Review

The cardiovascular effects and implications of peroxynitrite

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Abstract

Nitric oxide is an endogenous autacoid produced primarily by the vascular endothelium. Under basal conditions, nitric oxide undergoes a rapid biradical reaction with superoxide anions to form peroxynitrite. This reaction, and hence the formation of peroxynitrite is augmented in inflammatory-like conditions such as ischemia-reperfusion injury when both substrates are present in high concentrations. Peroxynitrite has been implicated as a physiologically active toxic metabolite of nitric oxide leading to vascular and myocardial dysfunction. Recent evidence, however, has suggested that peroxynitrite may actually have beneficial properties under in vivo biological conditions when thiol-containing agents (glutathione, albumin, cysteine) agents are available to convert the peroxynitrite anion to nitrosothiols and related products demonstrating antineutrophil and cardioprotective properties. The dichotomy of physiologically relevant properties of peroxynitrite has important clinical applications with respect to nitric oxide therapy for cardiac, vascular, cerebral and pulmonary disease states. This review summarizes the biological properties of peroxynitrite relevant to the cardiovascular system.

Keywords: Heart; Endothelium; Pathophysiology; Nitric oxide; Cardiovascular surgery; Endothelial function; Ischemia; Reperfusion

1. Introduction

In 1990, Beckman et al. described the generation of the potentially toxic nitric oxide metabolite, peroxynitrite anion, formed from the near diffusion limited biradical reaction between nitric oxide and superoxide anion [1]. This led to an explosion of research into both the chemical and physiologic actions of peroxynitrite, and the relationship of peroxynitrite to the well documented biological effects of both nitric oxide and superoxide anion [2–5]. With the increased interest in using nitric oxide therapy to treat a number of human diseases, the understanding of the physiological properties unique to peroxynitrite as an important nitric oxide metabolite is of crucial importance. Nitric oxide, also called nitrogen monoxide [6], is a free radical gas formed by the five electron oxidation of the guanidino moiety of l-arginine to the products nitric oxide and citrulline by the monoxygenase enzyme, nitric oxide synthase. The vascular endothelium is of primary impor-

tance in the synthesis of nitric oxide and regulation of nitric oxide synthase activity, although myocytes and neutrophils also produce nitric oxide. These same cell types also generate superoxide anions when stimulated by cytokines, ischemia–reperfusion, hypoxia–reoxygenation, and under disease conditions such as angiotensin-dependent hypertension. When generated by the same cell, or by closely interacting cells, nitric oxide and superoxide anion are in close proximity to each other, leading to the possible formation of peroxynitrite, especially under conditions favoring superoxide anion “bursts” (ischemia–reperfusion, inflammation, hypercholesterolemia, and angiotensin-induced hypertension) [7,8]. Nitric oxide is the only currently known biological molecule produced in high enough concentrations to react fast enough with superoxide (forming peroxynitrite) to outcompete endogenous superoxide dismutase [9,10]. The formation of peroxynitrite may be a double edged sword. On the one hand, nitric oxide neutralizes a potentially deleterious species of oxygen radical, the superoxide radical. On the other hand, the reaction consumes the
potentially cardioprotective molecule, nitric oxide, and produces a potentially deleterious metabolite, peroxynitrite [11–14]. It is not surprising, therefore, that peroxynitrite itself has been implicated as both a toxic compound causing tissue injury and a protective agent improving cellular or organ function. These contrasting physiological effects (deleterious versus beneficial) may be due to the environmental availability of “detoxifying” agents which can serve as catalysts, intermediates, and precursors for formation of nitrosylated products or nitric oxide. The detoxification reactions, in fact, occur simultaneously with tyrosine nitration of biological proteins, causing abnormalities in enzyme function and other protein-dependent mechanisms. Hence, the balance of these opposing and competing reactions can be shifted toward injury or protection, depending on the availability of these substrates in the biologic environment (Fig. 1) [15]. The result is a complex series of reactions between peroxynitrite and vascular endothelium, vascular smooth muscle, and myocytes, which initiates a wide spectrum of physiologic effects, both beneficial and injurious (Fig. 2). The objective of this review is to summarize the information available pertaining to the cardiovascular effects of peroxynitrite formation, its physiologic effects, and the subsequent clinical implications it may have.

2. Physiologically relevant biochemical properties of peroxynitrite

Peroxynitrite is formed from the biradical reaction of nitric oxide and superoxide at the rapid rate of 5–6.7·10^9 M^−1 s^−1 [3,6]. Peroxynitrite itself is not a free radical because of the shared nature of the two unpaired electrons on superoxide and nitric oxide which form a new bond [9,10,16,17]. Peroxynitrite is capable of hydroxylating and nitrating aromatic compounds, and inducing cellular injury by (a) lipid peroxidation [18], (b) DNA fragmentation similar to that of apoptosis [19,20], (c) damage to proteins and plasma lipids [15], (d) depletion of important plasma antioxidants such as glutathione and cysteine, and (e) nitration of proteins leading to cellular and organ dysfunction [2,3]. The mechanisms by which peroxynitrite carries out these potentially deleterious actions are generally thought to proceed in part via a highly reactive intermediate, ONOO−, which can nitrate and hydroxylate phenolic compounds, most importantly tyrosine residues and alter the activity of key proteins and enzymes [21]. The nature of this reactive intermediate is under current investigation, but it potentially exists as a relatively long-lived caged radical which can recombine to form nitrate [22]. A detailed description of the chemistry of the biologic intermediates of peroxynitrite is beyond the scope of this physiologic review, but can be found in numerous other sources [6,22–24].

In addition to the reactive intermediate ONOO−, peroxynitrite itself reacts with numerous biological compounds in vivo, the products of which may determine its ultimate physiologic effects. The most rapid reaction in vivo appears to be with carbon dioxide [22]. In this reaction, carbon dioxide acts as a catalyst in a series of reactions involving the free radicals CO2 and NO2 to produce a nitronium ion (NO2^+) which can then facilitate nitration of proteins and alter tissue and organ function [22]. The absence of carbon dioxide may explain the relatively poor ability of authentic peroxynitrite to nitrate...
proteins in vitro. However, in the presence of carbon dioxide, this nitration reaction takes place at a much more rapid rate. Other compounds such as hemoglobin, nitric oxide, glutathione, albumin, and cysteine are also capable of reacting with peroxynitrite [25]. The reactions with thiol-containing substances form products such as nitrosothiols which can be utilized by tissues in a beneficial manner (i.e. vasodilatation to improve blood flow). There has been a large amount of debate as to the physiologic feasibility for these reactions to occur in vivo given the more rapid reaction of peroxynitrite with other known biologic compounds, particularly carbon dioxide (Table 1). Although reactions with hemoglobin and thiol compounds appear to occur at a slower rate than the reaction of peroxynitrite with carbon dioxide in vitro, numerous investigators have demonstrated an active role for these compounds in detoxifying authentic peroxynitrite or a reactionary intermediate of peroxynitrite metabolism [26–32] (see Section 3). The inconsistency between chemical properties of peroxynitrite determined in in vitro studies and the in vivo physiological effects is still not resolved, and is under active investigation.

Production of peroxynitrite can be substantially accelerated by increasing the substrates nitric oxide or superoxide anion. Ischemia–reperfusion, with subsequent activation of neutrophils and increased neutrophil–endothelial cell interaction, is associated with an initial burst of superoxide radical production which is sustained at lower levels during the later phases of reperfusion [33,34]. The superoxide anion is generated not only by activated neutrophils, but also by the vascular endothelium via xanthine oxidase and NAD(P)H oxidase activity, and by the mitochondrial respiratory chain [35–43]. The release of nitric oxide by the endothelium and neutrophils is increased by stimulation of both endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) activity secondary to the reintroduction of molecular oxygen and accumulation of intracellular calcium. The release of nitric oxide at this time coincides with the release of superoxide anion during early reperfusion [44]. Hence, the substrates for peroxynitrite production are placed in close proximity in the microenvironment created by the neutrophils adherent to the endothelial cells, with the potential for activating high local concentrations necessary to induce tissue injury.

### 3. Detoxification pathways for peroxynitrite

The reaction between peroxynitrite and thiol-containing biological substance has physiological implications because these reactions lead to the formation of nitrosothiol components which potentially “detoxify” the anion without forming other noxious substances. These thiol-related reaction products may have physiologic actions relevant to the attenuation of inflammation and ischemia–reperfusion injury. The primary physiological participants in peroxynitrite detoxification include glutathione, albumin, cysteine, and hemoglobin moieties. The resulting thiol intermediates may subsequently regenerate nitric oxide; both nitrosothiols and nitric oxide may exert physiological effects consistent with nitric oxide-mediated stimulation of guanylate cyclase (i.e. vasodilatation) and attenuation of neutrophil functions (i.e. reduced adherence to stimulated endothelium). Mayer et al. [45] in 1995 reported that cultured endothelial cells treated with peroxynitrite (1 mM) demonstrated a thiol-dependent stimulation of soluble guanylate cyclase activity. This guanylate cyclase stimulation subsequently increased cyclic-GMP (cGMP) production to levels comparable to cells treated with a nitric oxide donor. Support for a central role for glutathione as the predominant thiol responsible for regeneration of authentic nitric oxide was demonstrated when depletion of endothelial cell glutathione levels by diethyl maleate (DEM) correlated with a subsequent reduction in cGMP production in a concentration-dependent manner. As additional evidence for a nitric oxide regeneration pathway of peroxynitrite action, the levels of nitric oxide in the media of these experiments paralleled the change in cGMP [45]. This in vitro data has been substantiated by other studies demonstrating the generation of a nitrosylated product from peroxynitrite via a thiol intermediate having physiological properties similar to nitric oxide [26–32].

Other pathways of peroxynitrite metabolism not involving the generation of thiol intermediates have been demonstrated which may also explain its decomposition. Graves et al. [46] utilized L-penicillamine to attenuate the effects of S-nitrosothiols in order to unmask thiol-independent pathways for detoxification of peroxynitrite. In an in vivo rat model, administration of peroxynitrite in the presence of L-penicillamine (to block interactions between S-nitrosothiol and its receptor) produced no significant systemic vasodilation or other hemodynamic effects while peroxynitrite, in the absence of L-penicillamine, caused vasodilation. These data implicate the involvement of a thiol-independent mechanism for the vasodilator properties of peroxynitrite. These studies did not identify the specific pathway for peroxynitrite detoxification, but did suggest a

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<tr>
<th>Substrate</th>
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<tr>
<td>Carbon dioxide</td>
<td>(4 \times 10^5)</td>
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<td>Oxyhemoglobin</td>
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potential role for hyperpolarization of the vascular smooth muscle by activation of ATP-sensitive potassium channels [46]. The redundancy and diversity of potential pathways for peroxynitrite metabolism may contribute to the controversies surrounding its seemingly diverse physiological actions (see Section 4).

4. Cardiovascular effects of peroxynitrite

4.1. Effects of peroxynitrite on blood and blood elements

Peroxynitrite has been found to exert oxidant effects on both cell membranes and cytoplasmic organelles of leukocytes, erythrocytes, and platelets. In addition, in vitro peroxynitrite can initiate peroxidation and oxidation reactions in plasma [20,47–53]. In vivo, however, peroxynitrite is often generated in an environment (i.e. plasma) rich in antioxidants (i.e. ascorbate, urate, cysteine, albumin, glutathione) which can potentially attenuate its detrimental effect. The addition of peroxynitrite to plasma results in a concentration-dependent depletion of antioxidants [15]. Van der Vliet et al. [15] determined that peroxynitrite (0.25–1 mM) added to human plasma significantly decreased levels of ascorbate and urate which continued for up to 60 min following administration of peroxynitrite. In addition, the cysteine residues on plasma albumin were also found to contribute to a thiol detoxification pathway of peroxynitrite. In this pathway, peroxynitrite caused a loss of sulfhydryl groups in a concentration-dependent fashion, an effect which became exponential after dialysis of soluble factors such as bicarbonate. Furthermore, this reaction to form nitrosothiols took place even in the presence of other antioxidants such as ascorbate and urate. Interestingly, bicarbonate did not have a significant effect on the reaction between thiols and peroxynitrite despite its relatively rapid rate of reaction in vitro as mentioned above, indicating that the in vivo milieu allows for some of the slower reactions of peroxynitrite (i.e. with glutathione, hemoglobin, albumin) to occur.

The interaction between neutrophils and the endothelium in a plasma environment is yet another potential site for the actions of peroxynitrite. Human neutrophils exposed to circulating proinflammatory factors such as platelet activating factor, tissue necrosis factor-α, complement fragments, through programmed cell death. The simultaneous production of superoxide and nitric oxide by neutrophils may provide sufficient precursors for peroxynitrite formation independent of, and in addition to, the superoxide anion and nitric oxide produced by endothelium or parenchymal tissue [1], Carreras et al. [49] used chemiluminescence to detect a synchronous increase in production of superoxide and nitric oxide by neutrophils activated with phorbol ester (PMA) which was paralleled by a simultaneous increase in peroxynitrite. Peroxynitrite, therefore, is a likely byproduct of the respiratory burst that occurs with increased neutrophil interaction with the endothelium and subsequent accumulation in ischemic-reperfused tissue. Hence, peroxynitrite may participate in the neutrophil-mediated inflammatory response through its direct and indirect oxidant effects.

The immunologic function performed by neutrophils and macrophages is dependent on the generation of toxic radical species to achieve their bactericidal effects. It has been postulated that peroxynitrite may contribute to the cytotoxicity in the host defense responses. Ischiropoulos et al. [50] have demonstrated that activated macrophages produce nitric oxide in sufficient quantity to form peroxynitrite if sufficient superoxide anion is available. Indeed, Ischiropoulos et al. [50] demonstrated convincingly that a majority of the nitric oxide produced by the activated macrophage converts to peroxynitrite despite the presence of superoxide dismutase, indicating a rapid conversion of nitric oxide to peroxynitrite that out-competes superoxide dismutase. Others have documented the in vitro production of peroxynitrite by leukocytes in response to bacteria known to be infectious to humans such as Escherichia coli.

Curiously, despite the possibility that white blood cells can utilize peroxynitrite as a cytocidal oxidant to eliminate infectious and cancerous cells [49,50], leukocytes can also initiate their own demise through apoptosis, purportedly induced in part by peroxynitrite [19,20]. Hence, peroxynitrite is a factor partially governing the longevity of the white cell life cycle [19,51]. Several studies describe apoptosis and membrane breakdown in transformed leukocytic HL-60 cells exposed to peroxynitrite [19,20,51]. In contrast to the HL-60 cells, the apoptotic process could not be triggered in either normal human umbilical vein endothelial cells or normal peripheral blood mononuclear cells at similar concentrations of peroxynitrite and may, therefore, be specific to leukocytes [20]. These observations suggest that leukocytes utilize peroxynitrite to effect opposing actions: to initiate the demise of other cells targeted for destruction, such as infectious or cancerous cells, or to initiate its own noninflammatory demise through programmed cell death.

Since erythrocytes lack a nucleus and other intracellular organelles, the effects of peroxynitrite are confined to the plasma membrane through lipid peroxidation and nitration of membrane proteins. Soszynski and Bartosz [47] investigated the effects of peroxynitrite on red blood cell membrane structures. In an elegant set of experiments, these investigators were able to show that peroxynitrite at concentrations ranging between 0.1–2 mM induces oxidation of membrane sulfhydryl groups coincident with a transient decrease in intracellular glutathione concentra-
tion. In addition, peroxidation of membrane lipids and aggregation or nitration of membranous proteins were observed following exposure of red blood cells to peroxynitrite. In these experiments [47], however, red blood cell hemolysis did not occur to any significant degree, suggesting that biochemical changes in the membrane did not compromise membrane integrity. In contrast, Kondo et al. [48] demonstrated that red blood cell hemolysis was significantly increased in the presence of lower concentrations of peroxynitrite (10% hemolysis at 100 μM, 30% at 500 μM). The hemolytic effect was significantly inhibited in the presence of the endogenous antioxidants glutathione, N-acetylcysteine, and albumin. These observations may support a role for erythrocytes as a significant intravascular source of glutathione capable of detoxifying peroxynitrite and attenuating peroxynitrite-induced injury to erythrocytes themselves, and perhaps to surrounding parenchymal tissue.

### 4.2. The effects of peroxynitrite on the vascular wall

The recognition that nitric oxide functions as a primary regulator of vascular reactivity is not new [13,59–69]. However, the vasoactive and other physiological properties of nitric oxide are significantly influenced by the presence of superoxide anion, by virtue of the diffusion limited biradical reaction between the two molecules, not only under pathological conditions (i.e. ischemia–reperfusion, hypertension), but also under basal conditions [70,71]. The physiological consequences of peroxynitrite generated by the biradical interaction between nitric oxide and superoxide anion relevant to the vascular endothelium are multiple and include (1) a decrease in the nitric oxide available for G-protein stimulation, (2) reduction in nitric oxide available to exert antineutrophil effects, and (3) neutralization of superoxide anion, thereby limiting endothelial and vascular smooth muscle injury. The net outcome of these often opposing effects depends upon the concentration of peroxynitrite achieved in the compartment of interest.

As the primary source of vascular nitric oxide and a significant source of superoxide anion, the endothelium is of particular interest as a potential generator of peroxynitrite. For several years, debate had been raised around the question of whether peroxynitrite could be formed in vivo in sufficient quantity (high nM to low μM) to exert a significant physiological effect on the vascular endothelium, vascular smooth muscle, and perivascular tissue. Achieving physiologically relevant concentrations seemed unlikely since (1) the circulating concentration of nitric oxide under basal conditions is relatively low (1–20 nM/L), (2) an equimolar concentration of superoxide is required for generation of peroxynitrite, and (3) the short half life (~1 s) of peroxynitrite at physiologic pH may prevent tissue accumulation, limit diffusion distance, and reduce interaction with biological molecules [72]. However, under pathologic conditions, such as hypercholesterolemia and hypertension, and during the early moments of reperfusion after ischemia, both nitric oxide and superoxide anions are generated at greater than basal levels, thereby favoring enhanced production of peroxynitrite. The physiological consequences of overproduction of peroxynitrite and superoxide anion was initially confirmed in cell culture preparations by Kooy and Royall [73] and Thom et al. [74] who demonstrated that peroxynitrite is produced in the endothelial microenvironment following agonist (bradykinin, A23187) stimulated production of endothelial nitric oxide in the presence of concomitant exogenous superoxide anion. This generation of peroxynitrite in the endothelial microenvironment may be sufficient to exert physiologic effects.

In vitro studies have demonstrated the capability of peroxynitrite to directly alter the intracellular second messenger pathways in both the endothelium and vascular smooth muscle. Peroxynitrite exerts its effects on the endothelium by triggering intracellular second messenger pathways to increase levels of cGMP (similar action to nitric oxide) which can then stimulate vascular smooth muscle relaxation [45]. Although the relaxation of vascular smooth muscle by peroxynitrite also occurs as a direct effect in the absence of the endothelium, the relaxation of vascular smooth muscle is potentiated by peroxynitrite’s effect on the intact endothelium. Liu et al. [29] reported that isolated coronary artery rings dilated at 100 nM peroxynitrite when the endothelium was intact, but required greater than 1 μM peroxynitrite when the endothelium was denuded. Furthermore, maximal dilation in the endothelialized segments was nearly 100% at a concentration of 10 μM, but reached only ~80% in vessels without endothelium. These studies imply that vascular dilation is facilitated by the endothelium, but that the predominant vasorelaxation response induced by peroxynitrite is independent of intact endothelium.

The mechanism underlying this endothelial facilitated vasodilation induced by peroxynitrite was investigated by Wu et al. [31] in 1994. Similar to the observations of Liu et al. [29]. 100 μM peroxynitrite induced a prolonged vasodilation in bovine pulmonary artery segments denuded of endothelium [31]. Interestingly, nitric oxide production detected by chemiluminescence mirrored the vascular response to peroxynitrite; that is, with increasing concentrations of peroxynitrite over time, there was a coincident and parallel increase in both vascular relaxation and nitric oxide levels (~20 pM at 0 min versus ~250 pM at 60 min). The increase in nitric oxide production was attenuated by depletion of cellular glutathione levels using 7 mM DEM, again implying thiol-related detoxification of peroxynitrite to a nitrosothiol or related product as an important degradation pathway, and suggesting furthermore that glutathione in vascular tissue contributes to the production of products with vasorelaxant properties. Depletion of endogenous cellular glutathione in disease states such as ischemia–reperfusion [75] and hypercholes-
Coronary artery endothelial damage induced by ischemia–reperfusion is expressed as (a) an initial increase in nitric oxide release followed by a prolonged reduction in the release of nitric oxide [44] commensurate with the severity of injury, (b) attenuated vasodilator responses to agonist stimulators of nitric oxide synthase (agonist stimulated function) indicative of impaired release of nitric oxide, and (c) compromised basal antineutrophil properties of the endothelium, i.e., an attenuated ability to inhibit adherence of stimulated or unstimulated neutrophils. In addition, myocardial ischemia–reperfusion is also known to deplete glutathione levels [78,79], ostensibly attenuating not only the endogenous glutathione–glutathione peroxidase antioxidant defense system but also the detoxification of peroxynitrite. Depletion of endogenous glutathione would favor build up of perivascular peroxynitrite, and would decrease production of nitrosothiols or other reaction products by the glutathione pathway (Fig. 1).

Nossuli et al. [72] reported preserved endothelial function in ischemic-reperfused coronary vessels exposed to authentic peroxynitrite (1 μM) in an in vivo feline regional ischemia model of 90 min left anterior descending (LAD) coronary artery occlusion and followed by 270 min of reperfusion. Ischemic-reperfused LAD coronary arteries from untreated (vehicle) hearts demonstrated endothelial dysfunction vis-a-vis impaired relaxation responses to incremental concentrations of acetylcholine, but no attenuated responses to the smooth muscle dilator sodium nitroprusside, suggesting endothelium-specific dysfunction. In contrast, 1 μM authentic peroxynitrite administered intraventricularly at reperfusion significantly reduced this postischemic endothelial dysfunction. In addition, in the group treated with authentic peroxynitrite, neutrophil adhesion to the endothelium of postischemic coronary artery segments as an index of basal function was significantly reduced (~90 PMN (polymorphonuclear leukocytes) mm⁻² in vehicle versus ~28 PMN mm⁻² in peroxynitrite groups). The vasculoprotective effect of peroxynitrite was shown to be concentration-dependent by Nossuli et al. [80] with no attenuation of postsischemic responses to agonist-stimulated relaxation until 2 μM peroxynitrite. However, 20 μM peroxynitrite was associated with endothelial dysfunction (Fig. 3).

In general agreement with the study by Nossuli et al. [72], Lefer et al. [81] using an intestinal ischemia/reperfusion model examining ischemic-reperfused mesenteric arteries, showed that the in vivo mechanism of peroxynitrite-mediated attenuation of neutrophil adherence to the endothelium involved a reduction of endothelial p-selectin expression, a critical molecule in the initial tethering of neutrophils to the endothelium. Inhibition of p-selectin surface expression has been known to be an important mechanism in the attenuation of neutrophil–endothelial cell interactions, and consistent with the antiinflammatory effects exerted by nitric oxide during ischemia–reperfusion. It was hypothesized in these in vivo experiments [81] that peroxynitrite was being converted through in vivo detoxification and degradation pathways to products with the characteristics similar to nitric oxide.

In that same study, Lefer et al. [81] used organ chambers to document that peroxynitrite in the nanomolar range caused no direct relaxation of normal preconstricted (9,11-methanoepoxy PGH₂ (prostaglandin-H₂)) rat aortic rings, but significant relaxation was demonstrated at 50 μM peroxynitrite consistent with relaxation of pulmonary vessels reported by Wu et al. [31] and Liu et al. [29]. The concentration of peroxynitrite used in the study by Lefer et al. [81] was 1 μM, which was based on previous studies showing the effectiveness of peroxynitrite in mediating vasodilation. However, the study by Nossuli et al. [72] indicated that higher concentrations of peroxynitrite (20 μM) were required to attenuate endothelial dysfunction. This discrepancy may be due to differences in the experimental models used or the specific components of the vasculature studied. Overall, these studies highlight the complex role of peroxynitrite in vascular function and suggest that further research is needed to fully understand its mechanisms of action.

Fig. 3. Relaxation of preconstricted coronary artery segments to endothelial receptor-dependent agonist acetylcholine in ischemic LAD and nonischemic LCx. Graph shows improved relaxation with 2 μM peroxynitrite in the ischemic LAD, but impaired relaxation in both nonischemic LCx and ischemic LAD with 20 μM peroxynitrite added (*= P < 0.05 compared to vehicle and 20 μM groups; **= P < 0.05 compared to both vehicle and 2 μM groups). VEH=vehicle. LAD=left anterior descending coronary artery; LCx=Left circumflex coronary artery. (Data adapted from Nossuli et al. [80].)
al. [81] did not impair acetylcholine stimulated vasorelaxation, suggesting that there was no endothelial or vascular smooth muscle dysfunction caused by direct exposure to peroxynitrite. The apparently greater resistance to peroxynitrite-induced injury at 50 μM concentration in the study by Lefer et al. [81] compared to injury produced at 20 μM peroxynitrite reported by Nossuli et al. [80] may be related to sensitization of the endothelium by ischemia–reperfusion. In addition, Lefer et al. [81] showed that 100 nM and 1 μM authentic peroxynitrite attenuated adherence of unstimulated neutrophils to superior mesenteric artery endothelium stimulated by thrombin or hydrogen peroxide, but not in a concentration-dependent manner. Furthermore, peroxynitrite completely inhibited neutrophil rolling and adherence along rat mesenteric venules activated by thrombin as visualized by intravital microscopy. These antineutrophil effects may be related to a decrease in expression of immunohistochemically identified p-selectin on these coronary venules exposed to peroxynitrite (800 nM) similar to the findings of Nossuli et al. [80]. These effects of peroxynitrite are similar to the antineutrophil properties of nitric oxide or nitrosylated molecules [82].

Whether the vasorelaxation and antineutrophil effects of authentic peroxynitrite are due to production of nitric oxide directly was investigated by Nossuli et al. [80]. In this study, hemoglobin, a known scavenger of nitric oxide, did not attenuate the vasorelaxation effects of 10–40 μM peroxynitrite in de-endothelialized coronary artery rings, nor did it prevent the inhibition of neutrophil adherence to the endothelium of coronary artery segments. Therefore, the vasorelaxant and antineutrophil effects of peroxynitrite may not be ascribed to authentic nitric oxide. However, the cardioactive and cardioprotective effects of peroxynitrite may be derived from the formation of a nitrosylated product, i.e., nitrosoglutathione [80]. In this study, reduced glutathione was converted to form nitrosoglutathione in the presence of 1–1000 μM peroxynitrite in vitro. The appearance of nitrosoglutathione was paralleled by the disappearance of reduced glutathione. Therefore, the physiological effects of peroxynitrite on vascular endothelium and the myocardium may depend on the concentration used exogenously or achieved endogenously, as well the presence or absence of pathways leading to the reduction of peroxynitrite and production of nitrosylated compounds.

A preliminary report from Lopez et al. [75] suggested that the presence or absence of detoxification reactions may be responsible for the divergent and inconsistent physiological effects of peroxynitrite in in vitro (i.e., crystalloid) compared to in vivo (i.e., blood) environments. Ronson et al. [83] investigated this question by assessing endothelium-dependent vasodilator responses in a canine model of ischemically injured hearts administered either a crystalloid or a blood cardioplegia solution as the variable with 5 μM peroxynitrite (BCP-) compared to unsupplemented blood cardioplegia group (BCP), but impaired relaxation in crystalloid cardioplegia group with peroxynitrite (Pleg+) compared to crystalloid cardioplegia alone (Pleg) (*P<0.05). (Data from Ronson et al. [83]).

Fig. 4. Relaxation of preconstricted postischemic coronary artery segments to endothelial receptor-dependent agonist acetylcholine. Graph shows improved relaxation in blood cardioplegia group supplemented with 5 μM peroxynitrite (BCP+) compared to unsupplemented blood cardioplegia group (BCP), but impaired relaxation in crystalloid cardioplegia group with peroxynitrite (Pleg+) compared to crystalloid cardioplegia alone (Pleg) (*P<0.05). (Data from Ronson et al. [83]).
tive effects of peroxynitrite are possibly mediated by a nitrosylated intermediate. The protective properties of peroxynitrite may be attenuated in tissues depleted of endogenous detoxifying agents such as glutathione, or environments lacking other detoxifying substances.

4.4. Peroxynitrite in ischemic-reperfused myocardium

Reperfusion of ischemic myocardium is the definitive treatment to attenuate myocardial injury. However, peroxynitrite itself causes additional tissue damage mediated by an inflammatory-like response and other factors (edema, calcium overload). Mediators responsible for the inflammatory-like component of postischemic myocardial injury are numerous and include superoxide anion [39, 84–86], hydrogen peroxide [87], and hydroxyl radicals [43, 84, 87]. These reactive oxygen species are produced to a great extent in a "respiratory burst" occurring during reperfusion, primarily generated by activated neutrophils [37, 88]. However, reactive oxygen species can also be produced by the increased activity of xanthine oxidase and NAD(P)H oxidase, and by abnormalities in mitochondrial oxidative processes [89]. Accordingly, peroxynitrite production in the ischemic-reperfused heart has been shown by Yasmin et al. [90] to peak in the early seconds of reperfusion at a time coincident with the generation of both nitric oxide and superoxide radicals [4]. Liu et al. [4] confirmed the generation of peroxynitrite in the postischemic myocardium in vivo during reperfusion following 20 min of regional LAD occlusion. Peroxynitrite levels paralleled the production of both nitric oxide and superoxide anions in both ischemic-reperfused and nonischemic regions consistent with its equimolar stoichiometry. Hence, peroxynitrite, as well as the hydroxyl-like intermediate and peroxynitrous acid, are present at critical time points during the instigation and evolution of postischemic injury, and may be mediators of biological injury.

Although peroxynitrite and its related metabolites may be formed during ischemia–reperfusion, certain thiol-containing compounds such as glutathione, albumin and cysteine [31, 91] which are normal constituents of blood and tissues, may ultimately convert peroxynitrite to nitrosothiols [27, 80]. These nitrosoylated agents may protect the heart by potentially preventing toxic build up of peroxynitrite in postischemic myocardium, and by generating protective nitrosothiols or nitric oxide [45, 31]. Numerous ex vivo and in vivo studies, some of which are reviewed below, have investigated the impact of peroxynitrite on postischemic myocardial contractile function and chamber compliance.

Ma et al. [2, 3] investigated the effect of physiologically relevant concentrations of peroxynitrite on left ventricular contractile function in crystalloid perfused (Langendorff) rat hearts contracting against an intraventricular balloon with fixed volume. Peroxynitrite generated either by the combination of S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and pyrogallol [2], or by the peroxynitrite generator SIN-1 (3-morpholinosidnonimine) [3] induced ventricular dysfunction manifested as significantly decreased left ventricular developed pressure [2, 3]. Creatine kinase release as a marker of myocardial morphologic injury was also accentuated in groups exposed to exogenously generated peroxynitrite [3]. The degree of functional abnormality was decreased with the addition of glutathione [3]. Similar results have been obtained with endogenous peroxynitrite generation following hypoxia–reoxygenation in rats [92] or with exogenously administered peroxynitrite [17]. Therefore, in crystalloid perfused hearts, peroxynitrite demonstrated deleterious effects which were attenuated by the antioxidant agent glutathione. However, it was suggested in the report by Ma et al. [3] that glutathione may also be acting to detoxify peroxynitrite to substances with cardioprotective actions similar to those of nitric oxide.

The mechanisms responsible for the contractile dysfunction associated with peroxynitrite may be related, in part, to reported inhibition of oxidative metabolism. Peroxynitrite has been shown to attenuate oxygen consumption and mitochondrial respiration of in vitro cardiomyocytes, resulting in decreased energy production and the potential for impaired contractility [93]. A potential site of action for peroxynitrite-induced attenuation of mitochondrial respiration is myocardial aconitase, which has been shown to be significantly more sensitive to peroxynitrite than to nitric oxide [32, 93]. Parenthetically, thiols have been shown to limit the damage to the aconitase enzyme caused by peroxynitrite as a potential mechanism for protection by glutathione in the ischemic-reperfused myocardium [32].

In contrast to the above in vitro studies, in vivo studies have reported largely beneficial effects on contractile function, infarction, and other physiologic end-points [72, 75]. Using a well-characterized feline model of 90 min LAD ischemia followed 4.5 h of reperfusion, Nossuli et al. [72] demonstrated a significant reduction in infarct size from 30% to 14% with infusion of 1 μM peroxynitrite at the time of reperfusion. In a follow-up study, Nossuli et al. [80] reported a similar reduction of infarct size in the same model with 2 μM exogenously administered peroxynitrite, but observed an increased infarct size at 20 μM peroxynitrite (Fig. 5). The decreased infarct size with 2 μM peroxynitrite was associated with better endothelial-dependent vasorelaxation, while 20 μM was associated with worse postischemic endothelial function, consistent with experiments mentioned in an earlier section. Therefore, in addition to the presence or absence of detoxification pathways, the physiological effects of peroxynitrite in vivo may be related to the concentration achieved, being protective at lower concentrations and deleterious at higher concentrations. It is tempting to speculate that these in vivo cardioprotective effects of peroxynitrite are mediated, in part, by inhibition of neutrophil-induced damage and accumulation in ischemic-reperfused myocardium as sug-
crystalloid cardioplegia with the addition of 5 mM peroxynitrite, an effect lost with 20 μM peroxynitrite. In contrast, hearts administered thiols, nitric oxide and others. This limitation of the in vivo cardioplegia solution compared to blood cardioplegia formations with blood components such as carbon dioxide, the groups with 5 mM on the myocardium. Similar to be linked with antineutrophil properties. However, these observations of Nossuli et al. [72,80], these hearts studies did not determine the etiology of these dichotomies. Elevated myocardial levels of addition, this study provides evidence that the cardio- protective effect on postischemic contractile function may perhaps detoxify peroxynitrite. However, whether detoxification by thiol substances was involved in the pivotal effects of peroxynitrite was not investigated in this study. To further test the cardiac effects of peroxynitrite in blood versus crystalloid environment on the myocardium, Ronson et al. [83] performed an in vivo canine experiment in a surgically relevant model of antecedent normothermic cardioplegia. Plegisol supplemented with 500 μM ONOO− had significantly less recovery of left ventricular myocardium was lower in the peroxynitrite supplemented blood cardioplegia group compared to the blood cardioplegia group without peroxynitrite, consistent with the antineutrophil effects of authentic peroxynitrite reported earlier by Lefer et al. [81]. In contrast, neutrophil accumulation (myeloperoxidase activity) in postischemic left ventricular myocardium was lower in the peroxynitrite supplemented blood cardioplegia group compared to the blood cardioplegia group without peroxynitrite, consistent with the antineutrophil effects of authentic peroxynitrite reported earlier by Lefer et al. [81].

Similar to the duality in physiological effects exerted on the endothelium based on the biological environment (i.e. blood versus crystalloid, presence or absence of detoxification agents), a plurality in physiological consequences is observed on myocardial end-points. In the study by Lopez et al. [75], peroxynitrite was observed to be cardioprotective when the isolated rat heart was perfused with blood, while it was cardiotoxic when the heart was perfused with crystalloid Krebs—Henseleit buffer. This observation implied that substances inherent in the blood perfusate could perhaps detoxify peroxynitrite. However, whether detoxification by thiol substances was involved in the pivotal effects of peroxynitrite was not investigated in this study.

To further test the cardiac effects of peroxynitrite in blood versus crystalloid environment on the myocardium, Ronson et al. [83] performed an in vivo canine experiment in a surgically relevant model of antecedent normothermic ischemia and cardiopulmonary bypass, in which a crystalloid environment was achieved with crystalloid cardioplegia, and a blood environment was achieved with blood cardioplegia. Peroxynitrite (5 μM) was added to each cardioplegia formulation and compared to its unsupplemented counterpart. Elevated myocardial levels of nitrotyrosine in the supplemented group suggested that peroxynitrite was delivered to the myocardium. Similar to the observations of Nossuli et al. [72,80], these hearts showed improved postischemic left ventricular systolic function (end-systolic pressure—volume relation, Fig. 6) in the groups with 5 μM peroxynitrite added to the blood cardioplegia solution compared to blood cardioplegia without peroxynitrite. In contrast, hearts administered crystalloid cardioplegia with the addition of 5 μM authentic peroxynitrite had significantly less recovery of left ventricular systolic performance than hearts arrested with crystalloid cardioplegia without peroxynitrite. However,

![Fig. 5. Myocardial area at risk (AAR) and infarct size after regional coronary artery occlusion and reperfusion. The figure shows no differences in AAR between groups, but significantly smaller infarct size in hearts treated with 2 μM peroxynitrite, an effect lost with 20 μM peroxynitrite. AN=Area necrosis, VEH=vehicle. (Data adapted from Nossuli et al. [80].) ](https://academic.oup.com/cardiovascres/article-abstract/44/1/47/275389/55)

![Fig. 6. Postischemic left ventricular function after 90 min of reperfusion measured as end-systolic pressure—volume relation (ESPVR) expressed as percent of baseline values. Systolic function is improved in the BCP(+) group compared to BCP(−) group, but is diminished in the Pleg(+) group compared to the other groups (*=P<0.05 versus blood(−) and Pleg(−), respectively). PLEG(−)=Plegisol without ONOO−; PLEG(+)=Plegisol supplemented with 5 μM ONOO−; BCP(−)=blood cardioplegia without ONOO−; BCP(+)=blood cardioplegia supplemented with 5 μM ONOO− (final concentration). (Data adapted from Ronson et al. [83].) ](https://academic.oup.com/cardiovascres/article-abstract/44/1/47/275389/55)
Fig. 7. Postischemic neutrophil accumulation determined by left ventricular tissue myeloperoxidase activity (MPO). BCP(−)=blood cardioplegia without ONOO−; BCP(+)=blood cardioplegia with 5 μM ONOO (final concentration); PLEG(−)=crystalloid cardioplegia without ONOO−; PLEG(+) = crystalloid cardioplegia with 5 μM ONOO− (final concentration). Increased myocardial neutrophil accumulation is evident in the Pleg(+) compared to the Pleg(−) group, and decreased in the BCP(+) versus the BCP groups (*=P<0.05). (Data adapted from Ronson et al. [83].)

5. Summary

Peroxynitrite is a physiologically active product of the reaction between nitric oxide and superoxide anion which has profound effects on the vascular endothelium, blood and its formed elements, and on the myocardium. The corelease of nitric oxide and superoxide anion by vascular endothelium and neutrophils with the resultant generation of peroxynitrite makes this anion a likely participant in vascular and myocardial injury associated with ischemia–reperfusion, hypercholesterolemia, and hypertension in which neutrophil–endothelial cell interactions are critical events in the pathogenesis of the disease. Although purely chemical considerations suggest that peroxynitrite can competitively react with few molecules other than carbon dioxide and heme proteins [22], significant biological responses to the anion or its degradative products have been reported. The exact nature of the intermediates or products exerting physiological effects on the vasculature, inflammatory cells, and cardiac tissue remains both controversial and elusive. The biological effects of peroxynitrite exposure are greatly dependent on the tissue concentration achieved and on the biological environment in which it is present. Both deleterious and beneficial effects can be observed in vivo dependent on the tissue concentration or the availability of detoxifying agents for conversion of peroxynitrite to another form, perhaps a nitrosothiol or nitric oxide itself. High tissue concentrations induce injury to the vascular endothelium, and produce contractile dysfunction and necrosis in myocardium jeopardized by ischemia–reperfusion injury. In biological media devoid of thiol substances a concentration-dependent injury is observed. However, in in vivo settings, physiologically relevant concentrations (up to 5 μM) exert protection to vascular endothelium and myocardium, and a reduction in neutrophil events, which are properties with striking similarity to those exhibited by nitric oxide. In point, evidence suggests that the physiological effects of peroxynitrite in vascular and myocardial tissue are partly determined by the presence or absence of sufficient detoxification pathways involving the nitrosylation of thiol moieties, nitrosothiols themselves, or regenerated nitric oxide attenuating vascular and cardiac tissue damage. In the absence of these detoxification pathways, however, oxidation of membrane lipids, DNA, and biologically active proteins may prevail and lead to tissue damage.

Nitric oxide therapy is currently being evaluated in experimental and clinical settings to attenuate myocardial ischemia–reperfusion injury, adult respiratory distress syndrome, and many other pathologic processes. However, enthusiasm for this therapeutic approach may be dampened by any untoward and deleterious effects. The conflicting reports on the cardioprotective effects of nitric oxide may be explained, in part, by the subsequent generation of peroxynitrite and its transformation into biological molecules capable of exerting physiologically relevant effects. The formation of peroxynitrite secondary to the application of nitric oxide therapy has direct implications on the benefits gained for treatment of vascular disease, ischemia–reperfusion, myocardial infarction, and atherosclerosis.

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