High oxygen tension constricts epineurial arterioles of the rat sciatic nerve via reactive oxygen species

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Submitted 30 October 2006; accepted in final form 17 May 2007

Sakai N, Mizuno R, Ono N, Kato H, Ohhashi T. High oxygen tension constricts epineurial arterioles of the rat sciatic nerve via reactive oxygen species. Am J Physiol Heart Circ Physiol 293: H1498–H1507, 2007. First published May 18, 2007; doi:10.1152/ajpheart.01190.2006.—Microcirculation of the sheath of the sciatic nerve fiber was investigated by using an intravital microscope, and changes in the diameter of the epineurial arterioles in response to highly oxygenated Krebs-bicarbonate solution were evaluated. Superfusion of low-oxygen (0%) Krebs-bicarbonate solution (LKS) onto rat sciatic nerves did not affect changes in the diameter of the arterioles. Nifedipine, a Ca2⁺-channel blocker, caused a dose-dependent dilation of the epineurial arterioles in LKS. In contrast, superfusion of high-oxygen (21%) Krebs-bicarbonate solution (HKS) onto rat sciatic nerves significantly constricted the epineurial arterioles in a time-dependent manner. The HKS-induced constriction of the epineurial arterioles was significantly reduced by treatment with 120 U/ml superoxide dismutase (SOD) alone or 5,000 U/ml catalase alone. In the presence of 120 U/ml SOD plus 5,000 U/ml catalase, 10⁻⁴ M tempol, 10⁻⁶ M diphenyleneiodium, 2 × 10⁻⁴ M apocynin, or 10⁻⁶ M allopurinol, the HKS-induced constriction of the epineurial arterioles completely disappeared. These results suggest that superfusion of highly oxygenated solution onto rat sciatic nerves constricts the epineurial arterioles through reactive oxygen species (ROS), including superoxide and hydrogen peroxide, and that production of superoxide involves a NADPH oxidase- or xanthine oxidase-dependent pathway. In conclusion, ROS play significant roles in the regulation of microcirculation of rat sciatic nerves in vivo.

BLOOD SUPPLY TO PERIPHERAL nerves is important to maintain the functions of the nerve fibers. The disturbance of blood circulation within sciatic nerves produces dysfunction such as suppression of motor nerve conduction velocity (2, 3). It has become clear that pathogenesis of diabetic neuropathy is associated with a reduction of blood flow in sciatic nerves in diabetic animals and humans (14, 34). Thus understanding the regulation of blood flow in sciatic nerves is quite important for diagnosis and treatment of diabetic neuropathy.

Laser Doppler flowmetry and hydrogen clearance are methods available to study the blood flow in sciatic nerves in vivo (2–4, 16, 17, 30, 33, 35, 46). From the use of these methods, it is known that the epineurial and endoneurial arterioles of sciatic nerves play crucial roles in the regulation of microcirculation. Although these methods are capable of measuring local blood flow in the sheath of sciatic nerves in vivo, changes in the diameter of the arterioles in the sheath of sciatic nerves could not be identified. It is necessary to measure changes in the diameter of the arterioles in vivo, because the arterioles and small arteries are resistant vessels, and changes in the diameter of the arterioles determine local blood flow in organs and tissues (6). There is, however, little information investigating changes in the diameter of the sheath of sciatic nerve arterioles in vivo by using an intravital microscope.

It has been reported that reduction of ACh-mediated nitric oxide (NO)-dependent dilation of epineurial arterioles (33) and reactive oxygen species (ROS)-mediated decrease in blood flow of sciatic nerves were observed in streptozotocin-induced diabetic rats (2, 4). These results suggest that ROS may contribute to the regulation of blood flow in the microcirculation of sciatic nerves. Thus the purpose of the present study was first to measure changes in the diameters of the epineurial arterioles of rat sciatic nerves in vivo by using an intravital microscope. Then we investigated the effects of ROS, induced by superfusion with highly oxygenated solution, on changes in the diameter of the epineurial arterioles of rat sciatic nerves in vivo.

MATERIALS AND METHODS

Animals. Seven-week-old male Wistar rats (n = 78; SLC Japan) were used for the present study. The rats were housed in an environment controlled vivarium and were fed a standard-pellet diet and water ad libitum. The Animal Ethics Committee, Shinshu University School of Medicine, in accordance with the principles and guidelines of the Japanese Physiological Society, approved all experimental protocols.

Surgical procedures. The rats were anesthetized with a subcutaneous injection containing a mixture of 2% a-choralose and 10% urethane (0.7 ml/100 g body wt) for the in vivo experiments (24, 31, 45). Additional anesthetics were administered (0.1 ml) when the rats showed body movement, hyperventilation, or increase in arterial blood pressure. The rats were under spontaneous breathing conditions with air throughout the experiments. To measure arterial blood pressure, the left femoral artery was cannulated with an elongated polyethylene tubing (no. 15; Igarashi) that was connected to a pressure transducer (Becton Dickinson) and an amplifier (6M52; Sanei). Changes in arterial blood pressure during experiments were monitored with a MacLab data-acquisition system (ADInstruments) and a personal computer (Power Macintosh 8500/1200; Apple) (23, 31, 45). To visualize the right sciatic nerves of the rats, skin incisions were made in the right thigh, and the bundle of gluteus maximus muscle was gently opened, similar to the methods of other investigators (35). Connective tissues around the sciatic nerve were carefully removed by using microsurgical instruments and wet-cotton swabs under a dissecting microscope (SX-40; Olympus). After these procedures, rats were placed on the stage of an intravital microscope (E600; Nikon).

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with a warm heating pad maintaining the body temperature (36–37°C). Experiments were terminated if mean arterial blood pressure fell below 80 mmHg, and data from these rats were discarded from analyses.

**Observation of rat sciatic nerve microcirculation.** We measured changes in the diameter of the epineurial arterioles of rat sciatic nerves (26 ± 7 μm; n = 78). The images of the epineurial arterioles were obtained through a water-immersion objective lens (×10; Nikon), a photo-eye lens (×2.5; Nikon), and a monochrome chilled charge-coupled device camera (C5985; Hamamatsu Photonics). The images were also recorded on a videocassette recorder (BR-S800; Victor) through a video timer (VTG-33; FOR-A) and were displayed on a television monitor (Trinitron Super Pitch; Sony). A video caliper (26) enabled us to measure changes in the diameter of epineurial arterioles of sciatic nerves.

In the present study, we used Krebs-bicarbonate solution (pH 7.40 ± 0.01 and 37°C) containing different concentrations of oxygen (low = 0% and high = 21%) for superfusion onto the sciatic nerves of rats. Low-oxygen Krebs-bicarbonate solution (LKS) and high-oxygen Krebs-bicarbonate solution (HKS) were bubbled with gas mixtures of 5% CO2–95% N2 and 21% O2–5% CO2–74% N2, respectively. The P02 values of the superfusion solution were measured with an oxygen electrode and an isolated dissolved oxygen meter (ISO2; World Precision Instruments). The P02 values of LKS and HKS were ~5 and ~140 mmHg, respectively, under the present experimental conditions. The Krebs-bicarbonate solution, containing (in mM) 120.0 NaCl, 5.9 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 NaH2PO4, 5.5 glucose, and 25.0 NaHCO3, was superfused over the sciatic nerve. The rate of superfusion was maintained at 6 ml/min throughout the experiments. Experimental protocols were made after equilibration for 30 min.

In the first protocol, to investigate vasoreactivity of the epineurial arterioles of sciatic nerves in response to vasconstrictor and vasodilator, the effects of norepinephrine (NE; 10−9–10−6 M) or nifedipine (a Ca2⁺-channel blocker; 10−8–10−5 M) on changes in the diameter of epineurial arterioles were studied in the presence of LKS. Each concentration of NE or nifedipine was superfused onto sciatic nerves until the maximum response was obtained.

In the second protocol, the effects of repeated superfusions of HKS onto the sciatic nerve on changes in the diameter of epineurial arterioles were studied for 10 min.

In the third protocol, the effects of superoxide dismutase (SOD; 120 U/ml) plus catalase (5,000 U/ml) on changes in the diameter of epineurial arterioles were studied under the condition of LKS for 10 min. The concentration of SOD plus catalase used in the present study was effective in significantly reducing ROS-mediated responses (45).

In the fourth protocol, the HKS-induced constrictions of the epineurial arterioles were studied in the absence and presence of SOD (120 U/ml) alone, catalase alone (5,000 U/ml), SOD (120 U/ml) plus catalase (5,000 U/ml), tempol (a cell membrane-permeable superoxide scavenger; 10−5 M) (8), inhibitors of NADPH oxidase (dimethylphenylenediamine (DPI; 10−6 M) or apocynin (2 × 10−4 M)) (15, 27), or allopurinol (a xanthine oxidase inhibitor; 10−4 M) (12) for 10 min. Sciatic nerves were pretreated with each drug for 30 min.

In the fifth protocol, the effects of Nα-nitro-l-arginine methyl ester (l-NAME, an inhibitor of NO synthase; 10−6–3 × 10−5 M) on changes in the diameter of epineurial arterioles were studied under LKS conditions. Additionally, in the presence of l-NAME (10−5 M), the effects of S-nitroso-N-acetylpenicillamine (SNAP, an NO donor; 10−9–10−6 M) on changes in the diameter of epineurial arterioles were studied under LKS conditions. Each concentration of l-NAME or SNAP was superfused onto sciatic nerves until the maximum response was obtained.

In the final protocol, the HKS-mediated responses of epineurial arterioles were studied before and after treatment with 10−5 M l-NAME, and the 10−5 M l-NAME-mediated responses of epineurial arterioles were also studied before and after the treatment with HKS. Additionally, in the absence and presence of l-NAME (10−5 M), the effects of SNAP on changes in the diameter of epineurial arterioles were studied under the HKS conditions. Each concentration of l-NAME or SNAP was superfused onto sciatic nerves until the maximum response was obtained.

Superfusion of the sciatic nerves with all the drugs used in the present study did not affect the arterial pressure of the rats.

**Drugs.** All salts (Wako), NE, nifedipine, SOD, catalase, DPI, allopurinol, l-NAME (Sigma), SNAP (Cayman Chemical), tempol (Aldrich), and apocynin (Calbiochem) were used in the present study. Nifedipine was dissolved with ethanol as a stock solution. DPI, apocynin, allopurinol, SNAP, and tempol were dissolved with DMSO as a stock solution. The stock solutions and other drugs were directly diluted with Krebs-bicarbonate solution. The concentration of ethanol or DMSO used in the present study did not affect the diameter of sciatic nerve arterioles or blood pressure in rats. Concentrations of the drugs were expressed as final concentration in the superfusion solution. All drugs were prepared on the day of the experiment.

**Data analyses.** Changes in the diameter of the epineurial arterioles of rat sciatic nerves were normalized by the diameter obtained before superfusion at ~5 min and were expressed as a percentage of the diameter. The data are presented as means ± SD, and n indicates the number of preparations. Significant differences (P < 0.05) were determined through the paired Student’s t-test, ANOVA, or repeated-measurement ANOVA followed by the Student-Newman-Keuls post hoc test, as appropriate.

**RESULTS**

Effects of NE or nifedipine on the diameter of epineurial arterioles of sciatic nerves in the presence of LKS. Superfusion of NE (10−9–10−6 M) onto sciatic nerves in the presence of LKS caused a dose-dependent constriction of the epineurial arterioles, and 3 × 10−7 M NE completely constricted the arterioles, causing termination of blood flow. Thus percent diameter changes in the epineurial arterioles in the absence and presence of 3 × 10−7 M NE were 100 ± 0% (n = 4) and 0 ± 0% (n = 4; P < 0.05 vs. absence), respectively (Fig. 1A).

Superfusion of nifedipine (10−8–10−5 M) onto sciatic nerves in the presence of LKS caused a dose-dependent dilatation of the epineurial arterioles, and 10−5 M nifedipine induced the maximum dilator response. Thus percent diameter changes in the epineurial arterioles in the absence and presence of 10−5 M nifedipine were 100 ± 0% (n = 5) and 133 ± 27% (n = 5; P < 0.05 vs. absence), respectively (Fig. 1B). These results indicate that the epineurial arterioles of rat sciatic nerves showed myogenic activity (78 ± 16% of the nifedipine-induced maximum diameter) under the present experimental conditions.

Effects of LKS or HKS on the diameter of epineurial arterioles of sciatic nerves. Figure 2 shows representative images of sciatic nerve epineurial arterioles superfused with LKS (top) or HKS (bottom). The diameter of the epineurial arteriole superfused with LKS did not significantly change for 30 min (Fig. 2A, 0 min and Fig. 2B, ~23 min). On the other hand, superfusion of HKS onto the sciatic nerve caused a marked constriction of the epineurial arteriole (Fig. 2C, 0 min and Fig. 2D, ~30 min).
2D, ~5 min). The HKS-induced vasoconstriction returned to the control diameter after the replacement of LKS. The HKS-induced constriction of the epineurial arterioles was reproducible. Thus Fig. 3 shows summarized data of percent diameter changes in the sciatic nerve epineurial arterioles after repeated superfusions with HKS. Superfusion of HKS onto sciatic nerves caused a time-dependent constriction of the epineurial arterioles, and percent diameter reached a minimum at ~6 min after the superfusion. There were no significant differences in percent diameter changes in the epineurial arterioles between the first (43 ± 29% at 10 min; n = 8) and second (36 ± 25% at 10 min; n = 8) superfusions with HKS.

**Effects of SOD plus catalase on the diameter of epineurial arterioles superfused with LKS.** Superfusion with LKS in the presence of 120 U/ml SOD plus 5,000 U/ml catalase (100 ± 0% at 0 min and 97 ± 5% at 10 min; n = 6; Fig. 4) did not affect percent diameter changes in the sciatic nerve epineurial arterioles even after the treatment with SOD plus catalase for 30 min.

**Effects of SOD alone, catalase alone, SOD plus catalase, or tempol on HKS-induced vasoconstriction of sciatic nerve epineurial arterioles.** In the presence of LKS, there were no significant differences in percent diameter changes in the epineurial arterioles between before (100 ± 0%) and after (108 ± 17%) the treatment with 120 U/ml SOD alone (n = 4). The HKS-induced vasoconstriction of the epineurial arterioles was significantly reduced by the treatment with 120 U/ml SOD alone (Fig. 5A). Thus percent diameter changes in HKS-induced vasoconstriction in the absence and presence of 120 U/ml SOD alone after the superfusion at 10 min were 52 ± 13% (n = 4) and 91 ± 11% (n = 4; P < 0.05 vs. absence), respectively (Fig. 5A).

In the presence of LKS, there were no significant differences in percent diameter changes in the epineurial arterioles be-
epineurial arterioles completely disappeared following treatment with $10^{-6}$ M DPI (Fig. 6A). Thus percent diameter changes in HKS-induced vasoconstriction in the absence and presence of $10^{-6}$ M DPI after the superfusions at 10 min were 54 ± 12% ($n = 4$) and 100 ± 13% ($n = 4$; $P < 0.05$ vs. absence), respectively (Fig. 6A).

In the presence of LKS, there were no significant differences in percent diameter changes in the epineurial arterioles between before (100 ± 0%) and after (115 ± 23%) the treatment with $2 \times 10^{-4}$ M apocynin ($n = 4$). The HKS-induced vasoconstriction of the epineurial arterioles completely disappeared following treatment with $2 \times 10^{-4}$ M apocynin (Fig. 6B). Thus percent diameter changes in the HKS-induced vasoconstriction in the absence and presence of $2 \times 10^{-4}$ M apocynin after the superfusions at 10 min were 40 ± 27% ($n = 4$) and 97 ± 2% ($n = 4$; $P < 0.05$ vs. absence), respectively (Fig. 6B).

In the presence of LKS, there were no significant differences in percent diameter changes in the epineurial arterioles between before (100 ± 0%) and after (111 ± 11%) the treatment with $10^{-6}$ M allopurinol ($n = 4$). The HKS-induced vasoconstriction of the epineurial arterioles was significantly reduced by the treatment with $10^{-6}$ M allopurinol (Fig. 6C). Thus percent diameter changes in HKS-induced vasoconstriction in the absence and presence of $10^{-6}$ M allopurinol after superfusion at 10 min were 35 ± 21% ($n = 4$) and 98 ± 15% ($n = 4$; $P < 0.05$ vs. absence), respectively (Fig. 6C).

Effects of L-NAME on the diameter of epineurial arterioles of sciatic nerves in the presence of LKS. Superfusion of L-NAME ($10^{-6}$–$3 \times 10^{-5}$ M) onto sciatic nerves in the presence of LKS caused a dose-dependent constriction of the epineurial arterioles, and $10^{-5}$ M L-NAME reached the minimum diameter. Thus percent diameter changes in the epineurial arterioles completely disappeared following treatment with $10^{-6}$ M DPI (Fig. 6A). Thus percent diameter changes in HKS-induced vasoconstriction in the absence and presence of $10^{-6}$ M DPI after the superfusions at 10 min were 54 ± 12% ($n = 4$) and 100 ± 13% ($n = 4$; $P < 0.05$ vs. absence), respectively (Fig. 6A).

In the presence of LKS, there were no significant differences in percent diameter changes in the epineurial arterioles between before (100 ± 0%) and after (115 ± 23%) the treatment with $2 \times 10^{-4}$ M apocynin ($n = 4$). The HKS-induced vasoconstriction of the epineurial arterioles completely disappeared following treatment with $2 \times 10^{-4}$ M apocynin (Fig. 6B). Thus percent diameter changes in the HKS-induced vasoconstriction in the absence and presence of $2 \times 10^{-4}$ M apocynin after the superfusions at 10 min were 40 ± 27% ($n = 4$) and 97 ± 2% ($n = 4$; $P < 0.05$ vs. absence), respectively (Fig. 6B).

In the presence of LKS, there were no significant differences in percent diameter changes in the epineurial arterioles between before (100 ± 0%) and after (111 ± 11%) the treatment with $10^{-6}$ M allopurinol ($n = 4$). The HKS-induced vasoconstriction of the epineurial arterioles was significantly reduced by the treatment with $10^{-6}$ M allopurinol (Fig. 6C). Thus percent diameter changes in HKS-induced vasoconstriction in the absence and presence of $10^{-6}$ M allopurinol after superfusion at 10 min were 35 ± 21% ($n = 4$) and 98 ± 15% ($n = 4$; $P < 0.05$ vs. absence), respectively (Fig. 6C).

Effects of DPI, apocynin, or allopurinol on HKS-induced vasoconstriction of sciatic nerve epineurial arterioles. In the presence of LKS, there were no significant differences in percent diameter changes in the epineurial arterioles between before (100 ± 0%) and after (100 ± 3%) the treatment with $10^{-6}$ M DPI ($n = 4$). The HKS-induced vasoconstriction of the epineurial arterioles completely disappeared following treatment with $10^{-6}$ M DPI (Fig. 6A). Thus percent diameter changes in HKS-induced vasoconstriction in the absence and presence of $10^{-6}$ M DPI after the superfusions at 10 min were 54 ± 12% ($n = 4$) and 100 ± 13% ($n = 4$; $P < 0.05$ vs. absence), respectively (Fig. 6A).
ial arterioles in the absence and presence of $10^{-5}$ M 1-NAME were $100 \pm 0\%$ ($n = 5$) and $32 \pm 15\%$ ($n = 5$; $P < 0.05$ vs. absence), respectively (Fig. 7A).

Effects of SNAP on the diameter of epineurial arterioles of sciatic nerves in the presence of 1-NAME under LKS. Superfusion of 1-NAME ($10^{-5}$ M) onto sciatic nerves significantly constricted the epineurial arterioles. Superfusion of SNAP ($10^{-9}$–$10^{-6}$ M) onto sciatic nerves in the presence of 1-NAME under LKS caused a dose-dependent dilation of the epineurial arterioles, and $10^{-6}$ M SNAP returned the 1-NAME-induced constriction to the preconditions. Thus $10^{-6}$ M SNAP-induced percent diameters of the epineurial arterioles in the presence of 1-NAME under LKS were $114 \pm 9\%$ ($n = 4$; $P < 0.05$ vs. before SNAP in the presence of 1-NAME; $40 \pm 10\%$; Fig. 7B).

Effects of 1-NAME on the diameter of epineurial arterioles of sciatic nerves in the presence of HKS. Figure 8A shows summarized data of percent diameter changes in the epineurial arterioles superfused with $10^{-5}$ M 1-NAME before and after the superfusion with HKS. Superfusion with $10^{-5}$ M 1-NAME caused a significant constriction of the epineurial arterioles in the absence of $10^{-5}$ M 1-NAME. The HKS-induced vasoconstriction of the epineurial arterioles was significantly enhanced by the additional treatment with $10^{-5}$ M 1-NAME. Thus percent diameter changes in HKS-induced vasoconstriction in the absence of 1-NAME, the presence of HKS alone, and HKS plus $10^{-5}$ M 1-NAME were $100 \pm 0\%$ ($n = 5$), $42 \pm 17\%$ ($n = 5$; $P < 0.05$ vs. absence), and $23 \pm 19\%$ ($n = 5$; $P < 0.05$ vs. absence or HKS alone), respectively.

Effects of HKS on 1-NAME-mediated constriction of epineurial arterioles of sciatic nerves. Figure 8B shows summarized data of percent diameter changes in the epineurial arterioles superfused with $10^{-5}$ M 1-NAME before and after the superfusion with HKS. Superfusion with $10^{-5}$ M 1-NAME caused a significant constriction of the epineurial arterioles in the absence of HKS. The $10^{-5}$ M 1-NAME-induced vasoconstriction of the epineurial arterioles was significantly enhanced by the additional treatment with HKS. Thus percent diameter changes in 1-NAME-induced vasoconstriction in the absence of L-NAME, the presence of 1-NAME alone, and the presence of 1-NAME plus HKS were $100 \pm 0\%$ ($n = 4$), $56 \pm 11\%$ ($n = 4$; $P < 0.05$ vs. absence), and $26 \pm 20\%$ ($n = 4$; $P < 0.05$ vs. absence or 1-NAME alone), respectively.
Effects of SNAP on the diameter of epineurial arterioles of sciatic nerves in the absence or presence of L-NAME under HKS. In Fig. 9A, superfusion of HKS onto sciatic nerves significantly constricted the epineurial arterioles. Superfusion of SNAP (10^{-9}–10^{-6} M) onto sciatic nerves in the presence of HKS caused a dose-dependent dilation of the epineurial arterioles, and 10^{-6} M SNAP returned the HKS-induced constriction to the pre-HKS conditions. Thus 10^{-6} M SNAP-induced percent diameters of the epineurial arterioles in the presence of HKS were 103 ± 19% (n = 5; P < 0.05 vs. before SNAP in the presence of HKS; 39 ± 2%; Fig. 9A).

In Fig. 9B, superfusion of HKS onto sciatic nerves significantly constricted the epineurial arterioles, and the additional treatment with 10^{-5} M L-NAME enhanced the HKS-mediated constriction. Superfusion of SNAP (10^{-9}–10^{-5} M) onto sciatic nerves in the presence of 10^{-5} M L-NAME under HKS caused a dose-dependent dilation of the epineurial arterioles, and 10^{-5} M SNAP returned the L-NAME-induced constriction under HKS to the pre-HKS conditions. Thus 10^{-5} M SNAP-induced percent diameters of the epineurial arterioles in the presence of 10^{-5} M L-NAME under HKS were 98 ± 3% (n = 4; P < 0.05 vs. before SNAP in the presence of L-NAME under HKS; 17 ± 24%; Fig. 9B).

DISCUSSION

The major findings in the present study are as follows: 1) superfusion of LKS onto rat sciatic nerves did not affect changes in the diameter of the arterioles; 2) superfusion of nifedipine, a Ca^{2+}-channel blocker, onto rat sciatic nerves in the presence of LKS significantly dilated the epineurial arterioles, indicating that the epineurial arterioles showed myogenic activity in vivo; 3) superfusion of HKS onto sciatic nerves for 10 min caused a marked constriction of the epineurial arterioles, and the HKS-mediated responses were reproducible; 4) in the presence of LKS, superfusion of SOD plus...
catalase onto sciatic nerves did not affect the vasoactivity of the epineurial arterioles; 5) in the presence of SOD alone or catalase alone, the HKS-induced vasoconstriction of the epineurial arterioles was significantly reduced; 6) the HKS-induced vasoconstriction of the epineurial arterioles also completely disappeared following treatment with SOD plus catalase; and 7) treatment with DPI, apocynin, or allopurinol caused a significant reduction of the HKS-induced vasoconstriction of the epineurial arterioles. These results suggest that superfusion of high-oxygen solution onto rat sciatic nerves constricts the epineurial arterioles through ROS, including superoxide and hydrogen peroxide, and that production of superoxide involves a NADPH oxidase- or xanthine oxidase-dependent pathway, and thus ROS may play significant roles in the regulation of microcirculation of rat sciatic nerves in vivo.

We assume that the present experimental superfusion with high-oxygen solution is similar to clinical environments during surgery on peripheral nerves, because surgical areas are usually exposed to an atmosphere of ~20% O₂. Therefore, reducing oxygen tension around peripheral nerves in surgical areas may be helpful for maintaining microcirculation of the nerves, and low-oxygen treatment of the nerves may facilitate patient recovery from surgical trauma after surgery. Although the present study used two concentrations of oxygen (0% and 21%), further investigation will be needed to evaluate the oxygen concentration-dependent effects on the mechanical activity of sciatic nerve arterioles in vivo.

**Myogenic activity of epineurial arterioles in rat sciatic nerves.** Blood supply to peripheral nerves is one of the important factors in maintaining physiological functions such as motor-nerve conduction velocity. Thus loss of blood supply to peripheral nerves frequently causes dysfunction. Recently, it has become clear that pathogenesis of diabetic neuropathy is associated with a reduction of blood flow in sciatic nerves in diabetic animals and humans (14, 34). Laser Doppler flowmetry and hydrogen clearance are useful methods for investigating hemodynamics of sciatic nerves (2–4, 16, 17, 30, 33, 35, 46). Although these methods have the advantage of directly measuring local blood flow of peripheral nerves, mechanical activity such as changes in diameter of the epineurial arterioles could not be identified through in vivo experiments. In the present study, intravital microscopic observation enabled us to measure changes in the diameter of the epineurial arterioles of rat sciatic nerves in vivo.

It is well known that resistant vessels such as arterioles and small arteries strongly contribute to the regulation of local...
blood flow in tissues and organs. The nature of myogenic activity of the resistant vessels plays a significant role in maintaining microcirculation in various vascular beds (6). Regulation of myogenic activity in peripheral nerve arterioles, however, is not fully understood. Wang et al. (38) demonstrated that isolated epineurial arterioles of rats (active and passive diameters were 55 ± 6 and 84 ± 6 μm, respectively, at 60 mmHg) show intrinsic myogenic reactivity in vitro. The diameters of sciatic nerve epineurial arterioles used in the present study were smaller (26 ± 7 μm; n = 78) and from a more distal location than those used in the Wang et al. studies (38). In the present study, superfusion of nifedipine (a Ca\(^{2+}\)-channel blocker) onto sciatic nerves in the presence of LKS significantly dilated the epineurial arterioles. These results may suggest that the epineurial arterioles of rat sciatic nerves possess intrinsic myogenic activity in vivo and that vasoactivity may contribute to the regulation of local blood flow in the microcirculation of sciatic nerves as an autoregulatory function.

In vivo studies with a laser Doppler flowmetry and hydrogen clearance also indicate the regulatory mechanisms of local blood flow in sciatic nerves. Thus exogenous application of vasoactive physiological substances onto sciatic nerves strongly affects local blood flow (16, 17, 46). In the present study, superfusion of NE onto sciatic nerves of rats caused a dose-dependent constriction of the epineurial arterioles. A dose of 3 × 10\(^{-7}\) M NE completely constricted the epineurial arterioles and terminated local blood flow in sciatic nerves. The NE-induced responses obtained in the present study were quite compatible with other reports (16, 38). It has been reported that endoneurial arterioles control the blood flow of sciatic nerves as well as the epineurial arterioles (2, 46). In the present study, we could not demonstrate vasoactivity of the endoneurial arterioles in sciatic nerves of rats. Thus further investigation will be needed to evaluate changes in the diameter of endoneurial arterioles of sciatic nerves by using an intravital microscope system and appropriate tracers such as fluorescent dyes.

**HKS-induced vasoconstriction of sciatic nerve epineurial arterioles.** In the present study, we demonstrated that superfusion of HKS onto sciatic nerves of rats caused a marked constriction of the epineurial arterioles and that superfusion of HKS for >15 min caused irreversible disturbance to sciatic nerve microcirculation, including termination of blood flow and blood clotting. On the other hand, superfusion with LKS did not affect the diameters of epineurial arterioles in the present study. Wang et al. (38) used a physiological buffer solution bubbled with 95% N\(_2\)-5% CO\(_2\) to measure in vivo blood flow of rat sciatic nerves with laser Doppler flowmetry, as did the present study, which superfused them with LKS. In a study using hydrogen-clearance methods, other investigators (3) filled the measuring area of sciatic nerves with mineral oil. These results suggested that a low-P(O\(_2\)) environment around sciatic nerves may permit us to measure blood flow and changes in diameter of epineurial arterioles in vivo. In other words, an oxidant stress around sciatic nerves may disrupt normal blood flow in sciatic nerve microcirculation.

In the present study, rats were under spontaneous breathing conditions with air throughout the experiments. Thus P(O\(_2\)) at arterial blood in sciatic nerve microcirculation in vivo may coincide with the physiological range: ~100 mmHg or less as far as existence of arterial blood flow. Superfusion of LKS (~5 mmHg) onto the sciatic nerve only allowed blood flow in sciatic nerve microcirculation. We could not measure P(O\(_2\)) at epineurial arteriolar blood of sciatic nerves in the present study. The present study, however, suggests that P(O\(_2\)) in the extraluminal space of epineurial arterioles has been low, whereas P(O\(_2\)) in the intraluminal space and the arteriolar wall may be maintained under the physiological range due to the existence of arteriolar blood flow. We also speculate that P(O\(_2\)) around sciatic nerves may be lower, because sciatic nerves were tightly surrounded with skeletal muscles and related tissues in vivo.

It is well known that reduction of oxygen supply to organs and tissues greatly affects ATP production in the cells through intracellular respiration. Intracellular concentration of ATP frequently contributes to the regulation of vasomotor activity. Also, ATP or its metabolite such as adenosine released from the cells plays significant roles in vascular functions (37, 43). Thus further investigation will be needed to evaluate involvement of P(O\(_2\))-ATP-dependent mechanisms for regulation of sciatic microcirculation in vivo.

**Involvement of ROS in HKS-induced vasoconstriction of sciatic nerve epineurial arterioles.** It is clear that ROS, including singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radicals, participate in the pathogenesis of many disorders such as inflammation, diabetes mellitus, neurodegenerative disease, and dysfunction of the cardiovascular system, as well as the regulation of physiological functions (7, 32, 40–42). Singlet oxygen is an electronically excited state of oxygen that has an electron in a higher-energy orbital. Singlet oxygen has a very short half life (10\(^{-6}\) s) and is not a radical, but its action as an oxidant is considered to be one of the most damaging among ROS (13, 44). Superoxide is generated by xanthine oxidase in the cytosol and is also formed from mitochondrial electron chains and NADPH oxidase (40–42). Superoxide is promptly changed to hydrogen peroxide by SOD (within ~10\(^{-9}\) s), and subsequently hydrogen peroxide is catalyzed to H\(_2\)O and O\(_2\). It is known that histidine and SOD plus catalase are scavengers of singlet oxygen and superoxide-hydrogen peroxide, respectively. Thus these substances are widely utilized for inhibiting ROS-mediated responses in vivo and in vitro studies.

In the present study, superfusion of HKS onto rat sciatic nerves caused a marked constriction of the epineurial arterioles, and HKS-induced vasoconstriction was ~50% of the maximum diameter. The HKS-induced responses were similar to NE-induced responses (ranging between 3 × 10\(^{-8}\) and 10\(^{-7}\) M). Treatment with SOD alone or catalase alone significantly reduced the HKS-induced constriction of epineurial arterioles. The HKS-induced constriction of epineurial arterioles completely disappeared in the presence of SOD plus catalase. Hydrogen peroxide constricted rat mesenteric (10), skeletal muscle (5), and mouse tail arterioles (25), whereas it dilated human atrial (23) and cat and piglet pial arterioles (18, 39). In rat skeletal muscle arterioles, exogenously administered hydrogen peroxide elicited a biphasic effect on arteriolar diameter, causing constriction at lower and dilation at higher concentrations (5). These results suggest that the mode of hydrogen peroxide-mediated responses may be specific to certain vascular beds and/or concentrations produced.
Treatment with tempol (a cell membrane-permeable superoxide scavenger) completely returned the HKS-induced constriction of epineurial arterioles to the preconstricted level. Furthermore, in the presence of DPI, apocynin, or allopurinol, the HKS-induced constriction of epineurial arterioles completely disappeared. These results suggest that superfusion with the high-oxygen solution used in the present experiments markedly constricts the epineurial arterioles of rat sciatic nerves through superoxide and hydrogen peroxide, that production of superoxide involves a NADPH oxidase- or xanthine oxidase-dependent pathway, and that ROS may play significant roles in the regulation of rat sciatic nerve microcirculation in vivo. For inhibition of NADPH oxidase, we used DPI and apocynin in the present study. Although these flavoproteins have been widely used for the inhibition of NADPH oxidase, nonspecific effects such as an inhibition of xanthine oxidase may be involved. Recently, it has been reported that gp91ds-tat, a synthesized peptide, is useful for specific blocking of NADPH oxidase (29). Thus further investigation will be needed to evaluate the selective effects of gp91ds-tat on the NADPH-mediated ROS-induced responses.

ROS signaling modulates the function of vascular smooth muscles under physiological conditions (20, 40–42). Treatment with SOD alone, catalase alone, SOD plus catalase, tempol, DPI, apocynin, or allopurinol did not affect the epineurial arterioles of sciatic nerves in the presence of LKS. Thus there is no effective basal production of ROS such as superoxide and hydrogen peroxide to constrict the epineurial arterioles in the presence of LKS. On the other hand, in pathophysiological models of streptozotocin-injected diabetic rats, preventing superoxide formation restores vasodilatation of the epineurial arterioles of sciatic nerves (4). In addition, it has been reported that production of hydrogen peroxide in skeletal muscle arterioles of type 2 diabetic mice (db/db mice) is enhanced and increases the basal arteriolar tone (9). Thus it is possible that qualitative and quantitative changes in scavenging ROS of the epineurial arterioles of sciatic nerves may occur during diabetes, disturbing responsiveness of oxygen sensors (NADPH oxidase and xanthine oxidase) to oxygen.

It has been reported that changes in the oxygen tension strongly affect production of endothelium-derived vasoconstrictor and vasodilator substances through NO synthase, cytochrome P-450-dependent enzymes (19, 21, 22, 28) and also alter the bioavailability of these vasoactive substances in microcirculation. Thus it is possible that the HKS-mediated constriction of epineurial arterioles of sciatic nerves may involve the oxygen tension-dependent vasoactive substance-mediated responses.

Rules of endogenous and exogenous NO for epineurial arterioles of sciatic nerves in the presence of LKS or HKS. It is well known that NO is a quite important molecule in the regulation of cardiovascular functions (11, 36) under physiological and pathophysiological conditions, and the interaction between NO and ROS modulates or changes their own functions (20, 40–42). In the present study, superfusion of L-NAME onto sciatic nerves in the presence of LKS caused a dose-dependent constriction of the epineurial arterioles, and $10^{-5}$ M L-NAME reached the minimum diameter. SNAP significantly reduced the L-NAME-induced constriction of the epineurial arterioles in the presence of LKS and then returned to the control condition. These results suggest that epineurial arterioles of sciatic nerves significantly produce endogenous NO and that application of exogenous NO significantly reverses L-NAME-mediated constriction of the epineurial arterioles due to inhibition of endogenous NO production.

To evaluate interaction between NO and ROS in the present study, we studied the effects of L-NAME on the HKS-induced constriction of epineurial arterioles in sciatic nerves and then, conversely, the effects of HKS on the L-NAME-induced constriction of epineurial arterioles in sciatic nerves. The epineurial arterioles superfused with HKS could furthermore constrict after additional treatment with L-NAME (Fig. 8A), suggesting that NO synthase is still in the active state in the presence of HKS. The epineurial arterioles preconstricted with L-NAME could furthermore constrict after additional treatment with HKS (Fig. 8B), suggesting that HKS-induced ROS-mediated constriction still worked after inhibition of NO synthase. In addition, exogenous application of SNAP (an NO donor) completely abolished the HKS- or HKS plus L-NAME-induced constrictions of the epineurial arterioles in the present study (Fig. 9, A and B). Collectively, these results suggest that ROS induced by superfusion with HKS are unlikely to restrain bioavailability of NO synthase and exogenous NO in epineurial arterioles of rat sciatic nerves in vivo.

Grants
This study was supported by Grants-in-Aid for Scientific Research (17500303, 17659406, and 19209044) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

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