Ischemia and Reperfusion-Induced Histologic Changes in the Rat Retina

Demonstration of a Free Radical-Mediated Mechanism

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Histologic alterations of ischemia- and reperfusion-induced retinal damage are critically dependent on the duration of the period of ischemia. Male Sprague Dawley rats were anesthetized, and a suture was placed behind the globe including the central retinal artery. Because it was desirable that untreated eyes show a great histologic change due to reperfusion-induced damage (in order that maximum scope would exist for demonstration of any protective effect of a drug treatment), a preliminary series of studies established the time-induced characteristics for the retina with transient regional ischemia. Eyes (n = 6–12 in each group) were subjected to 30, 60, or 90 min of ischemia followed by 0.5, 1, 2, 4, and 24 hr of reperfusion, respectively. The 30-min ischemia followed by reperfusion did not result in any histologic changes; 60-min ischemia followed by reperfusion induced a moderate retinal edema which returned to the preischemic value after 24 hr of reperfusion. The 90-min ischemia followed by reperfusion further aggravated retinal edema and increased the migration of neutrophil leukocytes. Even after 24 hr of reperfusion, the retinal edema had not disappeared although an attenuation was observed. In this study, the rats were treated with superoxide dismutase (SOD-PEG, 15 × 10³ U/kg) or EGB 761 (100 mg/kg) for 10 days (chronic treatment). The SOD and EGB 761 significantly reduced the development of reperfusion-induced retinal edema and significantly prevented the neutrophil leukocyte infiltration. Both also had a protective effect against reperfusion-induced injury when these agents were administered just before reperfusion (“late” administration). These results indicate that SOD and EGB 761 could diminish the reperfusion-induced histologic changes and inhibit the development of retinal edema and infiltration of neutrophil leukocytes. Invest Ophthalmol Vis Sci 32:1471–1478, 1991

Reactive oxygen intermediates, such as superoxide anion, hydrogen peroxide, and hydroxyl radical, are produced sequentially in the univalent reduction of molecular oxygen to water. During normal aerobic metabolism, these intermediates are generated at various cellular and subcellular sites which involve mitochondrial respiration, catecholamine oxidation, purine catabolism, and the phagocytic activity of neutrophils. Since these intermediates can be cytotoxic,1–3 endogenous antioxidant mechanisms have evolved in aerobic cells to eliminate radicals before they can cause cell damage. The two major protective mechanisms are the antioxidant enzyme systems,4 eg, superoxide dismutase (SOD), catalase, and the peroxidases, and the organic free-radical scavengers, eg, ascorbate and vitamin E.5,6

During ischemia and reperfusion in a number of tissue (eg, intestine,7,8 central nervous system,9 and heart10,11), oxygen-derived free radicals are thought to play an important role in the genesis of tissue injury. Endogenous scavenging mechanisms may be overwhelmed by a burst of radical production occurring during reperfusion or reoxygenation. We tested the importance of free radicals in reperfusion-induced changes in retina and used a model of retinal ischemia followed by reperfusion in rats to investigate the effects of SOD and an extract of Ginkgo biloba (EGB 761, Tanakan IPSEN, Paris, France) which probably act as a free-radical scavenger.12

Materials and Methods

Induction of Ischemia and Reperfusion

Male Sprague Dawley rats (270–320 g body weight) were anesthetized with pentobarbital (50 mg/kg). Their pupils were maximally dilated with atropine sulfate (1%), and the lids were retracted by sutures. At
the onset of each experiment, peritomy was done, and a suture was placed behind the globe (using an operating microscope; Wild Leitz, Heerbrugg, Switzerland) including the central retinal artery, ciliary arteries, and the retrobulbar connective tissue.13,14 Both ends of the ligature were then passed through a small plastic tube. Regional ischemia could be induced at any time by pulling the suture while pressing the tube against the surface of the central retinal artery. The ischemia could then be maintained for any desired period by clamping the tubing and the sutures. Reperfusion could be initiated by unclamping and removing the plastic tube. The successful induction of ischemia and the adequacy of reperfusion were confirmed visually with an ophthalmoscope. All animals were treated humanely in accordance with the ARVO Resolution on the Use of Animals in Research.

Fixation of Eyes for Histology

For fixation of the eye, a cannula was introduced through the heart into the aorta, and the right ventricle of the heart was opened to allow the blood escape. For washing out the blood before fixation, a buffered solution of 0.9% NaCl (100 ml) was used. A short (approximately 20 sec) washing-out period was optimal since prolonged perfusion before fixation may cause artifacts of the nervous tissue; 100 ml of the fixative solution (Bouin aqueux) followed immediately any interruption of the NaCl perfusion. Then the eye was enucleated and rapidly cut open and divided into two halves by coronal section through the ora serrata, the vitreous was removed, and the posterior half of the eye was immersed in fixateur (Bouin aqueux). After this postfixation, the tissue was dehydrated in graded series of ethanol and embedded in paraffin. Sagittal sections of 7 μm were cut and stained with hematoxylin and eosin.

Experimental Time Course

Three basic protocols were used.

Selection of ischemic duration: Histologic alterations to ischemia- and reperfusion-induced retinal damage are critically dependent on the duration of the period of ischemia.15-18 Since we required that untreated eyes show a great histologic change to reperfusion-induced damage (in order that maximum scope would exist for the demonstration of any protective effect of drug treatment), we did a preliminary series of studies to establish the time-induced characteristics for a retina with transient regional ischemia. Eyes underwent 30 min, 60 min, or 90 min of regional ischemia followed by 30 min, 60 min, 2 hr, 4 hr, or 24 hr of reperfusion, respectively.

Studies involving the chronic administration of drugs: Rats (n = 6–12 in each group) were treated with 15,000 U/kg of SOD intravenously (Sigma, St. Louis, MO) or 100 mg/kg of EGB 761 (orally), respectively, for 10 days. The relatively short pharmacologic half-life (6–10 min) of SOD was a drawback to the study of this scavenger in our experimental protocols. Therefore, a SOD in which the enzyme was conjugated to polyethylene glycol (PEG-SOD; Sigma) was used for all studies. This conjugated form of SOD has a pharmacologic half-life of 30 hr.19 The untreated (control) group received a daily dose of vehicle, and after the last treatment, the animals were anesthetized, and the surgical procedure was done as described. In these studies, the eyes were subjected to 90 min of regional ischemia followed by 4 or 24 hr of reperfusion. At the end of the reperfusion periods, the animals underwent transcardial perfusion followed by fixation of their eyes for histologic studies. When 24 hr of reperfusion was used, precautions were taken to prevent infection of the eyes; neomycin was dropped into the operated eye (five times per day). After 24 hr of reperfusion, the rats were reanesthetized, their eyes were examined under the operating microscope and ophthalmoscope, and transcardial perfusion followed by fixation was done.

Studies involving the "late" administration of drugs: To assess whether the effect of SOD against reperfusion-induced injury was direct, acting during the reperfusion period, or indirect, arising as a consequence of the effects of the drug on the eye during ischemia, some eyes were studied with drugs administered 5 min before the induction of reperfusion. The administration of EGB 761 was an exception; this treatment was administered orally before the induction of anesthesia. The surgical procedure and the duration of ischemia and reperfusion were similar to that described in the section on chronic administration of drugs.

Quantitation of Histologic Changes by Statistics

In previous reports, ischemia- and reperfusion-induced cell swelling was well recognized and documented in the inner plexiform layer of the eye,18,20 and the average thickness for each eye was measured in sagittal sections at or near the optic nerve and expressed in microns, using by a video-plan computer analyzer (Imstar, Paris, France). The identification of neutrophil leukocyte migration was also observed after 24 hr of reperfusion, and this phenomenon was classified as the presence or absence of neutrophil leukocytes.

Statistical analysis were based on the guidelines described by Wallenstein et al.21 A Gaussian-distributed
variable was expressed as the mean ± the standard error of the mean of retinal edema. A one-way analysis of variance was done to test for any differences between the mean values of groups. If a difference was found, the groups were compared using Fisher’s test. An analogous procedure was followed for binomially distributed variables (eg, presence or absence of neutrophil leukocytes). To compare individual groups, an overall chi-square test for a 2 X n table was constructed, followed by a sequence of 2 X 2 chi-square tests. A probability of less than 5% was considered significant.

Results

Selection for Duration of Ischemia

To demonstrate a protective effect of any interventions, we required that untreated rats have large histologic changes in the retina during reperfusion. To ensure this, within the experimental time course and conditions defined for this study, we undertook a preliminary series of studies in which eyes (n = 6–12 in each group) were subjected to 30, 60, or 90 min of regional ischemia followed by 0.5, 1.0, 2.0, 4.0, and 24 hr of reperfusion. The results (Table 1) reveal that, after 30 min of ischemia followed by reperfusion, no induction of retinal edema occurred. When the ischemic duration was extended to 60 min, reperfusion of the previously ischemic retina increased the edema formation in the inner plexiform layer. Maximum vulnerability occurred after 1 and 2 hr of reperfusion; during the remainder of the experiment, the thickness of the retinal plexiform layer returned to the control value. Increasing the ischemia to 90 min caused serious retinal edema, observed at a maximum value of 4 hr of reperfusion; this retinal edema did not return to the pres ischemic value even after 24 hr of reperfusion. Another important observation was the appearance of neutrophil leukocytes after 90 min of ischemia followed by 24 hr of reperfusion. We also observed many changes in the inner nuclear layer; there were more pyknotic nuclei, vacuolated spaces, and degenerative changes in the ganglion cells. These histopathologic changes were limited mostly to the inner part of the retina; only small alterations were seen in the photoreceptor layer using the light microscope. These histologic findings can be seen in the Figure 1. For the following studies, therefore, a 90-min ischemia followed by 4 and 24 hr of reperfusion was selected.

Studies Involving the Chronic Administration of Drugs

The eyes underwent a 90-min ischemia followed by 4 and 24 hr of reperfusion, respectively. The results (Fig. 2) demonstrate that SOD and EGB 761 significantly reduced reperfusion-induced edema formation after 4 hr of reperfusion (Fig. 2A) from its control value of 133 ± 5 µm to 87 ± 3 and 81 ± 8 µm, respectively. A similar reduction was also observed after 24 hr of reperfusion (Fig. 2B).

Studies Involving the “Late” Administration of Drugs

In the previous section we reported that SOD and EGB 761, when administrated chronically, reduced the reperfusion-induced edema formation in the inner plexiform layer after 4 and 24 hr of reperfusion. In this section we compared this result with that found in eyes in which SOD was administrated 5 min before the onset of reperfusion (“late” administration). The administration of EGB 761 was an exception; this drug was administrated orally before the induction of anesthesia. Thus, during ischemia (90 min), there was time enough for absorption of the drug from the gastrointestinal tract. The results show (Fig. 3) that, despite the late administration of SOD and EGB 761, the agents were equally as effective as when they were administrated chronically before the induction of ischemia. This provides strong evidence that SOD and EGB 761 exert their protective effects directly during the reperfusion period.

Table 1. Relationship between duration of ischemia and reperfusion-induced edema formation in rat retina

<table>
<thead>
<tr>
<th>Duration of ischemia (hr)</th>
<th>Reperfusion (hr)</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
<th>4.0</th>
<th>24</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>47 ± 4</td>
<td>51 ± 5</td>
<td>52 ± 3</td>
<td>47 ± 3</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>46 ± 3</td>
<td>48 ± 3</td>
<td>51 ± 3</td>
<td>49 ± 2</td>
<td>48 ± 2</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>79 ± 3†</td>
<td>96 ± 3†</td>
<td>96 ± 3†</td>
<td>59 ± 3*</td>
<td>54 ± 3</td>
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<tr>
<td>1.5</td>
<td></td>
<td>101 ± 4†</td>
<td>103 ± 3†</td>
<td>111 ± 4†</td>
<td>133 ± 5†</td>
<td>112 ± 4†</td>
</tr>
</tbody>
</table>

*P < 0.05.
†P < 0.001.

Eyes were subjected to 30, 60, or 90 min of regional ischemia followed by 0.5, 1.0, 2.0, 4.0, or 24 hr of reperfusion. The thickness (µm) of the inner plexiform layer was measured because the manifestation of retinal edema was well recognized in this layer of tissue.
n = 12 in each group, mean ± SEM.

Comparisons were made to the sham-operated values (0 hr of ischemia, occlusion was not carried out) at fixed time points.
In the late-administration groups, a protective effect was also observed (Fig. 4B), but this reduction was not as great as in the chronically treated rats.

Discussion

Retinal ischemia of sufficient severity and duration results in the death of the affected cells. Initially the ischemic retinal cells enter a phase of reversible injury, documented by the fact that timely reperfusion results in the survival of retinal cells otherwise destined to die. With an increasing duration of ischemia, an increasing number of retinal cells in the area at risk die despite reperfusion. These cells are said to have entered a phase of irreversible injury because they undergo necrosis even though the initial cause of injury has been removed. When and if reperfusion injury occurs, what mechanisms contribute to its development, and what adjunctive therapies, when applied before or during the reperfusion phase, will be benefi-

Fig. 1. Sagittal sections of the rat retina showing the structure of layers in control (untreated) eyes. (A) Nonischemic rat retina; (B) 90 min of regional ischemia followed by 4 hr of reperfusion; (C) 90 min ischemia followed by 24 hr of reperfusion. NLs: neutrophil leukocytes.

Effects of the Drugs on Leukocyte Migration

Since neutrophil leukocyte infiltration was observed after 90 min ischemia followed by 24 hr of reperfusion (Fig. 1C), we examined the effects of the drugs on the migration of neutrophils. As Figure 4 depicts, SOD and EGB 761 significantly reduced the incidence of neutrophil migration from its control value of 100% to 17% (P < 0.001) and 25% (P < 0.05), respectively, in the chronically treated rats (Fig. 4A).

Fig. 2. Effects of chronic treatment by SOD and EGB 761 on the extent of retinal edema after 90 min ischemia followed by 4 hr (A) and 24 hr (B) reperfusion, respectively, in the inner plexiform layer. Comparisons were made to the appropriate time-matched control (C) values. Mean ± SEM, **P < 0.001.
Fig. 3. Effects of "late" administration (5 min before reperfusion) of SOD and EGB 761 on the extent of retinal edema after 90 min ischemia followed by 4 hr (A) and 24 hr (B) reperfusion, respectively, in the inner plexiform layer. Comparisons were made to the appropriate time-matched control (C) values. Mean ± SEM, ***P < 0.001.

Fig. 4. Effects of chronic (A) and "late" (B) administration of SOD and EGB 761 on the incidence of neutrophil migration after 90 min ischemia followed by 24 hr of reperfusion. Comparisons were made to the time-matched control (C) value. *P < 0.05, **P < 0.01, ***P < 0.001.

Pentobarbital, as a general anesthetic agent, is widely used to introduce anesthesia in different experimental animals to study the histologic changes induced by retinal ischemia, although a number of investigators used different anesthetic agents. Under our experimental conditions, sham-operated rats also underwent pentobarbital anesthesia. Therefore the effects observed with SOD and EGB 761 could not be attributed to barbiturate anesthesia.

Our results clearly demonstrate that SOD and EGB 761, an extract of Ginkgo biloba, can reduce the damage to the rat retina of reperfusion-induced injury after 90 min of regional ischemia dramatically. Thus, after 90 min of ischemia followed by 4 hr of reperfusion, at the concentrations of 15,000 U/kg SOD and 100 mg/kg EGB 761, these agents reduced reperfusion-induced edema formation (which was expressed as the thickness of the inner plexiform layer in microns) to 87 ± 3 and 81 ± 8, respectively, from its control value of 133 ± 5. A similar reduction was found in the development of retinal edema after 90 min of ischemia followed by 24 hr of reperfusion. Although limited by observation in the rat retina in vivo, these results provide further support for the hypothesis that free radicals could play a role in the genesis of reperfusion-induced injury (through a membrane-permeability modification effect) and that inhibition or scavenging of these highly cytotoxic intermediates may provide an effective means of controlling this potentially dangerous retinal injury. The major finding to emerge our study was that the late administration (SOD was given before 5 min of reperfusion) and of EGB 761 (EGB 761 was orally administered just before the anesthesia) still resulted in a reduction in the thickness of the inner plexiform...
layer, referred to as attenuation of the retinal edema. Thus, although it might be argued that in the late-administration studies, these drugs exerted some effect during the last few minutes before reperfusion, it seems more likely that they exerted a direct effect during the reperfusion phase. Therefore, although the duration of ischemia and reperfusion is a primary determinant of the development of retinal edema to reperfusion-induced injury, SOD and EGB 761 can alter this preconditioned state during the subsequent reperfusion phase. It would be interesting to ascertain whether anti-free-radical interventions, such as allopurinol and catalase, can achieve a true protective effect, interfering with retinal edema formation during reperfusion periods.

Our study provides some information concerning the identity of the free radicals that may be involved in the genesis of reperfusion-induced injury. On the basis of previous study with SOD, which prevented the formation of hydroxyl radicals, it is tempting to propose that oxygen-derived free radicals may be involved. With regard to the mechanism by which free radicals may precipitate reperfusion damage (and other aspects of ischemic injury), it is well known that free radicals can cause severe membrane injury by initiating various reactions, including lipid peroxidation, which may alter membrane integrity and permeability characteristics. It is also well established that free radicals can cause the functional impairment of membrane channels and ionic pump mechanisms (not only in the retina), disturbing normal retinal function. Induction of such injury as a consequence of a "burst" of free-radical production at the onset of reperfusion may result in catastrophic perturbations of retinal function. This, together with the heterogeneity of injury and the recovery that characterizes early reperfusion, may create an ideal scenario for establishing edema formation and consequent precipitation of vision disturbances. However, before attributing such mechanisms to the genesis of reperfusion-induced injury, there is a clear need for more direct proof of free-radical involvement. This would involve using techniques such as electron spin resonance and subcellular membrane characterization to demonstrate the production of free radicals at the appropriate time and show that, under the conditions of our study, the SOD is acting as a highly effective free-radical scavenger, preventing the membrane injury that occurred in the absence of the agent. A number of triggers, in addition to free radicals, have been suggested as responsible for the genesis of reperfusion-induced retinal damage. These include stimulation of the platelet-activating factor, the release of lysophosphatides, and disturbances of potassium, sodium, and calcium conductance. One or more of these factors may trigger the reperfusion-induced retinal dysfunction, but that under normal circumstances, oxygen radicals may be the most common trigger. If this is true, then it would be reasonable to expect these triggers to be associated with different ischemic time-reperfusion vulnerability profiles. Thus, lysophosphatides, for example, might require a longer period of ischemia before they become a significant factor in reperfusion-induced retinal damage. To investigate such a possibility, it would be necessary to compare the relative susceptibility of retinal damage generated by reperfusion after different ischemic durations to different types of intervention. Ultrastructural changes were not examined in our study, but we found that short periods of ischemia (15 min) resulted in no observable ultrastructural changes. Vacuolization was the result of mitochondrial swelling severe enough in eyes subjected to 90 or 120 min of ischemia to cause the mitochondria to rupture. The smooth endoplasmic reticulum was condensed, a change which was focal in eyes that underwent the short period of ischemia, but more widespread in eyes subjected to 60 min of ischemia or more. At this stage of ischemia, electron microscopy revealed simple focal swelling of nerve fibers in the center of the lesion. In the ganglion cells there was depletion of cytoplasmic organelles at the side of the axonal hillock, reduction of ribosomes, swelling of mitochondria, and enlarged endoplasmic reticulum with clumping of nuclear chromatin. The inner plexiform layer showed marked swelling, and the outer nuclear layer contained swollen cells with almost empty cytoplasm, fragmented cytoplasmic matrix, and a few disintegrated mitochondria and vacuoles. It was apparent that the ganglion and bipolar cells were equally sensitive to anoxia, the astrocytes were the most resistant, and Müller fibers were intermediate.

Under certain conditions of altered physiology, the neutrophil leukocytes may react in a manner that leads to tissue injury. We also focused attention on the potential role of these cells as a determinant of the ultimate extent of reversible retinal injury after ischemia and reperfusion and identified those factors which modulate the inflammatory response to retinal cell injury. The tissue damage resulting from retinal ischemia activates a cascade of events which represents an inflammatory response that occurs independently of any improvement in retinal reoxygenation. We propose that the inflammatory response and the invading leukocytes contribute to the ultimate extent of retinal injury. Therefore, interventions directed against these cells or against the cytotoxic products produced by them should result in a redu
tion in the extent of tissue damage associated with retinal ischemia and/or reperfusion. Our studies indicate that the infiltration of ischemic tissue by these leukocytes begins and increases progressively after 90 min of ischemia followed by 24 hr of reperfusion; 30 or 60 min of ischemia followed by 24 hr of reperfusion did not lead to any such migration.

The agent SOD has a direct effect on reperfusion injury, scavenging the cytotoxic free radicals and significantly reducing the migration of these leukocytes (thus preventing the additional production of free radicals by the neutrophils). The EGB 761 also inhibited the migration of these cells and afforded protection against reperfusion-induced retinal damage. Therefore we conclude that the extract of *Ginkgo biloba* contains some natural free-radical scavengers which are able to bind these highly cytotoxic free radicals, preventing the reperfusion-induced retinal damage.

In conclusion, neutrophils can release cytotoxic products extra- or intracellularly without themselves undergoing destruction. Neutrophils can release various mediators capable of promoting tissue injury, including proteolytic enzymes, platelet-activating factor, arachidonic acid metabolites, and activated species of oxygen. Among the last mentioned group of neutrophil-derived products, superoxide anion, hydrogen peroxide, hydroxyl radical, and hypochlorous anion may be considered to be the most significant cytotoxic products derived from the metabolism of molecular oxygen. We propose that the generation of these cytotoxic metabolites of oxygen in the microenvironment formed between the adherent activated leukocytes and the altered retinal cells leads to an increase in tissue permeability and cell damage which is "explosive" after reperfusion because the neutrophils are directed and attracted to the reperfused region under the influence of the local accumulation of chemotactants. Therefore, we suggest that the extension of cell death on reperfusion of the ischemic retinal cells is mediated, in part, by the neutrophil-derived cytotoxic products of oxygen metabolism. It should be obvious that, in the absence of reperfusion, all cells that are ischemic will undergo irreversible changes and that reperfusion is essential for survival of the cells that may be viable up until the time of reperfusion. However, a portion of the still viable retinal cells will undergo further injury due to the cytotoxic actions of the reactive-oxygen species. These observations provide support for the hypothesis that, under some conditions of regional retinal ischemia and reperfusion, neutrophils adhere to the vessel wall and retinal cells at sites of inflammation and release toxic products capable of damaging the adjacent cells in the reperfused region.

The immediate extrapolation of our results to clinical use should be viewed cautiously because of: (1) the absence of retinal artery disease in animal models where there are higher flows in normal arteries than when they are diseased, (2) the speed of reperfusion, which is much faster during experimental retinal artery occlusion than in humans, and (3) the different enzyme activities in animals; eg, xanthine oxidase is present in some laboratory animals but not in humans.

**Key words:** arterial occlusion, SOD, EGB 761, free radicals, rat

**Acknowledgments**

The authors thank Mrs. M. Millerin and Mrs. C. Betin for expert technical assistance.

**References**