Targeted Increase in Cerebral Blood Flow by Transcranial Near-Infrared Laser Irradiation

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Background and Objective: Brain function is highly dependent on cerebral blood flow (CBF). The precise mechanisms by which blood flow is controlled by NIR laser irradiation on the central nervous system (CNS) have not been elucidated. In this study, we examined the effect of 808 nm laser diode irradiation on CBF in mice.

Study Design/Materials and Methods: We examined the effect of NIR irradiation on CBF at three different power densities (0.8, 1.6 and 3.2 W/cm^2) and directly measured nitric oxide (NO) in brain tissue during NIR laser irradiation using an amperometric NO-selective electrode. We also examined the contribution of NO and a neurotransmitter, glutamate, to the regulation of CBF by using a nitric oxide synthase (NOS) inhibitor, N^g-nitro-L-arginine methyl ester hydrochloride (L-NAME), and an *N*-methyl-D-aspartate (NMDA) receptor blocker, MK-801, respectively. We examined the change in brain tissue temperature during NIR laser irradiation. We also investigated the protection effect of NIR laser irradiation on transient cerebral ischemia using transient bilateral common carotid artery occlusion (BCCAO) in mice.

Results: We showed that NIR laser irradiation (1.6 W/cm^2 for 15–45 minutes) increased local CBF by 30% compared to that in control mice. NIR laser irradiation also induced a significant increase in cerebral NO concentration. In mice that received L-NAME, NIR laser irradiation did not induce any increase in CBF. Mice administered MK-801 showed an immediate increase but did not show a delayed additional increase in local CBF. The increase in brain tissue temperature induced by laser irradiation was estimated to be as low as 0.8°C at 1.6 W/cm^2, indicating that the heating effect is not a main mechanism of the CBF increase in this condition. Pretreatment with NIR laser irradiation improved residual CBF and reduced the numbers of apoptotic cells in the hippocampus.

Conclusion: Our data suggest that NIR laser irradiation is a promising experimental and therapeutic tool in the field of cerebral circulation research. Lasers Surg. Med. 42:566–576, 2010. © 2010 Wiley-Liss, Inc.

Key words: cerebral blood flow; near infrared laser; MK-801; forebrain ischemia; nitric oxide

INTRODUCTION

Brain function is highly dependent on cerebral blood flow (CBF). Human brain weight is about 2% of the whole body weight. However, CBF reaches about 15% of cardiac output. Improved outcome results when reduced CBF are prevented or respond to treatment not only in brain ischemia but also in traumatic brain injury, degenerative disease such as Parkinson's disease and Alzheimer's disease [1–6].

Photobiostimulation effects of near-infrared (NIR) laser irradiation have been known for almost 40 years [7]. Many studies have shown increased blood flow in various organs during and after NIR laser irradiation [7–10]. Recently, it has been reported that NIR laser irradiation is effective for controlling cerebral ischemia in vivo and clinically [11–14]. However, the precise mechanisms by which blood flow is controlled by NIR laser irradiation on the central nervous system (CNS) have not been elucidated. The vasodilatory action of NIR laser irradiation is most likely mediated by nitric oxide (NO). In this study, we examined the effect of 808 nm laser irradiation on CBF in mice and directly measured NO in brain tissue during NIR laser irradiation using an amperometric NO-selective electrode [15]. We also examined the contribution of NO and a neurotransmitter, glutamate, to the regulation of CBF by using a nitric oxide synthase (NOS) inhibitor, N^g-nitro-L-arginine methyl ester hydrochloride (L-NAME), respectively. It is currently accepted that *N*-methyl-D-aspartate (NMDA) receptor interacts with NOS in the neurotransmitter pathway

No work resembling the enclosed article has been published or is being submitted for publication elsewhere. We certify that we have each made a substantial contribution so as to qualify for authorship. We have disclosed all financial support for our work and other potential conflicts of interest.

All authors have seen and agree with the contents of the manuscript.

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Accepted 26 April 2010
Published online 15 July 2010 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/lsm.20938

[16]. To gain an insight into potential mechanisms linking neurotransmission with increased CBF, the effect of an NMDA receptor blocker, MK-801, was investigated. The thermal effect has been frequently discussed on the mechanisms of the effect of NIR laser irradiation. Therefore, we examined the change in skull surface and brain tissue temperature during NIR laser irradiation. We also investigated the protective effect of NIR laser irradiation on transient cerebral ischemia using transient bilateral common carotid artery occlusion (BCCAO) in mice [17,18]. We evaluated the CBF during BCCAO and apoptosis in the cerebral cortex and dorsal hippocampus 96 hours after the reperfusion by using in situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) of fragmented DNA.

MATERIALS AND METHODS

Animals

The institutional animal care committees of National Defense Medical College approved all experimental procedures. Extreme care was taken throughout the study to minimize pain and discomfort. Male C57BL/6J mice 9–11 weeks in age and weighing 23–27 g (CLEA Japan, Inc., Tokyo, Japan) were allowed free access to food and water before experimental use.

NIR Laser Irradiation and Cerebral Blood Flow Measurement

Mice were administered 20 mg/kg sodium pentobarbital intraperitoneally. Rectal temperature was maintained at approximately 37.0°C, until consciousness was regained, by using a heating pad (Digital Thermo Control Meter DT-102; Inter Medical, Nagoya, Japan) connected to a rectal thermistor. The head of each mouse was fixed with a head clamping device (Stereotaxic instrument SR-5M; Narishige, Tokyo, Japan). The skull was exposed after a midline scalp and periosteum incision with lidocaine local anesthesia. A mild dose of sodium pentobarbital was used to minimize its effects on CBF [19,20]. Therefore, local anesthesia was also used for surgery. Eight hundred eight nanometers CW diode laser (B&W Tek, Inc., Newark, DE) was applied to the left hemisphere transcranially (2 mm posterior to and 3 mm left of the bregma; Fig. 1a). The exposure field was set to 3 mm in diameter. The duration of laser irradiation was 45 minutes. To determine the appropriate power density (PD) of NIR laser irradiation, we irradiated the brain with the laser at three different PDs (0.8 W/cm^2, $n = 6$; 1.6 W/cm^2, $n = 9$; 3.2 W/cm^2, $n = 6$: NIR laser irradiation group), and laser power was checked using a photodiode-type laser power meter (PD300; Ophir Optronics Ltd, Jerusalem, Israel) before and after every irradiation. CBF in the cortex was measured semiquantitatively for both hemispheres with a non-invasive and non-contact laser Doppler blood perfusion imager (PeriScan PIM II, PeriMed, Stockholm, Sweden). By scanning the tissue with a low-power laser beam, color images of blood perfusion in the scanned area were created (Fig. 1b,c). CBF values were recorded before and at 15, 30, and 45 minutes after starting NIR laser irradiation. Indirect arterial blood pressure was monitored by using a non-preheating blood pressure monitor (MK-2000ST, Muromachi, Tokyo, Japan). Respiratory rates were also recorded. CBF and physiological parameters were also measured for sham-operated mice ($n = 9$: sham group) in the same way as described above without NIR laser irradiation.

Measurement of Brain Tissue Temperature

Information on tissue temperature is essential for understanding the mechanisms underlying the effect of laser irradiation on tissue. The temperature of tissue subjected to laser irradiation is often measured with a thermocouple directly inserted into the tissue exposed to laser light or into tissue in the vicinity of the laser irradiation area. In these cases, however, scattered laser light can directly hit the thermocouple, possibly causing an error in measurement. Thus, we estimated the temperature of brain tissue exposed to laser light by inserting a thermocouple (diameter, 300 μm; type-T;

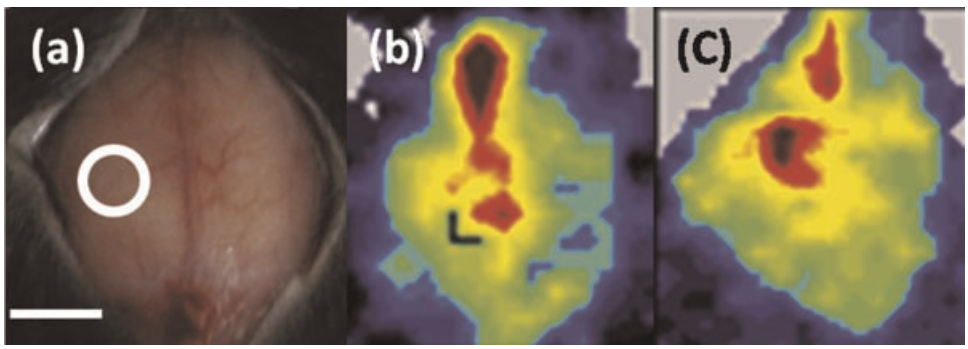

Fig. 1. Representative examples of the exposure field and perfusion images. **a**: The exposure field was set to 3 mm in diameter (2 mm posterior to and 3 mm left of the bregma). Scale bar is 5 mm in length. At the time of measurement, perfusion images were obtained (**b**: pre, **c**: 15 minutes after NIR laser irradiation). Targeted increase in cerebral blood flow of the irradiated field was observed (c).

HYP1-30-1/2-T-G-60-SMP-M Omega, USA) into the brain tissue at the center of the laser irradiation area immediately (<2 seconds) after turning off the laser at a time of interest; the temperature is referred to as T_c. This method enables accurate temperature estimation for the brain exposed to laser light, since the response time of the thermocouple (<1 seconds) is much shorter than the thermal relaxation time for the tissue. A cranial window with a diameter of 5 mm was made in the parietal bone of the right hemisphere. The exposed cortex was irradiated with laser light at a PD of 1.1 or 2.2 W/cm^2, corresponding to 1.6 or 3.2 W/cm^2 for transcranial irradiation, respectively, since transmittance of the skull was estimated to be about 70%. In addition, we continuously monitored the temperature of tissue in the vicinity of the laser irradiation area with another thermocouple of the same type; the temperature is referred to as T_v. As described later, we confirmed that T_v was almost the same as T_c under the present experimental conditions, indicating that the effect of scattered laser light on T_v is negligibly small. We also confirmed that the temperatures obtained by open-skull measurements described above were almost equal to those obtained by intact-skull measurements under corresponding conditions.

Direct Measurement of Nitric Oxide (NO)

We directly measured nitric oxide in the brain tissue during NIR laser irradiation by an amperometric NO-selective electrode (IMN-111, Inter Medical) [15]. All mice were prepared in the same way as that described above. A microelectrode sensor of 0.5 mm in diameter was inserted through the small burr hole of the skull to the brain 1.5 mm in depth just under the exposure field of NIR laser irradiation (PD, 1.6 W/cm^2, $n = 4$; PD, 0.8 W/cm^2, $n = 4$). Measurement was started before irradiating NIR laser. In sham control mice ($n = 4$), NO was measured in the same way as that in the NIR laser irradiation group without laser irradiation. NO was also measured during NIR laser irradiation in N^g-nitro-L-arginine methyl ester hydrochloride (L-NAME)-treated mice ($n = 4$).

Pharmacological Treatment

L-NAME was obtained from Sigma (St. Louis, MO), and MK-801 was obtained from Banyu (Tokyo, Japan). Mice received L-NAME (500 mg/L) in their drinking water for 5 weeks ($n = 9$: NAME group) as described by Deckel et al. [21]. Next, we investigated the effect of MK-801 on CBF increase by laser irradiation to examine the relationship between CBF increase and neurotransmission by laser irradiation. Three different doses of MK-801 (0.1, 1.0, and 10 mg/kg; $n = 8$, $n = 9$, and $n = 9$, respectively) were given intraperitoneally at 1 hour prior to 45-minute NIR laser irradiation (PD: 1.6 W/cm^2). Since MK-801 is known to induce hypothermia [22], rectal temperature was carefully monitored before, during and after NIR laser irradiation. CBF was measured before and at 15, 30, and 45 minutes after starting NIR laser irradiation.

Surgical Procedures for Transient Bilateral Common Carotid Artery Occlusion (BCCAO)

Animals were administered 20 mg/kg sodium pentobarbital intraperitoneally. Under local anesthesia, the bilateral common carotid arteries were exposed after a ventromedial cervical skin incision. The bilateral common carotid arteries were encircled loosely with a 4/0 silk thread to enable later occlusion with a non-traumatic small aneurysm clip. The distal portions of the arteries were inspected to confirm the absence of blood flow after applying the clips. CBF was measured sequentially just before, during 30 seconds and 1, 2, 5, 10, and 15 minutes, and at 30 seconds and 5 minutes after BCCAO. Rectal temperature, indirect arterial pressure and respiratory rate were also recorded as described above. To confirm the effect of pretreatment by NIR laser irradiation, we conducted 1.6 W/cm^2 NIR laser irradiation to the left hemisphere transcranially for 30 minutes before BCCAO ($n = 13$, NIR laser irradiation+BCAAO group). The control mice ($n = 13$, BCCAO control group) were also subjected to 15-minute BCCAO without pretreatment by NIR laser irradiation.

Anatomical Study of Cerebrovasculature

Variation in collateral circulation affects residual CBF following BCCAO. In all 26 mice that were subjected to BCCAO, the existence of the posterior communicating artery (P-com A) which connects the posterior circulation of the brain from the vertebral arteries with the anterior circulation from the carotid arteries in the circle of Willis was examined. The mice with P-com A have a bypass flow from posterior circulation through P-com A while conducting BCCAO. Therefore, these animals were excluded from further analysis. Ninety six hours after the reperfusion, the mice were given 100 mg/kg sodium pentobarbital used for overdose, and were perfused through the heart with 30 ml of saline solution (0.9%), followed by 30 ml of paraformaldehyde (4%) in 0.1 mol/L phosphate buffer (pH 7.4), and then injected with 1.5 ml colored silicon via the left cardiac ventricle. The brains were removed carefully, and the circle of Willis was examined.

Histological Study

To examine histological change due to NIR laser irradiation, randomly selected animals that were irradiated with NIR laser (PD, 1.6 W/cm^2, $n = 4$) were perfused transcardially in the same way as that described above. The brains were removed and embedded in paraffin after fixation in the same fixative for 24 hours at 4°C. Serial coronal sections (5 μm in thickness) were stained with hematoxylin-eosin.

In Situ Labeling of DNA Fragmentation

To evaluate the effect of NIR laser irradiation against transient BCCAO in mice, we carried out in situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) of fragmented DNA by using an apoptosis in situ detection kit (Wako Pure Chemical Industries, Osaka, Japan). After the anatomical study of cerebrovasculature, the brains without P-com were

embedded in paraffin after fixation in the same fixative for 24 hours at 4°C. Serial coronal sections (5 μm in thickness) were made. The procedures were performed as recommended by the manufacturer (http://www.wako-chem.co.jp/siyaku/info/gene/pdf/apotosisdkit.pdf).

The TUNEL-positive cells in the irradiated cerebral cortex and CA1 subfield in the dorsal hippocampus were counted in photomicrographs.

Statistical Analysis

All data are expressed as means ± SD. All data were analyzed by statistical software (Prism4.0; GraphPad Software, San Diego, CA). The Mann–Whitney *U*-test was used to compare CBFs between two groups. Analysis of variance followed by Bonferroni correction was employed to compare the value for physiological parameters. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Effect of NIR Laser Irradiation on CBF, Physiological Variables, and Histology

A representative sequence of laser Doppler perfusion imaging is shown in Figure 1c,d. Targeted increase in CBF of the irradiated field was invariably observed. The effects of NIR laser irradiation at different PDs on CBF are shown in Figure 2. At PDs of 0.8, 1.6, and 3.2 W/cm^2, CBF at the irradiated hemisphere was increased by 9.1 ± 5.7%, 30.4 ± 3.1%, and 30.5 ± 5.6% respectively. These results indicated that NIR laser irradiation at 1.6 W/cm^2 could increase CBF most efficiently. Therefore, we selected the PD of 1.6 W/cm^2 for irradiation in subsequent studies. Arterial blood pressure and respiratory rates were similar and showed no significant differences among groups.

Histology of the mouse brain with 45-minute NIR laser irradiation ($n = 4$) revealed no detectable ablation, coagulation or bleeding change on and around the exposure field.

Brain Tissue Temperature During Laser Irradiation

Brain temperature T_v (continuously monitored tissue temperature in the vicinity of the laser irradiation area) and brain temperature T_c (tissue temperature at the center of the laser irradiation area immediately after turning off the laser) were compared at PDs of 1.1 and 2.2 W/cm^2, corresponding to 1.6 and 3.2 W/cm^2 for transcranial irradiation, respectively. T_c and T_v were 33.1 and 33.2°C at 1.1 W/cm^2, respectively, and they were 36.7 and 36.2°C at 2.2 W/cm^2 at the same measurement time point. At both PDs, the difference between T_v and T_c was within 0.5°C, indicating that the effect of scattered laser light on T_v was negligibly small and that tissue temperature of the brain exposed to laser irradiation can be monitored by measuring T_v. Figure 3 shows a typical time course of the brain temperature T_v for irradiation at 1.1 W/cm^2. Mean T_v for about 10 minutes before laser irradiation was 32.9°C. After starting irradiation, T_v slightly increased and reached an equilibrium in about 25 seconds; the rise of mean

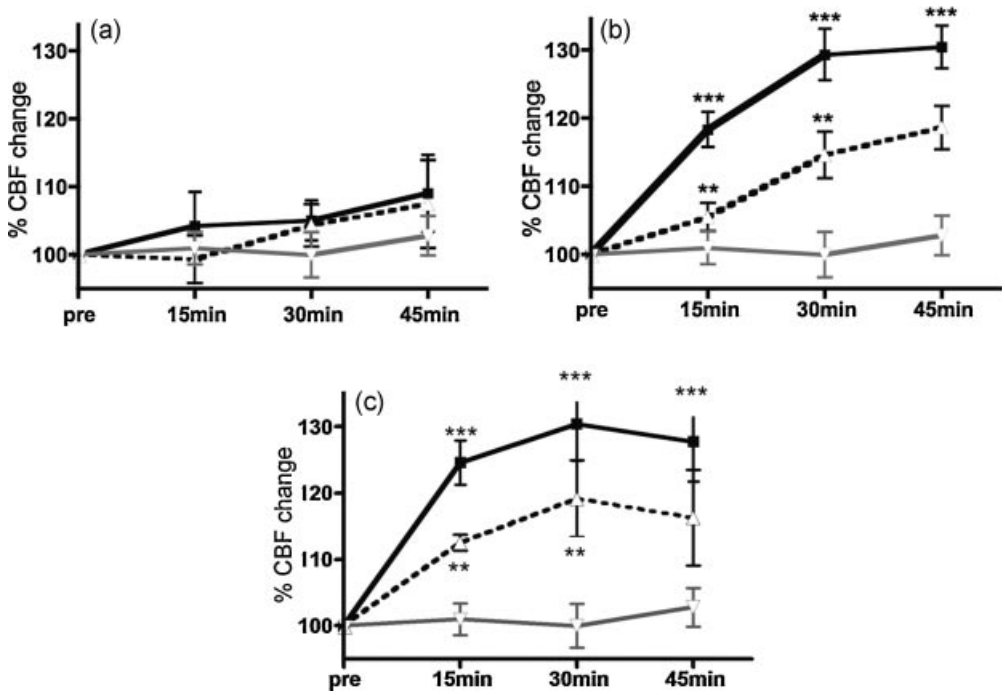

Fig. 2. Effect of NIR laser irradiation at three different power densities (PDs). At PDs of 0.8 (**a**), 1.6 (**b**), and 3.2 W/cm^2 (**c**), there were increases in CBF of 9.1 ± 5.7%, 30.4 ± 3.1% and 30.5 ± 5.6%, respectively, in the irradiated hemisphere. Each black solid line indicates the change in CBF in the irradiated hemisphere. Each dotted line indicates the change in CBF in the non-irradiated hemisphere. Each gray solid line indicates the changes in CBF in the sham group. There were significant differences between the BCF of each hemisphere and that of the sham group (**$P<0.01$, ***$P<0.001$).

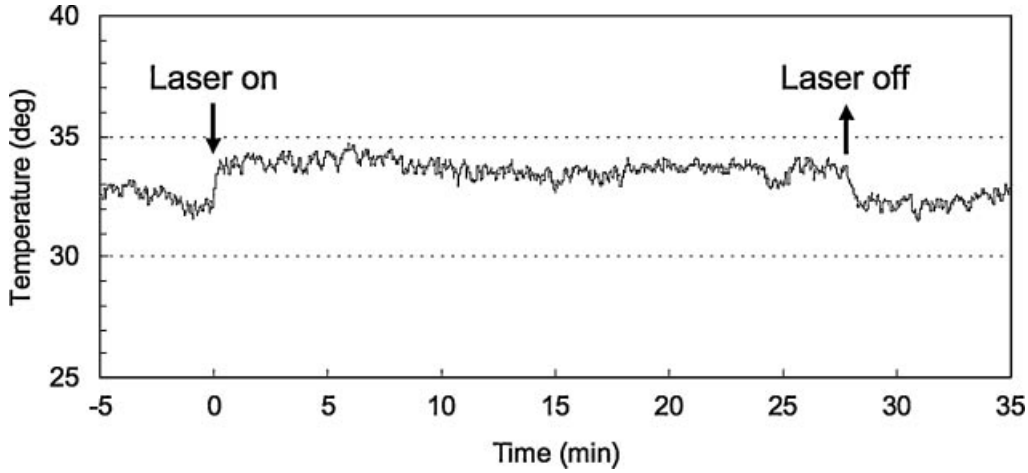

Fig. 3. Change in the brain tissue temperature during NIR laser irradiation. This is a typical time course of the brain temperature T_v for irradiation at 1.1 W/cm^2. Mean T_v for about 10 minutes before laser irradiation was 32.9°C. After starting irradiation, T_v slightly increased and reached an equilibrium in about 25 seconds; the rise of mean temperature T_v during laser irradiation for about 30 minutes was as low as 0.8°C.

temperature T_v during laser irradiation for about 30 minutes was as low as 0.8°C. At 2.2 W/cm^2, on the other hand, the rise of mean temperature T_v during irradiation for 45 minutes was ~3.8°C.

NO Concentration

Changes in NO concentration are shown in Figure 4. In the sham control mice, NO concentration was unchanged (Fig. 4a). All mice showed an immediate increase in NO

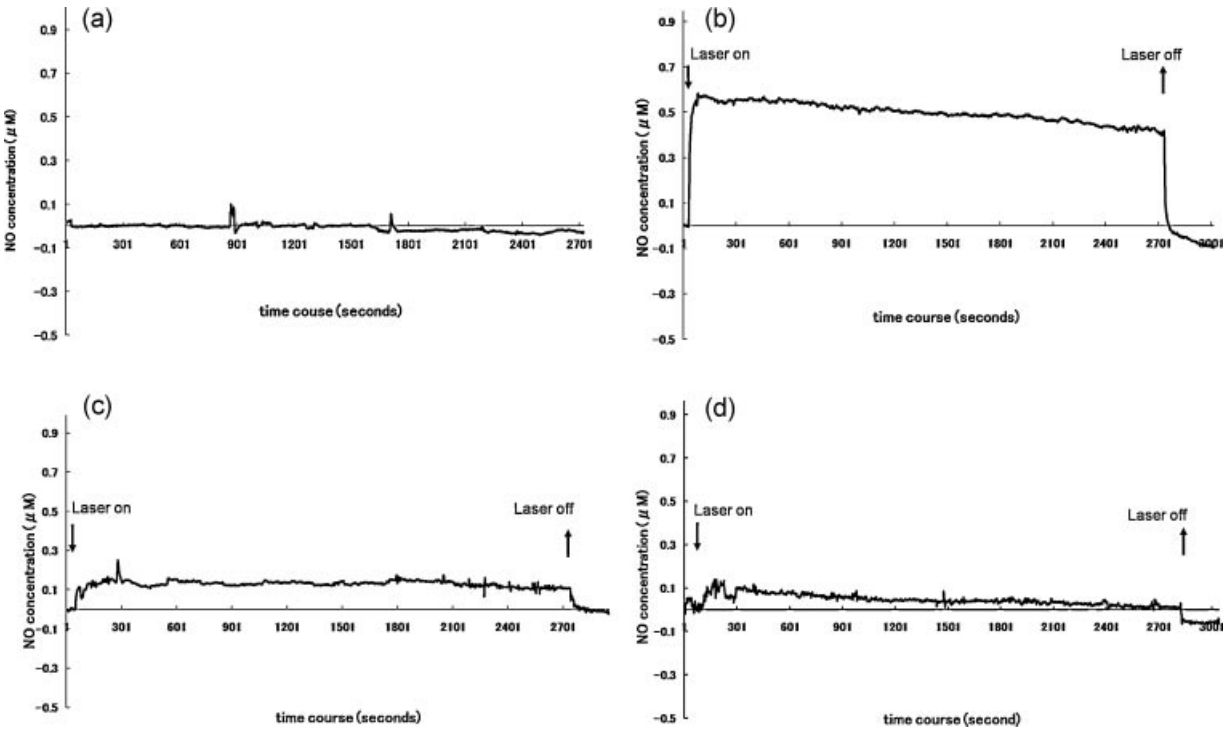

Fig. 4. Representative examples of change in nitric oxide (NO) concentration during NIR laser irradiation. In the sham control mice, NO concentration was unchanged (**a**). All mice showed an immediate increase in NO concentration after the start of NIR laser irradiation at PD of 1.6 W/cm^2, and NO returned to a resting level after the end of NIR laser irradiation (**b**). NO levels were significantly reduced by L-NAME treatment (**d**). Mice irradiated with NIR laser at PD of 0.8 W/cm^2 showed a small increase in NO concentration, only 15% of that in mice irradiated with NIR laser at PD of 1.6 W/cm^2 (**c**).

concentration after the start of NIR laser irradiation at a PD of 1.6 W/cm^2, and NO returned to a resting level after the end of NIR laser irradiation (Fig. 4b). NO levels were significantly reduced by L-NAME treatment (Fig. 4c). Mice irradiated with NIR laser at a PD of 0.8 W/cm^2 showed a small increase in NO concentration, only 15% of that in mice irradiated with NIR laser at a PD of 1.6 W/cm^2 (Fig. 4d).

Pharmacological Treatment

The effect of L-NAME on CBF during NIR irradiation. Almost no increase in CBF was observed in the L-NAME group. The increase in CBF was only $5.0 \pm 2.1\%$ at 45 minutes after starting NIR laser irradiation (Fig. 5a). Deckel et al. [21] investigated NOS activity in mice orally administered several doses (0, 5, 10, 50, 100, and 500 mg/L) of L-NAME and they reported that NOS activity was reduced dose-dependently as L-NAME concentration in the drinking water was increased. Therefore, we used the concentration of 500 mg/L in drinking water in this study. The absence of stimulatory effects of light in L-NAME treated mice indicates the involvement of NOS in the process.

The effect of MK-801 on CBF during NIR laser irradiation. The CBF was increased by approximately 20% by NIR laser irradiation in the three different MK-801 dose groups dose-independently (Fig. 5b). There was no significant difference among the three groups. Delayed additional increases in CBF at 30 and 45 minutes after starting NIR laser irradiation were not observed in the three groups. There were significant differences in CBF at 45 minutes after starting NIR laser irradiation in the MK-801 groups (0.1 and 10 mg/kg) compared with that in the NIR laser irradiation group. MK-801 is a non-competitive NMDA receptor blocker, and it suppresses glutamatergic synaptic transmission. The NMDA receptor interacts with neuronal NOS in the neurotransmitter pathway [16]. Suppression of glutamatergic neurotransmission by MK-801 did not influence the early increase but blunted the delayed additional increase in CBF by NIR laser irradiation. This result indicates that the delayed additional increase in CBF by NIR laser irradiation might be related to neuronal activation.

Arterial blood pressure, rectal temperature and respiratory rate were similar and showed no significant differences among groups.

Effect of Pretreatment by NIR Laser Irradiation for BCCAO in Mice

Pretreatment with NIR improves CBF in mice subjected to BCCAO. In the anatomical study of cerebrovasculature, P-com A was detected in 4 mice in the NIR laser irradiation group and in 5 mice in the control group. These 9 mice were excluded from the analysis. Mice in

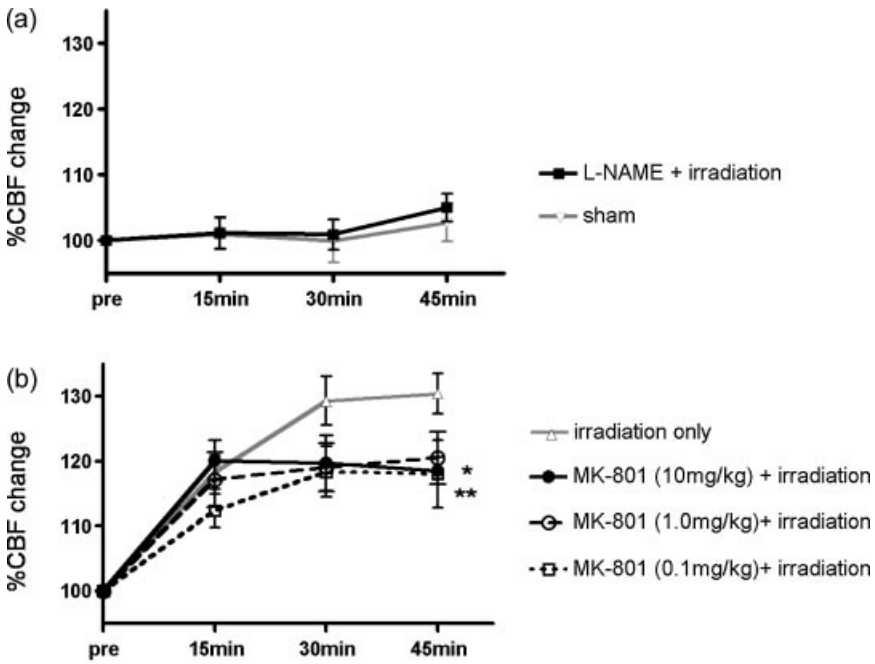

Fig. 5. Effects of L-NAME and MK-801 on CBF during NIR laser irradiation. Almost no increase in CBF was observed in the L-NAME group ($n = 9$). The increase in CBF was only $5.0 \pm 2.1\%$ at 45 minutes after NIR laser irradiation (**a**). CBF was increased by approximately 20% by NIR laser irradiation in all three MK-801 dose groups (**b**). Delayed additional increases in CBF at 30 and 45 minutes after NIR laser irradiation were not observed. CBFs at 45 minutes after NIR laser irradiation in the MK-801 groups (0.1; $n = 8$ and 10 mg/kg; $n = 9$) were significantly different from CBF in the NIR laser irradiation group ($n = 9$). (*$P < 0.05$; NIR laser irradiation group vs. 10 mg/kg MK-801 group, **$P < 0.01$; NIR laser irradiation group vs. 0.1 mg/kg MK-801 group).

which P-com A was observed showed high residual CBF more than 40% of pre-occlusion values in the same hemisphere. Residual CBF in mice without P-com was less than 30% of that in the control group during BCCAO. Sequential CBF before, during and after BCCAO is shown in Figure 6. Pretreatment by NIR laser irradiation significantly improved residual CBF during BCCAO in mice.

Physiological parameters during BCCAO. Physiological parameters are presented in Figure 7. Body temperatures, respiratory rates and heart rates were similar and showed no significant differences among the groups (Fig. 7a–c). However, there was a significant difference in arterial blood pressure (BP) between the laser irradiation group and control group (Fig. 7d).

NIR laser irradiation protects against transient brain ischemia and DNA fragmentation. Neuronal damage 96 hours after the reperfusion in both groups was evaluated using DNA fragmentation assay. However the frequency of damaged cell labeled with TUNEL staining in the laser irradiation group ($n = 9$) was lower than that in the control group ($n = 8$) (Fig. 8). The percentage of the TUNEL-positive cells to the total number of cells in the cortex was $9.1 \pm 8.3\%$ in the NIR laser irradiation group, and $27.0 \pm 20.0\%$ in the control group ($P<0.05$) (Fig. 8d). The percentage of the TUNEL-positive cells in the CA1 subfield was $44.8 \pm 26.6\%$ in the NIR laser irradiation group, and $84.0 \pm 19.0\%$ in the control group ($P<0.01$) (Fig. 8e).

DISCUSSION

Many studies have shown increased blood flow in various tissues during and after NIR laser irradiation [7]. However, to the best of our knowledge, there has been no study in which the relationship between NIR laser irradiation and CBF was examined. The results of this study indicate that NIR laser irradiation with the appropriate power density is effective for increasing CBF and that NO is involved in the mechanism of CBF increase in response to NIR laser irradiation, and also suggest a protective effect of NIR laser irradiation for transient ischemia.

The Effect of NIR on CBF

We examined the effect of NIR laser irradiation on CBF at three different densities (0.8, 1.6, and 3.2 W/cm^2). CBF at the irradiated hemisphere was increased by $9.1 \pm 5.7\%$, $30.4 \pm 3.1\%$, and $30.5 \pm 5.6\%$ respectively. These results indicated that NIR laser irradiation at 1.6 W/cm^2 could increase CBF most efficiently. The results obtained at different PDs indicate the possible existence of a threshold for a profound induction of NO by NIR irradiation.

Temperature of Irradiated Brain Tissues

The rise in temperature of the brain tissue exposed to laser light was found to be as low as $\sim 1^\circ$C under irradiation conditions enabling the most efficient CBF increase (1.6 W/cm^2, 30 minutes). Therefore, the CBF increase would not be due to the heating effect. Many studies have shown increased blood flow in various organs during and after NIR laser irradiation [7–10]. The biological effects of NIR laser irradiation are wavelength-specific and they are not therefore attributable to thermal effects in many cases. For neuronal tissue, photobiomodulation effects have been reported under a wide variety of light irradiation conditions, PD ranging from 7 mW/cm^2 to $\sim$5 W/cm^2 and total energy density ranging from 0.9 J/cm^2 to $\sim$4,300 J/cm^2 [11–14,23–25]. For example, NIR laser irradiation at 808 nm significantly reduced neurological

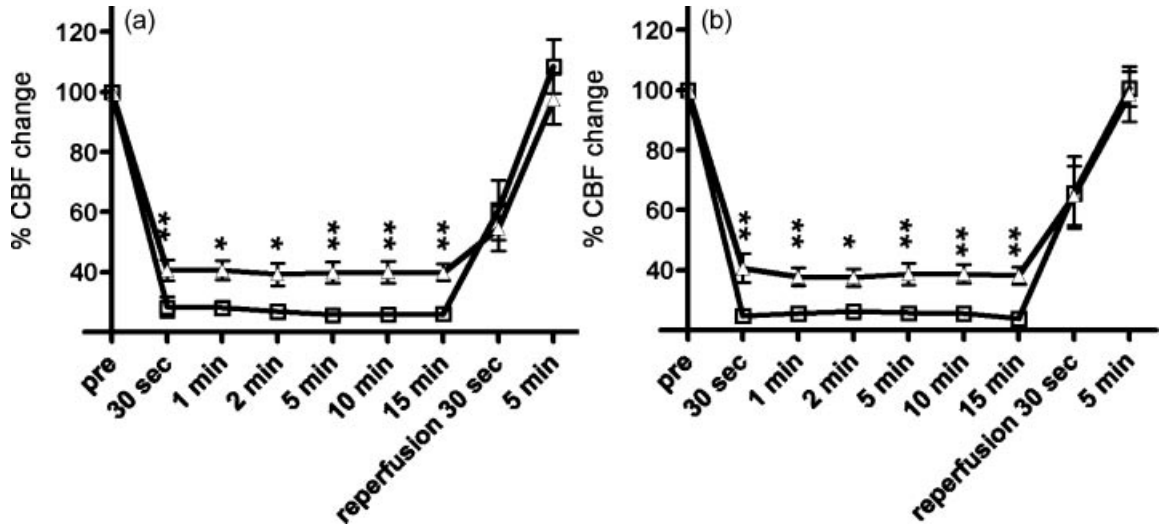

Fig. 6. Effect of NIR laser irradiation for 15-minute BCCAO. Pretreatment by NIR laser irradiation increased residual CBF following bilateral carotid occlusion in mice. The reduction in CBF was significantly (*$P<0.05$, **$P<0.01$) suppressed compared with that in the control group during BCCAO. Open triangles indicate the NIR irradiation group. Open squares indicates the control group. **a**: Irradiated hemisphere, (**b**) non-irradiated hemisphere.

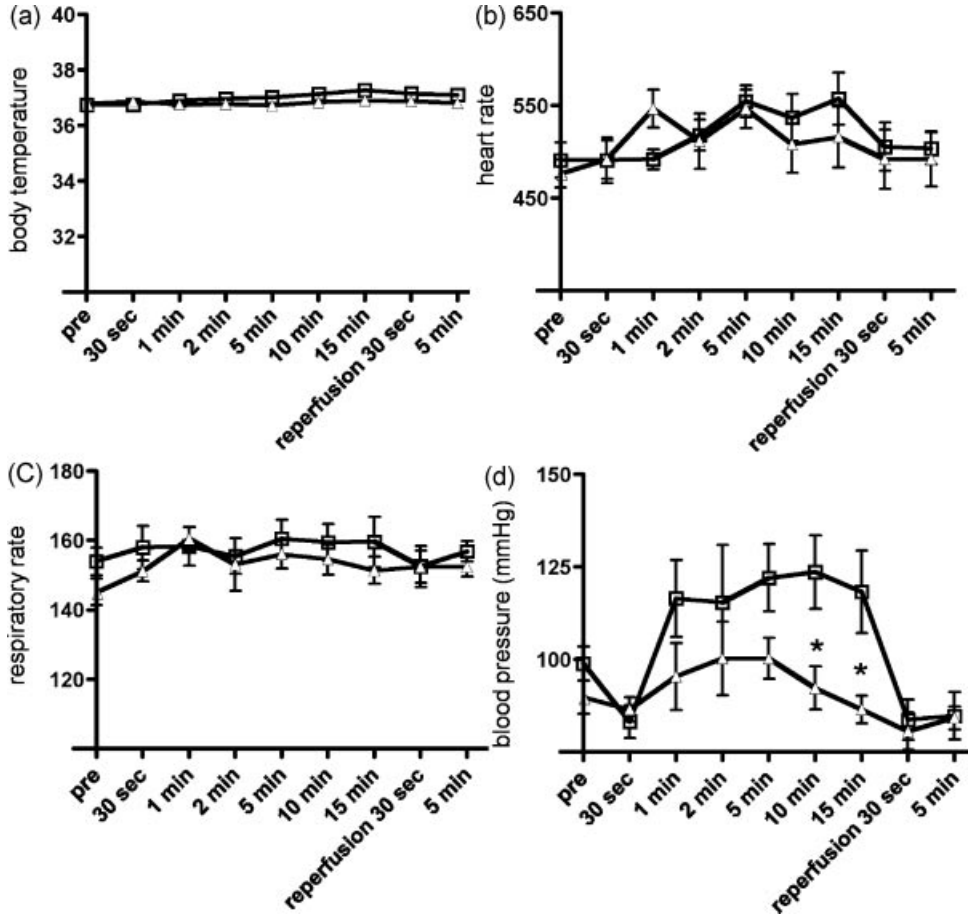

Fig. 7. Changes in physiological parameters. Body temperatures (**a**), respiratory rates (**b**) and heart rates (**c**) were similar and showed no significant differences among the groups. However, there was a significant difference in arterial blood pressure (**d**) between the two groups (*$P<0.05$). In the control group, the blood pressure increased just after BCCAO and returned to the baseline value after the reperfusion. On the other hand, in the pretreatment by NIR laser irradiation group, the blood pressure increase was mild, and blood pressure gradually returned to the baseline value before removal of the clip. Open triangles indicate the NIR irradiation group. Open squares indicates the control group.

deficits in animal stroke models and human stroke patients at 7–25 mW/cm^2 and 0.9–15 J/cm^2 [11–13,23]. For acute rat spinal cord injury (SCI), NIR laser irradiation at 810 nm promoted axonal regeneration and functional recovery at 530 mW/cm^2 and ~1,600 J/cm^2 [24]. For the rat normal brain, an increase in cerebral ATP content was observed with 830-nm laser irradiation at 4.8 W/cm^2 and ~4,300 J/cm^2 [25]. In the present study, we observed the most efficient increase in CBF at 1.6 W/cm^2 (Fig. 2), at which the rise in temperature of tissue was as low as ~1°C. Thus, heating effect would not be a main mechanism for the increase in CBF in this condition. At 3.2 W/cm^2, however, the temperature rise reached ~4°C and heating effect cannot therefore be excluded in the mechanism of CBF increase, although thermal damage was not observed in tissue by histological analysis. Further study is needed to fully understand the mechanism by which CBF is increased by NIR laser irradiation in the present animal models.

Effect of NIR on NO Concentration and Pharmacologic Inhibitors

In tissues other than brain, some studies have shown a relationship between the effect of NIR laser irradiation on blood flow increase and NO [7–10]. In this study, we examined this relationship for the brain by administering several inhibitors and directly monitoring NO concentration in the brain tissue.

NO is a free radical gas that is a powerful regulator of circulation (as an endogenous vasodilator) and a neurotransmitter (helping in the processing of nerve signals as they cross synapses). Due to its chemical properties, high diffusibility and short half-life, NO is a unique molecule in the brain [26]. NO is a gas and thus passes freely through the cell membrane. Therefore, neurons without synaptic connection can be influenced by diffusing NO. However, the half-life of NO is very short, that is, a few seconds [27], and the effect of NO is therefore localized.

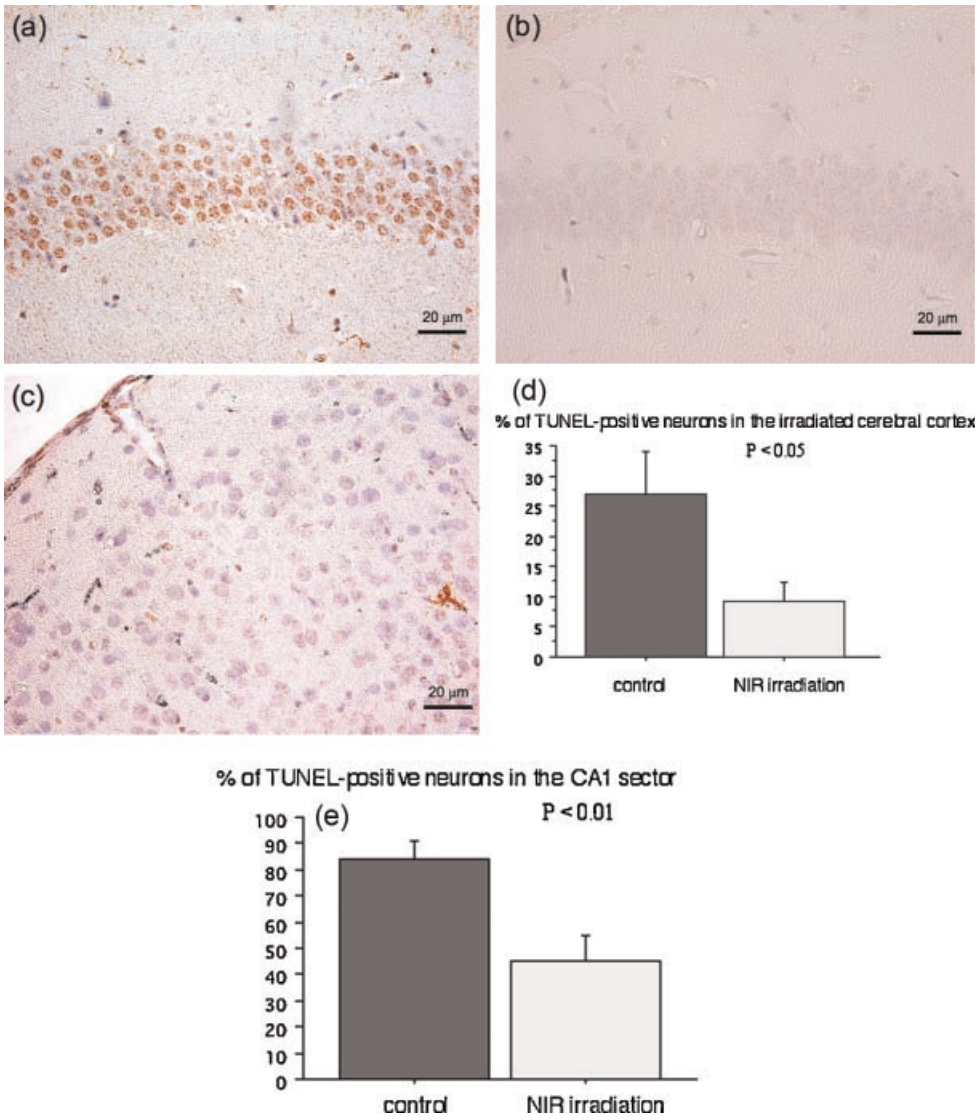

Fig. 8. The effect of NIR laser irradiation against transient ischemia on DNA fragmentation TUNEL staining in the CA1 subfield of dorsal hippocampus in the control group (**a**), and in the NIR irradiation group (**b**). TUNEL staining in the irradiated cerebral cortex in the NIR irradiation group indicates no increase in DNA fragmentation (**c**). A significant reduction in the percentage of the TUNEL positive cells in the cerebral cortex (**d**) and in the CA1 pyramidal cell layers (**e**) was observed in the NIR irradiation group ($n = 9$) compared with that in the control group ($n = 8$).

Mice irradiated with NIR laser at a PD of 1.6 W/cm^2 showed a significant increase in CBF (by 30% of control values). In the L-NAME-treated mice, on the other hand, the increase in CBF was suppressed to baseline values. Similarly, NO concentration was increased by NIR laser irradiation at a PD of 1.6 W/cm^2, and this response was also suppressed by L-NAME. These results indicate that CBF increase depends on NOS activity and NO concentration. Photobiomodulation effects on neuronal tissue and cells often show unique wavelength dependence, and photoactivation of cytochrome *c* oxidase in mitochondria is therefore considered as the possible mechanism [7,28]. It is known that NO competes with O_2 in the mitochondrial respiratory chain [29,30], by which respiration is regulated. Cytochrome *c* oxidase is a dominant photoreceptor in the NIR spectral region [31], and NIR laser irradiation can release NO from cytochrome *c* oxidase [7,32,33], resulting in increased blood flow. This is called the NO hypothesis [32,34] to explain the effect of increased blood flow induced by NIR laser irradiation; the wavelength-dependent effect can be interpreted by the absorption spectrum of cytochrome *c* oxidase [7,8,28,31]. The present study clearly showed that NIR laser irradiation was accompanied by increased NO content in tissue, which supports the NO

hypothesis for the effect of increasing CBF by NIR laser irradiation.

There was no significant change of NOS immunoreactivity after NIR (data were not shown). NO may be produced by cells in the absence of NOS (NOS-independent pathway) as described by Martin et al. [35].

Mice treated with MK-801 at three different doses showed a significant suppression in delayed additional increase of CBF compared with that in the control group dose-independently. There was no significant difference among these three groups. MK-801 is a non-competitive NMDA receptor blocker, and it suppresses glutamatergic synaptic transmission. As Andresen et al. [36] reported in their article, the mechanism responsible for coupling blood flow to neural activity is an intense area of investigation, and it is currently accepted that the NMDA receptor interacts with neuronal NOS in the neurotransmitter pathway [16]. NIR laser irradiation may influence neurons, astrocytes, and endothelial cells. Different forms of NOS in these cells may participate in NO production following NIR laser irradiation. Suppression of glutamatergic neurotransmission by MK-801 did not influence the early increase but blunted the delayed additional increase in CBF by NIR laser irradiation. This result indicates that the delayed additional increase in CBF by NIR laser irradiation might be related to neuronal activation. Our results suggest that an appropriate duration of NIR laser irradiation activates the CNS.

NIR Pretreatment of BCCAO Treated Mice

In this study, pretreatment by NIR laser irradiation significantly improved residual CBF during BCCAO and neuronal damage. The increase in CBF induced by NIR irradiation may be useful for temporal clinical practice (e.g., enhancement of ischemic tolerance before conducting temporal occlusion of the cerebral artery in microsurgery). We therefore investigated the effect of pretreatment with NIR irradiation for BCCAO in mice. For development of a highly reproducible BCCAO model, it is crucial to confirm the patency of P-com A, which connects posterior circulation of the brain from the vertebral arteries with anterior circulation from the carotid arteries in the circle of Willis [17,18,37,38]. In previous studies, P-com A was detected in 20.0–38.9% of C57BL6 mice [17,18,38]. In this study, we detected P-com A in 9/26 (34.6%) of the mice. Mice with P-com A showed high residual CBF (more than 40% of pre-occlusion values) in the same hemisphere, whereas residual CBF in mice without P-com A was less than 30% in the control group during BCCAO. These values are higher than the values measured by a laser Doppler flow meter in a BCCAO model in mice [7,18,37,38]. The blood flow measurement technique that we used is qualitative and is not good for distinguishing very low blood flows. However, we confirmed patency of P-com A in all mice by an anatomical study. Therefore, these relative values are considered acceptable. Moreover, this technique has the advantage of enabling measurement in a wider area than that with a laser Doppler flow meter. This technique thus enables simultaneous measurements in both hemispheres.

Pretreatment by NIR laser irradiation significantly improved residual CBF during BCCAO in mice not only in the irradiated hemisphere but also in the non-irradiated hemisphere. Thirty-min NIR laser irradiation at a PD of 1.6 W/cm^2 increased CBF by 15% in the opposite hemisphere. Although scattered light may possibly influence the opposite hemisphere, further study is needed to understand the mechanism by which CBF is increased in the opposite hemisphere. Interestingly, the recovery of CBF after cessation of BCCAO in the NIR laser-irradiated mice was more gradual than that in the BCCAO control group. This result can be partially explained by the fact that NO surge after the reperfusion was mitigated by pretreatment with NIR laser irradiation.

Changes in BP were significantly different among the groups. In the BCCAO control group, BP increased just after BCCAO and returned to the baseline value after the reperfusion. In the NIR laser irradiation+BCCAO group, on the other hand, the BP increase was mild, and BP gradually returned to the baseline value before removal of the clip. The increased residual CBF following NIR laser irradiation would result in the elimination of systemic reactive hypertension after BCCAO.

Neuronal damage 96 hours after the reperfusion in both groups was evaluated using DNA fragmentation assay. Excessive NO has negative effects on neurons. However, the number of the TUNEL positive cells in CA1 pyramidal cell layers and in the irradiated cerebral cortex was significantly reduced compared with that in the control group. These results suggest a protective effect of NIR laser irradiation for transient ischemia. NIR laser irradiation may have a protective role against ischemic insult.

CONCLUSION

The results of our study suggest that targeted increase of cerebral blood flow is achieved by NIR laser irradiation and that it is concerned with NOS activity and NO concentration. NIR laser irradiation under appropriate conditions would enable activation of the central nervous system. This can hopefully be applied to protect the brain from transient ischemia.

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