Blue Laser Light Increases Perfusion of a Skin Flap Via Release of Nitric Oxide from Hemoglobin

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It has recently been shown that nitrosyl complexes of hemoglobin (NO-Hb) are sensitive to low-level blue laser irradiation, suggesting that laser irradiation can facilitate the release of biologically active nitric oxide (NO), which can affect tissue perfusion. The aim of this study was to evaluate the therapeutic value of blue laser irradiation for local tissue perfusion after surgical intervention. Blood was withdrawn from a rat, exposed to NO and infused back to the same rat or used for in vitro experiments. In vitro, an increase of NO-Hb levels (electron paramagnetic resonance spectroscopy) up to 15 μM in rat blood did not result in the release of detectable amounts of NO (NO selective electrode). Blue laser irradiation of NO-Hb in blood caused decomposition of NO-Hb complexes and release of free NO. Systemic infusion of NO-Hb in rats affected neither systemic circulation (mean arterial pressure) nor local tissue perfusion (Doppler blood flow imaging system). In contrast, a clear enhancement of local tissue perfusion was observed in epigastric flap when elevated NO-Hb levels in blood were combined with local He-Cd laser irradiation focused on the left epigastric artery. The enhancement of regional tissue perfusion was not accompanied by any detectable changes in systemic circulation. This study demonstrates that blue laser irradiation improves local tissue perfusion in a controlled manner stimulating NO release from NO-Hb complexes.

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INTRODUCTION

Decreased peripheral blood flow related to impaired microcirculatory vasodilatation has been shown to occur in certain disease states including peripheral vascular disease, diabetes mellitus, hypercholesterolemia, hypertension, chronic renal failure, abdominal aortic aneurysmal disease, and venous insufficiency, as well as in menopause, advanced age, and obesity (1). Impaired peripheral blood flow appears also as a serious complication of transplantation (2) and plastic surgery (3), often resulting in transplant loss. Nitric oxide (NO) is one of the most important physiological regulators of the microcirculation (4–7), which activates vasodilatation via activation of cGMP-dependent pathway (8). The main source of NO for vasodilatation is the endothelial NO synthase (eNOS). The expression of this enzyme is regulated by a range of transcriptional and posttranscriptional mechanisms generating NO in response to a number of stimuli (9). However, in pathological states (i.e., ischemia) the generation of NO by eNOS is often impaired (10). NO donors as a source of exogenous NO have been shown to improve ischemia/reperfusion injury (11). In blood, NO reacts quickly with Hb. This interaction follows two pathways, namely NO-mediated oxidation of oxyHb to methemoglobin yielding NO_3^- and, secondly, the binding of NO to Hb yielding nitrosyl complexes of hemoglobin (NO-Hb). Both reactions have very high rate constants, $3.7 \times 10^7\ M^{-1}\ s^{-1}$ and $2.6 \times 10^7\ M^{-1}\ s^{-1}$, respectively (12), suggesting that under physiological conditions, there is no free NO in blood. Gow and coauthors (12,13), however, have shown that oxygen drives the conversion of nitrosylhemoglobin to S-nitrosohemoglobin (SNO-Hb), which was suggested to act as an endogenous NO donor and physiological regulator of blood pressure, releasing NO (14). Other data, however, suggest that SNO-Hb is not stable enough and releases nitrate (15), rather than turning back to NO-Hb. Despite these conflicting data on the role of SNO-Hb, the NO-Hb complexes are not considered to be a source of NO due to very high affinity of NO to heme iron.

A few years ago it was shown that NO-Hb complexes in protein solution are photosensitive and can release NO during exposure to laser radiation (16,17). Two years later it was shown that the exposure of smooth muscle to UV and visible light facilitates muscle

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relaxation and NO was supposed to be a mediator of this relaxation (18). The aim of this study was to clarify whether photo-dissociation of NO-Hb following vasodilatation can be a vasodilatory mechanism in in vivo systems. We have chosen a skin flap model for in vivo testing of this mechanism. Obligatory period of ischemia is an important problem in flap surgery and microcirculation was expected to improve.

MATERIALS AND METHODS:

Animals

The Animal Protocol Review Board of Vienna City Government approved the experimental protocol. All experimental procedures were performed under the conditions described in the guide for the care and use of laboratory animals of the National Institute of Health (publication NIH 86-23, revised 1985). Male Sprague Dawley rats (mean weight 397 g ± 17 SD) were initially anesthetized in an inhalation box provided with isoflurane (1.5 – 2.5 Vol%), oxygen (300 mL/min), nitrous oxide (750 mL/min), and air (3 L/min). Thereafter animals were transferred to a temperature-controlled surgical plate (rectal body temperature was maintained between 37.0 and 37.8°C). Anesthesia was maintained via an inhalation mask with the same scheme of anesthesia as mentioned above. Then the entire abdomen and the cervical region were carefully shaved followed by depilation. In all animals the jugular vein was cannulated and 1 mL/h of Ringer's solution was administered for fluid substitution and to maintain a constant blood pressure. A small incision in the right groin provided access to the right femoral artery, which was also cannulated. The catheter introduced in the femoral artery was used to withdraw the blood, to monitor systemic blood pressure, and as the port for administering the NO-Hb preparation. Later the incision was sutured using the interrupted sewing technique. Systemic mean arterial pressure (MAP) was measured with a Cardiosys (Experimetria, Hungary).

Enriching RBC and Whole Blood with NO-Hb Complexes

The blood was collected in a heparinized vial from the right femoral vein and used either for RBC preparation or for enriching with NO-Hb. NO-Hb complexes in erythrocytes or whole blood were produced during incubation of erythrocytes/whole blood with the gaseous NO dissolved in saline solution under anaerobic conditions. Oxygen was removed from RBC/blood during incubation under gaseous nitrogen for 20 min. The diffusion of gases into the RBC/blood was facilitated using a shaking table to provide gentle mixing of the cells suspension with gas. Then saturated NO solution (approx. 2 mM) was added to erythrocytes/whole blood by a syringe injection avoiding contact with air and the obtained mixture incubated for 10 min. Finally, the remaining free NO was removed from RBC/blood during another 10 min incubation under nitrogen. The levels of NO-Hb complexes were determined by means of EPR spectroscopy (Figure 1) and were on average as high as 105 μM of NO; the variation between preparations reached 30%. The blood enriched with NO-Hb was administered back to the blood circulation via the femoral artery as a bolus injection. To obtain NO solution, saline was bubbled first for 30 min with nitrogen, and then kept for 15 min under NO gas (Messer, Germany). A shaking table facilitated the diffusion of NO into saline. The admixtures occurring in NO gas were removed by bubbling through 10% NaOH. Samples containing NO-Hb and deoxyhemoglobin (obtained before exposure to NO) were subjected to the EPR measurement to determine NO levels.

NO-Hb Detection by EPR

Determination of NO-Hb levels was performed in RBC and whole blood withdrawn from the right femoral vein

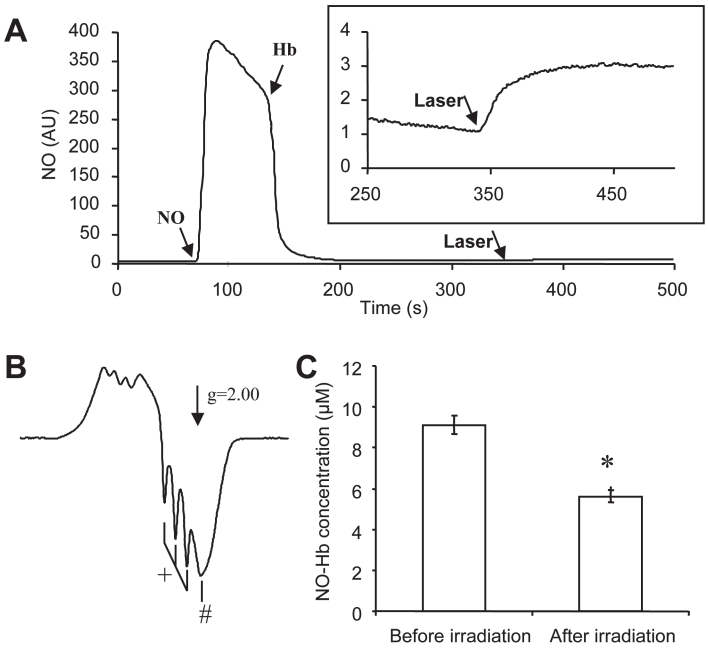

Figure 1. Effect of RBC and laser irradiation on the levels of NO and NO-Hb in a buffered oxygen free saline solution. (A) Kinetics of NO levels upon addition of Hb and laser irradiation; (B) NO-Hb EPR spectrum; + and # indicate the features of 5- and 6- coordinated NO-Hb complexes. The magnitudes of corresponding peaks were used to estimate the amounts of both complexes; (C) NO-Hb concentration in solution before and after irradiation with a 40 mW He-Cd laser. * significantly different $P < 0.01$.

after bolus infusion of either NO-Hb solution or control solution, and whole blood from the right femoral artery after HeCd laser irradiation again for both groups. The concentrations of nitrosylhemoglobin complexes in blood samples were measured by means of electron paramagnetic resonance (EPR) spectroscopy. Blood samples were placed in 1 mL syringes, frozen and stored at liquid nitrogen temperature until use. For measurement, samples were pressed out of the syringes and moved to a finger-tip liquid nitrogen Dewar. EPR spectra were recorded on a Bruker EMX EPR spectrometer (BioSpin GmbH Rheinstetten/Karlsruhe, Germany) at liquid nitrogen temperature under the following settings: microwave frequency 9,429 GHz, microwave power 30 mW, modulation frequency 100 kHz, modulation amplitude 6 G. For quantification of NO-Hb levels in blood samples, NO-Hb standards were prepared using NO_2^- solutions reduced by dithionite in the presence of hemoglobin as described before (29). Briefly, 100 mg of dithionite were mixed with 300 μL Hb and a range of NO_2^- concentrations (0 to 10 μM). The nitrosyl-hemoglobin signals in blood were compared with the standard solutions.

NO Detection by NO-Electrode

To evaluate the amount of free NO in suspension of RBC or blood containing NO-Hb, erythrocytes or whole blood enriched with NO-Hb were introduced into the NO-electrode measuring chamber (NO-chamber, WPI, USA). To remove air oxygen from the samples, gaseous nitrogen was bubbled through the buffer for 15 min before NO solution and RBC/blood were added. The samples were irradiated with laser light through the channel made in a NO-chamber for the second electrode. The calibration procedure was made in accordance with manufacturer instructions. To create a calibration curve, 20 μL of 50 μM $NaNO_2$ was added to 10 mL calibration solution (0,1 M H_2SO_4 + 0,1 M KI). This was repeated 3 times to show the stability of the NO electrode.

Axial Epigastric Flap

To observe the effect of NO-Hb and laser irradiation on vasomotility (vasodilatation) we chose to use the axial epigastric flap model. This model provides the possibility to exclude the influence of surrounding arterial inflow, studying only the definite inferior epigastric vessel tree. The dimensions of the flap were the same in all animals (4 × 8 cm^2) whereas the position of the flap varied according to the entrance of the left epigastric artery into the flap. The flap borders were outlined with a surgical marker. Surgical flap harvesting was performed cranially to caudally by blunt dissection technique. The entire flap was attached only left to the inferior epigastric neurovascular bundle. The fasciomyocutaneous flap was then sutured back to its original anatomical orientation with non-resorbable sutures using interrupted technique. The caudal incision of the flap was partly left open to ensure easy access to the left inferior epigastric artery for laser irradiation.

Superficial Perfusion Measurement

The Laser Doppler Imaging System (Moor, UK) was used to illustrate and evaluate flap perfusion. A low intensity (2mW) laser light beam (wavelength of 632.8nm) was scanned across the surface of the skin in a raster fashion by a moving mirror, thus giving a two-dimensional image of flap perfusion. Laser beam movements without any skin contact came from a standard working distance of 20 cm. Scan modus was set at 10 ms/pixel, and a resolution of 256 × 256 pixels was chosen. Perfusion values were recorded as colored pixels, giving the color-coordinated 2-D image of the flap perfusion. Three scans each lasting 4 min were acquired for each phase of experiments. The first phase, background, started 30 min after flap harvesting. During NO-Hb/saline bolus and infusion (Infusion phase), again three scans each lasting 4 min were acquired. The same scan protocol was used for the irradiation period (Irradiation phase) as well as for the period after irradiation (Post Irradiation phase). The laser beam was focused through the small caudal incision on the left epigastric artery directly distal to the origin from the femoral artery. Acquired images were further processed and evaluated with an image analyzing software tool, provided by the manufacturer of the LDI system. Numeric results were calculated in percentage of perfusion units (%PU). The mean of the three scans in the baseline period was set at 100%. The single baseline scans as well as the subsequent scans are referred to this mean baseline and expressed as percentage of mean baseline.

cGMP Determination

Whole blood samples were diluted in ethanol 1:3 (w/w) and put on ice for 30 min. Diluted ethanol/sodium chloride-solution 2:1 (volume/volume) was added to the diluted blood samples so that the final dilution of the samples in ethanol was 1:10.

After this procedure, the samples were mixed, centrifuged, and the supernatant was used for cGMP measurements via enzymimmunoassay. The determination procedure was carried out in accordance to manufacturer instructions, protocol #2 (c-GMP Enzymimmunoassay Biotrak (EIA) System, Amersham Biosciences Europe GmbH, Freiburg, Germany).

Statistics

All data are presented as means ± SEM. Statistical analysis for in vitro experiments was performed by two-tailed Student test and for in vivo experiments using one-way ANOVA test followed by post hoc test for least significant difference (LSD). Significance was based on a value of $P < 0.05$. The calculations were made with the software MS Excel (Microsoft Corp.) and SPSS 11.5 for Windows (SPSS Inc.).

RESULTS

Interaction between NO-Hb Complexes and Laser Irradiation

Addition of NO solution to the chamber containing buffer results in a quick

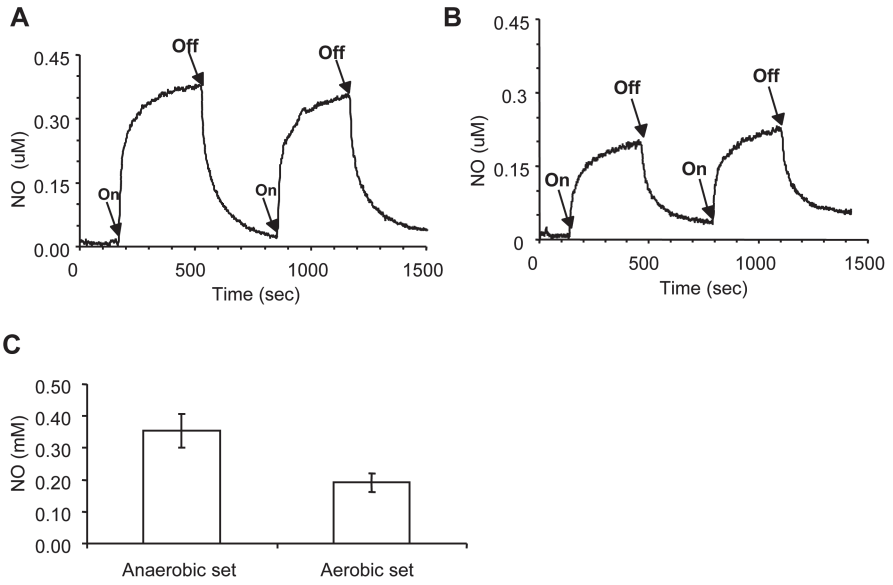

Figure 2. Kinetics of NO release and reabsorption triggered by He-Cd laser (40 mW) irradiation in erythrocytes enriched with NO-Hb.
(A) Anaerobic irradiation; (B) Aerobic irradiation; (C) Difference in NO concentration in solution due to switching the laser ON and OFF.

rise of the NO levels followed by a decay of NO levels, likely due to interaction with medium components and residues of oxygen. Addition of RBC results in an immediate disappearance of NO from the medium (Figure 1A). Thereafter a signal characteristic for NO-Hb was determined in the sample (Figure 1B). The same signal can be detected if whole blood is used instead of RBC (data not presented). He-Cd laser irradiation applied to the sample resulted in an increase in NO levels as detected by electrode technique (Figure 1A, inset) and in a decay of NO-Hb EPR signal (Figure 1C).

In details, the kinetics of photolytic release of NO was studied by means of NO-electrode. When NO-Hb containing erythrocytes were placed into the NO-electrode chamber, no free NO release was seen. With the start of laser irradiation, free NO was rapidly increased until a steady state was reached (Figure 2A & B). As soon as the laser light was turned off, the concentration of free NO decreased. This shows the reversible character of the photodissociation of NO-Hb. This phenomenon could be monitored both in anaerobic and aerobic conditions. However, the steady state concentration of NO was higher in anaerobic conditions (Figure 2C). To prove that NO-Hb is decreasing due to laser exposure, we looked at the samples incubated a total of 2 min with different exposure times. Table 1 shows that the levels of NO-Hb complexes decrease depending on the dose of irradiation. Moreover, the analysis of the shape of the spectrum shows that the changes are mostly on the account of 5-coordinated iron (Table 1).

Table 1. Effect of laser irradiation on the integral intensity and features of NO-Hb signal in RBC suspension enriched with NO.

Sets of samples (AU)	Incubation without laser (s)	Incubation under laser (s)	Integral intensity of NO-Hb signal (AU)	The ratio of EPR signal features of 5 to 6 coordinated NO-Hb
1	0	0	100.0 ± 1.0	0.46 ± 0.07
2	120	0	99.8 ± 0.7	0.42 ± 0.04
3	90	30	97.0* ± 1.38	ND
4	40	80	94.0* ± 2.25	ND
5	0	120	90.1* ± 0.35	0.26* ± 0.03

Effect of NO-Hb Infusion on Parameters of the Systemic Circulation

To clarify whether exposure of whole blood to NO can activate the NO-dependent signaling pathway, we determined cGMP levels in sham and NO-Hb enriched blood before infusion (Figure 3A) and in blood withdrawn from rats 12 min after infusion of sham and NO-Hb enriched blood (Figure 3B). Despite the fact that cGMP levels were significantly higher in fresh blood obtained from rats, there was no significant difference between sham and NO-Hb enriched blood in either case. There also was no difference in MAP determined 12 min after infusion of sham NO-Hb enriched blood (Figure 3C).

Effect of NO-Hb Infusion Combined with Laser Irradiation on Parameters of the Systemic Circulation

Systemic levels of cGMP and MAP were used to determine whether NO-Hb infusion combined with laser irradiation activate NO-dependent vasodilatation systemically. Following the protocol displayed in the scheme (Figure 4), we monitored the systemic blood pressure every 4 min and determined cGMP levels both 12 min after NO-Hb infusion and 12 min after the onset of laser irradiation. Figure 5 shows that there was no significant difference either between control and NO-Hb treated rats or before and after laser irradiation. In contrast to cGMP levels and MAP, the NO-Hb levels in blood decreased after laser irradiation (6.9 ±

1.7 nmol/mL vs. 3.5 ± 1.2 nmol/mL, respectively). In control rats, as well as before infusion of blood enriched with NO-Hb, the levels of endogenous NO-Hb were not detectable.

Effect of NO-Hb Infusion Combined with Laser Irradiation on the Local Tissue Perfusion

Laser Doppler images revealed no differences in superficial perfusion between control group and NO-Hb group during the baseline period (Figure 6, 7). Flap perfusion was stable during the infusion period for both the NO-Hb group and the control group (Figure 6,7). Irradiation of the left epigastric artery caused an immediate increase in perfusion in the NO-Hb group whereas flap perfusion did not change in the control group and remained at infusion period level. The second flap scan in the NO-Hb group reached a statistically significant level in perfusion compared with pre-irradiation level, which was also true for the 3rd scan with further increase of perfusion (Figure 6,7). The subsequent 1st scan in the post-irradiation period showed a decrease but still a statistically significant difference to control values. The last two scans revealed a continued decrease in perfusion and ended close to values observed in the control group.

DISCUSSION

Interaction of NO-Hb and Laser in Vitro

It has been previously shown that laser irradiation can induce dissociation of NO-Hb complexes in pure protein solution (16,17). In this study we have shown that this process can be induced also in RBC suspension (Figure 1,2). Moreover we have demonstrated that NO released from NO-Hb by laser can escape RBC. This was confirmed by detection of NO with NO-electrode in RBC medium and in blood. We have also shown that the release of NO is a reversible process, which takes place only under laser irradiation. After irradiation was accomplished, the levels of NO quickly decayed to undetectable levels.

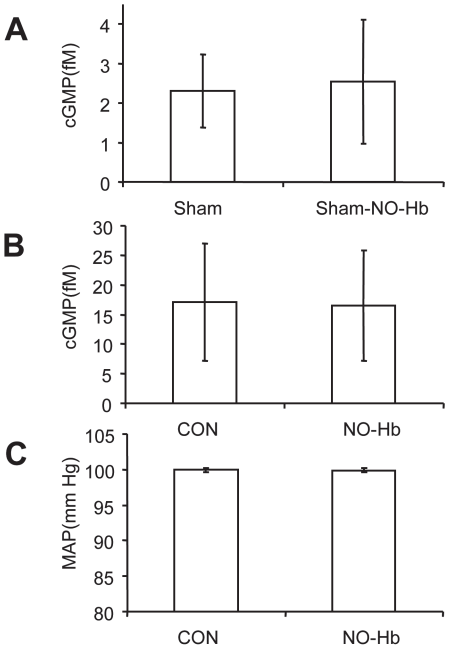

Figure 3. Effect of NO-Hb on cGMP levels and MAP.
(A)Effect of enriching with NO on the cGMP levels in sham blood (Sham) and in sham blood enriched NO-Hb (Sham-NO-Hb). (B) Effect of the infusion of sham and NO-Hb enriched blood on cGMP levels in the systemic circulation of rats. CON—resuscitation of sham blood; NO-Hb—resuscitation of sham blood enriched NO-Hb. (C) Effect of the infusion of sham and NO-Hb enriched blood on the MAP in systemic circulation of rats. CON—resuscitation of sham blood; NO-Hb—resuscitation of sham blood enriched NO-Hb.

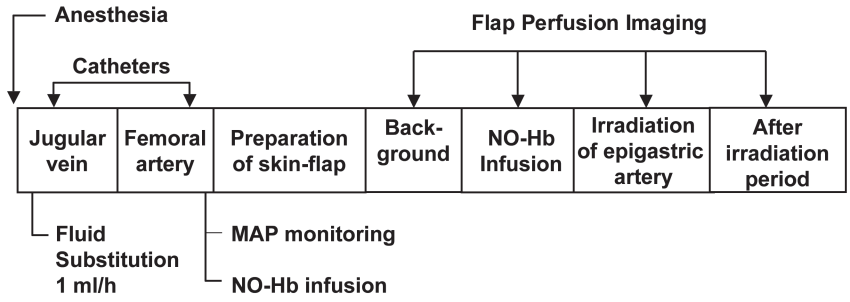

Figure 4. Experimental protocol: surgery and monitoring of systemic circulation (MAP) and local tissue perfusion (Doppler imaging).

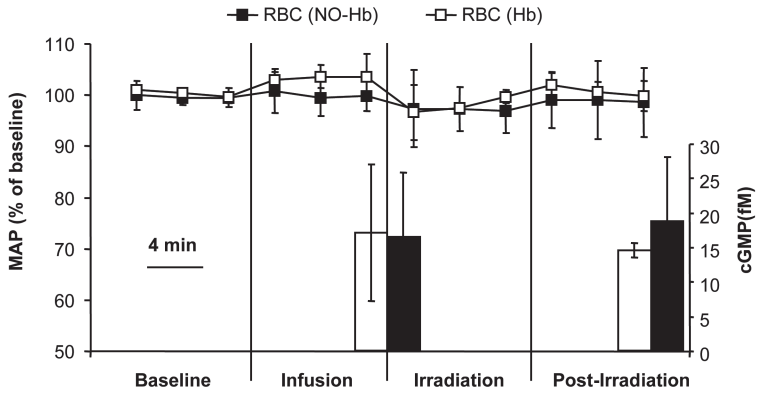

Figure 5. Effect of laser irradiation on the systemic levels of cGMP and MAP
Open bars/boxes—cGMP/MAP of rats receiving sham blood, respectively.
Closed bars/boxes—cGMP/MAP of rats receiving NO-Hb enriched blood, respectively.

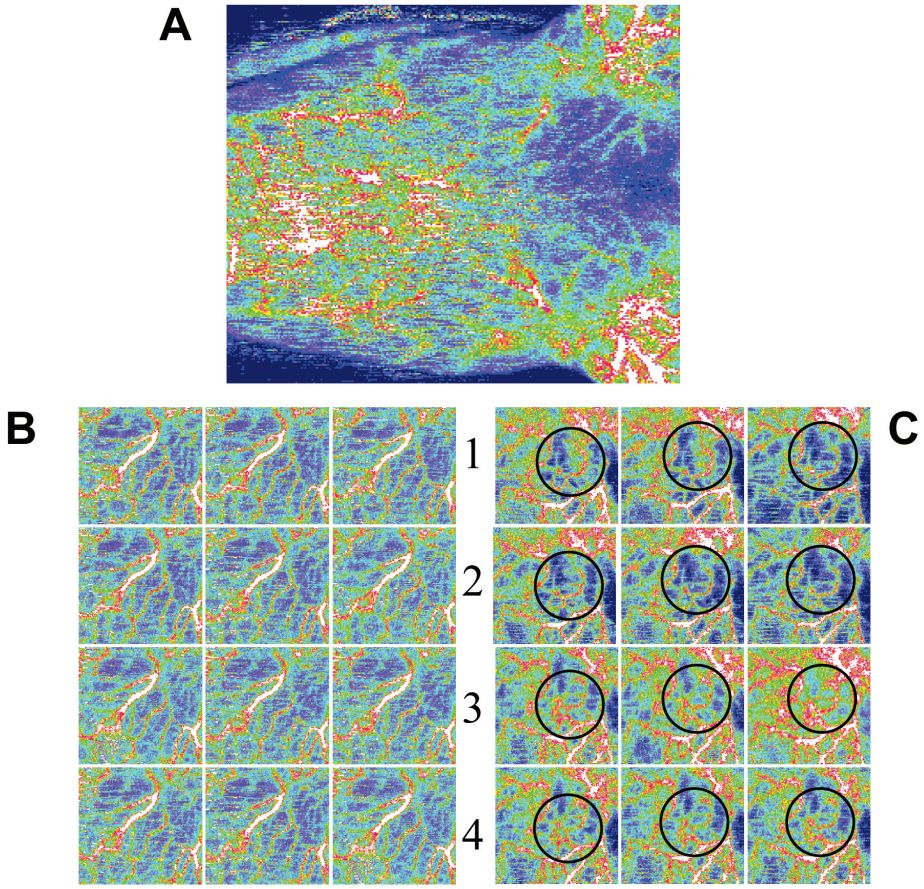

Figure 6. (A) Overview of superficial perfusion of the rodent epigastric area. (B) Control group with no alterations in perfusion (C) NO-Hb Group with increase in local perfusion during laser irradiation. Circles indicate the region where the maximal changes were observed. Quantification, however, was performed for whole region displayed in the figures. 1st column—Background, 2nd column—Infusion of NO-Hb, 3rd column—Irradiation of epigastric artery, 4th column—After irradiation period.

Because EC_{50} of guanylyl cyclase is approx. 2 nM (19), which is below the detection limit of the NO-electrode, it is not presumed that the remaining NO can not alter circulation systemically. The data presented in Table 1 show that irradiation influences the 5-coordinate iron nitrosyl complexes. 5-coordinate Fe(II)NO moieties is a signature of the T-state conformation, which does not lead to formation of SNO-Hb (7). This is an important point because it suggests that laser irradiation facilitates the release of NO from nitrosyl complexes of hemoglobin rather than the formation of S-nitroso-hemoglobin prior to NO release.

Effect of NO-Hb Infusion and Laser Irradiation on Systemic Circulation

We did not observe a decrease in MAP upon addition of NO-Hb, in spite of Gladwin et al (20) recently reporting a direct NO release from heme. However, looking just at Figure 5, a trend to lower blood pressure in animals receiving NO-Hb is seen. This trend could probably become significant with increasing NO-Hb levels. In such a situation NO-Hb complexes may influence systemic circulation, but this was not a focus of the present study. Nevertheless, this shows a compelling possibility to influence both systemic blood pressure and local tissue perfusion by means of infusion of NO-Hb. To clarify the question of whether photodissociation of NO-Hb can contribute to systemic circulation, we have chosen a model of skin flap perfusion, which makes possible a precise irradiation of the epigastric artery. In the experiments with flap perfusion, we have shown that neither infusion of NO-Hb nor infusion of NO-Hb combined with laser irradiation decreases blood pressure or elevates cGMP levels in circulating blood (Figure 3). Stable blood pressure indicates that spontaneous NO liberation from nitrosyl-hemoglobin does not occur in vivo, which is in line with recently published data (21). In contrast to unaltered MAP and cGMP levels, the concentration of NO-Hb complexes in blood decreased in response to laser irradiation. This indicates that NO is released from Hb but does not contribute to systemic circulation, probably due to the short lifetime of this molecule. However, it can be assumed that NO released from NO-Hb is able to influence the local circulation.

Effects of NO-Hb Infusion and Laser Irradiation on Local Circulation

We expected that the effect of laser irradiation should be limited to a small area in the vicinity of the irradiation point. Therefore we followed local circulation in the flap before and after laser irradiation by means of an LDI system. Neither infusion of NO-Hb alone, nor laser irradiation without NO-Hb infusion, did not influence local circulation. Only a combination of NO-Hb infusion and laser irradiation resulted in a remarkable increase of blood flow. We observed the main effect of epigastric artery irradiation directly distal to the laser focus. This indicates a targeted and strictly local limited irradiation effect, which can explain the unchanged cGMP levels in systemic circulation. Locally produced cGMP was simply diluted in systemic blood to undetectable concentrations. Our data suggest that laser irradiation induces the release of NO from nitrosyl complexes of hemoglobin followed by increased perfusion

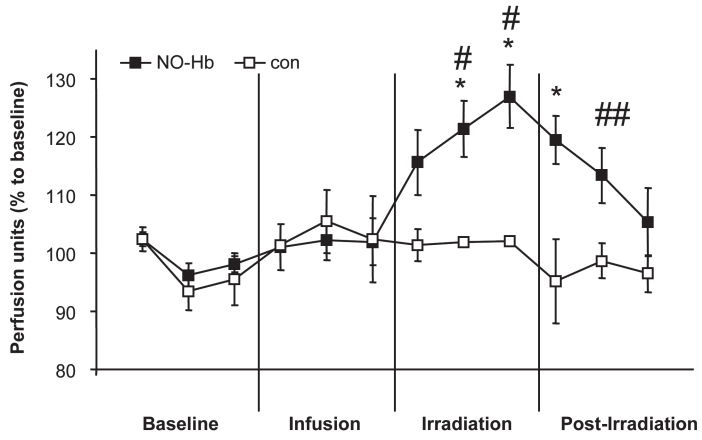

Figure 7. Quantification of laser Doppler scans of the epigastric flap in the rat.

of the tissue. However, we do not know the precise mechanism(s) underlying this effect. One could consider two possibilities. The first is that released NO diffuses to the vessel wall and induces vasodilatation. Another possible mechanism is recently reported in a series of publications of Marc Gladwin et al. considering nitrite as an intermediate in such a signaling pathway (20,22,23). It is difficult to say which of those pathways is operating predominantly in our experimental model; it is likely dependendent on many factors, primarily oxygen levels, which facilitate formation of nitrite from NO.

Possible Medical Applications

Our study clearly shows an increased local superficial flap perfusion by single spot irradiation using He-Cd laser combined with NO-Hb infusion. The perfusion area can be located precisely by positioning the laser beam on a corresponding blood vessel. In addition, the perfusion time can be determined exactly by turning the laser on and off. Thus, this study provides a new approach to improve local blood supply in a controlled manner, by local laser irradiation inducing NO release from NO-Hb complexes. This is the first report on an approach to regulate tissue perfusion in a precisely selected part of the body, which does not interfere with systemic blood circulation. The indications for such treatment can be flap surgery (pre-, post-surgery), non-surgical delay strategies or impaired blood supply in problematic superficial regions (chronic ulcers). In this study we infused exogenous NO-Hb complexes into control rats. However, these complexes can appear in the circulation, deriving from endogenous sources of NO. Therefore, it can be expected that under pathological circumstances, the effect of laser irradiation can occur without infusing NO-Hb. Such applications of laser irradiation are known as low level laser therapy (24–28).

Contribution to Low-Level Laser Therapy

Low-level laser therapy (LLLT) has received particular attention during the last decade. It has been reported to facilitate wound healing (24), particular venous leg ulcers (25), improve pain relief (26,27), rheumatoid arthritis (28) and a number of other disorders. However, recent attempts to review the results of LLLT have shown large variations in results due to a large heterogeneity of patients (26) and the absence of clear rules on how to choose the right irradiation conditions (28). At present, there are only empiric methods to choose the settings of LLLT. There is no objective method for immediate monitoring of the efficiency of LLLT and therefore little knowledge about mechanisms underlying LLLT is available. Our study shows that the photodissociation of NO-Hb may be a mechanism underlying effects of laser irradiation. This study provides a new approach to improve local blood supply in a controlled manner, by local laser irradiation-induced NO release from NO-Hb complexes. This is the 1st report on an approach to regulate tissue perfusion in a precisely selected part of the body which does not interfere with systemic blood circulation.

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