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Thrombospondin-1-CD47 blockade and exogenous nitrite enhance ischemic tissue survival, blood flow and angiogenesis via coupled NO-cGMP pathway activation

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Abstract

Tissue ischemia and ischemia-reperfusion (I/R) remain sources of cell and tissue death. Inability to restore blood flow and limit reperfusion injury represents a challenge in surgical tissue repair and transplantation. A central regulator of blood flow, reperfusion signaling and angiogenesis is nitric oxide (NO). De novo NO synthesis requires oxygen and is limited in ischemic vascular territories. Nitrite (NO₂⁻) has been discovered to convert to NO via heme-based reduction during hypoxia, providing a NO synthase independent and oxygen-independent NO source. Furthermore, blockade of the matrix protein thrombospondin-1 (TSP1) or its receptor CD47 has been shown to promote down-stream NO signaling via soluble guanylate cyclase (sGC) and cGMP-dependant kinase. We hypothesized nitrite would provide an ischemic NO source that could be potentiated by TSP1-CD47 blockade enhancing ischemic tissue survival, blood flow and angiogenesis. Both low dose nitrite and direct blockade of TSP1-CD47 interaction using antibodies or gene silencing increased acute blood flow and late tissue survival in ischemic full thickness flaps. Nitrite and TSP-1 blockade both enhanced in vitro and in vivo angiogenic responses. The nitrite effect could be abolished by inhibition of sGC and cGMP signaling. Potential therapeutic synergy was tested in a more severe ischemic flap model. We found that combined therapy with nitrite and TSP1-CD47 blockade enhanced flap perfusion, survival and angiogenesis to a greater extent than either agent alone, providing approximately 100% flap survival. These data provide a new therapeutic paradigm for hypoxic NO signaling through enhanced cGMP mediated by TSP1-CD47 blockade and nitrite delivery.

Keywords

nitric oxide; nitrite; thrombospondin-1; CD47; ischemia

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Introduction

The technical aspects of soft tissue surgery and microsurgery invariably induce dramatic changes in regional blood flow and produce some degree of ischemia and ischemia/reperfusion (I/R) injury [1]. Severing of vascular networks and connections during surgery initiates an interval of ischemia,[2] the duration of which and the sensitivity of the affected tissue determines the degree of tissue loss.[3] The results of this process in many instances are tissue necrosis and delayed wound healing [4;5]. Ischemic tissues experience decreased vessel and capillary caliber, leukocyte retention and activation, endothelial cell dysfunction, and inflammatory mediator production [6]. Restoration of flow to ischemic tissue incurs additional damage through the production of free radicals by xanthine oxidase and NADPH oxidase [7; 8] with the degree of reperfusion injury directly related to the length of the ischemic interval [9].

Nitric oxide is a bioactive gas produced constitutively by many cell types [10]. Its pro-survival roles include the regulation of vascular tone through relaxation of the smooth muscles cells of arteries,[11] promotion of endothelial cell health, inhibition of inflammatory cell activation, and blocking of thrombus formation. NO thereby maximizes blood flow to ischemic tissues and minimizes I/R injury and tissue loss [12;13]. Recently it was discovered that the matrix glycoprotein thrombospondin-1 (TSP1), via engaging the cell surface receptor CD47, blocks the pro-survival signaling of NO [14;15;16]. Inhibiting TSP1-CD47 signaling maximizes NO signaling and can increase tissue blood flow [17;18;19;20]. While TSP inhibition will enhance cGMP signaling, these effects are downstream of NO production. NO production becomes limited during anoxic conditions since NO synthase requires oxygen as a substrate [21;22]. At low oxygen tension, however, nitrite (NO₂-) can be reduced to NO by several cellular heme proteins including myoglobin and xanthine oxidoreductase [23;24]. The hypoxic conversion of nitrite to NO promotes potent cytoprotection in part via the dynamic modulation of mitochondrial reactive oxygen species production following reperfusion. This cytoprotective effect of nitrite has been observed in animal models of ischemia-reperfusion of the heart, liver, and brain [25]. Nitrite also increases angiogenesis in murine models of chronic ischemia [26].

We hypothesized TSP1-CD47 blockade and supplementation of nitrite could have additive activities to prevent tissue loss to ischemia. We examined this using a murine model of dorsal random myocutaneous flaps, which experience predictable and significant amounts of ischemic tissue necrosis. We show that nitrite alone increases ischemic myocutaneous flap survival with enhanced blood flow. Importantly, the combined treatment of nitrite and TSP1-CD47 blockade increases flap survival and flap blood flow significantly beyond either therapy alone.

Methods

Animals

C57BL/6 male mice aged 14 to 18 weeks were housed under pathogen free conditions and had ad libitum access to filtered water and standard rat chow. Handling and care of animals was in compliance with the guidelines established by the Animal Care and Use Committees of the National Cancer Institute. Male Sprague-Dawley rats (250–500 g; Harlan) were used in accordance with the IACUC of the University of Pittsburgh.

Reagents

1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was obtained from Cayman Chemicals (Ann Arbor, MI). Sodium nitrite, allopurinol and potassium hexacyanoferrate were obtained from Sigma Aldrich (St Louis, MO). A rat monoclonal antibody to murine CD47, Ab 301, was

prepared as described.[27] The TSP1 monoclonal antibody clone A6.1 was purchased from Neomarkers/Laboratory Vision (Fremont, CA). An isotype-matched control IgG2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Type I collagen (PureCol) was purchased from Inamed Biomaterial (Fremont, CA). A translation-blocking antisense morpholino oligonucleotide complementary to human and murine CD47 (CGTCACAGGCAGGACCCACTGCCCA) and a 5-base mismatch control (CGTgACAGcCAcGACCgACTGCgCA) were obtained from Gene Tools (Philomath, OR).

Nitrite preparation

Sodium nitrite was dissolved in sterile Millipore filtered water to obtain a 1 mol/L (M) stock which was then serially diluted to a final concentration of 600 μ mol/L (μ M). Solutions were prepared fresh daily and kept in sealed sterile eppendorfs to minimize atmospheric exposure. Animals received a single bolus dosage of 48 nmols/animal (achieving an approximate blood level of 10 μ M[25]) or vehicle via intraperitoneal (i.p.) injection.

Random flap model

C57BL/6 mice were matched for sex and age. Anesthesia was induced by and maintained with inhalation 1.5% isoflurane and a 50:50 mixture of oxygen to room air. Body temperature was monitored via rectal probe and maintained at 37 ° C with a heating pad and lamp during the procedure. The dorsal surface was clipped of hair and depilated with Nair®. The skin was then cleansed with surgical soap and alcohol and the animals draped. Using sterile technique, 1 × 3 cm random myocutaneous flap incorporating the panniculus carnosus and skin were raised. Flap dimensions were marked on the animal skin surface with the aid of a template to insure consistency in dimension. Flaps were orientated parallel to the long axis of the animal with flap bases directed caudal and secured after mobilization with interrupted 5-0 nylon sutures. A minimum number of sutures were employed to limit the effect of suturing on tissue survival. Animals were awakened and returned to individual cages and allowed ad libitum access to food and water. On postoperative day seven, the animals were again anesthetized with inhalation isoflurane and the flaps evaluated.

Estimation of survival area in flaps

The necrotic area of the flap was determined by color, refill, eschar, and the pin-prick test. The outlines of viable and nonviable areas were traced using transparent film and the area of flap necrosis versus total flap area determined by comparison with the original flap template. The cut weight of the flap tracing was compared the weight of the original template utilizing the method described by Ueda [28]. Flap survival area was presented as the ratio of flap survival on postoperative day seven divided by the original flap area multiplied by 100%.

Treatment groups

A total of 65 animals, 5 per group, underwent flap surgery and received the following: untreated, vehicle alone (sterile saline), nitrite alone, ODQ alone, Ab A6.1 alone, IgG2 control Ab alone, CD47 morpholino alone, control mismatch morpholino alone, nitrite + ODQ, nitrite + Ab A6.1, nitrite + IgG2 control Ab, nitrite + CD47 morpholino, nitrite + control mismatch morpholino. All treatment agents were administered just prior to the initiation of surgery. Nitrite, ODQ and the respective vehicle controls were given via i.p. injection. The TSP1 monoclonal antibody clone A6.1 or a matched isotype control antibody were given by direct injection of the surgical flap. A CD47 targeting morpholino or a mismatched control morpholino were also administered by direct injection of the surgical flap.

Laser Doppler analysis

Flap blood flow was measured using laser Doppler imaging (MoorLD1-2λ, Moor Instruments, Devon, England). Briefly, animals were placed on a heating pad, and anesthesia was provided by 1.5% inhalation isoflurane in a 50:50 mixture of oxygen to room air. Core temperature was maintained via heat lamp at 35.5 °C and monitored by rectal probe. After a 15 minute equilibration to the experimental set-up, analysis of blood flow was performed. The following instrument settings were used: override distance 21 cm; scan time 4 msec/pixel. Results are expressed as the percent change from baseline control of the region of interest (ROI).

Analysis of flap vascularity

On the 7th post-operative day animals were anesthetized with 1% isoflurane in a 50:50 mixture of oxygen to room air. Core temperature was monitored by rectal probe and maintained at 37° C via heat lamp. Flap sutures were removed and flaps gently inverted exposing the vascular network of the deep undersurface of each flap. Image acquisition of flap vascularity was performed with an Olympus C-5500 digital camera (Melville, NY). Analysis of images was performed to quantify the degree of macro-vascularity of each flap using the following grading system – a vessel was defined as the segment of blood vessel between two points of vessel branching. Only vessels filled with red (oxygenated) blood were counted. Results represent the mean ± SD of 5 flaps from each treatment group.

Tissue cGMP measurements

On post-operative day seven dorsal myocutaneous flaps were excised and necrotic tissue removed. The remaining viable portion of each flap was then snap frozen in liquid nitrogen, pulverized and resuspended in lysis buffer. The mixtures were then sonicated in a cold room at 4° C and centrifuged at 13000 rpm for 10 minutes. Aliquots from the cleared supernatant were then used for determination of tissue cGMP via standard immunoassay (Amersham, GE Healthcare, UK). Results were normalized to total protein which was determined for each sample via a BCA protein assay (Pierce, Rockford, IL). Results represent the mean ± SD of 5 flaps from each treatment group.

Nitrite reduction to NO in ischemic tissue flaps

Male Sprague-Dawley rats underwent surgical mobilization of 2 × 6 cm random myocutaneous flaps. Flaps were excised and immediately homogenized in a sucrose containing buffer. NO generation from tissue homogenates (10 mg/ml) treated with nitrite (10 mM) in the presence or absence of allopurinol (500 μM) and potassium hexacyanoferrate (500 μM) was measured in vessels in which the suspension and headspace were purged with helium and the vessel connected in line to a NO chemiluminescence analyzer.

Explant angiogenesis assay

Muscle biopsies 1 mm³ in size were harvested from the pectoralis major muscle of 10 week old C57BL6 mice and explanted into a three dimensional type I collagen matrix as described [14]. Explants were incubated in the presence of endothelial growth medium (Lonza, Switzerland) with 2% FCS and the indicated treatment agents. Cell migration through the extracellular matrix was measured 96 hours post-explantation. Results represent the mean ± SD of at least three separate experiments.

Histology

Tissue units were excised, fixed in 10% buffered paraformaldehyde, paraffin embedded, and sectioned at a thickness of 5 μm. Sections were then stained with hematoxylin and eosin (H&E). Review of each slide was performed by an independent observer unaware of the origin of each

tissue slide to quantify the micro-vascularity of each flap. Patent and functional microvessels were defined as those having an identifiable lumen filled with red blood cells. Results represent the mean $\pm$ SD of 5 flaps from each treatment group.

Statistics

Results are presented as the mean $\pm$ SD with significance calculated by the Student's *t* test (two-tailed) or ANOVA analysis as appropriate using a standard software package (Origin). Significance was assigned a *p* value ≤ 0.05 .

Results

Nitrite supplementation increases ischemic myocutaneous flap survival

Untreated or vehicle treated random dorsal myocutaneous flaps experienced $60.5 \pm 4\%$ (not shown) and $63.6 \pm 7.11\%$ necrosis respectively (Fig. 1A). In contrast systemic nitrite supplementation (48 nmols/animal i.p. at the time of surgery) significantly decreased tissue necrosis ($47 \pm 4\%$, *p* = 0.0017). Immediate and significant enhancement of flap blood flow in nitrite treated animals compared to vehicle treated flaps was confirmed by laser Doppler imaging in the first post-operative hour (Fig. 1B). Follow up analysis 72 h after surgery demonstrated persistence of enhanced flap flow in the nitrite treated group (data not shown).

Nitrite is reduced in ischemic soft tissue flaps to NO in a xanthine oxidoreductase independent manner

Recently xanthine oxidoreductase (XOR) has been reported to be capable of reducing nitrite to NO [29]. NO levels were determined in ischemic myocutaneous tissue flaps via chemiluminescence with and without XOR inhibition with allopurinol (Fig. 1C, D). Treatment of flap tissue with nitrite resulted in a rapid increase in NO production which was not significantly suppressed by treatment with allopurinol. Interestingly, a dramatic reduction in nitrite conversion to NO was achieved in ischemic flaps treated with potassium hexacyanoferrate suggesting a possible heme based mechanism.

Nitrite increased ischemic tissue survival is blocked by the sGC inhibitor ODQ

NO signals in part by stimulating soluble guanylate cyclase (sGC) [30;31] leading to increased cellular cGMP. cGMP, through activation of the cGMP-dependent kinase, leads to vascular smooth muscle cell relaxation, arterial dilation and increased tissue blood flow. ODQ is a chemical inhibitor of the heme moiety of sGC [32] and blocks NO-stimulated increases in cellular cGMP. Random flaps were created in C57BL/6 mice. Animals received vehicle, nitrite, vehicle plus ODQ or nitrite plus ODQ (20 μ g/g i.p.). Flap survival and blood flow were assessed 7 days following surgery. Flap necrosis increased substantially in animals treated with nitrite plus ODQ ($62 \pm 3\%$) versus nitrite alone ($42 \pm 4.5\%$, *p* = 0.0381). Similarly, flaps treated with vehicle plus ODQ showed more necrosis than flaps treated with vehicle alone (72 ± 6 versus $64 \pm 4\%$, *p* = 0.0073). Importantly, ODQ treatment significantly abrogated the ability of nitrite to restore blood flow in ischemic flaps (Fig. 2B).

TSP1 blockade enhances ischemic tissue survival

Random dorsal 1×3 cm myocutaneous flaps were treated with a monoclonal TSP1 antibody (clone A6.1, 2.4 μ g in 200 μ l of sterile saline injected into the flap) and showed significantly less tissue necrosis ($31.5 \pm 2.57\%$) versus flaps treated with a matched isotype control IgG2 antibody ($64.4 \pm 6\%$, *p* < 0.0001) (Fig. 3A, B) or vehicle treated flaps (data not shown since differences in flap necrosis between untreated and vehicle treated animals were not statistically significant).

TSP1 blockade and nitrite therapy synergize in preventing ischemic tissue survival

Flaps treated with a TSP1 blocking monoclonal antibody (clone A6.1) showed enhanced ischemic tissue survival. Likewise flaps treated with systemic nitrite showed enhanced tissue survival. However, ischemic flaps treated with combined therapy had the least degree of tissue necrosis (6.8 ± 6 , $p < 0.0001$) versus untreated flaps, vehicle treated or flaps receiving only single agent treatment (Fig. 3A, B). Further analysis suggested Ab A6.1, in a dose dependent manner, enhanced nitrite effects on tissue survival though significance was not reached until a maximum dose of $2.4 \mu\text{g}$ per tissue unit (Fig. 3C). Interestingly tissue cGMP levels, which were determined at 7 days post-operatively from the viable portion of each flap, were greatest in tissue units from treated flaps with flaps treated with both a TSP1 antibody and nitrite demonstrating the greatest tissue cGMP levels among all groups ($56 \pm 6 \text{ fmol}/\mu\text{g}$ protein, $p = 0.0144$) (Fig. 3D).

CD47 suppression via gene silencing and exogenous nitrite synergize in enhancing ischemic tissue survival

Morpholino oligonucleotides are stable reagents that allow temporary gene silencing of a target protein with minimal to no non-specific effects. The ability of the morpholino sequence to suppress CD47 protein in cultured cells [17] and murine and porcine tissue has been recently reported [33]. Random dorsal $1 \times 3 \text{ cm}$ myocutaneous flaps were injected with an oligonucleotide morpholino targeting CD47 alone ($10 \mu\text{M}$ in $200 \mu\text{l}$ of sterile saline) or in combination with systemic nitrite ($48 \text{ nmoles/animal i.p.}$). Those animals that received the CD47 targeting morpholino alone, though demonstrating increased flap survival, still had residual flap necrosis ($32 \pm 6\%$) (Fig. 4A, B). In contrast, the combination of a CD47 suppressing morpholino with systemic nitrite resulted in further enhancement of ischemic tissue survival and blood flow with minimal tissue necrosis ($14 \pm 3.5\%$, $p = 0.0001$). Results achieved with combined therapy were comparable to those previously published in random myocutaneous flaps performed in age and sex matched CD47 null animals [18] this despite a more stringent ischemic challenge secondary to an increase in flap length in the present series of experiments from a width to length ratio of 1:2 to 1:3. A mismatch control morpholino did not increase ischemic tissue survival above untreated or vehicle treated levels.

Nitrite and TSP1-CD47 blockade separately and together enhance angiogenic response in a wound healing environment

Consistent with the ability of nitrite to stimulate angiogenesis in a hind limb ischemia model [26], vascular cell outgrowth and migration through a three dimensional collagen matrix was increased in explants treated with exogenous nitrite (Fig. 5A, B). Nitrite alone enhanced angiogenic response in a dose dependent manner. Antibody blockade of TSP1 or CD47 alone also increased angiogenic response when compared to basal or low dose nitrite treatment (data not shown). Finally, the combination of a CD47 targeting antibody and nitrite or a TSP1 targeting antibody and nitrite resulted in greater vascular cell outgrowth than any single treatment agent alone.

Nitrite and TSP1-CD47 blockade combine to maximize ischemic soft tissue macrovasculature

Increased tissue perfusion and wound healing requires both an angiogenic response and functional macro-vessels. Consistent with the significant degree of tissue necrosis untreated random myocutaneous flaps had the lowest vascularity index (Fig. 6A, B). Blockade of TSP1-CD47 signaling with a monoclonal TSP1 antibody resulted in increased flap vascularity. Nitrite therapy alone also tended to increase the flap vascularity index. However, combination therapy increased flap vascularity by more than 3 times over untreated flaps ($74.3 \pm 8/\text{flap}$ versus $23 \pm 6.4/\text{flap}$, $p < 0.0001$) (Fig 6B).

Functional micro-vascularity is increased in ischemic flaps following TSP1-CD47 blockade and nitrite treatment

Quantification of the functional micro-vessels in ischemic flaps demonstrated greater micro-vessels density after nitrite or TSP1 antibody treatment alone (Fig. 7A, B). Importantly, in terms of tissue perfusion, combined treatment resulted in a 56.3% increase in micro-vessel density. Typical of myocutaneous flaps the functional macro- (see Fig. 6A, B) and micro-vascularity is demonstrated adjacent to the muscle compartment of the tissue unit.

Discussion

Reconstructive surgery and microsurgery have extended greatly the ability to restore complex wounds both functionally and aesthetically. However, wound healing complications and tissue/flap loss remain significant problems. Tissue necrosis and loss are also found as primary clinical manifestations of several chronic diseases including coronary artery disease, peripheral artery disease, diabetes and stroke.

Nitric oxide is a bioactive gas continuously produced by the endothelial component of the vascular system [10]. Among its several pro-survival roles NO regulates tissue blood flow through modulation of arterial tone. NO activates sGC in the vascular smooth muscle cells (VSMC) of arteries [11]. Activation of sGC leads to uncoupling of the contractile apparatus of VSMC which in turn results in blood vessel dilation and increased tissue blood flow. The matrix cellular protein TSP1 potently blocks NO-driven sGC activation in vascular cells [34]. This process necessarily requires TSP1 engagement of the cell surface receptor CD47 [16]. Animals lacking either TSP1 or CD47 display increased tissue blood flow in response to NO and enhanced tissue survival under ischemic conditions [17;18].

Nitrite has been identified as a novel reservoir of biologically active NO under conditions of ischemia and hypoxia and provides organ protection to I/R injury in the heart, liver and brain [35]. However, its role in modulating soft tissue responses to ischemia and I/R injury has not been explored. A topical ointment containing nitrite and citric acid applied to cutaneous flaps was reported to modestly enhance flap survival [36] and to increase wound healing in a diabetic mouse model [37], the therapeutic effects presumptively being obtained by conversion of nitrite to NO locally through acidic disproportionation. We herein demonstrate that *systemic* administration of nitrite can enhance myocutaneous flap survival to ischemia. A single bolus of nitrite administered at the time of flap surgery significantly enhanced tissue survival, flap blood flow and tissue cGMP. Pre-treatment with ODQ abolished increases in tissue survival and blood flow obtained with nitrite therapy. ODQ is known to irreversibly inactivate the heme moiety of sGC [38] suggesting the therapeutic benefit obtained with nitrite includes increased sGC activation and cGMP production. However, ODQ has off target effects and has been reported to also suppress activation of other hemoproteins [39] some of which could be involved in hypoxic reduction of nitrite to NO. However the authors have recently reported enhanced tissue protection/function and NO production obtained with nitrite therapy that was abolished following administration of either ODQ, the NO scavenger PTIO or Rp-8-Br-cGMP (an inhibitor of cGMP-dependent kinase)[40;41].

In the present report ischemic soft tissue NO levels were enhanced by ex vivo treatment with nitrite and this process was not suppressed with allopurinol suggesting that hypoxic reduction of nitrite to NO in ischemic myocutaneous flaps is not dependent upon XOR activity. In contrast, a near 80% reduction in NO signal was achieved in similar ischemic tissue flaps treated with nitrite in the presence of ferricyanide suggesting a possible role for heme in the conversion of nitrite to NO under these conditions. These results are in contrast with the recent report that demonstrates XOR mediated changes in nitrite stimulated cardiovascular effects

[29]. However this report did not differentiate between NO-dependent and NO-independent effects arising from nitrite treatment.

No complications were noted to arise from systemic nitrite administration. This is not unexpected since nitrite reduction to NO occurs along a pH and oxygen gradient [42]. Likewise reductase conversion of systemically administered nitrite is localized to regions of low blood flow, and decreased pH and oxygen, conditions specifically found in ischemic tissues thus minimizing systemic effects upon blood pressure and cardiac response. However, it is possible that systemic nitrite administration might produce an alteration in blood pressure in this model. In contrast, the local administration of therapeutic agents that block TSP1-CD47 signaling, through enhancing regional blood flow in soft tissue flaps, would be unlikely to alter blood pressure.

NO supplementation has been applied to several models of tissue ischemia/wound healing both through administration of NO-releasing agents such as isosorbide dinitrate [18;43;44;45] or through administration of the nitric oxide synthase substrate L-arginine with enhanced tissue survival [46;47] and wound breaking strength [48] reported. However, ischemic tissue flaps treated with either primary NO donors or NOS substrate demonstrate residual flap necrosis [49] suggesting that TSP1-CD47 inhibition of NO signaling remains a barrier to complete tissue survival through exogenous NO supplementation. In the present report we found that concurrent blockade of TSP1-CD47 signaling and nitrite supplementation increased, in an additive manner, ischemic tissue survival beyond what any single agent achieved. We also found in an ex vivo assay that simulates the angiogenic sprouting and cell migration of wound healing increased response to both nitrite and monoclonal antibodies targeting TSP1 and CD47. That combined therapy demonstrated additive rather than synergistic increases in ischemic tissue survival may reflect the robust improvement each agent (either nitrite or a TSP1 monoclonal antibody) provided alone. At the doses employed each individual agent achieved close to 75% tissue survival. When combined ischemic tissue survival approached 100%. The murine dorsal surface reasonably accommodates a soft tissue flap 3 cm in length and limits our flap width to length ratio to 1:3. A greater flap width to length ratio in a larger rodent would perhaps allow for demonstration of synergistic rather than mere additive increases in tissue survival with combination therapy.

Both CD47 Ab/nitrite and TSP1 Ab/nitrite in combination proved to be in the present model of myocutaneous flap ischemia nearly equal in effectiveness. However, targeting CD47 may be theoretically more beneficial since platelets represent a constant potential reservoir of preformed TSP1 [50]. Of great clinical relevance combined therapy with nitrite and a TSP1-CD47 blocking antibody produced significantly more functional micro- and macro-vascularity in ischemic soft tissue flaps than either treatment alone. The results of the present work highlight that a multi-directional approach of delivery of the NO pro-drug nitrite and blocking TSP1-CD47 inhibition of NO signaling achieves maximum tissue protection from ischemic injury with enhancement of angiogenesis and macro- and micro-vascularity (Fig. 8).

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References

1. Kerrigan CL, Stotland MA. Ischemia reperfusion injury: a review. *Microsurgery* 1993;14:165–175. [PubMed: 8479314]
2. Howell ST, Seaber AV, Urbaniak JR. Microcirculatory responses to vascular washout following ischemia. *Microsurgery* 1989;10:264–268. [PubMed: 2593795]
3. Kerrigan CL, Zelt RG, Daniel RK. Secondary critical ischemia time of experimental skin flaps. *Plast Reconstr Surg* 1984;74:522–526. [PubMed: 6484039]
4. Pang CY, Forrest CR, Mounsey R. Pharmacologic intervention in ischemia-induced reperfusion injury in the skeletal muscle. *Microsurgery* 1993;14:176–182. [PubMed: 8479315]
5. Riccio M, Pangrazi PP, Campodonico A, Scalise A, Marchesini A, Talevi D, Carboni A, Bertani C, Bertani A, Mazzanti L. Combined use of WEB2170 and HBO therapy can reduce ischemia and reperfusion injury to the skeletal muscle in a rabbit model. *Microsurgery* 2007;27:43–47. [PubMed: 17206621]
6. Carroll WR, Esclamado RM. Ischemia/reperfusion injury in microvascular surgery. *Head Neck* 2000;22:700–713. [PubMed: 11002326]
7. Knight KR, Angel MF, Lepore DA, Abbey PA, Arnold LI, Gray KA, Mellow CG, O'Brien BM. Secondary ischaemia in rabbit skin flaps: the roles played by thromboxane and free radicals. *Clin Sci (Lond)* 1991;80:235–240. [PubMed: 1850683]
8. Angel MF, Mellow CG, Knight KR, Coe SA, O'Brien BM. A biochemical study of acute ischemia in rodent skin free flaps with and without prior elevation. *Ann Plast Surg* 1991;26:419–424. [PubMed: 1952713]discussion 425–6
9. Buttemeyer R, Philipp AW, Mall JW, Ge B, Scheller FW, Lisdat F. In vivo measurement of oxygen-derived free radicals during reperfusion injury. *Microsurgery* 2002;22:108–113. [PubMed: 11992497]
10. Ignarro LJ. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. *J Physiol Pharmacol* 2002;53:503–514. [PubMed: 12512688]
11. Moncada S, Higgs EA. Nitric oxide and the vascular endothelium. *Handb Exp Pharmacol* 2006:213–254. [PubMed: 16999221]
12. Moncada S. Nitric oxide in the vasculature: physiology and pathophysiology. *Ann N Y Acad Sci* 1997;811:60–67. [PubMed: 9186585]discussion 67–9
13. Isenberg JS. Nitric oxide modulation of early angiogenesis. *Microsurgery* 2004;24:385–391. [PubMed: 15378585]
14. Isenberg JS, Ridnour LA, Perruccio EM, Espey MG, Wink DA, Roberts DD. Thrombospondin-1 inhibits endothelial cell responses to nitric oxide in a cGMP-dependent manner. *Proc Natl Acad Sci U S A* 2005;102:13141–13146. [PubMed: 16150726]
15. Isenberg JS, Wink DA, Roberts DD. Thrombospondin-1 antagonizes nitric oxide-stimulated vascular smooth muscle cell responses. *Cardiovasc Res* 2006;71:785–793. [PubMed: 16820142]
16. Isenberg JS, Ridnour LA, Dimitry J, Frazier WA, Wink DA, Roberts DD. CD47 is necessary for inhibition of nitric oxide-stimulated vascular cell responses by thrombospondin-1. *J Biol Chem* 2006;281:26069–26080. [PubMed: 16835222]
17. Isenberg JS, Hyodo F, Matsumoto K, Romeo MJ, Abu-Asab M, Tsokos M, Kuppusamy P, Wink DA, Krishna MC, Roberts DD. Thrombospondin-1 limits ischemic tissue survival by inhibiting nitric oxidemediated vascular smooth muscle relaxation. *Blood* 2007;109:1945–1952. [PubMed: 17082319]
18. Isenberg JS, Romeo MJ, Abu-Asab M, Tsokos M, Oldenberg A, Pappan L, Wink DA, Frazier WA, Roberts DD. Increasing survival of ischemic tissue by targeting CD47. *Circ Res* 2007;100:712–720. [PubMed: 17293482]
19. Isenberg JS, Pappan LK, Romeo MJ, Abu-Asab M, Tsokos M, Wink DA, Frazier WA, Roberts DD. Blockade of Thrombospondin-1-CD47 Interactions Prevents Necrosis of Full Thickness Skin Grafts. *Ann Surg* 2008;247:180–190. [PubMed: 18156939]
20. Isenberg JS, Hyodo F, Pappan LK, Abu-Asab M, Tsokos M, Krishna MC, Frazier WA, Roberts DD. Blocking thrombospondin-1/CD47 signaling alleviates deleterious effects of aging on tissue responses to ischemia. *Arterioscler Thromb Vasc Biol* 2007;27:2582–2588. [PubMed: 17916772]

21. Ostergaard L, Stankevicius E, Andersen MR, Eskildsen-Helmond Y, Ledet T, Mulvany MJ, Simonsen U. Diminished NO release in chronic hypoxic human endothelial cells. *Am J Physiol Heart Circ Physiol* 2007;293:H2894–H2903. [PubMed: 17720765]
22. Takemoto M, Sun J, Hiroki J, Shimokawa H, Liao JK. Rho-kinase mediates hypoxia-induced downregulation of endothelial nitric oxide synthase. *Circulation* 2002;106:57–62. [PubMed: 12093770]
23. Gladwin MT, Raat NJ, Shiva S, Dezfulian C, Hogg N, Kim-Shapiro DB, Patel RP. Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation. *Am J Physiol Heart Circ Physiol* 2006;291:H2026–H2035. [PubMed: 16798825]
24. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov* 2008;7:156–167. [PubMed: 18167491]
25. Shiva S, Sack MN, Greer JJ, Duranski M, Ringwood LA, Burwell L, Wang X, MacArthur PH, Shoja A, Raghavachari N, Calvert JW, Brookes PS, Lefer DJ, Gladwin MT. Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. *J Exp Med* 2007;204:2089–2102. [PubMed: 17682069]
26. Kumar D, Branch BG, Pattillo CB, Hood J, Thoma S, Simpson S, Illum S, Arora N, Chidlow JH Jr, Langston W, Teng X, Lefer DJ, Patel RP, Kevil CG. Chronic sodium nitrite therapy augments ischemia-induced angiogenesis and arteriogenesis. *Proc Natl Acad Sci U S A* 2008;105:7540–7545. [PubMed: 18508974]
27. Lindberg FP, Gresham HD, Schwarz E, Brown EJ. Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane-spanning domains implicated in alpha v beta 3-dependent ligand binding. *J Cell Biol* 1993;123:485–496. [PubMed: 7691831]
28. Ueda K, Nozawa M, Miyasaka M, Akamatsu J, Tajima S. Sulfatide protects rat skin flaps against ischemia-reperfusion injury. *J Surg Res* 1998;80:200–204. [PubMed: 9878314]
29. Casey DB, Badejo AM Jr, Dhaliwal JS, Murthy SN, Hyman AL, Nossaman BD, Kadowitz PJ. Pulmonary vasodilator responses to sodium nitrite are mediated by an allopurinol-sensitive mechanism in the rat. *Am J Physiol Heart Circ Physiol* 2009;296:H524–H533. [PubMed: 19074675]
30. Papapetropoulos A, Rudic RD, Sessa WC. Molecular control of nitric oxide synthases in the cardiovascular system. *Cardiovasc Res* 1999;43:509–520. [PubMed: 10690323]
31. Cary SP, Winger JA, Derbyshire ER, Marletta MA. Nitric oxide signaling: no longer simply on or off. *Trends Biochem Sci* 2006;31:231–239. [PubMed: 16530415]
32. Lee YC, Martin E, Murad F. Human recombinant soluble guanylyl cyclase: expression, purification, and regulation. *Proc Natl Acad Sci U S A* 2000;97:10763–10768. [PubMed: 10995472]
33. Isenberg JS, Romeo MJ, Maxhimer JB, Smedley J, Frazier WA, Roberts DD. Gene silencing of CD47 and antibody ligation of thrombospondin-1 enhance ischemic tissue survival in a porcine model: implications for human disease. *Ann Surg* 2008;247:860–868. [PubMed: 18438125]
34. Isenberg JS, Frazier WA, Roberts DD. Thrombospondin-1: a physiological regulator of nitric oxide signaling. *Cell Mol Life Sci* 2008;65:728–742. [PubMed: 18193160]
35. Gladwin MT, Schechter AN, Kim-Shapiro DB, Patel RP, Hogg N, Shiva S, Cannon RO 3rd, Kelm M, Wink DA, Espey MG, Oldfield EH, Pluta RM, Freeman BA, Lancaster JR Jr, Feelisch M, Lundberg JO. The emerging biology of the nitrite anion. *Nat Chem Biol* 2005;1:308–314. [PubMed: 16408064]
36. Gribbe O, Gustafsson LE, Wiklund NP. Transdermally administered nitric oxide by application of acidified nitrite increases blood flow in rat epigastric island skin flaps. *Eur J Pharmacol* 2008;578:51–56. [PubMed: 17976572]
37. Weller R, Finnen MJ. The effects of topical treatment with acidified nitrite on wound healing in normal and diabetic mice. *Nitric Oxide* 2006;15:395–399. [PubMed: 16731016]
38. Schrammel A, Behrends S, Schmidt K, Koesling D, Mayer B. Characterization of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one as a hemesite inhibitor of nitric oxide-sensitive guanylyl cyclase. *Mol Pharmacol* 1996;50:1–5. [PubMed: 8700100]
39. Zhao Y, Brandish PE, DiValentin M, Schelvis JP, Babcock GT, Marletta MA. Inhibition of soluble guanylate cyclase by ODQ. *Biochemistry* 2000;39:10848–10854. [PubMed: 10978171]

40. Pellegrino D, Shiva S, Angelone T, Gladwin MT, Tota B. Nitrite exerts potent negative inotropy in the isolated heart via eNOS-independent nitric oxide generation and cGMP-PKG pathway activation. *Biochim Biophys Acta*. 2009
41. Crawford JH, Isbell TS, Huang Z, Shiva S, Chacko BK, Schechter AN, Darley-Usmar VM, Kerby JD, Lang JD Jr, Kraus D, Ho C, Gladwin MT, Patel RP. Hypoxia, red blood cells, and nitrite regulate NOdependent hypoxic vasodilation. *Blood* 2006;107:566–574. [PubMed: 16195332]
42. Kim-Shapiro DB, Schechter AN, Gladwin MT. Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics. *Arterioscler Thromb Vasc Biol* 2006;26:697–705. [PubMed: 16424350]
43. Amadeu TP, Seabra AB, de Oliveira MG, Monte-Alto-Costa A. Nitric Oxide Donor Improves Healing if Applied on Inflammatory and Proliferative Phase. *J Surg Res*. 2007
44. Witte MB, Kiyama T, Barbul A. Nitric oxide enhances experimental wound healing in diabetes. *Br J Surg* 2002;89:1594–1601. [PubMed: 12445072]
45. Masters KS, Leibovich SJ, Belem P, West JL, Poole-Warren LA. Effects of nitric oxide releasing poly(vinyl alcohol) hydrogel dressings on dermal wound healing in diabetic mice. *Wound Repair Regen* 2002;10:286–294. [PubMed: 12406164]
46. Ozyazgan I, Ozkose M, Baskol G. Nitric oxide in flow-through venous flaps and effects of L-arginine and nitro-L-arginine methyl ester (L-NAME) on nitric oxide and flap survival in rabbits. *Ann Plast Surg* 2007;59:550–557. [PubMed: 17992151]
47. Zhu H, Ka B, Murad F. Nitric oxide accelerates the recovery from burn wounds. *World J Surg* 2007;31:624–631. [PubMed: 17308846]
48. Shi HP, Most D, Efron DT, Witte MB, Barbul A. Supplemental L-arginine enhances wound healing in diabetic rats. *Wound Repair Regen* 2003;11:198–203. [PubMed: 12753601]
49. Engel H, Sauerbier M, Germann G, Kuntscher MV. Dose-dependent effects of a nitric oxide donor in a rat flap model. *Ann Plast Surg* 2007;58:456–460. [PubMed: 17413891]
50. Lawler J. Thrombospondin-1 as an endogenous inhibitor of angiogenesis and tumor growth. *J Cell Mol Med* 2002;6:1–12. [PubMed: 12003665]
51. Isenberg JS, Jia Y, Fukuyama J, Switzer CH, Wink DA, Roberts DD. Thrombospondin-1 inhibits nitric oxide signaling via CD36 by inhibiting myristic acid uptake. *J Biol Chem* 2007;282:15404–15415. [PubMed: 17416590]

Figure 1. Nitrite increases ischemic soft tissue survival

(A) Random dorsal myocutaneous flaps in C57BL/6 mice were created. Animals were randomized into three groups, untreated, vehicle alone and nitrite (48 nmols/animal i.p.). Flap survival was evaluated 7 days after surgery. (B) Acute changes in blood flow were determined via laser Doppler during the first post-operative hour. * = $p < 0.05$ compared to vehicle. (C) Nitric oxide levels were determined via chemiluminescence from acutely excised ischemic soft tissue flaps following nitrite treatment (10 mM) in the presence or absence of allopurinol (500 μ M) or potassium hexacyanoferrate (500 μ M). (C) Representative raw data and (D) quantification of NO generation under the indicated treatment conditions. Results presented are the mean $\pm$ SD, $n = 3$ in each treatment group. * = $p < 0.05$.

Figure 2. Nitrite enhancement of ischemic flap survival is abrogated by inhibition of sGC activation (A) Random dorsal myocutaneous flaps 1×3 cm in dimensions were created in male C57BL/6 mice. Animals received nitrite (48 nmols/animal) or nitrite and ODQ (20 μ g/g i.p.). Flap survival was analyzed on post-operative day 7. Results presented are the mean $\pm$ SD of 5 animals in each group. * = $p < 0.05$ compared to nitrite alone. (B) Flap blood flow was assessed during the immediate post-operative interval via laser Doppler. Results presented are the mean $\pm$ SD of 5 animals in each group. * = $p < 0.05$ compared to nitrite plus ODQ.

Figure 3. Myocutaneous tissue survival to ischemia is increased by combined TSP1 targeting and nitrite

(A) Random dorsal myocutaneous flaps 1 × 3 cm in dimension were created in male C57BL/6 mice and evaluated on post-operative day 7. Animals received an IgG2 control Ab, a TSP1 monoclonal antibody (clone Ab A6.1) (both at 2.4 μg per tissue unit), nitrite (48 nmols/animal) plus an IgG2 Ab, or nitrite plus the A6.1. Representative flaps were photographed. (B) Flap survival is expressed as percent of total and was determined as described in the methods. Results are the mean ± SD of 5 animals in each group. * = p<0.05 compared to isotype control Ab or Ab A6.1 alone. (C) A dose range of TSP1 antibody A6.1 (0.3, 0.6, 1.2 and 2.4 μg per tissue unit) was tested against an optimum amount of nitrite (48 nmols/animal) and flap survival

determined. * = $p < 0.05$ compared to nitrite alone. **(D)** Tissue cGMP was determined by ELISA from flaps on post-operative day 7 and results normalized to total protein. Results are the mean $\pm$ SD of 5 animals in each group. * = $p < 0.05$ compared to Ab A6.1 alone.

Figure 4. Concurrent CD47 suppression and nitrite supplementation increases ischemic tissue survival beyond single agent therapy

(A) Random dorsal myocutaneous flaps 1 × 3 cm in dimension were created in sex and age matched C57BL/6 mice. Animals received a CD47 suppressing morpholino or a mismatch control morpholino alone or in combination with nitrite at the time of surgery. (B) Flap survival was assessed on post-operative day seven. Results represent the mean ± SD of 5 animals in each group. * = $p < 0.05$ compared to CD47 morpholino alone.

A.

B.

Figure 5. Nitrite and TSP1-CD47 blocking antibodies increase muscle explant angiogenic response (A) Pectoralis major muscle biopsies (1 mm³) excised from C57BL/6 mice were embedded in 3D type I collagen matrix and incubated in growth medium $\pm$ nitrite (0.1 – 10 μ M) $\pm$ TSP1 monoclonal antibody (clone Ab 6.1 (1 μ g/ml)) or $\pm$ a CD47 monoclonal antibody (clone 301 (1 μ g/ml)). (B) Angiogenic response was quantified 4 days post-explantation. Photomicrographs were obtained at 10x. Results represent the mean $\pm$ SD of three separate experiments.

Figure 6. Nitrite and TSP1-CD47 blockade enhances macro-vascularity of random ischemic soft tissue flaps

(A) Random dorsal myocutaneous flaps 1 × 3 cm in dimensions were created in male C57BL/6 mice. Animals received the indicated treatments at reported dosages (see Fig 2). On the 7th post-operative day animals were anesthetized and the flap undersurface digitally photographed. (B) Images were later quantified as described in the methods section to yield a vascularity index. Results presented are the mean ± SD of 5 animals in each treatment group. * = p<0.05 compared to a matched isotype control Ab.

Figure 7. Nitrite and TSP1-CD47 blockade enhance micro-vascularity of random ischemic soft tissue flaps

(**A**) Random dorsal myocutaneous flaps were excised, fixed and stained with hematoxylin and eosin. (**B**) Flaps were then visualized under 10x magnification and patent and blood filled vessels were counted. Results are from 5 flaps in each treatment category. * = $p < 0.05$ compared to a matched isotype control Ab. Blue arrows indicated vessels containing red blood cells.

Figure 8. Nitrite and TSP1-CD47 signaling pathways converge at sGC

Tissue ischemia arises from a decreased blood flow. As blood flow decreases tissue perfusion and oxygen drop and pH increases. In this microenvironment nitrite acts as an NO prodrug being converted by endogenous reductase activity to NO. Thus exogenous nitrite selectively delivers *exogenous* NO to areas where it is most needed. NO activates sGC to increase cGMP synthesis. cGMP acts on several downstream targets in endothelial cells to stimulate pro-angiogenic responses and in vascular smooth muscle cells to dilate blood vessels. At physiological concentrations, TSP1 acts primarily through its receptor CD47 to limit sGC activation [16]. At nM concentrations, TSP1 also signals through CD36 to inhibit the same responses [51], but CD47 is also necessary for these signals to inhibit cGMP signaling. Blockade of TSP1-CD47 signaling with monoclonal antibodies to TSP1 (Fig. 3) and CD47 or gene silencing of CD47 (Fig. 4) increases the pro-survival activities of NO and enhances angiogenesis, tissue perfusion and blood flow and survival from ischemia.