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Mechanisms of I/R-induced Endothelium-Dependent Vasodilator Dysfunction

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Abstract

Ischemia/reperfusion (I/R) induces leukocyte/endothelial cell adhesive interactions (LECA) in postcapillary venules and impaired endothelium-dependent, NO-mediated dilatory responses (EDD) in upstream arterioles. A large body of evidence has implicated reactive oxygen species (ROS), adherent leukocytes, and proteases in postischemic EDD dysfunction in conduit arteries. However, arterioles represent the major site for the regulation of vascular resistance, but have received less attention with regard to the mechanisms underlying their reduced responsiveness to EDD stimuli in I/R. Even though leukocytes do not roll along, adhere to, or emigrate across arteriolar endothelium in postischemic intestine, recent work indicates that I/R-induced venular LECA is causally linked to EDD in arterioles. An emerging body of evidence supports the view that I/R-induced EDD in arterioles occurs by a mechanism that is triggered by LECA in postcapillary venules and involves the formation of signals in the interstitium elicited by the proteolytic activity of emigrated leukocytes. This activity releases matricryptins from or exposes matricryptic sites in the extracellular matrix (ECM) that interact with the integrin $\alpha v \beta 3$ to induce mast cell chymase-dependent formation of angiotensin II (Ang II). Subsequent activation of NAD(P)H oxidase by Ang II leads to the formation of oxidants which inactivate NO, resulting in arteriolar EDD dysfunction. ROS generation also promotes eNOS uncoupling in the vascular wall, exacerbating impaired EDD responses. This work establishes new links between LECA in postcapillary venules, signals generated in the interstitium by emigrated leukocytes, mast cell degranulation, and impaired EDD in upstream arterioles. Given the importance of the endothelium in regulating vascular tone, these fundamentally important findings have enormous implications for our understanding of blood flow dysregulation in conditions characterized by I/R.

Keywords

Leukocyte adhesion; leukocyte emigration; matrix metalloproteinases; calpains; matricryptins; extracellular matrix; mast cells; endothelium-dependent vasodilation; myeloperoxidase; NADPH oxidase

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Introduction

Ischemia/reperfusion (I/R) is a localized pathology that often leads to multi-organ dysfunction and shock (Granger and Korthuis, 1995; Kalogeris, Baines, Krenz & Korthuis, 2016; Kalogeris, Baines, Krenz & Korthuis, 2012; Rosario, Waldo, Becker, Schmid-Schonbein, 2004; Boros, 2003). One of the earliest events in the pathogenesis of I/R is microvascular dysfunction that is accompanied by a diminution in nitric oxide (NO) production by the endothelium. This pronounced reduction in NO bioavailability has been implicated as a trigger for leukocyte/ endothelial cell adhesive interactions (LECA) and increased permeability in postcapillary venules (Boros, 2003; Granger, 2003; Granger and Korthuis, 1995; Kalogeris et al, 2012; Kalogeris 2016; Kurose, Wolf, Grisham & Granger, 1994; Rosario, Waldo, Becker, Schmid-Schonbein, 2004). Reperfusion of ischemic tissues also alters vasoconstrictor responses and produces deficits in endothelium-dependent, NO-mediated vascular reactivity (EDD) in arteries and arterioles (Banda, Lefer & Granger, 1997; Cuccocrea, Mazzon, Costantino, Seraino, De Sarro & Caputi, 2000; Faraci & Didion, 2004; Gao, Korthuis & Benoit, 1994; Gao, Korthuis & Benoit, 1996; Gao, Benoit & Korthuis, 1996; Hansen, 1998; Hayward and Lefer, 1998; Hayward & Lefer, 1999; Hayward, Campbell, Shin, Scalia & Lefer, 1999; Hein, Zhang, Wang, Chang, Thengchaisri & Kuo, 2003; Kalogeris et al, 2012; Kalogeris et al, 2016; Lefer & Ma, 1994; Lefer & Aoki, 1990; Lefer, Tsao, Lefer & Ma, 1991; Ma, Tsao & Lefer, 1991; Ma, Lefer, Lefer & Rothlein, 1992; Ma, Weyrich, Lefer, Buerke, Albertine, Kishimoto, et al, 1993; Ruh, Schmidt & Vogel, 2003; Tsao, Aoki, Lefer, Johnson & Lefer, 1990; Tsao, Ma & Lefer, 1992; Touyz, 2004; Weyrich, Ma, Lefer, Albertine & Lefer, 1993; Wolin, Gupte & Oekler, 2002; Woodman, Hart & Sobey, 1997; Yokohama, Korthuis & Benoit, 1996; Zhao, Nakamura, Wang, Wilcox, Shearer, Guyton et al, 1999).

In conduit arteries, it appears that superoxide and other reactive oxygen species (ROS) play a major role the development of EDD dysfunction after I/R (Faraci & Didion, 2004; Gourdin et al, 2009; Granger, 2006; Kalogeris et al 2012; Kalogeris et al 2017; Mikhed, Daiber & Steven, 2015; Touyz, 2004; Widlansky & Gutterman, 2011; Wolin et al, 2002). Based on studies conducted in adhesion molecule-deficient mice or in animals treated with anti-adhesion reagents it appears that adherent neutrophils also contribute to postischemic impaired vasorelaxation to acetylcholine in conduit arteries (Banda et al, 1997; Hayward & Lefer, 1999; Hayward et al, 1999; Lefer & Aoki, 1990; Ma et al, 1991, 1992, 1993; Tsao et al, 1992; Weyrich et al, 1993). In sharp contrast to conduit arteries, leukocytes do not or only very rarely roll along, adhere to, or emigrate across postischemic arteriolar endothelium in most peripheral tissues, as they do in postcapillary venules (Kalogeris et al, 2012; Kalogeris et al, 2016). Thus, a role for these inflammatory phagocytes in the postischemic impairment in EDD responses that occurs in arterioles had been largely ignored. However, an emerging body of evidence suggests that there may be a mechanistic link between postcapillary venular LECA and EDD in upstream arterioles (Banda et al, 1997; Dai & Korthuis, 2017).

Although the latter studies indicate that LECA in postcapillary venules are causally linked to EDD in upstream arterial vessels, the mechanisms coupling these manifestations of I/R-induced microvascular dysfunction are only now becoming understood. The purpose of this review is to summarize the evidence implicating roles for ROS, proteases, and neutrophils in

the pathogenesis of I/R-induced EDD dysfunction in conduit arterial vessels versus arterioles.

Leukocyte-Mediated Postischemic Endothelial Dysfunction in Arterioles and Venules

Endothelial cells (EC) lining the microvasculature are particularly vulnerable to the deleterious effects of I/R (Granger & Korthuis, 1995; Granger 2003; Kalogeris et al 2012; Kalogeris 2016). Prolonged ischemia (or hypoxia in cell culture models) results in depletion of intracellular energy stores, disrupted cytoskeletal architecture, EC swelling, diminished production of certain bioactive agents (e.g., prostacyclin, NO) and accelerated formation of others (e.g., endothelin, Ang II, thromboxane A2, chemotactic mediators). Hypoxic exposure induces the expression of some endothelial genes (e.g., adhesion molecules, cytokines) while suppressing others (e.g., eNOS, thrombomodulin). Reoxygenation or reperfusion exacerbates these EC responses, which occur rapidly and often without obvious morphologic evidence of cell injury in the initial period after blood flow restitution (Granger & Korthuis, 1995; Granger 2003; Kalogeris et al 2012; Kalogeris et al 2016). Even though all EC in the microcirculation are exposed to the detrimental effects of I/R, the characteristics of EC dysfunction are manifested in a site-specific manner within the functionally distinct segments of the microvasculature, as outlined below.

Postcapillary Venular Responses to I/R

Most of the inflammatory responses elicited by I/R can be attributed to EC activation in postcapillary venules (Granger & Korthuis, 1995; Granger 2003; Kalogeris et al 2012; Kalogeris 2016). Characteristic features of postcapillary venular responses to I/R include enhanced oxidant production, leukocyte rolling and stationary adhesion, transendothelial leukocyte migration, platelet-leukocyte aggregation, platelet/endothelial cell adhesion, and protein extravasation. Mast cells and macrophages also release substances that can amplify the inflammatory response to I/R (Boros, Kaszaki & Nagy, 1991; Bortolotto, Morrison, Han & Messina, 2004; Dai & Korthuis, 2011; Granger & Korthuis, 1995; Granger 2003; Kalogeris et al 2012; Kalogeris et al, 2016; Kanwar & Kubes, 1994; Kanwar & Kubes, 2004; Kanwar, Hickey & Kubes, 1998; Steiner, Gonzalez & Wood; 1985). Neutrophil recruitment in response to I/R occurs by a complex, highly dynamic and well-coordinated series of steps, as depicted in Figure 1. Experiments using immunoneutralization approaches or adhesion molecule-deficient mice have established the selectins as dominant receptors for leukocyte rolling in postischemic tissues, while the interaction between the β 2-integrins (CD11/CD18) on leukocytes and intercellular adhesion molecule-1 (ICAM-1) on EC accounts for the establishment of stationary adhesive interactions (Granger & Korthuis, 1995; Granger 2003; Kalogeris et al 2012; Kalogeris et al, 2016). Leukocyte emigration requires ICAM-1, platelet-endothelial cell adhesion molecule-1 (PECAM-1), CD99, and a number of junctional adhesion molecules (JAMs). Diapedesis occurs preferentially across regions of the endothelium where there are gaps in the underlying pericyte investment and the basement membrane matrix exhibits by low expression of its constituent molecules (Voisin & Nourshargh, 2014). P-selectin expression follows a biphasic course after I/R, with an initial rapid increase due to mobilization from pre-formed pools, followed by a slower

and sustained increase that is protein synthesis-dependent. E-selectin and other adhesion molecules such as ICAM-1 require more time (>3 hr) for full expression on activated EC, again by protein synthesis-dependent mechanisms.

Leukocyte recruitment to postischemic tissues is promoted by ROS generated by xanthine oxidase in venular endothelium and other enzymes (e.g., NADPH oxidases in the vascular wall and adherent leukocytes, mitochondrial sources) which participate in the formation of proinflammatory stimuli (e.g., PAF, LTB₄, Ang II, and activated complement components), modify the expression of adhesion molecules on the surface of leukocytes and endothelial cells (e.g., CD11b/CD18, P-selectin and ICAM-1), and reduce tissue levels of the potent anti-adhesive agent NO (Granger & Korthuis, 1995; Granger 2003; Kalogeris et al 2012; Kalogeris et al, 2016) (Figure 1). One of the earliest signs of microvascular dysfunction induced by I/R is a reduction in the restrictive properties of the endothelial barrier in postcapillary venules, an event strongly associated with postischemic oxidant stress-induced LECA (Granger & Korthuis, 1995; Granger 2003; Kalogeris et al 2012; Kalogeris et al, 2016). Recent work has also shown that leukocyte diapedesis invoked by LTB₄ induces leakage of plasma proteins across postcapillary venules by a mechanism involving neutrophilic TNF secretion (Finsterbach, Voisin, Beyrau, Williams & Nourshargh, 2014).

Arterial and Arteriolar Responses to I/R

Impaired endothelium-dependent, NO-mediated relaxation of VSM to mechanical signals (e.g., shear stress) and receptor-dependent vasodilator agonists (e.g., acetylcholine) are the primary postischemic manifestations of endothelial cell dysfunction in arteries and arterioles (Banda et al 1997; Faraci & Didion, 2004; Gourdin, Bree & De Kock, 2009; Hayward & Lefer, 1999; Hein et al, 2003; Kalogeris et al, 2012; Kalogeris et al, 2016; Lefer & Ma, 1994; Ma et al, 1991, 1992, 1993; Ruh et al, 2003; Tsao et al, 1990; Touyz, 2004; Weyrich et al, 1993; Wolin et al, 2002). Since relaxation to endothelium-independent vasodilators (eg, nitroprusside, papaverine) is preserved in postischemic vessels, it appears that vascular smooth muscle (VSM) dysfunction does not account for the impairment in arterial and arteriolar responsiveness. Rather, overproduction of superoxide by endothelium exposed to I/R accounts for the inability of arterioles to exhibit NO-dependent vasodilation since treatment with superoxide dismutase (SOD), SOD mimetics, and other antioxidants restore this response. Oxidative stress induced by I/R also contributes to impaired vasoconstrictor responsiveness (Gao et al, 1994; Gao et al, 1996; Gao et al, 1996; Yokohama et al, 1996). Work conducted in porcine coronary arterioles suggests that I/R inhibits NO-mediated vasodilation by increasing arginase activity, which reduces the availability of L-arginine to nitric oxide synthase for NO synthesis (Hein et al, 2003). However, since neither arginase inhibition nor L-arginine supplementation completely restored NO-mediated vasodilator responses, postischemic NO quenching by ROS and/or reduction in eNOS expression likely account for a significant proportion of EDD dysfunction.

LECA in Postcapillary Venules IS Required for the Development of Arteriolar EDD

Since leukocytes do not roll along, adhere to, or emigrate across arteries or arterioles in the postischemic intestine (Granger & Korthuis, 1995; Granger 2003; Kalogeris et al 2012; Kalogeris et al, 2016) (or most other tissues, the heart being a notable exception (Hayward and Lefer, 1999; Ma et al, 1991, 1992, 1993; Tsao et al, 1990; Weyrich et al, 1993), a role for venular leukocyte adhesion in arteriolar endothelium-dependent vasoregulation has been largely ignored. However, the recent demonstration that arterial tissue obtained from mice that are genetically deficient in P-selectin, CD11/CD18, or ICAM-1 do not exhibit EDD after I/R compared to wild-type animals (Banda et al, 1997; Zamboni, Stephenson, Roth, Suchy & Russell, 1997) suggests that LECA interactions in postcapillary venules may be causally linked to endothelial dysfunction in upstream conduit arteries. In support of this concept, we have recently demonstrated that both ACH and flow-dependent vasodilation are impaired in intestinal arterioles isolated from wild-type but not P-selectin deficient mice (Dai & Korthuis, 2017). An identical pattern of response was noted when arteriolar responses to ACH were observed by intravital microscopic examination in the intact mesenteric microcirculation of neutropenic rats. Similar observations have been made in hypercholesterolemia models and following stroke (Kim, Carter & Harris, 2007; Petrault, Ouk, Gautier, Laprais, Gele, Bastide, et al, 2004). Our previous work shows that sensitivity to vasoconstrictor stimuli is reduced in postischemic intestinal arteries and arterioles, effects that were mitigated by antioxidant treatment but were not abolished by neutrophil depletion (Gao et al, 1994; Gao et al, 1996). These are important observations since arterioles are primarily responsible for controlling the distribution of blood flow within the microcirculation and suggest that leukocyte adhesion is required specifically for the production of EDD dysfunction in postischemic intestinal arterioles, but not responses to vasoconstrictor stimuli. As noted above, studies conducted in coronary arteries obtained after myocardial I/R support these conclusions (Banda et al, 1997; Hayward and Lefer, 1999; Ma et al, 1991, 1992, 1993 Tsao et al, 1990; Weyrich et al, 1993; Zamboni, Stephenson, Roth, Suchy & Russell, 1997). However, a critical difference between these vascular beds is that I/R induces neutrophil adherence to coronary arteries but not arterioles in other vascular beds.

While it is important to re-emphasize that we have not observed leukocyte rolling or adhesion in arterioles of the postischemic mesentery or intestine, there are conditions wherein such adhesive interactions occur in arterioles. As noted above, leukocytes do adhere to aortic tissue and coronary arteries and arterioles after I/R and may contribute to EDD in those vessels (Banda et al, 1997; Cuccocrea et al, 2000; Hayward and Lefer, 1999; Ma et al, 1991, 1992, 1993; Tsao et al, 1990; Weyrich et al, 1993; Zamboni, Stephenson, Roth, Suchy & Russell, 1997). Monocytes clearly adhere to large artery endothelium in hypercholesterolemic animals and humans, especially in regions containing fatty streaks and disrupted flow patterns. In addition, cigarette smoke exposure induces leukocyte adhesion in arterioles in the dorsal skin-fold of hamsters (Lehr, Frei & Arfors, 1994). In the cat mesentery, reductions in shear rate, as occurs in ischemia, produce pronounced increases in the number of adherent leukocytes in postcapillary venules (Perry & Granger, 1991). On the

other hand, leukocyte adhesion in arterioles was occasionally observed, but the magnitude of the reduction (by >80-90%) in shear rate required to produce this effect was much greater and the numbers of adherent cells were far lower in arterioles (~10% of that noted in venules). We emphasize that we do not observe leukocyte adherence to arterioles during ischemia or reperfusion in rodents, perhaps because shear rate is reduced to 25% of control during ischemia in our model. Leukocyte adhesion is also occasionally observed in intestinal arterioles of diabetic animals (Lash & Bohlen, 1991) and has been noted after nitric oxide synthase inhibition or angiotensin II (Ang II) or TNF exposure (Alvarez & Sanz, 2001; Alvarez, Piqueras, Bello, Canet Moreno, Kubes, et al, 2001; Alvarez, Cerda-Nicolas, Nabah, Mata, Issekutz, Panes, et al, 2004; Nabah, Mateo, Cerda-Nicolas, Alvarez, Martinez, Issekutz, et al, 2005; Piqueras Kubes, Alvarez, O'Connor, Issekutz, Esplugues, et al, 2000; Sumagin & Sarelius, 2006). Whether leukocytes adhering to arteriolar walls in these models participate in EDD dysfunction has not been evaluated.

Interestingly, the effects of nitric oxide synthase inhibition are mediated by Ang II (Nabah et al, 2005). Ang II has also been shown to upregulate arteriolar P-selectin, E-selectin, ICAM-1 and VCAM-1 expression when assessed 4 hrs after reperfusion (Alvarez et al, 2004). While these observations have potentially important implications for our work, it should be noted that one hour of exposure to Ang II fails to promote leukocyte adhesion in arterioles, but does induce significant adhesion in venules over this time frame (Alvarez & Sans, 2001; Alvarez et al 2001; Alvarez et al 2004; Petnehazy, Cooper, Stokes, Russell, Wood & Granger, 2006; Piqueras et al, 2000; Yusof, Kamada, Gaskin & Korthuis, 2007). Rather, leukocyte adhesion did not occur in arterioles unless they were exposed to Ang II for four hours (Alvarez et al 2004). This may explain why we fail to note arteriolar leukocyte adhesion in our model as all responses are monitored within 90 min of reperfusion. These Ang II-induced postcapillary adhesive interactions occur by a mast cell-*independent* mechanism (Alvarez et al, 2001; Piqueras et al, 2000), an observation consistent with our postulate that mast cell degranulation occurs secondary to leukocyte emigration (see discussion of the role of mast cells in I/R-induced EDD dysfunction below) and is a cause rather than an effect of Ang II generation. In addition, it should be noted that mononuclear cells appear to be the major adherent cell type in response to Ang II (Alvarez et al, 2004), while neutrophils are the principal infiltrating leukocyte subtype during the first 60-120 min of intestinal reperfusion (Oliver, Specian, Perry & Granger, 1991). EDD dysfunction is noted in mesenteric arterioles after hemorrhagic shock, an effect that was not abolished by neutrophil depletion (Zakaria, Garrison, Kawabe & Harris, 2004). Thus, a role for neutrophils in EDD cannot be extrapolated to all conditions characterized by I/R.

Interestingly, leukocyte rolling may be more frequent in pulmonary arterioles than in venules under control conditions (Kuebler Kuhnle, Groh & Getz, 1994; Yamaguchi, Nishio, Sato, Tsumura, Ichihara, Kudo, et al 1997). It is not clear why leukocyte adhesion occurs in arterioles under the aforementioned conditions, but not in our model, but most likely relates to the different interventions used to elicit arteriolar leukocyte adhesion (diabetes, cigarette smoke exposure vs I/R), the different organs (heart, skeletal muscle vs gut) and/or species (cat vs murine or rat mesentery) studied, level of shear stress reduction required to elicit occasional arteriolar leukocyte adhesion, the presence of a much larger population of mast cells in the small intestine vs skeletal muscle, time frame over which responses occur (4 hrs

continuous exposure to elevated Ang II are required to elicit leukocyte adhesion to arterioles), or dose of interventions (Ang II) or conditions (eg systemic shock vs local I/R) tested.

Whatever the explanation, an important and often overlooked fact is that the endothelium appears to be protected from a misguided attack by adherent leukocytes. It has been suggested that the fluid dynamics of a leukocyte's intravascular environment may provide constant modulating signals that activate certain leukocyte functions, while simultaneously inhibiting others (Moazzam, DeLano, Zweifach & Schmid-Schonbein, 1997). As a result of these actions, it appears that proinflammatory signals activate a behavioral program in leukocytes that contributes to the development of leukocyte/endothelial interactions and promotes extravasation. However, protective mechanisms existent in the vasculature and in the leukocyte have evolved to keep leukocytes from secreting mediators at the wrong place or at an inappropriate time. For example, intracellular and plasma antioxidants or endogenous protease inhibitors neutralize the toxicity of mediators secreted by adherent leukocytes. In addition, shear stress may downregulate neutrophilic oxidant production and protease release by adherent cells but activates calcium and other ion channels important in regulating cell motility, thereby protecting the endothelium from leukocyte-derived mediators, while allowing their extravasation (Moazzam, DeLano, Zweifach & Schmid-Schonbein, 1997). However, once emigrated, the leukocyte is removed from shear stress-induced inhibition, and the extravasated cells can now mobilize their cytotoxic arsenal to direct a focused attack on extracellular matrix components. This postulate is consistent with our hypothesis that events occurring secondary to leukocyte emigration modify EDD processes in arterioles (see Figure 2 and discussion below). Since inhibition of leukocyte rolling or adhesion also prevents emigration, the potential role of postcapillary venular leukocyte emigration in the development of EDD in arterioles may be blocked by anti-adhesion interventions or is absent in mice deficient in P-selectin.

While the aforementioned studies provide strong support for the concept that events initiated by leukocytes adhering to postcapillary venules contribute to I/R-induced EDD in upstream arteries and arterioles, the mechanisms underlying this effect are entirely unclear. There are at least three potential explanations for ability of leukocyte/endothelial cells adhesive interactions in postcapillary venules to influence endothelial function in upstream arteries and arterioles. First, an ascending propagated signal could be initiated by leukocytes adhering to postcapillary venules in postischemic tissues via cell-cell communication between adjacent microvascular cells to upstream arterioles (Banda et al, 1997; Segal & Duling, 1986). Interestingly, after exposure to I/R, adherent leukocytes express connexin 43 that is localized to their points of contact with endothelial cells (Jara, Boric & Saez, 1995). It has been suggested that this may allow for heterotypic intercellular gap junctional communication to regulate diapedesis, but also perhaps be a source for initiating signaling to upstream arterioles. This may represent a less likely explanation because of the long distance the conducted response would have to travel, especially if a decrement in the conducted response occurs as the signal is propagated, as has been reported for conducted vasodilatory responses to acetylcholine in arterioles (Segal and Duling, 1986). Moreover, conducted responses to agonists appear to be quite sensitive to ischemia, with enhanced spatial decay (as reflected by reductions in mechanical length constants) occurring in response to arterial

or venous occlusion (de With, Haug, van der Heijden & Segal, 2005; Lin & Duling, 1994). More importantly, connexin expression is very sparse or absent in venules (Tallini, Brekke, Shui, Doran, Hwang, Nakai, et al, 2007; Schmidt, Wolfle, Boettcher & de Wit, 2008), an observation consistent with our demonstration that gap junction inhibitors do not prevent the leukocyte-dependent arteriolar EDD (Dai and Korthuis, 2017). Second, transfer of a highly diffusible mediator produced by adherent leukocytes or the underlying endothelium in the postcapillary venule to adjacent arterioles with countercurrent flow could account for this response (Kim, Carter & Harris, 39). However, anatomic considerations preclude this as a likely explanation for the aforementioned observations in the aorta and superior mesenteric artery and for arterioles not arranged in parallel with venules. A third possibility is release of inflammatory mediators that produce hyperreactive vasoconstrictor responses that limit NO-dependent dilation in arterial vessels, as has been observed in atherosclerotic plaques (Sato, Shirai, Hontani, Shinooka, Hasegawa, Kichise, et al, 2017). Similar responses have been suggested to play a role in vascular smooth muscle hyperreactivity associated with variant angina (Lanza & Maseri, 2000). This explanation may not pertain to our observations because atherosclerosis is not present. Mast cell-derived mediators produce vasoconstriction of hamster cheek pouch arterioles after I/R, but fail to modify NO-dependent dilator responses to acetylcholine in skeletal muscle (Keller, 1997). In addition, our previous work indicates that mesenteric vessels become hyporesponsive to vasoconstrictor stimuli such as norepinephrine, vasopressin and Ang II (Gao et al, 1994, 1996, 1996; Yokoyama et al, 1996). On the other hand, intestinal arterioles exhibit reduced vascular caliber after I/R or intermittent hypoxia, an effect that is likely due to reduced bioavailability of NO and/or H₂S (Gonzalez Bosc, Osmond, Giermakowska, Pace, Riggs, Jackson-Weaver, et al 2017; Kanagy, Szabo & Papapetropoulos, 2017; Kurose et al, 1994; Osmond & Kanagy, 2014).

Two key observations led us to propose a fourth possibility (Figure 2) which we believe provides the most likely explanation for the ability of LECA in postcapillary venules to induce EDD in upstream arterioles. First, we and others have demonstrated that prevention of I/R-induced leukocyte rolling or stationary adhesion completely prevents leukocyte emigration across postcapillary venules (Kalogeris et al, 2012; 2016). Second, tantalizing evidence suggests that proteolytic fragments of extracellular matrix proteins alter vasoreactivity in arterioles (Gloe, Sohn, Meininger & Pohl, 2002; Hein, Platts, Waitkus-Edwards, Mousa & Meininger, 2001; Hill & Meininger, 2012; Martinez-Lemus, Wu, Wilson, Hill, Davis, Davis & Meininger, 2003; Martinez-Lemus, Sun, Trache, Trzeciakowski & Meininger, 2005; Mogford, Davis, Platts & Meininger, 1996; Wu, Mogford, Platts, Davis, Meininger & Davis, 1998). These observations led us to hypothesize that the oxidative/proteolytic activity of emigrating leukocytes may lead to the formation of nascent mediators or exposure of previously cryptic effectors in the interstitium that directly, or through activation of downstream mediators, produce impaired arteriolar responses to endothelium-dependent agonists and shear stress, which will be discussed next (Figure 2).

Mediators Generated by the Proteolytic Activity of Emigrated Leukocytes are Essential for the Development of EDD Dysfunction in I/R

In order to produce tissue injury, adherent leukocytes must extravasate into the tissues (Granger & Korthuis, 1995; Kalogeris et al, 2012, 2016). This removes leukocytes from the inhibiting effects of shear stress imposed by the flowing blood (Moazzam et al, 1997), thereby allowing the infiltrating leukocyte to disrupt subendothelial basement membrane components, hydrolyze extracellular matrix (ECM) proteins, and injure parenchymal cells by releasing cytotoxic oxidants and/or hydrolytic enzymes (Granger & Korthuis, 1995; Kalogeris et al, 2012, 2016; Welbourne, Goldman, Paterson, Valeri, Shepro & Hechtman, 1987). This postulate is supported by our observation that treatment with phalloidin, which we have previously shown completely prevents postischemic leukocyte emigration without influencing I/R-induced leukocyte rolling or adhesion, oxidant production or protease release by activated leukocytes, CD18 clustering, leukocyte adherence to plastic, or leukocyte emigration across inert membranes (Asako, Wolf, Granger & Korthuis, 2002; Korthuis, Carden, Kvietys, Shepro & Fuseler, 1991; Shigematsu, Ishida, Gute & Korthuis, 1999), prevented EDD in postischemic rat mesentery and in vessels isolated from murine intestine after I/R. These observations suggest that adherent leukocytes must emigrate into the tissues to influence vasodilator responses to ACH in arterioles. Since ACH responses were similar in arterioles of control rats (no-ischemia) in the presence and absence of phalloidin, it does not appear that this agent directly influences the responses to acetylcholine. However, it is possible that flow-dependent vasodilation in isolated arterioles may be influenced by this agent, which acts to prevent leukocyte emigration by stabilizing F-actin in endothelial cells, since cytoskeletal elements play an important role in transmitting mechanical signals in response increased shear stress.

It is important to note that we have not observed a single leukocyte to adhere to arteriolar endothelium in our model of intestinal I/R. However, because leukocytes do adhere to the endothelium in other conditions, we determined whether perfusion of isolated arterioles with neutrophils and subsequently exposed to PAF plus LTB₄ plus Ang II for 60-90 min would induce adhesion. These agents failed to induce arteriolar leukocyte adhesion, even when pseudo-shear rates were concomitantly reduced to 25% of control, and did not impair ACH responses. Similarly, PAF plus LTB₄ alone (ie, in the absence of neutrophil perfusion) failed to alter arteriolar ACH responses. Our latter observations are particularly relevant to I/R, because PAF, LTB₄, and Ang II have been shown to play a major role in postischemic leukocyte adhesion in venules (Granger & Korthuis, 1995; Kalogeris et al, 2012, 2016; Petnehazy et al, 2007; Yusof et al, 2007). These data indicate that leukocytes do not adhere to arteriolar endothelium in our model and also indicate that neutrophils flowing down arterioles do not modify ACH responses, even in the presence of activating factors and reduced shear rates.

Although much attention has focused on the role of reactive oxygen metabolites, less emphasis has been placed on elucidating the role of leukocyte proteases in the pathogenesis of I/R injury. However, it is clear that hydrolytic enzymes released by leukocytes, such as collagenase, elastase and matrix metalloproteinase-9, degrade important structural

components of the endothelial glycocalyx, basement membrane, and ECM, thereby contributing to leukocyte-dependent microvascular barrier dysfunction in postischemic tissues (Altshuler, Kistler & Schmid-Schonbein, 2016; Carden & Korthuis, 1996; Granger & Korthuis, 1995; Kalogeris et al, 2012, 2016; Schmid-Schonbein, 2016).

Among the proteases involved in the pathogenesis of I/R-induced microvascular dysfunction and tissue injury, matrix metalloproteinase-9 (MMP-9; 92 Kd gelatinase, gelatinase B) appears to play a prominent role. The MMP family consists of at least 20 zinc-containing serine proteases that participate in tissue and vascular remodeling, tumor metastasis, angiogenesis, and contribute to several phases of inflammation (Fujimura, Gasche, Morita-Juimura, Massengale, Kawase & Chan, 1999; Gasche, Jujimura, Morita-Fujimura, Copin, Kawase & Chang, 1999; Rosario et al, 2004; Yang and Rosenberg, 2015). These structurally complex endoproteases are also known as matrixins or metzincins and are found in many cell types, including fibroblasts, osteoclasts, keratinocytes, leukocytes, and endothelial cells. MMPs are secreted by cells in an inactive form (zymogen) that can be activated by proteolytic cleavage. Recent evidence indicates that MMPs can also be activated by hypochlorous acid (HOCl), a potent oxidant formed by neutrophilic myeloperoxidase (MPO) (Fu, Kassim, Parks & Heinecke, 2001; Yang & Rosenberg, 2015). MPO also inactivates tissue inhibitor of matrix metalloproteinases-1, allowing this neutrophilic enzyme to disinhibit MMP activity (Wang, Rosen, Madtes, Shao, Martin & Heineke, 2007). On the other hand, HOCl can also restrain the activity of some MMPs, providing a mechanism to fine tune proteolysis during inflammatory states (Fu, Kao, Bergt, Kassim, Huq, d'Avignon, et al, 2004). MMP-9 is expressed by endothelial cells during reperfusion (Gasche et al, 1999; Fujimura et al, 1999). Neutrophils infiltrating postischemic intestine also release MMP-9, which leads to extracellular matrix turnover (Rosario et al, 2004). MMP-9 has been implicated in parenchymal cell injury induced by myocardial infarction, hemorrhagic stroke, sepsis, and in liver and intestinal I/R (Camp, Tyagi, Aru, Hayden, Mehta & Tyagi, 2004; Fujimura et al, 1999; Gasche et al, 1999; Heo, Lucero, Abumiya, Koziol, Copeland & del Zoppo, 1999; Kuyvenhoven, Molenaar, Verspaget, Veldman, Palareti, Legnani, et al, 2004, Rosario et al, 2004; Smith and Gabler, 1995). Indeed, Rosario et al (2004) demonstrated that MMP-9 is highly expressed in the wall of the postischemic small intestine and appears to be derived from infiltrating neutrophils. Furthermore, these investigators demonstrated that pancreatic trypsin promotes rapid proteolytic activation of MMP-9 in the wall of the small intestine after I/R, allowing activated monomers to dimerize to the active form of the protease in postischemic gut, which in turn, amplifies the inflammatory response to I/R (ie, exacerbated postischemic leukocyte rolling, adhesion, and infiltration) and was associated with extensive morphologic mucosal damage.

Proteolytic digestion of ECM proteins has been shown to reveal hidden (latent) bioactive epitopes (e.g., the conserved amino acid sequence Arg-Gly-Asp or RGD) or release of proinflammatory mediators, collectively referred to as matricryptic effectors (Davis, 2010; Davis, Bayless, Davis & Meininger, 2000). With regard to vascular function, Meininger, Davis and coworkers (Gloe, Sohn, Meininger & Pohl, 2002; Hein, Platts, Waitkus-Edwards, Mousa & Meininger, 2001; Hill & Meininger, 2012; Martinez-Lemus, Wu, Wilson, Hill, Davis, Davis & Meininger, 2003; Martinez-Lemus, Sun, Trache, Trzeciakowski & Meininger, 2005; Mogford, Davis, Platts & Meininger, 1996; Wu, Mogford, Platts, Davis, Meininger &

Davis, 1998) were the first to demonstrate that synthetic RGD-containing peptides and proteolytic degradation products of extracellular matrix proteins such as collagen type I alter arteriolar tone. Furthermore, they demonstrated that synthetic RGDs interact with the integrin $\alpha_v\beta_3$ (and other integrins) to alter myogenic tone. These studies led us to postulate that proteolytic digestion of ECM proteins by leukocytes infiltrating the postischemic intestine may modify arteriolar vasoregulatory responses in postischemic tissues or in other inflammatory states by an integrin-dependent mechanism. Support for this concept is provided by the observations that treatment with broad-spectrum anti-proteases, MMP-selective inhibitors or agents targeting $\alpha_v\beta_3$ prevented postischemic arteriolar EDD dysfunction (Dai and Korthuis, 2017). Moreover, ACH responses were preserved in MMP-9-deficient mice, but not MMP-2/- mice, subjected to I/R compared to wild-type animals. Importantly, arterioles isolated from non-ischemic rat mesenteries demonstrated EDD dysfunction when incubated with postischemic intestinal lymph (as a surrogate for interstitial fluid) draining reperfused intestines of donor rats, but not when exposed to lymph obtained from rats treated with protease inhibitors during I/R. The latter studies support the concept that signals generated in the interstitium after I/R act to disrupt EDD function. In addition to these effects, MMP inhibition with doxycycline has been shown to improve EDD function by inhibiting oxidative stress and improving NO bioavailability (Castro, Rizzi, Ceron, Guimaraes, Rodrigues, Bendhack et al, 2012). Studies conducted with MMP inhibitors or in MMP-9 deficient mice also show that endothelial MMPs influence vascular reactivity (Cena, Lalu, Rosenfeldt & Schulz, 2008; Su, Palen, Lucchesi & Matrougui, 2006).

Mast Cells Contribute to I/R-induced Arteriolar EDD

Because mast cell density is very high in gastrointestinal tissues (Nilsson, Costa, Metcalfe, 1999), it is not surprising that these perivascular cells play a much more prominent role in the inflammatory response to I/R in the small bowel (Bortolotto et al, 2004; Kanwar et al, 1998). Intestinal mast cells degranulate during reperfusion (Kanwar & Kubes, 1994, 1994; Kanwar et al, 1998; Miller & Pemberton; 2003), but mast cell degranulation does not influence postischemic P-selectin expression in the first 10-30 min of reperfusion (Eppihimer, Russell, Anderson, Epstein, Laroux & Granger, 1997). This time frame is consistent with our contention that leukocyte extravasation must occur first to produce mediators that contribute to mast cell degranulation and subsequent induction of P-selectin expression. However, treatment with mast cell stabilizers is effective in preventing I/R-induced mucosal barrier disruption, increased vascular permeability, and leukocyte infiltration in the small intestine of mice and rats after reperfusion for 60 min (Kanwar & Kubes, 1994, 1994, Kanwar et al, 1998), a time frame that allows for neutrophil diapedesis-dependent mast cell degranulation to occur first.

Two types of mast cells are present in small intestine: connective tissue mast cells, which are located in the submucosa and muscularis layers of the bowel wall and in the mesentery, and mucosal mast cells, which as the name implies reside in the mucosa (Nilsson et al, 1999). These two types of mast cells release different mediators and may respond to distinct stimuli (Nilsson et al, 1999). For example, chymase, a chymotrypsin-like serine protease that catalyzes the formation of Ang II, is mainly contained in secretory granules of connective tissue mast cells and is released only by these mast cells, and not mucosal mast cells, in rats

and humans (Miller & Pemberton, 2003; Nilsson et al 1999). In normal tissues, mast cell chymase is stored in an inactive form, but acquires the ability to form Ang II following secretion from degranulating mast cells (Dell'Italia & Husain, 2004; Gaboury, Johnston, Niu & Kubes, 1995; Jin, Takai, Okamoto, Muramatsu & Miyazaki, 2004; Miller & Pemberton, 2003; Miyazaki and Takai, 2001; Nilsson et al 1999; Richard, Hurel-Merle, Scalbert, Ferry, Lallemand, Bessou, et al, 2001). Importantly, this enzyme can account for as much as 80-90% of Ang II formation in the heart and is a significant source of the peptide in damaged arterial vessels, indicating that chymase can become the chief source of Ang II in injured tissues. Thus, mast cell chymase is an important source Ang II in injured vessels but because Ang II derived from this enzymatic source does not influence blood pressure, it appears that the effects of chymase-derived Ang II are restricted to the local environment (Miyazaki & Takai, 2001; Richard et al, 2001; Dell'Italia & Husain, 2004; Suzuki et al, 1998).

Given this background and the facts that: 1) mast cells can be activated to release chymase and other proinflammatory mediators on exposure to RGDs, a secretory process that appears to be mediated by $\alpha_v\beta_3$ (Nilsson et al, 1999; Shimuzu, Irani, Brown, Ashman & Schwartz, 2001), 2) mast cell chymase has been implicated lipopolysaccharide-induced vasomotor dysfunction in arterioles, an effect that is mediated by Ang II-dependent production of reactive oxygen species (Suzuki, Coughney Gao & Rubinstein, 1998), and 3) mast cell chymase also plays an important role in activating pro-MMP-2 and -9, which may serve to amplify the effect of MMPs in I/R (Ishida, Takai, Murano, Nishikawa, Inoue, Murano, et al, 2008; Tchougounova, Lundequist, Fajardo, Winberg, Abrink & Pejler, 2005), we postulated that leukocyte-derived MMP-9 acts to expose latent matricryptic effectors in the extracellular matrix which interact with β_3 integrin binding sites on mast cells, thereby stimulating chymase secretion by mast cells (Figure 2). Chymase-dependent formation of Ang II in turn activates NAD(P)H oxidase to produce superoxide, quenching NO and leading to impaired vascular responses to endothelium-dependent vasodilators (Figure 2). This hypothesis is supported by the observations that treatment with a mast cell stabilizer prevented the I/R-induced impairment in ACH-induced vasodilation in mesenteric arterioles, as did treatment with a chymase inhibitor. Importantly, postischemic mast cell degranulation in the rat mesentery was attenuated in animals treated with phalloidin (which prevents leukocyte emigration), protease inhibitors, or β_3 immunoneutralization (Dai & Korthuis, 2017). In contrast, superfusing the mesentery with compound 48/80 promoted mast cell degranulation that was not inhibited by phalloidin and was associated with EDD. These latter observations suggest that mast cell degranulation per se can promote EDD in arterioles and that phalloidin does not directly influence mast cell degranulation. Taken together, our results suggest that the proteolytic activity of emigrating leukocytes may generate effectors that promote mast cell degranulation via the integrin $\alpha_v\beta_3$, and that chymase release by mast cells modifies postischemic arteriolar responses to ACH. This integrin is also upregulated on cerebral microvessels after stroke (Abumiya, Lucero, Heo, Tagaya, Kosiol, Copeland et al, 1999), suggesting the possibility of a more direct effect of $\alpha_v\beta_3$ on EDD function since ligation of this integrin promotes vasodilation (Mogford et al, 1996).

Ang II-dependent, NAD(P)H Oxidase-mediated Production of Reactive Oxygen Species (ROS) Contribute to eNOS Uncoupling and EDD in Arterioles after I/R

Overproduction of superoxide by postischemic endothelium largely accounts for the inability of arterioles to exhibit endothelium-dependent, NO-mediated relaxation since treatment with superoxide dismutase and other antioxidants restore this response in arterial tissue (Kalogeris et al, 2012, 2016). Although superoxide generation appears to be pivotal in producing postischemic EDD dysfunction, it also participates in the generation of other ROS that may influence arteriolar function (Cifuentes & Pagano, 2006; Gourdin, Bree & De Kock, 2009; Lyle & Griendling, 2005; Mehta & Griendling, 2007; Pagano and Gutterman, 2007; Rey, Cifuentes, Kiarash, Quinn & Pagano, 2001; Rey Li, Carretero, Garvin & Pagano, 2002; Touyz, 2004; Wolin et al, 2002). Reaction of superoxide with NO generates peroxynitrite (ONOO-) and other reactive nitrogen oxide species which are potentially deleterious (see next paragraph). Dismutation of superoxide spontaneously or by enzymatic action of SOD produces hydrogen peroxide, which can react with reduced transition metals to form the highly reactive hydroxyl radical. Hydrogen peroxide can also be metabolized by neutrophilic myeloperoxidase (MPO) to form hypochlorous acid and other oxidizing species. MPO can also act as an NO oxidase, thereby reducing the bioactivity of NO and increasing oxidative stress, thereby contributing to EDD dysfunction (Baldus, Heitzer, Eiserich, Lau, Mollnau, Ortak et al, 2004; Tian, Ding, Peng & Lu, 2017).

Despite the wealth of information supporting a role for oxidants in arteriolar EDD dysfunction, the source of superoxide that inactivates NO remains unclear. In addition to mitochondrial sources, superoxide and/or hydrogen peroxide can be formed by many enzymes. Two of the most important sources in normal vessels are cytochrome P450 and the NAD(P)H oxidases, while xanthine oxidase, uncoupled eNOS, and mitochondrial respiratory chain appear to play an important role in postischemic tissues (Kalogeris et al, 2012, 2016). It is well-known that xanthine oxidase-derived oxidants play an important role in the genesis of I/R injury by participating in the formation of pro-inflammatory mediators such as PAF and LTB₄ and by inactivating NO, which normally produces powerful anti-adhesive effects. The net effects of these actions are to promote leukocyte adhesion and emigration in postcapillary venules. Superoxide formed by the NAD(P)H oxidases appears to play a more dominant role in impaired EDD noted in arterial vessels in a variety of pathologic states. An interesting and important feature of eNOS is its ability to produce superoxide under certain conditions, such as L-arginine or tetrahydrobiopterin (BH₄) deficiency, dephosphorylation of threonine 495, and formation of eNOS monomers (Forstermann, 2006; Moens & Kass, 2006; Munzel, Daiber, Ullrich & Mulsch, 2005). Functional eNOS converts L-arginine and O₂ to NO and L-citrulline, an enzymatic process that consumes NADPH and requires Ca²⁺/calmodulin, flavin adenine dinucleotide, flavin mononucleotide, and BH₄. When uncoupled by absence of L-arginine or BH₄, electrons normally flowing from the reductase domain of one subunit to the oxygenase domain of the other subunit are diverted to molecular oxygen rather than L-arginine or the cofactor BH₄, resulting in superoxide production. Thus, oxygen reduction and NO formation are uncoupled

and superoxide is generated from the eNOS oxygenase domain by dissociation of the ferrous-dioxygen complex.

The NAD(P)H oxidases (Nox) of the cardiovascular system are expressed in endothelial cells, vascular smooth muscle cells, and fibroblasts in the vascular wall, as well as in cardiac myocytes (Lyle & Griendling, 2005; Mehta & Griendling, 2007; Touyz, 2004; Wolin et al, 2002). While the cardiovascular oxidases are similar to the neutrophil NADPH oxidase, they represent a unique family of enzymes (Nox1-5). The vascular Nox's are low output, slow-release enzymes relative to the phagocytic enzyme and exhibit moderate constitutive activity that is absent in neutrophils. Phagocytic NADPH oxidase consists of 4 major subunits: a membrane associated complex of 2 subunits, gp91^{phox} and p22^{phox}, and a cytosolic complex with two components, p67^{phox} and p47^{phox}. Under baseline conditions, these components are kept apart, rendering the enzyme inactive. On stimulation, p47^{phox} becomes heavily phosphorylated and, along with p67^{phox}, moves to the cell membrane and binds the membrane complex, allowing for activation. The membrane portion then transfers an electron from NADPH to molecular oxygen, forming superoxide. The vascular forms use NADH and NADPH to drive superoxide production and vary in their subunit composition and cellular location (Lyle & Griendling, 2005; Mehta & Griendling, 2007; Touyz, 2004; Wolin et al, 2002). One of the most important attributes of the vascular Nox's is its responsiveness to hormones (eg, Ang II, thrombin, lactosylceramide, PAF, IL-1, TNF, etc), shear forces, and local metabolic factors (eg, lactate), with the oxidase of each cell type exhibiting differential responsiveness to such stimuli (Lyle & Griendling, 2005; Mehta & Griendling, 2007; Touyz, 2004; Wolin et al, 2002).

Of particular interest to our work is the fact that Ang II increases Nox activity in vascular smooth muscle, an effect mediated by lipoxygenase metabolites of arachidonic acid (Lyle & Griendling, 2005; Mehta & Griendling, 2007; Touyz, 2004; Wolin et al, 2002). Moreover, the intestinal vasculature exhibits extraordinarily high concentrations of Ang II receptors (Riaz, Wang, Schramm, Sato, Menger, Jeppsson et al, 2004; Gunther, Gimbrone & Alexander, 2004). Work from our laboratory also implicates Ang II in postischemic inflammatory responses in the small intestine (Yusof et al, 2007). In this study, I/R increased Ang II levels while AT1 receptor blockade, chymase inhibition, and a CGRP receptor antagonist attenuated postischemic leukocyte adhesion. Superfusion of control intestine (no ischemia) with Ang II for 2 hr induced leukocyte adhesion, an effect prevented by concomitant Nox inhibition. Ang II may also influence I/R-induced fluid accumulation via its effect to induce $\alpha_v\beta_3$ integrin-mediated dependent collagen contraction by fibroblasts (Nunohiro, Ashizawa, Graf, Hsueh & Yanoh, 1999), an action that has been suggested to create highly negative interstitial fluid pressure, thereby facilitating edema formation (Reed, Berg, Gjerde & Rubin, 2001). Applying a force to integrins has been shown to activate Nox by an Ang II-dependent mechanism (Browe & Baumgaarten, 2004, 2006). Of high significance to our central hypothesis (Figure 2) is the growing body of evidence supporting the notion that the products of the Nox's and eNOS (superoxide and NO, respectively) rapidly interact to form ONOO⁻, which can oxidize the essential NOS co-factor, BH₄, to biologically inactive products (Forstermann, 2006; Moens & Kass, 2006). As a consequence, oxygen reduction uncouples from NO synthesis, thereby transforming functional, NO-producing eNOS into a dysfunctional superoxide-producing enzyme. Indeed, Nox-driven

eNOS uncoupling by oxidation of BH₄ has been implicated in the vascular dysfunction associated with hypertension, hypercholesterolemia, and diabetes, which can be attenuated by administration of exogenous BH₄ (Elrod, Duranski, Langston, Greer, Tao, Dugas, et al 2006; Forstermann, 2006; Moens & Kass, 2006; Munzel Daiber, Ullrich & Mulsch, 2005; Stokes, Dugas, Tang, Garg, Guidry, Bryan, 2009). Moreover, myocardial infarct size, cardiac superoxide generation, and coronary artery endothelial dysfunction are attenuated by provision of exogenous BH₄, findings which support the concept that eNOS uncoupling contributes to I/R injury in the heart (Dumitrescu, Biondi, Xia, Cardounel, Druhan, Ambrosio et al, 2007; Forstermann, 2006; Moens & Kass, 2006). Taken together, these studies suggest the possibility that Nox-derived superoxide reduces the bioavailability of NO and thus EDD during I/R by at least two mechanisms related to the formation of peroxynitrite: 1) superoxide combines with NO formed by a still functional eNOS, producing ONOO⁻, an effect that effectively scavenges NO as it is produced, thereby quenching the direct effects of NO to produce vasodilation, and 2) ONOO⁻ produces eNOS uncoupling secondary to BH₄ oxidation, transforming a functional eNOS into a dysfunctional, superoxide producing enzyme. This fuels a futile cycle wherein uncoupled eNOS-derived superoxide contributes to BH₄ oxidation and leads to further eNOS uncoupling.

I/R-induced superoxide production has been implicated in EDD dysfunction in coronary and skeletal muscle arteries, and we have obtained evidence extending this concept to intestinal arterioles. Superfusing the mesentery with the cell-permeant SOD mimetic (Mn-TBAP), BH₄, or the Nox inhibitor apocynin attenuated impaired arteriolar responses to acetylcholine that are induced by I/R. The apocynin data not only provides a second line of evidence supporting a role for oxidants in the development of impaired responses to acetylcholine but also suggest that Nox may represent an important source of reactive oxygen species in vessels subjected to I/R, while the eNOS cofactor studies support a role for uncoupled eNOS. Interestingly, topical application of candesartan, an Ang II AT1 receptor antagonist was as effective as Mn-TBAP and apocynin in preventing I/R-induced arteriolar dysfunction. It is difficult to determine from this data whether the treatments directly exert their effects on arteriolar responses or to their ability to prevent I/R-induced leukocyte adhesion. However, arteriolar ROS production was attenuated by neutrophil depletion as well as by addition of Mn-TBAP, candesartan, or apocynin to the solution bathing mesenteric arterioles after I/R. More importantly, ROS production by naïve arterioles incubated with postischemic lymph was attenuated by addition of Mn-TBAP, candesartan, or apocynin to the lymph bathing the vessels. Taken together, these studies strongly support the notion that there is a link between effectors produced in the interstitium by the proteolytic activity of emigrated leukocytes and impaired responses of arterioles to endothelium-dependent, NO-mediated vasodilator responses (Figure 2). The fact that treatment with Mn-TBAP, BH₄, apocynin, or candesartan restores ACH-dependent responses in postischemic mesentery and prevents oxidant production in arteriolar vessels exposed to postischemic lymph supports our hypothesis that emigrating leukocytes generate effectors that induce oxidant production in arterioles by an Ang II-dependent, Nox-mediated mechanism that leads to eNOS uncoupling. Taken together, these data suggest that I/R-induced, Ang II-dependent Nox oxidase activation may be a source of superoxide that promotes eNOS uncoupling, inactivates NO and induces EDD

dysfunction. The fact that L-NIO partially reduced, but SOD mimetic and apocynin treatment, largely abolished postischemic superoxide generation in arterioles bathed in postischemic lymph supports this view. We also measured Ang II levels in plasma draining the ischemic region and demonstrated that I/R induced a 3-fold increase that was prevented by prior neutrophil depletion.

Summary and Future Directions

A large body of evidence indicates that neutrophils and ROS derived from Nox and uncoupled eNOS contribute to I/R-induced EDD dysfunction in conduit arteries and arterioles. However, until very recently, it was not clear whether these contributing factors were mechanistically linked in arterioles, where LECA is not observed after I/R. More recent work has implicated a role for mast cells and MMPs in postischemic arteriolar dysfunction and has tied these disparate findings into a cohesive mechanism (Figure 2). Neutrophils participate in the development of postischemic arteriolar EDD dysfunction by a mechanism that is initiated by adhesive interactions between these inflammatory phagocytes and postcapillary venular endothelial cells. As neutrophils migrate across postcapillary venular walls and gain access to the interstitial space, they become activated and release a variety of hydrolytic enzymes, including matrix metalloproteinase-9. The enzymatic activity of this and other proteases appears to cleave components of the extracellular matrix, thereby exposing matricryptic sites or releasing matricryptins that activate mast cells by an $\alpha v \beta 3$ integrin-dependent mechanism. Ensuing mast cell degranulation releases chymase, an enzyme that catalyzes the conversion of Ang I to Ang II. Ang II-dependent Nox activation results in ROS production, which interacts with NO produced by eNOS in response to shear stress or endothelium-dependent vasodilator agonists, producing peroxynitrite (and other reactive nitrogen oxide species). Peroxynitrite may exacerbate EDD dysfunction by producing eNOS uncoupling secondary to BH₄ oxidation, transforming a functional eNOS into a dysfunctional, superoxide-producing enzyme. This fuels a futile cycle wherein uncoupled eNOS-derived superoxide contributes to BH₄ oxidation and leads to further eNOS uncoupling. Understanding this pathway provides important insights into the pathogenesis of arteriolar dysfunction in I/R that may extrapolate to other conditions characterized by enhanced leukocyte infiltration into the tissues.

While this is an appealing hypothesis that links together adhesive events occurring in postcapillary venules to signals generated in the interstitium that ultimately act to impair responses to endothelium-dependent stimuli in upstream arterioles, it also provokes a number of questions for future studies. For example, it is not clear what role protease-dependent receptor cleavage (Schmid-Schonbein, 2016; Mazor & Schmid-Schonbein, 2015) might play in postischemic EDD dysfunction. Moreover, interesting recent work has demonstrated that pentraxin 3, a soluble pattern recognition receptor that plays an important role in innate immune responses, induces EDD dysfunction via a P-selectin/MMP-dependent pathway that may feed into our hypothesized mechanism (Carrizzo, Lenzi, Procaccini, Damato, Biagioni, Ambrosio et al, 2015). The role of neutrophil extracellular traps (NETs) in I/R-induced microvascular dysfunction and tissue injury has been established (Albadawi, Oklu, Raacke Malley, O'Keefe, Uong, Cormier et al, 2016; Ge, Zhou, Ji, Lu, Zhang, Zhang et al, 2015; Jansen, Emal, Teske Dessing, Florquin & Roelofs, 2017; Nakazawa, Kumar,

Marschner, Desai, Holderied, Rath et al, 2017; Pallet, 2017), but their potential contribution to EDD dysfunction in arterial tissues is unknown. Interestingly, pentraxin 3 is a key component of NETs (Liu, Chuang, Tsai & Yu, 2014), suggesting a possible linkage to our hypothesized mechanism for neutrophil-initiated EDD dysfunction in I/R.

In addition to MMPs, another group of proteases, the calpains, have been shown to participate in agonist-induced EDD (Hein, Rosa, Ren XU & Kuo, 2015), improve collateral-dependent perfusion and reduce infarct size after myocardial ischemia (Pötz, Sabe, Abid & Sellke, 2015; Sabe, Pötz, Elmadhun, Liu, Feng, Abid, et al, 2016), and contribute to EDD dysfunction associated with risk factors for cardiovascular disease (Chen, Zhao, Ni, Tang, Shan, Cepinskas, et al, 2014; Cheng, Jiang, Pansuria, Fang, Mai, Mallilankaraman, et al, 2015; Kishore, Benedict & Cheng, 2015; Nangle, Cotter & Cameron, 2006; Stalker, Gong & Scalia, 2005; Randriamboavonjy, Kyseolova, Elgheznavy, Zukunft, Wittig & Fleming, 2017; Stalker, Skvarka & Scalia, 2003; Wang, Peng, Zhang & Liu, 2008) or activation of Ang II type 1 receptors (Scalia, Gong, Berzins, Freund, Feather, Landesberg et al, 2011). However, the role of these proteases in I/R-induced EDD dysfunction has not been evaluated. Similarly, while inhibition of MMP activity, neutrophil depletion, prevention of NETosis, and ROS scavenging have been shown to protect against vascular hyporeactivity in endotoxemia in vivo or in arterial tissue exposed to bacterial toxins (Buerke, Sibelius, Grandel, Buerke, Grimminger, Seeger, et al, 2002; Castro, Cena, Cho, Walsh & Schulz, 2012; Cena, Lalu, Rosenfelt & Schulz, 2008; Cena, Lalu, Cho, Chow, Bagdan, Daniel, et al, 2009; Gardiner & Andrews, 2012; Nin, El-Assar, Sanchez, Ferruelo, Sanchez-Derrer, Martinez-Caro, et al 2011; Wenceslau, McCarthy, Szasz, Gouloupoulou & Webb, 2015), it is not clear whether these pathologic events are mechanistically linked as described for I/R.

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Abbreviations

ACH	acetylcholine
Ang II	angiotensin II
AT1-R	Ang II type 1 receptor
BH4	tetrahydrobiopterin
EC	endothelial cell
ECAM	endothelial cell adhesion molecule
ECM	extracellular matrix
EDD	endothelium-dependent, NO-mediated vasodilator
eNOS	endothelial nitric oxide synthase
eNOS	endothelial nitric oxide synthase
H₂O₂	hydrogen peroxide
I/R	ischemia/reperfusion
JAMs	junctional adhesion molecules A, B, & C

Knockout	-/-
LECA	leukocyte/endothelial cell adhesive interactions
LTB₄	leukotriene B ₄
MAb	monoclonal antibody
MMP	matrix metalloproteinase
MPO	myeloperoxidase
NO	nitric oxide
Nox	NAD(P)H oxidase
O₂⁻	superoxide anion
ONOO⁻	peroxynitrite
PAF	platelet-activating factor
RGD	Arg-Gly-Asp peptide
RNOS	reactive nitrogen oxide species
ROS	reactive oxygen species
SOD	superoxide dismutase
TNF	tumor necrosis factor

Figure 1.

The recruitment of leukocytes to ischemic sites involves a complex cascade of highly integrated steps. When leukocytes exit capillaries they are moved to the postcapillary venular walls (margination) by hydrodynamic dispersal forces. In the presence of activating factors, the margined leukocyte can form weak adhesive interactions that permit rolling along the endothelial surface. The rolling leukocyte can establish stationary adhesive interactions and then crawl along the endothelium to sites along the venular wall where the underlying basement membrane exhibits low expression of constituent molecules and where gaps between pericytes exist. The adherent leukocyte sends out pseudopods to move through or between adjacent endothelial cells to gain access to the subendothelial cell space, crawls along pericytes surrounding the venular endothelium until it encounters a gap between these cells, which it can use as a route of transit into the tissue space. Once in the tissue space, the emigrated leukocyte releases its cytotoxic arsenal of reactive oxygen species and hydrolytic enzymes to cleave extracellular matrix components and direct a focused attack on parenchymal cells. Figure from Kalogeris et al, 2016.

Figure 2.

Rather than adhering directly to arterial vessels to produce arteriolar EDD dysfunction after I/R, the mechanism by which activated neutrophils disrupt NO-dependent vasoregulation requires adhesive interactions and subsequent emigration across postcapillary venular walls. Upon entry into the extravascular compartment, these inflammatory phagocytes release proteases, such as matrix metalloproteinase-9 (MMP-9), to generate signals within the interstitium that provoke the release of mast cell-derived chymase. In turn, chymase catalyzes the conversion of angiotensin I (Ang I) to angiotensin II (Ang II), which instigates NADPH oxidase-dependent production of reactive oxygen species (ROS). This oxidative stress is exacerbated by postischemic eNOS uncoupling, which results in the production of superoxide instead of NO (not shown). Postischemic ROS may also be derived from xanthine oxidase and mitochondrial sources (not shown). I/R-induced ROS production quenches NO produced in response to mechanical stimuli (e.g., shear stress) or EDD agonists (e.g., acetylcholine), resulting in impaired EDD responses in arterioles. Figure modified from Dai & Korthuis, 2011.