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# Protective effect of polyethylene glycol-superoxide dismutase on leukocyte dynamics in rat retinal microcirculation under lipid hydroperoxide-induced oxidative stress

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**Abstract**

The levels of lipid hydroperoxide (LHPs) in vitreous are elevated in a variety of retinal disorders. Recently, we have shown that increased levels of LHPs in the vitreous enhanced leukocyte-endothelium interaction in the retina, which should contribute to the initial disturbance of the retinal microcirculation. Based upon the previous work, the purpose of the present study was to investigate the effect of polyethylene glycol-superoxide dismutase (PEG-SOD), one of the important enzyme antioxidants, on leukocyte-endothelial interaction in the retinal microcirculation under LHP-induced oxidative stress. Male Brown-Norway rats weighing approximately 250 g were used. LHP(18:2) was made from linoleic acid (LA) with lipoxygenase and 10 μg of LHP dissolved in 5 μl of sodium borate buffer (SBB, 0.02 M) was slowly injected into the vitreous using a 33-gauge needle. PEG-SOD (5000 units/kg) was given intravenously 5 min before LHP injection. At 2, 4, 6, 12, 24 and 48 hr after the vitreous injections, we evaluated the number of rolling leukocytes along the major retinal veins and the number of leukocytes that accumulated in the retinal microvasculature with acridine orange digital fluorography. In LHP-treated rats, leukocyte rolling along the major retinal veins was maximal at 6 hr after LHP injection. The number of rolling leukocytes in the PEG-SOD-treated rats was decreased to 5.5% of those in the LHP-treated rats at 6 hr after LHP injection ($P<0.01$). No rolling leukocytes were observed in either control or vehicle-treated eyes. The number of accumulated leukocytes in LHP-treated eyes started to increase at 12 hr, and peaked at 24 hr which was significantly higher than in both control and vehicle-treated eyes ($P<0.01$). The number of accumulated leukocytes in the PEG-SOD-treated rats was reduced by 88.0% at 24 hr ($P<0.01$). Intravenous injection of PEG-SOD significantly inhibited the leukocyte rolling and its accumulation under LHP-induced oxidative stress. These results suggest that PEG-SOD might attenuate various retinal microcirculatory disorders associated with LHP.


*Keywords:* lipid polyethylene-hydroperoxide; leukocyte; glycol-superoxide dismutase; retinal microcirculation

## 1. Introduction

Free radical and lipid peroxide formation, which can cause oxidative stress-induced damage to cell membranes, are initiated by various factors. As the eye is always exposed to initiators such as oxygen, light, ultraviolet ray and x-irradiation, the relationship between free radicals and ocular diseases has attracted much attention. Many studies have suggested important roles of free radicals and lipid peroxides in various ocular diseases including keratitis, cataract, uveitis, retinal degeneration, diabetic retinopathy, retinopathy of prematurity and retinal ischemic diseases (Lakatos et al., 1982; Sery and Petrillo, 1984; Bhuyan et al., 1986; Armstrong et al., 1992; Alio et al., 1995; Spaide et al., 1999).

The retina contains a high proportion of polyunsaturated fatty acids, which are susceptible to lipid peroxidation.

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Lipid hydroperoxide (LHPs) derived from oxidized unsaturated fatty acids are prominent intermediates of propagative reactions induced by activated species such as hydroxyl radical, lipid oxyl or peroxyl radicals, singlet oxygen, and peroxynitrite (Girotti, 1998). It is also known that lipid peroxidation injury to the endothelial cell membrane provides a signal or serves as a marker that can be recognized by circulating polymorphonuclear leukocytes (Del Maestro et al., 1981). Patel et al. (1991) reported that reactive oxygen species like $H_2O_2$ and LHP increased the expression of P-selectin and the adherence of neutrophils to endothelial cells in vitro. Recently, we have shown that oxidative stress induced by 18:2 LHP injection in the vitreous enhanced leukocyte-endothelium interaction in the retinal microcirculation in vivo using acridine orange digital fluorography (Tamai et al., 2002).

LHP is associated with a variety of retinal disorders such as diabetic retinopathy (Armstrong et al., 1992; Augustin et al., 1993), Eales' disease (Bhooma et al., 1997), proliferative vitreoretinopathy (Boker et al., 1994), retinopathy of prematurity (Lakatos et al., 1982), and age-related macular degeneration (Spaide et al., 1999). In fact, in the vitreous samples from patients with proliferative diabetic retinopathy (Augustin et al., 1993; Verdejo et al., 1999) or proliferative vitreoretinopathy (Boker et al., 1994; Verdejo et al., 1999), LHP levels were shown to be significantly elevated. In those patients, antioxidant activity was reduced compared with normal counterparts (Verdejo et al., 1999). It was demonstrated that levels of superoxide dismutase (SOD), one of the important enzyme antioxidants were notably reduced in diabetic patients (Kernell et al., 1992) and patients with Eales' disease which induces retinal vascular occlusion, inflammation and neovascularization (Sulochana et al., 1999). Therefore, we hypothesize that increasing the levels of SOD may reduce retinal microcirculatory disorders associated with LHP. In this study, we have investigated the effect of intravenous injection of SOD on rat retinal microcirculatory disorders under LHP-induced oxidative stress in terms of leukocyte dynamics in vivo. We used here polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) that has a longer half-life in plasma ($>30$ hr) than native SOD and has been proven to be effective against ischemic conditions (Pyatak et al., 1980; Tamura et al., 1988; Chi et al., 1989).

## 2. Materials and methods

### 2.1. Animal model

Male Brown–Norway rats weighing approximately 250 g were used. Only one eye of each rat was used. Rats were anesthetized with a mixture (1:1) of xylazine hydrochroride (4 mg/kg) and ketamine hydrochroride (10 mg/kg). The pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochroride. LHP (18:2) was made from linoleic acid (LA) with lipoxygenase and 10 μg of LHP dissolved in 5 μl of sodium borate buffer (SBB, 0.02 M) was slowly injected into the vitreous using a 33-gauge needle (Browne and Armstrong, 2002). Vehicle-treated rats were given the same amount of LA dissolved in 5 μl of SBB. PEG-SOD-treated rats were injected intravenously with 5000 units/kg of PEG-SOD (Sigma-Aldrich) 5 min before LHP injection. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### 2.2. Acridine orange digital fluorography

Acridine orange digital fluorography was performed as previously described (Kimura et al., 1995; Nishiwaki et al., 1995). In this technique, a scanning laser ophthalmoscope (Rodenstock Instrument), coupled with a computer-assisted image analysis system, makes continuous high-resolution images of the fundus stained with the metachromatic fluorochrome, acridine orange (AO; Wako Pure Chemical), which emits a green fluorescence when it interacts with DNA. The spectral properties of AO-DNA complexes are very similar to those of sodium fluorescein, with an excitation maximum at 502 nm and an emission maximum at 522 nm (Darzynkiewicz and Kapuscinsky, 1990). An argon blue laser was used as the illumination source, with a regular emission filter for fluorescein angiography.

Immediately before acridine orange digital fluorography, rats were anesthetized, and the pupils were dilated. A contact lens was used to retain corneal clarity throughout the experiment. Each rat had a catheter inserted into the tail vein, and was placed on a stereotaxic platform. Body temperature was maintained at $38 \pm 0.5$ °C. Immediately after AO (0.1% solution in saline) solution was infused intravenously, leukocytes stained selectively among circulating blood cells were observed with the scanning laser ophthalmoscope. Nuclei of vascular endothelial cells were also stained. AO was injected continuously through the catheter for 1 min at a rate of 1 ml/min. The fundus was observed to evaluate the leukocyte dynamics in the retinal microcirculation for 5 min after AO injection in a 40° field. AO easily infiltrates through vessel walls and diffuses into the retina due to its membrane permeability. Accordingly, a few minutes after AO injection was completed, fluorescence of circulating leukocytes was faint, due to washout. In contrast, leukocytes that had been trapped in the retinal microcirculation remained fluorescent for approximately 2 hr, being recognized as distinct fluorescent dots 30 min after AO injection. At 30 min after the injection of AO, the fundus was observed again to determine leukocyte accumulation in the retinal microcirculation. The obtained images were recorded on digital videotape at a rate of 30 frames/sec for further image analysis. After the experiment, rats were killed with an anesthetic overdose, and the eye was enucleated to determine a calibration factor with which to