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Review

The role of neutrophils in myocardial ischemia–reperfusion injury

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Abstract

Reperfusion of ischemic myocardium is necessary to salvage tissue from eventual death. However, reperfusion after even brief periods of ischemia is associated with pathologic changes that represent either an acceleration of processes initiated during ischemia per se, or new pathophysiological changes that were initiated after reperfusion. This 'reperfusion injury' shares many characteristics with inflammatory responses in the myocardium. Neutrophils feature prominently in this inflammatory component of postischemic injury. Ischemia–reperfusion prompts a release of oxygen free radicals, cytokines and other proinflammatory mediators that activate both the neutrophils and the coronary vascular endothelium. Activation of these cell types promotes the expression of adhesion molecules on both the neutrophils and endothelium, which recruits neutrophils to the surface of the endothelium and initiates a specific cascade of cell–cell interactions, leading first to adherence of neutrophils to the vascular endothelium, followed later by transendothelial migration and direct interaction with myocytes. This specific series of events is a prerequisite to the phenotypic expression of reperfusion injury, including endothelial dysfunction, microvascular collapse and blood flow defects, myocardial infarction and apoptosis. Pharmacologic therapy can target the various components in this critical series of events. Effective targets for these pharmacologic agents include: (a) inhibiting the release or accumulation of proinflammatory mediators, (b) altering neutrophil or endothelial cell activation and (c) attenuating adhesion molecule expression on endothelium, neutrophils and myocytes. Monoclonal antibodies to adhesion molecules (P-selectin, L-selectin, CD11, CD18), complement fragments and receptors attenuate neutrophil-mediated injury (vascular injury, infarction), but clinical application may encounter limitations due to antigen–antibody reactions with the peptides. Humanized antibodies and non-peptide agents, such as oligosaccharide analogs to sialyl Lewis^x, may prove effective in this regard. Both nitric oxide and adenosine exhibit broad spectrum effects against neutrophil-mediated events and, therefore, can intervene at several critical points in the ischemic–reperfusion response, and may offer greater benefit than agents that interdict at a single point in the cascade. The understanding of the molecular processes regulating actions of neutrophils in ischemic–reperfusion injury may be applicable to other clinical situations, such as trauma, shock and organ or tissue (i.e. vascular conduits) transplantation.

Keywords: Neutrophils; Endothelium; Nitric oxide; Adenosine; Infarction; Reperfusion injury; Inflammation; Monoclonal antibodies

1. Introduction

Undoubtedly, reperfusion of ischemic myocardium is necessary to salvage tissue from ultimate death. However, reperfusion after even brief periods of ischemia submits to the axiom '. . . for every action, there is a reaction.' Although the biochemistry and physiology of the host's response to injury has evolved as an immunological defense against bacterial and other invaders, the heart's inflammatory reaction to injury may not be optimal, or even appropriate, for its own healing in the case of myocardial ischemia. At the same time that reperfusing blood halts the ischemic process by supplying oxygen and nutrients, a cascade of events with properties similar to the inflammatory response is rapidly initiated. However, this inflammatory-like response to ischemia–reperfusion, me-

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diated largely by neutrophils, is mounted against host tissue, such as the endothelium and myocardium, and this 'normal' response has no system of checks and balances to distinguish 'self' tissue from 'non-self' and to adjust itself accordingly. Neutrophils play a central role in this inflammatory-like response to reperfusion by releasing oxidants and proteases that damage or kill tissues, and release inflammatory products that amplify the recruitment and activation of greater numbers of neutrophils into the effected myocardium, thereby extending the severity of tissue damage. Hence, neutrophils are intimately involved in the pathogenesis of myocardial infarction, vascular endothelial dysfunction, damage to the genetic apparatus, apoptosis and other manifestations of lethal injury in the acute phase following reperfusion. This 'reperfusion injury' involves a well-orchestrated series of interactions between neutrophils and the vascular endothelium via specific adhesion molecules on both cell types; these interactions are initiated in the immediate peri-reperfusion period, and may continue during the ensuing hours and days following reperfusion. These specific events appear to be critical and are a prerequisite to the eventual expression of tissue damage. The understanding of these physiological processes is interesting in and of itself, but, more importantly, it forms a basis for the therapeutic strategies addressing neutrophil-mediated myocardial reperfusion injury discussed in the latter part of the manuscript.

If one accepts the concept of reperfusion injury, and the central involvement of neutrophils in this process, then one may recognize both a window of opportunity during which drug therapy can be initiated, and an appropriate target. This opportunity to intervene is available to cardiologists at the time of coronary catheterization or entry into the emergency care facility, and to cardiac surgeons using cardioplegia as a vector for drug delivery or for various intravenous drugs in cases of off-pump cardiac surgery. Actually, reperfusion provides a broad gateway to treating the multiple, and often redundant, mechanisms involved in postischemic injury. The neutrophil-mediated inflammatory cascade during reperfusion represents one such important target for therapeutic intervention because of the pivotal role played by the neutrophil in deleterious events following reflow. However, effective therapeutic strategies targeting the cause or propagation of the myocardium's response to ischemic–reperfusion injury, rather than to its symptoms, are predicated on the knowledge of its molecular basis. This article discusses the role of the neutrophil in the inflammatory component of ischemic–reperfusion injury, and potential therapeutic strategies targeting neutrophil-mediated reperfusion injury of the heart. The majority of the discussion focuses on lethal injury following prolonged ischemia, and focuses little on non-lethal injury (i.e. myocardial 'stunning') because the predominant data (but not the entirety) suggest that neutrophils do not participate in the pathogenesis of this manifestation of injury.

2. Mechanisms of neutrophil-mediated injury

Neutrophils have been implicated as a primary mechanism underlying ischemic–reperfusion injury. The propensity to injure the myocardium and its component cells (notably the coronary vascular endothelium, microvasculature, myocytes) stems from the myocardium's primary responses to proinflammatory mediators, which leads to a redirection of the normal inflammatory response geared towards neutralizing host invaders to one that attacks the host tissue. The processes involved in inducing tissue injury by neutrophils include oxygen free radical generation, degranulation and release of proteases, and release of arachidonic metabolites and other proinflammatory mediators.

2.1. Oxygen free-radical generation

Superoxide anions are generated from the neutrophil-membrane-associated NADPH oxidase [1,2], which can be activated by soluble proinflammatory cytokines [*N*-formyl peptides, C5a, platelet activating factor (PAF)] and particulate stimuli. Adherence of neutrophils to biological surfaces, and circulating inflammatory mediators such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) prime the cells and greatly increase their response in vitro. Neutrophils stimulated by proinflammatory mediators produce superoxide anions, hydrogen peroxide and, ultimately, hydroxyl radicals in a respiratory burst characterized by a high metabolic activity and consumption of oxygen. Hydrogen peroxide is formed from dismutation of superoxide anions after the release of myeloperoxidase from azurophilic granules. A sensitive target of oxygen free-radical injury is the vascular endothelium. Oxygen free radicals promote the release of proinflammatory mediators from endothelial cells and other sources, which leads to the expression of adhesion molecules on endothelium [3]. Endothelial damage mediated by oxyradicals results in increased permeability [4–6], increased adherence of neutrophils [7], attenuated release of endothelium-derived factors with anti-neutrophil properties, such as nitric oxide and adenosine, and overexpression of endothelium-derived pro-inflammatory factors. In addition, oxygen free radicals oxidize low density lipoprotein (LDL) to pro-atherogenic products, which may represent a link between oxyradical generation by neutrophils and heart disease. Furthermore, superoxide anions derived from neutrophils may be a substrate in the formation of peroxynitrite, derived from the biradical reaction between neutrophil-derived superoxide anion and nitric oxide [8–13]. However, hypochlorous acid is the predominant cytotoxic molecule derived from neutrophils. Its cytotoxicity derives from production of powerfully oxidizing chloramines. In addition to directly injuring tissue, oxygen free radicals may provide a leukotactic signal by (a) stimulating the generation of complement, (b) inducing expression of

P-selectin on endothelium and (c) inducing surface expression of PAF on endothelium.

2.2. Degranulation products

Neutrophils degranulate to release proteases, collagenases, lipoxygenases, phospholipases, and myeloperoxidase (Table 1). The serine protease, elastase, is a major contributor to neutrophil-mediated damage, due partially to the effect of its highly cationic nature on membrane charge distribution. Elastase also hydrolyzes the extracellular matrix components elastin, fibronectin and collagen types III and IV.

2.3. Arachidonic acid metabolites and platelet activating factor

Neutrophil activation stimulates phospholipase A_2 and generates leukotriene B_4 (LTB_4) and PAF. LTB_4 and PAF are potent stimulants of neutrophil chemotaxis, degranulation and adhesion to endothelial cells, which may thereby amplify neutrophil recruitment and neutrophil-mediated injury [14]. PAF also stimulates platelets, which can then synergize with neutrophils to amplify injury [15,16]. Cytokine-induced neutrophil activation increases not only adherence properties [14] but also cytoskeletal rigidity, which, in turn, prevents conformation of shape to capillary dimensions. Hence, activated neutrophils embolize in precapillary vessels, thereby contributing to microvascular resistance and 'no-reflow' [17]. Other causes of embolization include homotypic aggregation of platelets and neutrophils, and endothelial cell swelling.

3. Neutrophils and endothelial dysfunction from reperfusion injury

Endothelial dysfunction plays a critical role in the pathogenesis of reperfusion injury in the myocardium [18–21]. This role stems from the close proximity of the endothelium to neutrophils and other inflammatory cell types at the vascular interface during the critical early phase as well as the later phase of reperfusion. The interaction between neutrophils and endothelial cells is mediated by a well orchestrated sequence of interactions between adhesion molecules on both the endothelium and neutrophils. These adhesion molecules are categorized into three families: (a) selectins, (b) β_2-integrins and (c) the immunoglobulin superfamily. The selectins (P-selectin, L-selectin, E-selectin) are glycoproteins involved in the interactions between neutrophils and the endothelium early in reperfusion. P-selectin is not constitutively expressed on

Table 1
Neutrophil-derived products involved in inflammatory responses to ischemia–reperfusion

Product	Source	Physiological effect
Oxidants		
Superoxide anion	Neutrophil (PMN) membrane NADPH oxidase	Endothelial dysfunction, adhesion molecule expression, tissue edema, vasoconstriction, neutralization of NO, Ca^{2+} dyshomeostasis, contractile dysfunction
Hydrogen peroxide	Dismutation of $^{-}O_2^{\cdot}$, conversion to HOCl	Lipid peroxidation trigger; P-selectin expression
Hypochlorous acid	PMN–azurophilic granule, myeloperoxidase; reaction with $H_2O_2 \rightarrow$ singlet oxygen	Chlorination, oxidation; formation of chloramines; predominant cytotoxant
Hydroxyl radical	PMN; Haber-Weiss reaction	Biological membrane damage
Proteinases		
Elastase		Hydrolysis of extracellular matrix proteins (elastin, fibronectin), collagen types III and IV
Collagenase		
Eicosanoids, lipids		
Phospholipase A_2		
Leukotriene B_4		chemotaxis, adhesion, degranulation, $^{-}O_2^{\cdot}$
Platelet activating factor	Thrombin-stimulated endothelium	Generation; actions species-sensitive
Thromboxanes (A_2, B_2)		Vasoconstriction
Adhesion molecules		
L-selectin		Shed after activation
sialyl $Lewis^x$		Counterligand to P-selectin
PSGL-1		Counterligand to P-selectin
β_2-Integrins (CD11a, CD11b, CD11c; CD18)		Constitutively expressed; activated by chemotactic stimulants; CD11b/CD18 binds ICAM-1 and C3bi; initiates firm adherence to endothelium (EC)

the surface of endothelial cells, but is stored in Weibel-Palade bodies. P-selectin expression on the surface of endothelial cells can be induced by proinflammatory mediators such as oxygen radicals [22], thrombin [23], complement components, histamine and hydrogen peroxide. After ischemia, P-selectin surface expression peaks after 10–20 min of reperfusion, and is subsequently shed to soluble fragments in blood [24–26]. Weyrich et al. [26] demonstrated that P-selectin was maximally expressed in feline arterioles and venules after 90 min of ischemia and 20 min of reperfusion. Longer periods of reperfusion are associated with a gradual decrease in the detected levels of P-selectin, representing shedding of the selectin. In contrast to P-selectin, L-selectin is constitutively expressed on the surface of neutrophils, and may be the counterligand for P-selectin during early reperfusion [27]. Recently, a high affinity glycoprotein ligand for P-selectin, termed P-selectin glycoprotein ligand-1, PSGL-1 [28], has been identified, which may mediate, in part, neutrophil rolling on purified P-selectin [28] and on intact endothelium. The third member of the selectin family, E-selectin, is expressed on the surface of endothelial cells. It is expressed later in reperfusion (4–6 h) and may therefore be involved in later reperfusion events (discussed below).

The β_2-integrins (CD11/CD18 complex) are a family of heterodimeric glycoproteins that are constitutively expressed on the surface of neutrophils. There are three distinct α-chains (CD11a, CD11b, CD11c) and a common β subunit. The CD11b/CD18 complex is stored in secondary granules in neutrophils. Activation of neutrophils by a number of proinflammatory mediators, including PAF, involves an increase in surface expression of CD11/CD18 complexes (CD11b/CD18, CD11c/CD18), which is achieved, in part, by rapid translocation from granules to the membrane surface, or by an increase in adhesive avidity to the respective counterligands, which involves a conformational change and conversion from a low affinity state to a high affinity state with exposure of functional epitopes. Increased surface expression and affinity state of perhaps the major complex CD11b/CD18 is triggered after the rolling phase of neutrophils on the endothelium, a step that is a prerequisite for firm adherence mediated by interaction with its counterligand ICAM-1 on the endothelium.

ICAM-1, VCAM-1 and platelet-endothelial cell adhesion molecule-1 (PECAM-1) are members of the immunoglobulin superfamily. ICAM-1 is the counterligand for CD11/CD18 on neutrophils and is constitutively expressed on the surface of vascular endothelial cells. ICAM-1 is upregulated by cytokines 2–4 h after stimulation in vitro or after myocardial ischemia–reperfusion [29,30] and coincides with the upregulation of CD11/CD18. PECAM-1 is expressed constitutively on the surface of platelets, leukocytes and endothelial cells, and is localized to the intercellular junctions of the latter [31,32]. PECAM-1 may be involved in the transendothelial migration of neutrophils [33,34]; monoclonal antibodies to PECAM-1 have been reported to inhibit neutrophil transendothelial migration [33–35], with subsequent reduction of injury (infarction) [36].

Neutrophils are recruited to the reperfused myocardium by chemotactic factors that are released by the myocardium during ischemia [37,38] and begin to interact with the endothelium through a process of 'rolling' (Fig. 1). Rolling along the endothelial surface is mediated by P-selectin on the endothelium and sialylated glycoprotein on the neutrophil, most likely sialyl Lewis^x or the sialomucin P-selectin glycoprotein ligand-1 (PSGL-1) [28,39]. This initial loose adherence is an obligatory step that is necessary for later firm adherence mediated by the CD11/CD18 complex and ICAM-1, leading to transendothelial migration into the myocardial parenchyma and their physiological sequellae (no-reflow, necrosis) [27,40–42]. After initial tethering of neutrophils by endothelial P-selectin, a well orchestrated sequence of neutrophil–endothelial cell interactions evolves, with the endothelial expression of adhesion molecules, such as E-selectin and ICAM-1, and expression of adhesion counterligands on the neutrophils, such as CD11/CD18, which allow firm adherence of neutrophils to the endothelium. Platelet activating factor [43,44] and LTB_4 [44] can increase the surface expression and adhesiveness of CD11/CD18 on neutrophils, while IL-1 [45] and TNF-α [45] increase ICAM-1 expression on the endothelium. Weyrich et al. [26] demonstrated that ICAM-1 levels were increased by ischemia–reperfusion. While levels of ICAM-1 remained at a relatively low level for 120 min of reperfusion, there was a significant rise in expression after 150 and 270 min.

Neutrophil adherence to the coronary endothelium induces functional injury to the endothelium [46–50]. Co-incubation of neutrophils and healthy coronary artery rings with thrombin (or hydrogen peroxide or histamine) in organ chambers causes contraction of the artery, due to neutralization of the vasodilator nitric oxide by superoxide anion, and injury-induced impairment of nitric oxide release (Fig. 2). The degree of vasocontraction is paralleled by the number of neutrophils adherent to the coronary artery endothelium. In addition, the vasocontraction responses could be attenuated by the antibody to P-selectin, PB1.3, and accentuated by the nitric oxide synthase inhibitor, L-nitro-arginine. Fig. 3 shows endothelial dysfunction after incubation of neutrophils with thrombin-stimulated endothelium from coronary arteries. Thrombin upregulates P-selectin and has no direct stimulatory effect on neutrophils. Thrombin treatment in the absence of neutrophils induces no discernable alterations in agonist-stimulated vasorelaxation responses. In contrast, co-incubation of neutrophils with thrombin-stimulated coronary artery endothelium is associated with a significant decrease in the concentration–response vasorelaxation curve, with a characteristic decrease in maximal response to the highest concentration of acetylcholine used. In contrast, vasorela-

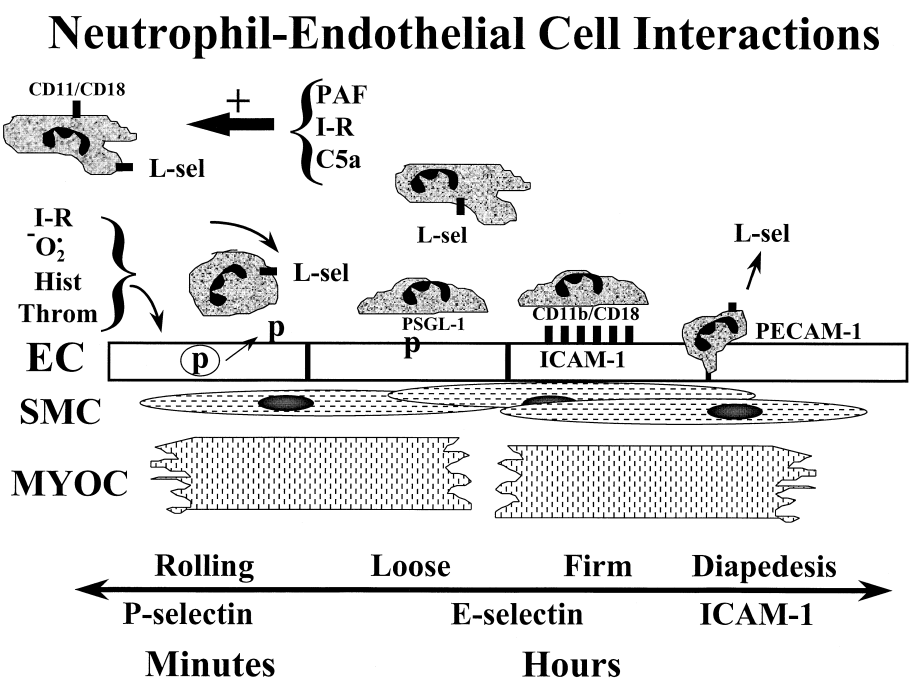

Fig. 1. Diagram of the interactions between neutrophils and endothelium (EC) with reference to the adhesion molecules mediating the interactions over the early and later time course. Neutrophils can be recruited towards the endothelium by proinflammatory mediators such as platelet activating factor (PAF), complement components (C5a) or ischemia–reperfusion (I–R). In addition, the endothelium is activated by proinflammatory mediators such as thrombin (Throm), histamine (Hist) or superoxide anions ($-O_2^{\cdot}$), resulting in surface expression of P-selectin (p). The early response of rolling involves selectin-mediated [P-selectin, L-selectin (L-sel)] loose adherence, while later firm adherence and diapedesis are mediated by ICAM-1 on endothelium and CD11b/CD18 on neutrophils, and by PECAM-1. MYOC=myocytes; SMC=smooth muscle cells.

xation responses to the smooth muscle dilator sodium nitroprusside is unaltered by ischemia or exposure to activated neutrophils. Therefore, neutrophils induce endothelium-specific damage to receptor-dependent and receptor-independent vasodilator function. Other neutrophil activators, such as PAF, can stimulate neutrophils to cause damage to both the endothelium and to vascular smooth muscle, possibly by protease activity.

In vivo ischemia and reperfusion cause injury to the vascular endothelium, expressed as a reduction in basal

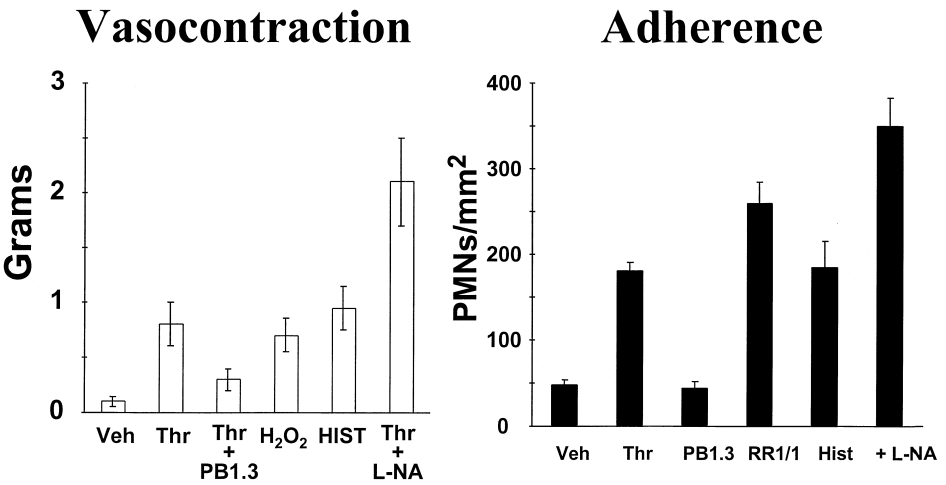

Fig. 2. Left panel: Vasocontraction (grams of tension) induced after incubation of thrombin (Thr)-stimulated coronary artery endothelium with unstimulated neutrophils. Segments were also stimulated with hydrogen peroxide (H_2O_2) and histamine (HIST). Studies with the P-selectin antibody PB1.3 indicate that the adherence process is mediated by P-selectin, and addition of the nitric oxide synthase inhibitor L-nitro-arginine (L-NA) suggests that blockade of basal nitric oxide production increases the vasoconstriction effect of neutrophils by attenuation of nitric oxide release. Right panel: Adherence of fluorescently labeled neutrophils (PMNs) on the endothelial surface of thrombin-stimulated coronary artery segments. Increased adherence after thrombin stimulation was attenuated by the P-selectin antibody PB1.3 but not the anti-ICAM-1 antibody RR1/1. The alternative stimulator, histamine, also increased adherence, while the nitric oxide synthase inhibitor, L-NA, accentuated neutrophil adherence to histamine-stimulated endothelium.

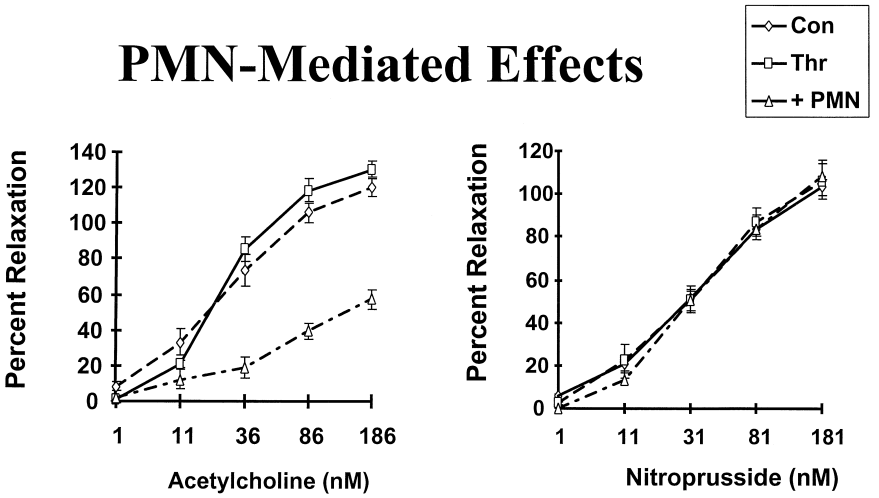

Fig. 3. Effect of preincubation of neutrophils with thrombin (2 U/ml)-stimulated normal canine coronary artery segments on concentration-dependent vasodilator responses to the endothelium-dependent receptor-dependent agonist acetylcholine (left panel) and receptor-independent smooth muscle dilator sodium nitroprusside (right panel). Coronary arteries were preconstricted with the thromboxane A_2 mimetic U46619. Con=control unstimulated vessels; Throm=thrombin-stimulated vessels; +PMN=neutrophil incubation with unstimulated coronary artery endothelium.

and stimulated NO release [48,51–53] and, hence, attenuate the response to agonist stimulators of eNOS [48,54,55], which is, in large part, dependent on neutrophils. In models of transient coronary occlusion (<2 h), endothelial dysfunction is not evident immediately after the ischemic period in the absence of reperfusion. However, endothelial dysfunction is progressively expressed, starting as early as 2.5 min after the start of reperfusion [51], and persists for hours [18,51] to days [56] after reperfusion. Tsao and Lefer [48] investigated the effect of ischemia and reperfusion on endothelial function. Free radical production is dramatically increased during the early moments of reperfusion, which can be attenuated by recombinant human superoxide dismutase (rhSOD), suggesting that free radicals may play a major role in the endothelial dysfunction that occurs early in reperfusion. The early loss of endothelial function, expressed as an impaired release of nitric oxide, is associated with a progressive increase in neutrophil adherence to the endothelial surface of ischemic–reperfused coronary arteries (Fig. 4a and b). Endothelial dysfunction may persist for days following reperfusion [57]. Models of in vivo global ischemia generally agree with endothelial dysfunction being observed only after reperfusion [52,53] unless more prolonged periods of ischemia are imposed [52]. As with regional ischemia–reperfusion, this endothelial dysfunction after global ischemia is expressed as a reduction in basal (i.e. adherence of unstimulated neutrophils to ischemic–reperfused coronary artery endothelium) as well as agonist-stimulated vasorelaxation responses. Endothelial dysfunction is often associated with morphological abnormalities in endothelial structure, including intracellular vacuolization, detachment from the basement membrane with exposed subendothelial matrix, loss of endothelial cell membrane integrity and attachment of neutrophils.

4. Neutrophil accumulation within the area at risk

One of the earliest sequellae of reperfusion is the ‘no-reflow’ phenomenon. In a study of the no-reflow phenomenon in canine hearts, Kloner et al. [58] demonstrated that extended periods of ischemia (90 but not 40 min of ischemia) followed by reperfusion were associated with a lack of blood flow to the subendocardium. Microscopic examination of the myocardium within this ‘no-reflow zone’ revealed severely damaged capillary structure. Engler et al. [59] demonstrated the presence of leukocytes in the vessels within the ‘no-reflow’ zones of the myocardium after ischemia and reperfusion, and an overall accumulation of leukocytes in the area at risk [60]. Following these initial observations, Engler et al. [61] demonstrated that removal of neutrophils from the perfusing blood reduced the extent of no-reflow and concomitantly reduced myocardial edema formation. Subsequently, other groups have also implicated the accumulation of neutrophils as a major cause of the no-reflow component of reperfusion injury as well as other manifestations of reperfusion injury, including ventricular arrhythmias and infarct size [62–64].

Neutrophils are recruited to the reperfused myocardium by chemotactic factors released by the myocardium during ischemia [37,38]. Many of these substances, including TNF-α, IL-8, IL-6, PAF, complement and leukotrienes, will initiate the processes of adherence to the endothelium described above. A link has been established between the accumulation of neutrophils and the development of reperfusion injury during the early reperfusion period. This link has been substantiated by several studies that investigated the time course of neutrophil accumulation and progression of injury. In 1988, Smith et al. [65] subjected rats to 30 min of regional myocardial ischemia followed by reperfusion of up to 96 h. These investigators correlated

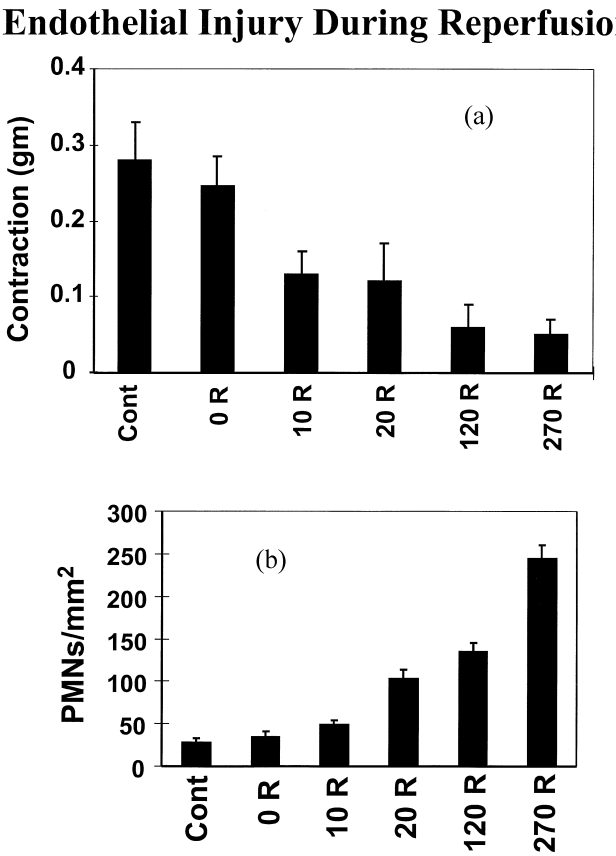

Fig. 4. Progressive effects of reperfusion on endothelial function in feline coronary arteries subjected to coronary artery occlusion and reperfusion. Panel (a): Vasocontraction responses to feline coronary artery rings exposed to nitric oxide synthase inhibitor after 0 to 270 min of reperfusion. The extent of contraction reflects the loss of basally released nitric oxide. Note that the control ring, when exposed to the nitric oxide synthase inhibitor, has the largest contractile response, suggesting a loss of vasodilation induced by basal nitric oxide release. After ischemia and reperfusion, the contractile response is diminished, suggesting the loss of basally released NO. Panel (b): Adherence of fluorescently labeled neutrophils (PMNs) to ischemic and reperfused coronary arteries after 0 to 270 min of reperfusion. Cont=control coronary arteries. R=minutes of reperfusion to which the vessels were exposed after occlusion. (Data from Murohara et al. [93]).

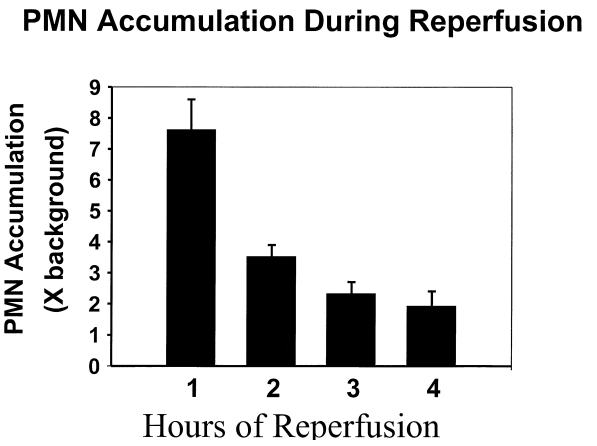

Fig. 5. Rate of neutrophil (PMN) accumulation in the area at risk myocardium after 1–4 h of reperfusion in a canine model of coronary artery occlusion. HR=hours of reperfusion. The rate of neutrophil accumulation is highest in the first hour of reperfusion. (Data from Dreyer et al. [66]).

the degree of morphologic injury (creatine kinase) to neutrophil accumulation assessed by both histology and analysis of myeloperoxidase (an enzyme specific to neutrophil azurophilic granules) activity during ischemia and reperfusion. This study demonstrated that: (a) neutrophils accumulated within the area at risk early in the reperfusion period; (b) their activity peaked during the first 24 h of reperfusion and (c) there was a positive correlation between myeloperoxidase activity and creatine kinase release at 24 h of reperfusion, after which time, the correlation was lost.

Subsequent to the study of Smith et al. [65], Dreyer et al. [38] identified a compound released by ischemic–reperfused canine myocardium into cardiac lymph fluid that was able to activate neutrophils isolated from healthy dogs. This endogenous compound was able to induce morphologic changes in neutrophils, orient neutrophils along a chemotactic gradient, induce expression of β_2 integrin adhesion molecules on neutrophils (i.e. CD11/CD18) and induce adherence of neutrophils to endothelial monolayers. The neutrophil stimulation by the cardiac lymph was found to be most potent when collected at 1 h of reperfusion and decreased to basal levels by 6 h of reperfusion. Dreyer et al. [66] further delineated the early time course of neutrophil accumulation in myocardium by studying the events that occurred in the first 4 h of reperfusion by using radiolabeled neutrophils to track actual accumulation and localization. This study demonstrated that neutrophils accumulated at the fastest rate early in the reperfusion period (Fig. 5), in agreement with the temporal progression of adherence to the coronary endothelium shown above by others [19], with preferential accumulation in the subendocardial region. Neutrophils continued to accumulate over 4 h of reperfusion, but at progressively slower rates, further supporting the theory that neutrophils play an important role in the early events of reperfusion. These early events involve the initial contact between neutrophils and the endothelium in coronary arterioles and venules, and the process of neutrophil rolling [67].

5. Role of neutrophils in late reperfusion injury

Studies have demonstrated that the evolution of infarction is a dynamic process that takes place during the early phase of reperfusion (<6 h) after a brief period of ischemia. Frame et al. [68] found that binding of anti-cardiac myosin antibody, an indicator of progressive myocyte membrane disruption characteristic of necrosis, was increased after 45 min of reperfusion compared to that

after 60 min of ischemia. Farb et al. [69] also demonstrated that, in a rabbit model of 30 min ischemia followed by 180 min reperfusion, infarct size (delineated by horseradish peroxidase uptake into myocytes) was significantly increased compared with that after ischemia alone. However, Rochitte et al. [70] recently reported a progressive increase in tissue injury and microvascular obstruction during 2, 6 and 48 h of reperfusion. During the first 6 h of reperfusion, neutrophils are mainly localized to the intravascular space, while the majority of neutrophils at later time points are found in the interstitial compartment. This observation is consistent with histopathologic findings that neutrophils are sequestered in the intravascular compartment during early reperfusion (4–6 h) [71]. Early postischemic damage to the vascular endothelium mediated by neutrophils may be initiated by direct contact with the endothelium and, ultimately, cause damage to myocytes (infarction) by subsequent diffusion of cytotoxic inflammatory mediators before direct neutrophil–myocyte contact occurs in 4–6 h. However, later reperfusion injury responsible for infarct extension may involve transendothelial migration of neutrophils and subsequent neutrophil-mediated myocyte injury. Therefore, the neutrophil-mediated response to ischemia and reperfusion leading to vascular and myocyte damage may extend beyond the short term (4–6 h) of reperfusion.

6. Anti-neutrophil therapy

Neutrophils feature so prominently in the etiology of ischemia–reperfusion injury in lethal models of myocardial ischemic–reperfusion injury that therapy directed towards the processes of neutrophil activation, adherence to endothelium, emigration into the parenchyma and the release of cytotoxic products are all candidate targets towards which pharmacological therapy could be directed. The proximal-most processes of activation and adherence may be more efficient targets for therapy compared to more distal processes, or towards the symptoms of injury; the latter approach does not address the mechanisms by which the symptoms are presented. The attenuation of neutrophil-mediated injury manifested as infarction, vascular dysfunction and blood-flow defects and, in some cases, contractile dysfunction have been used as indirect evidence of the key role played by neutrophils in ischemic–reperfusion injury.

Therapy directed against neutrophil-mediated components of ischemic–reperfusion injury include (a) neutrophil depletion, (b) direct inhibitors of neutrophil activation, (c) neutrophil-specific anti-adhesion therapy with antibodies and (d) endothelium-specific anti-adhesion therapy. The ability of any of these interventions to attenuate neutrophil-mediated damage is dependent on the role that neutrophils play in the pathophysiology of the end-point of observation (infarction, contractile dysfunction, vascular dysfunction), and the severity of the injury and, hence, the ability to 'rescue' tissue by any intervention. For example, neutrophils may not play a major role in contractile dysfunction following brief non-lethal ischemia ('stunning') [72–74]. Brief non-lethal ischemia may be viewed as primarily an oxidant-mediated injury, not an inflammatory injury [75] and, regardless of its etiology, there may be insufficient stimulation of activating chemotactic factors, local production of complement and cellular adhesion molecules necessary to recruit neutrophils into the ischemic–reperfused myocardium. Furthermore, the degree of endothelial injury may be insufficient to promote neutrophil adherence and amplify neutrophil recruitment [76]. Alternatively, the actions of neutrophils may not alter the physiology of the endpoint of interest, i.e., contractile function. However, the contractile dysfunction following brief global ischemia in which infarction may not be evident, but in which increased creatine kinase activity suggests some degree of structural damage, has been observed to be neutrophil-dependent [77,78]. In other models of lethal injury, the role of neutrophils in the pathogenesis of endothelial dysfunction and infarction is well documented. Therefore, the model and endpoints must be taken into careful consideration when designing and interpreting experiments testing anti-neutrophil therapy to be sure that neutrophils affect the physiology of the endpoint of interest.

6.1. Neutrophil depletion

Neutrophil depletion can be achieved by several methods, including chemotherapy to induce systemic neutropenia [72,79], administration of anti-neutrophil antiserum (antibodies) or by passing systemic or regional (i.e. coronary) blood supply through neutrophil-clearing filters [62,72,80,81]. Neutrophil filters have also been used to filter blood or blood cardioplegia during cardiac surgery [82,83]. The reported benefits of local or systemic neutropenia in models of severe ischemia include reduction of postischemic arrhythmias [81], an attenuation of post-ischemic 'no-reflow' in the myocardial area at risk [61,63], a decrease in postischemic microvascular permeability [84], reduction in infarct size with neutrophil depletion before reperfusion [62,80] or at the time of reperfusion [63]. However, with shorter periods of ischemia, no reduction in postischemic blood flow defects has been reported because neutrophil adherence and plugging may not be triggered by modest ischemic periods [74]. Although neutrophil-specific filters are relatively efficient at removing neutrophils, they do effect other cell types, such as platelets, and they activate complement or release other vasoactive substances (adenosine) that may modulate neutrophil actions by mechanisms other than direct removal.

6.2. Anti-adhesion molecule therapy

Interference with the neutrophils themselves or with the initial adherence to coronary vascular endothelial cells is

an effective therapeutic target since this step is a prerequisite for neutrophil-mediated damage. Early work by Romson et al. [62] demonstrated a reduction in infarct size and neutrophil accumulation by administration of an 'anti-neutrophil antibody'. Interference at the earliest step of 'rolling', by blocking either P-selectin on the endothelium or L-selectin on the neutrophil with monoclonal antibodies, has been reported to result in cardioprotection. Blockade of P-selectin with monoclonal antibodies such as PB1.3 reduces infarct size and associated neutrophil accumulation in the area at risk and attenuates endothelial dysfunction [40,85,86]. Blockade of L-selectin on neutrophils with DREG-200 [87,88] gave a similar profile of cardioprotection to myocardial and vascular endothelium as P-selectin blockade. Intervention with the neutrophil adhesion molecule CD18 using antibodies such as MAb R15.7 [89,90] was reported to reduce neutrophil–endothelial cell interactions and neutrophil accumulation [66,89] associated with ischemia–reperfusion. In the study by Ma et al. [89], MAb R15.7 reduced myocardial infarct size, attenuated neutrophil accumulation within the area at risk and attenuated endothelial cell dysfunction. Interference with ICAM-1, the counterligand to CD18, using monoclonal antibodies (RR1/1), has also been reported to reduce infarct size [91,92], neutrophil accumulation and to attenuate endothelial dysfunction or postischemic blood-flow defects [92]. Other studies have used antibodies against CD11, CD18, the CD11/CD18 complex or L-selectin on the neutrophil to decrease superoxide-induced adhesion of neutrophils to endothelial cells, [22,93] reduce adherence to myocytes [94] and to reduce ischemia–reperfusion injury [90,95]. Therefore, direct interference with the early and prerequisite interactions between neutrophils and endothelium has been effective in reducing postischemic injury. In addition to suggesting a therapeutic treatment, these observations further substantiate the role of neutrophils in mediating the distal events of infarction and blood-flow defects that are characteristic of severe postischemic injury.

Antibody therapy has several potential limitations in its clinical application. First, the limited half-life of antibodies in the systemic circulation may address only the early phase of reperfusion and not the later phase (>4–6 h) of reperfusion. Hence, antibody therapy may not sustain long-term effects, as demonstrated by Gill et al. [96] for an antibody to sialyl-Lewis^x, which had shown short-term reduction of infarct size [97,98]. Drugs with similar short half-lives that are not administered continuously over several days following reperfusion may suffer this limitation, unless attenuation of the early phase prevents triggering or amplification of subsequent secondary processes. Second, systemic antibodies may be generated to the therapeutic antibody, thereby forming an antigen–antibody complex. Humanized peptide receptor antagonists for various proinflammatory mediators and adhesion molecules, as well as non-active mimetics of adhesion molecules (decoys), may also offer a potential therapeutic strategy.

6.3. Nitric oxide therapy against neutrophil-mediated damage

NO is an autacoid, formed primarily by the vascular endothelium by the conversion of L-arginine and molecular oxygen to citrulline, and is subsequently released into the intravascular compartment, the perivascular compartments and interstitium. The close proximity of newly released nitric oxide to these compartments that are active in the pathogenesis of ischemia–reperfusion injury places NO in a unique position to modulate biochemical reactions and cell–cell interactions that are characteristic of ischemic–reperfusion injury. The broad range of physiological actions relevant to ischemic–reperfusion injury and other cardiovascular disease states is shown in Fig. 6. Nitric oxide has direct effects on both the neutrophil and the endothelium. In addition, NO attenuates activation of mast cells and platelets [99,100] with which the neutrophils synergize during ischemia–reperfusion [15]. Platelets potentiate the activation and interaction of neutrophils with the endothelium by releasing thromboxane A_2 (pro-adhesion, pro-diapedesis), platelet derived growth factor (chemoattractant, chemotaxis), platelet factor 4 (chemotactic), serotonin (pro-adherence), adenosine (chemotactic at low concentrations, inhibitory at higher concentrations [101]) and IL-8 [16]. The direct effects of NO on the neutrophil include inhibition of superoxide anion production, degranulation and adherence to coronary artery endothelium [49,102]. Inhibition of superoxide generation by neutrophils may involve a direct inhibition of membrane-bound NADPH oxidase activity [103]. In addition, nitric oxide neutralizes superoxide anions in a very rapid and essentially irreversible biradical reaction to form peroxynitrite [10–13].

In addition to direct inhibitory effects on neutrophils, nitric oxide also has direct inhibitory effects on the vascular endothelium, which subsequently attenuates its interaction with neutrophils. Nitric oxide attenuates the upregulation of P-selectin, E-selectin and ICAM-1 [43,104], adhesion molecules involved in the loose and firm adherence of neutrophils to endothelium. The attenuation of P-selectin expression involves, in part, the inhibition of P-selectin messenger RNA and synthesis of the P-selectin glycoprotein [105], which may imply that nitric oxide attenuates not only the immediate surface expression of adhesion molecules but also their longer-term expression following ischemia and reperfusion. Through inhibition of superoxide anion generation and adherence to vascular endothelium, nitric oxide attenuates neutrophil-mediated damage to coronary artery vascular endothelium [49,106–113].

As a result of its potent anti-neutrophil and anti-inflammatory properties, NO has been reported to exert

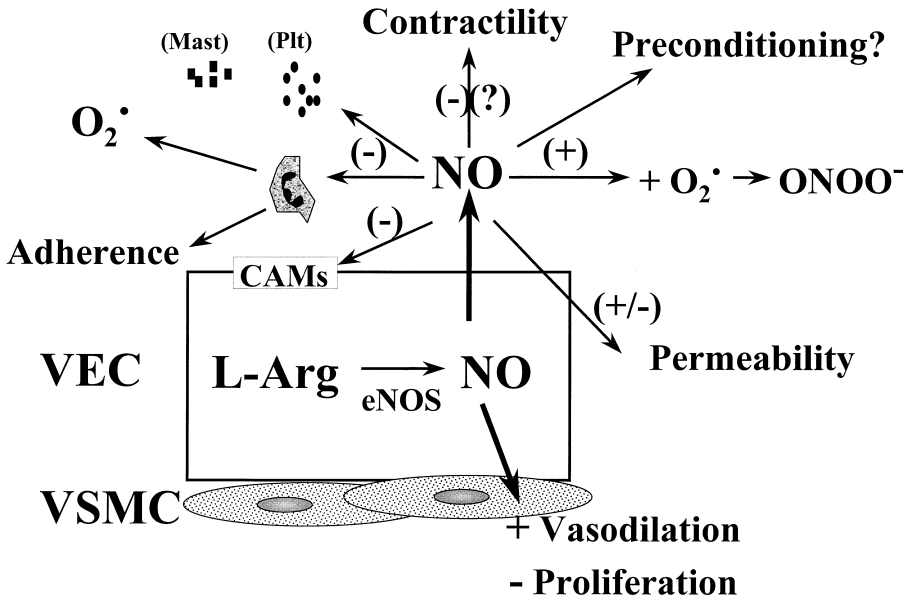

Fig. 6. Schematic diagram showing the physiological effects of nitric oxide (NO) derived from the vascular endothelium (VEC) on cell adhesion molecules (CAMs), neutrophils (not labeled), mast cell and platelet (Plt) aggregation, and on the production of metabolites such as peroxynitrite (ONOO−) and vascular smooth muscle (VSMC) dilation. (+)=stimulatory; (−)=inhibitory; L-arg=L-arginine; eNOS=endothelial-derived nitric oxide.

potent cardioprotection from ischemic–reperfusion injury. Authentic nitric oxide [99,114] or direct nitric-oxide donors [106,108,115–119] have been reported to reduce infarct size and, also, to preserve coronary artery endothelial basal (static neutrophil adherence) and agonist-stimulated (relaxation responses) function. L-Arginine, the precursor of nitric oxide, increases nitric oxide generation and release, and reduces postischemic injury (infarct size, endothelial dysfunction) in a manner similar to that of nitric oxide [55,120–122]. Conversely, inhibition of endothelial nitric oxide synthase using analogs such as L-nitro-arginine increases the extent of injury [123,124]. Intravenous [122] and intracoronary [55] L-arginine, administered at the time of reperfusion, was shown to significantly decrease both postischemic coronary artery endothelial dysfunction and infarct size. The reduced infarct size was associated with a decrease in neutrophil adherence to coronary artery endothelium and neutrophil accumulation in the area at risk. In a study by Nakanishi et al. [55], infusion of 10 mM L-arginine into the left anterior descending (LAD) coronary artery starting at the time of reperfusion resulted in a significant reduction of infarct size (Fig. 7). However, infarct size reduction was not observed with 10 mM D-arginine, the non-metabolized enantiomer of L-arginine. Myeloperoxidase activity, a marker of neutrophil accumulation, increased significantly in the area at risk in the untreated group, and was significantly reduced by intracoronary L-arginine (Fig. 7B). Postischemic endothelial vasorelaxation responses to acetylcholine were significantly increased in the L-arginine treated group compared to both the vehicle group and the D-arginine group (Fig. 7C). Although L-arginine does not directly inhibit superoxide anion generation by activated PMNs, it does inhibit adherence of unactivated PMNs to thrombin-stimulated coronary artery endothelium (Fig. 8). Furthermore, endothelial dysfunction associated with the adherence of PMNs is attenuated by L-arginine. Therefore, the cardioprotective effects of L-arginine may involve, in large part, an attenuation of PMN-mediated actions that would otherwise culminate in vascular injury and infarction.

In contrast to the reported cardioprotective effects of nitric oxide, reports have implicated nitric oxide in promulgating injury because of its actions as a radical, or via the generation of potentially deleterious metabolites such as peroxynitrite ($ONOO^-$) and its intermediary product with hydroxyl radical-like actions (NOOH*) [125–132]. Therefore, a duality of opposing physiological actions is associated with endogenous and exogenous nitric oxide therapy, and the neutrophil as a generator of both nitric oxide and superoxide anion as substrates for peroxynitrite [8,9] may be directly involved in this controversy. A detailed discussion on the physiological effects of peroxynitrite has been presented elsewhere [10,12,133].

6.4. Adenosine, neutrophil function and neutrophil-related cardioprotection

Adenosine is a cardioprotective autacoid that is present in small quantities (less than 1 μM) in the normal myocardium [134–136] and is transiently increased during ischemia by hydrolysis of high-energy phosphates (ATP, ADP, AMP). The physiological tissue levels of adenosine are regulated by the production and release of adenosine

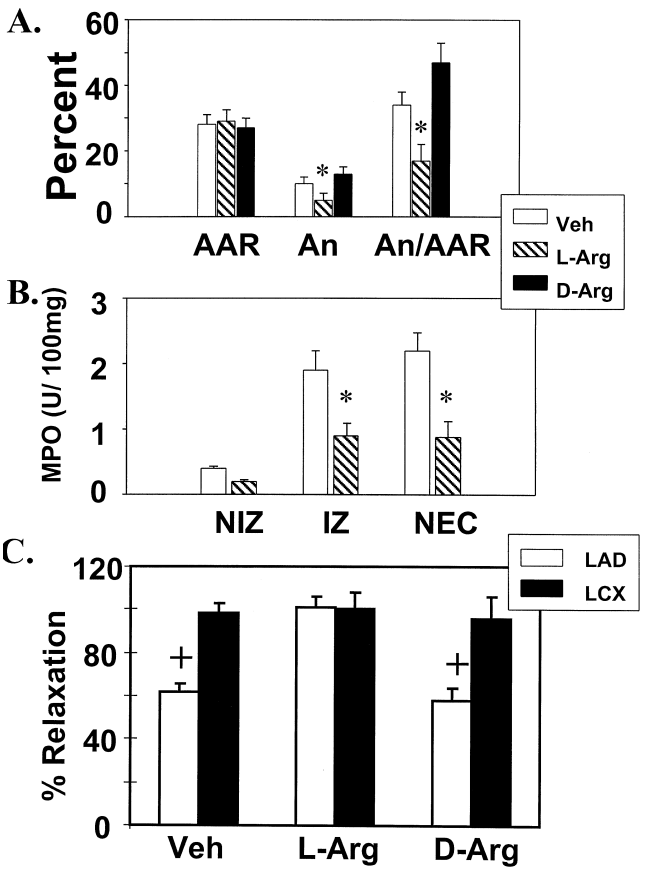

Fig. 7. Myocardial infarct size, neutrophil accumulation and coronary artery endothelial injury in a canine model comprising 60 min of coronary artery occlusion and 270 min of reperfusion. Panel A: Percent area at risk (AAR), area of necrosis (An) and necrosis/risk ratio (An/AAR). Group legend is on the right side between panels A and B. Panel B: Myeloperoxidase (MPO) activity as a marker of neutrophil accumulation in myocardium from the nonischemic zone (NIZ), non-necrotic area at risk (IZ) and non-necrotic area at risk.*$P<0.05$ vs. Veh (saline group) and D-arginine (D-Arg), if applicable. Panel C: Peak endothelial relaxation responses to acetylcholine of ischemic–reperfused left anterior descending (LAD) and nonischemic left circumflex (LCX) coronary arteries. +=$P<0.05$ vs. LCX responses. Results are expressed as the mean±SEM. (Data from Nakanishi et al. [55]).

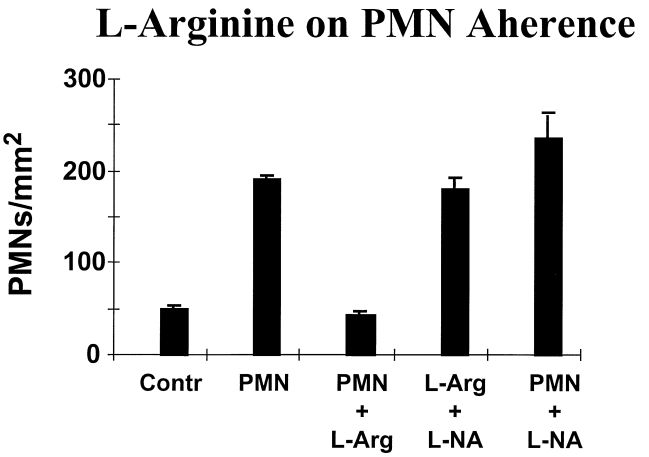

Fig. 8. Adherence of fluorescently labeled canine neutrophils (PMNs) to normal canine coronary artery segments in organ chambers in which the endothelium has been unstimulated (Contr) or stimulated with thrombin (thrombin, 2 U/ml). Thrombin stimulation increased adherence (fluorescent microscopy) of PMNs to the endothelial surface threefold (PMN). L-Arginine (10 mM) decreased adherence to control levels, which was reversed by coincubation with the nitric oxide synthase inhibitor, L-nitro arginine (L-NA; L-Arg+L-NA). L-NA by itself increased PMN adherence through inhibition of endogenous NO. *$P<0.05$ vs. unstimulated control endothelium and L-arginine. Results are expressed as means±SEM. (Data from Sato et al. [49])

by cardiac myocytes, the endothelium, neutrophils and other cell types. Adenosine interacts with specific purinergic receptors (Table 2) on the endothelium, myocytes or neutrophils to elicit a wide range of physiological responses that are not unlike those of NO. Therefore, adenosine can exert a broad spectrum of effects on key components (neutrophils, endothelium) and compartments (intravascular, interstitial, myocyte) involved in ischemia and reperfusion injury. The target of these receptor-mediated interactions has implications as to the time course of administration of therapeutics.

Cronstein et al. [137] reported that adenosine inhibited superoxide generation by neutrophils activated by a number of physiological stimuli, including fMLP, A23187 and concanavalin A. Later studies determined that this inhibitory effect was mediated by the A_2 adenosine receptor [137–141]. Studies from our laboratory confirmed the attenuation of superoxide generation in a concentration-dependent manner by the A_2 receptor mechanism [64,141]. Furthermore, the selective A_{2a} agonist CGS-21680 attenuates superoxide production in a similar manner to adenosine. However, the A_3 adenosine receptor does not seem to regulate neutrophil superoxide-anion generation [142].

Unlike the potent inhibitory effects on superoxide production, adenosine has only modest effects on neutrophil degranulation [137,138,143]. Inhibition of degranulation is probably mediated by activation of the adenosine A_{2a} receptor subtype [138]. The role of the A_3 receptor on degranulation is not clear at present.

Many studies have demonstrated that adenosine attenuates the adherence of neutrophils to endothelial cells [139–141,144]. However, adenosine has opposing effects on neutrophil adherence related to the concentration-dependent stimulation of the A_1 or A_{2a} adenosine receptors. At lower concentrations, adenosine increases the adherence of neutrophils to endothelium [139] by A_1 receptor-mediated effects. However, higher concentrations inhibit neutrophil adherence by A_{2a} receptor mechanisms. This dual action has been confirmed by Felsch et al. [140]. Adenosine attenuates adherence by a down-regulation of β_2-integrin (CD11b/CD18) [145] expression and inhibition of L-selectin shedding human neutrophils mediated through the A_{2a} receptor [146]. Data also suggest that adenosine may inhibit the release of cytokines involved in responses to ischemia and reperfusion (IL-6 and IL-8)

Table 2
Effects of adenosine receptor subtype activation on neutrophil functions

Receptor subtype	Physiologic effect	Timing of effect
A_1	Increased adherence to endothelium	Ischemia
A_{2a}	Decreased adherence to endothelium	Reperfusion
A_{2a}	Decreased superoxide generation	Reperfusion
A_{2a}	Decreased degranulation	Reperfusion
A_3	Decreased adherence to endothelium (?)	Not identified
A_3	Decreased degranulation (modest to none)	Not identified

[147]. In addition, Bullough et al. [148] demonstrated that adenosine also inhibits neutrophil adherence to myocytes.

Reduction of neutrophil–endothelial cell interactions by exogenously applied adenosine or adenosine receptor agonists, as well as endogenously produced adenosine, was first shown by Cronstein et al. [149] and later by Zhao et al. [141] to reduce injury to the vascular endothelium, primarily by A_{2a} receptor mechanisms. The effects of the A_3 receptor on neutrophil adherence are largely unknown. A preliminary report by Jordan et al. [142] demonstrated a reduction in neutrophil adherence to coronary vascular endothelium by A_3 receptor activation [142]. Therefore, adenosine may play an important role in modulating local inflammatory responses by its ability to either upregulate or suppress the actions of neutrophils at the site of injury. At concentrations achieved during ischemia or pharmacologically, the inhibitory responses predominate, resulting in suppression of superoxide anion generation, degranulation and neutrophil adherence.

The role of adenosine in modulating reperfusion injury in vivo has been extensively investigated, based largely on the purine's potent anti-neutrophil properties. Olaffson et al. [150] first demonstrated a reduction in infarct size and improvement in regional function at 24 h reperfusion when adenosine was administered at the onset of reperfusion. Microscopic analysis demonstrated a significant reduction in neutrophil accumulation and preservation of endothelial morphology in the ischemic–reperfused myocardium in the group given adenosine at reperfusion. Subsequent studies have shown the beneficial effects of adenosine when given at the onset of reperfusion, including reduction of infarct size [151,152], preservation of post-ischemic coronary flow reserve [153] and blood flow [151], post-ischemic regional contractile performance [151,153], and reduction in neutrophil accumulation in the area at risk [153]. The in vivo reduction in reperfusion injury has been attributed primarily to A_{2a} receptor-mediated processes [64,154,155].

In summary, adenosine has a broad spectrum of physiological effects that make it suitable as a cardioprotective agent with effectiveness in all three windows of opportunity (pretreatment, during ischemia and reperfusion) and against numerous targets, including the neutrophil. The duration of the physiological actions seem to extend well beyond its plasma half-life. In addition, adenosine reduces reperfusion injury by inhibiting the neutrophil and the endothelium directly, and their interactions with each other, largely by A_{2a}-receptor mechanisms. Although the studies of Olafsson et al. [150] suggest that a single treatment with adenosine results in sustained (days) reduction of infarction, studies are needed to investigate the effects of adenosine treatment on endothelial function, neutrophil migration and accumulation, and attenuation of later phase reperfusion injury events related to neutrophils and other inflammatory mediators.

6.5. Anti-complement therapy against neutrophil-mediated damage

The complement cascade, particularly the alternative pathway, is activated during myocardial ischemia–reperfusion and is a major contributor to the pathologic sequellae of cardiopulmonary bypass [156–161]. Complement fragments, such as the anaphylatoxins C3a and C5a, are generated and released both locally [162] and systemically [163,164] and the membrane attack complex is deposited on cell membranes [165]. Ivey et al. [166] demonstrated that the release of the complement fragment C5a is associated with reperfusion, and C5a generation was associated with increased neutrophil accumulation in the area at risk. In ischemia–reperfusion, complement induces injury directly, independent of neutrophils, and acts as chemoattractant and activator of neutrophils and other inflammatory cell types. Direct injury may be induced by C5a and via assembly of the membrane attack complex, thereby increasing cell permeability and cellular edema, and increasing the release of histamine and PAF.

Tissue damage mediated by neutrophils can be initiated by complement fragments, notably C5a, which are potent stimulators of neutrophil superoxide production, and adherence to coronary artery endothelium [167]. Complement increases the expression of CD18 on the neutrophil [168,169] and increases P-selectin expression on the surface of the endothelium [170]. The chemoattractant properties of C5a cause neutrophil accumulation in vascular beds [171] and induce neutrophil-mediated reperfusion injury. Shandelya et al. [172] showed that C5a or plasma factors, most likely C5a, were necessary to induce neutrophil-mediated postischemic contractile dysfunction. Inhibition of the complement cascade by inhibiting C1-esterase activity during reperfusion (responsible for cleavage of C1 into C1r and C1s chains, and initiation of the complement cascade by C1s) has been shown by Buerke et al. [173] to

reduce infarct size and attenuate neutrophil accumulation in the area at risk, with associated improvement in coronary endothelial function. Amsterdam et al. [174] reported that preischemic infusion of a monoclonal antibody against C5a to reduce bio-availability of this fragment reduced infarct size in a porcine model of LAD occlusion and reperfusion. However, the C5a antibody did not reduce the accumulation of neutrophils in the area at risk, although it reduced C5a-stimulated neutrophil aggregation, degranulation and superoxide anion formation in vitro. In a surgical model of reperfusion in which regional ischemia and cardiopulmonary bypass act as dual triggers for complement activation, Riley et al. [167] used a C5a receptor antagonist to reduce infarct size. Concomitant with decreased infarction, there was a significant decrease in neutrophil accumulation, as assessed by myeloperoxidase activity and improved postischemic (postcardioplegic) regional contractile function in the area at risk (similar to the improved function observed by Amsterdam et al. [174]). In a similar model, Tofukuji et al. [175] demonstrated preservation of postcardioplegic endothelial function when an anti-C5a antibody was given prior to the onset of cardiopulmonary bypass.

Complement receptor type 1 (CR-1) is an endogenous membrane-bound (red blood cells and leukocytes) glycoprotein regulator of complement activation (both alternative and classical pathways) through dissociation of the C3 and C5 convertases. A reduction in ischemic–reperfusion injury has been reported with the peptide-soluble complement receptor-1 (sCR-1). sCR-1 is able to inhibit both the classical and alternative pathways of the complement cascade. sCR-1 was reported to block complement-mediated free-radical generation by neutrophils [172]. Weisman et al. [176] reported a significant reduction in infarct size with sCR-1 in the rat model, which was associated with a reduction in both neutrophil accumulation and deposition of the membrane attack complex in the area at risk. Similar reports have confirmed this cardioprotection with sCR-1 [172,177,178].

Recently, heparin and heparin derivatives have shown benefit in reducing myocardial ischemia–reperfusion injury. Heparin is a glycosaminoglycan produced by mast cells and basophils, and is found as a major proteoglycan component on the glycocalyx of vascular endothelium. In addition to its potent anticoagulant activity, heparin inhibits the complement cascade at several sites, notably at the level of C3 convertase, resulting in attenuation of complement activation and leukocyte-mediated effects. Heparin inhibits neutrophil adhesion to coronary vascular endothelium, superoxide generation and chemotaxis, but, interestingly, it does not attenuate neutrophil degranulation. Black et al. [179], using heparin or *N*-acetyl heparin administered just before reperfusion, reported a significant reduction in infarct size. Gralinski et al. [180], using the low molecular weight polysulfated heparin derivative LU 51198, and Friedrichs et al. [181], using *N*-acetyl heparin in a model of complement-mediated (human plasma) contractile dysfunction in an isolated-perfused rabbit heart preparation, observed a reduction of postischemic contractile dysfunction. Kouretas et al. [182] have suggested that the cardioprotective effects manifested as preserved postischemic contractile function involve nitric oxide, perhaps through attenuation of coronary artery endothelial dysfunction effecting nitric oxide release.

7. Other anti-inflammatory therapy

In addition to these newer therapeutic approaches to reduce reperfusion injury, conventional anti-inflammatory treatments may also have potential benefit. Steroids have been used to suppress inflammation for decades and there is substantial evidence that they may reduce some of the mechanisms of injury induced by ischemia and reperfusion. Suzuki et al. [183] demonstrated that hydrocortisone decreases the level of histamine-induced leukocyte adherence. Accordingly, leukocyte adherence induced by adrenalectomy was reduced with hydrocortisone therapy, suggesting a relation between steroid levels and leukocyte adhesion. Node et al. [184] reduced infarct size and postischemic arrhythmias in dogs treated with 17β-estradiol. This protection appeared to be related to the production of nitric oxide and/or the activation of a calcium-dependent potassium channel. Other classic anti-inflammatory compounds, such as aspirin and ibuprofen, have equivocal actions on reducing postischemic damage. Seemingly equal numbers of studies have shown either a benefit [185–188] or no effect [189,190] of these and other anti-inflammatory agents. However, it is difficult to determine the true potential of these agents because of the wide range of endpoints used to determine their effectiveness, including infarct size, ischemic and reperfusion arrhythmias.

8. Summary remarks

Neutrophils play an active role in myocardial ischemia–reperfusion injury. Interactions between neutrophils and the coronary vascular endothelium, mediated by adhesion molecules on both cell types, are critical initial steps in the initiation of the inflammatory-like response. Therapeutics directed at specific stages in this inflammatory cascade are effective in truncating the response, the benefit being a reduction in endothelial cell dysfunction and microvascular blood flow defects, myocyte injury culminating in infarction and, in some cases, contractile dysfunction. Since the activation, propagation and amplification reactions of the neutrophil-mediated inflammatory response to ischemia and reperfusion are in a cascading sequence and are exquisitely redundant, the more proximal the point in these molecular interactions (i.e. neutrophil–endothelial cell

interactions) at which therapeutics can intervene, the less likely that the point of interdiction will be circumvented. Hence, agents that interfere with the early P-selectin-mediated phase of 'rolling' effectively truncate the subsequent sequential steps as well as amplification steps in the early phase of reperfusion. In addition, agents with broad spectrum actions, like adenosine and nitric oxide (or organic NO-donor agents), that interdict at several points in neutrophil responses (such as superoxide generation, release of inflammatory mediators, expression of adhesion molecules) will likely circumvent the redundant inflammatory pathways and provide effective cardioprotection from ischemia–reperfusion injury. A greater understanding of the basic science underlying neutrophil-mediated responses to ischemia–reperfusion injury will be key in the development of new strategies and therapeutics to attenuate the consequences of coronary artery disease, angioplasty and cardiac surgery, and to intervene in disease states (diabetes, hypertension, hyperlipidemia) that intensify the neutrophil-mediated responses to ischemia–reperfusion.

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