

## Original article

## Enhancement of nitric oxide release from nitrosyl hemoglobin and nitrosyl myoglobin by red/near infrared radiation: Potential role in cardioprotection

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## ABSTRACT

Nitric oxide is an important messenger in numerous biological processes, such as angiogenesis, hypoxic vasodilation, and cardioprotection. Although nitric oxide synthases (NOS) produce the bulk of NO, there is increasing interest in NOS independent generation of NO *in vivo*, particularly during hypoxia or anoxia, where low oxygen tensions limit NOS activity. Interventions that can increase NO bioavailability have significant therapeutic potential. The use of far red and near infrared light (R/NIR) can reduce infarct size, protect neurons from methanol toxicity, and stimulate angiogenesis. How R/NIR modulates these processes *in vivo* and *in vitro* is unknown, but it has been suggested that increases in NO levels are involved. In this study we examined if R/NIR light could facilitate the release of NO from nitrosyl heme proteins. In addition, we examined if R/NIR light could enhance the protective effects of nitrite on ischemia and reperfusion injury in the rabbit heart. We show both in purified systems and in myocardium that R/NIR light can decay nitrosyl hemes and release NO, and that this released NO may enhance the cardioprotective effects of nitrite. Thus, the photodissociation to NO and its synergistic effect with sodium nitrite may represent a noninvasive and site-specific means for increasing NO bioavailability.

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### 1. Introduction

Nitric oxide (NO) is enzymatically generated during the oxygen-dependent conversion of L-arginine to L-citrulline by the nitric oxide synthases (NOS). These enzymes are present or are inducible in numerous cell types, including vascular endothelium, neuronal cells, macrophages and cardiomyocytes [1]. Although NOS are primarily responsible for the bulk of NO production, there is increasing interest in NOS independent generation of nitric oxide *in vivo*, particularly during hypoxia or anoxia, where low oxygen tensions limit the activity of the NOS enzymes [2]. Nitrite, and to some extent nitrate through its reduction to nitrite, have been identified as additional physiological sources of NO [3]. Identified nitrite reductases include deoxyhemoglobin [4,5], deoxymyoglobin [6], xanthine oxidoreductase [7], cytochrome c oxidase [8,9] and NOS itself [10]. In the case of hemoglobin, the nitrite reductase activity is related to the redox potential of the heme iron, and is significantly greater in the 'R' conformation than in the 'T' conformation allowing maximal nitrite reduction at approximately 50% oxygen saturation [11–13]. Once formed, a significant portion of the

NO generated will bind to vacant deoxygenated hemes to yield the product iron–nitrosyl hemoglobin (HbNO) or iron–nitrosyl myoglobin (MbNO) [6,14]. The extremely slow off-rate of NO from these ferrous heme complexes renders them largely inert in terms of NO storage, although it has been suggested that processes which oxidize the heme iron could facilitate NO release [6]. These observations suggest any exogenous stimulation that could enhance NO release from these stable complexes could potentiate the NO-dependent effects of nitrite.

There is tremendous interest in the use of light for clinical applications. Wavelengths in the red to near infrared (R/NIR) spectrum between 630 and 830 nm have been extensively studied *in vivo* and *in vitro*. In this range there is improved tissue penetration due to the absence of melanin absorption in the skin [15]. Water exhibits minimal light absorption, thereby reducing radiant heat production in the exposed tissue to below 0.5 °C [16,17]. Near infrared light has been used to reduce infarct size, protect neurons from methanol toxicity, heal chemotherapy induced mucositis, and stimulate angiogenesis [18–21]. Increased nitric oxide is a postulated effector in the mechanism by which near infrared light exerts its actions, but how nitric oxide becomes bioavailable is not well understood. There is strong evidence that cytochrome c oxidase can act as a photoreceptor allowing the photolytic dissociation of any bound nitric oxide [22,23]. Additionally, the nitric oxide present in the heme-Cu<sup>II</sup>a<sub>3</sub> can be photolysed by visible light [24].

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In this study we examined if R/NIR light could facilitate the release of NO from nitrosyl heme proteins. In addition, we examined if R/NIR light could enhance the protective effects of nitrite on ischemia/reperfusion injury in the isolated rabbit heart. We show in both chemical systems and in heart tissue that R/NIR light can decay nitrosyl hemes and release NO, and that this released NO may enhance the cardioprotective effects of nitrite. We suggest that R/NIR exposure may enhance the efficacy of nitrite-dependent protective strategies.

## 2. 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*.

All experiments were carried out in 10 mM phosphate buffered saline pH 7.40 (PBS). Diethylenetriamine pentaacetic acid (DTPA) was added to all buffers at a concentration of 1 mM for chelation of metal ions.

### 2.1. R/NIR source

The 670 nm LED light source with power output up to 100 mW/cm<sup>2</sup> was obtained from Quantum Devices. Power output was measured with a light meter (X97 Irradiance meter, GigaHertz-Optik). The light sources were placed 2.5 cm from their target in all experiments.

### 2.2. Generation of nitrosyl hemoglobin

Oxyhemoglobin (OxyHb) was prepared from fresh human blood as previously described [25]. The oxyHb solution (approximately 200  $\mu$ M) was placed in a long-neck quartz cuvette closed with an airtight rubber stopper. The sample was deoxygenated using gentle agitation with alternating vacuum assisted removal of oxygen and introduction of argon gas, and monitored spectrophotometrically. After deoxygenation, the NO donor, PROLI NONOate (Cayman Chemical) was added, from a deoxygenated 10 mM stock solution in 100 mM NaOH, and the UV/Vis spectrum (HP 8453) was acquired between 450 and 700 nm to verify conversion to nitrosyl hemoglobin. HbNO concentration was determined using an extinction coefficient of 12.5 mM<sup>-1</sup> cm<sup>-1</sup> at 575 nm [26].

### 2.3. Generation of nitrosyl myoglobin

Metmyoglobin (metMb) from horse skeletal muscle (Sigma) dissolved in PBS containing 1 mM DTPA was degassed and saturated with high purity argon, then reduced to deoxymyoglobin (deoxyMb) by titration with sodium dithionite (Sigma). Myoglobin reduction was followed spectrophotometrically. When myoglobin reached the fully deoxy state, excess of highly concentrated PROLI NONOate (Cayman Biochemicals) dissolved in 100 mM NaOH was added. Solutions were made daily, and stored under argon.

### 2.4. Ozone chemiluminescence measurement of nitric oxide

HbNO or MbNO (20  $\mu$ M) in PBS containing DTPA (1 mM) and Antifoam emulsion B were added to a purge vessel, equilibrated at 30 °C in line with a chemiluminescence NO gas analyzer (Sievers NO analyzer; GE Analytical Instruments) for the detection of NO. The vessel was purged with argon gas. The purge vessel was irradiated with the R/NIR placed 2.5 cm from the reaction vessel. NO was detected by chemiluminescence after reaction with ozone. In addition, oxyHb was deoxygenated in the purge vessel before addition of nitrite

(1 mM). This mixture was then subjected to irradiation and generated NO was detected after reaction with ozone. For quantification purposes, a standard curve was constructed using PROLI NONOate.

### 2.5. UV-visible spectrum analysis

HbNO (final concentration 20  $\mu$ M) was added to a quartz cuvette under aerobic conditions and irradiated with the R/NIR light source placed 2.5 cm above the cuvette. Visible absorption spectra were taken every 2.5 s and deconvoluted using least squares analysis as previously described [13]. The standard reference spectra used for the least squares analysis were oxyhemoglobin, deoxyhemoglobin, iron–nitrosyl-hemoglobin, and methemoglobin. All experiments were performed in triplicate.

### 2.6. Electron spin resonance of MbNO

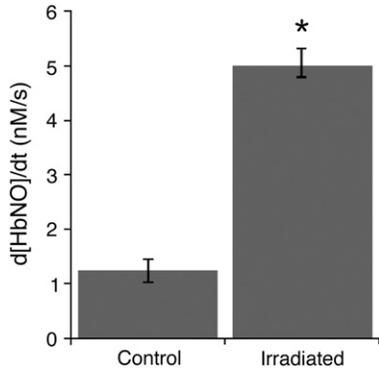
Rabbit myocardium from the control and ischemic zones was pulverized in liquid nitrogen and packed in an EPR sample tube. Tissue was kept frozen at all times. The tissue was examined by EPR (Bruker Elexsys) using the following conditions: temperature, 100 K; modulation amplitude: 5G; microwave power: 5 mW; scan width: 400G; scan time 41.94 s, time constant: 20.48 ms. The remaining control and ischemic tissue after pulverization was irradiated with red light (670 nm, 11 J total energy) before EPR analysis. All experiments were performed in triplicate.

### 2.7. Myocardial ischemia reperfusion experiments

Male New Zealand white rabbits weighing between 2.5 and 3.0 kg were anesthetized with intravenous sodium pentobarbital (30 mg/kg) as previously described [27]. Briefly, a tracheostomy was performed through a midline incision, and each rabbit was ventilated with positive pressure using an air–oxygen mixture (FiO<sub>2</sub> 30%). Arterial blood gas tensions and acid–base status were maintained within a normal physiological range by adjusting the minute ventilation. A thoracotomy was performed at the left fourth intercostal space, a prominent branch of the left anterior descending coronary artery (LAD) was identified, and a silk ligature was placed around this vessel approximately halfway between the base and the apex for the production of coronary artery occlusion and reperfusion. Coronary artery occlusion was verified by the presence of epicardial cyanosis and regional dyskinesia in the ischemic zone, and reperfusion was confirmed by observing an epicardial hyperemic response. Hemodynamics were continuously recorded on a polygraph throughout each experiment.

For the *in vivo* generation of MbNO using nitrite in rabbit hearts, 50 mg of sodium nitrite dissolved in 0.9% saline was infused into the left atrium during 30 min LAD occlusion. After occlusion, a 5 min reperfusion was performed to ensure adequate tissue penetration of the sodium nitrite into the ischemic zone. Since MbNO generation requires nitrite in the presence of hypoxia, a 5 min reocclusion period was performed. The heart was excised at the end of reocclusion and snap-frozen in liquid nitrogen.

To assess the effect of R/NIR on cardiac ischemia and reperfusion injury, all rabbits underwent a 30 min LAD occlusion followed by 3 h of reperfusion. In six separate groups, rabbits ( $n = 6$  to 7 per group) were randomly assigned (Latin square design) to receive 0.9% saline (control Group 1), or R/NIR irradiation (670 nm at 60 mW/cm<sup>2</sup>) was applied to the heart for 3 min followed by a 3 min resting period. Group 2 received 3 cycles of irradiation (32.4 J) prior to the onset of ischemia. Group 3 received 5 cycles of irradiation prior to ischemia (54 J). Group 4 received 0.4 mg/kg sodium nitrite (Sigma) dissolved in 0.9% normal saline i.v. 30 min prior to ischemia. Group 5 received 4 mg/kg sodium nitrite in 0.9% normal saline as an i.v. 30 min prior to ischemia. Group 6 received a 0.4 mg/kg sodium nitrite i.v. and then 3 cycles of R/NIR irradiation (32.4 J) prior to the onset of ischemia.



**Fig. 1.** UV-visual absorbance data was collected from control HbNO (20  $\mu$ M), and HbNO exposed to 14 J of light at 670 nm ( $n=3$ ). The decay rate of HbNO was calculated from linear regression analysis using basis spectra for methHb and HbNO. Irradiation produced a 4-fold increase in the rate of methHb conversion.  $p<0.05$ .

Cardioprotection studies were repeated using infusions of the nitric oxide scavenger carboxy-PTIO (Alexis Chemicals). Group 1 received 5 cycles of irradiation (54 J) prior to ischemia, with an infusion of c-PTIO at 0.17 mg/kg/min during the entire period of irradiation. Group 2 received 0.4 mg/kg sodium nitrite i.v. and then 3 cycles of irradiation (32.4 J) with a c-PTIO infusion of 0.17 mg/kg/min during the irradiation period prior to the onset of ischemia.

To assess the effect of R/NIR when applied upon reperfusion, additional rabbits underwent a 30 min LAD occlusion followed by 3 h of reperfusion. In four groups ( $n=7$ /group) rabbits were randomly assigned. Group 1 received 3 min of irradiation (60 mW; 11 J) at the time of reperfusion, with light exposed to the myocardium just prior to the release of the ligature. Group 2 received 3 min of irradiation (3 mW; 0.5 J) Group 3 received 0.4 mg/kg sodium nitrite (Sigma) dissolved in 0.9% normal saline i.v. 30 min prior to ischemia. Group 4

received 0.4 mg/kg sodium nitrite i.v. 30 min prior to ischemia and then 3 min of irradiation (3 mW; 0.54 J) upon reperfusion.

To assess the role of nitric oxide scavenging on R/NIR cardioprotection, 2 additional groups were added. Group 1 received an infusion of c-PTIO (0.17 mg/kg/min) over the 3 min period of irradiation (60 mW/cm<sup>2</sup>) at the time of reperfusion. Group 2 received 0.4 mg/kg sodium nitrite i.v. 30 min prior to ischemia and then c-PTIO (0.17 mg/kg/min) over the 3 min of irradiation (0.54 J) at reperfusion.

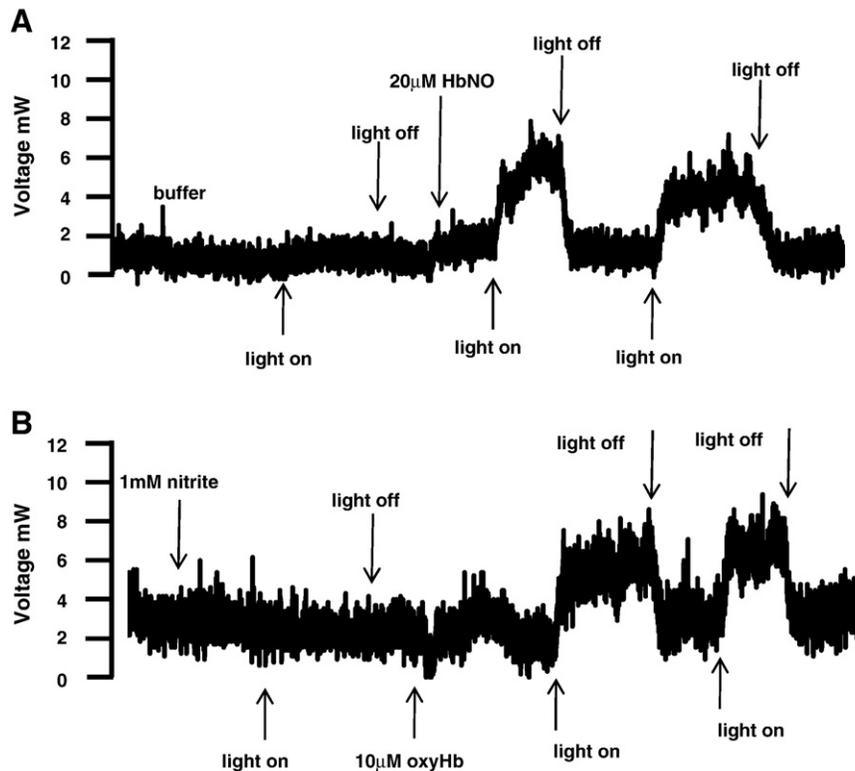
Myocardial infarct size was determined in the following manner. The LAD was reoccluded at the completion of each experiment and 3 ml of patent blue dye was injected i.v. The left ventricular area at risk for infarction was separated from surrounding normal areas (stained blue), and the two regions were incubated at 37 °C for 20 min in 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M phosphate buffer adjusted to pH 7.4. Infarcted and noninfarcted myocardium within the area at risk were carefully separated and weighed after storage overnight in 10% formaldehyde. Myocardial infarct size was expressed as a percentage of the area at risk. Rabbits that developed intractable ventricular fibrillation and those with an area at risk less than 15% of total left ventricular mass were excluded from subsequent analysis.

Statistical analysis of data within and between *in vivo* groups was performed with analysis of variance (ANOVA) for repeated measures followed by Bonferroni's modification of Student's *t* test [28]. Statistical analysis of data *in vitro* was with a Student's *t* test. Changes were considered statistically significant when  $p<0.05$ . All data are expressed as mean  $\pm$  standard deviation (SD).

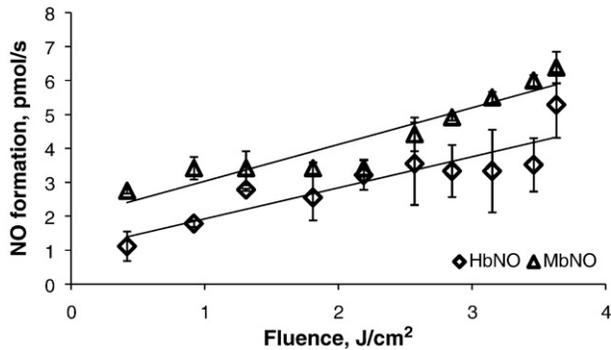
### 3. Results

#### 3.1. The effect of 670 nm light on the absorption spectra of nitrosyl hemoglobin

In oxygenated solution, HbNO slowly converts to methHb as a result of the release of NO. To examine if R/NIR irradiation could accelerate



**Fig. 2.** Ozone chemiluminescent detection of nitric oxide generated from 670 nm. (A) A tracing where 20  $\mu$ M HbNO was exposed to light (18 mW/cm<sup>2</sup>). Peak height increased only with the application of light to HbNO. (B) 1 mM sodium nitrite could not be directly photolysed by 670 nm energy (18 mW/cm<sup>2</sup>), however once oxyHb was added to the sodium nitrite and the solution was deoxygenated with argon gas to generate HbNO, the same application of 670 nm light energy resulted in NO detection.



**Fig. 3.** Quantification of NO release from HbNO by R/NIR as a function of power. 20  $\mu$ M HbNO and MbNO were irradiated with 670 nm light at different fluences. NO release was quantified by extrapolation to a standard curve generated from Prol-NONOate. The rate of HbNO and MbNO decay increases with greater fluence.

this process, HbNO (20  $\mu$ M) was irradiated with 670 nm light and visible spectra were obtained every second for 300 s. Spectra were deconvoluted by multiple linear regression analysis using basis spectra for metHb and HbNO. Fig. 1 shows the effect of 670 nm irradiation on the decay rate of HbNO. The background rate of HbNO conversion to metHb was increased 4 fold due to 47 mW/cm<sup>2</sup> (14 J) of irradiation. These data suggest that this light source facilitates the release of NO from nitrosyl heme proteins.

### 3.2. Effect of R/NIR light on NO release from nitrosyl hemoglobin

In order to examine and quantify NO release, 20  $\mu$ M HbNO was added to a glass purge vessel attached to a chemiluminescence analyzer and irradiated with 670 nm light. As shown in Fig. 2A, irradiation of buffer in the purge vessel of the NO analyzer did not generate any chemiluminescence signal. However, after injection of HbNO, a chemiluminescence signal was detected upon initiation of irradiation, and the signal returned to baseline upon cessation of irradiation in a repeatable manner. No signal was observed if either HbO<sub>2</sub> or nitrite were added to the purge vessel and irradiated (data not shown and Fig. 2B). This data strongly suggests that 670 nm light is able to liberate NO from HbNO. Similar results were obtained using MbNO (data not shown). Interestingly, if nitrite was added to the purge vessel, the addition of oxyHb resulted in an increase in the

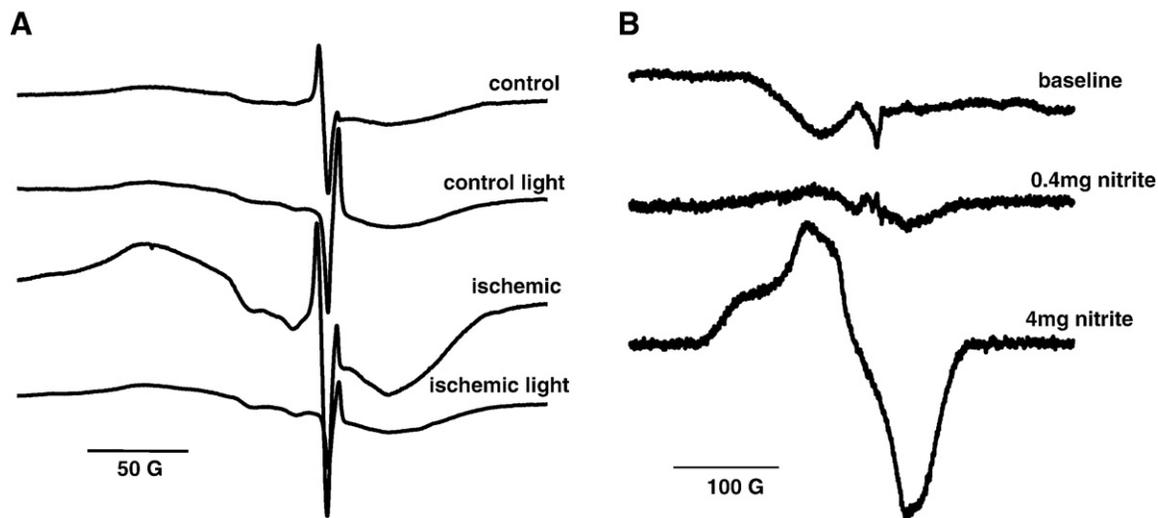
**Table 1**  
Hemodynamics recorded during R/NIR and nitrite induced cardioprotection.

	Baseline	Occlusion	Reperfusion			
			3 min	1 h	2 h	3 h
<i>HR (min<sup>-1</sup>)</i>						
Control	253 ± 35	246 ± 40	229 ± 26	225 ± 28	221 ± 29	212 ± 31
NIR5x	264 ± 37	245 ± 10	240 ± 23	235 ± 21	230 ± 25	221 ± 21
NIR3x	244 ± 31	204 ± 15	209 ± 15	208 ± 20	204 ± 22	189 ± 10
.4Nitr	253 ± 29	250 ± 24	234 ± 20	237 ± 19	222 ± 19	227 ± 23
4.0Nitr	260 ± 25	245 ± 20	238 ± 17	233 ± 16	222 ± 12	218 ± 16
.4Nitr+NIR3x	248 ± 29	217 ± 29	219 ± 28	212 ± 32	203 ± 22	198 ± 22
3mWRad-Post	253 ± 21	233 ± 21	220 ± 12	221 ± 15	209 ± 12	201 ± 11
60mWRad-Post	241 ± 13	222 ± 18	210 ± 18	206 ± 21	199 ± 17	196 ± 18
3mWRad-Post+.4Nitr	244 ± 27	219 ± 23	214 ± 25	214 ± 26	206 ± 30	201 ± 31
<i>MAP (mm Hg)</i>						
Control	74 ± 10	58 ± 9	57 ± 6	60 ± 9	61 ± 11	59 ± 10
NIR5x	74 ± 14	55 ± 8	67 ± 10	68 ± 12	66 ± 7	66 ± 7
NIR3x	76 ± 8	56 ± 11	59 ± 11	63 ± 7	58 ± 7	64 ± 9
.4Nitr	69 ± 15	61 ± 12	60 ± 9	59 ± 9	60 ± 9	60 ± 9
4.0Nitr	77 ± 13	60 ± 10	59 ± 11	55 ± 7	59 ± 12	62 ± 12
.4Nitr+NIR3x	73 ± 9	55 ± 9	58 ± 9	57 ± 8	58 ± 6	59 ± 4
3mWRad-Post	67 ± 6	64 ± 2	64 ± 5	67 ± 4	66 ± 5	67 ± 6
60mWRad-Post	71 ± 9	64 ± 10	65 ± 11	63 ± 9	61 ± 10	63 ± 11
3mWRad-Post+.4Nitr	69 ± 6	55 ± 7	56 ± 9	59 ± 7	58 ± 9	59 ± 5
<i>RPP (min<sup>-1</sup> · mm Hg · 10<sup>-3</sup>)</i>						
Control	21.2 ± 4.3	16.7 ± 3.9	15.4 ± 2.3	15.7 ± 2.4	16.0 ± 3.6	14.6 ± 3.5
NIR5x	22.1 ± 4.3	15.7 ± 2.1	18.4 ± 3.2	18.5 ± 3.8	17.6 ± 2.9	16.9 ± 2.5
NIR3x	21.0 ± 3.5	13.4 ± 1.8	14.3 ± 2.7	15.1 ± 1.5	13.6 ± 1.4	13.8 ± 1.9
.4Nitr	20.4 ± 6.1	17.7 ± 4.8	16.4 ± 2.4	16.2 ± 2.8	15.6 ± 2.7	15.7 ± 2.7
4.0Nitr	22.5 ± 3.8	17.2 ± 3.4	16.5 ± 3.1	15.1 ± 0.9	15.3 ± 2.6	15.5 ± 2.3
.4Nitr+NIR3x	20.6 ± 3.2	14.3 ± 2.8	15.0 ± 2.7	14.4 ± 3.1	13.7 ± 1.4	13.6 ± 1.1
3mWRad-Post	19.5 ± 2.4	16.9 ± 1.6	16.2 ± 1.8	17.0 ± 1.6	15.9 ± 1.7	15.5 ± 1.6
60mWRad-Post	19.5 ± 2.8	16.5 ± 3.0	15.8 ± 3.2	15.3 ± 3.3	14.3 ± 2.9	14.2 ± 3.0
3mWRad-Post+.4Nitr	19.2 ± 2.8	14.3 ± 2.5	14.1 ± 2.7	14.7 ± 2.7	14.1 ± 3.3	13.9 ± 2.9

Data are mean ± SD.

Abbreviations: HR = heart rate; MAP = mean arterial pressure; RPP = rate pressure product.

chemiluminescence signal during irradiation, which returned to baseline upon cessation of irradiation. If the light was subsequently cycled on and off, a robust signal was observed only in the presence of irradiation. An interpretation of these results is that the combination of oxyHb and nitrite, in the anaerobic environment of the purge



**Fig. 4.** (A) EPR signals generated from myocardial tissue exposed to 30 min of ischemia while infused with sodium nitrite. The signal from the ischemic zone is highly characteristic of MbNO. This signal is dramatically reduced after exposure to 18 mW/cm<sup>2</sup> of light at 670 nm (11 J). The non-ischemic control tissue has reduced MbNO signal present, with minimal reduction by light (18 mW/cm<sup>2</sup>; 11 J). (B) EPR signals from blood collected during nitrite infusions. The characteristic HbNO signal is present at 0.4 mg/kg bolus of sodium nitrite. The signal is increased with a greater dose of sodium nitrite (4 mg/kg).

**Table 2**

Hemodynamic data recorded during R/NIR and nitrite cardioprotection in the presence of the NO scavenger c-PTIO.

	Baseline	Occlusion	Reperfusion			
			3 min	1 h	2 h	3 h
<i>HR (min<sup>-1</sup>)</i>						
cPT&5xPre	258 ± 22	218 ± 17	213 ± 10	215 ± 19	201 ± 26	204 ± 31
cPnit&3xPre	245 ± 30	219 ± 20	219 ± 23	221 ± 37	220 ± 31	213 ± 27
cPT&60mWpO	245 ± 24	236 ± 29	226 ± 35	224 ± 26	221 ± 23	214 ± 26
cPnit&3mWpO	253 ± 25	235 ± 42	245 ± 21	238 ± 28	237 ± 32	215 ± 28
<i>MAP (mm Hg)</i>						
cPT&5xPre	68 ± 15	60 ± 11	60 ± 9	65 ± 11	69 ± 9	57 ± 16
cPnit&3xPre	69 ± 5	53 ± 3	51 ± 6	49 ± 4	50 ± 3	53 ± 3
cPT&60mWpO	64 ± 12	53 ± 12	53 ± 6	54 ± 9	53 ± 6	59 ± 13
cPnit&3mWpO	67 ± 11	59 ± 9	61 ± 5	67 ± 6	60 ± 5	57 ± 3
<i>RPP (min<sup>-1</sup> mm Hg · 10<sup>-3</sup>)</i>						
cPT&5xPre	20.2 ± 5.3	15.3 ± 3.5	14.9 ± 2.5	16.3 ± 3.6	16.0 ± 3.8	14.1 ± 5.6
cPnit&3xPre	21.8 ± 1.0	13.7 ± 1.6	13.5 ± 2.9	13.4 ± 3.0	13.4 ± 1.7	13.3 ± 2.3
cPT&60mWpO	20.1 ± 3.0	14.8 ± 3.6	14.2 ± 2.7	14.4 ± 3.3	13.9 ± 2.1	14.8 ± 3.8
cPnit&3mWpO	19.7 ± 4.1	16.7 ± 4.4	17.4 ± 1.6	18.2 ± 1.2	16.6 ± 2.8	14.3 ± 1.3

Data are mean ± SD.

Abbreviations: HR = heart rate; MAP = mean arterial pressure; RPP = rate pressure product.

vessel generates NO via the nitrite reductase activity of deoxyHb. A significant proportion of the liberated NO is autocaptured by hemoglobin to form HbNO, and irradiation with 670 nm light liberates a portion of this autocaptured NO. Fig. 3 shows a quantification of the amount of NO released from both HbNO and MbNO as a function of irradiation power. The range of NO formation rates (between 1 and 6 pmol/s) corresponds to an HbNO decay rate of between 0.5 and 3 nM/s in the 2 ml volume of the purge vessel, agreeing well with the data obtained by spectrophotometry (Fig. 1).

### 3.3. Effect of R/NIR light on nitrosyl heme proteins in myocardium

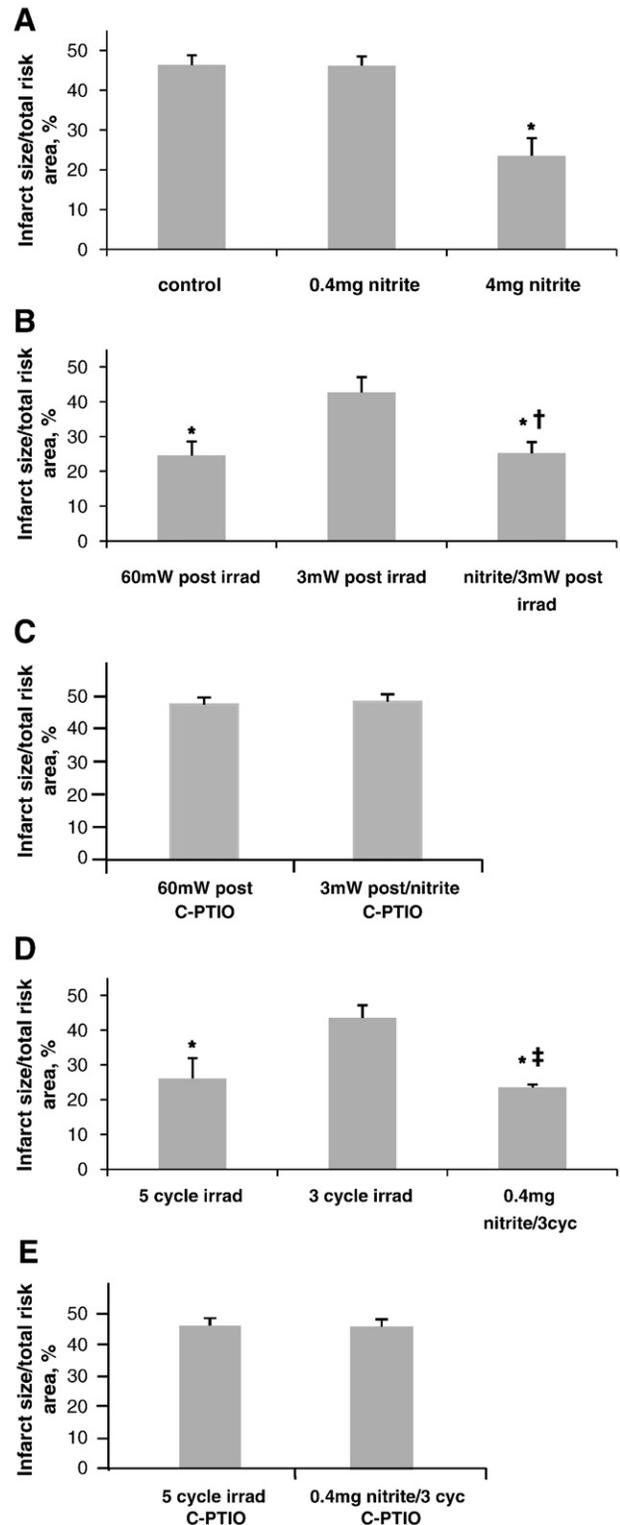
Sodium nitrite was infused into the left atrium of an anesthetized rabbit undergoing left coronary artery occlusion. Myocardium was snap-frozen and processed as described in the Materials and methods section. Tissue from the right, non-ischemic portion of the heart (control in Fig. 4A) showed a low level of nitrosyl heme formation as measured by EPR spectroscopy. In contrast, tissue from the ischemic region exhibited a large increase in the heme nitrosyl signal. It is likely that the major origin of this heme nitrosyl signal is the reduction of nitrite to NO by cardiac nitrite reductases (including deoxymyoglobin and xanthine oxidoreductase) and the capture of nascent NO by deoxymyoglobin. When R/NIR at 670 nm (18 mW/cm<sup>2</sup>; 11 J) was applied to the same tissue samples, the MbNO signal was reduced in the ischemic zone by ~60%, suggesting dissociation of the heme-NO

**Fig. 5.** Effect of R/NIR on myocardial ischemia and reperfusion injury. (A) The control group received normal saline prior to ischemia. Infarct size was significantly reduced compared to the control in the 4 mg/kg sodium nitrite. There was no significant reduction in infarct size compared to the control in the group receiving 0.4 mg/kg sodium nitrite. (B) Infarct size as a % of area at risk in rabbits undergoing LAD occlusion was significantly reduced compared to control when exposed to high dose (60 mW/cm<sup>2</sup>) R/NIR at reperfusion. Low-dose (3 mW/cm<sup>2</sup>) R/NIR exposure on reperfusion did not decrease infarct size. When 0.4 mg/kg of sodium nitrite is administered prior to ischemia, and low-dose R/NIR light is given at reperfusion, infarct reduction is similar to high dose R/NIR. (C) Infusion of C-PTIO abolished R/NIR cardioprotection at reperfusion in both the high dose R/NIR and the low-dose R/NIR with 0.4 mg/kg sodium nitrite. (D) 5 cycles of R/NIR at 670 nm (11 J/cycle) prior to ischemia can significantly reduce infarct size compared to saline control. There was no significant reduction in infarct size compared to the control when 3 cycles (11 J/cycle) of R/NIR light was administered prior to ischemia. However a significant reduction in infarct size could be obtained when 0.4 mg/kg sodium nitrite was combined with 3 cycles of light exposure. This suggests the effect of near infrared light on infarct size is enhanced by addition of nitrite. (E) Infusion of c-PTIO abolished the preconditioning effects of 5 cycles of R/NIR and 3 cycles of R/NIR with 0.4 mg/kg sodium nitrite. \**p*<0.01 compared to control; †*p*<0.01 compared to 60 mW post irradiation; ‡*p*<0.01 compared to 5 cycles R/NIR.

bond upon irradiation. The EPR signal generated from the control region was decreased slightly with the same application of R/NIR light.

### 3.4. Effect of R/NIR light on myocardial ischemia and reperfusion injury

To examine if photolytic release of NO stores by R/NIR light is cardioprotective during ischemia and reperfusion injury, we examined the effect of R/NIR exposure *in vivo* with nitrite infusion. The cardioprotective effects of nitrite have been well documented in the



literature [29–31]. All hemodynamic data has been provided, with no significant hemodynamic changes between the studied groups (Tables 1 and 2).

Fig. 4B shows the level of HbNO formed in blood after infusion of the rabbit with 0, 0.4 and 4 mg/kg nitrite. In the absence of nitrite the signal largely consisted of a copper (II) signal (likely derived from plasma ceruloplasmin and erythrocyte superoxide dismutase). Upon infusion of 0.4 mg/kg nitrite, after subtraction of the copper background signal, the formation of a nitrosyl heme spectrum was observed. This signal was markedly enhanced after administration of 4 mg/kg nitrite.

Coronary artery ischemia and reperfusion in control rabbits receiving saline (Fig. 5A), caused infarction in 46% of the area of risk. If the rabbit was given a i.v. bolus of 4 mg/kg sodium nitrite before the onset of ischemia, cardioprotection was observed after reperfusion (Fig. 5A). A lower dose of sodium nitrite (0.4 mg/kg) was found to be ineffective (Fig. 5A), again in agreement with published studies [31].

As shown in Fig. 5B, exposure of the rabbit cardiac preparation to 60 mW/cm<sup>2</sup> (11 J) of 670 nm light, in the absence of nitrite, at the time of reperfusion resulted in a significant reduction in infarct size whereas a lower power radiation (3 mW/cm<sup>2</sup>, 0.5 J) was ineffective. Interestingly, when low-dose radiation (3 mW/cm<sup>2</sup>) was combined with low-dose nitrite (0.4 mg/kg) a synergistic effect was observed suggesting that R/NIR is able to potentiate the cardioprotective effects of nitrite. The infusion of carboxy-PTIO (c-PTIO), a nitric oxide scavenger, throughout the irradiation period abolished the protective effects of R/NIR and R/NIR with low-dose nitrite (Fig. 5C). To verify thermal effects were not contributing to our observations, temperature at the heart surface was measured, with a temperature elevation <0.5 °C after light exposure.

To examine if R/NIR light can precondition the myocardium from ischemia and reperfusion injury, a similar protocol was performed with irradiation occurring prior to ischemia (Fig. 5D). In this protocol the irradiation was administered in short bursts in a similar manner to ischemic and anesthetic pre-conditioning protocols [27,32]. It was found that 3 cycles of irradiation (see Materials and methods section) of 670 nm light (60 mW/cm<sup>2</sup>) exposure were not cardioprotective, whereas 5 cycles significantly preconditioned myocardium, markedly reducing infarct size. Again, low-dose nitrite (0.4 mg/kg) enhanced the ability of low-dose irradiation to condition the heart against injury. When c-PTIO was infused during the irradiation period, the effect of R/NIR and combined R/NIR with low-dose nitrite (0.4 mg/kg) on infarct size reduction was abolished (Fig. 5E).

In combination these data suggest that the ability of R/NIR light to liberate nitric NO from tissue stores contributes to cardioprotection, and that nitrite, by potentially increasing the size of these NO stores, in the form of increases in MbNO and HbNO, enhances cardioprotection at lower doses of R/NIR.

#### 4. Discussion

This investigation provides evidence that electromagnetic energy at a wavelength of 670 nm can release NO from the nitrosylated forms of hemoglobin and myoglobin, and that this augmented NO release can be cardioprotective. R/NIR light accelerated the conversion of HbNO to metHb by a factor of about 4. HbNO slowly auto-oxidizes to metHb and nitrate due to the slow release of NO from the heme iron, followed by oxygen binding to the vacant heme and rapid reaction of NO with the newly formed oxyHb. This latter reaction generates metHb. The increased rate of conversion of HbNO to metHb in the presence of R/NIR suggests an increased rate of release of NO from this complex. We directly measured NO after irradiation of HbNO and MbNO using ozone-dependent chemiluminescence. This method utilizes the reaction between NO and ozone to generate light and can detect as little as 1 pmol of NO [33]. HbNO is injected into a sealed vessel, is purged with argon, and the gas stream is fed to the reaction cell of a chemiluminescence analyzer. Any NO that is liberated will

either re-bind to deoxyhemoglobin or be swept out of solution in the gas phase. Importantly, no signals were observed upon irradiation after injection of oxyHb or nitrite alone. However, the combination of oxyHb (which is rapidly deoxygenated under the anaerobic conditions of the purge vessel) and nitrite, resulted in a measurable release of NO, consistent with the earlier observation of Gladwin et al. [5]. It is expected under these conditions that the nitrite reductase activity of hemoglobin will release NO and that a significant proportion of this NO will be autocaptured by deoxyHb to form HbNO [5]. Irradiation of this HbNO solution resulted in a robust formation of NO. This experiment raises the possibility that R/NIR light could be used to augment the effects of nitrite as an NO-releasing agent.

Oxygenated derivatives of hemoglobin and myoglobin react with NO with a rate constant of approximately  $4\text{--}5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  [34]. This reaction converts NO into nitrate and has been suggested to be a major route of NO destruction in mammalian systems [34]. In contrast, the reaction of NO with deoxyHb and deoxyMb forms a quasi-stable nitrosyl derivative where the NO is directly bound to the ferrous heme [5,6,35]. While the rate constant of formation of these derivatives from NO is similar to the rate of reaction of NO with oxyHb, their stability is derived from an extremely slow off-rate that is measured in tens of minutes to hours [36]. The half time of HbNO in humans *in vivo* has recently been measured to be about 20 min [37]. In addition, MbNO has been detected in myocardium as a result of iNOS induction [38,39]. Of interest is the fact that HbNO and MbNO are major products of the nitrite reductase activities of these heme proteins. Both deoxyHb and deoxyMb can reduce nitrite to NO via a process that is strongly pH dependent, occurring more rapidly under acidic conditions [13]. The initial product of this reaction is NO, and this reaction has been shown to be involved in vasodilatory responses and cardioprotection, giving a plausible mechanism for hypoxia, or ischemia, driven NO generation [5,6]. However, a proportion of the nascent NO is captured by a local deoxygenated heme, and in the test-tube, the final end products of this reaction are an equal mixture of HbNO and metHb [35]. It is worth noting that the relative rates of nitrite reduction are T-state Hb << R-State Hb < Mb [6]. Consistent with other metalloporphyrin–ligand interactions, MbNO and HbNO were found to be photodissociable at wavelengths up to 608 nm, however their quantum yield is extremely low ( $\phi = 0.001$ ) [40,41].

We observed the generation of measurable levels of MbNO in ischemic myocardium during infusion of nitrite, as determined by EPR spectrometry. After 670 nm light exposure there was a large reduction in the MbNO EPR signal, strongly suggesting the photolysis of MbNO by 670 nm light in this tissue.

Results of the present investigation demonstrate that 670 nm light and nitrite can act synergistically to precondition and postcondition myocardium against ischemia and reperfusion. We hypothesize that the mechanism of synergy involves the photolysis of NO stores generated from the administration of nitrite. Based on experiments with purified HbNO, MbNO and ex vivo cardiac MbNO it is conceivable that these proteins represent examples of such stores. The mechanism of cardioprotection by NO both during the pre-conditioning window and during reperfusion has been extensively examined and discussed in the literature and is an area of on-going debate. It is likely that many components of the cardiomyocyte response to ischemia/reperfusion are modulated by NO, including mitochondrial and sarcolemmal ATP-sensitive potassium channels, mitochondrial Ca<sup>2+</sup> uptake upon reperfusion, and apoptotic signaling pathways [42]. Interestingly NO generated by nitrite reductase activity of deoxymyoglobin has been demonstrated to regulate mitochondrial respiration through modulation cytochrome c oxidase activity [6]. Regardless of the mechanistic intricacies it has become clear that increasing the steady state NO level as a result of pharmacological intervention is a cardioprotective strategy and it appears as though R/NIR irradiation may contribute to this process. It must be acknowledged that other pathways of action of R/NIR cannot be discounted.

The clinical promise for the combined use of R/NIR light and nitrite is of considerable interest. R/NIR light facilitates localized NO

bioavailability in a site-specific manner secondary to increased concentrations of HbNO and MbNO in ischemic regions. The combination has the advantage over well utilized nitric oxide donors sodium nitroprusside and nitroglycerin, because nitrite does not induce tolerance [3]. Nitroglycerin requires conversion to nitrite by the P450 mitochondria aldehyde dehydrogenase, which leads to the development of tolerance [3].

The far red/near infrared region of the electromagnetic spectrum (between 630 nm and 1000 nm) has a number of properties that are attractive to possible physiological and clinical use. The potential for tissue damage is limited because the photon energy in this region is non-ionizing. The thermal effect in this range is also minimized by the low absorption of light energy by water and melanin in this range and this improves overall penetration of the light into tissues. There has been intense clinical interest in the use of irradiation therapy in tissue healing. It has been shown that R/NIR light heals mucositis in pediatric patients undergoing chemotherapy, decreases infarct size, and has a neuroregenerative effect in methanol induced retinal toxicity [18,19,21].

An alternative or parallel mechanism to explain the biological activity R/NIR is the release of NO from nitrosylated cytochrome *c* oxidase. Cytochrome *c* oxidase has been identified as a mediator of some biological effects of R/NIR [18]. It has been suggested that the mechanism by which cytochrome *c* oxidase exerts its action is via NO. Nitric oxide binds to the fully and partially reduced heme  $a_3$  CuB binuclear center and will decrease mitochondrial oxygen consumption [43]. This  $\text{cyt } a_3^{3+}\text{-NO}$  species is photosensitive and application of light at 500 nm was able to photolyse NO from the complex, which led to the reversal of mitochondrial respiratory inhibition [24]. This observation led to speculation that application of red light or near infrared light could release NO and make it available for other biological applications e.g. altering gene expression, vasodilation [22,23]. Although it is plausible this reaction could occur, the relative amounts of NO which would be bound to the enzyme and available for release would be quite limited [44]. Additional complexity to the role of NO and cytochrome *c* oxidase inhibition, is that the oxidized heme  $a_3$  CuB center can form a complex with nitrite, which is insensitive to light. Nitrite bound to the  $\text{cyt } a_3^{3+}$  dissociates from the binuclear site and appears not to undergo further reduction to NO [45–47]. Thus, conditions which favor formation of the photosensitive nitrosyl species are low  $\text{O}_2$  tensions, and high concentrations of reduced cytochrome *c*, which are present after ischemia [26]. Conversely, high  $\text{O}_2$  and low concentrations of reduced cytochrome *c* favor nitrite formation, which occurs under physiological conditions [45]. It might be speculated that the direct actions of light-dependent reversal of NO mediated cytochrome *c* oxidase inhibition, and thus mitochondrial respiration, could be effective in modulating reperfusion injury, however it is not clear if this would be protective or damaging [6]. NO induced inhibition of cytochrome *c* oxidase during reperfusion has been suggested to be beneficial for preventing cardiac injury [6,48,49].

The photodissociation of HbNO and MbNO by R/NIR light has clinical importance. Increasing evidence of the clinical benefits to R/NIR light in a variety of tissues may have a common mechanism that includes generation of significant amounts of NO. The possibility of exerting biological effects on remote organ systems in a noninvasive manner through excitation of NO stores in the blood or muscle is feasible, and site specificity for ischemic regions is highly likely.

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