In Vivo Evaluation of Retinal Injury After Transient Ischemia in Hypertensive Rats

Fumitaka Hirose, Junichi Kiryu, Kazuaki Miyamoto, Kazuaki Nishijima, Shinsuke Miyahara, Hideto Katsuta, Hiroshi Tamura, Yoshihito Honda

Abstract—A number of studies have suggested that hypertension affects the pathogenesis of inflammatory reactions in various organs. The objective of this study was to evaluate the effects of hypertension on leukocyte–endothelial interactions after transient retinal ischemia. Transient retinal ischemia was induced for 60 minutes in spontaneously hypertensive rats (SHR) and in age-matched normotensive Wistar-Kyoto rats (WKY). At 4, 12, 24, 48, and 72 hours after reperfusion, flat-mount retinas were prepared to evaluate the density of leukocytes that had been accumulated in the retina. Intercellular adhesion molecule-1 (ICAM-1) mRNA levels were studied by semiquantitative polymerase chain reaction and ICAM-1 protein levels were studied by enzyme-linked immunosorbent assay. At 14 days after reperfusion, the retinal damage and the effect of superoxide dismutase on the damage were evaluated histologically. In SHR, the number of accumulated leukocytes peaked at 48 hours after reperfusion, and it was upregulated to 5.2-fold, as compared with that of WKY (P<0.001). ICAM-1 mRNA expression and ICAM-1 protein levels were increased significantly in the ischemia-reperfused retina in SHR compared with WKY (P<0.05). Histological examination demonstrated marked increase in the retinal ischemia/reperfusion damage in SHR (P<0.01) and a significant amelioration of the damage by treatment with superoxide dismutase in SHR (P<0.05). Oxidative stress may thus be an important mechanism for the deterioration seen in ischemia/reperfusion injury in the SHR retina. (Hypertension. 2004; 43:1-5.)

Key Words: rats, inbred SHR ■ ischemia ■ leukocytes ■ oxidative stress ■ cell adhesion molecules

Hypertension causes various disturbances in many organs, including eyes. Epidemiologic studies have reported hypertension to be a risk factor for diabetic retinopathy,1 age-related macular degeneration,2 and retinal artery and vein occlusion,3,4 which are the leading causes of blindness and visual disability among the elderly in many countries.5 In addition, severe hypertension per se can induce hypertensive retinopathy, which can lead to visual loss.6

It has been reported that transient ischemia in spontaneously hypertensive rats (SHR) leads to a greatly enhanced inflammatory reaction with organ injury in the heart,7 brain,8 and mesentery,9 as compared with age-matched Wistar-Kyoto rats (WKY). These reports then raise the question of whether hypertension may also be deleterious to ischemia/reperfusion injury in the retina via enhanced inflammatory reactions. The answer remains unclear.

Leukocytes play a central role in ischemia/reperfusion injury because they produce oxygen-derived free radicals that can cause tissue injury.10,11 Some studies showed that the antiradical effect of superoxide dismutase (SOD), which has been commonly used as a free radical scavenger, on the production of oxygen free radicals in ischemia/reperfused rat retina.12 Leukocytes accumulate into tissues through the interactions with vascular endothelial cells and the interactions are mediated by intercellular adhesion molecule-1 (ICAM-1), which are expressed on vascular endothelium.13 We previously demonstrated in retina that the inhibition of ICAM-1 leads to decrease the leukocyte–endothelial interactions and ischemia/reperfusion injury.14

The retinal microcirculation can be observed noninvasively, because the optic media, which consists of the cornea, lens, vitreous body, and retina, are transparent. In addition, every retina has an equal, limited, and relatively flat area. Therefore, the retinal microcirculation is suitable for quantitative analysis. In the present study, we evaluated the pathogenesis of hypertension and its effect on leukocyte dynamics, ICAM-1 expression, and retinal damage after transient retinal ischemia. In addition, we examined the effect of SOD on the retinal ischemia/reperfusion injury in SHR.

Methods

Animal Model
Male SHR (12 weeks old; n=78) and age-matched male normotensive WKY (n=78) were obtained from Japan SLC (Hamamatsu, Japan). Thirty-six rats from each strain were used for the retinal vessel diameters, blood pressures, leukocyte count in peripheral

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blood, and retinal flat-mount experiments. Twenty-four rats from each strain were used for ICAM-1 expression evaluation. Eighteen rats from each strain were used for histological procedures. Each rat was anesthetized with xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg), as previously described. Transient retinal ischemia for 60 minutes was induced in the right eye of each rat by ligation of optic sheath, as previously described.

Because the pharmacological half-life (6 to 10 minutes) of SOD is relatively short, SOD conjugated to polyethylene glycol (PEG-SOD; Sigma, St Louis, Mo) was used in our experimental protocols, because it has a pharmacological half-life of 30 hours. The rats were treated with 15 000 U/kg of PEG-SOD intravenously 5 minutes before the induction of reperfusion. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Evaluation of Blood Pressures, Major Retinal Vessel Diameters, and Leukocyte Count in Peripheral Blood**

Each rat was anesthetized and the pupil of the right eye was dilated, as previously described. Systolic blood pressure was monitored at each time point in the tail of each rat by means of a sensing cuff attached to a blood pressure analyzer (IITC, Woodland Hills, Calif). Peripheral blood sample was analyzed by a hematology analyzer (ERMA, Tokyo, Japan).

**Flat-Mount Preparation for Evaluation of Leukocyte Accumulation**

After the evaluation of major retinal vessel diameters and blood pressures, we evaluated leukocyte accumulation in the ischemia-reperfused retina. Acridine orange (Wako Pure Chemicals, Osaka, Japan) (0.1% solution in saline) was injected continuously through a catheter for 1 minute at a rate of 1 mL/min. Acridine orange emits green fluorescence when it interacts with DNA; nuclei of circulating leukocytes are thereby stained. Accordingly, a few minutes after acridine orange injection was stopped, the fluorescence of circulating leukocytes was faint because of the washout effect. In contrast, leukocytes that had accumulated in the retina remained fluorescent for 2 hours. At 30 minutes after injection of acridine orange, the retina was carefully removed and a flat-mount was prepared using a mounting medium for fluorescence (Vector Laboratories, Burlingame, Calif). The retina was then examined using a fluorescence microscope. For quantitative analysis, the number of accumulated leukocytes in high-power fields (×200) was counted and expressed as the average number per field (0.50 mm²) in 4 peripapillary areas examined.

**Semiquantification of ICAM-1 Gene Expression by Reverse-Transcription Polymerase Chain Reaction Method**

The eyes were enucleated at 24 hours after reperfusion. The retina was carefully isolated. Polymerase chain reaction was performed as previously described. The primers were AGCCTCAGGCCTAA-GAGGAC (sense) and AGGGTCCCAGAGGGTCTA (antisense) for ICAM-1, and GCCATCTGACCGAAGTGA (sense) and GGCATCTGCTTGCTGAACTG (antisense) for β-actin, which was used as the internal standard.

**Enzyme-Linked Immunosorbent Assay for ICAM-1**

The eyes were enucleated at 48 hours after reperfusion and enzyme-linked immunosorbent assay was performed. The retina was carefully isolated, placed in 150 μL of lysis buffer (20% glycerol, 10 mmol/L KCl, 1 mmol/L MgCl₂, 0.1% Triton, 300 mmol/L NaCl, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, 20 mmol/L HEPES [pH 7.9]) and homogenized. The lysate was centrifuged at 14 000 rpm for 15 minutes at 4°C, and the ICAM-1 levels in the supernatant were determined with the ICAM-1 kit (Quantikine; R&D Systems, Minneapolis, Minn) according to the manufacturer’s protocol. Total protein was determined using the bicinchoninic acid (BCA) kit (Bio Rad, Hercules, Calif) and was used to normalize the ICAM-1 protein levels.

**Histological Procedures**

To investigate the effects of hypertension and the protective effect of SOD on retinal ischemia/reperfusion injury, we performed a quantitative histological analysis. After 14 days of reperfusion, the eyes were collected and stained with hematoxylin and eosin, and then the thickness of the overall retina was measured as previously described.

**Statistical Analysis**

Data are reported as the mean ± SEM. The data were analyzed by 1-way ANOVA, using a post hoc test with Fisher protected least-significance procedure. Unpaired t-tests were used to compare results between groups at matched follow-up time points after reperfusion. Differences are considered to be statistically significant when the P values are <0.05.

**Results**

**Blood Pressures and Leukocyte Counts in Peripheral Blood**

The Table indicates the physiological variables for both rat strains used in these experiments. The systolic blood pressures were significantly higher in SHR than in WKY (P<0.0001) throughout the course of the ischemia/reperfusion injury. The leukocyte counts in the peripheral blood were significantly lower at 4 and 48 hours after reperfusion in SHR than in WKY (P<0.05); however, no significant differences of the leukocyte counts between both strains were found in the control, at 12, 24, and 72 hours after reperfusion.

<table>
<thead>
<tr>
<th>Parameters Control</th>
<th>4 Hours</th>
<th>12 Hours</th>
<th>24 Hours</th>
<th>48 Hours</th>
<th>72 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mm Hg)</td>
<td>115.8±6.8</td>
<td>83.5±15</td>
<td>118.5±7.2</td>
<td>117.5±4.0</td>
<td>120.3±4.7</td>
</tr>
<tr>
<td>WBC (×10³/μL)</td>
<td>7.8±1.5</td>
<td>10.6±1.3</td>
<td>10.8±1.6</td>
<td>9.7±0.7</td>
<td>11.9±1.3</td>
</tr>
<tr>
<td>SHR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>189.3±6.1*</td>
<td>170.2±14*</td>
<td>177.3±12*</td>
<td>196.7±5.7*</td>
<td>212.3±7.2*</td>
</tr>
<tr>
<td>WBC (×10³/μL)</td>
<td>8.0±0.7</td>
<td>7.2±0.7†</td>
<td>10.5±1.0</td>
<td>10.2±0.9</td>
<td>7.7±0.4†</td>
</tr>
</tbody>
</table>

Values are mean±SEM. WKY indicates Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; SBP, systolic blood pressure; WBC, peripheral leukocyte count.

‡P<0.01 vs WKY values at each time point; †P<0.05 vs WKY values at each time point.
Major Retinal Vessel Diameters

Figure 1 indicates changes of major retinal vessel diameters in the rats at various time points after reperfusion. The diameters of major retinal arteries and veins of SHR were smaller at all time points compared with those of WKY. In arteries, slight vasoconstriction occurred gradually after reperfusion in both strains, reaching 0.85-fold (not significant) in WKY and 0.88-fold (not significant) in SHR at its minimum, compared with those of controls. In contrast, venous dilation was observed after reperfusion, reaching 1.4-fold in WKY ($P_{0.0008}$) and 1.5-fold in SHR ($P_{0.0007}$) at its maximum, compared with those of controls.

Leukocyte Accumulation

Figure 2 shows the leukocytes accumulated in the retina after transient ischemia. In control rats, only a few leukocytes were recognized in the retina, whereas accumulated leukocytes increased in the retina after transient ischemia. Figure 3 indicates changes in the number of leukocytes accumulated in the retina. The number of accumulated leukocytes peaked at 12 hours after reperfusion in WKY and at 48 hours after reperfusion in SHR. The number of accumulated leukocytes at 48 hours after reperfusion in SHR increased by 1.6-fold ($P_{0.033}$) compared with that in the WKY at 12 hours after reperfusion, and by 5.2-fold ($P_{0.0007}$) compared with that in the WKY at 48 hours after reperfusion.

The number of accumulated leukocytes in SOD-treated SHR and WKY at 48 hours after reperfusion was 45.5% ($P_{0.0071}$) and 94.7% (not significant), respectively, of that in the vehicle-treated rats of each strain at 48 hours after reperfusion. The data demonstrated significant protection effect of SOD on the leukocyte accumulation after transient ischemia in SHR.

Gene Expression and Protein Levels of ICAM-1 in the Retina

The levels of ICAM-1 mRNA expression in retinal ischemia/reperfusion-induced rats of both strains and in sham-operated SHR were evaluated as a ratio of the average values of sham-operated WKY. There were no significant differences in ICAM-1 mRNA expression between sham-operated WKY and SHR. In contrast, ICAM-1 mRNA expression in the
Retinas of the SHR at 24 hours after reperfusion was increased by 2.1-fold \((P<0.0136)\) compared with those of the sham-operated SHR, and it was increased by 2.9-fold \((P=0.0008)\) compared with those of WKY at 24 hours after reperfusion. This finding was substantiated by enzyme-linked immunosorbent assay. The ICAM-1 protein level in the vehicle-treated SHR at 48 hours after reperfusion was upregulated to 2.39-fold \((P<0.0001)\) of the sham-operated SHR and upregulated to 1.15-fold \((P=0.0157)\) of the WKY at 48 hours after reperfusion.

**Histological Study**

Histological examination showed differences of the thickness of the retinas among the 3 groups (sham-operated, vehicle-treated, and SOD-treated rats) in both strains (Figure 4). There were no significant differences in thickness of sham-operated retinas between each strain. The thickness of the retinas in the vehicle-treated SHR was significantly reduced to 72.0% \((P=0.0012)\) of that in the sham-operated SHR and 84.3% \((P=0.0084)\) of that in the vehicle-treated WKY. The data demonstrated significant greater ischemia-reperfused retinal damage in the vehicle-treated SHR. With treatment by SOD, however, structure of the postischemic retinas was preserved in SHR. The thickness of the retinas in SOD-treated SHR was 85.1% (not significant) of that in the sham-operated SHR and 118.2% \((P=0.018)\) of that in the vehicle-treated SHR. There were no significant differences in thickness of SOD-treated retinas between each strain. The data demonstrated significant protection effect of SOD on retinal damage after transient ischemia in SHR.

**Discussion**

Leukocytes play an important role in the pathogenesis of many inflammatory conditions. In the present study, we quantitatively evaluated the effects of hypertension in vivo on leukocyte accumulation and retinal damage after transient retinal ischemia. We demonstrated that the number of leukocytes accumulated in the retinas was significantly increased and that ischemia/reperfusion-induced retinal damage is more severe in SHR. Experimental studies have shown that inflammatory damage to tissues can be reduced by preventing leukocyte participation.\(^{14}\) Accordingly, increased leukocyte accumulation in the retinas of SHR compared with that of WKY might cause significantly more ischemia/reperfusion-induced retinal damage in SHR.

Previous studies have shown that the level of ICAM-1 expression is significantly higher in the microvasculature of SHR than in that of WKY.\(^{20}\) Our findings also showed that ICAM-1 mRNA expression and ICAM-1 protein level in the retinas after transient ischemia of SHR was increased compared with those of WKY. In addition, ICAM-1 has been reported to play an important role in leukocyte–endothelial cell interactions during ischemia/reperfusion, and that inhibition of the adhesion molecule markedly decreases leukocyte accumulation and retinal damage subsequent to transient ischemia.\(^{14}\) Therefore, increased ICAM-1 mRNA expression and ICAM-1 protein level in the retinas of SHR might lead to increased leukocyte accumulation in the retinas of SHR compared with the one seen in WKY.

A significant amount of NO produced by SHR may be consumed by enhanced superoxide anion.\(^{21}\) Both leukocyte accumulation and the production of oxygen-free radicals may contribute to tissue injury after transient ischemia.\(^{22,23}\) Schmid-Schonbein et al\(^{24}\) have shown that organ injury in SHR might be related to a greater proportion of spontane-
ously activated granulocytes and an increased spontaneous production of xanthine oxidase-derived oxidants in the microvasculature. Endogenous scavenging mechanisms may thus be overwhelmed by a burst of radical production during reperfusion or reoxygenation.

In this study, with treatment by SOD, retinal damage during ischemia/reperfusion injury was reduced in SHR. SOD is known to play an important role as a free-radical scavenger and can lead to a reduction of leukocyte migration after transient ischemia, and thereby can prevent the production of oxygen free radicals by leukocytes, especially in SHR. Szabo et al. showed the effects of SOD on ion shifts after retinal transient ischemia in SHR. They reported that oxidative stress is one possible factor involved in the pathogenesis of retinal ischemia/reperfusion damage in SHR, and SOD reduced the retinal reperfusion-induced ionic imbalance. From these results and our results, it is clear that retinal injury after transient ischemia in SHR is closely related to oxidative stress and can be reduced by treatment with SOD.

In addition, nitric oxide has been reported to play an important role in vasodilation. Our current data show that the diameters of major retinal arteries and veins of SHR are smaller than those of WKY after transient ischemia. These findings might be explained by reduced bioavailability of nitric oxide-derived from the endothelial cells in SHR.

Perspectives
We demonstrated that SHR show increased leukocyte accumulation in the retinas and greater retinal damage after transient ischemia compared with WKY. These findings may be based partially on oxidative stress produced by leukocytes accumulated in the postischemic retina. These considerations warrant further investigation to develop a novel therapy for the many ocular ischemic diseases associated with hypertension by focusing not only on blood pressure control but also on oxidative stress. In addition, because the retina is part of the central nervous system, we may be able to extrapolate the results of the present study for the benefit of future studies of postischemic brain injury.

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References
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