Potential Benefits of Peroxynitrite

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Abstract: Peroxynitrite (PN) is generated by the reaction of nitric oxide (NO) and superoxide in one of the most rapid reactions in biology. Studies have reported that PN is a cytotoxic molecule that contributes to vascular injury in a number of disease states. However, it has become apparent that PN has beneficial effects including vasodilation, inhibition of platelet aggregation, inhibition of inflammatory cell adhesion, and protection against ischemia/reperfusion injury in the heart. It is our hypothesis that PN may serve to inactivate superoxide and prolong the actions of NO in the circulation. This manuscript reviews the beneficial effects of PN in the cardiovascular system.

INTRODUCTION

The endothelium releases a variety of relaxing and contracting factors that regulate the underlying smooth muscle [1]. One such molecule, nitric oxide (NO) is produced by the endothelium and participates in the modulation of blood flow, thrombosis and inflammation [2]. Although NO has been labeled as a toxic environmental pollutant, it is not toxic in low concentrations [2, 3]. However, when produced in elevated amounts during inflammatory conditions, NO becomes toxic, as the reaction of NO with another oxidant, superoxide, forms the powerful oxidant, peroxynitrite (PN) [4, 5].

$$O_2^- + NO^\bullet \rightarrow ONOO^-$$
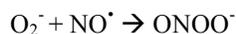
Moreover, it is hypothesized that this molecule determines the role of NO in either physiologic *vs* pathologic processes [3], as many of the pathological effects of NO are mediated by PN [3, 6-8]. These pathologic effects of PN are discussed in excellent reviews elsewhere [3, 9-19].

In contrast, PN has been shown to have beneficial effects similar to those of NO and has been shown to produce vasorelaxation [20, 21], inhibit platelet aggregation [22, 23], and can limit ischemia-reproduction injury [24-26]. In this review, we describe the progress made in understanding the beneficial role of PN in the cardiovascular system.

VASORELAXANT PROPERTIES OF PEROXYNITRITE

Reperfusion of ischemic tissue initiates production of NO and superoxide, with subsequent formation of PN that can be a significant factor in propagating ischemic injury in some, but not all, organs studied [5, 27-32]. Following the observations that PN formation may have a role in increasing coronary vasoconstriction during reperfusion of the ischemic coronary vascular bed [28]; Liu, Beckman *et al.* investigated the effect of PN in isolated canine coronary artery rings precontracted with PGF2a [23]. They found that PN concentrations of 10 nM to 30 μM produced consistent, rapid, and dose-dependent relaxation, regardless of the presence of the endothelium. However, PN was 7- to 10-fold less potent when compared to NO under the same experimental conditions [23], and when compared to NO-induced relaxation in other studies [33-36]. PN-induced coronary artery ring relaxation was reproducible with repeated administration of PN and the relaxation was reversible by washing with a standard bicarbonate-buffered Krebs-Henseleit solution. Although not directly reported, these findings suggest that tachyphylaxis to repeated administration of PN was not observed. When PN was allowed to decompose for 20 min, a marked decrease in relaxation response was observed. Sodium nitrite also produced a dose-dependent relaxation, but was slower in onset and that higher concentrations were required [23]. To investigate the role of NO oxidation, the addition of superoxide dismutase (SOD) which reduces the breakdown of NO [37] was found to potentiate PN-induced relaxation. In contrast, the addition to the bath of hemoglobin or methylene blue, NO scavengers [1, 38, 39], significantly decreased PN-induced relaxation [23]. The enhanced relaxation to PN by SOD and the inhibition of relaxation to PN by hemoglobin or methylene blue suggest that the effect of PN could be mediated by the formation of NO or by the formation of a nitroso-derivative [23]. PN has been shown to increase the formation of cyclic GMP in cultured vascular smooth muscle cells [40]. PN can be oxidized to a nitrosodioxyl radical that is in equilibrium with NO and molecular oxygen, which may contribute to the relaxing effects of PN

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[41]. However, responses to NO are reduced by superoxide and the interaction of NO with superoxide may decrease the concentration of NO available [42, 43]. This finding suggests that the decreased response to PN when compared to NO maybe due to decreased NO bioavailability. The finding that PN relaxes coronary arteries are in contrast to studies showing coronary artery vasoconstriction by PN [28]. These experiments involve short-term exposure of the coronary rings to PN, whereas long-term PN exposure can induce myocardial injury [32].

Wu, Pritchard *et al.* also investigated the role of PN in isolated endothelium-denuded bovine pulmonary arterial smooth muscle rings [21]. In this study, potassium-preconstricted pulmonary arterial rings were relaxed by 0.1μM concentrations of PN. Decomposed PN and sodium nitrite, the major decomposition product of PN [44], produced slower onset of responses and are in agreement with Liu, Beckman *et al.* [23]. Moreover when repeatedly washed, the ring preparations could be contracted to their initial tone level with potassium chloride; and in endothelium-intact preparations incubated for 30 min with a high concentration of PN, this prior treatment with PN did not significantly alter relaxation responses to acetylcholine, nor did the presence of the endothelium alter PN-induced relaxations. These findings suggest that PN does not alter vascular responses in this preparation. The mechanism of PN vasorelaxation was studied with the addition of methylene blue and LY-83583, and these inhibitors of soluble guanylate cyclase [45, 46] reduced PN-induced relaxation. The use of DMSO, a tissue-permeable scavenger of hydroxyl radical [5, 47], did not affect relaxation responses to PN. These findings suggest a role of soluble guanylate cyclase with PN, and support the findings of Liu, Beckman *et al.* where the administration of sodium hydroxide solutions did not alter responses to PN [23]. It was hypothesized that PN acts by two mechanisms. PN appears to initially induce relaxation *via* the generation of NO, as small quantities of NO generated from PN were detected by chemiluminescence analysis [48, 49]. However, decomposition products of PN also appear to contribute to the prolonged relaxation response, as sodium nitrite produced similar responses. Nitrite is thought to relax vascular smooth muscle by stimulation of soluble guanylate cyclase, after it is reduced to NO [50]. However, the small amounts of NO generated by PN or by nitrite appear unlikely to mediate responses produced by PN. A more plausible mechanism is that PN liberates NO by generating nitrosothiols by reacting with tissue sulfhydryls, since the addition of glutathione (GSH), a tripeptide thiol [51], produced a time-dependent increase in NO generation. Nitrosothiols have been shown to release NO and to stimulate guanylate cyclase [35, 52-54]. Moreover, pretreatment with diethyl maleate, an agent known to deplete tissue GSH [55], reduced both relaxation and formation of NO upon exposure to PN. It was concluded that PN reacts with arterial smooth muscle to generate NO that leads to initial relaxation of vascular smooth muscle, but that the decomposition products of PN also contribute to the prolonged response by the generation of a nitrosothiol-like compound that continues to release NO over prolonged periods [21].

Exposure of vascular endothelium to bacterial toxins and cytokines, as well as to macrophages and neutrophils, can generate large quantities of NO and superoxide, and these molecules and their major metabolite, PN, may be responsible for the observed hyporesponsiveness in the systemic circulation to vasoconstrictors used in the management of septic shock [2, 56-58]. Villa, Salas *et al.* examined coronary perfusion pressure response to PN and the effects of PN on responses to other agents [59]. In separate series of experiments, dose-dependent vasodilator responses to PN (0.3-1000 μM) were generated that was maximal at the 100 μM dose. Repeated infusions of midrange doses of PN (30 μM and 100 μM) produced significant vasodilation, however the development of tachyphylaxis to PN was observed when 3 to 4 doses were administered [59]. The vasodilator effect was abolished by administration of oxyhemoglobin, an NO scavenger [38], whereas decomposed PN did not induce vasodilation [59]. In contrast to the findings of Liu, Beckman *et al.* and Wu, Pritchard *et al.* [21, 23], infusion of PN at doses of 3 μM and at 1000 μM significantly reduced the vasodilator responses to acetylcholine, isoproterenol, and to a S-nitrosothiol, S-nitroso-N-acetyl-DL-penicil-lamine (SNAP). However, infusions of PN at midrange doses (10, 30, and 100 μM) did not produce a significant reduction in the vasodilator response to these agents. The authors suggested that with the midrange doses of PN, the release of NO from PN protected against cytotoxic damage, whereas with the 1000 μM dose, the vascular damage induced by this high dose of PN overcame a protective effect of NO generated from PN [59]. The additional findings that oxyhemoglobin reduced vasodilator responses to acetylcholine, isoproterenol, and SNAP suggest that local formation of S-nitrosothiols may be responsible for the vasodilator actions of PN, as studies have shown that in the presence of small concentrations of human plasma, albumin, or glutathione, PN is converted to S-nitrosothiols or to other NO donors that subsequently release NO [22]. Finally, depressor responses to PN were long lived in this preparation and the vasodilator responses to isoproterenol were markedly depressed for up to 75 min following exposure to 3 μM PN [59]. In contrast, coinfusion of SNAP with 3 μM PN prevented the effect of PN seen in the response to these vasodilators. The findings of tachyphylaxis and vascular dysfunction seen with PN are in contrast to the results of Liu, Beckman *et al.* [23] and of Wu, Pritchard *et al.* [21]. Although PN initially produced vasodilatation, tachyphylaxis rapidly developed, and these results and other studies [5, 22, 44, 57, 58], provided experimental evidence in support of the hypothesis that PN may lead to irreversible endothelial-dependent and endothelial-independent vascular damage [59].

PN reacts with GSH to form S-nitrosothiols that generate NO, which may be a detoxification pathway [22], and has been shown to be a relaxant in isolated or intact vasculature [21, 23, 59], and that this action of PN may be due to nitrosylation of tissue thiols [21]. Moro, Darley-Usmar *et al.* examined the role of PN on isolated rabbit aortic strips [60] denuded of endothelium and superfused in a cascade bioassay [61]. The addition of PN (30–300 μM) to the superfusate resulted in concentration-dependent relaxations of all three detector strips and these observed relaxations were inhibited by oxyhemoglobin [61]. These results confirmed previous studies in bovine pulmonary arterial rings [21], canine coronary arterial rings [23], and in the coronary vasculature in the isolated rat heart [59]. However, the results obtained in this series of experiments did not demonstrate the development

of tachyphylaxis in contrast to the findings in the isolated rat heart preparation [59]. These differences in results could be due to the dilutional effects of the medium or buffers used in this series of experiments, whereas PN was directly injected into the isolated rat heart preparation [59]. As relaxations to PN were similar in the cascade bioassay, it was deduced that a stable NO donor was formed from the reaction of PN with the Krebs buffer in the superfusate. The authors repeated the experiments incubating PN in Krebs buffer without glucose and found that PN had little relaxant effect suggesting that PN reacted with glucose in the Krebs buffer to form compounds that caused relaxation [61]. The finding that the duration of responses to PN was greater than 20 seconds suggested that the observed vasorelaxations arise from the formation of compounds that can act as NO donors [5, 22, 62]. Moreover, the addition of D-glucose, or L-glucose to PN in the superfusate, suggests that PN can react with organic molecules, including sugars and bicarbonate, to form an NO donor [5, 41, 58, 61, 63]. However when electrochemically assayed, the reaction between PN and glucose to generate NO required both Cu^{++} and L-cysteine, a requirement similar to that found for other organic nitrates, such as nitroglycerin (GTN), or to nitrites such as amyl nitrite [64-66]. Additionally, PN reacted with L-glucose as well as to other molecules with functional alcohol groups such as fructose, glycerol, or glyceraldehyde [61].

In pathologic situations, recruited macrophages and neutrophils can be activated to produce both superoxide and NO as part of the inflammatory response [2, 57, 58]. The balance between these two radicals, and formation of PN may result in pathologic effects with alteration in vascular reactivity and blood flow in the human placenta. Prior studies have shown the placental villus has the ability to generate and respond to NO [67]. Holcberg, Kossenjans *et al.* studied the interactions of NO and superoxide in the vasculature of isolated perfused human placental cotyledons preconstricted with the thromboxane agonist, U46619 [68], with fetal perfusion pressure used as an index of vascular response [69]. It was observed that infusion of superoxide (~0.53 μM/L) produced a mild vasoconstriction in the perfused placental cotyledons, and that the infusion of NO produced vasodilatation, but that the infusion of both radicals together produced a greater vasodilatation than NO alone [69]. It was concluded that although superoxide decreased the vasodilator action of NO, the interaction of the two radicals produced a more potent vasodilator, which may be PN in the placental vasculature [69].

Excessive production of NO and nitrogen metabolites have been shown to worsen lung injury in a hyperoxic animal model, as the administration of L-arginine produced pulmonary hypertension and edema, whereas the administration of L-NAME, a NOS inhibitor [56], or SOD, which scavenges superoxide [42], attenuated the pathologic responses to of L-arginine, suggesting that NO, superoxide, or PN may contribute to acute lung injury during hyperoxia [70]. Certainly, the production of NO in large quantities by cytokine-induced NOS has been implicated in vascular dysfunction seen in septic shock and other inflammatory states [56, 71], but how these pathological changes in vascular tone are mediated are not fully known [71]. The generation of NO and superoxide can lead to formation of PN and hydroxyl radicals [58]. Kooy, Royall *et al.* investigated the role of PN in the pathology of vascular dysfunction in inflammatory

states in a mesenteric, renal, and hindquarters flow velocity model in the rat [72]. These authors found that PN in repeated iv doses of 10 μM/kg decreased mean arterial pressure and hindquarters and mesenteric vascular resistance. However, with repeated exposure to PN, a progressive decrease in the response to PN was observed, with a progressive development of increased systemic arterial pressure. Following the development of tachyphylaxis to PN, there were decreased responses to vasodilating and vasoconstricting catecholamines. The authors found that PN is a potent systemic vasodepressor but that tachyphylaxis rapidly developed. PN may be an important factor in vascular pathology seen in sepsis and other inflammatory conditions [72].

Vascular dysfunction, a common feature of the systemic inflammatory response syndrome (SIRS) [73], may be mediated by the pathological production of NO or indirectly through formation of secondary reaction products [56, 74]. The development of tachyphylaxis following repeated administrations to PN, and the subsequent development of hypertension along with vascular depression in response to exogenous catecholamines [72], led Kooy & Lewis to further examine the role of PN in the development of systemic hypertension [75]. The effects of repeated administrations of PN 10 μM/kg iv in anesthetized rats with Doppler flow probes initially produced marked reductions in mean arterial pressure and significant decreases in hindquarters and mesenteric resistance, but with minor decreases in renal resistance [75]. However, subsequent injections of PN produced smaller responses so that by the 10th injection, changes in mean pressure were approximately 12% of control, changes in hindquarters resistance were approximately 17% of control, and changes in mesenteric resistance were approximately 12% of control [75]. Moreover during these experiments, a gradual sustained increase in mean arterial pressure and in vascular resistance developed within 30-60 min, and was most prominent at 2 hrs. By comparison, these hemodynamic values did not change over time in control animals [75]. It was suggested that endogenous PN might deplete reduced thiols, by decreasing S-nitrosothiol formation, such as S-nitrosocysteine, or alter the process by which S-nitrosothiols relax vascular smooth muscle. The most abundant source of free sulfhydryl groups in mammalian tissue is cysteine, and is readily available for the formation of nitrosothiols [76]. Although S-nitrosothiols are hypothesized to be responsible for the vasorelaxant properties of PN [21] and of NO [54, 77], and as PN is a potent oxidant capable of sulfhydryl oxidation [44], inhibition of S-nitrosothiols might promote increases in vascular tone [75]. Finally, in these experiments, the increases in tone were unlikely due to augmented sympathetic neurogenic vasomotor tone as the catecholamines used in these experiments, norepinephrine and epinephrine, were found to produce substantially smaller responses in PN-tolerant rats [75].

The production of NO and oxygen radicals during ischemia or hypoxia followed by reperfusion may contribute to cerebral injury [29]. During cerebral ischemia/hypoxia calcium accumulates, which upon oxygen resupply during reperfusion activates neuronal NO synthetase that oxidizes arginine and produces NO. NO reacts with superoxide, which is also produced during reperfusion, to form PN, and also the powerful and cytotoxic oxidants, hydroxyl radical and nitrogen dioxide [29]. Superoxide, hydrogen peroxide,

and hydroxyl radicals are reported to be strongly vasoactive [21, 23, 78-80]. Brain metabolism regulates cerebral blood flow, and studies suggest that changes in extracellular potassium concentration link brain metabolic activity with blood flow [81]. Wei, Kontos *et al.* examined the effects of PN and the role of potassium channels following topical application of hydrogen peroxide, superoxide, and PN in doses of 1, 3 and 5 µM to cerebral arterioles of the anesthetized cat with cranial windows [82]. Neither the topical application of LY-83583, a soluble guanylate cyclase inhibitor [21, 83, 84], nor tetraethylammonium (TEA), a calcium-activated potassium channel inhibitor [81, 85], affected the vasodilator action of hydrogen peroxide. However glyburide, an ATP-sensitive potassium channel inhibitor [83], inhibited the vasodilator response to hydrogen peroxide or PN, whereas LY-83583 had no effect [82]. In contrast, the vasodilation observed from superoxide was only significantly reduced by TEA [82]. These authors found that vasorelaxant action of both hydrogen peroxide and PN were dependent on activation of ATP-sensitive potassium channels, whereas the vasodilator action of superoxide was dependent upon opening of calcium-activated potassium channels. In contrast to the findings of Wu, Pritchard *et al.* and of Liu, Beckman *et al.* [21, 23] activation by PN at low concentrations of soluble guanylate cyclase does not appear to play a role in the cerebral vasodilator actions of hydrogen peroxide, superoxide, or PN; whereas at higher non-physiologic concentrations, an effect similar to that as reported in pulmonary and coronary vessels were observed [21, 23]. Their findings suggest that PN, as well as hydrogen peroxide, probably act through an oxidant mechanism and either reduce the concentration of ATP by interfering with cellular metabolism, or that these oxidants open ATP-sensitive potassium channels [82].

PN has been found to inhibit cellular respiration that may contribute to endothelial dysfunction [7, 86-91]. Recent studies have shown that NO at high concentrations, as well as PN, can activate poly(adenosine 5'-diphos-phoribose) synthase (PARS) [87, 89] which consumes energy, resulting in cell death through depletion of beta-nicotinamide adenine dinucleotide (NAD^+) and adenosine triphosphate (ATP) [92, 93]. PARS is involved in DNA repair [94, 95], cell differentiation [96, 97], and control of the cell cycle [98, 99]. Recent studies in rat aortic rings exposed to PN demonstrate a dose-dependent reduction in vascular contractility, with a significant increase in PARS activity [100]. As the pulmonary circulation is a prime site for PN formation [101-104], Chabot, Mitchell *et al.* investigated the role of PARS activation during PN stimulation in the pulmonary vascular bed of the rat, and the role of PN on vascular responses to endothelial-dependent and independent agents [105]. These authors observed under baseline conditions in their isolated pulmonary arterial ring preparation, PN (10 nM to 100 µM) had no significant effect on resting tone, whereas under elevated tone conditions with the potent thromboxane mimetic, U46619 [68, 106], PN produced concentration-dependent relaxations [105], whereas the responses to decomposed PN were significantly depressed when compared to fresh PN at the highest dose studied. Furthermore, PN relaxed precontracted pulmonary arterial rings at concentrations that did not induce endothelial dysfunction as responses to acetylcholine were reproducible when compared to control and in fact, at the highest concentrations studied in this preparation, PN aug-

mented relaxations induced by the endothelium-dependent vasodilator agent [105]. The vasodilator effect of PN on rat pulmonary arteries occurred in concentrations that were in agreement with values observed in canine coronary arteries [23], in rat coronary arteries [59], and in bovine pulmonary arteries [21]. The findings that PN did not decrease the response to acetylcholine, and at higher concentrations, enhanced responses to acetylcholine, suggest that PN does not impair the ability of the endothelium to release NO, and in fact may enhance responses [105]. In the presence of PARS inhibition with 3-aminobenzamide [93, 107], PN induced-relaxations were significantly inhibited, whereas PARS inhibition had no effect on the relaxation response to endothelium-derived NO [105]. In contrast, responses to PN were not affected by a NO synthase inhibitor or in the presence of SOD. In contrast to Liu, Beckman *et al.* [23], these findings suggest that superoxide has no role in mediating responses to PN, nor does PN act by stimulating the release of NO from the endothelium. However, the PARS inhibitor significantly reduced the relaxation response to PN. In contrast to the effects on PN, little to no effect on the relaxation produced by acetylcholine or by sodium nitrite was observed during PARS inhibition, whereas the relaxation increased following administration of sodium nitroprusside. The authors suggest that PN, but not to endothelial-derived NO, can activate PARS and result in rapid depletion of ATP with a consequent reduction in pulmonary vascular tone [105]. Although the PARS inhibitor blunted responses to PN, but not to acetylcholine, suggest that NO and PN induce relaxation by different mechanisms [56, 57]. The observations that PN is responsible for the vascular hyporesponsiveness together with other findings of PN toxicity [5, 7, 22, 44, 57-59, 90, 91, 100], suggest that inhibitors of PARS may reduce the persistent hypotension seen in sepsis or other inflammatory conditions without affecting the actions of endothelium-derived NO [105]. These findings are in contrast with Villa, Salas *et al.* that PN induced vascular dysfunction, including impairment of responses to acetylcholine [59].

NO has been reported to regulate coronary vascular tone through stimulation of soluble guanylate cyclase [20, 38]. PN reacts with thiols to form compounds, including nitroso-thiols, with reported vasodilator activity [22, 61, 108-110]. GSH synthesized from glutamate, cysteine, and glycine [111], is the most abundant group of nonprotein thiols found in animal cells, and is probably the most important intracellular antioxidant [111], as evolutionary evidence suggests that GSH evolved as a molecule to protect against oxidant toxicity [112]. GSH is oxidized to glutathione disulfide (GSSG) by GSH peroxidase, an important enzyme known for antioxidant actions, as GSH peroxidase protects the cell against reactive oxygen species including PN and can inhibit enzymes such as lipoxygenases, NO synthases, and NADPH oxidase [113-115]. GSH and GSSG have also been reported to relax isolated canine coronary arteries [116], however the actions of thiols in the control of coronary vascular tone in the intact heart are unknown. Cheung and Schulz examined the effects of GSH and related compounds, GSSG, GSH ethyl ester, and L-cysteine, in isolated rat hearts perfused with Krebs buffer by the Langendorff method [117]. GSH, GSSG, and GSH ethyl ester, but not L-cysteine, produced a concentration-dependent increase in coronary flow [117], and the data suggest that the vasodilation was mediated by

an NO-guanylate cyclase dependent mechanism as the NO synthase inhibitor, NG-monomethyl-L-arginine, prevented the GSH induced increase in coronary flow and attenuated responses to GSSG. Moreover, a specific soluble guanylate cyclase inhibitor [118], abolished vasodilator responses to GSH and to GSSG, and significantly decreased the increases in myocardial cGMP levels, suggesting a role for a soluble guanylate cyclase-dependent mechanism. Additionally, the vasodilator actions of GSH were significantly reduced by SOD [37], suggesting a role of an NO dependent mechanism. Inhibition of GSH reductase, the enzyme that regenerates GSH from oxidized GSSG, abolished GSSG-induced vasodilation [119]. Finally, neither the administration of glibenclamide, an ATP-sensitive potassium channel antagonist [120], or of a non-steroidal anti-inflammatory drug, indomethacin [121], affected the vasodilator actions of GSH and GSSG. The authors concluded that the observed coronary vasodilation from GSH and from GSSG in this model was mediated by an NO- and guanylate cyclase-dependent mechanism, induced by the reactions between GSH and PN to form S-nitrosoglutathione, a NO donor [117]. Endothelial cells have been shown to produce NO and superoxide [122], and that PN can be produced when stimulated [90]. Studies have shown that the vasodilator actions of PN depend upon reaction with thiols, in particular GSH, to form S-nitrosoglutathione [22, 109]. As both NO and superoxide depend on GSH for their vasodilator effects, these data suggest that there is a significant basal production of PN in the isolated heart [117]. Moreover, S-nitrosoglutathione is a potent NO donor that is active at nanomolar concentrations [109, 123].

Although several studies have shown that under physiological conditions, PN produces prolonged vasorelaxant responses [21, 23, 59, 61], however this relaxant action cannot be due to the direct action of PN, because of inherent instability at physiological pH [5, 124]. Moreover, Moro, Darley-Usmar *et al.* demonstrated that the vasorelaxant effects of PN were due to the formation of a secondary, more stable species following reaction with glucose [61]. Dowell and Martin further characterized the actions of PN in isolated rat aortic rings, and also investigated the reactions of PN with D-glucose and with related substances containing functional alcohol groups [125]. Under elevated tone conditions, micromolar concentrations (1 μM-100 μM) of PN induced a concentration-dependent relaxation of aortic rings that were maximal when the rings were denuded of endothelium. Prior incubation of endothelial-absent rings with hemoglobin, a scavenger of NO [1, 34, 64, 65] or incubation with methylene blue, attenuated the response to PN [125]. Although endothelium-present rings were significantly less sensitive to PN, incubation with the NOS inhibitor, L-NAME [126-128], in endothelium-intact rings enhanced this latter effect [125]. Administration of neutralized (decomposed) PN also produced smaller concentration-dependent relaxations, were similar to relaxations produced by comparable concentrations of sodium nitrite, and were without effect when residual nitrite was removed from the neutralized PN solution [125]. These findings were comparable to the findings of Liu, Beckman *et al.* who also observed that relaxation responses to decomposed PN was slower in onset and lower in magnitude in both endothelium-present and endothelium-denuded rings preparations [23]. Moreover, the authors

demonstrated that the observed relaxation from PN was reproducible over a 6 hour period and is in contrast with the development of tachyphylaxis as reported by Villa, Salas *et al.* [59]. Following washout of endothelium-containing ring preparations incubated with high concentrations of PN, there was no effect on subsequent relaxation response to acetylcholine, but a significant depression of subsequent contractions to phenylephrine was observed however, this suppression was reversed in 2 h, or with prior incubation with the nonselective soluble guanylate cyclase inhibitor, methylene blue [34, 54, 64] in the ring preparation [125]. The finding of no significant long-term modulation of phenylephrine-induced contractions by PN is in contrast to the results of Kooy, Royall *et al.* who found attenuation of responses to catecholamines following the development of tachyphylaxis to PN [72]. When D-glucose was added to PN, a more potent relaxant effect was produced in endothelium-absent rings when compared to PN alone [125], whereas the addition of D-glucose with neutralized PN, no increase in relaxant effect was observed [125]. The substitution of glycerol for D-glucose also reacted with PN to produce a potent vasorelaxant response. As thiols have been shown to enhance the release of NO by a number of compounds [35, 52-54], the effects of the thiol-containing amino acid, L-cysteine, were examined. L-cysteine in the presence of PN had no effect on aortic ring tone, but when added to the reaction products of PN and D-glucose or glycerol, L-cysteine potentiated the relaxant actions [125]. However, the addition of membrane impermeant sugars, mannitol or sorbitol, with PN demonstrated no new relaxant activity, but relaxation was potentiated by the presence of L-cysteine. In contrast, when PN was mixed with L-glucose (impermeant sugar) a new substance with potent relaxant properties was formed, which was also potentiated by the presence of L-cysteine [125]. Their findings are in contrast with Moro, Darley-Usmar *et al.* in that relaxation was dependent upon the concentration of glucose in the perfusion medium [61]. It is possible that the reaction between PN and glucose in these experiments forms an organic nitrate or nitrite, as prior experiments have shown that both GTN and amyl nitrite release NO in the presence of thiols, such as L-cysteine [65, 129, 130]. Moreover, PN had been shown to react with plasma thiols [63], and with albumin [57]. These experiments demonstrated that PN can react with glucose and other compounds with functional alcohol groups to form vasorelaxant species, but that PN as studied in this preparation is not dependent upon this reaction for expression of vasorelaxant activity [125].

Prior studies have shown that the actions of NO involve stimulation of guanylate cyclase [2, 21, 23], and of a role for tissue GSH in the vascular relaxation response to PN [21, 117, 131]. Moreover, PN induces accumulation of cGMP in a GSH-dependent manner in endothelial and smooth muscle cells, and PN produces thiol-dependent stimulation of purified guanylate cyclase [109]. Davidson, Kaminski *et al.* found that micromolar concentrations of nitrogen dioxide, a key metabolite of NO, produced prolonged relaxation of isolated endothelium-denuded rings of bovine pulmonary arteries precontracted with potassium [131]. Additionally, it was found that relaxation in response to nitrogen dioxide involved the nitrosation of GSH, and possibly other tissue thiols, to release NO over prolonged periods of time. Moreover, it was concluded that chronic exposure to PN and to

nitrogen dioxide causes tissue GSH depletion and the cytotoxic aspects of these reactive nitrogen species [29, 44, 57, 91, 132-135] are expressed [131]. In a subsequent study, Davidson, Kaminski *et al.* examined the role of endogenous PN formation in endothelium-denuded bovine pulmonary arterial rings during elevated levels of NO [136]. These authors questioned the role of the large concentrations of both PN and NO necessary to obtain vascular relaxation in various preparations [21, 23, 131]. The exposure of precontracted rings to nanomolar concentrations of NO (50 nM) produced prolonged relaxation and an increase in endogenous release of NO, which was reduced following depletion of tissue GSH with diethyl maleate (DEM) [21, 131]. Moreover, the induced NO relaxation in this preparation was reversed by methylene blue, a known guanylate cyclase inhibitor [21, 34, 38, 45, 54], or by oxyhemoglobin, a scavenger of NO [38, 59, 61]. The authors also found that by inhibiting superoxide production with diphenyliodonium, an agent that interferes with the catalytic cycle at the flavin site [137, 138], or by scavenging superoxide with SOD [20, 37, 38, 45, 46, 54, 76, 139] or with Tiron [140-142], the NO-mediated relaxation was reversed [136]. Moreover during exposure to exogenous NO, a marked increase in PN formation was detected, suggesting that PN participates in prolonging the initial relaxation to NO either through a thiol trapping of NO or through NO regeneration [136].

The role of tissue thiols as a target of PN is supported by the evidence that PN oxidizes plasma thiols [63], albumin [44], as well as glutathione in erythrocytes [143]. Glutathione enhances PN to release NO and activates soluble guanylate cyclase [21, 109] and the use of thiol-depleting agents block PN-induced elevation of cyclic GMP [109], and block the release of NO and relaxation in bovine pulmonary arteries [21]. Furthermore, PN has been shown to react with sugars and compounds with alcohol side groups to form nitrated or nitrosated derivatives with NO-releasing properties [61, 125] that can be enhanced by glutathione or L-cysteine [61, 125]. Enhanced release of NO from thiols has been shown with a wide variety of organic nitrates and nitrites [130]. However uncertainty exists as to which of the functional groups of L-cysteine, thiol, amino, and/or carboxylic acid are involved in the release of NO. Dowell and Martin investigated four groups of compounds; 1) analogues with a free thiol group with or without a free amino or carboxylic acid group such as D-cysteine, the optical isomer of L-cysteine; L-cysteine ethyl ester, an esterified carboxylic acid; N-acetyl-L-cysteine, a compound with an acetylated amine; and reduced glutathione, a tri-peptide containing L-cysteine; 2) analogues lacking a free thiol but retaining a free amino and carboxylic acid groups, such as S-methyl-L-cysteine, a methylated thiol group; oxidized glutathione, two molecules of glutathione conjugated through a disulphide bridge; and L-alanine, another amino acid that is similar in structure to L-cysteine but lacking the thiol functional group; 3) analogues retaining an amino group but lacking a free thiol or carboxylic acid group, such as L-cystine diethyl ester, a compound containing two molecules of L-cysteine ethyl ester conjugated through a disulphide bridge; and L-alanine ethyl ester, L-alanine with an esterified carboxylic acid group; and finally 4) N-acetyl-L-alanine, L-alanine with an acetylated amine group, a compound that lacks free thiol and amino groups but retains the carboxylic acid function. Dow-

ell and Martin [144] investigated the interactions of PN with these compounds in isolated endothelium-denuded rat aortic rings under similar conditions to that described by Martin, Furchgott *et al.* [145]. In these experiments, these authors found that a micromolar concentration of PN (1 μM) incubated for 10 min in the aortic ring preparation, followed by repeated washing, produced profound depression of phenylephrine-induced contraction, that could be maintained for at least 1 hour, but was reversible [144]. As PN has a short half-life [5, 124], these results suggest PN reacts to form NO-releasing found in the vascular rings or with constituents in the bathing medium. These results also confirmed that PN has little to no effect on long-term depression of endothelial-induced vasoconstriction [125], which are in contrast with the findings of Villa, Salas *et al.* [59]. As thiols enhance the release of NO from a variety of compounds [146], administration of reduced glutathione or of L-cysteine were without effect on control rings (phenylephrine-contracted), however, micromolar concentrations of PN, followed by the addition of L-cysteine, or analogues of L-cysteine that contain a free thiol group such as D-cysteine, L-cysteine ethyl ester, N-acetyl-L-cysteine or reduced glutathione, induced relaxation of similar magnitude to that observed with L-cysteine [144]. In contrast, analogues lacking free thiols such as S-methyl-L-cysteine, L-alanine, or oxidized glutathione, induced no relaxation to phenylephrine contracted rings following exposure to PN [144]. These data suggest that analogues containing free thiols are necessary for the formation of NO-releasing compounds [144]. Furthermore, these authors investigated analogues lacking a free thiol group, but retaining the amino group, such as L-cystine diethyl ester and L-alanine ethyl ester and found that these analogues once mixed with PN, formed products with potencies similar to that of analogues lacking only the thiol group [144]. Finally N-acetyl-L-alanine, a compound with a substituted amino group that lacks a free thiol, failed to generate relaxant activity following exposure to PN [144]. In summary, PN when combined with species containing a free thiol group generated similar relaxant responses, but when mixed with analogues lacking thiols, formed products with relaxant activities significantly less potent than when compared to L-cysteine, and that analogues with a free amino group, but no thiol or carboxylic group function, formed products with similar relaxant properties to compounds lacking only the thiol group. It appears that PN can react with L-cysteine to form novel relaxants in which the activity is derived from the formation of a S-nitrosothiol, and with less activity when forming a N-nitroso derivative [144].

Studies from several laboratories suggest that vascular smooth muscle relaxation induced by nitrogen-oxide containing vasodilators such as GTN, SNP and nicorandil are mediated by increases in the levels of the second messenger, cyclic GMP [34, 147]. It is also known that the cholinergic neurotransmitter, acetylcholine, also indirectly utilizes cyclic GMP as a second messenger to cause vasodilation [1]. However, the mechanisms by which cyclic GMP produce relaxation of vascular smooth muscle remain to be defined. The effect of cyclic GMP on smooth muscle calcium homeostasis such as plasma membrane Ca^{++} influx, sarcoplasmic reticulum Ca^{++} release, and plasma membrane Ca^{++} extrusion have been studied [148-151]. The role of potassium channels in hyperpolarization induced by nitrates and by acetylcholine

has been investigated [152, 153]. Benkusky, Lewis *et al.* examined the role of ATP-sensitive potassium channels in the response to PN in the pentobarbital-anesthetized rat [72, 75]. PN in doses of 1 to 10 µM/kg produced dose-dependent decreases in mean arterial pressure and in hindquarters and mesenteric vascular resistances, but with repeated 10 µM/kg injections, tachyphylaxis occurred [154]. Moreover, following the development of tachyphylaxis to PN, responses to acetylcholine and of prostacyclin were attenuated, whereas responses to an NO donor were not changed [154]. Responses to acetylcholine and to prostacyclin were depressed following the development of tachyphylaxis to PN. These results confirm earlier results that PN is a significant vasodilator in the systemic circulation of the rat, and that tachyphylaxis develops [72, 75], and subsequently induces hypertension [75]. Tachyphylaxis to PN also reduces responses to acetylcholine and to prostacyclin, by blocking smooth muscle ATP-sensitive potassium channels [154], as recent work has shown that PN may hyperpolarize vascular smooth muscle by activation of ATP-sensitive potassium channels [82].

A number of proteins with cysteine residues can be nitrosated [110, 155]. Both S-nitrosylated low-molecular weight thiols, such as S-nitrosoglutathione, and protein thiols, such as S-nitrosoalbumin, have been detected in biological fluids as a consequence of endogenous nitrogen-oxide metabolism and exogenous NO administration [156, 157]. S-nitrosothiols can serve as NO donors when activated by homolytic cleavage to form NO radicals [158, 159], and it is possible that the hemodynamic effects of organic nitrates and PN may involve the formation of biologically active S-nitrosothiols [21-23, 54, 59]. Prior investigations with L-penicillamine, a natural degradation product of all penicillins has structural resemblance to the amino acids, cysteine, methionine, valine, and threonine [160], have shown that this agent attenuated vasodilator responses to L-S-nitrosocysteine [161, 162] suggesting that L-penicillamine blocks S-nitrosothiol recognition sites. Graves, Lewis *et al.*, 1998 [163] examined the role of S-nitrosothiols and PN in pentobarbital-anesthetized rats [72, 75]. PN (0.5 µM-10 µM). The S-nitrosothiols (L-S-nitrosocysteine, L-S-nitroso-glutathione, S-nitrosoalbumin) produced dose-dependent decreases in mean arterial pressure and in mesenteric and hindquarter vascular resistances whereas decomposed PN produced minor effects [163]. Whereas the hypotensive and vasodilator effects of the S-nitrosothiols were significantly reduced following administration of L-penicillamine, the hemodynamic actions of PN were not affected by the S-nitrosothiol recognition site inhibitor [163]. These findings suggest that in the systemic circulation, formation of low-molecular-weight S-nitrosothiols are unlikely to be primarily responsible for the hemodynamic effects of PN [163]. These findings also give indirect support to the hypothesis that the mechanism of PN may be due to activation of soluble guanylate cyclase and the subsequent generation of cGMP, as these findings are supported by other studies showing that inhibition of guanylate cyclase markedly attenuated the relaxation observed by PN in isolated pulmonary arteries [21], and that SOD potentiated the vasorelaxant effects of PN [22], and/or PN-mediated vasorelaxation may occur through hyperpolarization of vascular smooth muscle through activation of ATP-sensitive potassium channels [163].

Reactive nitrogen and oxygen species, such as PN, are continuously generated and play important roles in pathologic processes [22, 56, 58, 164-166]. Endogenous antioxidants constitute the first line of defense against oxidant-induced injury, but under chronic pathologic conditions, these defenses can be overwhelmed allowing these reactive species to target tissues and impair important processes [63, 167-169]. A major extracellular antioxidant, uric acid, may minimize PN-mediated damage to tissues [63, 100, 170]. In this study, Skinner, White *et al.* examined the reaction between uric acid and PN in vessel reactivity studies in an isolated aortic ring model [171]. Uric acid was oxidized in the presence of micromolar concentrations of PN resulting in a previously unidentified uric acid derivative in human plasma [171]. This derivative resulted in endothelium-independent relaxation of rat aortic rings that was reversed by the administration of oxyhemoglobin, a NO scavenger [38, 59], or following administration of a cyclic GMP synthesis inhibitor [38, 40, 59, 172-174]. Endothelium-absent aortic ring segments displayed similar dose-response characteristics to the uric acid derivative when compared to endothelium-intact ring segments [171]. These results suggest that the NO-donating properties of the uric acid product are not mediated by NO release from the endothelium. NO release from the nitrated uric acid product was electrochemically confirmed and was not thiol-dependent [171], in contrast to the findings that organic nitrites release NO [66]. The authors suggested that uric acid is more susceptible to oxidation by PN than by other purines studied in this preparation (hypoxanthanine and xanthine), or in purine oxidation products (allantoin and parabanic acid) [171]. Finally, this uric acid nitration/nitrosation product may play a role in pathologic states by releasing NO, that can decrease vascular tone and increase tissue blood flow [171].

PN and hydrogen peroxide can generate hydroxyl radicals [5, 165, 175], that produce tissue injury [93, 115, 176-178], and produce pronounced cerebral arteriolar dilation in low concentrations [80, 82], as both radicals dilate cerebral arterioles by opening ATP-sensitive potassium channels [82]. Wei, Kontos *et al.* examined the roles of these radicals on vascular responses and how these radicals are modified by radical scavengers in a feline cranial window model [179]. The authors investigated the role of three antioxidants, dimethylsulfoxide (DMSO), salicylate, and L-cysteine, on cerebral arteriolar dilation following topical application of PN and hydrogen peroxide [179]. The effect of these antioxidants on the vasodilation caused by pinacidil and cromakalim, two known openers of ATP-sensitive potassium channels were examined [152, 180, 181]. The authors confirmed that PN and hydrogen peroxide produced cerebral arterial dilation by activation of potassium channels [82, 182] and that the responses were inhibited by the antioxidants [179]. However, the two water-soluble antioxidants, salicylate and L-cysteine, were more effective in inhibiting the vasodilatation from PN than that of hydrogen per-oxide, whereas the lipid-soluble agent, DMSO, was more effective in inhibiting the vasodilatation from hydrogen peroxide than PN, and that all three antioxidants inhibited the responses in low concentrations. Their findings suggest that the sites of action for these two reactive oxygen species may be different, as cells are defended against superoxide by SOD and are defended against hydrogen peroxide by catalase and glu-

tathione peroxidases [179]. Furthermore, these oxidants also inhibited responses to two potassium channel openers, pinacidil and cromakalim [179]. The variation in effectiveness of the antioxidants suggest that reactive species act at different sites, one in a water-soluble environment and the other in a lipid-soluble environment and can inhibit ATP-sensitive potassium channels [179].

The formation and action of PN can be detected by measurement of nitrotyrosine residues [183]. In normal pregnancy, faint nitrotyrosine staining is found in the endothelium of placental villous tissue, whereas, in preeclampsia and/or intrauterine growth restriction, moderate to intense nitrotyrosine staining is seen in villous vascular endothelium, as well as in surrounding vascular smooth muscle and villous stroma [184]. The presence of nitrotyrosine residues, particularly in the endothelium, may indicate the formation and action of PN, resulting in vascular damage that contributes to increased placental vascular resistance [184]. In preeclampsia, a major cause of maternal and fetal morbidity and mortality appears to be due to impaired vasodilator function and increased vasoconstrictor function due to disturbed endothelial barrier function [185]. Superoxide production is enhanced in pathologic processes [37, 46, 186], and has been shown to interfere with the actions of NO [37, 46, 165]. In addition to toxic effects, PN may also act as an endogenous modulator of endothelial cell function [21, 23]. Okatani, Watanabe *et al.* evaluated the role of superoxide and of PN in regulating tone in the human fetoplacental vasculature [187]. In this study, helical sections of human umbilical arteries with intact endothelium were obtained from healthy pregnant women. Under increased tension conditions induced by potassium chloride, the authors demonstrated that superoxide is a potent vasoconstrictor, whereas PN is a vasodilator. The authors suggested that the vasorelaxation induced by exogenously PN in this model has biological relevance in the regulation of the human umbilical artery [187]. Subsequent studies in this model demonstrated that the administration of melatonin reduced the vasorelaxant properties of SIN-1, 3-morpholinosydomine, a sydnonimine that is known to release superoxide and NO, and form PN under physiological conditions [58, 188]. These authors suggested that melatonin significantly suppresses the vasorelaxant effect of SIN-1 due to the scavenging of PN [189].

Superoxide generated by oxidative stress in vascular disease can decrease endothelial-dependent vasorelaxation [1, 37, 135, 190, 191]. Moreover, prior studies may only assay the effects of vasorelaxants on one vascular bed with little to no comparison of the responses in multiple vascular beds from the same species. Pagano, Griswold *et al.* compared responses to SOD in multiple vascular beds in the rabbit, and to observe the capacities of these vascular beds to produce superoxide as well as NO, and finally, to observe the responses of the vascular beds to elevated levels of superoxide [192]. Using a lucigenin chemiluminescence assay, these authors found that baseline superoxide levels were higher in the carotid arteries than in the thoracic aorta, however, vasodilator responses to acetylcholine were not significantly different. Following treatment with a SOD inhibitor, superoxide levels were significantly higher in the carotid artery and in the abdominal aorta when compared to the thoracic aorta. The authors found that SOD inhibition would significantly reverse the endothelial-dependent response to acetylcholine

in the thoracic aorta, but not in the carotid artery. Moreover, the SOD inhibition resistant relaxation to acetylcholine in the carotid artery was blocked by L-NAME, a NO synthase inhibitor. These results suggested that PN, or possibly a superoxide resistant NO donor, is involved in carotid artery relaxation. Their findings suggested that a greater production of nitrite and of superoxide occurs in the carotid artery and that PN is the relaxing agent that is resistant to higher levels of superoxide in this artery, and suggests that PN is a superoxide resistant mediator [192].

Prior studies have shown that the relaxant effect of PN may be due to its oxidant properties, the nitrosylation of tissue thiols, or stimulation of cGMP production, and activation of ATP-sensitive potassium channels [22, 29, 163, 183, 193]. However, the differences observed among these studies may be related to different experimental protocols and species used in the studies. The mechanisms underlying PN induced vasodilation in isolated blood vessels was studied by Li, Li *et al.*, 2004, in an isolated canine cerebral arterial model [194]. Under basal conditions, PN (10^{-10} to 10^{-6}M) reduced ring tension in this preparation. Moreover, under elevated tone conditions, PN was able to elicit concentration-dependent relaxation with or without the presence of endothelium, with no significant differences observed in the response [199]. These data were in agreement with studies in other arterial ring preparations from different animals [23, 125, 144], and are in agreement with the concept that the endothelium has no significant role in the modulation of PN responses [23]. However, these findings are in contrast to observations in a cerebral smooth muscle cell preparation and in middle cerebral arteries of the rat where PN induced contraction [195, 196]. In this study, the PN-induced relaxation was reproducible without the development of tachyphylaxis, suggesting that the action of PN is reversible and does not cause vascular damage. The addition of methylene blue or of 1H-[1,2,4]oxadiazolo-[4,3-alpha]quinoxalin-1-one (ODQ) to the ring preparations significantly reduced the relaxation induced by PN. The observation that methylene blue, a nonselective soluble guanylyl cyclase inhibitor [21, 34, 38, 45, 54], or of ODQ, a specific soluble guanylyl cyclase inhibitor [197, 198], markedly inhibited the relaxations induced by PN suggest that action of PN is mediated through a cGMP-dependent mechanism. The addition of calyculin A to the preparation abolished PN-induced relaxation. The findings with calyculin A, which can inhibit myosin-light-chain phosphatase [199], suggests that activation of myosin phosphatase is an additional mechanism by which PN can induce relaxation in this preparation. In these experiments the authors found that tetraethylammonium chloride, a nonspecific potassium channel inhibitor, significantly decreased PN-induced relaxations in a concentration-dependent manner, but that PN had no effect on rings that were precontracted by high concentrations of potassium chloride. These findings suggest that PN decreases muscle tone by activating potassium channels. Additionally, in this preparation, the authors examined the role of Ca^{++} and observed that PN could reduce the magnitude of calcium-induced contractions of high K^+-depolarized aortic rings in a concentration dependent manner. These findings suggest that PN may block the entry of extracellular calcium across the vascular smooth muscle membrane. In this series of experiments, the authors examined a role for NO-release and found that L-NMMA

and L-NAME, two nonselective NOS inhibitors, or L-arginine, a substrate for NO synthase, did not modulate PN-induced relaxation in this preparation. Finally, the effects of pharmacologic antagonists were examined in this study (diphenhydramine, cimetidine, methysergide, atropine, naloxone, and haloperidol), and it was found that none of the antagonists could influence PN-induced relaxation. In regard to mechanisms, it was observed that PN-induced relaxation in canine cerebral arteries is mediated by increases in cGMP, membrane hyperpolarization *via* potassium channel activation, activation of myosin light chain phosphatase, and interference with calcium entry and release [194].

The development of tachyphylaxis has been reported in some studies, but not in others [59, 61, 72, 75, 105, 194]. Additionally, PN has been reported to induce vasodilation by releasing vasoactive prostaglandins [200, 201]. Although PN causes vasodilation in a number of isolated arterial preparations [21, 23], the responses in the systemic and perfused hindquarters vascular beds and in the isolated perfused lung of the rat were not documented. We investigated the role of PN on vascular responses in these beds and observed that iv injections of PN produced dose-dependent decreases in hindquarters and systemic arterial pressure, whereas injections of PN into the lung perfusion circuit produced increases in pulmonary arterial pressure [202]. Moreover, responses to PN were rapid in onset, short in duration, and repeatable without exhibiting tachyphylaxis. Additionally, repeated injections of PN did not alter systemic, hindquarters, or pulmonary responses to endothelium-dependent vasodilators or to a number of vasoactive agonists that act by various mechanisms, and moreover, did not alter the hypoxic pulmonary vasoconstrictor response. Injections of sodium nitrate or nitrite or decomposed PN had little effect on vascular pressures. In these series of experiments responses to PN were not altered by the presence of meclofenamate, a nonselective cyclooxygenase inhibitor. These studies show that PN has significant pulmonary vasoconstrictor and hindquarters and systemic vasodepressor and vasodilator activities, and that repeated short-term exposure did not impair vascular responsiveness in these vascular beds, and finally that responses to PN are not dependent upon cyclooxygenase product release [202].

Studies have demonstrated that relaxation of feline cerebral arteries occurs upon topical administration of PN [82] and systemic administration of PN produce pronounced vasodilation in pentobarbital-anesthetized rats [154, 163, 203], and that this relaxation involves ATP-sensitive potassium channels [82, 154]. Moreover, tachyphylaxis develops following the administration of PN [59,72,154,163,203], and that the actions of a variety of G protein-coupled receptors agonists are substantially impaired [203]. These observed effects of PN upon G protein-coupled receptor-mediated responses may be related to functional amino acid residues on these proteins that are susceptible to oxidation or to nitration. Graves, Lewis *et al.*, 2005 examined the role that progressive oxidation or nitration of amino acids within ATP-sensitive potassium channels are responsible for the development of tachyphylaxis and that the loss of ATP-sensitive channel function may be due to agonist-induced activation of these channels [204]. Systemic injections of PN in the pentobarbital-anesthetized rat [154, 203], produced significant decreases in mean arterial pressure, and in mesenteric and

hindquarters vascular resistances, with progressive development of tachyphylaxis to PN [204]. In subsequent series of experiments following the development of tachyphylaxis induced by ten injections of PN, responses to the ATP-sensitive potassium channel agonist, cromakalim [152, 179], were reduced, whereas to sodium nitroprusside responses were unchanged. These findings suggest that the development of tachyphylaxis to PN involve the loss of ATP-sensitive potassium channel function that may be due to oxidation or nitration of amino acids found in these channels [204].

The vasorelaxant action of PN has many characteristics in common with NO [61], and this action may be due to oxidation or nitration of amino acids, nitrosylation of tissue thiols, or the stimulation of cGMP production and activation of ATP-sensitive potassium channels [22, 29, 163, 183, 204]. Prior studies in canine cerebral arteries demonstrated that the vasodilator effects of PN are mediated by elevation of cGMP levels, membrane hyperpolarization *via* potassium channel activation, activation of myosin-light-chain phosphatase, and interference with calcium entry [194]. However, the presence of these PN pathways in other blood vessels, such as the aorta is unknown. Li, Li *et al.*, 2005 evaluated the effects of PN in isolated rat aorta segments and under baseline tone conditions, PN failed to alter tension in the aortic rings [205]. However following contraction with phenylephrine, PN elicited concentration-dependent relaxations. No significant differences in relaxation were found in either endothelium-present or endothelium-absent rings. The addition of methylene blue or ODQ reduced relaxation induced by PN, whereas sildenafil significantly potentiated PN induced relaxation. The addition of tetraethylammonium chloride also significantly decreased relaxation induced by PN in a concentration-dependent manner. PN had no significant effect on rings precontracted with high potassium chloride solution. The addition of calyculin A significantly reduced PN mediated relaxation in a dose dependent manner, and PN significantly reduced calcium-induced contractions in potassium depolarized aortic rings. Finally, a variety of pharmacologic agonists and antagonists including L-NMMA, L-arginine, indomethacin, atropine, naloxone, diphenhydramine, cimetidine, glibenclamide, haloperidol, SOD, and catalase did not influence the relaxant responses to PN in this preparation. These finding suggest that PN-induced relaxation of rat aortic rings are mediated by elevations in cGMP levels, membrane hyperpolarization *via* potassium-channel activation, activation of myosin phosphatase activity, and interference with calcium entry [205]. These results confirm earlier studies in canine cerebral arteries and suggest that these mechanisms are found in several vascular beds and in different species [194].

Although the mechanism by which PN alters ATP-sensitive channels is not understood [82, 154], it is unlikely that the oxidant properties of PN are involved since oxidants have been shown to reduce ATP-sensitive potassium channel activity [206, 207]. Moreover, PN mediate vasodilator responses have been shown to be markedly attenuated by the ATP-sensitive potassium channel blocker, glibenclamide [208], but are not attenuated by L-penicillamine, in doses that can reduce the vasodilator responses to L-S-nitrosocysteine, a S-nitrosothiol [163]. Graves, Kooy *et al.* 2006 [209] examined the role of L-penicillamine in the re-

sponses to PN in their pentobarbital-anaesthetized rat model [72, 75]. These authors found that although the initial reductions in mean arterial pressure and in systemic and hindquarters vascular resistances elicited by PN were not decreased by L-penicillamine, the responses were much shorter in duration. Although the ATP-sensitive potassium channel agonist, cromakalim, decreased mean arterial pressure and vascular resistance; these responses were not modulated by L-penicillamine, suggesting this PN scavenger is not a nonselective inhibitor. These findings that the vasodilator actions of PN were markedly attenuated by ATP-sensitive potassium channel blocker, glibenclamide [208], led these authors to suggest that PN can dilate resistance arteries by ATP-sensitive potassium channel dependent and ATP-sensitive potassium channel independent mechanisms. Although PN may initiate vasodilation by formation of S-nitrosothiols; the sustained vasodilation is maintained by the activation of ATP-sensitive potassium channels [209]. Finally their findings found that L-penicillamine can scavenge PN and interfere with the PN-mediated vasodilator mechanisms [209].

In studies in isolated rat pulmonary artery rings, in isolated rat aortic rings, in feline coronary artery rings, or in rat pulmonary and systemic circulations, vasorelaxant responses to acetylcholine were not inhibited following exposure to PN, and tachyphylaxis to the vasorelaxant response to PN was not reported, and in one preparation, responses to acetylcholine were enhanced [25, 105, 125]. However, responses in other vascular beds of the cat are not well described, that led this laboratory to investigate responses of PN in the hindlimb vascular bed of the cat [202, 210]. Injections of PN into the perfusion circuit caused dose-dependent decreases in perfusion pressure. Inasmuch as blood flow was maintained constant, the decreases in perfusion pressure reflect decreases in hindlimb vascular resistance. The hindlimb vasodilator responses to PN were rapid in onset, short in duration, and the vasodilator responses to PN could be repeated without causing tachyphylaxis [210]. The absence of the development of tachyphylaxis to the vasodilator response to PN is in agreement with studies in the systemic and hindlimb vascular bed of the rat, and in isolated coronary arteries from the dog and cat [23, 25, 202]. Although PN has been reported to produce vascular dysfunction, the results of these studies observed that vascular responses of PN to could be repeated as subsequent injections, in doses up to 4000 nmol, had no significant effect on baseline perfusion pressure or on responses to vasoactive agonists [210]. Moreover, repeated short-term exposure to PN had no significant effect on vasodilator responses to acetylcholine, sodium nitroprusside, DEA/NO, or to albuterol. These data suggest that PN does not impair endothelial function in the hindlimb vascular bed. The results showing that vasodilator responses to acetylcholine are not inhibited are consistent with results in the hindquarters vascular bed or in aortic rings of the rat, in isolated cat coronary arteries, and in isolated rat pulmonary arteries where responses to acetylcholine were enhanced by the highest dose of PN studied [25, 105, 125, 202]. These data are in contrast to studies showing that responses to vasodilator agents, including acetylcholine, are reduced after the development of tachyphylaxis to PN [59, 154]. The explanation for differences in results is uncertain, but may be explained by the observation that in studies where responses to acetylcholine were preserved, tachyphylaxis to PN was not observed [25, 105, 125, 202]. The repeated ad-

ministration of PN did not alter vasoconstrictor responses to norepinephrine, U46619, or angiotensin II in the hindlimb vascular bed. These data are in contrast to studies in the anesthetized rat where regional vascular responses to iv injections or exposure to several catecholamines was reduced following systemic administration of PN [125, 203]. The reason for the difference in results is uncertain, but may involve the route of administration used. The results in the hindlimb vascular bed of the cat indicate that PN does not impair alpha- or beta-adrenergic, thromboxane A2, or angiotensin II receptor mediated responses or impair endothelial function under the conditions of these experiments [210]. The mechanism by which PN produces a vasodilator response in the hindlimb circulation was investigated and the role of NO synthase was examined. The role of NO synthase/soluble guanylate cyclase/cGMP dependent pathway in the vasorelaxant properties of PN has been investigated in a number of vascular preparations [21, 23]. However, studies attempting to investigate the role of soluble guanylate cyclase have been hampered by the lack of a potent and selective inhibitor of this enzyme in *in vivo* preparations. Methylene blue has been used but this compound is not selective [211]. However, information involving this pathway can be inferred with the use of selective guanylate or G-kinase inhibitors [212-217]. Our findings with the type 5 cGMP phosphodiesterase inhibitor on responses to PN and the two NO donors, suggest that our observations are in agreement with other studies in that PN acts by a NO-cGMP dependent mechanism [21, 194, 205]. These findings are in contrast with other studies reporting that the NO-cGMP pathway does not play a role, but rather the PARS pathway mediates the vasoactive properties of PN [105]. These results show that responses to PN were not altered by L-NAME in a dose that attenuated vasodilator responses to acetylcholine. These data suggest that responses to PN are not mediated by release of NO from the endothelium. The cyclooxygenase inhibitor, sodium meclofenamate, had no significant effect on the response to PN in a dose that attenuated responses to the prostaglandin precursor, arachidonic acid, suggesting that responses to PN are not dependent on the release of vasodilator products in the cyclooxygenase pathway. It has been reported that decreases in systemic arterial pressure and regional vascular resistance in response to PN are mediated by ATP-sensitive potassium channels and that tolerance to PN involves ATP-sensitive potassium channels in the anesthetized rat [208]. In the present study in the cat hindlimb vascular bed, vasodilator responses to PN were not inhibited by the K^+-ATP channel antagonist, U37883A, in a dose that attenuated responses to the K^+-ATP channel opener, levcromakalim. These data are in agreement with studies in precontracted rat aortic rings where glybenclamide did not influence the relaxant effects of PN, but are in contrast to studies showing that glybenclamide reduced vasodilator responses to PN in the rat [208, 218]. The explanation for the differences in results is uncertain, but again may involve route of administration, species, or vascular bed studied. The observation that the vasodilator responses to PN in the cerebral vascular bed of the cat with a cranial window was attenuated by glybenclamide suggest that the mechanism of the vasodilatory response may depend upon the vascular bed studied in the cat [82]. It has been reported that vasorelaxant responses to PN are dependent on the formation of NO donor-like compounds [21, 61, 219]. The reaction of PN with thiol groups on plasma proteins, sugars, and tissue substituents can

result in the rapid formation of an S-nitrosothiol NO donor compound that has vasodilator properties [61, 125, 219]. It has been hypothesized that the involvement of nitrosothiols which release NO can serve to prolong vasodilator responses to NO [21, 220, 221]. It has been reported that relaxation of isolated arterial segments in response to PN is mediated by the release of NO from an S-nitrosothiol, the activation of soluble guanylate cyclase, and the accumulation of cGMP in vascular tissue [21, 194, 205]. The role of S-nitrosothiol formation has been questioned and in other studies evidence has been presented to show that PN induced vasodilation is not mediated by the formation of S-nitrosothiols [82, 163]. However, the present studies do not provide information on the identity or chemical nature of the species formed from PN, but do provide information on the mechanism of the vasodilator response to PN. Although it is technically difficult to measure cGMP levels in resistance vessels in the hindlimb vascular bed of the cat or rat, the hypothesis that a cGMP dependent mechanism is involved can be investigated by the use of a type 5 cGMP phosphodiesterase inhibitor [213, 214]. In these experiments, the effects of zaprinast, a type 5 cGMP phosphodiesterase inhibitor, on responses to PN and several NO donors were investigated. These results show the hindlimb vasodilator responses to PN, and to the NO donors, sodium nitroprusside and DEA/NO, were enhanced in duration at a time when vasodilator responses to the beta 2 receptor agonist, albuterol, were not altered. These data along with the observation that the type 4 cAMP phosphodiesterase inhibitor, rolipram, did not alter the duration of the response to PN, provides evidence in support of the hypothesis that PN induced vasodilation in the cat circulation is mediated, at least, in part by a cGMP-dependent mechanism [222, 223]. Although experiments with zaprinast provide evidence in support of a cGMP dependent mechanism, additional support for this mechanism could be derived from experiments with inhibitors of guanylate cyclase and of cGMP dependent protein kinase. However, such agents are not currently available for use in the intact cat preparation [210].

ANTI-PLATELET AGGREGATION EFFECTS OF PEROXYNITRITE

Alterations in the bioavailability of endothelium-derived factors play a role in the early course of vascular diseases [224]. As endothelial dysfunction occurs early [225], and subsequently heralds pathologic changes in vascular structure and activation of platelets and leukocytes, these events lead to the development of atherosclerosis and thrombosis [226]. NO has been shown to inhibit platelet aggregation and vessel adhesion [1, 227], whereas superoxide reduces anti-platelet activities of NO [37]. PN is a cytotoxic molecule in inflammatory conditions [104], but the role of this downstream molecule to NO and superoxide had not been fully evaluated on platelet function. Moro, Darley-Usmar *et al.* 1994 investigated the role of this oxidant in suspensions of washed platelets and also in platelet-rich plasma obtained from human subjects [22]. These authors observed that PN (20 µM-200 µM), but not of decomposed PN, when added to washed platelet suspensions produced concentration-dependent increases in aggregation when measured by platelet-

ionized calcium aggregometer [22]. The additions of platelet aggregation inhibitors such as SNAP, prostacyclin, or indomethacin [228, 229] to the washed platelet suspensions had no significant effect on platelet aggregation induced by PN [22]. The addition of PN could also completely reverse the inhibitory effects of SNAP on collagen-induced platelet aggregation, whereas PN would significantly reverse (~50%) the antiaggregatory effects of indomethacin and of prostacyclin [22]. These results suggested that PN could counteract the platelet-inhibitory effects induced by stimulation of cGMP, cAMP, or by inhibition of thromboxane formation [22]. Moreover, the addition of catalase, superoxide dismutase, mannitol, desferrioxamine, or DTPA to the washed platelet suspensions had no effect on platelet aggregation induced by PN [22]. These results suggest that PN has a direct effect on platelets not due to the formation of hydrogen peroxide, superoxide, or hydroxyl radicals. In contrast, the addition of EGTA or RGDS to the washed platelet suspension completely inhibited PN-induced aggregation [22]. These findings that the proaggregatory action of PN can be prevented by RGDS, a IIb/IIIa receptor antagonist, or by EGTA, a calcium-chelating agent that can interfere with exposure of this IIb/IIIa receptor, suggest that PN induces platelet aggregation by directly stimulating platelet receptors [22]. However, these findings of PN-induced aggregation on washed platelets were in contrast to the effects of PN in platelet-rich plasma, where PN (20 µM-200 µM) acted as an inhibitor of platelet aggregation, and inhibited collagen-induced aggregation in platelet-rich plasma [22]. This novel effect of PN could be reversed by prior incubation with hemoglobin suggesting a role for NO formation. The first observation of the inhibitory effect of NO and NO donors on platelet aggregation was made in 1983 [230]. The addition of platelet poor plasma to washed platelets resulted in PN inhibition of platelet aggregation [22]. Moreover, reversal of the aggregatory effect of PN is observed in washed platelets following the additions of low concentrations of human serum albumin, or GSH, but this effect was not seen in the presence of oxidized glutathione (GSSH) [22]. Following the addition of GSH to solutions of PN, significant amounts of NO and S-nitrosoglutathione were detected by chemoluminescence. These findings suggest that PN can be modified and that plasma thiols are candidates since they counteract the actions of oxidants [112, 231]. NO and GSNO are formed in the reaction between PN and GSH with the formation of GSNO involving the hemolytic cleavage of PN yielding nitrites which can nitrosylate thiols [232]. These authors concluded that the actions of PN may be dependent upon the pathologic state and that thiols exert their protective actions by these mechanisms [22]. Moro, Darley-Usmar *et al.* 1995 further investigated the products of the reaction between D-glucose and PN on platelet inhibition [61], and found greater inhibition of aggregation when compared to PN alone, and that the platelet inhibition was dependent on the concentration of D-glucose [61]. Moreover, when L-glucose was substituted for D-glucose, the platelet inhibition were equally effective, and that these effects were reversed by oxyhemoglobin [61].

Platelet aggregation is inhibited by NO, which binds to the heme of platelet guanylate cyclase that increases cGMP

Table 1.   Studies Examining the Vasomotor Properties of PN

Authors	Model	PN Concentrations	Findings	Reference
Liu, Beckman et al.	Isolated canine coronary artery rings precontracted with PGF2a	10 nM to 30 μM	Vasodilation regardless of the presence of the endothelium PN was 7- to 10-fold less potent than NO Tachyphylaxis was not observed	[23]
Wu, Pritchard et al.	Isolated bovine pulmonary arterial smooth muscle rings, potassium-preconstricted Endothelium-intact preparations incubated for 30 min with a high concentration of PN	0.1 μM	Vasodilation regardless of the presence of the endothelium Incubation with PN did not significantly alter relaxation responses to acetylcholine	[21]
Villa, Salas et al.	Isolated perfused rat heart	0.3 μM to 1000 μM, single-dose infusions 30-100 μM	Dose-dependent vasodilation of coronary vascular bed Development of tachyphylaxis in response to 3 to 4 repeated infusions	[59]
Moro, Darley-Usmar et al.	Isolated rabbit aortic strips de-nuded of endothelium & super-fused in a cascade bioassay	30–300 μM	Vasodilation No observed tachyphylaxis Reacts with organic molecules to form an NO donor	[61]
Holcberg, Kossen-jans et al.	Isolated perfused human placental cotyledons preconstricted with the thromboxane agonist, U46619	PN precursor, superoxide 0.53 μM/L infusion combined with NO infusion	Greater vasodilatation observed with combination when compared to an infusion of NO alone	[69]
Kooy, Royall et al.	Mesenteric, renal, and hindquarters blood flow velocity in the rat	10 μM/kg	Initial vasodilation seen in mesenteric and hindquarters beds, but rapid development of tachyphylaxis with repeated administration Attenuation of responses to catecholamines following the development of tachyphylaxis to PN	[72]
Kooy & Lewis	Mesenteric, renal, and hindquarters blood flow velocity rat in the rat	10 μM/kg	Significant decreases in hindquarters and mesenteric resistance, but with minor decreases in renal resistance Development of a sustained increase in mean arterial pressure and in vascular resistance, following PN exposure	[75]
Wei, Kontos et al.	Cerebral arterioles of the anesthetized cat with cranial windows	1, 3, 5 μM	Dose-dependent vasodilation Glyburide inhibited the vasodilator response to hydrogen peroxide or PN	[82]
Lefer, Scalia et al.	Isolated rat aortic rings	1) 5 μM 2) 50 nM-500 nM	1) Significant relaxation 2) No detectable effect Macromolar range exerts significant vasorelaxant effects Does not impair endothelium dependent responses	[26]
Chabot, Mitchell et al.	Isolated pulmonary arterial ring preparation under elevated tone conditions following contraction with the potent thromboxane mimetic, U46619	10 nM to 100 μM	Vasorelaxation PN augmented the relaxations induced by the endothelium-dependent vasodilator, acetylcholine PARS inhibition significantly reduced the relaxation responses to PN	[105]
Dowell and Martin	Isolated rat aortic rings, under elevated tone conditions	1 μM-100 μM	Dose-dependent relaxation No development of tachyphylaxis No significant long-term modulation of phenylephrine-induced contraction by PN PN can react with glucose and other compounds with functional alcohol groups to form vasorelaxant species	[125]

(Table 1) contd.....

Authors	Model	PN Concentrations	Findings	Reference
Dowell and Martin	Isolated endothelium-denuded rat aortic rings, precoordinated with phenylephrine	1 μM	Profound depression of phenylephrine-induced contraction No effect on long-term depression of endothelial-induced vasoconstriction Analogues containing free thiols are necessary for the formation of NO-releasing compounds	[144]
Benkusky, Lewis et al.	Mesenteric, renal, and hindquarters blood flow velocity rat model	1 μM/kg - 10 μM/kg	Dose-dependent decreases in mean aortic pressure and in hindquarters and mesenteric resistance Rapid development of tachyphylaxis with attenuated responses to acetylcholine and prostacyclin	[154]
Graves, Lewis et al., 1998	Mesenteric, renal, and hindquarters flow velocity rat model	0.5 μM-10 μM	Dose-dependent decreases in mean aortic pressure and in hindquarters and mesenteric resistance S-nitrosothiols are unlikely to be primarily responsible for the hemodynamic effects of PN	[163]
Skinner, White et al.	Isolated aortic ring rat model	0.05 to 1 mM	Dose-dependent relaxation Uric acid derivative from PN and albumin resulted in endothelium-independent relaxation	[171]
Wei, Kontos et al.	Feline cranial window model	0.5 to 5 μM	Dose-dependent relaxation Water-soluble antioxidants are more effective in PN inhibition	[179]
Li, Li et al., 2004	Isolated canine cerebral arterial rings model, baseline tone and precoordinated with PGF2a	10^{-10} to 10^{-6}M	Dose-dependent relaxation with or without endothelium Endothelium has no significant role in the modulation of PN responses Activation of myosin phosphatase May block the entry of extracellular calcium across vascular smooth muscle membranes	[194]
Nossaman, Dabisch et al.	Isolated perfused lung of the rat Systemic and perfused hindquarters vascular beds	1.6 to 4.8 μM 37 to 3700 nM	Dose-dependent vasorelaxation in hindquarters Vasoconstriction in the lung No observed tachyphylaxis Not dependent upon cyclooxygenase product release	[202]
Graves, Lewis et al. 2005	Mesenteric, renal, and hindquarters blood flow velocity rat model	10 μM/kg	Development of tachyphylaxis involves the loss of ATP-sensitive potassium channel function	[204]
Li, Li et al., 2005	Isolated rat aorta rings model	10^{-8} to 10^{-4}M	Dose-dependent relaxation Mediated by elevations in cGMP levels, membrane hyperpolarization *via* potassium-channel activation, activation of myosin phosphatase activity, and interference with calcium movement and calcium entry	[205]
Graves, Kooy et al. 2006	Mesenteric, renal, and hindquarters blood flow velocity rat model	1 to 20 μM/kg	Dilates resistance arteries by ATP-sensitive potassium channel dependent and ATP-sensitive potassium channel independent mechanisms	[209]
Nossaman, Bivalacqua et al.	Hindlimb vascular bed of the cat	400 to 4000 nM	Vasodilator response not inhibited by K^+ ATP channel blocker Response mediated by a cGuP-dependent mechanism No impairment of endothelial function Repeated administration did not alter responses to norepinephrine, U46619, or angiotensin II	[210]

NO, nitric oxide; cGMP, cyclic guanosine monophosphate; ATP, adenosine triphosphate; PN, peroxynitrite; U46619, thromboxane A2 mimic.

formation and inhibits platelet activation [233]. Hydrogen peroxide is a weak activator of platelets [234], and may be formed when platelets are activated [235, 236]. Although PN has recently been shown to induce both platelet-stimulatory and -inhibitory effects [22, 61], and that both hydrogen peroxide and PN are formed under inflammatory conditions [237], the role of hydrogen peroxide and PN on platelet function are uncertain. Naseem and Bruckdorfer investigated this combination of radicals in platelet-rich plasma obtained from healthy human volunteers [238]. The addition of physiologically relevant concentrations of hydrogen peroxide did not inhibit thrombin or collagen-induced platelet aggregation, whereas the addition of NO was effective [238]. However, the simultaneous administration of NO and hydrogen peroxide to this suspension inhibited the agonist-induced aggregation that was greater than the inhibition caused by NO alone [238]. The addition of the NO scavenger, carboxy-PTIO [239], resulted in complete reversal of NO-induced inhibition of aggregation, even when hydrogen peroxide was simultaneously added with NO [238]. These findings suggest that there is enhanced NO platelet sensitivity in the presence of hydrogen peroxide, but that the inhibitory activity was dependent upon NO and suggest that hydrogen peroxide may have a positive role in the amplification of the action of NO [238].

The earlier reports by Moro, Darley-Usmar *et al.* 1994, 1995 demonstrated that PN could affect platelet aggregation under different experimental conditions, platelet-rich plasma and washed platelet suspensions, but were investigated with the use of one aggregating agent, collagen [22, 61]. Prior investigations [5, 21-23, 240] with PN were undertaken with PN synthesized from NaNO2 and H2O2 in an acid medium, with the resultant mixture containing contaminants that could alter experimental results. To further study the effects of PN on platelet aggregation in different media and to observe the effects of PN in the presence of more conventional platelet activators, Yin, Lai *et al.* examined the role of PN using a synthesis process that was free of H2O2 and NaNO2, on platelet-rich plasma, platelet-poor plasma, and with albumin obtained from human volunteers [241]. Increasing concentrations of PN (50 µM-200 µM) added to the presence of different platelet aggregation inducing agents, such as ADP, collagen, thrombin, and U46619, inhibited platelet aggregation in a dose-dependent manner [241]. Moreover, the authors observed that the addition of PN could reverse platelet aggregation previously induced by collagen, ADP and thrombin. These findings suggest that PN nitrosylates plasma proteins [241] that prevents phosphorylation of tyrosine residues needed for calcium influx or mobilization necessary for platelet aggregation [242]. As PN is a potent tyrosine nitrating agent [183], and that elevated levels of nitrotyrosine have been observed in inflammatory states [243], it is possible that PN induces nitration of tyrosine that blocks tyrosine phosphorylation and results in the inhibition of the primary phase of aggregation [241]. The anti-aggregatory effects of PN were dependent upon the presence of nitrite mixed with PN as complete inhibition of ADP-induced platelet aggregation was obtained with a lower dose of PN formulated with nitrite contamination when compared to the nitrite-free PN preparation [241]. However, PN that had been preincubated with either platelet-poor plasma or with albumin did not alter inhibition of aggregation when added to

platelet-rich plasma and were comparable to results with PN under initial experimental conditions. These latter findings suggest that PN inhibition of aggregation required protein nitrosylation [241] and not the release of NO as suggested by other studies [5, 21] and are supported by the observation that intracellular cGMP levels are only increased at the highest concentration (200 µM) of PN studied in this preparation [241].

Nitrosative stress and PN can generate NO donors [21, 22, 61]. These findings as well as other studies [25, 26], suggest that PN can exert a cytoprotective function, whereas PN oxidative stress worsens pathologic conditions [244-246]. Certainly, the imbalance between NO and superoxide has been implicated in inflammatory disease states [57, 165, 247-249]. PN can promote aggregation of platelets [22], however in the presence of low concentrations of plasma, PN can inhibit this aggregation [241]. PN may nitrate tyrosine residues in platelet proteins and interfere with platelet aggregation [250], whereas PN can also induce platelet aggregation [22, 61, 241, 251]. These studies as well as others suggest that PN can participate in both nitrosative and oxidative stress [63, 252]. Previous studies are unclear about the role of P-selectin in the aggregation of platelets [22, 61], and in some studies of platelet activation, P-selectin expression were decreased in the presence of PN [250]. P-selectin is an adhesion glycoprotein found on platelets and is rapidly expressed following platelet activation or by activated endothelium [253]. These dual actions of PN were investigated by Brown, Moro *et al.* on washed platelets and in human platelet-poor plasma by examining P-selectin expression, platelet calcium concentrations, and aggregation in platelets [254]. These authors observed that PN (50 µM-400 µM), but not decomposed PN, produced concentration-dependent increases in platelet P-selectin expression, and increases in platelet Ca^{++} concentration [254]. The results in washed platelets indicate that PN induces aggregation by mobilization of Ca^{++} from the extracellular compartment, as shown in earlier studies that pro-aggregatory effects of PN were inhibited by EGTA, a Ca^{++} chelating agent [22]. In contrast, PN (50 µM-400 µM), but not decomposed PN, inhibited collagen-induced P-selectin expression and when platelet-poor plasma was added to PN, the inhibition was further enhanced [254]. These data suggest PN produces a mediator that is capable of inhibiting P-selectin expression in platelets. The role of endogenous NO on the inhibitory effects of PN was examined, and preincubated washed platelets with the soluble guanylate cyclase inhibitor, ODQ [197,198,254], or with the NO scavenger, oxyhemoglobin [254]. In the presence of ODQ, PN-induced P-selectin expression was enhanced 2- to 3-fold achieving maximal activation in washed platelets, and ODQ reversed PN inhibition of collagen-induced P-selectin up-regulation [254]. In contrast, oxyhemoglobin, reversed PN-induced inhibition of collagen-induced aggregation. The data that collagen-induced P-selectin expression was sensitive to oxyhemoglobin or ODQ suggest activation of NO-soluble guanylate cyclase is important in modulating platelet aggregation [254]. PN has dual effects on platelets due to the conversion to NO or NO donors. PN (100 µM-3000 µM) produced a concentration-dependent depletion of platelet membrane thiols, but at concentrations higher than associated with the release of NO or a NO donor [254]. PN-induced platelet activation seems to be dependent upon thiol

oxidation and an increase in intracellular Ca^{++}. To investigate the actions of thiol oxidation, washed platelets were preloaded with the fluorescent Ca^{++}-sensitive probe, FURA-2, then washed and treated with PN. PN (200 µM-400 µM) produced a concentration-dependent increase in intracellular calcium levels, that only became significant at 300 µM or above [254]. These data suggest that PN can exert dual effects on platelets, activation or inhibition depending upon the conditions. PN-induced platelet activation seems to be dependent upon thiol oxidation and increase in intracellular Ca^{++}, whereas the inhibitory effects of PN is dependent upon the conversion to NO or a NO donor [254]. The inhibitory NO-dependent effects of PN occur at lower concentrations than the activating effects. The authors concluded that these observations are in agreement with other studies showing both damaging (activating) and cytoprotective (inhibiting) effects [254].

PN or PN-generating systems induce nitration of tyrosine residues in proteins *via* intermediate formation of tyrosyl radicals and nitrite [255]. Studies have shown repeated exposure of platelets to PN increase levels of nitration [250]. Moreover, the generation of tyrosyl radicals is a critical first step in the formation of thromboxane A2, a potent lipid mediator of platelet activation [234, 241]. As the effects of PN on COX protein nitration and on prostaglandin formation in platelets have not been investigated, Boulos, Jiang *et al.* investigated these effects in washed platelet suspensions obtained from human volunteers [256]. These authors observed that PN (100 µM-190 µM) would produce a moderate aggregatory effect, which was less than the effect obtained with arachidonic acid. However, treatment with lower PN concentrations (4 µM-50 µM) inhibited arachidonate-induced aggregation in a dose-dependent manner [256]. Preincubation with a polyphenolic tea antioxidant that inhibits tyrosine nitration [257], significantly reduced the inhibitory effect of PN on arachidonate-induced platelet aggregation. The exposure of COX-1 and COX-2 to PN generated COX proteins that were immunoreactive to 3-nitrotyrosine antibodies [256]. COX-1 and COX-2 contain 27 tyrosine residues, suggesting that PN nitration of COX is likely to impair formation of tyrosyl radicals necessary for enzymatic activity [256]. In contrast, PN stimulated prostaglandin biosynthesis when added to arachidonic acid and increased PGH2 levels or peroxide tone required for eicosanoid signaling. These results suggest that PN targets both COX and arachidonic acid and modulates eicosanoid synthesis. Reactive species are often generated in inflammatory conditions and the timing of these events is important in predicting the net effect of PN on platelets [256].

PN oxidizes sulfhydryl groups and induces membrane lipid peroxidation in the arterial wall [57, 188], and stimulates nitration or nitrosation leading to impaired cellular function and alteration in signaling pathways [191, 258-261]. Nowak and Wachowicz studied the actions of acute and chronic exposure of PN on platelet function in platelet-rich plasma obtained from pigs [262]. The addition of PN (0.031-0.062 mM-2 mM), but not decomposed PN to ovine platelets, produced immediate, concentration dependent inhibitory effect on thrombin- and to collagen-induced platelet adhesion, a first step in platelet activation [262]. Following

platelet incubation with PN (0.25 mM-2 mM), lipid peroxidation increased in a dose dependent manner, whereas significant changes in platelet proteins were not seen [262]. These data suggest that peroxidation of platelet lipids induced by PN changes membrane structure and affects specific receptors inhibiting platelet adhesion or aggregation [262]. These authors suggest that lipid peroxidation of platelet membranes by PN modifies signal transduction and alters responses to thrombin and collagen, however the PN concentrations used in these experiments were high and may not represent physiologic conditions [262].

The actions of PN on platelet function are concentration-dependent, PN stimulates platelet aggregation at high concentrations [22], whereas at lower concentrations, or in presence of plasma, inhibits platelet aggregation [22, 241, 254]. This inhibition is cGMP-dependent because PN nitrosates thiols to form nitrosothiols that release NO and inhibit platelet activation [21, 22, 254]. However, PN also nitrates phenols such as tyrosine and phenylalanine that affects cellular function [134, 263, 264]. Protein nitration may interfere with protein phosphorylation signaling pathways with recent studies showing that nitrated proteins form in platelets undergoing collagen-induced aggregation [265-267]. Low, Sabetkar *et al.* examined the effects of PN on platelet function and to further understand the role of the NO-cGMP pathway and protein nitration in washed platelets and in platelet-rich plasma from human volunteers [268]. PN (3 µM-10 µM), but not decomposed PN, inhibited thrombin-induced platelet aggregation, and the presence of ODQ, an inhibitor of soluble guanylate cyclase [197, 198, 254], did not influence the inhibition of thrombin-induced aggregation by PN [268]. The ability of PN to induce cGMP formation was compared to authentic NO solutions and these authors observed that PN, when compared to NO, did not significantly increase the synthesis of cGMP above basal levels. These data suggest that the platelet inhibiting action of PN is independent of a cGMP mechanism. In a separate series of experiments, incubation of washed platelets with PN for up to one hour before stimulation with thrombin or collagen resulted in a temporary (<30 min) inhibition of aggregation, and suggest that the actions of PN were transient and non-toxic as PN does not permanently impair the ability of platelets to respond to these agents [268]. The addition of PN to unstimulated platelets leads to a significant increase in nitrated cytosolic, but not in membrane, platelet proteins [268]. However, nitrotyrosine levels decreased to near normal after 60 minutes, suggesting that protein nitration by PN is reversible. Wide variation between human platelet preparations was observed in both the initial levels of nitration and in the speed of nitration loss. Finally, the influence of PN on platelet-signaling proteins was also investigated. The addition of thrombin to washed platelets increased a broad range of protein phosphorylation, however incubation of PN with washed platelets prior to thrombin stimulation led to decreases in protein phosphorylation, but that this inhibitory effect of PN was reversible in some proteins with platelet proteins recovering the ability to be phosphorylated within 15 min. These data found that low PN concentrations can reversibly inhibit aggregation by preventing phosphorylation of key signaling proteins [268].

Table 2.   Studies Examining the Anti-Platelet Aggregation Effects of PN

Authors	*Model*	*PN*	*Findings*	*Reference*
Moro, Darley-Usmar et al. 1994	1) Washed human platelet suspensions 2) Platelet-rich human plasma	20 μM-200 μM	1) Increases aggregation 2) Decreases aggregation Reversal of the aggregatory effect is observed in washed platelets following the additions of plasma, human serum albumin, or glutathione	[22]
Moro, Darley-Usmar et al. 1995	1) Washed human platelet suspensions 2) Platelet-rich human plasma	20 mM	Platelet inhibition was dependent on the concentration of D- or L-glucose	[61]
Yin, Lai et al.	1) Platelet-rich plasma 2) Platelet-poor plasma 3) Human albumin	50 μM-200 μM	Inhibited platelet aggregation in a dose-dependent manner to different aggregation agents, ADP, collagen, thrombin, and U46619 PN inhibition of aggregation requires protein nitrosylation	[241]
Brown, Moro et al.	1) Washed human platelets 2) Human platelet-poor plasma	50 μM-400 μM	1) Induces aggregation by mobilization of Ca^{++} from the extracellular compartment 2) Inhibited collagen-induced P-selectin expression 3) Inhibition of aggregation due to conversion to NO or NO donor 4) Inhibition *vs* aggregation is concentration dependent	[254]
Boulos, Jiang et al.	Washed human platelet suspensions	1) 100-190 μM 2) 4-50 μM	1) Moderate aggregatory effect 2) Inhibited aggregation in a dose-dependent manner Modulates eicosanoid synthesis Timing of reactive species generation is important in PN's effects on platelets	[256]
Nowak and Wachowicz	Ovine platelet-rich plasma	0.031-0.062 mM-2 mM	Concentration dependent inhibitory effect on thrombin- and to collagen-induced platelet adhesion Peroxidation of platelet lipids affects platelet aggregation	[262]
Low, Sabetkar et al.	1) Washed human platelets 2) Platelet-rich plasma from human volunteers	3 μM-10 μM	Wide variation between human platelet preparations was observed Low concentrations can reversibly inhibit aggregation by preventing phosphorylation of key signaling proteins	[268]

ADP, adenosine diphosphate; PN, peroxynitrite.

PROTECTIVE EFFECT OF PEROXYNITRITE IN ISCHEMIA REPERFUSION INJURY

At nanomolar concentrations, NO modulates a number of physiologic processes related to vascular reactivity and platelet function [228, 269], leukocyte-endothelial cell interactions [270], vascular permeability [271, 272], and is cytoprotective in ischemia-reperfusion injury [273, 274]. However, at higher micromolar concentrations, NO as well as PN, participates in cellular injury as seen in the lung [243], following the development of ischemia-reperfusion injury, endotoxic and hypovolemic shock [7, 275]. Although vascular injury is linked to formation of toxic products from activated neutrophils [104], and that endothelial cells, macrophages and neutrophils can generate PN and induce membrane lipid peroxidation [57], the effects of PN on neutrophil-endothelial cell interactions were largely unknown. Lefer, Scalia *et al.* investigated the effect of physiologic relevant concentrations of PN on neutrophil (PMN)-endothelial cell interactions [26]. In rat isolated aorta rings, PN (5 μM), but not decomposed PN, produced significant relaxation, whereas at concentrations of 50 nM-500 nM, detectable responses were not seen [26]. These data show that PN in a macromolar range can exert significant vasorelaxant effects in isolated vessels. The administration of acetylcholine following the administration of 5 μM of PN to the ring preparation produced a significant relaxation suggesting that PN does not impair endothelium dependent responses [26]. The effects of PN were observed on adherence to the endothelium of unstimulated PMNs following thrombin stimulation in isolated superior mesenteric artery. Thrombin stimulation increased PMNs adhesion rates by 7-fold, however, following the administration of PN (100 nM), PMNs adhesion rates were significantly reduced [26]. The effects of PN on leukocyte rolling and adherence in mesenteric postcapillary venules were also examined and that thrombin or hydrogen

peroxide markedly increased leukocyte rolling, but following administration of PN (800 nM), leukocyte rolling was significantly reduced [26]. However, these effects of PN were transient, as the addition of fresh thrombin at 2 hours to this preparation resulted in a return of leukocyte adherence and rolling, suggesting that PN does not irreversibly damage leukocytes or endothelial cells [26]. In separate series of experiments, the effects of PN (800 nM) following induction of P-selectin with L-NAME were examined and pretreatment with PN significantly inhibited the L-NAME-mediated increases in leukocyte rolling and adherence, confirming that NO is an important modulator of leukocyte adhesion to the endothelium [26, 270]. Moreover, pretreatment with PN significantly reduced surface expression of P-selectin following stimulation with either L-NAME or thrombin, suggesting that nanomolar concentrations inhibit leukocyte-endothelial cell interactions by a P-selectin dependent mechanism [26]. The role of PN in attenuating leukocyte-endothelial interactions was tested in a myocardial ischemia reperfusion model. Perfused rat hearts were subjected to 20 min of ischemia followed by 45 min of reperfusion with and without PMNs, and the hearts reperfused in the presence of PMNs resulted in a significant sustained reduction in coronary flow and impaired ventricular function [26]. However, infusion of PN resulted in a significant degree of cardioprotection and cardiac perfusion and ventricular performance improved when compared to PMNs perfused hearts, and that postischemic coronary flow protection was less that observed in PMNs-free perfused heart, whereas ventricular performance was comparable to the PMN-free heart preparation [26]. Histological and myeloperoxidase activity analysis demonstrated that PN significantly attenuated PMNs infiltration into heart tissue and suggest that PN protects the ischemic/reperfused rat heart in large part by inhibiting PMNs accumulation [26]. These data show that PN in nanomolar concentrations can inhibit leukocyte-endothelial cell interactions, exert cytoprotective effects and that inhibition of P-selectin is a key mechanism in modulation of PN-inhibited leukocyte-endothelial cell interactions [26].

Studies have shown that the extension of myocardial infarct into initially nonischemic border zone occurs during subsequent reperfusion of tissue [276]. Moreover, PN is released upon reperfusion of ischemic heart tissue [277]. Although PN has been reported to exert cytotoxic effects at high doses [22, 57, 115], prior studies have shown that at physiologic concentrations, PN reduces PMNs adhesion to the endothelium and attenuates PMNs-mediated contractile dysfunction seen in the isolated perfused rat heart [26]. However, similar studies *in vivo* are lacking. Nossuli, Hayward *et al.* 1997 examined the role of PN *in vivo* in physiologically relevant concentrations in a feline model of myocardial reperfusion injury [25]. In feline hearts subjected to ischemia reperfusion, ischemia was induced by tightening a reversible silk ligature around the LAD for complete coronary occlusion of flow for 90 min followed by reperfusion of ischemic myocardium for 270 min [25]. In a subgroup of animals, PN (1 µM/L) infusion was administered 10 minutes before reperfusion in cats subjected to myocardial ischemia and reperfusion. In those experiments, PN-treated animals demonstrated marked attenuation of cardiac necrosis when compared to control animals, but only in those animals administered the infusion *via* the intraventricular route,

whereas with an intravenous method of PN infusion, the extent of necrosis was not significantly different between groups [25]. Early endothelial dysfunction is a critical event in PMN-mediated vascular injury [26, 56, 278]. The authors examined the response of endothelial-dependent vasodilators, acetylcholine and the calcium ionophore, A23187, to the endothelial-independent vasodilator, sodium nitrite, in coronary artery rings from ischemic/reperfused and in control animals [25]. Coronary artery rings obtained from animals subjected to myocardial ischemia and reperfusion exhibited marked attenuation of responses to the endothelial-dependent vasodilators, when compared to the nonischemic controls animals, however, these rings retained full relaxation responses to sodium nitrite [25]. However in animals receiving intraventricular PN-infusion prior to the myocardial insult, the isolated coronary artery rings demonstrated a significantly improved relaxation suggesting that PN exerts a vasculoprotective effect on coronary vascular endothelium following ischemic reperfusion insult [25]. To further assess the role of neutrophil adhesion to the vascular endothelium, the extent of neutrophil adherence to arterial segments obtained from ischemic/reperfused vascular beds were examined and compared to arterial segments obtained from control cats. Few neutrophils were adhered to the endothelium of LAD coronary artery segments obtained from control nonischemic cats, whereas in LAD segments isolated from ischemic/reperfused cats exhibited a marked increase in neutrophil adherence. In ischemic/reperfused animals that received PN infusion, the degree of neutrophil-endothelial adherence was greater than non-ischemic control animals but less than LAD segments isolated from ischemic/reperfused animals [25]. These data support the hypothesis that PN can significantly modulate neutrophil-endothelial interactions in an *in vivo* setting of ischemic/reperfusion injury [25]. These authors next examined the role of P-selectin expression in coronary venules from myocardial tissue at risk for ischemia/reperfusion and compared with areas not at risk. In animals subjected to ischemia/reperfusion, a significant increase in area of coronary venules staining positive for P-selectin was observed, whereas in those animals that received PN intraventricular infusion, then the ischemia/reperfusion insult, a significant attenuation of P-selectin expression was observed in the coronary microvasculature to levels that were not significantly different that those observed in control nonischemic animals [25]. These data further support the observations that P-selectin plays a role in neutrophil adherence and that PN can modulate P-selectin expression [25, 26]. Similar results were obtained following stimulation of non-ischemic/reperfused coronary artery segments exposed to thrombin where a significant increase in neutrophil adherence was observed, and that prior PN-infusion significantly decreased neutrophil adherence to the endothelium [25]. These data provide evidence to support the hypothesis that physiologically relevant concentrations of PN, when directly infused into the left ventricle, can exert significant cardioprotective and vasculoprotective effects following myocardial ischemia/reperfusion, and that PN can partially attenuate neutrophil-endothelium interactions [25].

Earlier studies have shown cardioprotective and vasculoprotective effects of PN and a role in modulating neutrophil-endothelial adherence in myocardial/reperfusion injury models [25, 26], however, the maximally achievable concentra-

tions of PN to active these beneficial effects are not known. Moreover, the beneficial actions of PN may be due to the formation of S-nitrosothiols and PN may act as a NO carrier or PN can act as a direct NO donor. Nossuli, Hayward *et al.* 1998 examined the dose-response relationships following different concentrations of PN infusion in a model of myocardial ischemia/reperfusion and also investigated with the mechanisms responsible for these beneficial effects [279]. Animals were subjected to myocardial ischemia followed by reperfusion and were administered with different concentrations of PN (0.2 µM, 2 µM, or 20 µM) directly infused into the left ventricle beginning 10 min before and continued throughout the reperfusion period [279]. The infusions of PN were assessed in their ability to limit the progression of myocardial necrosis following reperfusion. Interestingly, only the middle dose (2 µM) statistically protected the myocardium from developing a substantial amount of necrosis [279]. These data suggest that the maximally effective concentrations of PN were in the lower micromolar range (2 µM) and the cardioprotective effects were not due to changes in hemodynamic, electrophysiological or hematologic variables [279]. In regard to endothelial injury, a significant decrease in response to acetylcholine, and the calcium ionophore, A23187, in animals receiving the cardiac insult, but in animals infused with the middle dose of PN (2 µM) endothelial function was preserved, whereas in animals receiving either the low or high dose of PN (0.2 µM or 20 µM), endothelial dysfunction was comparable to that observed in animals subjected to myocardial ischemia/reper-fusion [279]. The extent of neutrophil adherence to coronary artery segments obtained at the conclusion of these experiments was examined, and again in those animals receiving the middle dose of PN (2 µM), LAD segments exhibited a significantly lower neutrophil adherence that in those animals receiving the ischemia/reperfusion insult alone [279]. The role of surface expression of P-selectin was examined in this model, and the percentage of expression in the coronary microvasculature was significantly attenuated only with the infusion of PN at the 2 µM dose [279]. These data suggest that only the 2 µM dose was able to modulate surface expression of P-selectin, leukocyte adherence, and myocardial tissue protection and the significant cardioprotective mechanism may be due to reduced expression of adhesion molecules by PN on the vascular endothelium [279]. Coronary artery rings were denuded of endothelium, and challenged with PN (10 µM-40µM) and in the presence or absence of hemoglobin (100µM), a known NO scavenger [1,34,64,65], PN relaxed these rings in the presence or absence of Hgb. These findings suggest that PN does not directly donate NO under these conditions [279]. Finally, the role of PN leading to S-nitrosothiol formation was examined, and using a wide concentration range (1 µM-1000 µM), PN was incubated with reduced GSH in the presence of a copper chelator, with the reaction products analyzed by HPLC, and observed the production of S-nitrosothiols. PN nitrosated GSH to form GSNO [279].

Reperfusion of ischemic myocardium is associated with the development of dysrhythmias [280-283], however ischemic preconditioning protects the heart from subsequent ischemic damage [283, 284], reduces the incidence of dysrhythmias [285, 286], and has been shown to preserve contractile function [287]. NO and superoxide production were increased following reperfusion of ischemic tissue [288]. PN, the downstream molecule of these radicals, can also play a role in the pathophysiology of myocardial perfusion injury [289], although PN, at physiologic concentrations, has been shown to have cardioprotective effects [25, 26, 279]. Altug, Demiryürek *et al.* investigated the effect of exogenous PN on ischemic/reperfusion dysrhythmias in an isolated rat heart model [290]. The effects of three different infusions of PN (0.4 µM/ml/min, 4 µM/ml/min, and 40 µM/ml/min) were studied in a modified Langendorff preparation [291], and PN at the higher infusions of (4 µM/ml/min and 40 µM/ml/min) produced a significant increase in severe dysrhythmias whereas the lowest dose (0.4 µM/ml/min) produced a small number of ventricular ectopic beats. Moreover, PN at this dose, produced a significant reduction in the incidence of ventricular fibrillation when compared to control animals [290]. The administration of PN did not produce any significant change in coronary artery perfusion pressure or pressure-rate index measured throughout the course of the experiment [290]. These data suggest that the lowest dose of PN reduced the development of ischemia/reperfusion dysrhythmias in physiologic salt-perfused hearts [290], whereas the higher doses increased the incidence of dysrhythmias and partially support the findings of Schultz *et al.* [292], and of Naseem, Kontos *et al.* [293] that partial inhibition of the elevated endogenous NO production found in ischemic states can improve myocardial function and reduce the incidence of ischemia/reperfusion dysrhythmias [293]. The administration of urate did not change the beneficial effects of PN on reperfusion-induced dysrhythmias [290] and suggest that although uric acid is a scavenger of PN [294], urate administered in these experiments was ineffective in modifying the incidence of ischemia/reperfusion and are in agreement with other studies [295]. The prior administration of L-NAME reduced the incidence of ventricular fibrillation, but did not affect the incidence of irreversible ventricular fibrillation, whereas the administration of superoxide dismutase with catalase significantly increased the incidence of irreversible ventricular fibrillation [290]. These results suggest that PN, at low concentrations, may exert favorable effects in decreasing the incidence of ischemia/reperfusion-induced dysrhythmias [290]. Moreover, the use of a cell-permeant antioxidant that reacts rapidly with PN, abolished the beneficial effect of PN [296].

Ischemic/reperfusion injury in the lung is common following surgical procedures such as in lung transplantation, pulmonary embolectomy, or more commonly, following cardiopulmonary bypass [297, 298]. Prior studies have shown that ischemic preconditioning provides significant protection to the lungs [299-301] and that ischemic preconditioning of the lung can be protective against subsequent ischemia/reperfusion injury [302, 303], but underlying mechanisms are not well understood. Turan and Demiryürek investigated the effects of exogenous administration of PN in isolated rat lungs and to examine the contribution of endogenous PN formation on preconditioning [304]. Isolated lungs were perfused by Krebs-Henseleit solution and inflated with room air. Lungs were preconditioned with either ischemia for 5 min or an infusion of PN (10 µM), followed with 5 min reperfusion, and with 2h of ischemia and 10 min reperfusion. The application of ischemic preconditioning maintained vascular responses following ischemia/reperfusion, and an infusion of PN could mimic the beneficial effects of precondi-

Table 3.   Protective Effect of Peroxynitrite in Ischemia Reperfusion Injury

Authors	*Model*		*PN*	*Findings*		*Reference*
Lefer, Scalia et al.	A)	PMNs rolling and adherence to the endothelium	A1) 100-1000 nM	A1) PMNs adhesion rates were significantly reduced		[26]
			A2) 800 nM	A2) Leukocyte rolling significantly reduced		
	B)	myocardial ischemia reperfusion rat model	B1) 800 nM infusion	B1) Inhibiting PMNs accumulation, with significant cardio-protection, improved cardiac perfusion and ventricular performance		
				P-selectin inhibition		
Nossuli, Hayward et al. 1997	1)	Feline myocardial reperfusion injury model	1 µM/L infusion	1) Marked attenuation of cardiac necrosis		[25]
	2)	Harvested isolated coronary artery rings		2) Significantly improved ring relaxation		
				Attenuation of P-selectin expression		
Nossuli, Hayward et al. 1998	A)	Feline myocardial reperfusion injury model	0.2 µM, 2 µM, or 20 µM infusions	Middle dose (2 µM):		[279]
				A) Decreased myocardial necrosis and preserved endothelial function		
	B)	Harvested isolated coronary artery rings		B1) Lowered neutrophil adherence		
				B2) Lowered P-selectin expression		
				PN does not directly donate NO		
				PN nitrosates glutathione		
Altug, Demiryürek et al.	Modified Langendorff preparation rat model		0.4 µM/ml/min, 4 µM/ml/min, and 40 µM/ml/min	Lowest dose (0.4 µM/ml/min) significantly reducted ventricular fibrillation incidence		[290]
				Use of a cell-permeant antioxidant abolished beneficial effects		
Turan and Demiryürek	Krebs-Henseleit solution perfused rat lung model		10 µM infusion	PARS inhibition reversed beneficial effects		[304]
				Lung malondialdehyde levels were significantly decreased		

PMN; polymorphonuclear cells; PARS, poly(adenosine 5'-diphos-phoribose) synthase; PN, peroxynitrite.

tioning. However, the beneficial effects of PN infusion on vascular responses following ischemia/reperfusion injury could not be reduced in the presence of uric acid, a PN scavenging agent [167, 305, 306]. Moreover, the use of the PARS inhibitors, 3-aminobenzamid or nicotinamide, reversed the beneficial effects of ischemia and PN preconditioning [304]. The improved wet/dry weight ratios demonstrated that the ischemia preconditioning and PN infusion caused a marked reduction in lung edema [304]. Lung malondialdehyde levels were significantly decreased in ischemic and PN preconditioned groups [304]. The authors suggested that PN has a significant role in the protective effects of preconditioning [304].

CONCLUSION

Reactive oxygen species such as superoxide can react with NO in pathologic conditions leading to the formation of the potent oxidant, PN [307]. PN, when formed in inflammatory conditions, causes cytotoxic effects including lipid peroxidation and cell death [44, 57, 104, 307]. However, in the literature examined in this paper, PN has been shown to have beneficial effects in the cardiovascular system, but in other reviews, the biologic effects of PN are not beneficial [3, 9-19], hence the effects of PN can be paradoxical. These differences could well be due to the environment in which studies were conducted [308]. Studies in nonsanguineous environments have largely shown the cytotoxic properties of PN, whereas *in vivo* models or in blood-perfused *ex vivo* models, the beneficial effects of PN are observed, particularly when

examined in appropriate physiological concentrations. Nonsanguineous studies may result in the conversion of PN into deleterious mediators with hydroxyl radical-like activities, whereas in physiologic studies, the beneficial actions of PN may be due to reaction of PN with plasma, red cell glutathione, or plasma cysteine and albumin, with the production of NO or an NO-donor like compound [308].

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