# Ischemia-Reperfusion Causes Exudative Detachment of the Rabbit Retina

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**Purpose.** To characterize the activation of macroglial (Müller) and microglial cells, as well as neuronal cell degeneration, during ischemia-reperfusion in rabbit retina and to test the possible effect of triamcinolone acetonide on gliosis.

**Methods.** Transient retinal ischemia was produced by increasing intraocular pressure for 60 minutes. Triamcinolone (8 mg) was intravitreally applied immediately after the cessation of ischemia. At 3 and 8 days after reperfusion, the $K^+$ currents of acutely isolated Müller cells were recorded, and the $Ca^{2+}$ responses of Müller cells on stimulation of P2Y receptors were recorded fluorometrically in retinal wholemounts. Microglial/immune cells in the nerve fiber layer of retinal wholemounts were labeled with isolectin. To evaluate neuronal and Müller cell loss, the numbers of cells were counted in retinal slices.

**Results.** Transient ischemia caused exudative detachment of the central retina that was characterized by disruption of the pigment epithelial monolayer, the presence of scattered pigment epithelial and immune cells in the expanded subretinal space, and retinal folds. A significant loss of photoreceptor cells was observed at 8 days after reperfusion. At 3 and 8 days after reperfusion, Müller cell gliosis was apparent, as indicated by cellular hypertrophy, downregulation of $K^+$ channel expression, and an increased number of cells that displayed P2Y receptor-mediated $Ca^{2+}$ responses. The number of microglial/immune cells increased strongly after reperfusion. Intravitreal triamcinolone did not affect the parameters of Müller cell gliosis but decreased the number of microglial/immune cells.

**Conclusions.** Ischemia-reperfusion of the rabbit retina causes exudative retinal detachment that is characterized by a loss of photoreceptor cells, whereas the inner retina remains largely preserved. Micro- and macroglial cells are activated early during reperfusion, even before dropout of the photoreceptor cells. Intravitreal triamcinolone may decrease the degree of microglial/immune cell activation. (*Invest Ophthalmol Vis Sci.* 2005;46:2592–2600) DOI:10.1167/iovs.04-1402

Various ocular and systemic diseases (e.g., central retinal artery occlusion, carotid artery disease, diabetic retinopathy, hypertension, and possibly glaucoma) are accompanied by retinal ischemia, which is a common cause of visual impairment and blindness.[1] Retinal ischemia-reperfusion results in neuronal cell degeneration and in activation of glial cells. Neuronal cell degeneration is caused by oxygen and substrate deprivation during ischemia, as well as by reperfusion injury, mainly mediated by the formation of damaging free oxygen radicals[2–4] and by glutamate excitotoxicity[1,5,6] and is accompanied by opening of the barriers between blood and neural tissues.[7,8]

In the rodent retina, pressure-induced transient ischemia causes neuronal cell death predominantly in the ganglion cell (GCL) and inner nuclear (INL) layers, via necrosis and apoptosis.[9,10] The early neuronal degeneration results in a reduced thickness of the inner retinal tissue within 7 days after reperfusion.[11–15] In contrast, the outer nuclear layer (ONL) remains stable in thickness during this early postischemia stage, and no loss of photoreceptor somata is observed in the first week after reperfusion.[15,16] The ONL degenerates during later postischemia stages (2–4 weeks after reperfusion).[16] However, the susceptibility of the inner versus the outer retina to ischemic injury seems to be species dependent. A stronger sensitivity of the outer than the inner retina to ischemic damage has been reported in primate and rabbit retinas.[17–19] The reasons for the selective vulnerability of the inner rodent retina to transient ischemia are unclear; possibilities include the fact that the rat retina has a retinal blood supply in association with astrocytes, whereas this is not present to the same extent in the rabbit retina. The selective vulnerability of the inner rodent retina has been associated with various pathogenetic mechanisms: no-reflow problems in the postischemic retinal circulation,[12] elevated expression of the p53 protein that is presumably involved in apoptotic cell death of inner retinal neurons,[9] selective expression of glutamate receptors in inner retinal neurons,[1,20] and selective initial accumulation of leukocytes that release cytotoxic free radicals and cytokines within the inner retinal tissue.[21] Indeed, it has been found that shortly after transient ischemia in the rat retina, blood-derived leukocytes enter the inner retinal tissue and surround the neurons in the GCL. Invading leukocytes are assumed to attack and kill retinal neurons by a nitric oxide-dependent mechanism.[22] After this initial stage, activated microglial cells may contribute to ganglion cell degeneration, by a similar nitric oxide-dependent mechanism.[22] Excessive production of nitric oxide may be one causative factor in the loss of retinal ganglion cells in glaucoma.[23]

Reactive gliosis may have cytoprotective and cytotoxic effects on retinal neurons. Müller glial cells are implicated in cytokine-mediated protection of photoreceptor cells from death.[24,25] They release antioxidant substances such as glutathione, and they buffer the elevated extracellular $K^+$ and pro-

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tect neuronal cells from glutamate and nitric oxide toxicity, particularly by glutamate uptake and subsequent detoxification by glutamine synthesis.[26] In contrast, activated retinal glial cells may also exert cytotoxic effects by the expression of proinflammatory cytokines such as tumor necrosis factor,[27,28] by production of free radicals, and by dysregulation of the uptake of glutamate[29] and $K^+$ that may exacerbate neuronal hyperexcitation and glutamate toxicity.[26] In addition to macroglial cells, microglial cells, the resident immune cells of the neural tissue that serve important ameliorative functions such as the protection against bacterial infection and the phagocytosis of destroyed cells,[30] may have a role in neuronal cell death.[31] Microglial cells release a variety of potentially noxious substances, including reactive oxygen species, nitric oxide, and proinflammatory cytokines.[32,33] In the developing retina, microglial cells cause programmed neuronal cell death.[34] Furthermore, retinal microglial cells have been shown to induce degeneration of photoreceptor cells in vitro.[35] Treatment of the adult retina with microglia-inhibiting factors suppresses axotomy-induced neuronal degradation.[36] Retinal microglial cells may modify photoreceptor survival by controlling the neurotrophic factor production of Müller cells.[37] However, the protective or cytotoxic roles of microglial cells remain controversial.[31]

To investigate the cause of the vulnerability of the outer rabbit retina to ischemic injury[17,18] and to characterize early glial cell activation, ischemia-reperfusion of the rabbit retina was induced by transient elevation of the intraocular pressure. The degree of Müller cell activation was characterized by using two physiological parameters: (1) the expression level of the plasma membrane $K^+$ conductance, which is involved in the ionic homeostasis of the normal retina[38] and that has been shown to be downregulated in various retinal diseases including ischemia-reperfusion injury of the rat retina,[39–41] and (2) the intracellular $Ca^{2+}$ responsiveness on stimulation of purinergic P2Y receptors, which has been shown to be elevated in experimental injury of the rabbit retina.[42,43] To examine the activation of microglial cells, the density of microglial/immune cells in the nerve fiber layer (NFL) was investigated. Furthermore, the effect of intravitreal triamcinolone acetonide (9$\alpha$-fluoro-16$\alpha$-hydroxyprednisolone), an anti-inflammatory corticosteroid commonly used in the treatment of inflammatory eye diseases, on glial cell activation was investigated.

## Materials and Methods

### Animals and Ischemia Model

All experiments were performed in accordance with applicable German and British laws and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-four young adult rabbits (1.8–2.3 kg, both sexes) were anesthetized by intramuscular ketamine (50 mg/kg; Ratiopharm, Ulm, Germany) and xylazine (3 mg/kg; BayerVital, Leverkusen, Germany). The pupils of the eyes were dilated by topical tropicamide (1%; Ursapharm, Saarbrücken, Germany) and phenylephrine hydrochloride (5%; Ankerpharm, Rudolstadt, Germany). Transient retinal ischemia was induced in one eye of the animals, and the other eye remained untreated and served as the control. The anterior chamber of the test eye was cannulated from the cornea with a 30-gauge infusion needle connected to a bag containing normal saline. Intraocular pressure was increased to 125 mm Hg for 60 minutes by elevating the saline bag. Records of the dark-adapted electroretinogram that were made before and during high intraocular pressure revealed a decrease of the b-wave by approximately 80% (not shown). After cessation of the elevated intraocular pressure, triamcinolone acetonide (8 mg; stem solution in methanol, resuspended in 100 $\mu$L saline before use) was intravitreally applied to surgical and control eyes. After survival times of 3 or 8 days, the animals were anesthetized as described and were killed by intravenous embutramid mebezonium iodide (T61; 3 mL; Hoechst, Unterschleissheim, Germany), and the eyes were excised.

### $Ca^{2+}$ Imaging

Wholemounts of retinal pieces (3 × 3 mm) isolated from the retina ventrally to the visual streak were placed, vitreal surface up, in a perfusion chamber and were incubated for 1 hour at room temperature in extracellular solution containing two different calcium-sensitive fluorescence dyes, Fluo-4/AM (11 $\mu$M) and Fura-Red/AM (17 $\mu$M; Molecular Probes, Eugene, OR). The extracellular solution contained (mM) 110 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 $Na_2HPO_4$, 0.25 glutamine, 10 HEPES, 11 glucose, and 25 $NaHCO_3$, adjusted to pH 7.4 with Tris-base and bubbled with carbogen (95% $O_2$, 5% $CO_2$). After 10 minutes of washing by continuous perfusion of extracellular solution, adenosine 5′-triphosphate (ATP, sodium salt; Serva Electrophoresis, Heidelberg, Germany) was added by a rapid change of the perfusate. Fluorescence images were recorded with a confocal laser scanning microscope (LSM 510 Meta; Carl Zeiss Meditec, Oberkochen, Germany). The fluorescence dyes were excited at 488 nm; the emission of Fluo-4 was recorded with a band-pass filter between 505 and 550 nm, and the emission of Fura-Red was recorded with a 650 nm long-pass filter. Images were taken of the vitread surface of retinal wholemounts in an area of 230 $\mu m^2$.

### Microglial/Immune Cell Reactivity

To label microglial cells, acutely isolated retinal wholemounts were incubated in saline containing Cy3-tagged *Griffonia simplicifolia* agglutinin (GSA) isolectin I-$B_4$ (25 $\mu$g/mL) for 1 hour at room temperature, simultaneously with the calcium-sensitive dyes. The GSA lectin labels D-galactose residues that are expressed by both resting and activated microglial cells and by blood-derived immune cells in the rabbit retina. The Cy3 fluorescence was recorded with a 543-nm HeNe laser and a 560- to 615-nm band-pass filter. The degree of microglial/immune cell activation was estimated by counting the density of GSA lectin-stained cells at the vitreal surface (i.e., in the NFL).

### Electrophysiological Recordings

Müller cells from the retina ventrally to the visual streak were acutely isolated by using papain and DNase I-containing solutions, as described previously.[40] The cell suspensions were stored in serum-free modified Eagle's medium at 4°C (up to 4 hours) before use. Whole-cell voltage-clamp recording were obtained at room temperature (22–25°C) with an amplifier (Axopatch 200A; Axon Instruments, Foster City, CA) and ISO-2 software (MFK, Niedernhausen, Germany). Patch pipettes were pulled from borosilicate glass (Science Products, Hofheim, Germany) and had resistances between 4 and 6 MΩ when filled with the intracellular solution that contained (mM) 10 NaCl, 130 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 EGTA, and 10 HEPES (pH 7.2). The series resistance (10–18 MΩ) was compensated for by 30% to 50%; the signals were low-pass–filtered at 3 kHz (eight-pole Bessel filter), and the sampling rate was 15 kHz. The recording chamber was continuously perfused with bath solution consisting of (mM) 110 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$ 10 HEPES, 11 glucose, and Tris-base to adjust the pH to 7.4.

To evoke whole-cell $K^+$ currents, de- and hyperpolarizing voltage steps of 250-ms duration, with an increment of 20 mV, were applied from a holding potential of −80 mV. The membrane capacitance of the cells was measured by the integral of the uncompensated capacitive artifact evoked by a hyperpolarizing voltage step from −80 to −90 mV when $Ba^{2+}$ (1 mM) was present in the bath solution to block the predominant $K^+$ conductance. For recording the capacitive artifact, the sampling rate was 30 kHz and the frequencies above 10 kHz were cut off.

To evaluate the subcellular distribution of inward $K^+$ conductance, a solution containing 50 mM KCl was pressure ejected for 50 ms onto four different membrane regions of isolated cells. The evoked currents were recorded at a holding potential of −80 mV through a patch

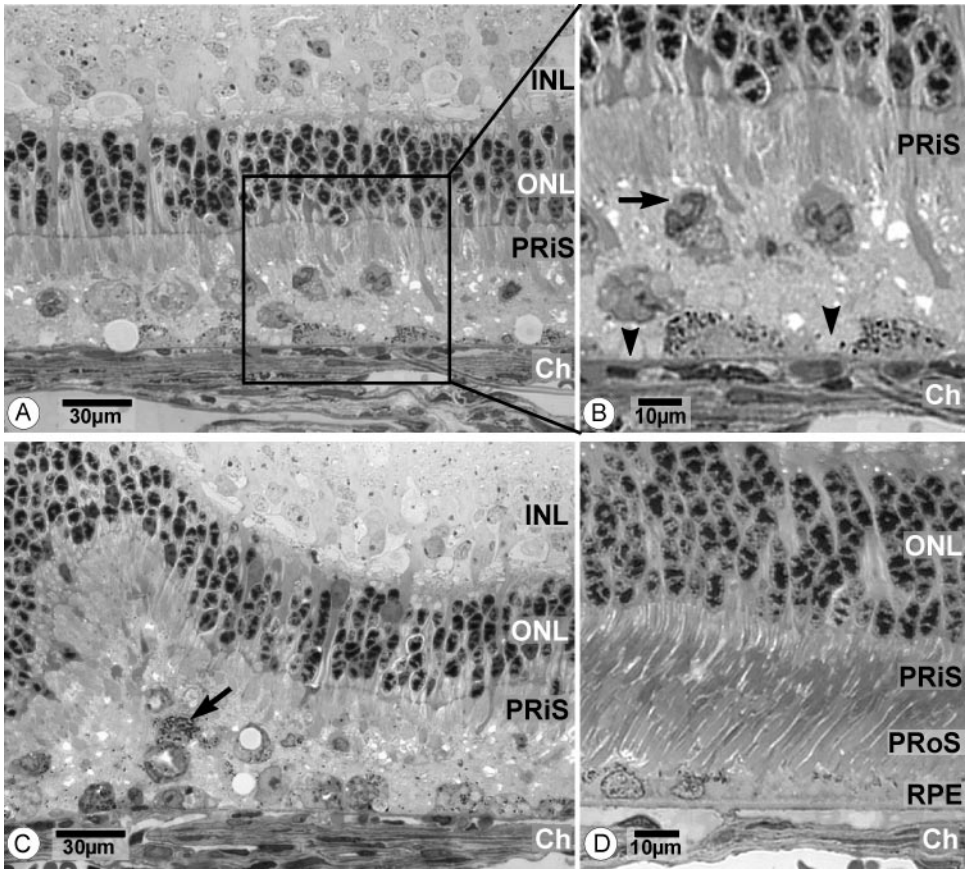


**FIGURE 1.** Ischemia-reperfusion of the rabbit retina caused exudative retinal detachment. Semithin sections of a 3-day postischemic retina (**A–C**) and a control retina (**D**), respectively. (**A**) Central part of the postischemic retina. The subretinal space was expanded and contained scattered cells of different morphologies. The photoreceptor outer segments were severely degenerated. (**B**) An enlarged retinal area from (**A**) showing disruption of the RPE monolayer (*arrowheads*) and putative blood-derived immune cells within the subretinal space (*arrow*). (**C**) Another part of the central retina displays a retinal fold and an expanded subretinal space containing dispersed RPE cells (*arrow*). (**D**) Central part of control retina with an intact interface between photoreceptor outer segments and RPE cells. Ch, choroidea; INL, inner nuclear layer; ONL, outer nuclear layer; PRiS, photoreceptor inner segments; PRoS, photoreceptor outer segments; RPE, retinal pigment epithelium.

pipette at the soma of the cells. To evoke outward currents, a solution containing 0.3 mM KCl was focally applied to the cells. Alterations of the KCl concentration were made by equimolar changes of the NaCl concentration.

## Histochemistry and Cell Counting

The animals were perfused with 2% paraformaldehyde in saline, the eyes were excised and fixed in 4% paraformaldehyde and embedded in paraffin, and 5-$\mu$m-thick sections through the eyes were produced in dorsoventral direction (through the optic nerve head). The sections were incubated overnight at 4°C with monoclonal anti-vimentin (clone VIM3B4, 1:200; Progen Biotechnik, Heidelberg, Germany), and developed with a biotinylated secondary antibody. To determine the Müller cell density, the number of vimentin-labeled Müller cell processes in the inner plexiform layer (IPL) was counted. The number of cell nuclei was determined in hematoxylin-stained slices. Five different retinal regions were examined: the visual streak, retinal areas 3 mm dorsal and ventral from the visual streak, and the retinal periphery at a 1-mm distance from the dorsal and ventral retinal edges.

## Semithin Retinal Sections

After pars plana sclerotomy, the excised eyes were fixed in a buffered mixture of 2% glutaraldehyde and 4% paraformaldehyde overnight, and postfixed in 1% osmium tetroxide for 2 hours. The tissues were rinsed, dehydrated in ethanol, and stained overnight in 70% ethanol saturated with uranylacetate. After further dehydration in absolute ethanol and propylene oxide, the samples were embedded in Araldite (502 Kit; Sigma-Aldrich, Steinheim, Germany) and sectioned by using an ultramicrotome (Nova; LKB, Turku, Finland). Semithin sections (0.5 $\mu$m) were stained with toluidine blue and visualized by using a conventional light microscope.

## Data Analysis

The electrophysiological data were not leak subtracted and were not corrected for liquid junction potentials, since these did not exceed 3 mV. The amplitude of the steady state whole-cell currents was measured at the end of 250-ms voltage steps. As a rough estimate of the resting membrane potentials, the zero-current potentials of the steady state I-V curves were determined. To evaluate the $Ca^{2+}$ responses, the fluorescence ratio $F_{\text{Fluo-4}}/F_{\text{Fura-Red}}$ was calculated, and the obtained values were normalized to the prestimulus value by calculation of the ratio $F/F_0$, where $F_0$ represents the baseline fluorescence before agonist application. The incidence of responding Müller cells was estimated by using a procedure previously described in detail.[44]

Statistical analysis was performed on computer (Prism; GraphPad Software, San Diego, CA). Significance was determined by Student's *t*-test for two groups or by ANOVA followed by comparisons for multiple groups. Data are expressed as the mean ± SD (histologic data) or SEM (electrophysiological and imaging data); *n* represents the number of retinal wholemounts (calcium imaging) and the number of isolated cells investigated (electrophysiological recordings).

# RESULTS

## Retinal Histology

The retinal histology was investigated at 3 and 8 days after reperfusion by using semithin retinal slices. At 3 days after reperfusion, an exudative (serous) retinal detachment was observed in central parts of the postischemic retinas (Figs. 1A–C), but not in the far periphery. In central parts of postischemic retinas, the monolayer of the retinal pigment epithelial (RPE) cells was severely disrupted. The subretinal space was expanded and contained scattered RPE cells and blood-derived

immune cells (Figs. 1A–C). Retinal folds were regularly observed in the detached areas (Fig. 1C). The outer segments of photoreceptors in the detached retina were in the process of degeneration. Areas with exudative retinal detachment and disrupted RPE were also observed at 8 days after reperfusion (not shown). There were hypertrophied Müller cell fibers but no other apparent histologic alterations in the inner layers of postischemic retinas (not shown).

### Müller Cell Density

To determine the density of Müller cells, retinal slices were immunostained against vimentin, and the density of stained Müller cell processes was counted in the IPL. Because the Müller cell density depends on the site of the retina (with a decreasing gradient from center to periphery),[45] slices of central and peripheral tissues were investigated, and the results were pooled. The density of Müller cells was not significantly altered at 3 or 8 days after reperfusion (12,438 ± 1,236 cells/mm$^2$) compared with the control cells (14,153 ± 449 cells/mm$^2$).

### Neuronal Cell Degeneration

At 3 days after reperfusion, the ONL displayed a certain disorganization of its histoarchitecture, with a less dense packing of photoreceptor cell nuclei compared with the control retinas. Single nuclei were displaced into the outer plexiform layer or extruded into the inner segment layer (not shown). At 8 days after reperfusion, the number of rows of photoreceptor nuclei in the ONL decreased from five to seven in control retinas to approximately two in postischemic retinas. Neuronal degeneration was estimated by counting the number of neuronal cell nuclei per Müller cell in slices from five different retinal regions. At 3 days after reperfusion, no decrease of neuronal cell numbers was observed (Fig. 2). However, at 8 days after reperfusion, the neuronal cell number in the postischemic retinas was decreased when compared with that in control retinas. This decrease was clearly significant in ventral retinal areas (Fig. 2A). In all retinal regions investigated, the number of neurons in the GCL was not significantly altered (Fig. 2B). Similarly, the number of neurons within the INL remained largely unaltered at 8 days after reperfusion. A significant reduction was found only in the ventral peripheral retina (Fig. 2C). In contrast, a relatively strong reduction of the number of photoreceptor cells was observed at 8 days after reperfusion, especially in the ventral retina (Figs. 2D, 2E). The data suggest that the inner retina remains largely preserved up to 8 days after reperfusion while the photoreceptor cells undergo degeneration, especially in ventral retinal areas.

### Müller Cell Reactivity: $K^+$ Currents

Electrophysiological records were made to reveal whether Müller cells change their $K^+$ channel expression after reperfusion. Examples of whole-cell current records of typical cells from control and 8-day postischemic retinas are shown in Figure 3A. The predominant plasma membrane conductance of Müller cells from control retinas is a $K^+$ current with a relatively weak inward rectification. Retinal ischemia-reperfusion caused a significant decrease in the density of inward $K^+$ currents in Müller cells to 47% ± 8% at 3 days ($P < 0.001$) and to 39% ± 5% at 8 days after reperfusion ($P < 0.001$), compared with the control (100%; Fig. 3B). In postischemic Müller cells of the rat, the outward $K^+$ currents have been found more reduced than the inward currents.[41] In contrast, in the rabbit cells, the ratio of outward to inward currents around the resting membrane potential of ~ −80 mV decreased only slightly, from 0.68 in control cells to 0.66 at 3 days and to 0.57 at 8 days after ischemia (Fig. 3C). The downregulation of the $K^+$ currents was not accompanied by alterations of the resting membrane potential (not shown). Significant Müller cell hypertrophy (measured as the whole-cell membrane capacitance in acutely isolated cells) was evident at 3 days after reperfusion (by 22% ± 7%; $P < 0.05$); however, after 8 days of reperfusion, the cell membrane area returned to near control levels (Fig. 3D).

The subcellular distribution of the $K^+$ conductance was determined by focal application of a high-$K^+$ solution onto four different plasma membrane domains of acutely isolated Müller cells, evoking inward $K^+$ currents at the holding potential of −80 mV (i.e., close to the resting membrane potential of the cells; Fig. 4A). In cells from control and postischemic retinas, the highest $K^+$ conductance was found at the end feet of the cells abutting the vitreous body in situ (Fig. 4B), as previously described.[46] After reperfusion, the relative decrease in $K^+$ conductance was equal in all membrane domains investigated (Fig. 4B). Similar alterations were observed when a low-$K^+$ solution, evoking outward $K^+$ currents, was ejected onto the cells (not shown), with amplitude decreases of the currents similar to the inward currents (e.g., the outward currents evoked at the end feet were: control, 145 ± 44 pA; 3 days after ischemia, 72 ± 7 pA; and 8 days after ischemia, 27 ± 11 pA, $P < 0.05$).

### Müller Cell Reactivity: ATP-Evoked $Ca^{2+}$ Responses

It has been shown that in wholemounts of normal adult rabbit retinas, only a small subpopulation of Müller cells respond to extracellular application of ATP with a transient increase of the cytosolic $Ca^{2+}$, via activation of purinergic P2Y receptors (Fig. 5A).[43] In the present study, approximately 15% of the Müller cells present in control tissues showed $Ca^{2+}$ responses to application of ATP (Fig. 5B). Retinal ischemia-reperfusion caused a significant increase of the incidence of Müller cells showing ATP-evoked $Ca^{2+}$ responses (Fig. 5A), with ~50% and ~59% of responding cells in 3- and 8-day postischemic retinas, respectively (Fig. 5B). The data suggest that an increasing number of Müller cells express functional P2Y receptors after transient ischemia.

### Microglial/Immune Cell Reactivity

Cell proliferation is a characteristic feature of microglial cell reactivity under various pathologic conditions.[30] Ischemia-reperfusion of the rabbit retina caused a strong increase in the number of GSA lectin-stained microglial/immune cells in the NFL of retinal wholemounts (Fig. 6A) that was more pronounced at 8 days than at 3 days of reperfusion (Fig. 6B).

### Effect of Triamcinolone

The effect of intravitreal triamcinolone on the degree of glial cell activation was investigated in 8-day postischemic retinas. Intravitreal triamcinolone did not change the density of $K^+$ currents in Müller cells isolated from control or postischemic retinas (Fig. 3B), the cell membrane capacitance of isolated Müller cells (not shown), or the number of Müller cells that showed $Ca^{2+}$ responses on purinergic receptor stimulation (Fig. 5B). The increase of the number of GSA lectin-stained microglial/immune cells in the NFL, however, was significantly decreased in the presence of intravitreal triamcinolone (Fig. 6B). Taken together, intravitreal triamcinolone did not affect Müller cell gliosis but may have decreased microglial cell proliferation and/or immune cell invasion.

## Discussion

The present study confirms previous observations[17,18] showing that the pattern of neuronal cell degeneration in the post-

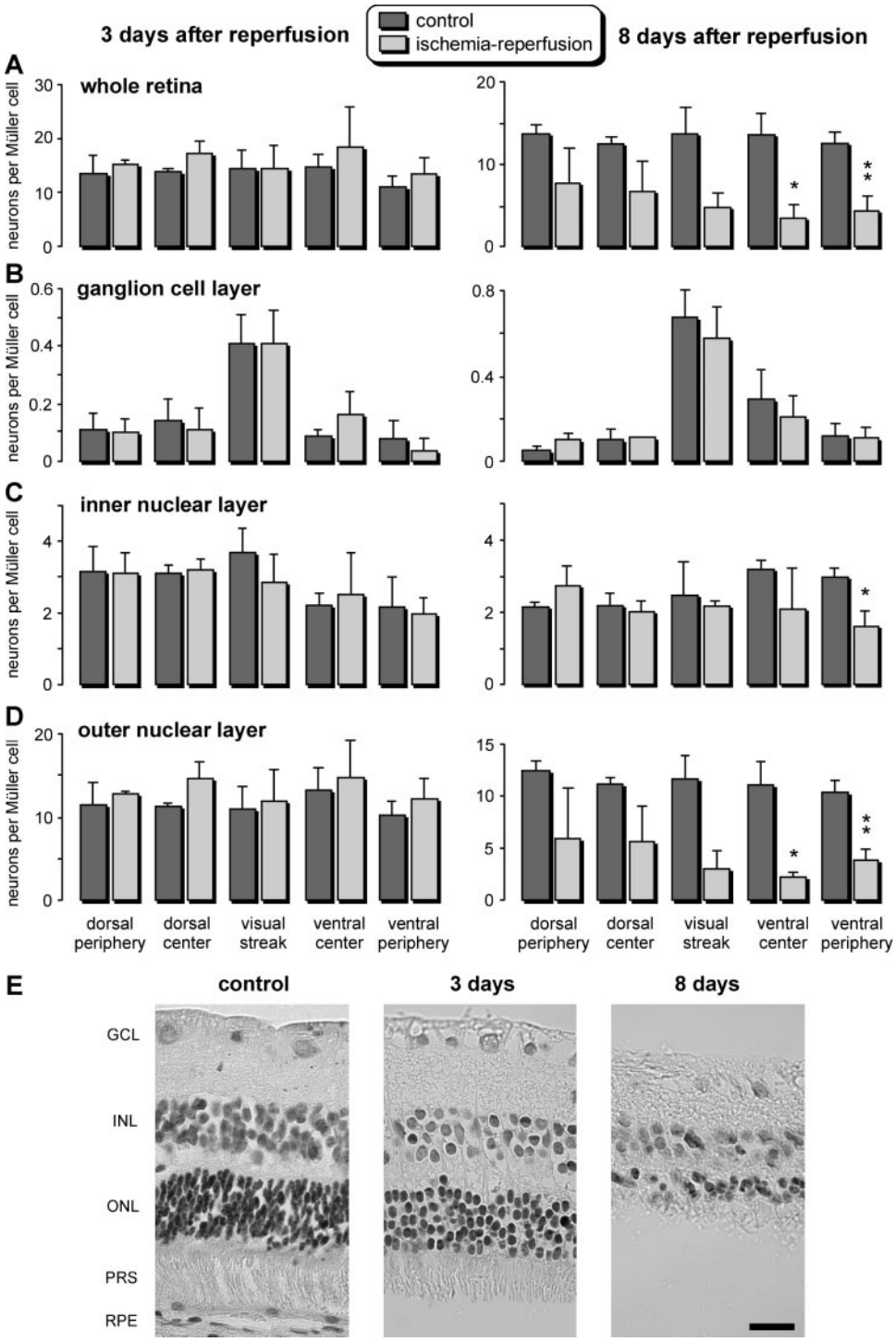


**FIGURE 2.** Ischemia-reperfusion of the rabbit retina caused selective loss of photoreceptor cells. (**A**–**D**) The number of neuronal cell nuclei per Müller cell in 3-day (*left*) and 8-day (*right*) postischemic retinas are shown. The nuclei were counted in five different retinal regions. Control: nontreated contralateral eyes. (**A**) All neuronal cell nuclei. (**B**) Nuclei in the GCL. (**C**) Neuronal cell nuclei in the INL. (**D**) Photoreceptor (PRS) cell nuclei. (**E**) Representative slices through the ventral center of the control, in 3- and 8-day postischemic retinas. Scale bar, 20 $\mu$m. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

ischemic rabbit retina is contrary to that observed in the postischemic rodent retina. Whereas the inner retina appeared to remain largely preserved up to 8 days after reperfusion, with no neuronal degeneration in the GCL and only a slight degeneration in the INL, the photoreceptor cells degenerated rapidly during the first week after reperfusion, especially in the ventral retina (Fig. 2). However, the reason for the vulnerability of the outer rabbit retina during ischemia-reperfusion is presently unclear. Our results show that ischemia-reperfusion causes a disruption of the RPE monolayer and the development of exudative retinal detachment that is accompanied by the invasion of leukocytes into the expanded subretinal space and by a degeneration of photoreceptor cells.

Though there is a large body of literature regarding the factors that may cause ischemia–reperfusion injury of the retina, the pathomechanisms of neuronal cell degeneration are still not fully understood. In addition to various other factors, two main mechanisms have been suggested[1]: cellular damage by free oxygen radicals, formed during reperfusion in the retinal tissue or released by infiltrating immune cells,[2–4,22] and cell injury by glutamate toxicity.[5,6] Glutamate toxicity has been implicated particularly in the degeneration of the inner retinal

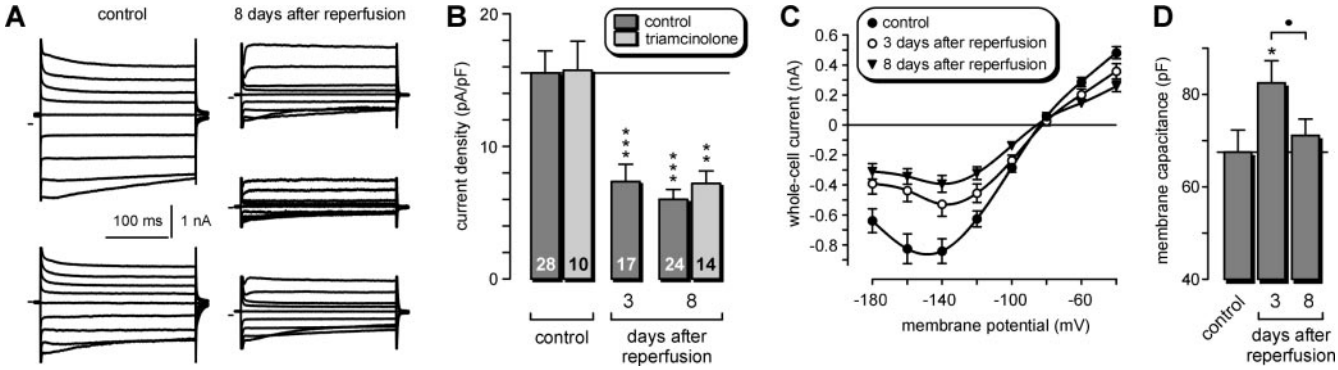


**FIGURE 3.** Ischemia-reperfusion of the rabbit retina causes downregulation of $K^+$ currents in Müller glial cells. (**A**) Examples of current recordings in cells from control (*left*) and 8-day postischemic (*right*) retinas. Voltage steps were applied from a holding potential of −80 mV to increasing de- and hyperpolarizing potentials between −160 and 0 mV. Small bars at *left* indicate zero-current levels. (**B**) Mean densities of the inward currents measured in voltage steps from −80 to −140 mV, depending on the duration of reperfusion. The number of cells is shown within the bars. (**C**) Mean I-V relationships of the whole-cell currents of Müller cells from control and postischemic retinas. The steady state currents were measured at the end of 250-ms voltage steps. (**D**) Mean cell membrane capacitance. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus the respective control. $^\bullet P < 0.05$.

layers, containing neurons that express ionotropic glutamate receptors.[1,20] In the present study, we investigated the retina of the rabbit that is (with the exception of the medullary rays) nonvascularized (i.e., all oxygen and glucose consumed by retinal neurons and glial cells is supplied via diffusion from the choriocapillaris). We observed no marked cell loss in the inner retina up to 8 days after reperfusion. (However, one should keep in mind that distinct changes of the inner retinal histoarchitecture may occur as early as 3 days after reperfusion, such as altered expression patterns of choline acetyltransferase and parvalbumin by amacrine cells,[47] as well as Müller cell and microglial activation [present data].) The present results suggest that the early vulnerability of the outer rabbit retina is due to retinal detachment and the infiltration of blood serum and leukocytes into the subretinal space. The release of free oxygen radicals such as nitric oxide and of cytotoxic cytokines by leukocytes has been implicated in the degeneration of the inner rodent retina during ischemia–reperfusion injury.[21,22] Glutamate toxicity is apparently not a main causative factor for neuronal degeneration in the postischemic rabbit retina.

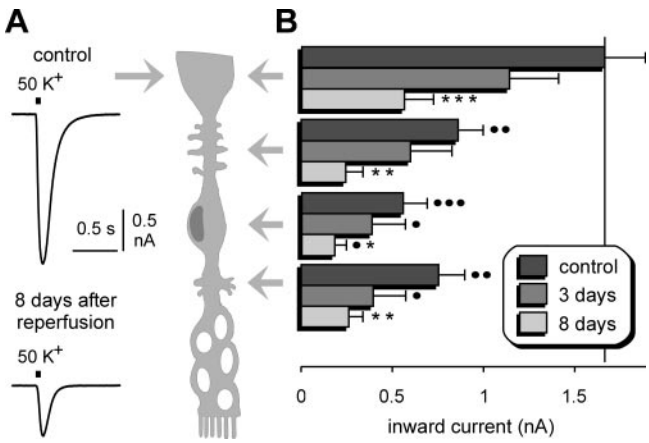


**FIGURE 4.** Subcellular distribution of the $K^+$ conductance in Müller glial cells. Focal ejection of a 50 mM-$K^+$ solution onto four different membrane domains was used to evoke inward $K^+$ currents. The control $K^+$ concentration was 3 mM. (**A**) Representative traces of inward currents of a cell from a control retina and from an 8-day postischemic retina. The high-$K^+$ solution was ejected onto the end feet of the cells; (□) ejection time. (**B**) Subcellular distribution of the inward $K^+$ conductance in cells from control ($n = 9$ cells), 3-day ($n = 7$), and 8-day postischemic retinas ($n = 11$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus control. $^\bullet P < 0.05$; $^{\bullet\bullet}P < 0.01$; $^{\bullet\bullet\bullet}P < 0.001$ versus end foot current.

The time courses, patterns, and degrees of both neuronal degeneration and glial reactivity in the present study are very similar to what was observed in a rabbit model of rhegmatogenous retinal detachment. During rhegmatogenous detachment, the number of photoreceptor cells was unchanged after 2 days but was significantly reduced after 1 week of detachment, when the inner retina remained largely preserved.[40,48] During rhegmatogenous detachment, the Müller cells displayed early reactivity with an increase of the ATP-induced $Ca^{2+}$ responsiveness and decrease of the $K^+$ currents (both beginning within 1 day of detachment) and maximum reactivity after 3 days of detachment.[43] Moreover, the extent of cellular hypertrophy is similar in Müller cells during rhegmatogenous[43] and serous detachment (Fig. 3D). The decrease in the cell membrane area observed at 8 days after reperfusion may be related to the dropout of photoreceptor cells that may cause a retraction of Müller cell processes that formerly surrounded the photoreceptor cell somata. The lack of ganglion cell loss up to 8 days after reperfusion, despite significant microglial (Fig. 6) and Müller cell activation (Figs. 4, 5) in the NFL and GCL, does not support the assumption that glial cell activation plays a significant role in neuronal degeneration after retinal ischemia.

Recently, we described a downregulation of the $K^+$ conductance of Müller cells during ischemia-reperfusion of the rat retina.[41] However, there are significant differences between Müller cells from postischemic rat and rabbit retinas: (1) The $K^+$ current downregulation in rat cells was accompanied by a significant increase in the inward rectification of the $K^+$ currents due to an almost complete absence of $K^+$ outward currents.[41] However, in rabbit cells, the inward rectification remained largely unaltered (Fig. 3C). (2) In contrast to Müller cells of the postischemic rat retina, which showed the strongest downregulation of the $K^+$ conductance in perisomatic membrane domains,[41] the cells of the postischemic rabbit retina decreased their conductance at all membrane domains to a similar degree and showed, similar to the cells from control retinas, their highest conductance at the end foot membrane (Fig. 4B). A similar rather uniform downregulation of the $K^+$ conductance at all membrane domains has been found recently in rabbit Müller cells during experimental rhegmatogenous detachment (Pannicke T, unpublished observa-

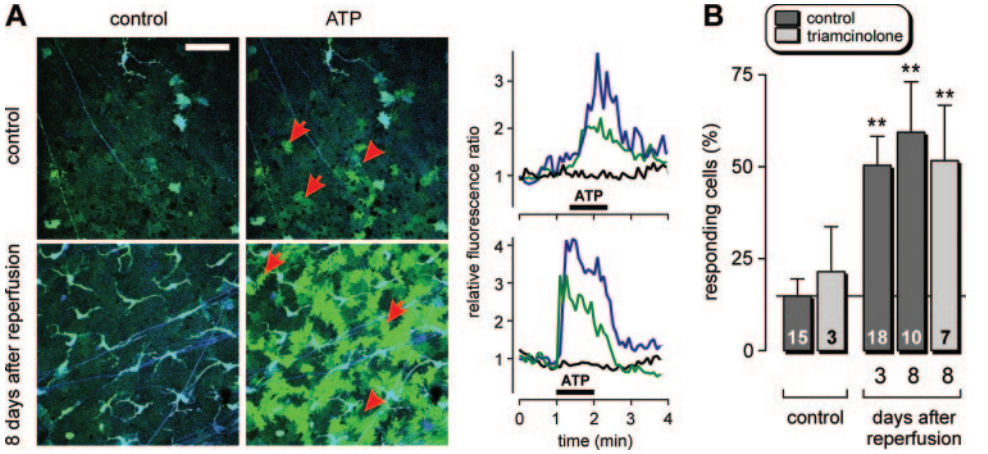


**FIGURE 5.** Ischemia-reperfusion of the rabbit retina caused upregulation of ATP-evoked $Ca^{2+}$ responses in Müller cell end feet. Confocal images were recorded by laser scanning microscopy at the vitread surface of acutely isolated retinal wholemounts (i.e., in the NFL). (**A**) The wholemounts from a control retina and an 8-day postischemic retina were stained with the calcium-sensitive dyes Fluo-4/AM (*green*) and Fura-Red/AM (*blue*). The images show dye-filled axon bundles and microglial/immune cells that lie among the end feet of Müller cells, before (*left micrographs*) and during the peak $Ca^{2+}$ response on extracellular application of ATP (200 $\mu$M; *right micrographs*). *Arrows*: end feet that showed $Ca^{2+}$ responses; *arrowheads*: nonresponding end feet; *right*: time-dependent changes of the relative fluorescence ratio of the end feet. A fluorescence ratio of 1 means no change in the cytosolic free-$Ca^{2+}$ level. Scale bar, 50 $\mu$m. (**B**) Mean number (±SEM) of Müller cell end feet that showed $Ca^{2+}$ responses to ATP, depending on the duration of reperfusion. The number of responding end feet is expressed as a percentage of all end feet investigated (100%). The number of investigated wholemounts is shown within the bars. $^{**}P < 0.01$ versus control.

tion, 2004). Although the reasons for these species differences are unclear, they may be explained by the dominant expression of $K^+$ channels in cell membrane domains that surround retinal vessels in the case of rat cells,[41] whereas rabbit Müller cells seem to express their $K^+$ channels more evenly in the whole cell membrane,[49] with the exception of a pronounced expression at the end foot membrane.[46] Furthermore, Müller cells from rat and rabbit retinas may express different subtypes of $K^+$ channels. Rodent Müller cells express both weakly rectifying Kir4.1 channels and strongly rectifying $K^+$ channels (e.g., Kir2.1),[50] whereas in rabbit cells, the virtually exclusive expression of the weakly rectifying Kir4.1 channel subtype in the entire cell membrane has been suggested.[49] However, such a difference in the patterns of Kir4.1 protein expression between rat and rabbit Müller cells remains to be demonstrated unequivocally, as well as the proposed specific downregulation of the Kir4.1 (but not of the Kir2.1) protein in rat Müller cells.

Triamcinolone acetonide is used clinically for the treatment of inflammatory diseases such as macula edema[51,52] and is presently tested in experimental models of various retinal diseases. Until now, the mechanisms of triamcinolone's rapid edema-resolving effect are incompletely understood, and research has been focused on mechanisms of inhibition of the development of vasogenic edema and inflammatory processes. Triamcinolone decreases the leakage of blood–retinal barriers,[53,54] decreases the paracellular permeability of cultured epithelial cells, and downregulates the inflammatory expression of endothelial adhesion molecules,[55] resulting in an inhibition of leukocyte-endothelial interactions in the ischemic retina (Mizuno S, et al. *IOVS* 2004;45:ARVO E-Abstract 3602). An inhibited leakage of the RPE barrier should counteract the development of serous detachment and, therefore, the activation of glial cells. However, we did not find an alteration of distinct parameters of Müller glial cell activation by intravitreal triamcinolone. Similarly, intravitreal triamcinolone did not modulate the glial cell activation in a rabbit model of rhegmatogenous detachment (Uckermann O et al., unpublished observation, 2004). The decreasing effect of triamcinolone on the density of microglial/immune cells in the NFL/ and GCL (Fig. 6B) may suggest an inhibition of immune cell infiltration.

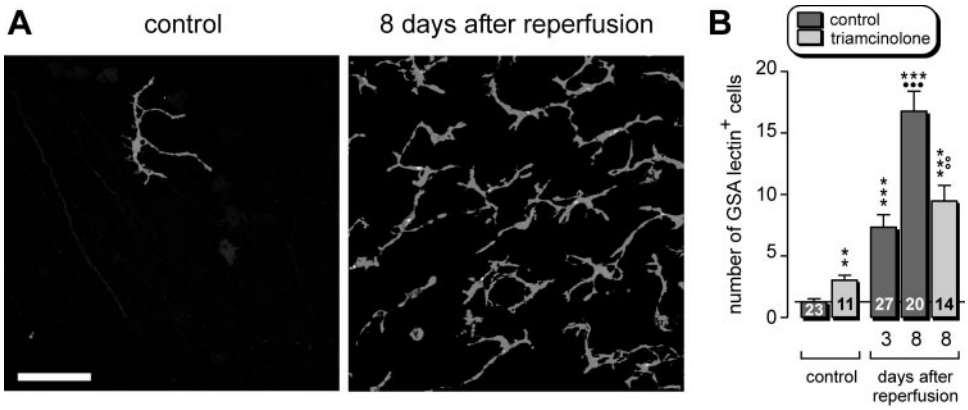


**FIGURE 6.** The density of GSA lectin-labeled microglial/immune cells at the vitreous surface of rabbit retinal wholemounts increased during ischemia-reperfusion. (**A**) Image of the vitreal surface (i.e., view of the NFL) of acutely isolated wholemounts from a control retina and from an 8-day postischemic retina, displaying microglial/immune cells labeled by GSA lectin. (**B**) Mean number (±SEM) of GSA lectin-labeled cells per unit area of vitreal retinal surface (230 $\mu m^2$). The number of investigated wholemounts is shown within the bars. $^{**}P < 0.01$; $^{***}P < 0.001$ versus control. $^{\bullet\bullet\bullet}P < 0.001$, 3 versus 8 days of reperfusion. $^{\bullet\bullet}P < 0.01$, significant blocking effect.

In summary, transient ischemia-reperfusion of the rabbit retina causes retinal detachment that may explain the vulnerability of the outer retina to ischemic injury. There is an early activation of Müller and microglial cells in the postischemic retina that is very similar to the glial cell activation previously observed during rhegmatogenous retinal detachment.[43] The postischemic rabbit retina may be a useful model to investigate the pathogenetic mechanisms and to test novel therapeutic approaches for serous retinal detachment.

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