HHS Public Access

Author manuscript

Ann N Y Acad Sci. Author manuscript; available in PMC 2015 April 29.

Published in final edited form as:

Ann N Y Acad Sci. 2010 October ; 1207: 83–88. doi:10.1111/j.1749-6632.2010.05737.x.

Reperfusion of chronic tissue ischemia: nitrite and dipyridamole regulation of innate immune responses

Christopher B. Pattillo, **Kai Fang**, **Justin Terracciano**, and **Christopher G. Kevil**

Department of Pathology, LSU Health Sciences Center-Shreveport, Shreveport, Louisiana

Abstract

Chronic and intermittent ischemic vascular disorders represent a burgeoning clinical challenge. Previous studies have focused on the idea that therapeutic angiogenesis strategies could alleviate tissue ischemia; however, it is now appreciated that vascular disease is not simply limited to vascular wall cells but also influenced by simultaneously occurring inflammatory responses. Our laboratory has discovered that pharmacological treatment of permanent tissue ischemia with dipyridamole significantly augments ischemic tissue reperfusion, angiogenesis, and arteriogenesis over time. We have found that the beneficial effects of dipyridamole therapy are due to its ability to increase tissue nitric oxide bioavailability that corrects tissue redox imbalance. Importantly, we have also discovered that dipyridamole treatment invoking nitric oxide (NO) production significantly downregulates various innate immune response genes during chronic ischemic tissue injury. These findings demonstrate that dipyridamole induced production of nitrite/NO significantly decreases inflammatory responses while increasing vascular growth in ischemic tissues.

Keywords

inflammation; angiogenesis; interleukin 10; toll like receptors nitric oxide

Introduction

Occlusive vascular diseases remain one of the leading causes of health problems in industrialized nations leading to ischemic tissue dysfunction. Remodeling of the vasculature through therapeutic angiogenesis and arteriogenesis has become a primary goal for the treatment of intermittent and chronic tissue ischemia. However, both clinical and basic science studies reveal complex regulation of ischemic revascularization that currently evades our ability to therapeutically enhance this response.

Angiogenesis is defined as the development of new microvessels from pre-existing capillaries. Hypoxia triggers a cascade of events leading to increased angiogenic activity that involves increased transcription factor activity (e.g., HIF-1), expression of various growth factors (e.g., VEGF-A, bFGF, etc.), and subsequent endothelial cell sprouting, proliferation,

Correspondence: Christopher G. Kevil, Ph.D., LSU Health Sciences Center-Shreveport, 1501 Kings Hwy, Shreveport, LA 71130, ckevil@lsuhsc.edu.

and directional migration[1]. The nascent microvasculature further develops through anastamoses and is enveloped by pericytes to form a mature microcirculatory unit. Through this process, increased angiogenic activity is essential to restore perfusion of oxygen and nutrients and exchange metabolic waste in ischemic tissues.

Arteriogenesis involves the development of arteries from collateral vessels and is not necessarily dependent on hypoxia for initiation[2]. Physical forces initiate arteriogenesis with fluid shear stress (FSS) being an important factor leading to vessel structural changes[3]. Integrins, tyrosine-kinase receptors, and ion channels act as sensors that detect changes in FSS to stimulate the endothelium, thus increasing arteriogenesis[4]. In addition, leukocyte infiltration into tissues also plays a key role in arteriogenesis. Following activation, endothelial cells produce chemokines such as monocyte chemoattractant protein-1 (MCP-1) to recruit monocytes that influence arteriogenesis activity[5]. Heil et al. demonstrated that monocyte infiltration is important in modulating arteriogenesis[5]. In this study, the authors showed that monocyte recruitment is important in regulating arteriogenesis activity in two different hind limb ligation models of tissue ischemia, such that a reduction in monocyte activity inhibits collateral vessel growth.

Leukocyte infiltration into ischemic tissues serves to activate several aspects of angiogenesis and arteriogenesis. Leukocyte recruitment involves numerous steps, including immune cell rolling, firm adhesion, and transmigration into the intravascular space. Proteins such as endothelial P-selectin and E-selectin adhere to leukocyte ligands, causing increased leukocyte rolling along the endothelial surface. Members of the immunoglobulin superfamily proteins, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), then regulate leukocyte firm adhesion to the endothelial cell surface. Once firmly adhered, additional adhesion molecules, including junctional adhesion molecules (JAM's) and platelet endothelial cell adhesion molecule (PECAM-1), mediate leukocyte transmigration across the endothelial cell barrier. After transmigration, leukocytes can release numerous different cytokines, chemokines, and proteases, which induce proliferation and migration of endothelial and smooth muscle cells. Interestingly, endothelial cell adhesion molecules themselves may also participate in angiogenic activity by regulating endothelial cell activation, signal transduction activity, and intracellular redox status[6–10].

As vascular blockage results in decreased blood flow to areas downstream of that vessel, functional reperfusion of ischemic tissue is rerouted around the area of blockage in healthy animals and patients[11]. However, specific beneficial approaches that facilitate reperfusion of ischemic tissue in disease states are poorly understood and likely contribute to the inability to translate past discoveries to the clinic. The femoral artery ligation model of the mouse (and other animals) provides a straightforward way in which to evaluate intervention strategies aimed at enhancing vascular growth and function. Studies using this model reveal that enhancing blood flow through pre-existing collateral vessels and modulation of various inflammatory responses impact vascular remodeling responses, thus highlighting diverse mechanisms participating in therapeutic arteriogenesis/angiogenesis[11–13].

Dipyridamole therapy enhances vascular development in a NO dependent fashion

Dipyridamole in combination with aspirin is used clinically as an anti-platelet drug decreasing risk for recurrent stroke. Dipyridamole itself is known to do more than platelet inhibition, with other biological actions being anti-inflammatory, anti-oxidant, and inhibiton of phosphodiesterases and potentiating the effects of nitric oxide[14–18]. We have recently tested the effect of dipyridamole on blood flow restoration in a model of chronic tissue ischemia[15]. We found that by day 5 following induction of chronic ischemia, ischemic limb perfusion was largely restored. The restoration in blood flow correlated with enhanced vascular density as well as cellular proliferation. Through the use of an endothelial nitric oxide synthase (eNOS) deficient mutant mouse strain, we were able to identify eNOS as playing an integral role in the revascularization process. We were able to demonstrate that dipyridamole augmented angiogenesis due to PKA dependent eNOS activation, which enhanced production of nitric oxide and subsequent plasma nitrite. These findings suggest that dipyridamole therapy may be beneficial in a clinical setting of chronic tissue ischemia by increasing eNOS and angiogenic activity. What made the effects of dipyridamole therapy unique was not only the fact that eNOS activity was elevated but that increased bioavailable NO emanated from non-ischemic tissue, leading to an increase in plasma nitrite anion, supporting the hypothesis of an endocrine nitrite/nitric oxide-dependent pathway proposed by Elrod et al.[19]

Nitrite anion therapy enhances vascular development effecting gene regulation of inflammation and cellular protection pathways

We have previously shown that sodium nitrite therapy is able to enhance tissue perfusion and angiogenesis in a model of chronic ischemia[20]. We found that nitrite therapy selectively increased ischemic tissue NO and enhanced angiogenesis in a NO dependent manner. Dipyridamole therapy also enhanced angiogenesis by enhancing nitric oxide/nitrite production. These findings squarely identify nitric oxide as a critical regulator of angiogenic potential in an ischemic setting. We subsequently performed whole genome array analysis on mice treated with sodium nitrite therapy at days 3 and 7 post-ligation[21]. Our gene array analysis showed that at day 3 post-ischemia, sodium nitrite treated animals had significantly decreased inflammatory gene expression concomitant with an increase in genes which function to protect and preserve skeletal muscle tissue. By 7 days post ischemia, cell survival and tissue development genes were upregulated in the nitrite treated groups. These gene array findings, combined with our previous report of nitrite dependent induction of angiogenesis, suggest that nitrite therapy may facilitate vascular remodeling by modulating inflammatory gene expression. Disruption of inflammatory gene expression may also act to protect tissue in ischemic regions via upregulation of genes influencing cardiovascular system development such as vascular endothelial cell cadherin, purinergic receptor P2Y, and vitronectin, thereby allowing increased vascular cell proliferation and development in these regions at later time points[21].

Dipyridamole or nitrite therapy alter innate immune gene expression

Shortly following ligation (i.e., 3 days post-ligation) and induction of permanent hind limb ischemia, increased inflammatory infiltrates occur within ischemic tissue[5]. This fact combined with our previous data above prompted us to perform a PCR array focused on innate immune response genes. To examine possible differences in innate immunity gene expression, we performed unilateral femoral artery ligation of the left hind limb in mice to establish chronic tissue ischemia as we have previously reported[15, 20, 22]. Animals were treated with either dipyridamole (200 mg/kg) or sodium nitrite (165 ug/kg) until three days post-ligation, at which time gastrocnemius muscle tissue from both limbs (ischemic and non-ischemic) were harvested for mRNA isolation. Quantitative real time PCR arrays for innate and adaptive immune responses were used to measure the effects of both treatments on gene expression.

Sodium nitrite therapy resulted in the downregulation of 21 genes and the upregulation of 5 genes involved in innate immune responses (Table 1). The five immune genes that were upregulated have a paucity of information associated with them regarding their influence on tissue ischemia or angiogenesis. The toll interacting protein (Tollip) is an inhibitory protein in the toll-like receptor pathway, whose upregulation would corroborate attenuation of innate immune responses[23]. Differential expression of innate immune response genes, combined with our knowledge that sodium nitrite therapy promotes endothelial cell proliferation, indicates that certain aspects of innate immunity may be anti-angiogenic, at least at early time points post-ischemia. TNF-receptor associated factor 6 (Traf6) upregulation inhibits TNF-alpha induced apoptosis by downregulating the presence of reactive oxygen species[24]. Ruckdeschel et al. implicated that Traf6 promotes macrophage survival in a NF-kappa B related cascade[25]. Clearly, Traf6 and its anti-apoptotic nature would work to preserve ischemic tissue until blood flow can be restored. In the same article by Ruckdeschel et al., interleukin-1 receptor-associated kinase 2 (Irak2) was identified as promoting macrophage apoptosis in a bacterial insult model of host immune response[25]. These two genes (Traf6 and Irak2) appear to have opposite effects such that the upregulation of both molecules appears counterproductive. However, this could be influenced by the temporal nature of chronic tissue ischemia, as differential gene expression occurs at both early and late time points. Traf6 may promote macrophage survival early in the angiogenic/arteriogenic process, allowing for the delivery of cytokines that could enhance the angiogenic potential of the tissue. At later time points, Irak2 expression could be increased to clear tissue of unnecessary macrophages recruited for cytokine delivery.

Nitrite therapy also significantly upregulated heat shock protein 90 alpha, class B member 1 (Hsp90ab1), and macrophage migration inhibitor factor (Mif). VEGF and fluid shear stress have both been reported to augment Hsp90ab1 binding to eNOS, where it promotes eNOS activation leading to increased NO production in blood vessels[26]. The fact that Hsp90ab1 gene expression is increased suggests that nitrite therapy could establish a positive feedback response, further promoting nitric oxide production in ischemic tissue. Mif has been shown to inactivate p53, which inhibits angiogenesis[27], implicating a possible novel role for Mif in regulating cytoprotection and inhibition of p53 mediated responses during ischemic insult. Together, identification of these five immune related genes indicates that modulation of

innate immunity gene expression during chronic tissue ischemia is beneficial for vascular remodeling and tissue reperfusion.

Dipyridamole or sodium nitrite therapy significantly downregulated expression of genes associated with innate immunity (Tables 1 and 2). Interestingly, either treatment approach substantially decreased IL-10 gene expression, which has been implicated in various aspects of angiogenesis and vascular remodeling. Previous reports suggest that increased IL-10 expression is associated with pathological angiogenesis seen in cancer and retinal hypoxia during development[28–31]. However, Silvestre et al. reported that IL-10 acts in an anti-angiogenic manner during hind limb ischemia, as genetic deficiency of the molecule augmented ischemic limb reperfusion, vascular remodeling, and VEGF expression[32]. Our findings are consistent with these reports and indicate that both nitrite and dipyridamole therapy likely augment reperfusion of ischemic tissue due to inhibition of IL-10 expression.

Dipyridamole and sodium nitrite therapy decreased Toll-like receptor 1 and 3 expression in ischemic tissue (Tables 1 and 2). This is a novel and surprising finding, as little to no information exists regarding the role or importance of Toll-like receptors for ischemia induced angiogenesis or vascular remodeling. However, a previous study by Lee et al. reported that Tlr-3 signaling may be involved in regulating endothelial cell adhesion molecule and cytokine expression[33]. Moreover, Müller et al. showed that candida albicans activates Tlr-3/MyD88 signaling, leading to endothelial cell IL-8 expression[34]. These data are consistent with a recent report demonstrating that genetic deficiency of MyD88 significantly impairs wound healing mediated by adenosine A2A receptors[35]. While these findings do not provide any clear indication as to how modulation of the Tlr pathway regulates ischemic angiogenesis, it may be that these molecules play a more dominant role in pathological angiogenic responses associated with chronic inflammation[6, 8]. Together, these findings highlight novel innate immune responses in regulating ischemic tissue revascularization and reperfusion that requires additional investigation in greater detail.

As we continue to understand the mechanistic basis of vascular occlusive diseases, it is important to keep in mind that pathogenic mechanisms are multifactorial involving different cell types and locally produced factors (e.g., cytokines/chemokines). Here we demonstrate that regulation of innate inflammation appears to critically influence the initiation and development of angiogenesis during chronic tissue ischemia. Sodium nitrite therapy for permanent vascular occlusion provides protection to ischemic tissue through anti-apoptotic gene regulation as well as altered innate inflammatory gene expression. Dipyridamole therapy also downregulates innate immune response genes, yet its effect is more modest in comparison to sodium nitrite therapy. In summary, both our clinical and molecular findings clearly demonstrate that alteration of innate immune response gene expression plays an important role in governing ischemia induced angiogenesis and vascular remodeling. These novel insights suggest that alteration of these immune response pathways may be a useful target for therapeutic manipulation. However, much more work is needed to understand precisely how innate immune responses modulate ischemic angiogenesis and vascular remodeling.

Acknowledgments

The authors would like to thank Dr. Wolfgang Eisert for helpful discussions and comments.

This work was supported by NIH grants HL080482 and HL094021, and a Boehringer Ingelheim research grant.

Disclosures

C.G.K. received research grant support from Boehringer Ingelheim.

References

1. Semenza G. Signal transduction to hypoxia-inducible factor 1. Biochem Pharmacol. 2002; 64:993–8. [PubMed: 12213597]
2. Heil M, et al. Arteriogenesis versus angiogenesis: similarities and differences. J Cell Mol Med. 2006; 10:45–55. [PubMed: 16563221]
3. Pipp F, et al. Elevated fluid shear stress enhances postocclusive collateral artery growth and gene expression in the pig hind limb. Arterioscler Thromb Vasc Biol. 2004; 24:1664–8. [PubMed: 15242864]
4. Davies PF, et al. Spatial relationships in early signaling events of flow-mediated endothelial mechanotransduction. Annu Rev Physiol. 1997; 59:527–49. [PubMed: 9074776]
5. Heil M, et al. Blood monocyte concentration is critical for enhancement of collateral artery growth. Am J Physiol Heart Circ Physiol. 2002; 283:H2411–9. [PubMed: 12388258]
6. Chidlow JH Jr, et al. Pathogenic angiogenesis in IBD and experimental colitis: new ideas and therapeutic avenues. Am J Physiol Gastrointest Liver Physiol. 2007; 293:G5–G18. [PubMed: 17463183]
7. Mousa SA. Cell adhesion molecules: potential therapeutic & diagnostic implications. Mol Biotechnol. 2008; 38:33–40. [PubMed: 18095189]
8. Noonan DM, et al. Inflammation, inflammatory cells and angiogenesis: decisions and indecisions. Cancer Metastasis Rev. 2008; 27:31–40. [PubMed: 18087678]
9. Vannini N, et al. Endothelial cell aging and apoptosis in prevention and disease: E-selectin expression and modulation as a model. Curr Pharm Des. 2008; 14:221–5. [PubMed: 18220832]
10. Langston W, et al. Regulation of endothelial glutathione by ICAM-1 governs VEGF-A-mediated eNOS activity and angiogenesis. Free Radic Biol Med. 2007; 42:720–9. [PubMed: 17291995]
11. Ziegler MA, et al. Marvels, mysteries, and misconceptions of vascular compensation to peripheral artery occlusion. Microcirculation. 17:3–20. [PubMed: 20141596]
12. Clayton JA, Chalothorn D, Faber JE. Vascular endothelial growth factor-A specifies formation of native collaterals and regulates collateral growth in ischemia. Circ Res. 2008; 103:1027–36. [PubMed: 18802023]
13. Dai X, Faber JE. Endothelial nitric oxide synthase deficiency causes collateral vessel rarefaction and impairs activation of a cell cycle gene network during arteriogenesis. Circ Res. 106:1870–81. [PubMed: 20431061]
14. Neri Serneri GG, et al. Enhanced prostacyclin production by dipyridamole in man. Eur J Clin Pharmacol. 1981; 21:9–15. [PubMed: 7037426]
15. Venkatesh PK, et al. Dipyridamole enhances ischaemia-induced arteriogenesis through an endocrine nitrite/nitric oxide-dependent pathway. Cardiovasc Res. 85:661–70. [PubMed: 20061326]
16. Aktas B, et al. Dipyridamole enhances NO/cGMP-mediated vasodilator-stimulated phosphoprotein phosphorylation and signaling in human platelets: in vitro and in vivo/ex vivo studies. Stroke. 2003; 34:764–9. [PubMed: 12624305]
17. Kusmic C, et al. Antioxidant effect of oral dipyridamole during cerebral hypoperfusion with human carotid endarterectomy. J Cardiovasc Pharmacol. 2000; 36:141–5. [PubMed: 10942153]
18. Selley ML, et al. Dipyridamole inhibits the oxidative modification of low density lipoprotein. Atherosclerosis. 1994; 111:91–7. [PubMed: 7840817]

19. Elrod JW, et al. Nitric oxide promotes distant organ protection: evidence for an endocrine role of nitric oxide. Proc Natl Acad Sci U S A. 2008; 105:11430–5. [PubMed: 18685092]
20. Kumar D, et al. Chronic sodium nitrite therapy augments ischemia-induced angiogenesis and arteriogenesis. Proc Natl Acad Sci U S A. 2008; 105:7540–5. [PubMed: 18508974]
21. Pattillo CB, et al. Genome expression profiling and network analysis of nitrite therapy during chronic ischemia: possible mechanisms and interesting molecules. Nitric Oxide. 22:168–79. [PubMed: 19963074]
22. Senthilkumar A, et al. Sildenafil promotes ischemia-induced angiogenesis through a PKG-dependent pathway. Arterioscler Thromb Vasc Biol. 2007; 27:1947–54. [PubMed: 17585066]
23. Schimming TT, et al. Association of toll-interacting protein gene polymorphisms with atopic dermatitis. BMC Dermatol. 2007; 7:3. [PubMed: 17362526]
24. Ichikawa D, et al. TNF-receptor associated factor 6-deficient fibroblast is sensitive to the TNF-alpha-induced cell death: involvement of reactive oxygen species. Biochem Biophys Res Commun. 2006; 351:93–8. [PubMed: 17055451]
25. Ruckdeschel K, Mannel O, Schrottner P. Divergence of apoptosis-inducing and preventing signals in bacteria-faced macrophages through myeloid differentiation factor 88 and IL-1 receptor-associated kinase members. J Immunol. 2002; 168:4601–11. [PubMed: 11971008]
26. Garcia-Cardena G, et al. Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature. 1998; 392:821–4. [PubMed: 9580552]
27. Hudson JD, et al. A proinflammatory cytokine inhibits p53 tumor suppressor activity. J Exp Med. 1999; 190:1375–82. [PubMed: 10562313]
28. Dace DS, et al. Interleukin-10 promotes pathological angiogenesis by regulating macrophage response to hypoxia during development. PLoS One. 2008; 3:e3381. [PubMed: 18852882]
29. Garcia-Hernandez ML, et al. Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and induction of tumour and vascular cell proliferation. Immunology. 2002; 105:231–43. [PubMed: 11872099]
30. Nagata J, et al. Correlation between interleukin 10 and vascular endothelial growth factor expression in human esophageal cancer. Int J Mol Med. 2002; 10:169–72. [PubMed: 12119553]
31. Sakamoto T, et al. Interleukin-10 expression significantly correlates with minor CD8+ T-cell infiltration and high microvessel density in patients with gastric cancer. Int J Cancer. 2006; 118:1909–14. [PubMed: 16287061]
32. Silvestre JS, et al. Antiangiogenic effect of interleukin-10 in ischemia-induced angiogenesis in mice hindlimb. Circ Res. 2000; 87:448–52. [PubMed: 10988235]
33. Lee MT, et al. Interferon-beta and adhesion molecules (E-selectin and s-intracellular adhesion molecule-1) are detected in sera from patients with retinal vasculitis and are induced in retinal vascular endothelial cells by Toll-like receptor 3 signalling. Clin Exp Immunol. 2007; 147:71–80. [PubMed: 17177965]
34. Muller V, et al. Candida albicans triggers activation of distinct signaling pathways to establish a proinflammatory gene expression program in primary human endothelial cells. J Immunol. 2007; 179:8435–45. [PubMed: 18056390]
35. Macedo L, et al. Wound healing is impaired in MyD88-deficient mice: a role for MyD88 in the regulation of wound healing by adenosine A2A receptors. Am J Pathol. 2007; 171:1774–88. [PubMed: 17974599]

Table 1

Sodium nitrite 3 day- changes in innate immunity gene expression

Gene Name	Fold Change	p-value
interleukin-1 receptor-associated kinase 2	1.65	0.030080
heat shock protein 90 alpha (cytosolic), class B member 1	1.47	0.040898
macrophage migration inhibitory factor	1.34	0.046379
toll interacting protein	1.25	0.019830
TNF receptor-associated factor 6	1.25	0.026126
hypoxanthine guanine phosphoribosyl transferase	−1.15	0.045531
interleukin 1 receptor-like 2	−1.24	0.029575
glucuronidase, beta	−1.27	0.032143
toll-like receptor 2	−1.39	0.034887
caspase 1	−1.49	0.019856
collectin sub-family member 12	−1.49	0.037695
complement factor properdin	−1.63	0.002484
interleukin 1 receptor accessory protein	−1.63	0.017015
platelet-activating factor receptor	−1.63	0.046303
Interleukin 1 Receptor Antagonist	−1.87	0.002026
toll-like receptor 6	−1.87	0.028804
heme oxygenase (decycling) 1	−1.96	0.032096
CD14 antigen	−2.01	0.013929
toll-like receptor 9	−2.01	0.034892
interleukin 1 receptor, type I	−2.2	0.003264
toll-like receptor 1	−2.65	0.001952
interleukin 1 receptor, type II	−2.71	0.027270
toll-like receptor 8	−2.71	0.034340
chemokine (C-C motif) receptor 3	−3.05	0.003930
interleukin 10	−3.19	0.028143
pro-platelet basic protein	−3.42	0.010765

Table 2

Dipyridamole 3 day- changes in innate immunity gene expression

Gene Name	Fold Change	p-value
interleukin 10	−2.73	0.025868
toll-like receptor 1	−2.07	0.036956
toll-like receptor 3	−1.6	0.039925