Far red/near infrared light-induced protection against cardiac ischemia and reperfusion injury remains intact under diabetic conditions and is independent of nitric oxide synthase

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INTRODUCTION

Restoration of blood flow to a region of previously ischemic myocardium (reperfusion) is a critical life-saving intervention against tissue necrosis, but reperfusion itself also results in significant damage to the myocardium. Many therapeutic strategies such as ischemic and volatile anesthetic pre- and postconditioning have been developed and are effective in healthy animal models but few have translated successfully to patients (Ludman et al., 2010). A major reason for the resistance to cardioprotection against infarction by physical or pharmacological stimuli is the advanced age and/or presence of comorbidities such as diabetes in patients. For example, endothelial dysfunction appears to contribute to the lack of protection by ischemic or anesthetic postconditioning in diabetes (Raphael et al., 2010; Przyklenk et al., 2011). Activation of endothelial nitric oxide synthase (eNOS) and pro-survival signaling pathways, together with alteration of mitochondrial bioenergetics, contribute to the mechanisms of various cardioprotective strategies against ischemia and reperfusion injury (Tsang et al., 2004; Mio et al., 2009; Ge et al., 2010).

Although nitric oxide synthases (NOS) produce a large part of endogenous nitric oxide (NO), there is considerable interest in NOS-independent generation of NO in vivo, particularly during hypoxia or anoxia, where low oxygen tensions limit NOS activity (Godecke, 2006; Hendgen-Cotta et al., 2008, 2010). Interventions that can increase NO bioavailability have significant therapeutic potential. Under hypoxic conditions, heme-containing proteins such as myoglobin (Mb) and hemoglobin (Hb) exhibit nitrite reductase activity which results in an increase in NO bound to the heme iron of Mb and Hb (Gladwin et al., 2006; Hendgen-Cotta et al., 2008). We have recently found that far red/near infrared...
light (NIR) both in purified systems and in myocardium can release NO from nitrosyl hemes (Lohr et al., 2009). Further, NIR protected cardiomyocytes and the rabbit heart from hypoxia and reoxygenation injury in a NO-dependent manner, reversible by NO scavenger cPTIO, and enhanced the protective effect of nitrite against ischemia and reperfusion injury of the rabbit heart (Lohr et al., 2009; Zhang et al., 2009).

NIR modulates biochemical systems by activating light-sensitive proteins harboring NIR-sensitive chromophores (Karuv, 1999; Desmet et al., 2006). Previous studies suggested that NIR promotes cell survival during physiologic stress (Eells et al., 2003; Liang et al., 2008; Zhang et al., 2009). Repeated photostimulation of the myocardium has been demonstrated to be beneficial against long-term reperfusion injury in the rat and dog (Oron et al., 2001). For example, low-energy infrared (803 nm) laser irradiation delivered to the epicardium was shown to reduce scar formation and myocardial infarct size several weeks after prolonged coronary artery occlusion in dogs and rats (Oron et al., 2001). Aside from the heart, the beneficial effects of NIR light treatment have been studied in particular in a model of traumatic brain injury as well as in wound healing (Ankri et al., 2010; Naeser et al., 2011). NIR light treatment also improved the collateral blood vessel grow in a mouse model (tight skin mouse) of scleroderma (Zaidi et al., 2013). Frequently, the beneficial effects of NIR light treatment have been associated with the stimulation of mitochondrial metabolism, particularly at the level of cytochrome c oxidase, complex IV of the electron transport chain (Karuv, 2008). However, in a model of cardiac ischemia and reperfusion injury it is difficult to perceive how acceleration of cytochrome c oxidase at the time of reperfusion conveys protection to the heart. Rather, a mild reversible inhibition of the electron transport chain has been shown to reduce reactive oxygen species production during reperfusion, thereby increasing cardiomyocyte survival (Burwell et al., 2009). NO inhibits electron transport through competitive binding at complex IV and 5-nitrosation at complex I (Piantadosi, 2012; Chouchani et al., 2013). Thus, we tested the hypothesis that brief exposure to NIR light at the time of reperfusion protects the heart in a wave length-dependent manner; and that this wave length dependence is paralleled by the release of NO from nitrosyl-heme proteins. We also examined whether NIR induced protection is maintained in a mouse model of acute hyperglycemia and diabetes (db/db) where protection by volatile anesthetics fail.

**MATERIALS AND METHODS**

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin. Furthermore, all conformed to the *Guiding Principles in the Care and Use of Animals* of the American Physiologic Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.

**MYOCARDIAL ISCHEMIA AND REPERFUSION INJURY IN MICE**

A murine model of myocardial ischemia and reperfusion injury was used as previously described (Ge et al., 2010). C57BL/6 (wild type) mice, as well as eNOS<sup>−/−</sup> and diabetic db/db mice were used for these experiments. Glucose (2 g/kg) was administered intraperitoneal 10 min before ischemia to produce hyperglycemia. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (100 mg/kg) and ventilated with room air supplemented with 100 % oxygen at a rate of 100 breaths/min with a tidal volume of approximately 0.25 ml using a rodent ventilator (Harvard Apparatus, South Natick, MA). Body temperature was maintained between 36.8 °C and 37.5 °C. Myocardial ischemia was produced by occluding the left coronary artery (LAD) for 30 min, and reperfusion was initiated by loosening the suture and continued for 3 h.

**EXPERIMENTAL PROTOCOL**

Mice were randomly assigned to receive no irradiation (control) or NIR irradiation applied to the epicardial surface (670 nm, 170 mW/cm²) with an LED array (NIR Products LLC, Milwaukee, WI) for 1 min before and through the first 4 min of reperfusion (energy-density equivalent to 51 J/cm²). Separate experiments were performed to evaluate the energy- and wavelength-dependence of NIR-mediated cardioprotection by varying the array (670, 740, 830 nm) and the output of the device.

C57BL/6, eNOS<sup>−/−</sup> mice and db/db mice were used to explore the dependence of NIR-mediated cardioprotection on eNOS and its efficacy in a diabetic animal. Acute hyperglycemia was induced by administration of D-glucose (2 g/kg) in C57BL/6 mice 10 min before ischemia. Mannitol (1.82 g/kg) was used for osmotic control in preliminary experiments in C57BL/6 mice both with and without NIR treatment and did not exhibit any significant effect compared to mice that did not receive mannitol. Pharmacological inhibition of eNOS was used to complement the experiments in eNOS<sup>−/−</sup> mice and thus, C57BL/6 mice received 1mg/kg, i.v. of the non-selective NOS inhibitor L-NAME prior to LAD occlusion and reperfusion.

**DETERMINATION OF MYOCARDIAL INFARCT SIZE**

For infarct size measurements, the heart was first stained by cannulation of the aorta with a 1 % solution of 2,3,5-triphenytetrazolium chloride. Then the suture previously placed around the left descending coronary artery was retied and diluted phthalo blue dye was injected through the same cannula. As a result of these procedures, the non-ischemic portion of the left ventricle was stained dark blue. Viable myocardium within the area at risk was stained bright red, and infarcted tissue was light yellow. The heart was then excised and ventricles were cut into 4–5 uniform transverse slices of 2 mm thickness using a mouse heart matrix. Slices were then analyzed by planimetry.

**NITROSYL HEMOGLOBIN (HbNO) AND NITROSYL MYOGLOBIN (MbNO) PREPARATION**

Oxyhemoglobin purified from human blood according to a published procedure (Rossi-Fanelli et al., 1961) was deoxygenated, or solution of metmyoglobin (from horse skeletal muscle, Sigma) was reduced in an anaerobic chamber with Na2S2O4 in phosphate buffered saline (PBS, pH 7.4). Then the heme proteins were nitrosylated by addition of equivalent concentration of highly concentrated PROLI NONOate (Cayman Chemicals, Ann
Arbor, Mi) dissolved in 0.1 N NaOH. The process was spectrophotometrically followed. Solutions were made daily, and used immediately.

**NO-DEPENDENT CHEMILUMINESCENCE ANALYSIS**

A Sievers 280i Nitric Oxide Analyzer (General Electric, Boulder, CO) was used to detect NO evolved from nitrosyl species as a consequence of NIR irradiation. HbNO or MbNO (3 ml of 10 μM) was placed into the purge vessel of the analyzer, and externally irradiated at various powers and wavelengths for 1 min. Detector response for NO liberated from known amounts of PROLI NONOate injected into PBS pH 7.4 was used as a basis of quantification.

**MEASUREMENT OF OXYGEN CONSUMPTION IN ISOLATED MITOCHONDRIA**

Rat heart mitochondria were isolated by differential centrifugation as previously reported (Pravdic et al., 2010). Mitochondrial oxygen consumption was measured with a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, UK) at 30 °C in respiration buffer containing mitochondria at a final concentration of 1 mg protein/mL. The mitochondrial respiration buffer was composed of 130 mM KCl, 5 mM KH2PO4, 20 mM MOPS, 2.5 mM EGTA, 1 mM Na2HPO4, and 0.1 % BSA, at pH 7.4. State 2 respiration was initiated with 5 mM pyruvate and 5 mM malate as substrates. The adenosine diphosphate (ADP)-stimulated oxygen consumption (state 3 respiration) was measured in the presence of 250 μM ADP. After hypoxia was reached mitochondria were incubated with deoxymyoglobin (40 μM; prepared from myoglobin with sodium dithionite as reducing agent) and sodium nitrite (20 μM) for 1 min and then exposed directly to NIR (170 mW/cm²) for another min. After that the chamber was opened to allow reoxygenation. A faster rate of reoxygenation of the chamber indicated an inhibition of respiration.

**STATISTICAL ANALYSIS**

Statistical analysis of data within and between groups was performed with analysis of variance (ANOVA) for repeated measures followed by the Student-Newman-Keuls test. Changes were considered statistically significant when $P < 0.05$. All data are expressed as mean ± standard deviation (SD) unless otherwise indicated.

**RESULTS**

The mouse was chosen as model to determine the efficacy of NIR-mediated protection against cardiac ischemia and reperfusion injury in order to expand our findings on NIR-induced protection in rabbits and due to the advantage of the large availability of genetically engineered animals. Exposure to NIR for the last min of occlusion and first 4 min of reperfusion significantly ($P < 0.05$) reduced infarct size at the highest chosen irradiance (170 mW/cm², corresponding to 51 J/cm²) compared to control experiments without NIR exposure (31 ± 7 vs. 51 ± 4 % of left ventricular area of risk, Figure 1). The effect of NIR on infarct size was energy dependent. Myocardial infarct size was 59 ± 5 and 39 ± 6 %, at an irradiance of 10 and 27 mW/cm², corresponding to 3 and 8.1 J/cm² respectively. Thus, the threshold of cardioprotection appeared to occur at an irradiance level of 30 mW/cm².

Importantly, no increase in epicardial surface temperature upon exposure to NIR was observed.

We then tested whether NOS is involved in the mechanism of NIR-induced protection. Pretreatment with the non-selective NOS inhibitor L-NAME had no effect alone (60 ± 6 % infarct size of area at risk), nor did it inhibit NIR-mediated reduction in infarct size (42 ± 5 %). Similarly, eNOS−/− mice were also protected against myocardial ischemia and reperfusion injury by NIR treatment (44 ± 5 % compared to 59 ± 4 % without treatment). An irradiance of 170 mW/cm² was applied in all cases. These data suggest that NIR-mediated cardioprotection is independent of the activity of NOS (Figure 2).

We recently reported (Lohr et al., 2009) that NIR light has the capacity to liberate NO from nitrosylated hemoglobin (HbNO) and myoglobin (MbNO). Here we examined the wavelength dependence of NO release and protection. A solution of
HbNO or MbNO (10 µM) was placed into the purge vessel of a chemiluminescence-based NO analyzer and subjected to irradiation for 1 min. We observed 2–3 times more NO released at 670 nm compared to 740 and 830 nm at 10 mW/cm² irradiance (Figure 3A). As NIR does not have to penetrate tissue in these experiments, less irradiance compared to what is required for protection of the in vivo heart is needed. There was no significant difference between NO liberation from HbNO and MbNO. A similar trend was found with the wavelength dependence of NIR-induced reduction of infarct size. In contrast to 670 nm no significant protection was observed at 740 and 830 nm (Figure 3B).

Mitochondria are a potential therapeutic target of NO produced at the time of reoxygenation (Chouchani et al., 2013). Therefore, experiments were designed to establish the net outcome of NIR-enhanced nitrite reductase activity on mitochondrial respiration after hypoxia. We measured the reoxygenation rate of a mitochondrial suspension after hypoxia in the presence of deoxymyoglobin and nitrite, with and without NIR (660 nm, 50 mW/cm²) (Figure 4). While Mb and nitrite induce inhibition of mitochondrial respiration alone (Shiva et al., 2007a,b; Hendgen-Cotta et al., 2008), we hypothesized that light enhances this inhibition through its action on MbNO formed as a consequence of nitrite reductase activity of heme. We found that NIR, while alone had no considerable effect, could potentiate the inhibition caused by Mb and nitrite at lower nitrite doses. It triggered a significantly faster reoxygenation and thereby a decrease in respiration rate in the presence of deoxymyoglobin and nitrite than solely deoxymyoglobin and nitrite would induce. A partial compensatory effect of NO bound to and released from complex IV cannot be excluded, however, in the investigated in vitro system Mb was present in wide excess over cyt c oxidase (0.64 mg/ml Mb vs. 1 mg/ml total mitochondrial protein), and NIR was switched off at the time of reoxygenation. Thus, NO released from Mb might partially bind to cytochrome c oxidase or mediate S-nitrosation of complex I at the beginning of reoxygenation, thereby accelerating reoxygenation and inhibiting respiration. The observed effect is relevant since a mild reversible inhibition of the mitochondrial electron transport chain during cardiac reperfusion has been shown to reduce reactive oxygen
species production. In the presence of NO scavenger cPTIO (10 μM), the NIR effect was reversed.

In diabetes, endothelial dysfunction, including defective NOS, is considered one of the causes for the failure of protective strategies such as ischemic or anesthetic pre- and postconditioning to reduce cardiac ischemia and reperfusion injury. Therefore, a NOS-independent mechanism of NO generation may allow NIR to reduce ischemia and reperfusion injury in the hyperglycemic or diabetic heart. Indeed, a similar degree of NIR-induced cardioprotection was observed in mice that were exposed to acute hyperglycemia (39 ± 4 % myocardial infarct size of area at risk vs. 52 ± 2 % without NIR) and in the diabetic db/db mouse (43 ± 4 vs. 61 ± 3 %) compared to the wild type mice (41 ± 3 % vs. 56 ± 3 %).

**DISCUSSION**

The current results demonstrate that a brief exposure to NIR immediately before and during early reperfusion protects the myocardium against infarction in an NOS-independent mechanism. Mitochondria are one potential therapeutic target of NIR-induced release of NO but other targets such as NO-sensitive guanylyl cyclase require further investigation. Importantly, NIR protects the hyperglycemic and diabetic heart. The absence of such protections has been one of the major hurdles in the implementation of pharmacological pre- and particularly postconditioning into the clinical setting.

In the nineteen nineties in Russia patients with coronary heart disease with prior myocardial infarction were exposed repeatedly to NIR by low-level laser therapy (LLLT) applied to the area of the heart on the skin. Lipid peroxidation was significantly reduced after NIR but little is known on whether cardiac function improved (Zubkova et al., 1993; Sorokina et al., 1997). In subsequent studies NIR was applied after chronic myocardial infarction in rat and dog models. NIR (803 nm, 6 mW/cm² at the surface of the myocardium for 3 min, at 4-6 different locations) was applied twice, 15 min and 3 days after myocardial infarction, through the open chest directly onto the myocardium in dogs, and through the intercostal muscles in rats. Both mortality and infarct size were significantly reduced compared to untreated animals (Oron et al., 2001). Irradiation with NIR after myocardial infarction in rats resulted in a significant improved mitochondrial bioenergetics, and an increase in an inducible heat shock protein (HSP70), vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) expression (Yaakobi et al., 2001). This was paralleled by a significant elevation in angiogenesis (Tuby et al., 2006). More recently, increased angiogenesis and collateralization upon NIR exposure (670 nm, 50 mW/cm², 10 min per day for 14 days) with LED have also been reported in the ischemic hind limb of mice and rabbits (Lohr et al., 2013). In a mouse model for systemic sclerosis, an autoimmune connective tissue disorder characterized by oxidative stress, impaired vascular function, and attenuated angiogenesis, NIR stimulated angiogenesis by increasing angiogenin and decreasing angiotatin expression in the ischemic hind limb (Zaidi et al., 2013).

In the present study we found that myocardial infarction can be prevented from occurring, or at least reduced by a one-time NIR treatment right at the time of reoxygenation. It appears highly unlikely that NIR-induced reductions in infarct size were attributed to increased collateral perfusion as NIR-induced angiogenesis typically occurs as a result of sustained stimulation over several days. Therefore, the underlying mechanism of protection is likely different. Under hypoxic conditions heme-containing proteins such as myoglobin (Mb) and hemoglobin (Hb) exhibit nitrite reductase activity which results in an increase in NO liberation (Gladwin et al., 2006; Hendgen-Cotta et al., 2008). The NO formed may subsequently react with available deoxyHb or deoxyMb to yield iron-nitrosyl Hb (HbNO) or iron-nitrosyl Mb (MbNO). Thus, HbNO and MbNO may represent a significant storage pool of NO in the heart. Here we have demonstrated both for purified hemoglobin and myoglobin that NIR can decay nitrosyl heme and release NO in a wavelength-dependent manner. Importantly, the highest NO release was recorded at 670 nm where protection against ischemia and reperfusion injury was present (Figure 3). This further suggests a distinct mechanism from the previously reported protection through repeated NIR treatment in the permanently ligated heart where longer wavelengths were equally protective. We previously reported in the ischemic rabbit heart, after infusion of sodium nitrite, a large increase in nitrosyl heme formation as measured by electro paramagnetic resonance spectroscopy (EPR). The MbNO signal was reduced in the ischemic zone by NIR treatment suggesting dissociation of the heme-NO bond upon irradiation (Lohr et al., 2009).

Frequently, the beneficial effects of NIR treatment have been associated with the stimulation of mitochondrial metabolism, particularly at the level of cytochrome c oxidase, complex IV of the electron transport chain and concomitant enhancement of ATP synthesis (Karu, 2008). NIR may directly affect cytochrome c oxidase activity through one of its redox active metal centers. In addition, it has been suggested that NIR exerts its action on cytochrome c oxidase by a mechanism via NO release. The activated cytochrome c oxidase may not only cause changes in electron transport chain activity, including ROS generation, but released NO is available for other biological processes such as vasodilation and gene expression. However, compared to potential NO release from HbNO or MbNO the relative amounts of NO in the case of cyt c oxidase is limited (Osipov et al., 2007). Further, it is difficult to perceive how acceleration of cytochrome c oxidase at the time of reperfusion conveys protection to the heart. Rather, a mild reversible inhibition of the electron transport chain has been shown to reduce reactive oxygen species production during reperfusion and increase cardiomyocyte survival (Burwell et al., 2009). This was confirmed in ischemic isolated mitochondria where, in the presence of deoxymyoglobin and sodium nitrite, a decrease in respiration was detected upon reoxygenation of mitochondria after application of NIR (Figure 4). NO signaling may lead to S-nitrosation of a cysteine residue in complex I that has been implicated in protection against cardiac ischemia and reperfusion injury (Cochain et al., 2013). Reversible S-nitrosation of complex I slows the reactivation of mitochondria during the crucial first minutes of the reperfusion of ischemic tissue, thereby decreasing ROS production, oxidative damage and tissue necrosis.
to the heart with a flexible fiber optic NIR probe. The probe in the esophagus or stomach (when advanced) is immediately adjacent to the left atrium and the inferior and posterior walls of the left ventricle. Thus, the anterior wall that is frequently affected by myocardial infarction may be as much as 6 cm away from the probe. Still, it may not be necessary for NIR light to penetrate the area at risk directly. A remote effect of NIR, comparable to remote preconditioning, might still provide protection and lead to a reduction of infarct size. Signaling factors such as heat shock proteins or NO may mediate such effect.

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FIGURE 5 | NIR is cardioprotective in the presence of diabetes or hyperglycemia. The exposed heart of wild type, db/db, and hyperglycemic mice was irradiated at 670 nm with 170 mW/cm² irradiance for 1 min during ischemia, and 4 min during reperfusion. Light exposure resulted in a similarly effective decrease of infarct size in all three cases. Values are means ± SD, *p < 0.05 when compared to untreated wild type.
