



Ascorbate attenuates red light mediated vasodilation: Potential role of S-nitrosothiols



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ABSTRACT

There is significant therapeutic advantage of nitric oxide synthase (NOS) independent nitric oxide (NO) production in maladies where endothelium, and thereby NOS, is dysfunctional. Electromagnetic radiation in the red and near infrared region has been shown to stimulate NOS-independent but NO-dependent vasodilation, and thereby has significant therapeutic potential. We have recently shown that red light induces acute vasodilatation in the pre-constricted murine facial artery via the release of an endothelium derived substance. In this study we have investigated the mechanism of vasodilatation and conclude that 670 nm light stimulates vasodilator release from an endothelial store, and that this vasodilator has the characteristics of an S-nitrosothiol (RSNO). This study shows that 670 nm irradiation can be used as a targeted and non-invasive means to release biologically relevant amounts of vasodilator from endothelial stores. This raises the possibility that these stores can be pharmacologically built-up in pathological situations to improve the efficacy of red light treatment. This strategy may overcome eNOS dysfunction in peripheral vascular pathologies for the improvement of vascular health.

1. Introduction

The endothelium regulates vascular tone and blood flow by secreting vasoactive substances in response to injury, infection, hypoxia and changes of metabolism [1,2]. However, under severe pathological conditions the endothelium becomes dysfunctional, and the production of vasorelaxants is attenuated [2,3]. Nitric oxide, largely generated from endothelial nitric oxide synthase (eNOS) [4], is a critical relaxing factor [5], as evidenced by the onset of vascular pathologies when eNOS function is impaired. Other sources of NO have been recognized, particularly in response to hypoxic situations, so as to counteract the deleterious effects of eNOS dysfunction and the oxygen requirements of this enzyme. The major pathway for this enzyme independent NO production is thought to be the reduction of inorganic nitrite to NO [6,7].

Nitric oxide and/or inorganic nitrite can generate additional nitrogenous products that can potentially act as sources of NO. S-

Nitrosothiols (RSNO) are generated from the nitrosative biological chemistry of NO [8] and have the potential to liberate NO in addition to their more well-established role in redox signaling pathways [9–12]. Dinitrosyl iron complexes (DNIC), another stable intracellular product of NO formation, are quantitatively more abundant than RSNO although their functional importance has not received as much attention. DNIC form spontaneously from the cellular labile iron pool, NO, and thiolate nucleophiles. Non-protein DNIC exist as an equilibrium mixture of mononuclear and binuclear forms. High thiol concentration e.g. cellular glutathione, drives the equilibrium towards the mononuclear form [13–17].

There has been significant interest in the use of red and near infrared light (R/NIR) to promote vasodilation in a number of pathological situations. R/NIR irradiation has been shown to be effective at transiently increasing blood flow and there are significant indications that NO is involved in this response. However, the source of NO has not been established, which limits its clinical applicability. Previously we

Abbreviation: eNOS, endothelial nitric oxide synthase; RSNO, S-nitrosothiols; R/NIR, red and near infrared light; GSNO, S-nitrosoglutathione; DNIC, dinitrosyl iron complex; GSH-DNIC, glutathione dinitrosyl iron complexes; Proli/NO, 1-(hydroxy-NNO-azoxy)-L-proline; CL, ozone-chemiluminescence signal; DHA, dehydroascorbate; DTPA, diethylenetriamine pentaacetic acid; NEM, N-ethylmaleimide; SE, standard error

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characterized the ability of 670 nm light to release NO from iron-nitrosyl heme proteins. Importantly, we showed such release exhibited a synergistic cardioprotective effect with inorganic nitrite, suggesting that R/NIR was more effective after nitrite-dependent build-up of nitrosyl myoglobin [18–20]. The liberation of NO from other heme nitrosyls, such as nitrosyl cytochrome c oxidase has also been implicated [21].

We have recently demonstrated that irradiation of isolated vessels with 670 nm light results in the release a substance from the vascular endothelium with vasodilatory activity and suggested that this substance was a small NO-containing molecule [22]. In this study we have further investigated the nature of this intermediate and conclude that it has characteristics closely aligned with RSNO, although a contribution from DNIC, cannot be ruled out.

2. Methods

2.1. Materials

All materials used were Sigma-Aldrich (St. Louis, MO) products, unless otherwise mentioned.

LED light sources with power supply were purchased from NIR Technologies, Waukesha, WI (used for chemiluminescence experiments), or from Quantum Devices Inc., Barneveld, WI (used for cell and pressure myography measurements). Power output was determined with X97 Optometer (Gigahertz Optic GmbH, Turkenfeld, Germany).

2.2. DNIC syntheses

Binuclear GSH-DNIC was synthesized from FeSO_4 (100 μM) and glutathione (200 μM) and 1-(hydroxy-NNO-azoxy)-L-proline (Proli/NO) (100 μM) in 15 mM Hepes pH 7.4. Solutions were degassed with nitrogen gas before the addition of Proli/NO.

2.3. Pressure myography

Vasodilation was measured with pressure myography. 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*. Segments of facial arteries (160–260 μm ID) from C57Bl6/J mice were transferred to a water-jacketed perfusion chamber and cannulated with two glass micropipettes at their in situ length. The arteries were bathed in the PSS-equilibrated solution maintained at pH 7.4 and 37 °C. The micropipettes were connected to a reservoir filled with physiological saline solution and the arteries were pressurized to 60 mmHg. The internal diameter of the arteries were measured with a video system composed of a stereomicroscope (Olympus CK 40), a charge-coupled device camera (Panasonic GP-MF 602), a video monitor (Panasonic WV-BM 1410), and a video measuring apparatus (Boeckeler VIA-100). After a 60 min equilibration period with or without sodium ascorbate (15 mM), the arteries were pre-constricted by ~ 50% of their resting diameter with a thromboxane A_2 analog, U-46619. The vessels were placed in the dark and once steady-state contraction was obtained were treated with 10 mW/cm² of 670 nm light for 5 min. The light sources were placed 2.5 cm from the target. Vasodilator responses to papaverine (10^{-4} M) or acetylcholine (10^{-10} – 10^{-4} M) were determined and expressed as percent maximal relaxation relative to U-46619 pre-constriction, with 100% representing the passive baseline diameter with papaverine or acetylcholine.

2.4. Ozone-based chemiluminescence

RSNO formation was detected with NO analyzer (Sievers Model 280i) using tri-iodide method [23]. Sulfanilamide was added to the samples to remove nitrite. Quantification was performed based on the detector response for known amounts of S-nitrosoglutathione (GSNO).

2.5. High performance liquid chromatography

Samples were separated on a Kromasil C-18 column (250 × 4.6 mm, 0.5 μM particle size) and eluted with methanol and 0.05% tetrafluoroacetic acid (TFA) with a gradient method using an Agilent 1100 chromatograph. The effluent was monitored with a diode array spectrophotometer detector at 310 nm (DNIC) and at 336 nm (GSNO). Quantification was performed using Agilent ChemStation software and known amounts of DNIC and GSNO [24].

2.6. Statistics

A Student's two-tailed *t*-test was used for statistics, and $p \leq 0.05$ was considered statistically significant. All data are reported as mean \pm standard error (SE) unless otherwise indicated.

3. Results

3.1. Red light-dependent dilation of facial artery: effects of ascorbate

We recently established that red light irradiation time-dependently increases dilation of pre-constricted murine facial artery in an NO-dependent, but eNOS-independent manner. Incubation of vessels with the NOS inhibitor L-NAME did not significantly affect vasodilation, however, the NO scavenger cPTIO abolished the effect [22]. These data suggest that red light-stimulated an NO-dependent vasodilation that was largely NOS-independent. Ascorbate, at high concentration has been shown to decompose RSNO [25], so to examine RSNO involvement in the mechanism of vasodilation, we pre-incubated vessels with sodium ascorbate (1-h, 15 mM) prior to irradiation. Both ascorbate-pretreated, and untreated vessels were subject to red light irradiation protocol that consisted of initial irradiation for 5 min, followed by a 10-min dark period. This was followed by an additional 5 min of irradiation followed by a final 5 min of dark (Fig. 1A). As we previously demonstrated, red light resulted in an increase in vessel diameter that ceased upon removal of the light, the second period of irradiation resulted in additional vasodilation which again ceased after the irradiation stopped. Ascorbate pre-treatment resulted in a significant diminution of red light-stimulated vasodilation during all phases of the protocol (e.g. 12% vs. 27% at 20 min) strongly suggesting that ascorbate interfered with the mechanism of vasodilation. Importantly, ascorbate treatment did not alter the response of the vessels to acetylcholine (Fig. 2) indicating that there was no disruption of endothelial/eNOS-dependent vasodilation in these vessels. We examined the effect of ascorbate on potential small-molecule “NO stores” by tri-iodide-mediated ozone-dependent chemiluminescence. This method will detect tri-iodide-releasable NO from cellular molecules. This method is often used to detect RSNO but will also detect iron nitrosyls and perhaps other nitrogenous molecules. Fig. 1B (raw data) and 1C (quantification) show a significant reduction in signal as a result of ascorbate treatment of the vessels, suggesting that an ascorbate-sensitive species may be responsible for the dilatory effect of red light.

In our previous study [22] we showed that irradiation of vessels at 670 nm generates a relatively stable transferable NO-dependent mediator which is released from the endothelium and could dilate a naïve vessel in the dark. We examined if ascorbate pre-treatment would prevent the release of this mediator. In Fig. 3 the facial artery was incubated with and without ascorbate, washed, and then exposed to red light for 10 min. The bathing solution was collected and transferred to a

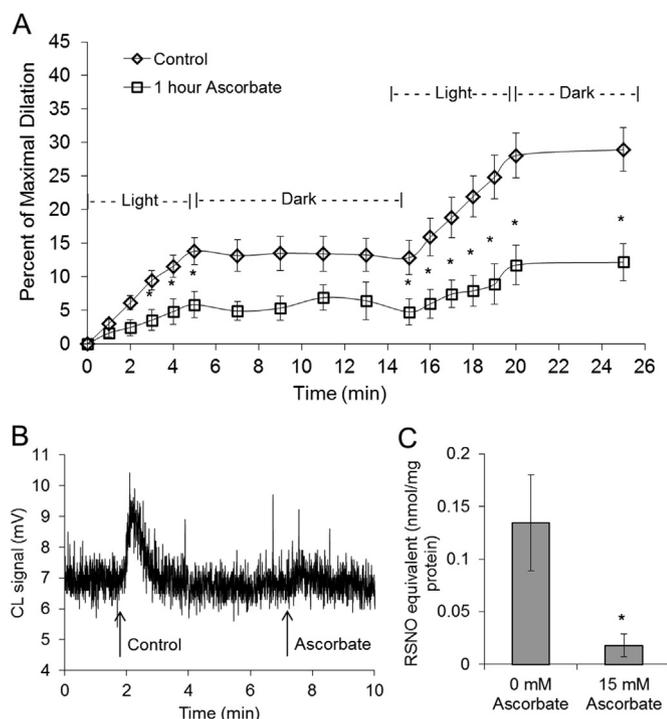


Fig. 1. Ascorbate effect on red light-dependent vasodilation. (A): Dilatation of isolated facial arteries of C57Bl/6 mice (pressurized to 60 mmHg before pre-contraction with U46619) with and without pre-incubation of sodium ascorbate (15 mM for 60 min) upon irradiation (670 nm, 6 J/cm²). Values are means \pm SE, N = 8, *p < 0.05. (B): Representative trace of triiodide-based chemiluminescence analysis of artery tissue homogenized in phosphate/DTPA/NEM pH 7.4. Arrows indicate injection of samples from control and ascorbate treated vessel, protein content: 19 and 25 μ g, respectively. (C): Quantification chemiluminescence data. N = 4, *p < 0.05.

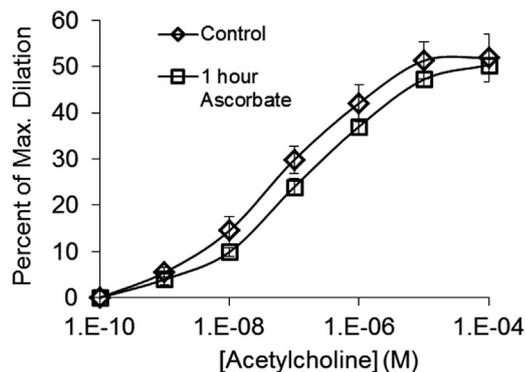


Fig. 2. Ascorbate effect on acetylcholine-induced vasodilation. Dilatation of isolated facial arteries of C57Bl/6 mice (pressurized to 60 mmHg before pre-contraction with U46619) as a function of acetylcholine concentration with and without pre-incubation of sodium ascorbate (15 mM for 60 min). Values are means \pm SE, N = 6.

naïve vessel in the dark. A reduced dilatation was observed in the donor vessel that was pre-treated with ascorbate, in agreement with Fig. 1. Vasodilation of the acceptor vessel was also significantly diminished if the donor vessel was pre-incubated with ascorbate. These data are consistent with the hypothesis that red light is able to liberate a vasodilatory species from endogenous stores that is depleted by ascorbate treatment.

3.2. Potential role of S-nitrosothiols

Tri-iodide chemiluminescence detects S-nitrosothiols, nitrosamines,

and heme-nitrosyls, and according to our latest findings also DNIC [24]. Nitrosamines and heme-nitrosyls are resistant to ascorbate [26,27]. The reducing effect of ascorbate on RSNO is well known [25] and is the basis of a common protein RSNO detection methodology, the biotin switch assay. The reaction is slow, but can be catalyzed by trace transition metal ions [28]. The effects of ascorbate on DNIC are currently unknown (see following section). To assess if ascorbate could affect RSNO mediated vasodilation, we examined the effect of ascorbate on GSNO-dependent vasodilation in pre-constricted murine facial arteries. GSNO stimulated a time and concentration-dependent dilatation of these vessels (Fig. 4). Pre-Incubation of GSNO (40 nM) with sodium ascorbate (15 mM, 60 min at 37 °C) significantly attenuated vasodilatation from 28% to 11% at 10 min time point. The reaction between GSNO and ascorbate is complex and pH dependent but these conditions GSNO decay will have an \sim 15 min half-life [29,30], thus the dilatation achieved after 60 min incubation is in good agreement with \sim 10 nM of remaining GSNO (Fig. 4B, dotted line). The effects of ascorbate are consistent with the hypothesis that a nitrosothiol is the active intermediate.

3.3. Potential role of DNIC

As mentioned above the effects of ascorbate on DNIC are currently unknown. To examine the possibility that DNIC could represent the transferable intermediate, we determined the effect of binuclear GSH-DNIC on vasodilatation, by administering pre-synthesized GSH-DNIC to a pressurized facial artery preparation. Consistent with previous studies in aortic ring baths [31], there was significant vasodilation over 10 min (Fig. 5A). As with GSNO, pre-incubation of DNIC with ascorbate diminished its vasodilatory response (Fig. 5A) suggesting ascorbate is also able to decompose DNIC to a form incapable of vasodilation. To further investigate this, we incubated binuclear GSH-DNIC (70 μ M) with and without sodium ascorbate (15 mM, 37 °C) and examined any spectral changes. As shown in Supplemental Fig. 1 we observed a decrease in the 300–500 nm region of the DNIC spectrum over time. However, when we examined the DNIC content by HPLC (Fig. 5B) ascorbate had no effect on DNIC levels over the 60 min of incubation. We additionally examined the effect of dehydroascorbate, the 2-electron oxidized form of ascorbate, as a potential contaminant of ascorbate. We did observe a slight reduction of DNIC levels with DHA but only at very high concentrations of 15 mM (Fig. 5B).

4. Discussion

We previously demonstrated that red light (670 nm) is able to dilate pre-constricted facialis arterial preparations by an eNOS-independent but nitric-oxide dependent mechanism [22]. We have rigorously controlled for heating effects and have concluded that any slight temperature increase resulting from the irradiation procedure has negligible effects on vasodilation [32]. In addition, the light generated a quasi-stable vasoactive substance in the bath solution surrounding the vessel that could be transferred to a second vessel to promote vasodilation. In this study, we have investigated the intracellular source of this signal. We show facialis vessels contain a low level of material that is able to release NO in a tri-iodide-based chemiluminescence assay. The identity of this material is uncertain but almost certainly consists of one or more nitroso or nitrosyl species. Nitrite per se is ruled out as all samples were treated with sulfanilamide to remove this anion. In an attempt to narrow down the identity of this species we pre-treated the vessel with a high concentration of ascorbate. Ascorbate will directly reduce RSNO [25], but not other nitroso compounds (O- and N-nitroso compounds), heme nitrosyls [26,27]. The effect of ascorbate in DNIC has not before been studied. Ascorbate decreased the level of tri-iodide-detectable material in the vessel and this was accompanied by a reduced ability of the vessel to dilate to red light. Importantly, the vessels responded normally to acetylcholine indicating that ascorbate did not

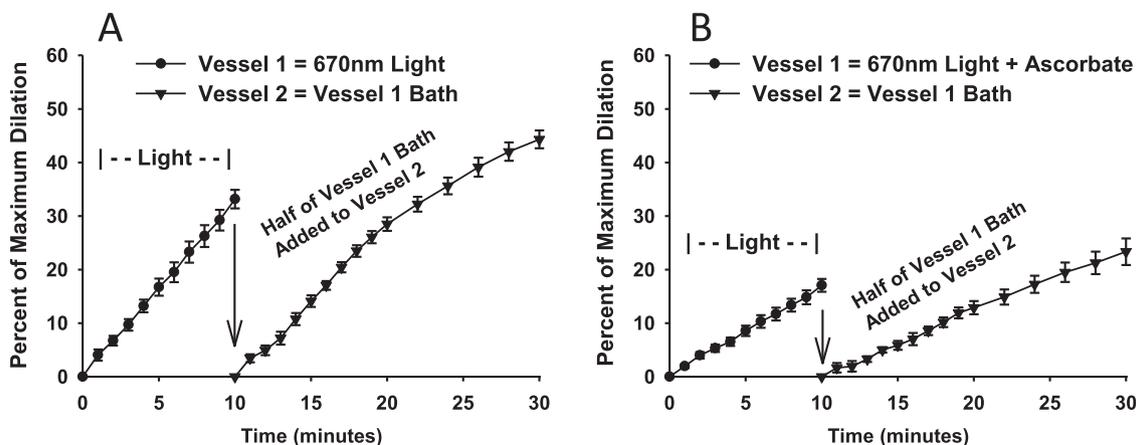


Fig. 3. Adoptive transfer. Facial arteries from C57Bl/6 mice were isolated and pressurized to 60 mmHg before pre-constriction with U46619 (n = 8). **A)** After 10 min of light exposure, the bath solution from vessel 1 was added to vessel 2. **B)** Preincubation with 15 mM ascorbate reduces dilation in both the treated vessel 1 (17.1%, SD 3.2, SE 1.2) as well as the bath transfer vessel 2 (23.4%, SD 6.6, SE 2.5).

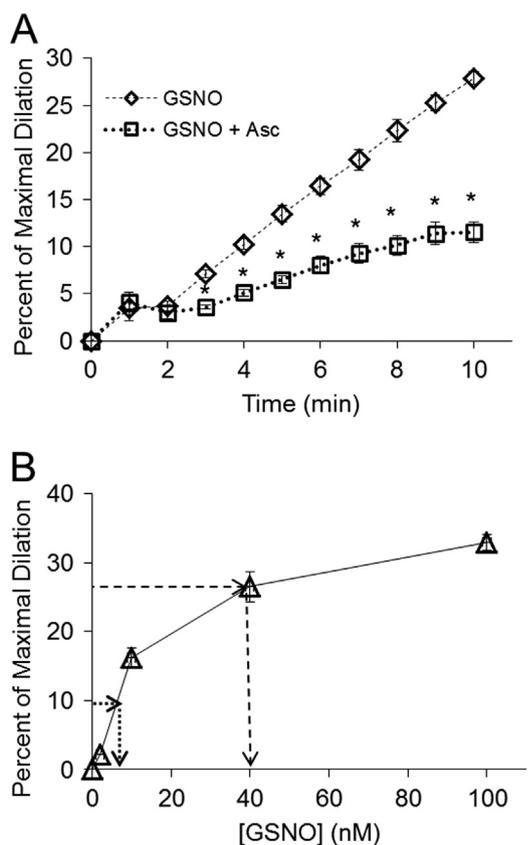


Fig. 4. GSNO-dependent vasodilation. Facial arteries from C57Bl/6 mice were isolated and pressurized to 60 mmHg before pre-constriction with U46619 (n = 8). **A)** Pre-constricted vessel was exposed to 40 nM GSNO (diamonds), or 40 nM GSNO pre-incubated with 15 mM ascorbate (squares). **B)** Pre-constricted vessel was exposed to increasing concentrations of GSNO. The maximal dilations after 10 min were plotted as a function of GSNO concentration. Arrows indicate GSNO concentrations at the maximal dilations on (A) panel without (dashed line) and with (dotted line) ascorbate treatment. Values are means ± SE, N = 4, *p < 0.05.

affect the canonical eNOS/NO/sGC pathway in these tissue preparations.

Ascorbate also prevented the formation of the transferable vasodilator, such that the ability of the bath solution to relax an untreated vessel, in the dark was diminished. These data suggest a functional relationship between the tri-iodide detectable substance within the

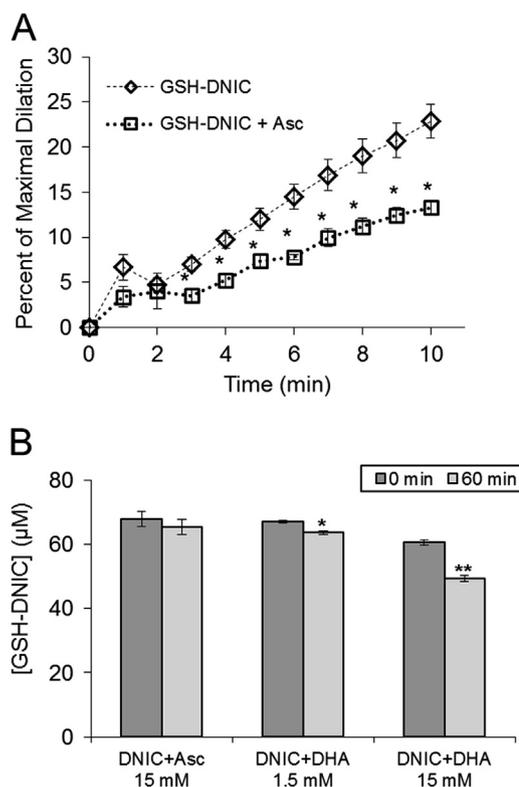


Fig. 5. DNIC-dependent vasodilation. Facial arteries from C57Bl/6 mice were isolated and pressurized to 60 mmHg before pre-constriction with U46619 (n = 8). **A)** Pre-constricted vessel was exposed to 40 nM GSH-DNIC (diamonds), or 40 nM GSH-DNIC pre-incubated with 15 mM ascorbate (squares). Values are means ± SE, N = 4, *p < 0.05 **B)** DNIC-GSH (70 µM) was incubated with sodium ascorbate (15 mM), or with dehydroascorbate (DHA, 1.5 mM and 15 mM) at 37 °C in 50 mM HEPES, pH 7.4. DNIC levels were determined at 0, and 60 min after separation with HPLC/detection at 312 nm. Values are means ± SE, N = 3.

vessel and the transferable vasodilator leading to the idea that light is able to stimulate the mobilization of this 'store' to elicit dilation distant from the irradiated tissue.

Ascertaining the identity of the transferable vasodilator is challenging. The levels released into the bath are very low, but, as shown in Figs. 4 and 5, sufficient to dilate the vessels under study. This somewhat precludes an analytical approach to detection. We have attempted to distinguish the functional effects of DNIC and GSNO by their sensitivity

to ascorbate but this has generated somewhat puzzling and ambiguous results. Vasodilation by low levels of GSNO and DNIC was diminished by pre-incubation with ascorbate. In the case of GSNO this is easily understandable as ascorbate has been shown to degrade GSNO over time [25,33] and this reaction has been used as positive identifier of S-nitrosation. However, the effect on DNIC is less clear. Mononuclear DNIC are thought to have 9 d-electrons with the partially filled d orbital giving rise to characteristic electron paramagnetic resonance spectrum for an $S = \frac{1}{2}$ complex. This complex is stable in the presence of high concentrations of excess thiol, suggesting reduction to the d-10 complex is not facile. In addition, when thiol is limiting DNIC exist as a paramagnetic binuclear complex that fully satisfies the 18-electron rule. Even with the caveat that protein DNIC are likely mononuclear due to steric constraints of the thiolic ligands, reduction of DNIC by ascorbate is a highly unlikely scenario. Our data indicate that ascorbate affected the UV spectrum of DNIC, but once separated by HPLC it reverted to the binuclear complex form with no loss of concentration. This perhaps suggest the ascorbate anion as a nucleophile at high concentration may participate in some level of reversible ligand exchange which reverts back to the thiolate ligand once the complex is resolved on-column. We have observed that mononuclear DNIC always resolve as binuclear DNIC on HPLC due to removal of excess ligand, which points to the dynamic nature of these transformations [24,34]. We additionally examined the effect of DHA as a putative contaminant oxidant in ascorbate. While DHA did appear to degrade DNIC at very high concentrations this could not explain the effect on vasodilation. We are left with the conclusion that ascorbate somehow interferes with DNIC-mediated dilation, either by directly altering the complex or by indirectly interfering with the bioactivity or bioactivation of DNIC. The mechanism by which DNIC elicit vasodilation is not resolved although NO clearly plays a role. Clearly more studies are required to understand the effects of ascorbate on DNIC activity in this system.

Clearly 15 mM Ascorbate is a high concentration and it is possible that additional biological reactions of ascorbate are occurring, such as reduction of disulfides or reaction with nitrite anion [35]. Ascorbate did not affect the ability of the vessel to dilate to acetyl choline, indicating that any such reactions do not alter the canonical EDRF pathway in this vessel preparation.

The concept of a store suggests a purposeful accumulation of a bioactive compound in an inactive form that can be released in times of need. It is rather grandiose to describe the tri-iodide-detectable material as a store, hence our use of quotation marks. However, in terms of the red light effect described here, it may be fortuitous that oxidation products of NO build up in the endothelium, blood, and muscle due to the activity of eNOS and nitrite metabolism; and which represents an effective storage pool of NO that can be liberated with a non-physiological photolytic stimulus. Our recent work suggest that red light may have a dual action in accessing this material, acting directly on the material but also allowing release to the extracellular space (Fig. 6)

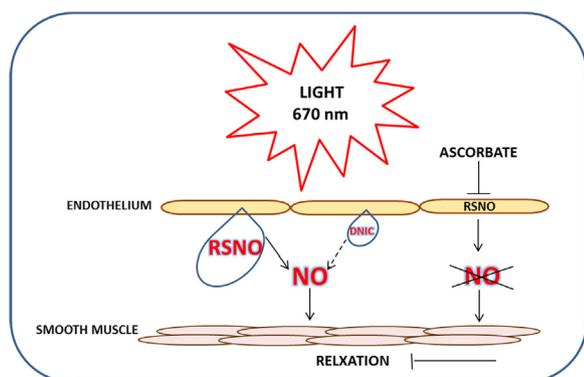


Fig. 6. Mechanism of light-mediated NO-dependent vasorelaxation.

[22,32]. The potential transport of released vasodilator into the blood stream could extend the reach of red light to clinically relevant tissue beds more distal and deeper than the surface.

The therapeutic consequences of this work are quite profound when consideration is given to the shared problem of insufficient nitric oxide production in many common vascular disorders (e.g. peripheral vascular disease, coronary artery disease, myocardial infarction) [36]. Oxidative stress from atherosclerosis, diabetes, or ischemia leads to reduced NOS activity [36,37]. Consequently, the vascular bed exhibits impaired vasodilation, increased platelet aggregation, and reduced capacity for ischemic cardioprotection [38]. Numerous clinical trials using NO supplementation have shown a benefit to improving the observed vascular dysfunction in these diseases [39]. The limitations of NO supplementation due to systemic changes in hemodynamics (i.e. hypotension), increased tolerance to NO donors, and patient reported adverse effects (i.e. headache and presyncope) reduce its long-term efficacy in chronic vascular disease. However, there is clear therapeutic advantage to exploit endogenous sources of NO, which does not display the tolerance effects observed in classical NO donors such as nitroglycerin [40]. What requires additional clarity are the mechanisms by which these endogenous compounds can achieve a maximal therapeutic benefit above their actions under homeostatic conditions. Furthermore, reconciliation between the known effects of these endogenous NO sources and the observed effects of red light may provide a unique intersection by which effective NO production can be leveraged in a site-specific manner. As depicted in Fig. 6, our data support the contention that red light energy stimulates release of a nitrogenous vasodilator that has many characteristics of RSNO from the endothelium which dilates the underlying smooth muscle. Most importantly, the released compound is semi-stable so as to stimulate vasodilation over minutes.

The penetration depth of red light is a limitation of its clinical applicability. Generally, is thought to be able to penetrate to about 0.5 cm. Our own modeling using Monte Carlo methods predict 10% of the energy present at the surface will reach 1.5–2 cm of depth (unpublished data). This depth would allow to impact peripheral arteries and any tissues in reach of fiber optics (i.e. coronary artery derived catheterization). As mentioned above, the light-dependent release of a vasodilator into the blood could effectively extend the reach of the light beyond the direct irradiation area.

Red light has been suggested to be beneficial in several pathological situations through increased NO formation. The early phase of wound healing is accelerated in rats treated with red light, which leads to significant reductions in wound area, and as healing progresses the impact of light on wound area decreases. This temporal regulation of light stimulated wound healing is consistent with NO regulated growth factor expression, e.g. basic fibroblast growth factor and vascular endothelial growth factor (VEGF) [41]. In addition, the magnitude of light stimulated NO production has significant implications for angiogenesis, as other studies attribute development of collateral vessels coincides with changes in NO levels [42]. Red light treatments also reduce the parenchymal damage observed in traumatic brain injury and stroke models, but also cardiac ischemia-reperfusion injury through NO mechanisms [18,19,43].

A clear corollary of this observation is that if we can bolster the level of red light sensitive vasodilatory material, it may be possible to improve the efficacy of red light therapy without relying on the endogenous level of the NO stores. In our previous study of cardiac reperfusion injury we observed a synergy between the protective effects of nitrite and red light [18]. The recognition that pre-treatment of patients with RSNO, nitrite or perhaps even other more common nitrovasodilators such as nitroglycerin, in combination with light exposure, may have additional benefits suggests an exciting advancement to expanding clinical use.

Author contribution

A.K, B.L. performed experimental work; N.L supervised and led this study; all authors helped design experiments and write the manuscript.

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Competing interest

The authors declare that there is no competing interest associated with the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.redox.2018.09.008](https://doi.org/10.1016/j.redox.2018.09.008).

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