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Original article

Far red/near infrared light treatment promotes femoral artery collateralization in the ischemic hindlimb

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ABSTRACT

Nitric oxide (NO) is a crucial mediator of hindlimb collateralization and angiogenesis. Within tissues there are nitrosyl-heme proteins which have the potential to generate NO under conditions of hypoxia or low pH. Low level irradiation of blood and muscle with light in the far red/near infrared spectrum (670 nm, R/NIR) facilitates NO release. Therefore, we assessed the impact of red light exposure on the stimulation of femoral artery collateralization. Rabbits and mice underwent unilateral resection of the femoral artery and chronic R/NIR treatment. The direct NO scavenger carboxy-PTIO and the nitric oxide synthase (NOS) inhibitor L-NAME were also administered in the presence of R/NIR. DAF fluorescence assessed R/NIR changes in NO levels within endothelial cells. In vitro measures of R/NIR induced angiogenesis were assessed by endothelial cell proliferation and migration. R/NIR significantly increased collateral vessel number which could not be attenuated with L-NAME. R/NIR induced collateralization was abolished with c-PTIO. In vitro, NO production increased in endothelial cells with R/NIR exposure, and this finding was independent of NOS inhibition. Similarly R/NIR induced proliferation and tube formation in a NO dependent manner. Finally, nitrite supplementation accelerated R/NIR collateralization in wild type C57Bl/6 mice. In an eNOS deficient transgenic mouse model, R/NIR restores collateral development. In conclusion, R/NIR increases NO levels independent of NOS activity, and leads to the observed enhancement of hindlimb collateralization.

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1. Introduction

Peripheral arterial disease (PAD) is an enormous clinical problem with 8 million Americans being treated for symptoms such as intermittent claudication and critical limb ischemia [1]. Therapies have focused on risk reduction, anti-platelet medications, and vasodilators such as cilostazol [2–4]. Surgical intervention with stent placement or bypasses of affected blood vessels is reserved for patients with severe critical limb ischemia and is often ineffective, therefore the risk for limb amputation remains high.

One treatment would be to stimulate collateral blood vessels within the ischemic limb and restore blood flow to the distal extremity. NO (nitric oxide) mediates collateralization through the production of growth factors e.g. vascular endothelial growth factor (VEGF), angiopoietin, and basic fibroblast growth factor (bFGF) [5–7]. NO is predominantly produced by enzymatic reduction of L-arginine to L-citrulline via nitric oxide synthases (NOSs). However there is increasing interest in NOS-independent NO generation, particularly during hypoxia or anoxia, when NOS is inactive [8,9]. Nitrite can be enzymatically reduced to NO by molecules such as deoxyhemoglobin [10–13], deoxymyoglobin [14], xanthine oxidoreductase [9,15], cytochrome c oxidase [16] and NOS itself [17]. The heme moiety in these proteins binds the newly formed NO, thus generating nitrosyl storage pools of potentially bio-available NO.

Previously, we identified that light in the far red/near infrared spectrum (670 nm, R/NIR) releases NO from nitrosyl hemoglobin and nitrosyl myoglobin. NO generated in this fashion was responsible for R/NIR induced cardioprotection, thereby supporting the concept that these storage pools of NO can be manipulated for therapeutic purposes [13]. Light exposure between 630 nm and 830 nm has been extensively studied in vivo and in vitro [18–21]. In this range there is improved tissue penetration due to limited melanin absorption [22]. Radiant heat production in R/NIR exposed tissue is below 0.5 °C, because of limited water absorption by light [23,24]. Based on this evidence, we propose that repetitive application of R/NIR (670 nm) will stimulate collateral blood vessel development independent of NOS. We further propose that this increase in NO is physiologically

Abbreviations: R/NIR, red/near infrared light wavelength peak at 670 nm; NO, nitric oxide; NOS, nitric oxide synthase; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor.

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significant so as to allow peripheral vascular collateralization in a NO deficient model.

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*.

2.1. R/NIR sources

The 670 nm LED light source with power output up to 100 mW/cm^2 was obtained from Quantum Devices and 60 mW/cm^2 light source from NIR Technologies. Power output was measured with a light meter (X97, GigaHertz-Optik) at the surface to report actual irradiance intensity. The light sources were placed 2.5 cm from their target in all animal experiments. In vitro, culture dishes and slides were placed directly on the light source. The irradiance intensities listed below reflect the values obtained at the skin surface for in vivo studies, or the bottom of the culture dish for in vitro studies.

2.2. Rabbit model of hindlimb ischemia

New Zealand White rabbits underwent unilateral femoral artery excision according to previously published protocols [25]. After a 5-day recovery, rabbits were randomized into 6 groups. Group 1 ($n = 6$) underwent ischemia alone and group 2 ($n = 6$) underwent ischemia with a daily R/NIR exposure (60 mW/cm^2 × 10 min = 36 J/day) for 14 days. Group 3 ($n = 6$) received 0.5 g/L of L-NAME (Sigma) in their drinking water for 14 days and Group 4 ($n = 6$) received L-NAME with R/NIR exposure. Group 5 ($n = 6$) received infusions of 0.17 mg/kg/min carboxy-PTIO (c-PTIO, Alexa chemical), a NO scavenger 1 min prior to R/NIR through 2 min post R/NIR. Group 6 ($n = 6$) received daily infusion of c-PTIO without R/NIR. After 14 days, rabbits were anesthetized, the left carotid was cannulated, and both femoral arteries were injected with 10 cm^3 Omnipaque contrast media, and imaged with radiographic fluorography. Collateral blood vessel number was directly counted by 2 independent and blinded reviewers.

2.3. Endothelial cell proliferation assay

Human umbilical vein endothelial cells (HUVECs) were grown to passages 4–6 in EGM media (Lonza), and then seeded (10,000 cells/well) on a 24 well culture plate (Costar). Cells were exposed to R/NIR (100 mW/cm^2/day) for 4 days. The exposure duration varied to generate an energy dose response curve for cell proliferation. After the last exposure, cells were washed with PBS, trypsinized and counted using a hemocytometer. Experiments were performed in quadruplicate.

2.4. Western analysis

Vessels from the ischemic and control limbs were excised, snap frozen in liquid nitrogen, and isolated using protease inhibitor supplemented NP40 lysis buffer supplemented with protease inhibitors. Protein extracts were measured by the BCA method (Pierce), resolved using standard SDS-PAGE, and transferred to nitrocellulose. Blots were probed with mouse anti-VEGF antibody (1:200, Santa Cruz). Membranes were stripped and re-probed with mouse alpha-actin (1:200, Santa Cruz). Densitometry was performed using Image J software. Four samples in each group were analyzed. Data was normalized to the non-ischemic limb.

2.5. Endothelial cell tube formation

Human umbilical vein endothelial cells (HUVECs) were grown to passages 4–6 in HUVEC media (Cell Applications). Fibrin gels were prepared according to previously published protocols [26,27]. HUVECs (40,000 cells/well), were seeded on the surface of the three-dimensional matrix and incubated 1 h prior to exposure with HUVEC media alone, Group 2 with 50 ng purified VEGF/well, Group 3 with L-NAME (1 mM), and Group 4 with c-PTIO (100 μM). One plate was exposed to R/NIR (50 mW/cm^2 × 150 s = 7.5 J), and a control plate was not exposed. Thirty minutes post-exposure, the media were exchanged for standard media in order to avoid cell death secondary to extreme chronic NO depletion. This process was repeated daily for a total of 4 exposures. Experiments were performed in quadruplicate.

2.6. NO detection by DAF-2 (diaminofluorescein) fluorescence

HUVECs (passages 4–6) were seeded (25,000 cells/chamber) on chamber slides (Nunc) and grown to 90% confluence. Individual cell chambers incubated for 30 min in DMEM, 1 mM L-NAME, or 100 μM carboxy-PTIO, before 4-amino-5methylamino-2′7′-diamiofluorescein diacetate (2 μM DAF-2, Invitrogen) were added prior to exposure. Cells were then irradiated for 7.5 J (150 s at 50 mW/cm^2), incubated for 30 min at 37 °C, and then mounted for confocal microscopy. Images were acquired using a Nikon Eclipse TE2000-U microscope equipped with EZC12 software. Fluorescence was monitored with excitation at 488 nm and emission at 510 nm. Images were analyzed using Nikon Element software to measure mean fluorescence intensity. Five images from each group were measured.

2.7. Mouse hindlimb collateralization model

C57Bl/6 mice ($n = 6$) or endothelial NOS knock-out mice (eNOS$^{-/-}$, $n = 6$) underwent femoral artery ligation and excision distal to the femoral profundus and proximal to the saphenous and popliteal arteries [28,29]. The mice were placed within a box containing a transparent floor which rested directly on the light source. Beginning immediately after surgery, C57Bl/6 or eNOS$^{-/-}$ mice were exposed to R/NIR light (60 mW/cm^2 × 10 min = 36 J/day) for 14 days. Additional groups of C57Bl6 and eNOS$^{-/-}$ mice underwent femoral artery ligation and excision with sodium nitrite supplementation (8.2 μg/kg/day, for 14 days, i.p.), and then further divided into receiving daily R/NIR or not receiving R/NIR [30].

2.8. Collateral blood flow

Laser Doppler flow imaging was performed on all mice for noninvasive measurement of blood flow (Moor Instruments, UK). Mice were anesthetized with 1.25% isoflurane, allowed to acclimate on a heating pad to maintain body temperature, and then scanned [28]. Flow intensities in both limbs were measured on Days 0, 3, 7, and 14. Data was expressed as the ratio of mean intensity between the ischemic limb and the contra-lateral control.

2.9. Statistical analysis

All values are expressed as mean ± SEM. Comparisons were made using a one way ANOVA with post-hoc Bonferroni *t*-test, or a Student's *t*-test. Values for $p < 0.05$ were considered significant.

3. Results

3.1. Application of R/NIR stimulates femoral artery collateralization

To assess the effect of R/NIR on femoral artery collateralization, a rabbit hindlimb ischemia model was employed. Fig. 1 summarizes