA, USA). Traverse, 10 micron-thick cryostat sections were cut and placed onto superfrost plus slides (Fisher Scientific, Pittsburgh, PA, USA). Sections were incubated with antibodies against uPA (1:100 dilution in PBS, Innovative Research, MI, USA), calretinin (amacrine cell marker, 1:100 dilution in PBS, Chemicon, CA, USA), and neurofilament light (ganglion cell marker, 1:100 dilution in PBS, Santa Cruz Biotechnology, Santa Cruz, CA, USA) to determine the tissue localization of these proteins. Sections were washed three times (15 min each) with Tris-HCl buffer (pH 7.4) and incubated with appropriate Alexa FluorR-568-conjugated secondary antibodies (1:200 dilution in PBS for NF-L and calretinin; Molecular Probes, Eugene, OR, USA) or Alexa FluorR-486-conjugated secondary antibodies (1:200 dilution in PBS for uPA) for 1 h at room temperature. Sections were washed again with Tris-HCl buffer (three times, 15 min each) and mounted with a coverslip. Sections were observed under a Nikon bright-field microscope equipped with epifluorescence; digitized images were obtained using a SPOT digital camera. Images were converted into gray scale images using Adobe Photoshop Software, versions 5.5 and 7.0 (Adobe system Inc., Mountain View, CA, USA).

Immunogold labeling of tPA

For tPA immunogold labeling (see Fig. 2), 10 micron-thick cryostat sections (prepared as described above) were incubated with a solution containing 10% BSA (in PBS, pH 7.4) and 0.3% Triton X-100 for 1 h at room temperature. Sections were washed three times with PBS (15 min each) and incubated overnight with polyclonal antibodies against tPA (1:100 dilution; Innovative Research, Southfield, MI, USA; antibody was diluted in a solution containing 1% BSA and 0.3% Triton X-100 in PBS). The next morning sections were
washed three times with PBS (30 min each) and incubated with goat anti-rabbit IgG conjugated to 5 nm gold particles (1:100 dilution; Accurate Chemical and Scientific Corporation, Westbury, NY, USA) in 1% BSA containing 0.3% Triton-X-100 for 1 h at room temperature. Sections were washed four times (20 min each) with PBS and four times (20 min each) with distilled water and silver enhancement was performed on the sections according to manufacturer’s instructions (Silver Enhancement Kit light Microscopy, Accurate Chemical and Scientific Corporation, Westbury, NY, USA). Finally, sections were mounted using standard aqueous mounting solution and observed under a bright-field microscope. Digitized images were obtained using a Spot® digital camera attached to the microscope and compiled using Photoshop software (Adobe System Inc.).

Apoptotic assay

Apoptotic cell death in retinal cross sections was determined using a commercially available kit. Briefly, 10 micron-thick cryostat sections (n=8–10 sections for each treatment from 4 independent experiments) prepared as described above and apoptotic cell death was detected by TdT-mediated dUTP nick-end labeling (TUNEL) assay, using an In situ cell death detection kit with fluorescein (Roche Biochemicals, Mannheim, Germany) and the protocol provided by the manufacturer. Tissue sections were examined using a Nikon microscope equipped with epifluorescence; digital images were obtained with a SPOT digital camera and compiled using Adobe Photoshop Software, versions 5.5 and 7.0 (Adobe System). The remaining number of TUNEL-positive cells in retinal cross sections was quantitated using image analysis software (Scion Corporation); results were represented as total number of TUNEL-positive cells, mean ± SE. Statistical significance was analyzed by using a nonparametric Newman-Keuls analog procedure (GB-Stat Software, Dynamic Microsystems).

Western blot analysis

Aliquots containing an equal amount of retinal protein extracts (25 μg) were mixed with gel loading buffer and separated on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred onto nylon membranes and nonspecific binding was blocked with 10% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Membranes were then probed with antibodies against tPA (1:1000 dilution; Innovative Research, Southfield, MI, USA), uPA (1:2000 dilution; Innovative Research), and plasminogen (1:2000; Innovative Research). After incubation with the primary antibodies, membranes were washed with TBS-T and incubated with appropriate horse radish peroxidase (HRP) -conjugated secondary antibodies (1:4000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature. Finally, the proteins on the membranes were detected using an ECL chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposing the membranes to X-ray film. Recombinant uPA, tPA, and plasminogen were coelectrophoresed as positive standards (data not shown).

Retrograde labeling of retinal ganglion cells

Ganglion cells were retrogradely labeled as described previously (65, 67). Briefly, 1.5 μL of a 5% solution of Aminostilbamidine (Molecular Probes) in PBS was injected into the superior colliculi of anesthetized mice using a stereotaxic apparatus. KA was injected into the vitreous humor 1 wk after Aminostilbamidine application. Various times after KA injection, the animals were anesthetized and their eyes were enucleated and fixed in 4% paraformaldehyde for 1 h at room temperature. Retinas were detached from the eye cups and rinsed with PBS (two times, 15 min each). After rinsing, retinas were overlaid on a glass slide and four small incisions were made at the periphery to flatten the retina. The retinas were mounted with coverslips using an aqueous mounting medium (containing an anti-fading agent; GEL/MOUNT; Biomeda Corporation, Foster City, CA, USA). Alternatively, retinas were detached from the eyecups, embedded in OCT compound and processed for preparation of retinal cross sections as described above. Ten micron-thick retinal cross sections were prepared and aminostilbamidine-positive ganglion cells were observed under a fluorescence microscope (Nikon, Tokyo, Japan). Retinal ganglion cells located approximately the same distance from the optic disk were observed under a fluorescence microscope (~155 sq. microns, 40× magnification) and photographed using a Spot® digital camera attached to the fluorescence microscope.

RESULTS

Kainic acid up-regulates tPA and uPA in the retina and contributes to retinal damage

To determine whether hyperstimulation of non-NMDA-type glutamate receptors modulates tPA and uPA levels in the retina, KA was injected into the vitreous humor in CD-1 mice and total retinal proteins were extracted. tPA and uPA activity in retinal protein extracts was determined by fibrinogen/plasminogen zymography assays. Zymography assays indicated a very low and constitutive level of tPA activity in control or PBS-injected eyes (Fig. 1A). In contrast, KA injected eyes showed a dose-dependent (Fig. 1A) and time-related (Fig. 1B) increase in tPA activity in the retina. tPA activity was increased as early as 6 h after KA injection, returned to lower levels by 1 day, and increased again at 2 days after KA injection (Fig. 1B). This type of tPA activity profile was consistently observed in four independent experiments as shown by arbitrary densitometric units (Fig. 1C). Western blot analysis of retinal protein extracts from a similar experiment confirmed the expression pattern of tPA in the retina (Fig. 1D). Although low levels of PAI-1 and PAI-2 were observed constitutively in retinal proteins extracted from uninjected control or PBS-injected eyes, Western blot analysis indicated no significant change in PAI-1 and PAI-2 proteins in KA injected eyes (at 6, 12, 24, 48, and 96 h) compared with uninjected control or PBS-injected eyes (data not shown). Zymography assays indicated the absence of uPA activity in retinal proteins extracted from uninjected control retinas (Fig. 1A, B). In contrast, intravitreal injection of KA led to a transient up-regulation in uPA activity in the retina. uPA levels were induced as early as 6 h after KA injection, reached a peak between 1 and 2 days, and returned to lower levels at day 4 (Fig. 1B, C).

To determine the cell types that synthesize tPA and uPA, immunohistochemical analysis was performed on retinal cross sections prepared at 6, 12, 24, 48, and 96 h
after KA injection. An intense tPA immunostaining (by immunogold labeling) was constitutively observed in the ganglion cells in uninjected control or PBS-injected eyes (Fig. 2A). A faint tPA immunostaining was observed in the anterior portion of the inner plexiform layer. tPA immunoreactivity present in ganglion cells was found in the extracellular space as early as 6 h after KA injection (possibly due to depolarization) and 1 day after KA injection most cells that expressed tPA disappeared in the ganglion cell layer (Fig. 2A; this observation correlated with a reduction in tPA activity in zymograms (Fig. 1B)). In addition, 2 days after KA injection tPA immunolocalization was noticed in cells that are scattered in the inner plexiform layer and in the ganglion cell layer. However, the morphological appearance of these tPA-immunoreactive cells was different from the morphology of either ganglion or amacrine cells. uPA was absent in retinal sections prepared from uninjected control or PBS-injected eyes (Fig. 2C) but uPA protein was transiently up-regulated in the nerve fiber layer after intravitreal injection of KA (Fig. 2C). An up-regulation in tPA (in ganglion cells) and an induction in uPA (in the nerve fiber layer) after KA injection was associated with the presence of TUNEL-positive apoptotic cells in the retina (Fig. 2B).

Twelve hours after KA injection, increased expression of tPA and uPA was associated with the appearance of increased number of TUNEL-positive cells in the ganglion cell layer and with few TUNEL-positive cells in the inner nuclear layer (Fig. 2B). At 1 day after KA injection, tPA expression was reduced in the retina because the majority of ganglion cells that expressed tPA have undergone apoptosis at this time, while increased expression of uPA was still observed in the nerve fiber layer in cells that resemble activated astrocytes. At this time, TUNEL-positive cells were observed in the inner and outer nuclear layers due to secondary retinal damage (Fig. 2B). At 48 h after KA injection, TUNEL-positive cells were found in the outer nuclear layer, although the expression of tPA and uPA does not correlate with localization of TUNEL-positive cells. At 48 h after KA injection, most of the tPA was expressed by cells that migrated into the inner nuclear and ganglion cell layers; these cells do not seem to be either ganglion cells or amacrine cells, but seem to be microglial cells.

Since cells in the ganglion cell layer and inner nuclear layer showed TUNEL-positive staining as early as 6–12 h, two different experiments were performed to determine the cell types that underwent apoptotic death (TUNEL-positive) in these two layers. In the first experiment, ganglion cells were retrogradely labeled with aminostilbamidine and KA was injected into the vitreous humor 1 wk after labeling. Retinas were removed from the enucleated eyes 1 day after KA injection, prepared as flat mounted retinas, and the loss of aminostilbamidine-positive ganglion cells was determined by fluorescence microscopy (Fig. 3A). Retinal cross sections were prepared 1 day after KA injection (from retrogradely labeled retinas) and the loss of aminostilbamidine-positive ganglion cells was determined by fluorescence microscopy (Fig. 3B). Retinal cross sections prepared from a similar experiment were immunostained with antibodies against neurofilament light (NF-L) (C) or calretinin (D). Note a decrease in the number of aminostilbamidine-positive ganglion cells, a decrease in nerve fiber layer-associated NF-L immunostaining, and a decrease in calretinin-positive immunostaining at 1 day after KA injection. Images in panel A are taken at 40× magnification. All other images were obtained at 20× magnification.

Figure 3. Effect of KA on retinal damage. Retinal ganglion cells were retrogradely labeled with aminostilbamidine 1 wk before KA injection, and their loss in retinal flat mounts (A) or retinal cross sections (B) was determined at 1 day after KA injection. B) Retinal cross sections prepared from uninjected control and KA injected eyes (at 1 day) from a separate experiments were immunostained with antibodies against neurofilament light (NF-L) (C) or calretinin (D). Note a decrease in the number of aminostilbamidine-positive ganglion cells, a decrease in nerve fiber layer-associated NF-L immunostaining, and a decrease in calretinin-positive immunostaining at 1 day after KA injection. Images in panel A are taken at 20× magnification. All other images were obtained at 40× magnification.

A multinucleated giant cell (MGC) was observed in the inner nuclear layer (Fig. 4A). The MGC was immunostained with antibodies against calretinin (Fig. 4B). Note absence of aminostilbamidine-positive retinas and ganglion cells (Fig. 4C). The loss of aminostilbamidine-positive ganglion cells was determined by fluorescence microscopy (Fig. 4D). Retinal cross sections prepared from a similar experiment were immunostained with antibodies against neurofilament light (NF-L) to determine ganglion cell loss (Fig. 3C). Examination of retinal cross sections indicated a significant decrease in the number of aminostilbamidine-positive and NF-L–positive ganglion cells 1 day after KA injection (Fig. 3A–C) compared with uninjected controls. Since amacrine cells in the retina can respond to hyperstimulation of glutamate receptors by KA, in a second experiment immunolocalization experiments were performed on retinal cross sections prepared 1 day after KA injection to determine whether KA causes loss of amacrine cells (Fig. 3D). Immunolocalization of retinal cross sections with calretinin antibodies (labels AII amacrine cells) indicated a decrease in the number of calretinin-positive amacrine cells in the ganglion cell layer (presumably displaced amacrine cells) and in the
inner nuclear layer (Fig. 3D). These results indicate that intravitreal injection of KA leads to an up-regulation in tPA and uPA activity in the retina and causes loss of ganglion cells and amacrine cells by an apoptotic mechanism.

Kainic acid induces plasminogen activation

One role of plasminogen activators (tPA and uPA) is to activate a physiological zymogen plasminogen into active plasmin. Active plasmin in turn can modulate extracellular matrix proteins such as laminin and contribute to neuronal cell loss by removing critical cell-matrix interactions. Although the data presented above show an increase in tPA and uPA activity (Fig. 1), it was not completely clear whether increased levels of plasminogen activators play a role in plasminogen activation in the retina. Therefore, retinal proteins extracted from KA- or PBS-injected eyes were subjected to Western blot analysis using an antibody that detects plasminogen and its active product plasmin (Fig. 4A, C). Casein zymography assays were performed using retinal protein extracts to determine whether plasmin, which might be generated from plasminogen, is proteolytically active (Fig. 4B). Western blot analysis indicated a low and constitutive level of plasminogen protein in retinal proteins extracted from control eyes (Fig. 4A). Western blot analysis indicated an increase in plasminogen levels and activation of plasminogen to plasmin in retinal proteins extracted from KA-injected eyes in a dose- (Fig. 4A) and time-related fashion (Fig. 4C). Casein zymography assays indicated that the lower molecular weight plasmin band is indeed proteolytically active (Fig. 4B). These results indicate that KA-mediated up-regulation in uPA and tPA associates with plasminogen activation in the retina.

rPAI-1 and tPA-STOP attenuate KA-induced retinal damage

Since an up-regulation in uPA and tPA and activation of plasminogen to plasmin were associated with retinal damage (Fig. 3), we reasoned that inhibition of either plasminogen activators or plasmin might protect retina against KA-induced damage. Three different experiments were performed to determine these possibilities. First, the effect of rPAI-1 and tPA-STOP (which can inhibit uPA and tPA) on uPA as well as tPA activity was determined by zymography assays on retinal proteins extracted from KA or KA plus rPAI-1 or KA plus tPA-STOP-injected eyes. Zymography assays indicated that higher concentration of rPAI-1 (5.0 μg) and tPA-STOP (4 μM) inhibited tPA and uPA activity in the retina (Fig. 5). In the second experiment, the effect of rPAI-1 and tPA-STOP or α2-antiplasmin (plasmin inhibitor) on KA-induced cell loss was determined by TUNEL assays at 2 days postinjection. Quantification of total number of TUNEL-positive cells in retinal cross sections indicated a significant increase in TUNEL-positive cells in the retina after KA injection, as expected (Fig. 6A). Significantly fewer TUNEL-positive cells were observed in retinal cross sections after injection of KA with rPAI-1 or tPA-STOP. In contrast, intravitreal injection of KA with α2-antiplasmin (4.0 μg) failed to offer significant protection against KA-induced cell loss (Fig. 6A). In the third experiment, the

Figure 5. Effect of rPAI-1 and tPA-STOP on tPA and uPA activity in the retina. Two days after intravitreal injection of KA (20 nmol) with or without indicated concentrations of rPAI-1 or tPA-STOP, retinal proteins were extracted and aliquots containing an equal amount of proteins (25 μg) were subjected to fibrinogen/plasminogen zymography. Note that rPAI-1 and tPA-STOP inhibited KA-induced tPA and uPA activity in the retina.
Figure 6. Effect of plasminogen activator and plasmin inhibitors on KA-induced retinal damage. A) Retinal cross sections were prepared 2 days after intravitreal injection of KA (20 nmol) with or without indicated concentrations of rPAI-1, tPA-STOP, and α-2-antiplasmin (A-2-AP) and apoptotic cell death was determined by TUNEL assays. The remaining number of TUNEL-positive cells in retinal cross sections was quantitated using Scion-image analysis software and the results from 4 independent experiments were represented as mean TUNEL-positive cells ± s.e. *P < 0.05 PBS vs. KA; NS, not significant, KA vs. KA plus A-2-AP (4.0 μg); $P < 0.05 KA vs. KA plus tPA-STOP (1.0 μM); NS, not significant, KA vs. KA plus rPAI-1 (0.25 μg); $P < 0.05 KA vs. KA plus tPA-STOP (4.0 μM), &P < 0.05 KA vs. KA plus rPAI-1 (5.0 μg). B) 2 days after intravitreal injection of KA (20 nmol) with or without indicated concentrations of rPAI-1 or tPA-STOP, retinal cross sections were prepared and immunohistochemical analysis was performed using antibodies against neurofilament light (NF-L) and calretinin. Note that rPAI-1 and tPA-STOP (but not α-2-antiplasmin) inhibited uPA and tPA and attenuated the loss of ganglion cells and amacrine cells in the retina.

DISCUSSION

Previous studies have indicated that retinal damage in diseases such as glaucoma and diabetic retinopathy might be due to hyperstimulation of ionotropic glutamate receptors (“excitotoxicity”) mediated by excessive levels of extracellular glutamate in the retina (1, 2, 13, 68, 69). The mechanisms involved in retinal damage, however, are poorly understood, and many are still speculative. Therefore, in this study we have determined the role of plasminogen activators in excitotoxic retinal damage.

We show that kainic acid that hyperstimulates non-NMDA type glutamate receptors can induce retinal damage through an up-regulation of ionotropic glutamate receptors (excitotoxicity) mediated by excessive levels of extracellular glutamate in the retina (1, 2, 13, 68, 69). The mechanisms involved in retinal damage, however, are poorly understood, and many are still speculative. Therefore, in this study we have determined the role of plasminogen activators in excitotoxic retinal damage.

The effect of rPAI-1 and tPA-STOP on retinal degeneration was determined by immunostaining of retinal cross sections with antibodies against ganglion cells (NF-L) and amacrine cells (calretinin). Examination of retinal sections with antibodies against ganglion cells (NF-L) was determined by immunostaining of retinal cross sections and plasminogen activators (both uPA and tPA) can cause retinal damage independent of plasminogen activation. To determine these possibilities, we injected KA with inhibitors of uPA and tPA (rPAI-1 and tPA-STOP) or an inhibitor of plasmin (α-2-antiplasmin) into the vitreous humor and determined the retinal damage by TUNEL assays.

We found that intravitreal injection of tPA-STOP not only inhibited the activities of tPA and uPA (zymography assays, Fig. 5) but offered significant protection against KA-induced retinal damage (TUNEL assays, Fig. 6). In contrast, intravitreal injection of KA with α-2-antiplasmin (4.0 μg) failed to offer significant protection against KA-induced retinal damage. These results suggest that KA-induced retinal damage is mediated by an increase in plasminogen activators (both uPA and tPA) but independent of plasminogen activation. For example, tPA can activate NMDA-type glutamate receptors as shown in the central nervous system (49, 70), and uPA can control calcium influx (71); both tPA and uPA might perform similar functions in the retina and contribute to excitotoxic retinal damage. Although at first glance this might seem an unlikely possibility because retinal damage in the ganglion cells was up-regulated after intravitreal injection of KA, and most of the tPA was released into the extracellular space in the inner retina after KA treatment. In contrast, uPA, which was absent in uninjected control retinas, was induced after intravitreal injection of KA and localized in the nerve fiber layer. Once up-regulated, tPA and uPA can perform distinct functions in the retina. They can contribute to retinal damage via activation of plasminogen into active plasmin. Plasmin in turn can contribute to retinal damage by modulating or degrading extracellular matrix proteins such as laminin (present in the nerve fiber layer); this in turn might result in detachment-induced apoptosis of ganglion cells and amacrine cells (66). Plasminogen activators tPA and uPA can cause retinal damage independent of plasminogen activation.
this study was determined after hyperstimulation of non-NMDA type receptors (by KA), ganglion cells and amacrine cells express both NMDA and non-NMDA type glutamate receptors; tPA up-regulated in the retina after KA injection can proteolytically process NMDA-type glutamate receptors and might contribute directly to retinal damage. Although we found an increase in uPA activity and protein levels and an association of uPA with plasminogen activation, little is known about the additional roles of uPA in the retina at this time. Studies aimed in this direction are necessary to delineate additional roles of plasminogen activators in the retina. Although inhibition of plasmin activity can attenuate detachment-induced apoptosis, in the presence of increased amounts of excitotoxin such as kainic acid and in the presence of increased levels of uPA and tPA, inhibition of plasmin alone does not seem to confer protection against retinal damage.

In a previous study we observed that the optic nerve ligation-induced retinal damage is dependent on plasminogen activation; in this study, we found that KA-induced retinal damage is independent of plasminogen activation. Although the exact mechanisms for the differential role of plasminogen activation in retinal damage are not clear, there is one major difference between the animal model employed in this study (intravitreal injection of kainic acid) and the animal model used in our previous study (optic nerve ligation). In our earlier study, relatively higher plasminogen levels were found in the retina after optic nerve ligation compared with levels found in the retina after kainic acid injection into the vitreous humor. The increase in plasminogen levels found in the retina after optic nerve ligation was due to the damage to the central retinal artery, compromise in blood retinal barrier (BRB, determined indirectly by albumin Western blot analysis) and leakage of plasminogen into the retina, whereas similar compromise in BRB was not observed after intravitreal injection of kainic acid (data not shown). Clearly, plasminogen activation seems to play a differential role in retinal damage depending on the model system used.

We made an intriguing observation in this study with regards to the activity and protein profile of tPA during time course experiments. Constitutive levels of tPA present in the uninjected control or PBS-injected eyes were increased as early as 6–12 h after KA injection, decreased by 24 h, and increased again at 48 h (Fig. 1). This observation can be explained by two possibilities. First, a reduction in tPA activity could simply be due to the loss of ganglion cells that contributed to tPA production in the retina. This possibility was supported by immunohistochemical analysis of tPA in Fig. 2. Data in Fig. 2 show that tPA present in the ganglion cells was gradually released into the extracellular space starting at 6–12 h possibly due to membrane depolarization (72). At 24 h after KA injection, the majority of the ganglion cells that expressed tPA were killed by KA, hence zymography and Western blot assays indicated reduced levels of tPA in the retina. Second, tPA remain-

PLASMINOGEN ACTIVATORS PROMOTE RETINAL DAMAGE
Although there is some evidence in the CNS regarding the role of tPA in neuronal damage (30, 32), the novelty of the observations made in this study is that up-regulation of not only tPA but also uPA plays a causative role in retinal damage in response to intravitreal injection of KA, which hyperstimulates glutamate receptors. As indicated above, in the absence of a clear understanding of the mechanisms involved in ischemia or excitotoxic retinal damage (due to hyperstimulation of glutamate receptors), the results provided in this study suggest that strategies aimed at reducing the activity of plasminogen activators might offer protection against retinal damage in blinding retinal diseases in which glutamate receptor-mediated excitotoxicity has been implicated as a causative factor (6, 8, 28, 68, 69).

This work was supported by National Institutes project grant EY13643 (to S.K.C.) and Vision Research Infrastructure Development Grant EY014803.

REFERENCES.


