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Reperfusion Syndrome: Cellular Mechanisms of Microvascular Dysfunction and Potential Therapeutic Strategies

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Reperfusion injury is the paradoxical and complex phenomenon of exacerbation of cellular dysfunction and increase in cell death after the restoration of blood flow to previously ischemic tissues. It involves biochemical and cellular changes causing oxidant production and complement activation, which culminates in an inflammatory response, mediated by neutrophil and platelet cell interactions with the endothelium and among the cells themselves. The mounted inflammatory response has both local and systemic manifestations. Despite improvements in imaging, interventional techniques, and pharmacological agents, morbidity from reperfusion remains high. Extensive research has furthered the understanding of the various pathophysiological mechanisms involved and the development of potential therapeutic strategies. Preconditioning has emerged as a powerful method of ameliorating ischemia reperfusion injury to the myocardium and in transplant surgery. More recently, postconditioning has been shown to provide a therapeutic counter to vasoocclusive emergencies. More research and well-designed trials are needed to bridge the gap between experimental evidence and clinical implementation.

Keywords: ischemia; reperfusion; endothelial dysfunction; microvascular dysfunction; nitric oxide; adhesion molecules; cytokines; preconditioning; postconditioning

Historically, the awareness of reperfusion dates back to 1941, when Bywaters and Beall[1] noted profound metabolic derangement and subsequent death after the release of crushed limbs in air-raid casualties. The concept of reperfusion leading to tissue injury was introduced by Hearse[2] in 1971. Haimovici[3] in 1979 described similar observations, which were referred to as the "myonephropathic metabolic syndrome." The full extent of reperfusion-induced tissue injury was described by Parks and Granger[4] in 1986. Reimer et al[5] introduced the term lethal reperfusion injury in 1989, as they described cell necrosis resulting from reperfusion and also the role of free radicals and neutrophils in reperfusion injury. Episodes of ischemia prime the tissue to injury by subsequent reperfusion via the metabolic and molecular mechanisms associated with endothelial and vascular dysfunction. Although ischemic injury is mainly caused by oxygen-deprived cellular necrosis, reperfusion produces an inflammatory response that both heightens local damage and leads to systemic insult manifested as systemic inflammatory response syndrome[6] or multiple-organ dysfunction syndrome.

Despite considerable research in understanding the pathophysiology of ischemia reperfusion injury

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(IRI), a "gold standard" test for the condition remains elusive. No single account suffices to explain the variations in manifestation of this injury in different individuals or different tissues. In this review, an attempt has been made to provide insight into the pathophysiology of IRI in light of recent developments. An overview of the molecular and cellular mechanisms involved in endothelial and microvascular dysfunction is provided. The mechanisms of ischemic preconditioning (IPC) are also reviewed, along with other potentially therapeutic methods.

Endothelial Dysfunction

Endothelium

Vascular endothelium is a monolayer of endothelial cells that lines the entire vascular tree. Endothelium is a versatile tissue and performs a wide variety of sensory, secretory, and metabolic functions,[7,8] allowing it to play a crucial role in IRI. Its strategic placement, defining the intravascular and extravascular spaces, allows it to regulate the transport across these spaces. This is particularly important, as endothelium is in constant contact with the blood and all of its cells. The endothelium is inactive in the resting state, its prime function being to facilitate blood flow by providing an antithrombotic surface. The endothelium is able to sense any changes in the internal milieu of the vessel wall and has a remarkable capacity to accommodate its function and structure accordingly. This allows it to be a pivotal defense mechanism involved in restoration of vascular homeostasis.

Disruptions in internal homeostasis can be caused by a variety of stimuli: for example, ischemia, reperfusion, local physical injury, shear stress, and inflammation. On exposure to these adverse stimuli, the endothelium gets activated.[9] This results in adhesion of neutrophils and platelets to the endothelium, causing it to create a proinflammatory and prothrombotic surface.[8] This involves a self-propagative set of reactions, culminating in an increased oxidant load and a loss of local vasodilatory mechanisms. All of these changes are cumulatively termed endothelial dysfunction, and this is the rate-limiting factor in the pathophysiology of IRI. Various mediators and interactions are discussed in detail later in this review.

Cell–Cell Interactions

Activation of endothelium leads to neutrophil recruitment via a multistep cascade consisting of leukocyte rolling, firm adhesion, and ultimately transendothelial migration (TEM). TEM occurs by both paracellular (at endothelial junctions) and transcellular (through the endothelial cells) routes in vivo.[10-12] These steps are facilitated by increased permeability of the injured endothelium, altered or increased expression of adhesion molecules and cytokines, and reduced availability of antiinflammatory mediators such as nitric oxide (NO).

Neutrophil-endothelial adhesion (Figure 1)[13] is regulated by a receptor-ligand mechanism between corresponding neutrophil and endothelial receptor. Neutrophil adhesion molecules are also known as integrins; each integrin is a heterodimer composed of heavy α and a light β chain. The group, which appears to play a key role in neutrophil adhesion and migration, is that which contains the common β_2 or the cluster difference (CD) 18 chain,[13,14] as shown by in vitro studies that demonstrated increased adhesion in human neutrophils to cultured endothelium when exposed to various activating agents such as C5a,[15] platelet activating factor,[16] and tumor necrosis factor-α (TNF-α).[17] A return to almost basal levels of adhesion and abolition of neutrophil migration across the endothelial monolayer were also noted with monoclonal antibodies directed at CD11b and CD18 chains. Although in vitro models have been successful, in vivo clinical trials have so far failed to show any reduction in reperfusion injury by blocking neutrophils. In a randomized, double-blind, placebo-controlled trial, an antibody against the leukocyte adhesion integrin, CD18/CD11b, was given to patients with symptoms of acute myocardial infarction before coronary angioplasty.[18] Radionucleotide studies failed to demonstrate any reduction in the infarct size in the treatment limb.

Expression of cell adhesion molecules (CAMs) in response to an inflammatory stimulus is a well-regulated process coordinated by specific regulatory proteins within the endothelium and the inflammatory cells. Nuclear factor-κB (NF-κB) is a latent gene regulatory protein[19] sequestered in the cytoplasm of most cells in an inactive form. It is implicated in most inflammatory responses and is particularly responsive to TNF-α and interleukin-1 (IL-1). After activated, this protein upregulates the genetic expression of CAM among various other proinflammatory mediators. Intercellular CAMs (ICAMs) are glycoproteins expressed on the surface of the endothelial cells,[20] which bind to integrins on the blood cells. They play a crucial role in leukocyte trafficking in IRI because of their interaction with

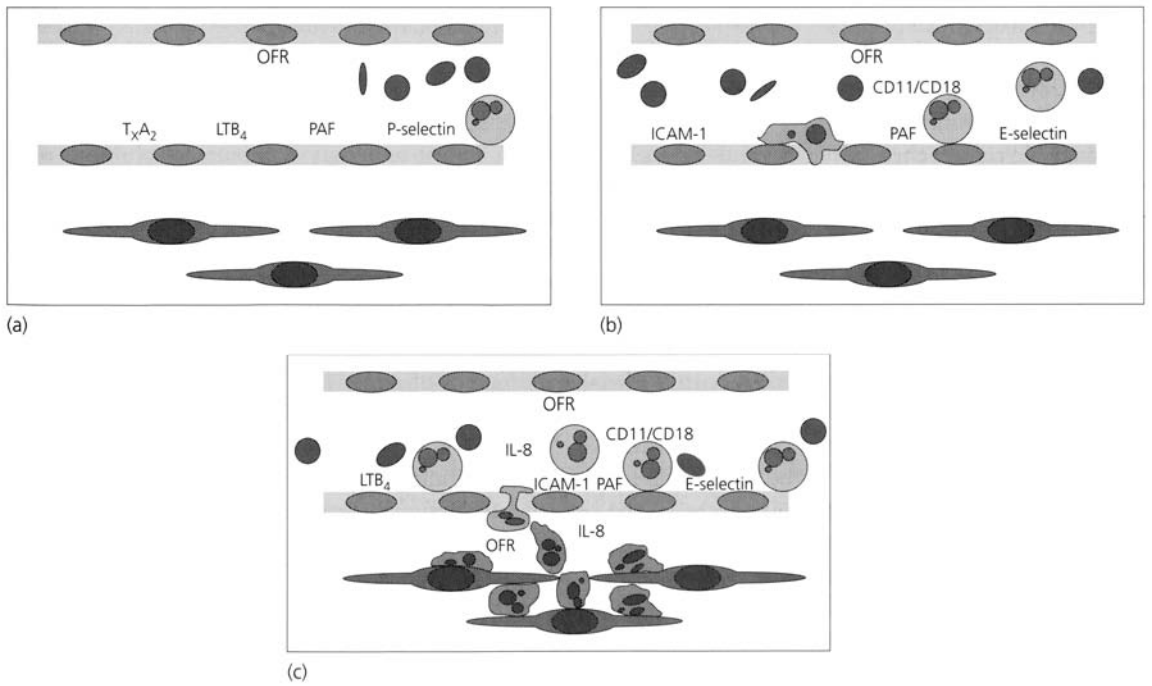

Figure 1. The role of neutrophils in the mediation of skeletal muscle reperfusion injury. (a) The creation of a local microenvironment that promotes neutrophil activation and P-selectin expression. (b) Neutrophil–endothelial adhesion via neutrophil integrins and endothelial adhesion molecules. (c) Release of further chemotactic mediators and neutrophil transmigration/sequestration within reperfused skeletal muscle. OFR = oxygen-derived free radicals. Reproduced with copyright permission from Blackwell Science Ltd.

leukocyte–endothelial CAM (expressed on the surface of leukocytes in addition to integrins), lymphocyte function associated antigen-1 (integrin adhesion protein expressed on the surface of activated T cells), and macrophage antigen-1.[21] A list of endothelial leukocyte CAMs is given in Table 1.[22]

Selectins are also a group of cell-surface carbohydrate binding proteins that facilitates TEM.[23] There are 3 members of the selectin family: L-selectin on leukocytes, P-selectin on platelets, and E-selectin on endothelial cells.[24] Selectins interact with integrins to accentuate endothelial–cellular adhesions. P-selectin has been found to play a key role in hepatic IRI by promoting neutrophil and platelet adhesions and has also been implicated as one of the dominant factors in leukocyte rolling in postcapillary venules.[25] L-selectin amplifies neutrophil capture within the microvasculature at the site of inflammation. Blocking of both P-selectin and L-selectin has been shown to reduce IRI in the liver.[26,27] This reduction is also seen when monoclonal antibodies against both macrophage antigen-1[28] and ICAM-1[29] are used in IRI in rat liver.

Selectin-mediated adhesion is dependent on extracellular calcium (Ca^{2+}). ICAM-1 occupancy has also been shown to trigger elevations in intracellular free calcium.[30] Besides being an important signaling pathway in cell–cell interactions, intracellular Ca^{2+} surges have been noted to correspond with cellular dysfunction caused by IRI. Significant increases in cytosolic and mitochondrial Ca^{2+} concentrations are noted in IRI in the liver.[31] Ca^{2+} has also been implicated in increased free radical production by the mitochondria in IRI.[32] The exact mechanisms of Ca^{2+} entry and increase within the cell in IRI are not yet fully understood. A possible mechanism is opening of the mitochondrial permeability transition pore[33,34] brought on by oxidative stress and increases in cytosolic calcium itself. Cyclosporin A, a specific inhibitor of mitochondrial permeability transition pore, has been demonstrated to prevent mitochondrial and liver dysfunction in reperfusion.[35,36] Intracellular acidosis, which prevails in ischemic states, itself contributes to calcium load. Disruption of Ca^{2+} homeostasis causes damage to the mitochondrial electron transport chain and triggers apoptosis by stimulation

Table 1. Endothelial–Leukocyte Cell Adhesion Molecules

Family	Members	Cluster Designation	Cellular Distribution	Counter-receptor/Ligand
Selectins	E-selectin	CD62E	Endothelium	PSGL-1, ? other carbohydrate-bearing structure(s) on leukocytes
	L-selectin	CD62L	Leukocytes	PSGL-1, ? inducible carbohydrate-bearing structure(s) on endothelium
	P-selectin	CD62P	Endothelium, platelets	PSGL-1, ? other carbohydrate-bearing structure(s) on leukocytes
Mucin-like	PSGL-1	CD162	All blood leukocytes	E-, L-, and P-selectin
β_1 integrins	$\chi_4\beta_1$	CD29	Monocytes, lymphocytes	VCAM-1
β_2 integrins	LFA-1	CD11a/CD18	Leukocytes	ICAM-1, ICAM-2, ICAM-3
	Mac-1	CD11b/CD18	Monocytes, neutrophils	
	P150, 95	CD11c/CD18	Monocytes, neutrophils	
β_7 integrins	$\chi_4\beta_7$		Lymphocytes	VCAM-1
Ig	ICAM-1	CD54	Endothelium, leukocytes, epithelial cells, fibroblasts, other cell lines	LFA-1, Mac-1
	ICAM-2	CD102	Endothelium	LFA-1
	ICAM-3	CD50	Leukocytes	LFA-1
	VCAM-1	CD106	Endothelium, smooth muscle cells	$\chi_4\beta_1, \chi_4\beta_7$
	PECAM-1	CD31	Endothelium, leukocytes, platelets	PECAM-1

ICAM = intercellular cell adhesion molecule; LFA-1 = lymphocyte function-associated antigen-1; PECAM-1 = platelet/endothelial cell adhesion molecule-1; PSGL-1 = P-selectin glycoprotein ligand-1; VCAM = vascular cell adhesion molecule.

of calcium-dependent proteases, nucleases, and phospholipids. This effect of extramitochondrial Ca^{2+} on amplification of mitochondrial damage in IRI is thought to be dependent on Ca^{2+} concentrations and the duration of hypoxia.[33]

Although Ca^{2+} is an important signaling mechanism in cell–cell adhesion, there is another set of messenger proteins coordinating the transmission of signal from the adhesion molecule to the cytoskeleton of the cell. This set of signaling proteins is the Rho protein family.[37] They are a subdivision of the Ras family of messenger proteins that link cell surface receptors to the actin cytoskeleton[38] within the cells. Rho proteins cause alterations in the cell cytoskeleton and cell behavior in response to external stimuli in IRI and regulate endothelial and smooth muscle cell contractility and function. They are increasingly being implicated in effecting every aspect of vascular biology, including endothelial barrier function, TEM, platelet adhesion, and inflammatory and wound healing processes.[39] Leukocyte-endothelial adhesion promotes Rho activation, and this in turn causes opening up of the endothelial gap junctions.[40] Rho proteins have also been implicated in facilitating production of free radicals,[41] but this role needs further investigation. There has been

increasing interest in understanding the precise role of Rho proteins in maintenance of vascular integrity, with a view to develop strategies to combat IRI.

Once stimulated, the inflammatory process tends to be self-propagative. Although this could be explained by the magnitude of the insult itself, the contribution of local factors cannot be ignored. One such factor is the CD40 ligand (CD40L), a cell surface molecule that interacts with its receptor, CD40 (membrane glycoprotein receptor), to form a CD40/CD40L ligand-signaling complex. CD40 receptor is expressed on a wide variety of cell populations,[42] including lymphocytes, macrophages, platelets, dendritic cells, endothelial cells, smooth muscle cells, and fibroblasts. Although initially implicated in atherosclerosis and advanced peripheral vascular disease,[43] there is an emerging body of evidence implicating it in IRI as well. Experiments on mouse brain[44] showed that CD40/CD40L signaling contributed to reperfusion-induced brain infarction. It promotes a prothrombogenic environment by enhancing platelet-platelet (homotypic) and platelet-leukocyte (heterotypic) aggregations.[45] In doing so, there is increased frequency of local ischemic episodes, thereby promoting the hypoxic and inflammatory environment. CD40/CD40L signaling also promotes interplay between inflammation and immunity in IRI. It promotes differentiation of helper T cells into T_H1 effector cells in the peripheral lymphoid organs and further activation of macrophages. T_H1 effector cells also secrete a variety of cytokines, further promoting inflammation and cell adhesion.

Cytokines

Cytokines are regulatory proteins that modulate inflammatory and immunological responses by binding to specific receptors. They are produced by leukocytes, endothelial cells, and almost every nucleated cell and act in both autocrine and paracrine fashion. Over 200 cytokines have been cloned to date; structurally, they can be classified into 9 main families (Table 2).[46] They facilitate all aspects of inflammation by altering cell proliferation, differentiation, and function. Although cytokines are intimately linked to inflammation, they are not specific for inflammation in IRI. The cytokine role in IRI is reflective of the inflammatory complex initiated after IRI. Cytokine production after IRI is transient with absent or low basal levels, and this has positive implications for establishing their role in the pathophysiology and as markers for the underlying disease process. They also act as harbingers of remote tissue injury following IRI. Elevated levels of TNF-α, IL-1, and IL-6 were detectable in serum and systemic organs such as lung and kidney after hind limb ischemia.[47,48] Hindlimb ischemia models also confirm the role of cytokines in increasing neutrophil recruitment by complementing integrin-induced neutrophil adhesion to the endothelium. Cytokines can be broadly classified as proinflammatory, antiinflammatory, or with ambivalent role. Identifying the specific role of cytokines with respect to IRI and vascular disease is the subject of ongoing research.

Table 2. Cytokine Families Based on Structural Features of Cytokines

IL-2/IL-2 family
IL-6/IL-12 family
Interferon-α/β family
Tumor necrosis factor family
IL-10 family
IL-17 family
Interleukin-1 family
TGF-β family
Chemokine family

IL = interleukin; TGF-β = transforming growth factor-β.

TNF-α is a proinflammatory cytokine that fulfills multiple biological functions. In the endothelial cell, it actively induces actin filament rearrangement, leading to cell damage and loss of tight junctions, manifested as leaky capillaries.[49,50] It has been found to have a negative ionotropic effect on the heart leading to hypotension, pulmonary edema, and metabolic acidosis. It has a pivotal role in septic shock[51] and could be responsible for some of the similar features seen after reperfusion and in associated multiple organ failure. TNF-α also causes expression of IL-1, IL-6, IL-8, and monocyte chemotactic protein-1.[52,53]

IL-1 has not been found to have a role in normal homeostasis in humans, but is a potent chemotactic agent[54] and increases the expression of ICAM-1 and vascular CAM-1 on endothelial cells.[55] It acts by binding to specific receptors: IL-1 receptor type 1 is one such receptor. In mice lacking IL-1 receptor type 1, there is a failure to develop proliferative lesions of vascular smooth muscle cells in mechanically injured arteries.[55] Although injections of IL-1 receptor antagonist in humans have resulted in

reduction in inflammation and joint destruction in rheumatoid arthritis,[56] no such results are available for its specific use in IRI.

IL-6 is produced by both lymphoid and nonlymphoid cells and regulates acute-phase response in inflammation and immune reactivity.[57] Other than inducing cell adhesion, it increases endothelial permeability. Its role in promoting oxidative burst in leukocytes is controversial. It is easily detected systemically and has been identified as an independent predictor of peripheral vascular disease progression.[58] It stimulates hepatic production of acute phase reactants such as C-reactive protein, α1-antitrypsin, fibrinogen, α1-acid glycoprotein and haptoglobin.[59] This explains elevated C-reactive protein levels corresponding to peaks in IL-6 levels as seen in peripheral arterial disease.

IL-8 is a potent chemokine. Significant levels of IL-8 have been noted in myocardial, skeletal, and renal IRI.[60-62] Leukocytes and endothelial cells produce IL-8, and there is a surge in its production after inflammation stimulated by IL-1 and TNF-α. Its production is also stimulated by hypoxic conditions via NF-κB–induced transcription in endothelial cells. Besides neutrophil chemotaxis and adhesion, it also causes degranulation of the neutrophils releasing their proteolytic enzymes. Because of its effect in oxidative bursts within the neutrophil, IL-8 has been suggested as the link between neutrophil adhesion and increase in free radical production, particularly in the post capillary venules.[63]

Cytokines have a diverse role in inflammation, and extensive research is being carried out to understand their specific roles in IRI, with a view to modulate their interaction at the receptor level for future therapies.[62] No successful trials have been reported so far.

Reactive Oxygen Species

There is a complex interplay among endothelial activation, inflammatory cell recruitment, and reactive oxygen species (ROS) production. In health, there exists a balance between the formation of these oxidizing chemical species and their effective removal by protective antioxidant mechanisms. Oxidative stress is disruption of this balance in favor of ROS production, and this constitutes one of the important mechanisms of endothelial dysfunction. ROS are involved in important signaling processes in various cardiovascular pathologies, including IRI. ROS-induced signaling is mediated via enhanced transcription of regulatory proteins such as NF-κB and hypoxia-inducible factor-1 within the endothelium.[64] This leads to increased transcription of CAM and vascular endothelial growth factor, specifically induced by activated hypoxia-inducible factor-1,[65-67] resulting in enhanced leukocyte and platelet adhesion to the endothelium. Leukocytic recruitment further accelerates ROS production.[10,68]

ROS include superoxide, hydrogen peroxide, and hydroxyl ions. They are produced by a variety of sources. Hypoxia causes alterations in various enzymes involved in energy metabolism in the cell and directly to the mitochondrial mechanisms themselves, causing uninhibited production of ROS on resumption of oxygen supply during reperfusion. Enzyme systems implicated in IRI include cytochrome oxidase, xanthine oxidase (XO), reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase, and the mitochondrial electron transport chain.

Under normal resting conditions, oxidative phosphorylation is the major source of energy production by mitochondria in the cell. This results in formation of ATP, which is the energy currency of the body. ATP is converted to ADP, and the energy released is used by the body. This complex process is carried out by the respiratory chain (electron transport chain) in the mitochondria. The respiratory chain is a set of enzymes embedded in the mitochondrial inner membrane. Each enzyme is responsible for a specific step, which causes release of electrons that are sequentially transferred down the chain; ATP is the end product. NADH dehydrogenase and cytochrome oxidase are 2 of the important enzyme complexes involved in the electron transfer chain. Small amounts of superoxides generated in this reaction are neutralized by the enzyme manganese superoxide dismutase (MnSOD).[69] IRI causes derangements in this enzyme function, leading to excess superoxides, which the rapidly dwindling stores of MnSOD fail to neutralize.

The XO system has been recognized as an important source of ROS. Hypoxia ablates mitochondrial ability to recycle ADP leading to a rise in its levels. This serves as an important substrate for the purine metabolite hypoxanthine. Normally, hypoxanthine is converted to xanthine by an enzyme, xanthine dehydrogenase. Hypoxia causes conversion of xanthine dehydrogenase to XO, inhibiting hypoxanthine metabolism and leading to buildup of its levels. On reperfusion, when oxygen is available, XO uses oxygen as its substrate leading to

conversion of hypoxanthine to urate and superoxides are produced as a by-product of this reaction.[70] The role of allopurinol,[71] besides many other XO inhibitors, has been investigated in IRI. No clinically significant results have been noted so far.

NAD(P)H oxidase is another enzyme system implicated in IRI. It is an important source of ROS in neutrophils, endothelial cells, and platelets.[72,73] NAD(P)H oxidase-induced ROS production is more aggressive in neutrophils (oxidative burst)[74] as compared with a slower release in endothelial cells. As described later, this enzyme system also interacts with NO[75] to produce reactive nitrogen species (RNS).

Reactive ferryl species have also been identified as contributors to oxidative stress.[76,77] This is particularly important in trauma settings when myoglobin and hemoglobin are released into the plasma. In vitro experiments have shown that under conditions as they prevail in IR injury, hemoglobin is oxidized to an intermediate reactive ferryl form and causes lipid peroxidation in endothelial cells.[78]

ROS induce apoptosis and cell necrosis.[79] They impair vasodilator responses[80]; in higher concentrations, they oxidize proteins and lipids and damage DNA. ROS-induced injury is at multiple levels of cellular function via a wide range of processes. One such mechanism is lipid peroxidation,[81,82] whereby they cause direct damage to cellular and organelle membranes resulting in structural damage and release of various autolytic enzymes. There is experimental evidence for the prevalence of this mechanism in almost every organ system exposed to IRI: the kidney,[83] retina,[84] lungs,[85] liver,[25] myocardium,[86] brain,[87] blood vessels,[86] and placenta.[88] Regardless of the nature of the free radicals, they attack the phospholipids of the cell and organelle membranes culminating in irreversible structural and functional damage. Polyunsaturated fatty acids are more vulnerable to this insult than monounsaturated fatty acids.[89] The by-products formed are biologically active mediators themselves; thus, they contribute to the self-perpetuating nature of this injury. The importance of identifying and quantifying the by-products of lipid peroxidation cannot be underestimated. Not only do these serve as markers of reperfusion injury, but they also give information about the oxidant load. One of the secondary by-products produced as a result of this process is malondialdehyde (MDA).[90] MDA levels have been successfully used as markers of oxidative stress in various disease conditions such as eclampsia and in renal IRI. There are significantly higher mean MDA levels and significantly lower superoxide dismutase levels[91] in eclamptics when compared with normotensive pregnant women. The role of MDA as a marker of oxidative stress in peripheral arterial disease remains unexplored. Table 3 gives a list of some of the by-products of lipid peroxidation.

Table 3. By-products of Lipid Peroxidation

Malondialdehyde (MDA)—aldehyde product of lipid peroxidation
9,11 octadecadienoic acid—conjugated diene
Isoluminol, ethane, pentane—volatile hydrocarbons
8-epi $PGF_{2\alpha}$ (isoprostanes)—prostaglandin isomers as by-product of arachidonic acid peroxidation

Antioxidants

Antioxidants can be defined as substances that when present at concentrations lower than the oxidizable substrate significantly delay or inhibit oxidization of that substrate.[92] IRI is a state of increased oxidant load compounded by inactivation or depletion of antioxidants. The cumulative effect of all these changes is a shift of balance in favor of oxidizing species–redox imbalance.

Antioxidant defenses can be classified as primary, secondary, or tertiary depending on whether they prevent free radical production, facilitate removal or neutralization of the formed free radicals, or repair the oxidatively damaged substances. Antioxidants can also be classified as enzymatic and nonenzymatic. The enzymatic oxidants are MnSOD, thioredoxin reductase, and glutathione reductase. The nonenzymatic antioxidants can be classified as lipid-soluble such as vitamin E or water-soluble such as vitamin C.[93] MnSOD and other antioxidant defenses such as ascorbate are rendered inactive by ROS and RNS.

Extensive research and trials have been undertaken in an attempt to explore antioxidant-based therapies. In hamster microcirculation experiments, it has been shown that pretreatment with MnSOD before reperfusion prevented lipid peroxidation, maintained ROS at normal levels, increased arteriolar diameter, and decreased leukocyte adhesion[94]; however, clinical trials with antifree radicals have failed to show encouraging results. Human superoxide dismutase was given in patients after acute myocardial infarction and before percutaneous transluminal angioplasty, but no improvement was

noted in ventricular function.[95] Some antioxidant trials have shown decrease in incidence of systemic complications such as acute respiratory distress syndrome and renal function. This was noted in an antioxidant trial on severe closed head injury patients.[96] No improvement, however, was noted in clinical outcome as regards head injury. A similar trial with mannitol (nonspecific free radical scavenger) in infrarenal aortic aneurysm repair showed a lower incidence of acute respiratory distress syndrome and renal failure postoperatively as compared with the placebo group.[97] Failure to curb reperfusion-induced damage in organ systems with antioxidant therapy probably explains the diversity of mechanisms by which the free radical mediated injury is pursued.

NO and RNS

NO is a free radical with a short biological half-life of only a few seconds. NO-mediated vasodilatation maintains the basal vascular tone under normal homeostatic conditions.[98] Besides the vasodilatory function, NO plays an important role in limiting neutrophil and platelet adhesion, aggregation, and activation. NO is discussed here because it is also an important source of free radical production in IRI. Free radicals generated from NO are referred to as RNS—peroxynitrite and NO radical. RI is a state of depleted and dysfunctional NO metabolism.

NO is generated in the endothelium from oxidation of arginine by an enzyme, NO synthase (NOS).[99] NAD(P)H and tetrahydrobiopterin (BH_4)[100] are important cofactors in this reaction. Although the NOS system is generating NO, the NAD(P)H oxidase system continues to produce superoxides in the same cell. Superoxides thus formed react with NO to form RNS,[101] peroxynitrite in particular (Figure 2). Peroxynitrite[102] is an important contributor to endothelial dysfunction. It promotes vasospasm and thrombosis and some of these effects are caused by peroxynitrite-induced derangements in prostaglandin synthesis (nitration of prostaglandin 12 synthase). Peroxynitrite causes negative feedback inhibition of NOS to produce NO. NO production is further hindered by peroxynitrite-mediated inactivation of cofactors required for NO production. BH_4 is oxidized to inactive metabolites. Although initially implicated in endothelial dysfunction secondary to atherosclerosis, BH_4 depletion has now been found to have similar effects in IRI and has therefore become a target for therapeutic manipulation. Tiefenbacher et al[103] showed preservation of endothelial-dependent vasodilatation after administration of serapterin (metabolic precursor of BH_4) and 6-methyl BH_4 (synthetic version of BH_4).

IRI, therefore, is a state of depleted NO and results in a shift of balance in favor of vasoconstrictive forces. This is compounded by the platelet and leukocytic adhesion to the endothelial cell. All of the mechanisms that lead to endothelial dysfunction in IRI in turn contribute to the creation of a proinflammatory and prothrombotic microenvironment, and this forms the template for progression to microvascular dysfunction.

Microvascular Dysfunction

Microvascular dysfunction is a spectrum of changes specific to vasculature in response to endothelial dysfunction. In keeping with the heterogeneity of the endothelium, it is manifested in a site-specific manner with a degree of variation in arterioles, capillaries, and venules.

Arterioles

Arteriolar vasoconstriction or a lack of arteriolar vasodilatation is noted in all organ systems after IRI. The arterioles exhibit a biphasic response to hypoxia-phasic contraction followed by tonic relaxation. The second phase of this process is the result of production of NO in response to hypoxia.[104] This response is significantly hampered in IRI[105] and is the underlying mechanism for arteriolar dysfunction.

Various mechanisms are responsible for decreased bioavailability of NO in IRI. ROS generated in IRI inhibit NO production. Negative feedback inhibition of NOS by peroxynitrite has been discussed previously. Arginine is the substrate for NO production. IRI causes competitive inhibition of NOS by increasing the activity of another enzyme, arginase, which uses the arginine pool instead.[106] RNS also cause depletion of cofactors required for production of NO.[107] Decreased levels of NO alone, however, do not provide a holistic explanation for loss of arteriolar vasodilation. Arteriolar tone is the net result of a delicate equilibrium between vasodilator and vasoconstrictor forces.[108] IRI causes accumulation of potent vasoconstrictors such as endothelins, and all of these processes occur in conjunction with inflammatory processes implicated in endothelial dysfunction.

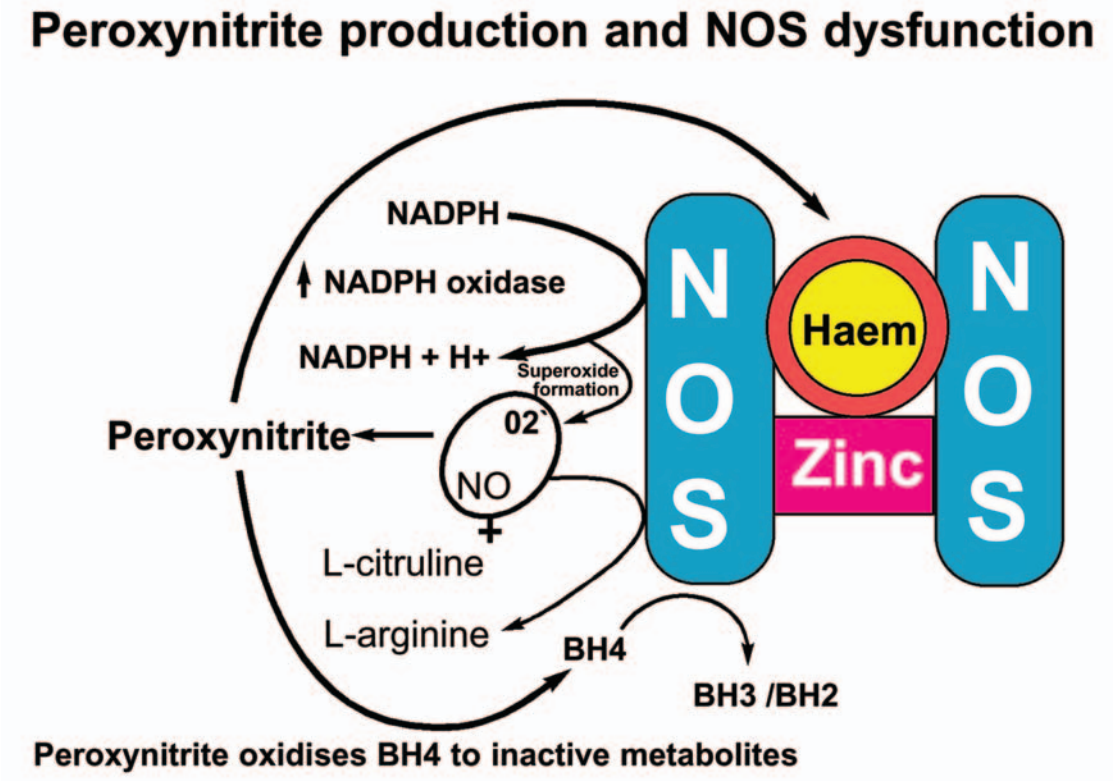

Figure 2. In the presence of high concentrations of oxygen free radicals, nitric oxide (NO) combines with unpaired oxygen to form peroxynitrite. Excessive peroxynitrite leads to oxidation of BH_4 to the inactive form BH_3 and to deactivation of the haem core, which uncouples NO synthase (NOS) and converts it into a dysfunctional enzyme producing yet more free radicals.

Endothelins are vasoconstrictor peptides produced by the endothelium in response to hypoxia.[109,110] Subjection of the endothelium to shear stress causes a similar response. They tend to bind to specific receptors on the vascular smooth muscle and cause a surge in intracellular Ca^{2+}, which increases the smooth muscle tone. Under physiologic conditions, NO counters the action of endothelins by restoring the basal intracellular Ca^{2+} levels, and this is lost in IRI. Leukocyte adhesion plays a prominent role in microvascular dysfunction in the capillaries and venules, but has not been considered a major component of arteriolar dysfunction. Increased platelet adherence facilitates maintenance of an ischemic environment by promoting microthrombi and hindering blood flow.

"No Reflow Phenomenon" and Capillaries

IRI-mediated surges in inflammatory mediators, and CAM expression initiates inflammatory and coagulation cascades, causing occlusion of capillaries.[111] This is known as "no-reflow," implying local blood flow failure. Although initially described after prolonged cerebral ischemia, this has been confirmed to exist in other tissues such as kidneys, liver, skeletal muscles, and myocardium. It manifests itself clinically as continued organ dysfunction after reperfusion, exemplified by failure of transplanted graft/organ, reperfusion arrhythmias and myocardial stunning in the heart, and persistent leg ischemia and compartment syndrome in the peripheries. No one mechanism accounts for this phenomenon, as it is due to a combination of various mechanical, biochemical, and cellular insults borne by the endothelial cells in the process of reperfusion. It is noted to be patchy in nature so that a given tissue has areas of perfusion and of ischemia. The severity of no-reflow depends on the severity and duration of the preceding ischaemia. This correlation has been confirmed in rat hind-limb studies,[112] wherein blood flow was reduced after reperfusion after a recorded period of ischemia. IRI causes a disruption in the endothelial barrier function, resulting in macromolecular leakage and interstitial edema (vascular

dysfunction).[113,114] In the clinical setting, this is commonly seen in the lungs, manifested as pulmonary edema and increased oxygen demand.[115]

Changes leading up to no-reflow in capillaries can be divided into extraluminal and intraluminal. Both mechanisms cause obliteration of the capillary lumen. Extraluminally, there is buildup of interstitial pressure as a consequence of the tissue edema, causing collapse of the vessel wall. Intraluminally, endothelial cells lining the capillaries tend to swell. Activated endothelium facilitates platelet[116] and leukocyte adhesion.[117] This promotes intravascular thrombosis and congestive occlusion of capillary lumen. Activated leukocytes are rigid, and with increased expression of adhesion molecules, there is slow transit throughout the capillaries. Leukocyte recruitment also accentuates redox imbalance, promoting endothelial damage mediated by free oxidants. All of these mechanisms, in addition to loss of NO-mediated vasodilation, lead to vessel wall collapse and further ischemia.

Attempts to counter no-reflow have included pretreatment with inhaled NO, which has been shown to limit barrier dysfunction in lung IRI.[118] Aerosolized prostaglandin E_1 and prostaglandin I_2 have a similar effect in the lung.[119] L-Arginine and antioxidant vitamins have been shown to increase the capillary diameter and decrease interstitial edema in rabbit hind limbs following reperfusion.[120] Anticomplement antibodies in animal experiments have been shown to ameliorate IRI-induced microcirculatory disturbances in the pancreas,[121] thus suggesting inflammatory response to ischemia in the capillaries.

Venules

Venules bear the brunt of the inflammatory changes in reperfusion, as the venular endothelial cells are the prime site of adherence of leukocytes and platelets. After endothelial injury, there is increased expression of transcription factors such as NF-κB. Once activated, this leads to changes in gene expression in the endothelial and inflammatory cells, leading to production of selectins and other mediators of inflammation. P-selectin expression increases rapidly after IRI. Functional blocking experiments in adhesion molecule-deficient mice show P-selectin to be the dominant receptor for leukocyte rolling in postischemic venules, where interaction between β_2 integrin on leukocytes and ICAM-1 on endothelial cells accounts for firmly adherent leukocytes.[122-125] At low blood flow, there is increased interaction between the neutrophils and endothelium[126] to promote adhesion and immigration across the vascular barrier. Low flow rates also promote leukocyte-platelet interactions.[127] CD40L signaling plays a key role in inflammation through the induction of CAM and tissue factors in the endothelial cells and by enhancement of production of proinflammatory cytokines.[128-130] Increased leukocyte concentration further promotes platelet adhesion, causing an increase in microvascular thrombosis. This phenomenon is well documented in hepatic, cerebral, and skeletal IRI. Increased leukocytic production of free oxidants, along with preexisting supply from the endothelium, leads to oxidant-induced endothelial damage. Restrictive properties of the endothelium are greatly hampered, and there is increased extravasation of albumin and plasma proteins. Trafficking of leukocytes in the postischemic venules is the rate-limiting step determinant of endothelial barrier function. Studies have shown the correlation between the amount of albumin leakage and the number of leukocytes that are adherent to endothelial cells.[131]

Potential Therapeutic Strategies

Progress made toward clearer understanding of the varied phenomena of the pathophysiology of IRI has catalyzed research to curb this adverse process. Some of the earlier methods of intervention have been outlined in Table 4. Preconditioning is a promising avenue to limit IRI-induced damage and can be classified as ischemic and pharmacologic preconditioning. Furthermore, recent trials with the novel mechanism of postconditioning have shown encouraging results.

IPC

IPC is a phenomenon in which brief periods of subtoxic ischemia induce protection against subsequent prolonged ischemia. Murray et al[132] first demonstrated this in 1986. Four cumulative 5-minute ischemic periods caused a 70% reduction in infarct size in a canine heart caused by a subsequent 40-minute ischemic insult as compared with controls. After this initial observation, the phenomenon was reported across different mammalian species, including humans.

Table 4. Ischemia Reperfusion Injury: Intervention Strategies

Free radical scavengers
Enzyme inhibitors
Receptor antagonists
Cell adhesion molecule blockade
Molecular manipulation of cells
Controlled reperfusion
Preconditioning: ischemic, pharmacological
Postconditioning

Protection provided by IPC is time dependent and occurs in 2 stages—early and late window of protection (WOP). Early WOP sets in minutes after IPC and is lost if the time between IPC and index ischemia extends to beyond 2 to 3 hours.[133,134] Immediate onset of protection suggests the role of preformed mediators in this process. Late WOP sets in about 12 to 24 hours after IPC and lasts up to 96 hours.[135] Late WOP is thought to involve de novo protein synthesis,[136] which contributes to the prolonged protection conferred by it. Early and late IPC share signal transduction cascades besides their respective signaling mechanisms, but the exact description of these processes is beyond the scope of this review.

Although the definite end effector of these signal cascades in IPC remains elusive, mitochondrial K_{ATP} channels have been implicated in this role.[137] This view is supplemented by the evidence gained from studies on isolated cell models of ischaemia, using nicorandil[138] and diazoxide[139] (selective K_{ATP} channel openers), which provided similar protection to IPC. These channels regulate mitochondrial and cytosolic Ca^{2+} and ATP concentrations and in turn regulate mitochondrial integrity and cellular apoptosis under conditions of oxidative stress. IPC induces protection by facilitating the adjustment of the energy balance of a cell to a new, more dormant state,[140] which is more congenial to survival in hypoxic conditions. Preconditioned canine hearts have been shown to consume ATP at a slower rate as compared with controls.[141] One of the mechanisms involved in achieving this new equilibrium is the promotion of a state of transient membrane arrest.[134] This preserves ATP used by the Na^+ pump, which otherwise uses substantial energy to maintain transmembrane potential.

IPC has also been shown to curb microvascular dysfunction by preservation of vasoregulatory function of the preconditioned endothelium.[142] This is facilitated by sustained NO production by the endothelium, inhibiting the effect of vasoconstrictor peptides, as evidenced in hepatic IRI.[25,143] Reduced expression of P-selectin and CAM is also noted[144]; these reduce leukocytic adhesion with a consequent reduction in capillary no-reflow.

IPC was introduced clinically as a useful cardioprotective adjunct in coronary artery bypass surgery (CABG).[145] Over time, there has been wider application in transplant and resection surgery, liver and lungs in particular. Fewer episodes of postoperative cardiac dysrhythmias after CABG[146-148] and reduced ionotropic requirement after liver resection[149] and CABG[150] have been noted, following the use of IPC. Although IPC has shown successful transition from laboratory to clinical setting, there are certain factors curtailing its wider application. Exact information is required about the timing of the index ischemia. This is important for planning the preceding ischemic episodes and is not possible in acute scenarios. There is no consensus regarding the number and duration of cycles of controlled ischemia required to impart the maximal benefit in heart or the liver. Most of the evidence is from animal (canine and rabbit) studies, and it could be inferred that a smaller heart requires shorter cycles and larger hearts with lower myocardial metabolism require cycles of longer duration.[126] There has also been disagreement about whether threshold ischemia is different in females and males.[151,152]

Overall, application of IPC remains limited to specialized centers with particular interest in this phenomenon. This can be attributed partly to conflicting clinical results generated from small, randomized trials. Although improvements have been shown in limiting a particular aspect of reperfusion—increased protective enzymes (MnSOD) in patients undergoing pneumonectomy[153] as compared with controls or decreased level of apoptotic sinusoidal lining cells in preconditioned livers[154]—the overall impact on improved clinical outcome remains unclear. In the absence of robust evidence, it is easy to understand reservations about applying a potentially harmful stimulus to a fragile donated organ. Evidence from larger multicentric trials would be useful in promoting wider application of this concept in clinical medicine.

Pharmacological Preconditioning

Much research has gone into the development of pharmacological preconditioning mimetics with potential clinical use. Cardiology has taken a clinical

lead in this field, and several of these agents, being used for purposes other than cardioprotection, have already been approved by the Food and Drug Administration.[155] Some of the drugs included in this group are the statins, adenosine, opioids, nitrous oxide inhalation therapy, and erythropoietin. The pathways of acetylcholine-induced and bradykinin-induced preconditioning and cardioprotection have been extensively studied.[156] Although pharmacologic preconditioning appears promising in smaller trials studying cellular and subcellular mechanisms of an individual agent, or studies on individual organs, or in animal studies, level 1 evidence from well-organized clinical trials is required to bring this research to fruition.

Postconditioning

Postconditioning can be defined as the process of limiting IRI-induced damage by application of repetitive short ischemic windows during early reperfusion. This is at slight variance from controlled reperfusion, as it involves application of repetitive ischemic episodes. Zhao et al[157] were the first to describe this observation in canine hearts in 2003 and coined the term postconditioning. Staat et al[158] were the first to describe the clinical application to human hearts. They described a 35% reduction in infarct size (as indicated by the creatinine kinase levels) in patients subjected to postconditioning. These findings are promising in clinical arenas where occluded vessels or unwarranted ischemia limited application of IPC. Although no foresight into time of ischemic event is required for postconditioning, it needs to be applied within minutes of reperfusion for maximal benefit.[136] Even a short delay of a few minutes leads to diminution of its effects. Although Yang et al[159] found that a delay of 10 minutes after reperfusion diluted postconditioning-induced protection, Philipp et al[160] found protection to be lost as early as 1 minute after reperfusion. Understanding of the mechanisms of postconditioning is still in an infant state, although the key pathways appear to be similar to IPC; unlike IPC, the role of mitochondria in postconditioning has not been fully elicited.

Clinically, postconditioning is well suited for vasculo-occlusive emergencies and elective surgical settings involving clamping of arteries and subsequent release. As with IPC, further evidence needs to be gained about this promising concept. It has also opened exciting research avenues into pharmacotherapeutic agents mimicking postconditioning and the possibilities of synergy in preconditioning-mediated and postconditioning-mediated protection.

Conclusion

IRI is responsible for significant morbidity and mortality. Complex interplay between local and systemic inflammatory processes culminates in endothelial dysfunction, and together with loss of NO-mediated vasodilatation, microvascular dysfunction sets in. ROS and RNS promote IRI, but due to the multifactorial etiology of IRI, antioxidant supplementation alone does not ameliorate IRI. Improved understanding of the pathophysiology of IRI has enabled development of models of various therapeutic strategies. Preconditioning has shown successful transition from laboratory to clinical settings. Postconditioning also shows potential in vaso-occlusive emergencies but further work is required to establish its efficacy in the clinical field.

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