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Pathophysiological roles of peroxynitrite in circulatory shock

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Summary

Peroxynitrite is a reactive oxidant produced from nitric oxide (NO) and superoxide, which reacts with proteins, lipids and DNA and promotes cytotoxic and pro-inflammatory responses. Here we overview the role of peroxynitrite in various forms of circulatory shock. Immunohistochemical and biochemical evidence demonstrate the production of peroxynitrite in various experimental models of endotoxic and hemorrhagic shock, both in rodents and in large animals. In addition, biological markers of peroxynitrite have been identified in human tissues after circulatory shock. Peroxynitrite can initiate toxic oxidative reactions *in vitro* and *in vivo*. Initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3-phosphate dehydrogenase, inhibition of membrane Na^+/K^+ ATP-ase activity, inactivation of membrane sodium channels, and other oxidative protein modifications contribute to the cytotoxic effect of peroxynitrite. In addition, peroxynitrite is a potent trigger of DNA strand breakage, with subsequent activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP), which promotes cellular energetic collapse and cellular necrosis. Additional actions of peroxynitrite that contribute to the pathogenesis of shock include inactivation of catecholamines and catecholamine receptors (leading to vascular failure), endothelial and epithelial injury (leading to endothelial and epithelial hyper-permeability and barrier dysfunction) as well as myocyte injury (contributing to loss of cardiac contractile function). Neutralization of peroxynitrite with potent peroxynitrite decomposition catalysts provides cytoprotective and beneficial effects in rodent and large animal models of circulatory shock.

Keywords

Nitric oxide; superoxide; endotoxin; inflammation; contraction; vascular dysfunction; poly (ADP-ribose) polymerase

Production and reactivity of peroxynitrite

Nitric oxide (•NO) and superoxide ($O_2^{\cdot-}$) rapidly react to form the toxic reaction product, peroxynitrite anion ($ONOO^-$) (1, 2). The oxidant reactivity of peroxynitrite is mediated by an intermediate with biological activity of hydroxyl radical, which is not hydroxyl radical

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per se, but, rather, peroxynitrous acid or its activated isomer. While •NO is a relatively stable and highly diffusible free radical, $O_2^{\cdot-}$ is much shorter lived and has restricted diffusion across biomembranes. Therefore, the sites of peroxynitrite formation are assumed to be spatially associated with the sources of $O_2^{\cdot-}$, such as the plasma membrane NAD(P)H oxidases or the mitochondrial respiratory complexes. For additional information on the chemistry, decomposition and reactivity of peroxynitrite, peroxynitrous acid and its activated isomer, see: (2–6).

Peroxynitrite is highly reactive. One of the key reactions of $ONOO^-$ in biological systems is its fast reaction with carbon dioxide (in equilibrium with physiological levels of bicarbonate anion), which leads to the formation of carbonate ($CO_3^{\bullet-}$) and nitrogen dioxide ($^{\bullet}NO_2$) radicals (yield approximating 35 %), which are one-electron oxidants. Nitrogen dioxide can undergo diffusion-controlled radical-radical termination reactions with biomolecules resulting in nitrated species such as nitrotyrosine (which is commonly used as a marker or 'footprint' of peroxynitrite; see below). Alternatively, ONOOH can undergo homolytic fission to generate one-electron oxidants hydroxyl ($^{\bullet}OH$) and $^{\bullet}NO_2$ radicals. The proton-catalyzed decomposition to form $^{\bullet}OH$ and $^{\bullet}NO_2$ radicals may become relevant in hydrophobic phases resulting in the initiation of lipid peroxidation processes (3, 4).

The activities of peroxynitrite include a rapid oxidation of sulfhydryl groups and thioethers, as well as nitration and hydroxylation of aromatic compounds, including tyrosine, tryptophan and guanine (7, 8). These reactions, when occurring during the reaction of peroxynitrite with enzymes, macromolecules and lipids, have been shown to influence numerous cellular functions (7–24). For instance, tyrosine nitration may lead to diminished function of the proteins, as has been shown or suggested in the case of superoxide dismutase (16) and neuronal tyrosine hydroxylase (15). Oxidation of critical sulfhydryl groups is responsible for the inhibition of mitochondrial and cytosolic aconitase (10, 13) and other critical enzymes in the mitochondrial respiratory chain (10, 13, 20, 21, 24) and disruption of the zinc-thiolate center at the active site of enzymes (11). There is also evidence that peroxynitrite can cause covalent modification of an active site thiol of glyceraldehyde-3-phosphate dehydrogenase (18). Peroxynitrite can also inhibit the activity of membrane Na^+/K^+ ATP-ase (9, 12, 14).

The reaction of peroxynitrite with lipids leads to peroxidation (malondialdehyde and conjugated diene formation) and formation of nitrito-, nitro-, nitrosoperoxo- and/or nitrated lipid oxidation adducts (25). Peroxynitrite also causes the oxidation of arachidonic acid, and the formation of F2-isoprostanes through the oxidation of low density lipoprotein (26). In addition, peroxynitrite has been shown to cause direct damage of pulmonary surfactant proteins (26–29).

Another important interaction of peroxynitrite occurs with nucleic acids, with the production of 8-hydroxydeoxyguanosine (30) or 8-nitroguanine (31). The mechanism of direct, peroxynitrite-induced the DNA strand breakage is probably related to abstraction of hydrogen atoms from the ribose of the DNA moiety, thereby opening the sugar ring (30, 32). However, in cells exposed to peroxynitrite, there is also an indirect mechanism of

peroxynitrite-induced DNA damage which involves the secondary production of mitochondrially derived oxidants and free radicals (33, 34).

The reactivity and decomposition pathways of peroxynitrite are strongly influenced by the chemical environment. In the presence of plasma, proteins, glucose or glutathione, peroxynitrite can form intermediates, which act as NO donors (35, 36). In plasma, peroxynitrite oxidizes ascorbic acid, uric acid, tyrosine, and -SH groups of plasma proteins (37, 38). There is a delicate balance between peroxynitrite-mediated oxidant processes and endogenous antioxidant pathways, which limit the reactivity of peroxynitrite (39). This is illustrated by the example of the endogenous antioxidant glutathione: pharmacological depletion of glutathione renders cells and animals extremely sensitive to the cytotoxic effects of peroxynitrite (40, 41).

Pathophysiological actions of peroxynitrite in cultured cells

Pharmacological studies demonstrate that peroxynitrite is more cytotoxic than NO or superoxide in a variety of experimental systems (10, 13, 24, 42, 43) and can induce both necrosis and apoptosis (4, 6, 44). While NO itself only exerts a limited effect on aconitase activity, peroxynitrite is a potent inhibitor of this enzyme under the same experimental conditions (10, 13). Furthermore, peroxynitrite - and not NO - is a potent initiator of DNA single strand breakage (45–47).

Peroxynitrite can induce marked alterations in cellular energetics and DNA integrity (Table 1). For instance, in pulmonary type II cells, peroxynitrite inhibits membrane Na^+/K^+ ATP-ase activity and sodium uptake (14), and similar effects were seen in intestinal epithelial cells as well (9). Profound inhibition by peroxynitrite of mitochondrial respiration has been observed in a variety of cell types (24, 43, 45, 46). Peroxynitrite exposure or endogenous generation of peroxynitrite in immunostimulated cells can also lead to depletion of intracellular NAD^+ and ATP levels in various cell types (46, 48–50).

Endogenous or exogenous peroxynitrite, is a potent trigger of DNA single strand breakage, which, in turn, activates the nuclear enzyme poly(ADP-ribose) polymerase (46). As overviewed elsewhere (6, 51–53), activation of PARP can rapidly deplete NAD^+, slowing the rate of glycolysis, electron transport, and ATP formation, resulting in cell dysfunction and cell death via the necrotic route. In addition to these effects on mitochondrial function, PARP can also poly(ADP-ribosyl)ate GAPDH (54, 55), and this effect can lead to an inhibition of glycolysis, as demonstrated in ischemic kidneys (55).

Peroxynitrite is a cytotoxic molecule (Fig. 1). Exposure to high concentrations of peroxynitrite leads to rapid cell death, associated with rapid energetic derangements. On the other hand, lower concentrations of peroxynitrite, after several hours, can lead to apoptotic cell death (56–62). The cellular dysfunction can manifest itself in suppressed cellular functions (e.g. reduction in mitochondrial respiration), but can also lead to increases in paracellular permeability, as demonstrated in intestinal epithelial cells (63, 64): this effect may have significant implications for the pathogenesis of intestinal barrier dysfunction associated with circulatory shock (see below).

Peroxynitrite formation has been implicated as an important participant in positive feedback cycles of injury in various diseases. For instance, peroxynitrite can promote the oxidation of cofactors either by direct or free radical dependent mechanisms. Peroxynitrite-mediated oxidation of tetrahydrobiopterin (BH_4) to 5,6-dihydrobiopterin (and subsequently to 7,8-dihydropterin) leads to the dysfunction (partial uncoupling) of NO synthase, since BH_4 is an essential co-factor of NO synthase. It has been proposed that low levels of BH_4 can, in turn, promote a cycle of its own destruction mediated by further peroxynitrite generation by the uncoupled NO synthase. This mechanism may contribute to vascular endothelial dysfunction induced by oxidative stress in various diseases (reviewed in (65). Reaction of NADH with peroxynitrite can result in the formation of NAD^+ and superoxide and, subsequently, hydrogen peroxide (66, 67). This reaction can induce both an imbalance in cellular pyrimidine nucleotide levels, as well as a positive feedback cycle of intracellular oxidant generation. Inactivation of mitochondrial electron transport enzymes increases the amounts of superoxide and hydrogen peroxide generated by the mitochondria (20), which may further contribute to cellular injury, in an additive or synergistic fashion (68). Moreover, the inactivation of manganese superoxide dismutase (MnSOD) by peroxynitrite due to nitration of critical tyrosine- 34 may also amplify mitochondrial injury (69). Nitration of cytochrome c results in a marked increase in its peroxidase activity, which may exacerbate oxidative damage to mitochondrial proteins and membranes after peroxynitrite exposure (70). The peroxynitrite-induced DNA damage and PARP activation cycle represents another amplification mechanism, which contributes to peroxynitrite cytotoxicity (6, 51–53). Peroxynitrite can also trigger the release of mitochondrial pro-apoptotic factors and trigger cytochrome c-dependent apoptosis in the cytosol through peroxynitrite-dependent oxidation of permeability transition pore components and also possibly cardiolipin oxidation (71).

Peroxynitrite can play a role in promoting pro-inflammatory cellular responses. Potential biological targets of peroxynitrite include membrane as well as cytosolic and nuclear receptors. Putative targets for peroxynitrite include the EGF receptor, β_1- and β_2-adrenoceptor, platelet-endothelial cell adhesion molecule-1, IRS-1 and the peroxisome proliferator-activated receptor gamma (PPARγ) (overviewed in (72). Not only receptors, but also receptors ligands are targeted for modification. For instance, the reaction of peroxynitrite with fibroblast growth factor-1 induces extensive cysteine oxidation, tyrosine nitration, and irreversible inactivation of protein activity (73). Finally, peroxynitrite can react with catecholamines and lead to their inactivation, which may contribute to the development of vascular failure in shock (see below).

Peroxynitrite may participate in reactions that upregulate the inflammatory responses at multiple levels. For instance, peroxynitrite has been shown to play a role in the promotion of the expression of ICAM-1 and P-selectin in human endothelial cells (74). In human neutrophils, peroxynitrite triggers the down-regulation of L-selectin expression, and up-regulation of CD11b/CD18 expression (75). These effects are likely to be mediated, at least in part, by the ability of peroxynitrite to trigger and enhance NF-κB mediated pro-inflammatory signal transduction pathways by modifying proteins associated with the activation of this transcription factor. Peroxynitrite is also able to affect other signal transduction pathways, including protein kinase C (76), MAP kinase (77), and src tyrosine kinases (78, 79). Peroxynitrite can also contribute to the enhanced production of pro-

inflammatory mediators by reduction of histone deacetylase HDAC2 activity through HDAC2 nitration (80). Elevated local levels of peroxynitrite during various forms of circulatory shock may upregulate localized inflammatory stress responses and possibly promote cellular and tissue injury (see below). Up-regulation of adhesion receptors by peroxynitrite may also result in an increased expression of endothelial adhesion molecules and such cells may represent a preferential site for adhesion and migration of neutrophils when simultaneously high concentrations of NO and neutrophil-derived superoxide are present.

Production of peroxynitrite in circulatory shock in animals and humans

The formation of peroxynitrite can be detected by its reactions (or 'footprints'). The first evidence for peroxynitrite formation, by (a) increased nitrotyrosine immunoreactivity and (b) increased oxidation of the fluorescent probe dihydrorhodamine 123 to rhodamine 123 was obtained in rat models of endotoxin shock and hemorrhagic shock (7, 81). Subsequent studies have confirmed the formation of nitrotyrosine in various experimental models of shock (82–88). It must be noted, that peroxynitrite is not the only species that can yield nitrated tyrosine: myeloperoxidase-dependent nitrative reactions can also result in the formation of the same species (89, 90). Indeed, there is now experimental evidence from myeloperoxidase-deficient experimental models that supports the existence of peroxynitrite-independent (and myeloperoxidase-dependent or myeloperoxidase-independent) mechanisms of tyrosine nitration in animal models of circulatory shock (91, 92). Recently, by using the novel, potent porphyrinic antioxidants ('peroxynitrite decomposition catalysts'), additional evidence has been obtained for the formation and pathophysiological significance of this species in circulatory shock (see below).

It is important to note that the markers of peroxynitrite generation have not only been documented in experimental models of shock, but also in human specimens obtained from patients suffering from circulatory shock. For instance, tyrosine nitration has been detected in the blood of septic patients (93, 94), in human samples after acute pulmonary injury (95), in chronic renal failure with patients with septic shock (96) and in myocardial and skeletal muscle samples after sepsis (97–101). There is a significant correlation between the degree of nitrotyrosine formation and the severity of the disease in human sepsis: in a preliminary study in a small set of patients with sepsis, Ohya and colleagues reported that plasma nitrotyrosine concentrations of the non-survivors and survivors were 0.7 nM versus 0.2 nM, respectively (93). In addition, Strand and colleagues have reported elevated levels of circulating nitrotyrosine in primary episodes of patients suffering from septic shock (94).

Role of peroxynitrite in the development of vascular changes in circulatory shock

One of the most important cardiovascular consequences of circulatory shock is the reduced responsiveness of arteries and veins to exogenous or endogenous vasoconstrictor agents (vascular hyporeactivity). This is usually coupled with a loss of endothelial function (reduced endothelium-dependent relaxations), as well as an increase of vascular permeability, leading to capillary extravasation and tissue edema. Many of the circulatory-

shock-associated vascular alterations have been attributed to the formation of oxygen-derived oxidants and free radicals and the expression of a distinct inducible isoform of NOS (iNOS) in the vascular smooth muscle cells (49,102–105). As peroxynitrite is capable of mimicking many of the vascular alterations associated with shock (endothelial dysfunction, vascular hyporeactivity), this species may play a significant pathogenetic role in the vascular alterations associated with circulatory shock.

In 1994, Moncada and colleagues have reported the ability of peroxynitrite to impair the ability of endothelium-dependent relaxant agents to produce vascular relaxations (8). The degree of the endothelial dysfunction induced by peroxynitrite is dependent on the antioxidant milieu, as exemplified by glutathione: depletion of endogenous glutathione exacerbates (40) whereas supplementation of glutathione protects (106, 107) against peroxynitrite-induced endothelial dysfunction. The notion that endogenous peroxynitrite participates in the impairment of endothelium-dependent relaxant functions is supported by indirect evidence, i.e. data demonstrating that neutralization of superoxide protects against the development of endothelial dysfunction in a rodent model of shock (81), as well as by data with more selective neutralizers of peroxynitrite in rodent models of shock (peroxynitrite decomposition catalysts; see also below) (108).

The impairment of endothelial function by peroxynitrite may contribute to the pathogenesis of organ failure in circulatory shock in many different ways: (a) it may exacerbate local vasospasm, may increase local neutrophil adhesion and migration into inflamed tissues; (b) it may exacerbate platelet activation and aggregation and (c) it may lead to hypo-perfusion of certain parts of various organs. Endothelial dysfunction induced by peroxynitrite may also be associated with increased endothelial permeability, and may lead to extravasation and local tissue edema. With respect to endothelial barrier dysfunction and peroxynitrite, a variety of cellular mechanisms have been described that may contribute to the deleterious effects of peroxynitrite, including PARP activation (109), disorganization of junctional proteins and dephosphorylation of phosphorylated focal adhesion kinase (FAK) at tyrosine 397 (109, 110) and an increase in protein phosphatase type 2A (111).

The mechanisms by which peroxynitrite may contribute to the impairment of vascular contractile function in circulatory shock are also multiple. Some of these mechanisms may be related to a direct impairment of vascular smooth muscle energy generation, via inhibition of mitochondrial function and/or activation of PARP (5, 81, 103) Other mechanisms may be related to nitration of F-actin in vascular smooth muscle leading to depolymerization and the subsequent loss of myogenic tone (112), and direct activation of potassium channels on the vascular smooth muscle (113, 114). An additional mechanism of peroxynitrite-mediated impairment of vascular function may involve inactivation of the sarcoplasmic reticulum Ca^{2+} pump function (115, 116). Other mechanisms that may contribute to the inhibition of contractile responses by peroxynitrite may be related to direct oxidative inactivation of vasoconstrictor catecholamines norepinephrine and dopamine, as well as inactivation of receptors for the vasoconstrictor hormones noradrenaline: such as peroxynitrite-mediated inhibition of α-adrenoceptor function and peroxynitrite-mediated inhibition of vasopressin receptors (117–121). Importantly, inhibition of superoxide production in rodent models of endotoxin shock increased the plasma levels of

noradrenaline, and decreased plasma levels of the inactive noradrenaline metabolite adrenochrome (122), indicating that a reactive oxidant-mediated (possibly peroxynitrite-mediated) mechanism is operative *in vivo* and consumes the endogenous catecholamines during circulatory shock.

Part of the vascular dysfunction elicited by peroxynitrite may be related to modulation of local mechanisms of vascular mediator production and coagulation. The selective nitration and inactivation of prostacyclin synthase by peroxynitrite may result in the accumulation of the intermediate PGH_2, which is capable to activate the thromboxane A_2 receptor on the surface of smooth muscle cells to promote vasoconstriction (123, 124). The nitration of prostacyclin-synthase thus functions as endogenous posttranslational switch that shuts off the prostacyclin-mediated vasodilatory, anti-aggregatory, and anti-adhesive conditions and may promote a pro-aggregatory and vasoconstrictive type vascular response in circulatory shock. Degradation of extracellular matrix proteins by peroxynitrite (125) may also contribute to pathophysiological vascular alterations, even though the potential role of this process in models of circulatory shock has not yet been explored.

Potential role of peroxynitrite in mediating myocardial hypocontractility in circulatory shock

Suppression of myocardial contractility is a common feature in patients with circulatory shock. Peroxynitrite is recognized as an endogenous myocardial depressant factor, with a potential role in the pathogenesis of myocardial hypocontractility in shock. The direct cytotoxic effects of peroxynitrite on cardiac myocytes have been demonstrated in multiple studies (126, 127). Infusion of peroxynitrite causes a reduction in myocardial contractility in isolated perfused hearts (128–131) and aggravates myocardial ischemic and reperfusion injury (132). The mechanism of peroxynitrite-mediated myocyte injury involves multiple pathways including nitration and inhibition of cardiac myofibrillar creatine kinase (133), alpha-actinin (134) and of myofibrillar proteins (135), activation of matrix metalloproteinases (127, 136, 137) and activation of PARP in the cardiac myocytes (138–140). Simultaneous generation of NO and superoxide, yielding peroxynitrite, has been demonstrated in hearts exhibiting myocardial dysfunction after endotoxemia (141). Neutralization of peroxynitrite mercaptoethylguanidine and 5,10,15,20-tetrakis(4-sulfonatophenyl)-porphyrinato iron (III) (FeTPPS), restored myocardial contractility in various models of shock and endotoxemia (142). There are no published data implicating the pathogenetic role of peroxynitrite in the myocardial dysfunction associated with human circulatory shock, but indirect evidence supports a potential relationship: tyrosine nitration has been demonstrated in cardiac specimens from patients with sepsis (97–101). PARP activation has also been demonstrated in patients who have died from septic shock: the extent of PARP activation shows a significant positive correlation with the release of cardiac enzymes in sepsis as well as with the extent of myocardial contractile dysfunction (143). Finally, studies conducted in human myocardial preparations exposed to endotoxin *in vitro* have indirectly implicated the potential role of peroxynitrite in the development of myocardial dysfunction during human sepsis (144). Taken together, the above data strongly

support the view that peroxynitrite acts as a myocardial depressant factor both in animals and humans suffering from circulatory shock.

Potential role of peroxynitrite in the development of hepatic dysfunction in circulatory shock

Hepatic dysfunction is another common pathophysiological event in patients with various forms of circulatory shock. Similar to other organs, the liver is both a source and a target of peroxynitrite. Multiple cell types of the liver, including hepatocytes, Kupffer cells, stellate cells, endothelial cells as well as infiltrating leukocytes have the capacity to generate nitric oxide, superoxide and peroxynitrite (145, 146). The respiratory burst oxidase of neutrophils, eosinophils, monocytes, and macrophages is an important source of superoxide and other reactive oxygen species. The major source of reactive nitrogen-derived radicals in circulatory shock is iNOS, an enzyme expressed in leukocytes, hepatocytes and the vascular smooth muscle cells. It has been demonstrated in several models of liver damage that NO and peroxynitrite contribute to the functional and morphological alterations. D'Ambrosio and colleagues demonstrated that S-nitroso-N-acetylpenicillamine-amine (SNAP), which generates NO, and 3-morpholinosydnonimine (SIN-1), which produces equal molar concentrations of superoxide and NO (resulting in peroxynitrite production), exhibit different levels of cytotoxicity in cultured human hepatocytes, with SIN-1 being markedly more cytotoxic than SNAP. Nitrotyrosine, a marker of peroxynitrite formation, was detected in hepatocytes treated with SIN-1 or SNAP. From these data it appears that hepatocytes generate significant amounts of intracellular superoxide, which reacts with the exogenous NO derived from SNAP to produce intracellular peroxynitrite, resulting in cytotoxicity. SIN-1 (and to a lesser degree SNAP) induced dose- DNA damage, as well as cell-cycle arrest in the S-phase, growth inhibition, and hepatocyte apoptosis. These data support the view that the functional and morphological changes observed in liver following chronic exposure to reactive nitrogen species are, in part, the result of mitochondrial and nuclear DNA damage (147). In another *in vitro* study Watanabe and colleagues observed the endogenously released NO and oxidative DNA alterations in hepatocytes co-cultured with splenic macrophages isolated from Wistar rats and incubated with either lipopolysaccharide (LPS) or interferon-gamma (148). Increased NO release, nitrotyrosine production and ratio of 8-hydroxy-deoxyguanosine (8-OH-dG) to deoxyguanosine (dG) were also noted in the hepatocytes. Part of the peroxynitrite-induced metabolic cellular dysfunction in hepatocytes resulted from an inhibition of mitochondrial respiration, in part via a direct mitochondrial action, and in part via activation of PARP (149).

Septic patients frequently suffer from acidosis. The stability and reactivity of many reactive nitrogen and oxygen species are dependent on the pH, which affects the degree of the resulting hepatocellular damage. Shu and colleagues demonstrated that acidification (pH 7.0) of the medium in normal and *C. parvum*-primed hepatocytes exposed to a mixture of pro-inflammatory cytokines and LPS produces a significant increase of peroxynitrite and hydroxyl radicals (150). Importantly, an enhanced degree of hepatocellular damage was noted in acidotic conditions, as compared to the responses at physiological (pH 7.4) or alkaline (pH 7.8) conditions. These results suggest that hepatocellular damage is partly

regulated by the surrounding pH: acidosis and reactive oxidant production are likely to act in concert to produce hepatocellular damage in circulatory shock.

In accordance with the above outlined *in vitro* studies, several independent *in vivo* studies suggest that peroxynitrite may also contribute to the hepatocellular damage in animal models of circulatory shock. Cimen and colleagues investigated the *in vivo* effect of bacterial LPS on Na^+,K^+-ATPase activity of guinea pig liver and investigated the possible contribution of various reactive nitrogen species (151, 152) and found a good correlation between the inhibition of Na^+,K^+-ATPase activity and the increase 3-nitrotyrosine levels in the livers of LPS-treated animals, suggesting that peroxynitrite may contribute to the inhibition of cell-membrane Na^+,K^+-ATPase.

Several series of studies, using various pharmacological interventions aimed at indirectly reducing peroxynitrite formation have resulted in improvements in hepatic function in various models of circulatory shock. These approaches included oxygen and nitrogen-derived radicals scavengers and neutralizers such as (−)-epitechin 3-O-gallate (153, 154), tempol (155–157) and Hypericum perforatum extract (158) or iNOS enzyme inhibitors such as N6-(iminoethyl)-L-lysine (159), aminoguanidine (160), and 1400W (108). The putative peroxynitrite scavenger uric acid has also improved hepatic function, as demonstrated in a rat model of hemorrhagic shock (159). The most definitive proof for the specific role of peroxynitrite in the pathogenesis of hepatic dysfunction in circulatory shock comes from studies by Cuzzocrea and colleagues who have examined the contribution of peroxynitrite formation in the pathophysiology of endotoxin-induced shock in the rat (108) using the peroxynitrite decomposition catalyst, 5,10,15,20-tetrakis(4-sulfonatophenyl) porphyrinato iron III chloride (FeTTPs). In this model, FeTTPs markedly attenuated the degree of hepatic injury, and significantly improved mortality rate.

‘Cytopathic hypoxia’ (a common feature of circulatory shock, where tissues (that are nominally adequately perfused) lose their ability to extract and utilize oxygen due to the inhibition of cellular metabolism (161, 162). The evidence demonstrating that peroxynitrite has the ability to suppress hepatocyte metabolism in circulatory shock may be consistent with the hypothesis that peroxynitrite contributes to the pathogenesis of cytopathic hypoxia in circulatory shock. However, to date, no studies have been published to directly test the effect of specific peroxynitrite decomposition catalysts on tissue oxygen utilization or arterio-venous oxygen differences in animal models of circulatory shock.

Potential role of peroxynitrite in the development of renal dysfunction in circulatory shock

Renal dysfunction is another common feature of circulatory shock. Peroxynitrite can be directly toxic to renal epithelial cells in culture (153, 163). Paller and colleagues have implicated the potential pathogenetic role of peroxynitrite in primary cultures of rat proximal tubular epithelial cells exposed to hypoxia and reoxygenation (164). Hypoxia and reoxygenation produced a marked increase in cellular generation of reactive oxidant species and triggered a significant degree of LDH release. Similar to the studies demonstrating the generation of peroxynitrite *in vivo* (see above), intracellular peroxynitrite generation was

assessed by measuring the conversion of dihydrorhodamine 123 to rhodamine 123. PARP inhibitors of various structural classes have also been demonstrated to exert protective effects in various models of cultured kidney epithelial cells exposed to pro-oxidant conditions or hypoxia-reoxygenation (165–167).

Several lines of *in vivo* studies also point to the potential pathogenetic role of peroxynitrite in the development of renal injury associated with circulatory shock. The antioxidant tempol proved to be also effective in reducing the renal dysfunction and injury associated with ischemia/reperfusion of the kidney (157), in a model of multiple organ injury (including renal dysfunction) associated with hemorrhagic shock (155) as well as in another rodent model of multiple organ injury induced by cell wall components of *S. aureus* (lipoteichoic acid and peptidoglycan) (156). Finally, the peroxynitrite decomposition catalyst FeTTPs was shown to attenuate endotoxin-induced renal injury in a rat model of endotoxic shock (108).

Potential role of peroxynitrite in the development of pulmonary dysfunction in circulatory shock

Similarly to cultured hepatocytes and kidney epithelial cells, peroxynitrite has the capacity to induce injury to pulmonary epithelial cells: an effect which occurs via a combination of mechanisms including direct metabolic inhibition, activation of PARP, activation of caspases and other cell death effector pathways (168–170). In addition (as mentioned earlier), peroxynitrite can induce damage to pulmonary surfactant (26–29), which may lead to pro-inflammatory changes and self-amplifying cycles of pulmonary injury in shock. In various animal models of circulatory shock, formation of nitrotyrosine has been demonstrated in pulmonary tissue sections (171–173). Furthermore, pharmacological neutralization of this species has been shown to reduce pulmonary histological damage and improve pulmonary oxygen function, as demonstrated by the effects of the metalloporphyrinic compound FP-15 in a rat model of pulmonary reperfusion injury (173) or the metalloporphyrinic compound WW-85 in a large animal model of systemic inflammation and pulmonary dysfunction induced by IL-2 (174).

As patients with septic shock are generally subjected to mechanical ventilation, part of the pulmonary injury in critical illness is not the result of the primary disease, but a iatrogenic effect. Even though there are attempts to minimize the development of VILI (ventilator-induced lung injury), it is a fact of life that VILI develops a significant number of patients with circulatory shock, and it is, therefore, connected to the pathogenesis of circulatory shock itself. Hence, we briefly mention in our review that several studies have investigated the molecular pathogenesis of VILI and have implicated the potential role of peroxynitrite and related reactive species (175).

Conclusions

Multiple lines of evidence indicates that peroxynitrite, a labile, cytotoxic species, (a) is produced in various forms of circulatory shock; (b) has the capacity to induce cell and organ damage including cellular metabolic suppression and cell death (apoptosis and necrosis); and (c) its pharmacological neutralization exerts beneficial effects in various models of

circulatory shock, as evidenced by peroxynitrite decomposition catalysts (Table 2), as well as compounds that act as combined inhibitors of iNOS and scavengers of peroxynitrite (176–180). The pathogenetic roles of peroxynitrite not only include the promotion of vascular and myocardial dysfunction and hepatic, renal and pulmonary dysfunction (key components of organ failure), but also intestinal dysfunction (86, 181), pancreatic injury (108, 182), as well as skeletal muscle dysfunction (183, 184). In addition, peroxynitrite may possibly also contribute to the pathogenesis of cellular metabolic failure ('cytopathic hypoxia'). Several studies also demonstrate a correlation between its formation and the severity of the disease in *human* circulatory shock. Based on these findings, the conclusion can be formed that peroxynitrite is a pathogenetic factor and a potential drug development target in circulatory shock. We must keep in mind, however, that peroxynitrite is a cytotoxic byproduct of nitric oxide, and nitric oxide exerts multiple vital physiological roles (Fig. 1). Therefore, selective neutralization of peroxynitrite formation (e.g. using catalytic inhibitors of superoxide formation or by compounds that promote the catalytic decomposition of peroxynitrite) appears to represent a preferred approach over non-selective pharmacological inhibition of NO generation.

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Abbreviations used

AIF	apoptosis-inducing factor
BH_4	tetrahydrobiopterin
cGMP	soluble guanylate cyclase (sGC)-cyclic guanosine-3′,5′-monophosphate
$CO_3^{\bullet-}$	carbonate
dG	deoxyguanosine
eNOS	endothelial nitric oxide synthase
FAK	phosphorylated focal adhesion kinase
FeTPPS	5,10,15,20-tetrakis(4-sulfonatophenyl)-porphyrinato iron (III)
FeTTPs	5,10,15,20-tetrakis(4-sulfonatophenyl) porphyrinato iron III chloride
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HDAC2	histone deacetylase
iNOS	inducible nitric oxide synthase
LNIL	N6-(iminoethyl)-L-lysine
LPS	bacterial lipopolysaccharide
MMPs	matrix metalloproteinases
MnSOD	manganese superoxide dismutase

NAD^+	Nicotinamide adenine dinucleotide
NO	nitric oxide
$^{\bullet}NO_2$	nitrogen dioxide radical
$O_2^{\bullet-}$	superoxide
8-OH-dG	8-hydroxy-deoxyguanosine
$^{\bullet}OH$	hydroxyl radical
$ONOO^-$	peroxynitrite anion
PAR	poly(ADP-ribose) polymer
PARG	poly(ADP-ribose) glycohydrolase
PARP	poly (ADP-ribose) polymerase
PPARγ	peroxisome proliferator-activated receptor gamma
PTP	permeability transition pore
SIN-1	3-morpholinosydnonimine
SNAP	S-nitroso-N-acetylpenicillamine-amine
VILI	ventilator-induced lung injury

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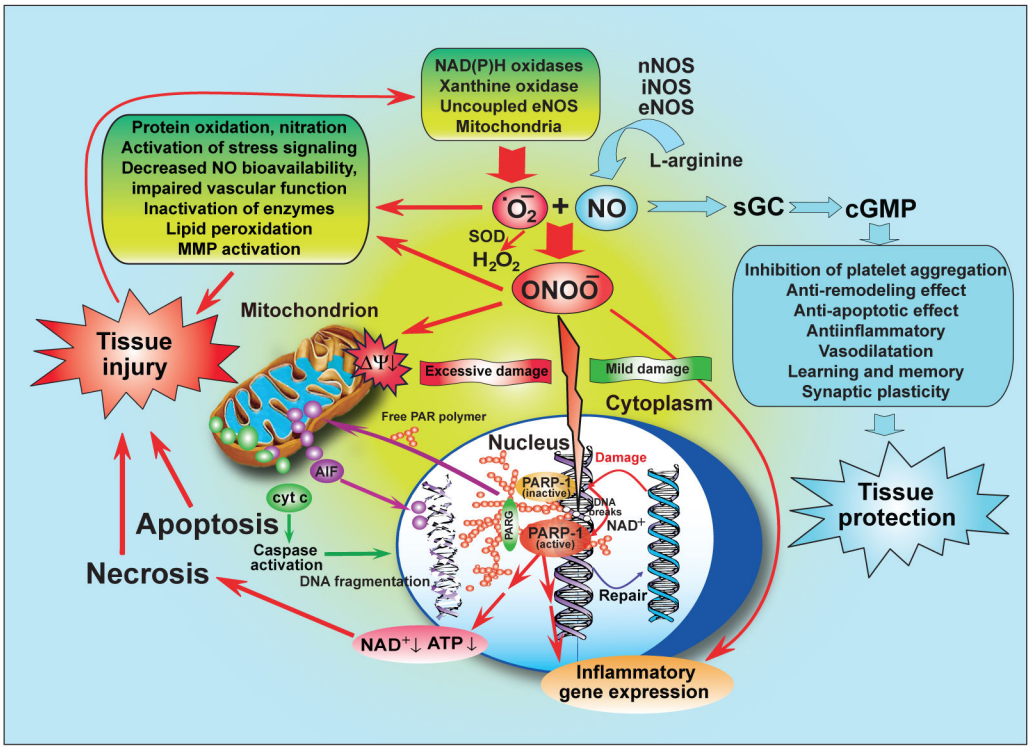

Figure 1. The nitric oxide – peroxynitrite - PARP pathway in circulatory shock

Nitric oxide (NO) by activating soluble guanylate cyclase (sGC)-cyclic guanosine-3′,5′-monophosphate (cGMP) signal transduction pathway mediates various physiological/beneficial effects including vasodilation, inhibition of platelet aggregation, anti-inflammatory, anti-remodeling and anti-apoptotic effects. In circulatory shock nitric oxide and superoxide ($\dot{O}_2^-$) react to form peroxynitrite ($ONOO^-$), which induces cell damage via lipid peroxidation, inactivation of enzymes and other proteins by oxidation and nitration and activation of stress signaling, matrix metalloproteinases (MMPs). Mitochondrial enzymes are particularly vulnerable to attacks by peroxynitrite, leading to reduced ATP formation and induction of mitochondrial permeability transition by opening of the permeability transition pore (PTP), which dissipates the mitochondrial membrane potential ($\Delta\Psi$). These events result in cessation of electron transport and ATP formation, mitochondrial swelling and permeabilization of the outer mitochondrial membrane, allowing the efflux of several pro-apoptotic molecules, including cytochrome c and apoptosis-inducing factor (AIF). In turn, cytochrome c and AIF activate a series of downstream effectors, which mediate caspase dependent and independent apoptotic death pathways. In addition to its damaging effects on mitochondria, peroxynitrite, in concert with other oxidants, causes oxidative injury to DNA, resulting in DNA strand breakage which in turn activate the nuclear enzyme poly(ADP-ribose) polymerase (PARP-1). Activated PARP-1 consumes NAD^+ to build-up poly(ADP-ribose) polymers (PAR) which are then metabolized by the activity of poly(ADP-ribose) glycohydrolase (PARG). Peroxynitrite, at least in part via overactivated PARP-1, may also facilitate the expression of a variety of inflammatory genes leading to increased

inflammation and associated tissue injury. (Reprinted from Am. J. Pathol 2008, 173:2–13 (5), with permission from the American Society for Investigative Pathology).

Table 1

Pathogenetic roles of peroxynitrite in circulatory shock

	Potential mechanisms
Vascular dysfunction	Catecholamine oxidation; inhibition of catecholamine receptors and suppression of vascular smooth muscle function (via mitochondrial inhibition and via PARP activation) leading to suppression of contractile function. Direct damage to vascular endothelial cells via nitrosative damage and via PARP activation. Inhibition of endothelial function via activation of neutrophils. Inhibition of vascular prostacyclin synthetase. These alterations may culminate in tissue edema and in an inadequate perfusion of tissues.
Myocardial dysfunction	Inhibition of myocyte cellular respiration, nitration and inhibition of cardiac myofibrillar creatine kinase, inhibition of alpha-actinin and of myofibrillar proteins, activation of matrix metalloproteinases and activation of PARP, leading to suppression of myocardial contractile function.
Gut epithelial failure	Epithelial cell injury via mitochondrial dysfunction, DNA damage, PARP activation, cellular energetic failure, secondary intracellular oxidant generation, resulting in increased epithelial paracellular permeability, bacterial translocation and secondary positive feedback cycles of injury.
Renal failure	Epithelial cell injury via mitochondrial dysfunction, DNA damage, PARP activation, cellular energetic failure, secondary intracellular oxidant generation.
Hepatic failure	Hepatocyte injury via mitochondrial dysfunction, DNA damage, PARP activation, cellular energetic failure, secondary intracellular oxidant generation, resulting in hepatocyte death.
Pulmonary dysfunction	Epithelial cell injury; damage to pulmonary surfactants, leading to impaired pulmonary oxygen exchange.
Systemic inflammation	Upregulation of signal transduction mechanisms; enhanced production of pro-inflammatory cytokines and chemokines, upregulation of neutrophil adhesion molecules, resulting in further exacerbation of the inflammatory response and organ failure.

Table 2

Effects of peroxynitrite neutralizing agents in animal models of shock

Decompositio n catalyst	Shock model	Main effects	References
FeTMPS, FeTMPyP	Endotoxin- induced duodenal and intestinal damage in rats.	Reduction of microvascular leakage, lipid peroxidation, and epithelial cell injury.	(185, 186)
FeTMPS	Splanchnic artery occlusion shock in rats.	Reduction of bowel injury and improvement of survival rate. Reduction of the intensity P-selectin and ICAM-1 expression.	(187)
FeTPPS	Endotoxin shock models in rats.	Improvement in myocardial contractile function. Protection against LPS-induced vascular failure, hypotension, tissue injury, and mortality.	(142) (108)
FP15	Rat and mouse models of endotoxic shock and polymicrobial sepsis.	Reduction in hepatic injury, improvement in survival rate.	Soriano and Szabo, unpublished observations.
WW85	IL-2-induced pulmonary injury and systemic inflammation in sheep.	Improvement in lung transvascular fluid flux, decreased lipid peroxidation, prevention of tachycardia, and reduction in fever.	(174)
	Mouse model of cecal ligation induced sepsis.	Improvement in survival rate.	Radermacher and Szabo, unpublished observations.
MnTCPP	Rodent models of endotoxic and zymosan induced shock.	Prevention of vascular dysfunction and cellular energetic alterations. Reduction in peritoneal exudation, polymorphonuclear migration and peroxynitrite formation. Improvement in cellular energetics *ex vivo.*	(81) (188)
MnTE-2-PyP	Cecal ligation and puncture in rats.	Preservation of mitochondrial function and diaphragmatic contractility.	(189)