Opioid Receptor Activation: Suppression of Ischemia/Reperfusion-Induced Production of TNF- α in the Retina

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PURPOSE. The detrimental role of TNF- α in ischemia-induced tissue damage is known. The authors study examined whether opioid receptor activation alters TNF- α levels in the postischemic retina.

METHODS. Retinal ischemia was induced by raising the intraocular pressure above systolic blood pressure (155–160 mm Hg) for 45 minutes. Rats were pretreated with the opioid receptor agonist morphine (1 mg/kg; intraperitoneally) before injury. Selected animals were pretreated with the opioid antagonist naloxone (3 mg/kg; intraperitoneally). Human optic nerve head (ONH) astrocytes and rat microglial cells were treated with morphine (0.1–1 μ M) for 24 hours and then treated with 10 μ g/mL or 30 ng/mL lipopolysaccharide (LPS), respectively. TNF- α was measured by ELISA. Opioid receptor subtypes in astrocytes and microglia were determined by Western blot analysis.

RESULTS. There was a time-dependent increase in TNF- α production; the maximum production occurred at 4 hours after ischemia and localized to the inner retinal regions. Ischemia-induced TNF- α production was significantly inhibited by morphine. In astrocytes and microglia, LPS triggered a robust increase in the release of TNF- α , which was significantly inhibited ($P < 0.05$) by morphine. Naloxone reversed the morphine-induced suppression of TNF- α production in vivo and in vitro. Both ONH astrocytes and microglial cells expressed δ -, κ -, and μ -opioid receptor subtypes.

CONCLUSIONS. These data provide evidence that the production of TNF- α after ischemia/reperfusion injury is an early event and that opioid receptor activation reduces the production of TNF- α . Immunohistochemistry data and in vitro studies provide evidence that ONH astrocytes and microglial cells are the primary sources for the TNF- α production under ischemic/inflammatory conditions. Activation of one or more opioid receptors can reduce ischemic/reperfusion injury by the suppression of TNF- α production. (*Invest Ophthalmol Vis Sci*. 2011;52:2577–2583) DOI:10.1167/iov.10.5629

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Retinal ischemia plays a pivotal role in a number of retinal degenerative diseases such as diabetic retinopathy, glaucoma, and retinal artery occlusion.¹ The sequence of events by which ischemia leads to retinal degeneration is not completely understood but likely involves both necrotic and apoptotic processes.² A variety of substances (e.g., glutamate, oxygen free radical, nitric oxide, proinflammatory cytokines) have been implicated in ischemic retinal injury.¹ However, recent studies have provided evidence that tumor necrosis factor (TNF)- α plays a central role in the pathogenesis of a number of degenerative retinal diseases.^{3–6} In addition, intravitreal injections of TNF- α into normal eyes leads to retinal ganglion cell death and optic nerve degeneration.^{7,8}

Tumor necrosis factor (TNF)- α is a proinflammatory cytokine that is rapidly upregulated after ischemic brain injury and in several neurodegenerative disorders, such as multiple sclerosis, Parkinson disease, and Alzheimer disease.^{9,10} In the central nervous system (CNS), TNF- α can be produced by invading immune cells, activated astrocytes, and microglia.^{4,11,12} TNF- α is synthesized as a monomeric transmembrane 26-kDa protein that is normally inserted into the membrane as a homotrimer.¹³ The membrane-associated form can be cleaved by the matrix metalloproteinase ADAM17 (TACE) to release the 17-kDa monomer or the 51-kDa trimer.¹⁴ Both the membrane-bound form and the soluble form are active, and the cellular response is mediated by TNFR1 (p55) or TNFR2 (p75) receptors. Responses to TNF- α are diverse, varying from the activation of the extrinsic apoptotic pathway to the activation of cytoprotective pathways such as NF- κ B, p38, JNK, ERK1/2, and PI3K/Akt. This response is determined by the receptor subtype activated and the adaptor proteins associated with the receptor after activation.

Opioids are powerful analgesics; however, they also have other important pharmacologic properties. For example, the opioidergic system can modulate stress responses within the immune system, cardiovascular and gastrointestinal tissues, and the CNS. Recently, we have demonstrated that opioid receptor activation is required for the development of ischemic preconditioning within the retina and that the administration of a broad-range opioid agonist, morphine, can reduce ischemic retinal injury.¹⁵ The cellular mechanisms that are involved in opioid-mediated retina neuroprotection are unknown. In the rheumatoid arthritis synovial cells and splenic lymphocytes, opioids have been shown to suppress the release of proinflammatory cytokines.^{16,17} Given the prominent role TNF- α plays in degenerative disorders in the retina and the brain, the present study examined whether opioid receptor activation can alter TNF- α levels in the postischemic retina.

MATERIALS AND METHODS

Animals

Adult male or female Brown Norway rats (3–5 months of age; 150–200 g; Charles River Laboratories, Inc., Wilmington, MA) were used in this study. Rats were kept under a 12-hour light/12-hour dark cycle. Animal

handling was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the study protocol was approved by the Animal Care and Use Committee at the Medical University of South Carolina.

Retinal Ischemia

Retinal ischemia was induced using methods as described previously.¹⁵ Briefly, rats were anesthetized with intraperitoneal ketamine (75 mg/kg) and xylazine (8 mg/kg) (Ben Venue Laboratories, Bedford, OH), and local analgesia of the cornea was induced using proparacaine (5 μ L; 0.5%, Akorn, Inc., Buffalo Grove, IL). Body temperature was maintained at 37°C by means of a heating pad (Harvard Apparatus, Holliston, MA). The anterior chamber was cannulated with a 30-gauge needle that was connected to a container of sterile normal saline through polyethylene tubing; subsequently, the reservoir was elevated to raise the IOP above systolic blood pressure (155–160 mm Hg) for 45 minutes. The IOP was monitored by an in-line pressure transducer connected to a computer. The contralateral eye was left untreated, serving as the control. Animals were treated with the opioid agonist morphine (1 mg/kg; intraperitoneally), or vehicle (saline). Selected animals were pretreated with the opioid antagonist naloxone (3 mg/kg; intraperitoneally) 1 hour before morphine administration. Doses and timing of morphine and naloxone administration were selected based on our previously published studies examining the neuroprotective response to these agents.¹⁵ In animals receiving morphine, naloxone, or both, contralateral eyes were used to assess the effects of these agents on normal retinal TNF- α .

Optic Nerve Head Astrocyte Cultures

Eight human eyes from four donors were obtained from Life-Point Ocular Tissue Division (Charleston, SC) to isolate optic nerve head (ONH) astrocytes as described previously.¹⁸ Briefly, to isolate primary ONH astrocytes, ONH tissue was dissected from each eye, freed of scleral tissue and central retinal vessels, and cut into four pieces. These pieces were then plated onto a collagen I-coated cell culture plate and allowed to grow for 2 to 3 weeks in Dulbecco's minimum essential medium (DMEM; Manassas, VA) containing 10% fetal bovine serum (FBS) and antibiotics. ONH astrocytes from the mixed cell culture were purified by immunopanning, as described elsewhere.¹⁹ Briefly, culture plates were coated with C5 neuroepithelial monoclonal antibody, which was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. The mixed population of cells derived from tissue explants was trypsinized and resuspended in DMEM and placed on the C5 antibody-coated plates for 40 minutes. Nonadherent cells were removed, and adherent cells were cultured in astrocyte growth medium (Cambrex, East Rutherford, NJ) containing 3% FBS. The purity of the astrocyte culture was determined by positive immunostaining for the astrocyte markers GFAP (glial fibrillary-acidic protein) and NCAM (a cell surface adhesion-molecule) in each batch. ONH astrocytes were pretreated with the opioid agonist morphine (0.1 μ M) for 24 hours, followed by treatment with 10 μ g/mL lipopolysaccharide (LPS) for 6 hours, for the measurement of TNF- α release. Cells from third to fifth passages were used in these studies. Each experimental condition was evaluated using astrocyte cultures obtained from at least three different donors.

Rat Retina Microglia Primary Cell Cultures

Microglial cells were isolated from the retinas of newborn rats within the first 24 hours of birth, as described earlier.²⁰ Primary cultures of microglial cells were obtained from four groups of neonate rats, and cells from each group were used in the present study. Briefly, retinas were collected and washed twice with ice-cold PBS and digested with 0.125% trypsin at 37°C for 3 minutes. Trypsin was inactivated by adding culture medium (DMEM/F12 [1:1] with 10% FBS, 100 U/mL penicillin G, and 100 μ g/mL streptomycin). Tissues were triturated with a plastic pipette and washed twice. Cells were filtered through a nylon mesh (mesh opening, 100 μ m; Nitex Sheets; Sefar America, Inc.,

Kansas City, MO), collected by centrifugation, resuspended in culture medium, and plated onto 100-cm² cell culture flasks at a density of 2×10^5 cells/cm². All cultures were maintained in a humidified incubator at 37°C and 5% CO₂ and fed on the third day, then fed once every 4 days. After 2 weeks, microglial cells were harvested in culture medium by shaking the flasks at 100 rpm for 1.5 hours. The cell suspension was centrifuged, and the detached cells were replated in culture medium at designated densities for each experiment. The purity of the microglia cultures was approximately 98%, as determined by immunocytochemical staining analysis for OX42, a macrophage/microglial cell marker. The morphology of microglial cells in culture was carefully examined by phase-contrast and fluorescence microscopy. Microglial cells were pretreated with the opioid agonist morphine (1 μ M) for 24 hours, followed by treatment with 30 ng/mL LPS for 6 hours for the measurement of TNF- α release.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the levels of TNF- α in retina extracts and conditioned media, using quantitative sandwich-ELISA technology (R&D Systems [Minneapolis, MN] for human samples and eBioscience [San Diego, CA] for rat samples). Retina extracts or conditioned media collected from Brown Norway rats, rat microglial cells, or human ONH astrocytes were incubated in 96-well plates coated with monoclonal antibody specific for TNF- α . After washing at each step, biotin-conjugated anti-rat or anti-human TNF- α (polyclonal) and avidin-HRP antibodies were added and incubated in accordance with the manufacturer's instructions. After washing, a substrate solution was added to each well. The enzyme reaction was terminated by adding stop solution (2N H₂SO₄). Absorbance was measured at 490 nm. Using a standard curve prepared from seven dilutions of recombinant TNF- α , each concentration of TNF- α was calculated for the retina extracts and conditioned media.

Immunohistochemistry

Eyes were enucleated 4 hours after ischemia, and immunohistochemistry was performed as described earlier.¹⁵ Eyes were then fixed in 4% paraformaldehyde (PFA) for 2 hours. After the anterior segment of the eye and the lens were removed, eyes were further fixed in 4% PFA for another 2 hours and then cryoprotected in 25% sucrose solution overnight at 4°C. Eyecups were washed in ice-cold phosphate-buffered saline and frozen in OCT embedding medium over dry ice. Eyes were either stored at -20°C or used to cut the cryosections. Ten-micron-thick cryosections were cut at -20°C, fixed in cold methanol for 10 minutes, and rinsed in 1 $\times$ Tris-buffered saline (TBS), pH 7.5. Tissues were permeabilized with 0.2% Triton X-100 in TBS and washed again with TBS. Tissues were then blocked with 5% BSA in TBS for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibodies (e.g., anti-TNF- α antibody [Armenian hamster IgG, 1:25; eBioscience] and anti-GFAP antibody [1:100; rabbit IgG, 1:100; Sigma-Aldrich, St. Louis, MO]) or serum. Cryosections were then washed with TBS and incubated with fluorescein-conjugated secondary antibody (anti-Armenian hamster IgG, 1:400; DyLight 488; and anti-rabbit IgG, 1:600 rhodamine; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at room temperature for 1 hour. Negative control slides were incubated with normal serum in the place of primary antibody. The sections were observed and photographed with a fluorescence microscope (Axioplan; Carl Zeiss, Thornwood, NY).

Western Blot Analysis

Equivalent amounts of retina extracts or cell lysates (15 μ g) were loaded onto 10% SDS-PAGE, and proteins were separated and transferred to nitrocellulose membranes as described earlier.¹⁵ The membranes were blocked with 5% nonfat dry milk followed by incubation for 12 hours at 4°C with appropriate primary antibodies (1:1000 dilution) selective for δ -, κ -, and μ -opioid receptors. After washing, membranes were incubated for 1 hour at 20°C with appropriate secondary antibodies (HRP-conjugated; dilution 1:3000). Prestained

molecular weight markers were run in parallel to identify the molecular weights of the opioid receptors. For chemiluminescence detection, the membranes were treated with enhanced chemiluminescent reagent, and the signal was monitored using an imaging system (Versadoc; Bio-Rad, Hercules, CA).

Statistical Analysis

Statistical comparisons were made using the Student's *t*-test for paired data or ANOVA using the Dunnett posttest for multiple comparisons (GraphPad Software, Inc., San Diego, CA). $P \leq 0.05$ was considered significant.

RESULTS

Change in TNF- α Levels in Response to Ischemia/Reperfusion Injury

Retinas were collected at 2, 4, 8, and 24 hours after ischemia. As shown in Figure 1, relatively low levels of TNF- α were detected in normal retinas (nonischemic). In postischemic eyes, a robust increase in TNF- α levels occurred by 2 hours, and a peak rise was observed at 4 hours (control eyes, 55 ± 10 ; ischemic eyes, 586 ± 126 pg/mg protein; $P < 0.05$; $n = 11-15$). Although TNF- α levels began to decline by 8 hours after ischemia, TNF- α levels remained significantly elevated up to 24 hours after retinal ischemia (control eyes, 53 ± 8 ; ischemic eyes, 207 ± 23 pg/mg protein; $P < 0.05$; $n = 3-6$).

To determine whether opioid receptor activation can alter TNF- α production induced by ischemia/reperfusion, a broad-range opioid receptor agonist, morphine (1 mg/kg; intraperitoneally), was administered 24 hours before the induction of ischemia/reperfusion injury. Retinas were again collected 2, 4, 8, and 24 hours after ischemia and analyzed for TNF- α production. As shown in Figure 2, ischemia-induced elevations in TNF- α levels were significantly ($P < 0.05$) reduced at all time points in animals that received 1 mg/kg morphine. However, morphine by itself did not significantly alter TNF- α levels in the contralateral eye at any time point (data not shown). To confirm that morphine-induced attenuation in TNF- α levels was mediated by opioid receptor activation, animals were pretreated with an opioid receptor antagonist, naloxone (3 mg/kg;

FIGURE 1. Ischemia/reperfusion-induced TNF- α production. Ischemia was induced by raising IOP above systolic blood pressure (155–160 mm Hg) in the ipsilateral eye for 45 minutes in Brown Norway rats. The contralateral eye was used as a control. Retinas were collected at 2 hours (4 rats), 4 hours (11 rats), 8 hours (4 rats), and 24 hours (3 rats), and retinal extracts were used to measure TNF- α by ELISA. Data are expressed as mean $\pm$ SE. * $P < 0.05$; $n = 3-11$.

FIGURE 2. The opioid receptor agonist morphine attenuates ischemia/reperfusion-induced TNF- α production from the rat retina. Brown Norway rats were treated intraperitoneally with 1 mg/kg morphine or with an equal volume of saline vehicle 24 hours before the induction of ischemic retinal injury. Retinas were extracted 2 hours (4 rats), 4 hours (12 rats), 8 hours (8 rats), and 24 hours (4 rats) after ischemia and were analyzed for TNF- α by ELISA. Data are mean $\pm$ SE. * $P < 0.05$; $n = 4-12$.

intraperitoneally), 1 hour before morphine administration. As shown in Figure 3, naloxone pretreatment reversed the morphine-induced inhibition in TNF- α production.

TNF- α Expression in Response to Ischemia/Reperfusion Injury

TNF- α expression was determined in the retinas of seven rats 4 hours after ischemia. The contralateral eye, which underwent no surgical manipulations, served as the nonischemic control. As shown in Figure 4, no staining for TNF- α was seen in normal

FIGURE 3. Effects of the opioid receptor antagonist naloxone on morphine-induced reduction in TNF- α production in ischemic retinas. Brown Norway rats were treated intraperitoneally with 3 mg/kg naloxone (11 rats) or with an equal volume of saline vehicle (7 rats) 1 hour before 1 mg/kg morphine (intraperitoneal) treatment. Twenty-four hours after the morphine treatment, ischemic retinal injury was induced by raising the IOP above systolic blood pressure (155–160 mm Hg) for 45 minutes. The contralateral eye was left untreated as the control. Retinas were extracted 4 hours after ischemia and analyzed for TNF- α by ELISA. Data are expressed as mean $\pm$ SE. * $P < 0.05$; $n = 7-11$.

FIGURE 4. Immunohistochemistry for TNF- α . Eyes of Brown Norway rats were enucleated 4 hours after ischemia. Contralateral eyes were used as the nonischemic controls. Cryosections of nonischemic and ischemic retinas were immunostained by anti-TNF- α and anti-GFAP antibodies. *Green*: staining for TNF- α ; *blue*: DAPI in the nuclei; *red*: GFAP. *Far right*: double labeling for TNF- α and GFAP. There was no positive staining when primary antibodies were omitted (not shown). Fluorescence microscopy. Scale bar, 20 $\times$.

(nonischemic) eyes. However, TNF- α staining in the ischemic retina increased with intense staining of select cells within the ganglion cell layer and light diffuse staining throughout the inner plexiform layers. Limited GFAP staining was seen in normal retinas, and it increased remarkably in the nerve fiber layer of ischemic retinas. As shown in Figure 4, intense cellular TNF- α staining was colocalized in cells that exhibited positive immunostaining for GFAP. No evidence of TNF- α staining was noted in Müller cells.

Effect of Opioid Receptor Activation on Glial Secretion of TNF- α

In the postischemic eye, TNF- α may be derived from invading immune cells, resident activated glial cells, or both. At times corresponding to the peak rise in TNF- α levels (4 hours), immunostaining for ED1 (an antigen expressed by monocytes and macrophages²¹) in the ischemic eye was limited and not different from that in the contralateral control eye (data not shown). Based on these results, we focused our *in vitro* studies on the effects of opioids in modulating the release of TNF- α from glial cells, astrocytes, and microglia. The TLR-4 agonist LPS was used to induce TNF- α release from astrocytes and microglia. Preliminary studies in our laboratory demonstrated that LPS induced TNF- α release from ONH astrocytes in a concentration-dependent manner, with maximum release occurring at a concentration of 10 μ g/mL (data not shown). As shown in Figure 5, nonstimulated ONH astrocytes showed a very low level of TNF- α . LPS triggered a robust increase in the release of TNF- α into the culture media. This increase could be seen as early as 3 hours after LPS treatment, with peak release occurring at 6 hours (control, 7 ± 3 pg/mg protein; 10 μ g/mL LPS, 135 ± 9 pg/mg protein; $P < 0.05$; $n = 8-9$) in ONH astrocytes (Fig. 5). To evaluate whether opioid receptor activation opposed the LPS-induced release of TNF- α from ONH astrocytes, cells were treated with the opioid receptor agonist morphine (0.1 μ M) for 24 hours, followed by LPS treatment for 6 hours. As shown in Figure 6, LPS-induced TNF- α release was significantly inhibited in the presence of morphine in ONH astrocytes (LPS, 108 ± 14 pg/mg protein; morphine + LPS, 34 ± 10 pg/mg protein; $P < 0.05$; $n = 12-13$). The inhibitory response of morphine on TNF- α production was reversed in the presence of the broad-range opioid receptor antagonist naloxone (LPS, 108 ± 14 pg/mg protein; morphine + LPS, 34 ± 10 pg/mg protein; naloxone + morphine + LPS, 89 ± 15 pg/mg protein; $P < 0.05$; $n = 4$; Fig. 6).

Previous studies in microglia found a similar time course for LPS-induced TNF- α release, with the maximum release measured at 6 hours.^{20,22} To evaluate whether opioid receptor activation opposes the LPS-induced release of TNF- α from microglial cells, cells were treated with the opioid receptor agonist morphine (1 μ M) for 24 hours, followed by LPS treatment for 6 hours. As shown in Figure 7, LPS-induced TNF- α release was significantly inhibited in the presence of morphine in microglial cells (LPS, 597 ± 91 pg/mg protein; morphine + LPS, 121 ± 10 pg/mg protein; $P < 0.05$; $n = 4$). The inhibitory response of morphine on TNF- α production was reversed in the presence of the broad-range opioid receptor-antagonist naloxone (LPS, 597 ± 91 pg/mg protein; morphine + LPS, 121 ± 10 pg/mg protein; naloxone + morphine + LPS, 606 ± 102 pg/mg protein; $P < 0.05$; $n = 4$; Fig. 7).

The expression pattern of opioid receptors in rat ONH, rat microglial cells, and human ONH astrocytes was determined using selective anti- δ -, anti- κ -, and anti- μ -opioid receptor an-

FIGURE 5. Time-course response of LPS on TNF- α release from human ONH astrocytes. Primary cultures of ONH astrocytes were obtained from four human donors (e.g., eight eyes), and cells of passages 3 to 5 were used in this experiment. ONH astrocytes were treated with LPS (10 μ g/mL) for the indicated time, and media were collected and concentrated. TNF- α released into conditioned media was measured using ELISA. Data shown in this figure were confirmed using ONH astrocytes obtained from at least three different donors. Data are expressed as mean $\pm$ SE. * $P < 0.05$; $n = 8-9$.

FIGURE 6. Effects of morphine on LPS-induced TNF- α release from ONH astrocytes. Primary cultures of ONH astrocytes were obtained from four human donors (e.g., eight eyes), and cells of passages 3 to 5 were used in this experiment. ONH astrocytes were pretreated with the opioid receptor antagonist naloxone (1 μ M) for 1 hour, followed by 0.1 μ M morphine treatment for 24 hours. After incubation, the media were replenished with media containing naloxone and morphine; then the cells were treated with LPS (10 μ g/mL) for 6 hours. The medium was collected at the end of the incubation, concentrated, and analyzed for TNF- α release by ELISA. Data shown in this figure were confirmed using ONH astrocytes obtained from at least three different donors. Data are expressed as mean $\pm$ SE. * P < 0.05; n = 4–13.

tibodies with Western blot analyses. As shown in Figure 8, all three opioid receptors were expressed in rat ONH, rat microglial cells, and human ONH astrocytes.

DISCUSSION

Retinal ischemia contributes to visual impairment and blindness in diseases such as diabetic retinopathy, glaucoma, and

FIGURE 7. Effects of morphine on LPS-induced TNF- α release from microglial cells. Primary cultures of microglial cells were obtained from four groups of neonate rats, and cells from each group were used in this experiment. Primary cultures of rat microglial cells were pretreated with the opioid receptor antagonist naloxone (10 μ M) for 1 hour, followed by 1 μ M morphine treatment for 24 hours. After incubation, the media were replenished with media containing naloxone and morphine; then the cells were treated with LPS (30 ng/mL) for 6 hours. The medium was collected at the end of the incubation and analyzed for TNF- α release by ELISA. Data are expressed as mean $\pm$ SE. * P < 0.05; n = 4.

FIGURE 8. Determination of opioid receptor subtypes in rat ONH, rat microglial cells, and human ONH astrocytes by Western blot analysis. ONH extract, cell lysate from microglial cells, or human ONH astrocytes (15 μ g proteins) were analyzed by Western blotting using selective anti- δ -, anti- κ -, or anti- μ -opioid receptor primary antibodies, followed by incubation with appropriate secondary antibodies (HRP-conjugated; dilution 1:3000). The signal was captured using enhanced chemiluminescent reagent and an imaging system. Data shown are representative of three independent experiments.

retinal artery occlusion.^{1,23} Ischemia is defined as an arrest of blood flow and a consequent reduction of oxygen supply. The supply of oxygen to the retina is controlled by retinal and choroidal blood flow. Ocular ischemia, induced by elevated IOP, caused by raising of the IOP above the systemic blood pressure, is frequently used as an animal model in retina research and has been described in numerous studies.^{1,15} This method produces global ischemia by obstructing both the retinal and the uveal circulation, as evidenced by the reduction in amplitude of the ERG waveforms and pallor of the fundus and iris. Retinal ischemia followed by reperfusion shows a clear thinning of the inner part of the retina. Moreover, the pathologic findings in the rat retina after ischemia/reperfusion injury bear striking resemblance to those found in the retina of the patient with glaucoma.²³

Opioids have been used clinically for centuries as analgesics. However, other biological effects induced by opioids include cytoprotection, immunomodulation, neuroendocrine regulation, and behavior modification. Most of these biological responses are presumed to be manifested through the activation of G-protein-coupled receptors. Three opioid receptors (delta, kappa, and mu) have been identified. The expression of opioid receptors has been shown in virtually all major organ systems, including the central nervous system, heart, skin, and eyes.^{15,24,25} Endogenously, these receptors are stimulated by the release of opioid peptides (endorphins, dynorphins, and enkephalins). In the eye, opioid receptors have been implicated in the regulation of iris function, aqueous humor dynamics, corneal wound healing, retinal development, and retinal neuroprotection.^{15,26,27}

Data shown in this manuscript demonstrated the production of TNF- α after ischemia/reperfusion injury is an early event that most likely initiates and activates a downstream signaling cascade, leading to inner retina cell death under ischemic conditions. Our data are comparable to other published findings. For example, immunohistochemistry data have shown an increase in the expression of TNF- α in the inner retina layer in a time-dependent manner after ischemic/reperfusion injury in Sprague-Dawley rats.²⁸ Here, TNF- α expression was detected as early as 3 hours after ischemia/reperfusion injury, peaked from 12 to 24 hours, and then dramatically decreased after 72 hours. Aydogan et al.²⁹ have shown that levels of TNF- α remain

>2.3-fold higher than the basal level for up to 7 days after ischemia in Brown Norway rats. Our data demonstrated a peak production of TNF- α at 4 hours after ischemia/reperfusion injury. By 8 hours, TNF- α concentration began to return to basal levels but remained significantly elevated up to 24 hours after ischemia/reperfusion injury.

Activation of opioid receptors by exogenous opioid agonists has been shown to protect the heart, brain, and retina against ischemic injury.^{15,25,27,30} Recently, we demonstrated a neuroprotective role for morphine in an acute model of ischemia/reperfusion against ischemic injury in the eye.¹⁵ To investigate the cellular events that are involved in morphine-induced retinal neuroprotection against ischemia/reperfusion injury, we determined the inhibitory actions of morphine on the production of proinflammatory cytokines, such as TNF- α . To determine whether the administration of exogenous opioid agonists can protect the retina against ischemic/reperfusion injury through the suppression of TNF- α production, rats were treated with a broad-range opioid agonist, morphine (1 mg/kg; intraperitoneally), 24 hours before the ischemic retinal injury. As shown in Figure 2, ischemia-induced TNF- α production was significantly inhibited in the morphine-treated animals. Furthermore, inhibitory actions of morphine on TNF- α production were antagonized in the presence of a broad-range opioid receptor antagonist, naloxone (Fig. 3). Taken together, these data provided evidence that opioid receptor activation likely protects the retina from ischemic injury, in part by the suppression of TNF- α production. To the best of our knowledge, this is the first report showing opioid receptor-mediated inhibition of TNF- α production in the ischemic retina. The application of these results to the treatment of acute ischemic retinal disorders (e.g., AION and central retinal artery occlusion) will require additional data regarding the efficacy of opioid agonists in reducing ischemic retinal injury when administered after ischemic injury. However, recent studies^{31,32} have provided evidence that chronic progressive retinal disorders involving small focal ischemic events or reduced perfusion, such as diabetic retinopathy and glaucoma, also involve glial activation and the secretion of TNF- α . Our results demonstrating that preischemic treatment with opioid receptor agonists can suppress glial TNF- α production support the idea that opioid agonists will be efficacious in slowing or reducing secondary degenerations after ischemia or degeneration associated with reduced ocular perfusion. However, additional evaluation in pathologically relevant models is required to determine not only efficacy but also which opioid receptor subtype(s) are involved in any neuroprotective action.

In previous studies, identification of the main source(s) of TNF- α production under stress/ischemic conditions had remained in question. A variety of cell types, including activated macrophages, astrocytes, microglia, and/or neuronal cells under stress/ischemic conditions, have been proposed for the enhanced production of TNF- α . In the present study, we provided evidence to delineate the major source(s) of TNF- α production under ischemic conditions. To evaluate whether circulating activated-macrophages secrete TNF- α into the ischemic retina, we stained retinal sections of normal and ischemic eyes with anti-ED-1 antibodies (a marker for activated macrophages). There was no positive staining with ED-1 antibodies after 4 hours of ischemia (when TNF- α was maximally produced; data not shown). Furthermore, immunohistochemistry data demonstrated colocalization of TNF- α and GFAP immunostaining in the ischemic retina sections, suggesting that glial cells are the primary source for the TNF- α production in ischemic conditions. Based on the data presented in this article, we hypothesized that resident glial cells, ONH astrocytes, and microglial cells are the major sources of TNF- α production and

the site of opioid agonist actions for TNF- α suppression under ischemic conditions.

To confirm this hypothesis, we used primary cultures of human ONH astrocytes and rat microglial cells. To evaluate whether ONH astrocytes and microglial cells are the primary sources for TNF- α production under ischemic/inflammatory conditions, we stimulated ONH astrocytes and microglial cells with the TLR-4 agonist LPS, which has frequently been used to mimic stress/inflammatory conditions in numerous cell types.^{33,34} Cultured ONH astrocytes and microglial cells activated by LPS have been used to study astrocyte and microglial cell behavior as an *in vitro* model of neuro-inflammation.

Our data also demonstrate that LPS produced a time-dependent increase in the TNF- α release from ONH astrocytes (Fig. 5), with peak release occurring at 6 hours. The concentration response studies in ONH astrocytes indicated a maximum release of TNF- α at 10 μ g/mL (data not shown). Studies have also shown that TNF- α is released in a time- and concentration-dependent manner; maximum release occurred at 6 hours²² with 30 ng/mL LPS in microglial cells.²⁰ To evaluate whether opioid receptor activation opposes the LPS-induced release of TNF- α from ONH astrocytes and microglia, cells were preincubated with the opioid receptor agonist morphine (0.1–1 μ M) for 24 hours, followed by LPS treatment for 6 hours. As shown in Figures 6 and 7, the LPS-induced TNF- α release was significantly inhibited in the presence of morphine. These data provided strong evidence that TNF- α is released from ONH astrocytes and microglial cells under stress/inflammatory conditions and that the TNF- α released by LPS is opposed by opioid receptor activation.

Ischemia/reperfusion injury involves many signaling mechanisms, resulting in necrotic and apoptotic cell death.² Delayed neuronal cell death in the brain, retina, and spinal cord injury secondary to transient ischemic injury occurs, in part, by apoptosis.^{35–37} Studies have shown that the inhibition of TNF- α leads to protection in models of ischemia/reperfusion in rat brain, mouse brain, and rat myocardium.^{38–40} TNF- α is upregulated rapidly in the brain after injury,¹⁰ and excessive synthesis of TNF- α after trauma has been correlated with poor outcome. It is believed that the hyperactivity of microglial cells in the CNS and retina may be responsible for the subsequent injury, including inflammation and neuronal degeneration. In the eye, *in vivo* neutralization of TNF- α during retinal ischemia by neutralizing TNF- α antibodies significantly preserves inner retinal function, as evidenced by ERG.²⁸ The use of opioid agonists to limit inflammatory cytokine expression in the retina may provide novel means to protect the retina from ischemic injury.

In summary, our study provides evidence that the production of TNF- α initiated by ischemic/reperfusion injury is an early event that subsequently leads to retinal ganglion cell death. Opioid receptor activation plays a central role in the suppression of TNF- α production and probably protects retinal function. Based on the data presented in this article, it is evident that ONH astrocytes and microglial cells are the primary sources of TNF- α under ischemic/inflammatory conditions in the eye. Additionally, both ONH astrocytes and microglial cells have functional opioid receptors that oppose the production of proinflammatory cytokines such as TNF- α under ischemic/inflammatory conditions. Retinal ischemia plays a central role in several ocular diseases, including glaucoma, a leading cause of visual impairment and blindness in the world. The findings presented support the concept that enhancement of opioidergic activity in the eye may present a viable neuroprotective strategy for the treatment of retinal diseases that exhibit an ischemic component in their etiology.

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