Published in final edited form as:
Front Biosci. ; 14: 4809–4814.

Role of peroxynitrite in the redox regulation of cell signal transduction pathways

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Abstract

Peroxynitrite is a potent oxidant and nitrating species formed from the reaction between the free radicals nitric oxide and superoxide. An excessive formation of peroxynitrite represents an important mechanism contributing to cell death and dysfunction in multiple cardiovascular pathologies, such as myocardial infarction, heart failure and atherosclerosis. Whereas initial works focused on direct oxidative biomolecular damage as the main route of peroxynitrite toxicity, more recent evidence, mainly obtained *in vitro*, indicates that peroxynitrite also behaves as a potent modulator of various cell signal transduction pathways. Due to its ability to nitrate tyrosine residues, peroxynitrite affects cellular processes dependent on tyrosine phosphorylation. Peroxynitrite also exerts complex effects on the activity of various kinases and phosphatases, resulting in the up- or downregulation of signalling cascades, in a concentration- and cell-dependent manner. Such roles of peroxynitrite in the redox regulation of key signalling pathways for cardiovascular homeostasis, including protein kinase B and C, the MAP kinases, Nuclear Factor Kappa B, as well as signalling dependent on insulin and the sympatho-adrenergic system are presented in detail in this review.

Keywords

Cardiovascular Diseases; Cell Signalling; Insulin; MAP kinase; Nitration; Nitric Oxide; Nuclear Factor Kappa B; oxidation; Peroxynitrite; Phosphorylation; Protein Kinase B; protein kinase C; Superoxide; Tyrosine; Review

2. INTRODUCTION: FORMATION, REACTIVITY AND CHEMICAL BIOLOGY OF PEROXYNITRITE

Peroxynitrite is a strong biological oxidant and nitrating compound formed from the extremely rapid, diffusion limited (rate constant k between 6.6 and $19 \times 10^9 M^{-1}sec^{-1}$) reaction between two parent radicals, the superoxide anion ($O_2^{\cdot -}$) and nitric oxide (NO) (1). The rate of this reaction is at least 3–8 times greater than the rate of $O_2^{\cdot -}$ decomposition by superoxide dismutase (SOD, $k=2.5 \times 10^9 M^{-1}sec^{-1}$), indicating that NO has the ability to drive $O_2^{\cdot -}$ away from its main detoxification pathway (1). The cellular sources of NO are restricted to the various isoforms of NO synthases, whereas $O_2^{\cdot -}$ can arise from the

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mitochondrial electron transport chain, NADPH oxidase and xanthine oxidase (1, 2), as shown in Figure 1. Further, NO synthase can also become an $O_2^{\cdot -}$ generator when it is "uncoupled", as may occur in conditions of oxidant stress or reduced substrate (L-arginine) or cofactors (tetrahydrobiopterin) availability (3). Importantly, the reaction of NO with $O_2^{\cdot -}$ essentially depends on the *ratio* of superoxide to NO rather than the *net amount* of these two precursors, implying that peroxynitrite is generated whenever approximately equal, but not necessarily augmented, fluxes of NO and $O_2^{\cdot -}$ are present (1).

Peroxynitrite easily crosses biological membranes, and despite a relatively short half-life (~10 ms), it can interact with target molecules in adjacent cells within one or two cell diameters (1, 2). It oxidizes target molecules either directly, through one or two-electron oxidation processes, (most notably thiols and iron-sulfur centers in proteins), or indirectly, via the generation of highly reactive radicals formed from the reaction of peroxynitrite with carbon dioxide, (which generates $CO_3^{\cdot -}$ and NO_2.), and from the homolysis of peroxynitrous acid (ONOOH) into OH. and NO_2. (1, 2). These radicals are strong one-electron oxidants and NO_2. can additionally trigger the nitration of tyrosine residues in proteins, forming 3-nitrotyrosine (4), as detailed below. Nitrotyrosine can easily be detected, and thus is helpful to assess the formation of peroxynitrite in biological systems (5), although it must be stressed that nitrotyrosine may also be formed independently from peroxynitrite, following the generation of NO_2. by various heme-peroxidases (myeloperoxidase and eosinophil peroxidase) in the presence of hydrogen peroxide (1, 6).

If formed in sufficient amounts to overcome cellular antioxidant defenses, peroxynitrite and its secondary radicals will inflict various oxidative damage to proteins, lipids and DNA. In turn these alterations inactivate metabolic enzymes, ionic pumps, and structural proteins, disrupt cell membranes and break nucleic acids, resulting in the dysfunction of multiple cellular processes and the induction of cell death through both apoptosis and necrosis, depending on the level of peroxynitrite (1, 2), as shown in Figure 1. A major pathway of peroxynitrite-dependent cytotoxicity is represented by the activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP) in response to DNA damage, which may trigger cell necrosis by ATP depletion, as indicated in figure 1 (see (7) for a detailed recent review on this particular topics). There is now substantial evidence that these multiple pathways of peroxynitrite-mediated cytotoxicity play key roles in the development and perpetuation of a number of cardiovascular and non cardiovascular diseases, as extensively reviewed recently (1, 2). A less well characterized consequence of peroxynitrite relies in the modulation of cell signal transduction. Increasing experimental evidence, mainly obtained in cellular models, indeed supports such novel roles of peroxynitrite and has changed our understanding of the pathophysiolgical roles of this species, as will be presented in detail in this review.

3. REDOX REGULATION OF CELL SIGNALLING BY PEROXYNITRITE

3.1. Tyrosine nitration and phosphotyrosine signalling

Protein tyrosine nitration is a covalent modification resulting from the addition of a nitro (-NO_2) group onto one of the two equivalent ortho carbons of the aromatic ring of tyrosine (4), which may produce three distinct effects on the affected proteins: loss of function, gain of function, or no effect (5). Widespread tyrosine nitration occurs in cells during *in vitro* exposure to peroxynitrite, affecting structural proteins, ion channels, metabolic enzymes, and proteins involved in apoptosis, to name only a few (see (5, 8, 9) for recent reviews on protein tyrosine nitration). The relevance of such observations *in vivo* remains, however, to be established, given that the yield of nitrotyrosine formation under conditions of elevated peroxynitrite generation *in vivo* (nitro-oxidative stress) remains largely smaller than what can be achieved *in vitro* with direct peroxynitrite exposure. Indeed, only 1 to 10 residues of tyrosine per 100,000 are found nitrated in plasma proteins under inflammatory conditions,

such as those observed in cardiovascular disease, although up to 10 times more 3-NT can be detected in tissues (9).

A major aspect of tyrosine nitration by peroxynitrite is the possibility of impairment of cellular processes depending on the generation of phosphotyrosine (1), as initially demonstrated in *in vitro* studies using various synthetic peptide models. For example, Tyr^{15} nitration of a synthetic peptide mimicking the tyrosine phosphorylation site of p34 cdc2 (a cyclin-dependent kinase required for completion of mitosis) fully prevented its phosphorylation by the Src kinase p56 Lck (10), while nitration of a single amino acid residue in synthetic substrates for the tyrosine kinases $p43^{v\text{-}alb}$ and $pp60^{src}$ reduced their phosphorylation by more than 50% (11). Similarly, peroxynitrite-induced tyrosine nitration at $Tyr^{686}$of a synthetic analogue of platelet-endothelial cell adhesion molecule 1 impaired its src-dependent phosphorylation and its binding to the tyrosine phosphatase SHP-2 (12). Further extending these observations, Saeki *et al.* showed that the peroxynitrite donor *3*-morpholinosydnonimine (SIN-1) triggered the nitration, while suppressing the phosphorylation, of the focal adhesion kinase (FAK) $p130^{cas}$ in human neuroblastoma SH-SY5Y cells, an effect abrogated by the tyrosine phosphatase inhibitor vanadate (13). Interestingly, vanadate also prevented the cell death triggered by SIN-1, suggesting that tyrosine nitration of $p130^{cas}$ might inhibit a key survival signalling pathway, possibly by interfering with the assembly of focal adhesion complexes (14).

The hypothesis that tyrosine nitration would essentially inhibit phosphotyrosine-dependent cell signaling has been largely reviewed following a series of investigations concluding that peroxynitrite rather promoted the process of tyrosine phosphorylation in a variety of cell types (15–23). Proposed mechanisms involve an imbalance between tyrosine phosphorylation and desphosphorylation, related to the activation of tyrosine kinases or the inhibition of tyrosine phosphatases, respectively, by peroxynitrite (1).

Firstly, Several receptor tyrosine kinases exhibit stimulated activity upon peroxynitrite exposure, triggering downstream phosphotyrosine-dependent signalling, as shown for the platelet-derived growth factor receptor (PDGFR) and the receptor for brain-derived neurotrophic factor (TrkB) in murine fibroblasts (24, 25), as well as the Epidermal Growth Factor Receptor (EGFR) in rat lung myofibroblasts (26). Secondly, peroxynitrite can directly enhance the activity of the non receptor tyrosine kinase Src, a family of proteins involved in signalling networks regulating cell metabolism, viability, proliferation, differentiation and migration (27), and whose catalytic activity is tightly regulated by the degree of phosphorylation of a specific C-terminal Tyr residue (Tyr^{527}): phosphorylation of this residue maintains the kinase inactive by inducing its interaction with an –SH2 domain within the protein, whereas its dephosphorylation triggers kinase activation by removing this interaction (28). Activation of Src by peroxynitrite, as reported in nervous cells (20, 29–31), endothelial cells (15) and erythrocytes (18, 20–22), remains incompletely understood in terms of molecular mechanism. However, according to Mallozzi *et al*, it might rely on the formation of a putative nitrated peptides, able to displace phosphorylated Tyr^{527} from its binding site in the SH2 domain (21, 32), thereby producing a conformational change identical to that produced by Tyr^{527} dephosphorylation.

Thirdly, peroxynitrite may also enhance phosphotyrosine signalling by impairing the activity of phosphotyrosine phosphatases. These proteins share a conserved cysteine residue within their active site, which is extremely sensitive to peroxynitrite-mediated oxidation and enzyme inactivation (19, 33–35). It has been proposed that, due to its structural similarities with phosphate anions, peroxynitrite might be attracted within the active site of the phosphatase, resulting in the oxidation of this critical cysteine-bound thiol and its subsequent inactivation (35).

In summary, at variance with the initial proposal that peroxynitrite would exclusively impair phosphotyrosine-dependent signalling by nitrating tyrosine, it has become evident that peroxynitrite may also upregulate such signalling depending on the cell type studied, the particular signaling pathway under investigation, and especially, the concentration of peroxynitrite used. Peroxynitrite has been generally associated with transient and reversible upregulation of phosphotyrosine at relatively low concentrations (10–200 microM), whereas higher peroxynitrite concentrations have been correlated with irreversible nitrotyrosine formation and suppression of phosphotyrosine signalling due to direct competition between nitration and phosphorylation of tyrosine. Such dichotomy has been well described in the band-3 protein, a membrane anion-exchange protein that mediates signalling events stimulating glycolytic activity in human erythrocytes (19). Relatively low peroxynitrite concentrations (10–100 microM) activated band-3 tyrosine phosphorylation through an inhibition of phosphotyrosine phosphatase, leading to GAPDH activation and lactate production. In contrast, higher peroxynitrite concentrations (200–1000 microM) induced band-3 tyrosine nitration and inhibited tyrosine phosphorylation, leading to irreversible suppression of glycolysis.

3.2. Peroxynitrite-mediated regulation of individual cell signalling pathways

3.2.1. Akt/protein kinase B—PKB (Akt) is a serine-threonine protein kinase that integrates cellular responses to growth factors and other extracellular signals, most significantly insulin. Activation of PBK/Akt crucially depends on phosphatidyl-inositol-3 kinase (PI3K) signalling through generation of 3′-phosphorylated phosphoinositides (36). PI3K, a member of the lipid kinase family, is composed of a catalytic subunit (p110) and an adaptor/regulatory subunit (p85). Upon receptor tyrosine kinase activation, the SH2 domain of PI3K associates with the receptor, triggering poly-phosphoinositides that activate target proteins including PKB/Akt, which harbour the protein-lipid binding domain pleckstrin-homology (PH) domain. Upon interaction with phosphoinositides, PKB/Akt translocates to the cell membrane where it is phosphorylated and activated by 3-phosphoinositide-dependent kinases 1 and 2 (PDK1 and PDK2). PKB/Akt then transits through the cytosol and eventually to the nucleus to regulate the activity of many substrates that influence cell survival, growth and differentiation (37). Akt plays essential roles in the cardiovascular system, sustaining potent cardioprotective activities in most pathological cardiac conditions (38), mediated by multiple downstream effectors, especially mTOR, glycogen-synthase kinase 3 (GSK3) and Pim-1 (39, 40). Furthermore, the PI3K-PDK-Akt axis ultimately culminates in the pleiotropic biological actions of insulin, notably in vascular function, by promoting nitric oxide (NO) release by phosphorylating serine (Ser) 1177 of endothelial NO synthase, thereby serving a critical role in the maintenance of physiological endothelial function.

Discordant data have been reported regarding the regulation of Akt signalling by peroxynitrite. Both authentic peroxynitrite and the peroxynitrite donor SIN-1 triggered PKB/Akt phosphorylation and activation, as reflected by increased phosphorylation of the PKB/Akt substrate GSK3 in human skin fibroblasts (24). Activation of PKB/Akt was here dependent on PDGFR and PI3K, because it was effectively inhibited by the specific inhibitors AG1295, respectively wortmannin and LY294002 (24). In a rat hepatoma cell line, peroxynitrite induced PI3K activation and PKB/Akt phosphorylation, leading to the activation of Nrf2/ARE transcription factor and the upregulated expression of the detoxifying enzyme glutathione-S-transferase (41). A similar conclusion was reached by Li *et al*, who demonstrated a peroxynitrite-dependent phosphorylation of Akt triggering the activation of Nrf2/ARE, and the subsequent upregulation of the antioxidant and cytoprotective enzyme heme-oxygenase-1 in neural cells, resulting in the limitation of peroxynitrite-mediated apoptosis (42). The mechanisms underlying Akt activation by

peroxynitrite in the above-mentioned studies has not been detailed, but two experimental works suggest that such activation rests on an oxidative, but not a nitrative, type of chemistry induced by peroxynitrite (43, 44).

In contrast with these findings, peroxynitrite inhibited Akt signalling in PC12 cells (45), endothelial cells (46–49), and Raw 264.7 macrophages (50). Restricted interactions of PI3K and phosphotyrosine proteins could account for these findings (51), since peroxynitrite nitrates a critical tyrosine residue in the p85 regulatory subunit of PI3K, preventing its association with the catalytic p110 subunit and subsequent PI3K activation (48, 50). Furthermore, as recently shown by Song and co-workers, Akt inhibition by peroxynitrite may depend on a complex cascade of signalling, whereby peroxynitrite would first enhance the nuclear shuttling of the zeta isoform of protein kinase C, followed by the nuclear export of the serine-threonine protein kinase and tumor suppressor LKB1, leading to the activation of the lipid phosphatase PTEN (Phosphatase and TENsin homologue deleted on chromosome 10), culminating into the impairment of PI3K/Akt signalling (46, 47). The inhibition of Akt by peroxynitrite has been associated with two major consequences, the first one being enhanced and accelerated apoptosis (46–48), notably in endothelial cells, the second one being the inhibition of insulin signalling (46). Given the well-known increase of vascular oxidative stress and peroxynitrite generation in diabetic vessels, these detrimental actions of peroxynitrite on Akt may play a critical role in the development and perpetuation of the endothelial dysfunction associated with diabetes (46, 52).

To sum up, peroxynitrite can either promote or inhibit the activation of the PI3K/PKB (Akt) pathway by inducing oxidation and tyrosine nitration reactions, respectively. The prevailing effect may depend on peroxynitrite concentrations, the cell type studied, and the chemical microenvironement. The *in vivo* relevance of peroxynitrite in the regulation of the PI3K/Akt pathway in the cardiovascular system remains to be addressed in future studies.

3.2.2. Mitogen-activated protein kinases—The mitogen-activated protein kinases (MAPKs) are the most important family of serine-threonine kinases, upon which many other signalling pathways converge. MAPKs are activated by dual phosphorylation at a specific tripeptide motif, mediated by a conserved protein kinase cascade involving MAPK kinases (MKK or MEK) and MAPK kinase kinases (MKKK or MEKK) (53). Upstream signalling pathways triggering MKKK activation mainly depends on the activation of growth factor receptors and small G-proteins such as Ras, Rac and Cdc42 (36, 53). The four major groups of MAPKs are extracellular signal-regulated protein kinase (ERK1/2), p38 MAPK (alpha, beta, ga,mma, delta), c-Jun NH_2-terminal kinase (JNK1, 2 and 3) and big MAP kinase (BMK or ERK5) (54, 55). MAP kinases become activated upon cell exposure to a wide range of stimuli, notably including oxidants and particularly peroxynitrite, as presented in the next sections.

3.2.2.1 Extracellular signal-regulated protein kinase (ERK): ERK plays central roles in signalling pathways activated by certain growth factors such as EGF (through the activation of EGFR, ras, Raf-1 kinase, and MEK1) (26). In addition, ERK can be activated by extracellular stress stimuli, such as oxidants and free radicals (36). In the heart, ERK represents a critical regulator of myocardial hypertrophy, as well as an important cardioprotective signalling molecule in various forms of stresses associated with myocyte apoptosis, eg myocardial ischemia-reperfusion and heart failure, as extensively reviewed in (38, 55).

The mechanism underlying ERK activation by oxidative stress is unclear. Direct activation of growth factor receptors, inhibition of MAPK phosphatases, and direct activation of ras, which acts as an oxidative stress sensor, might contribute to ERK activation by oxidants.

Data in isolated fibroblasts (26, 56), neutrophils (57, 58), endothelial and vascular smooth muscle cells (59–61), neural cells (31, 62), cardiomyocytes (63) and whole lung tissue (64) showed that, in addition to other known oxidants, peroxynitrite is also a powerful activator of ERK. However, distinct mechanisms may account for this effect in different cell types.

Indeed, activation of ERK depended on EGFR, Src tyrosine kinase and calcium calmodulin in PC12 cells (31), while requiring ras/Raf-1/MEK activation in human neutrophils (58). In endothelial cells, peroxynitrite activated ERK following the direct oxidative S-glutathiolation of p21ras at cysteine-118, leading to increase Raf-1 binding and downstream activation of ERK (59, 60). In contrast to these findings, we reported in H9C2 cardiomyocytes that peroxynitrite-mediated activation of ERK, although dependent on Raf-1 and MEK, was not due to upstream activation of p21ras (63). To address the respective roles of oxidation and nitration of tyrosine residues in peroxynitrite-mediated Raf-1/MEK/ERK activation, we took advantage of the fact that epicatechin, a flavanol extracted from green tea, inhibits tyrosine nitration much more efficiently than tyrosine oxidation. Our data showed that epicatechin prevented peroxynitrite-mediated protein nitration but did not affect ERK activation, indicating that oxidative chemistry of peroxynitrite primarily accounts for ERK activation in this setting (63). However, alternate mechanisms appear relevant in other cell types. For example, peroxynitrite-mediated ERK activation was entirely dependent on tyrosine nitration of MEK in rat lung myofibroblasts (62). Although peroxynitrite triggered EGFR and Raf-1 phosphorylation in these cells, this effect was dispensable for MEK/ERK activation. In some cell systems, activation of ERK upon peroxynitrite exposure was observed independently from any activation of the Raf-1/MEK pathway. Instead, it was related to the upstream activation of a calcium-dependent PKC isoform in rat-1 fibroblasts, and was due to the peroxynitrite-mediated release of Zinc from intracellular stores in primary oligodendrocytes (62).

Downstream events induced by the activation of ERK in response to peroxynitrite have remained poorly investigated so far. It has notably been associated with an enhancement of apoptotic cell death in primary murine neurons and astrocytes (65, 66), as well as in human neuroblastoma cells (67) and bronchial epithelial cells (68). A further consequence appears to be the activation of polymorphonuclear cells (PMNs). Indeed, ERK activated upon peroxynitrite exposure triggered the upregulation of beta2-integrin CD11b/CD18 in human PMNs, increased their adhesion to the endothelium (58), and enhanced their production of oxygen free radicals (57). Finally, the cascade peroxynitrite-p21ras-Raf-1-ERK (see above) in endothelial cells (59, 60) has been associated with the concomitant prevention of Akt activation in response to insulin (60), supporting its involvement in the resistance to insulin observed in type II diabetes, as exposed in detail below.

3.2.2.2. c-Jun NH_2-terminal kinase (JNK): JNK exists as three distinct isoforms (JNK-1, 2 and 3), activated in response to environmental stress stimuli, such as UV light, heat shock, osmotic stress, inflammatory cytokines, mechanical stress, and oxidants. JNK is activated by small G-proteins (ras/rac) through a signalling pathway that involves multiple MAPKKK, MKK1 and MKK4. These kinases are linked to each other by scaffold proteins (such as JIP-1) in specific signalling modules (69, 70). Activated JNK phosphorylates the proto-oncogene c-jun, which forms both homodimers and heterodimers with c-fos, leading to the activation of the AP-1 transcription factor. The JNK signalling pathway plays major roles in inflammatory responses and apoptosis. In analogy to ERK and p38 MAPK, divergent data link JNK activation to either cell death or survival in different cell types exposed to various stress stimuli for varying periods of time (36, 69). With specific respect to the heart, we recently provided evidence of a direct role of JNK as an effector of cardiac damage during myocardial infarction, by showing that a specific peptide inhibitor of JNK reduced apoptosis

and infarct size, while preventing myocardial dysfunction in rats exposed to myocardial ischemia and reperfusion (71).

Free radicals and oxidant species such as H_2O_2 can inactivate specific JNK phosphatases and activate upstream signalling molecules in the JNK signalling pathway (36). It is thus not surprising that JNK activation in response to peroxynitrite has been reported in a variety of cell types *in vitro*, including bronchial and alveolar lung cells (68, 72), rat cardiomyocytes (63), vascular endothelial and smooth muscle cells (73–75), liver epithelial cells (76), rat PC12 cells (77) and mouse fibroblasts (78). Peroxynitrite-mediated JNK activation has been associated with apoptotic cell death in murine alveolar C10 cells, in which JNK was activated upon the oxidation of the death receptor Fas. Peroxynitrite-mediated Fas oxidation resulted into Fas aggregation and the recruitment of the downstream effector FADD (Fas-associated death domain), finally converging to JNK-dependent apoptosis (72). In cardiovascular tissues information on peroxynitrite and the JNK pathway is scarce. We reported that cultured H9C2 cardiomyocytes display a strong phosphorylation of both JNK1 and JNK2 in the presence of graded concentrations of peroxynitrite, but the outcome of such activation was not investigated in this study (63). In the vasculature, peroxynitrite-dependent JNK activation has been shown to occur in endothelial cells exposed to laminar shear stress, which is essential to prevent the development of atherosclerosis, suggesting that peroxynitrite may represent a key molecular link between endothelial mechanical stress and endothelial function (73).

3.2.2.3. p38 MAP kinase: p38 MAPK includes 5 isoforms (alpha, beta$_1$, beta$_2$, gamma and delta) (α, β_1, β_2, γ and δ), at least, which are activated by environmental stress via upstream MAPKKK, MKK3 and MKK6. In the heart, p38 can be protective or detrimental to myocyte survival in a stressor-specific manner, exerts strong pro-inflammatory and negative inotropic effects, and also appears to play a protective role against cardiac myocyte hypertrophy and myocardial remodeling (see (55) for review). A further emerging function of p38 is represented by the regulation of proliferation in terminally differentiated cardiomyocytes (79).

The role of p38 alpha and beta isoforms in the regulation of cell survival under oxidative stress conditions has been studied extensively, in part owing to the availability of specific pharmacological inhibitors of these isoforms. Activation of p38 by various oxidant species induces cell cycle arrest and apoptosis in many cell types (36), and the outcome appears similar with peroxynitrite. Indeed, the later is a powerful trigger of p38 phosphorylation and activation in a wide number of cell types, notably from cardiac (63), vascular (48, 73, 80) and neural origin (31, 81–85). Upstream intermediates linking peroxynitrite and p38 appear largely cell specific, and involved, for instance, phospholipase A2 in bronchial epithelial cells (61), calcium-calmodulin kinase II and src in PC12 cells (31), or the release of zinc ions (Zn^{++}) from intracellular stores in neurons (84, 85). The latter mechanism was recently demonstrated in a cellular system relevant to the pathogenesis of cerebral ischemia and neurodegenerative diseases. These diseases are associated with excessive stimulation of N-methyl-D-aspartate (NMDA) receptors, enhanced neuronal NO generation, and subsequent toxicity. Addition of exogenous NO or NMDA (to increase endogenous NO) to cerebrocortical neurons led to peroxynitrite formation and consequent Zn^{++} release from intracellular stores. Free Zn^{++} induced respiratory block, mitochondrial permeability transition and p38 activation, followed by cell volume loss and apoptotic cell death, which were prevented in cells harboring a dominant-negative form of p38 (84, 85). Importantly, peroxynitrite is known to release free Zn^{++} by efficiently oxidizing Zn^{++}-sulfur bridges in mitochondrial proteins (84) and cytosolic enzymes of various cell types (86, 87). This suggests that the crosstalk between peroxnitrite, Zn^{++} and p38 observed in neurons might also occur in cells from tissues other than the brain.

3.2.3. Protein kinase C—PKC is a family of phospholipid-dependent serine/threonine kinases that participate in many signalling pathways regulating cell growth and differentiation, apoptosis, immune reactions and stress responses, especially to oxidative stress (36, 88). PKC consists of 11 isoforms subdivided in three subgroups with distinct mechanisms of activation: classical PKC isoforms (alpha, betaI, betaII, gamma) are activated by both calcium and diacylglycerol, novel PKC isoforms (delta, epsilon, eta, theta) are activated by diacylglycerol alone, and atypical PKC isoforms (lambda, iota, zeta) are activated by certain lipids but neither diacylglycerol nor calcium. Phosphorylation-activation of PKC induces its translocation to subcellular compartments, a process assisted by scaffold proteins (receptors for activated c kinase; RACKs) (89).

The role of PKC isoforms in cardiovascular diseases has been extensively investigated in recent years. Notably, it has been established that two calcium-insensitive PKC isoforms, PKC delta and epsilon, play opposing roles in ischemia/reperfusion injury of the heart: activation of PKCdelta during reperfusion triggers mitochondrial alterations leading to apoptotic cell death, whereas activation of PKCepsilon before ischemia protects mitochondrial function and diminishes apoptosis (90). In fact, activation of PKCepsilon represents a central mechanism of the cardioprotection afforded by so-called ischemic preconditioning, a brief sequence of short bouts of ischemia-reperfusion preceding a sustained ischemic event. During IPC, PKCepsilon is activated and translocates to cardiac mitochondria, resulting in the opening of mitoK_{ATP} (mitochondrial ATP-sensitive K^+) channels, the prevention of mitochondrial permeability transition pore opening, and protection against apoptosis (90).

Past evidence had been obtained that NO plays a major role in the process of PKCepsilon activation during ischemic preconditioning. Recent findings suggest that peroxynitrite, rather than NO, may be responsible for this effect, as indicated by Balafanova *et al.* (91). These authors demonstrated that stimulation of rat primary cardiomyocytes with an NO donor resulted in PKCepsilon activation, tyrosine nitration of PKCepsilon, and increased interaction of PKCepsilon to its anchoring protein RACK2. All these effects were suppressed by the peroxynitrite scavenger ebselen. Furthermore, peroxynitrite directly enhanced the binding of recombinant PKCepsilon and RACK2 in an *in vitro* assay. Comparable effects were detected in the intact myocardium in an *in vivo* model of NO-mediated cardioprotection in the rabbit, thereby delineating a novel signaling mechanism by which NO activates PKCepsilon in the heart via the generation of peroxynitrite (91).

In blood vessels, classical PKC isoforms regulate an array of physiological functions, and increased PKC activity (especially of the beta isoforms) has been associated with various acute and chronic vascular stresses, including hypoxia, ischemia-reperfusion, mechanical stress (most notably restenosis after angioplasty), atherosclerosis, and with diabetic vascular complications (92, 93). Importantly, all these forms of vascular stresses are associated with an enhanced formation of peroxynitrite in the vessel wall (1), and several line of evidence support a possible link between peroxynitrite and PKC activation in such conditions.

In bovine pulmonary endothelial cells, nitration of PKCalpha, associated with both its activation and translocation to the cell membrane occurs upon peroxynitrite stimulation. This effect is responsible for the downstream activation of cytosolic phospholipase A2 (cPLA2) and an increased release of arachidonic acid from the cell, implying that peroxynitite-dependent PKCalpha activation acts to modulate the production of vasoactive mediators from the endothelium (94). Furthermore, in retinal endothelial cell monolayers incubated with either high glucose or advanced glycation end-products, peroxynitrite formation occurs, leading to PKC activation and an increased permeability of the monolayers to radiolabelled iodide, substantiating an important role of peroxynitrite-PKC

signaling in altering normal endothelial physiology (95). Finally, peroxynitrite-mediated PKC activation has also been advocated to be involved in the development of vascular tolerance to nitroglycerin, a phenomenon which describes the progressive reduction of the vasodilating properties of nitrogycerin upon repeated exposure. Indeed, tolerance of rat aortic rings to nitroglycerine can be similarly prevented by the PKC inhibitor chelerythrine and by the peroxynitrite scavenger uric acid, whereas in bovine endothelial cells, exposure to nitroglycerin triggers the generation of peroxynitrite together with the activation and translocation of the two PKC isoforms alpha and epsilon (96).

3.2.4. Nuclear factor kappa B (NFkappaB)—The NFkappaB family of dimeric transcription factors regulates the expression of many genes involved in immune response, inflammation and cytoprotection from environmental stress. NFkappaB family members include p50 and p52 (as well as their p100 and p105 precursors), p65 (RelA), RelB and c-Rel. Under basal conditions, NFkappaB transcription factors are bound to inhibitory IkappaB proteins and sequestered in the cytoplasm. NFkappaB activation depends upon IkappaB phosphorylation and recognition by ubiquitinating enzymes. Proteasomal degradation of IkappaB proteins induces the release and nuclear translocation of IkappaB-bound NFkappaB transcription factors, which trigger the expression of target genes (see (97, 98). IkappaB proteins are phosphorylated by a protein kinase complex, IkappaB kinase (IKK), which is composed of two catalytic subunits, IKKalpha and IKKbeta, and a regulatory subunit, IKKgamma or NEMO (for review see (98). Many NFkappaB activation pathways converge on IKKalpha and IKKbeta. Inflammatory stimuli such as tumour necrosis factor alpha (TNFalpha) and lipopolysaccharides (LPS) trigger rapid NFkappaB activation in an IKKbeta-dependent manner (canonical pathway). Alternatively, a subset of TNF family members can induce the activation of a specific form of NFkappaB by a slower process dependent on the IKKalpha subunit (noncanonical pathway) (99).

Experimental evidence supports a pivotal role of NFkappaB in the pathophysiology of major cardiac diseases, including myocardial infarction and heart failure. In animal models of myocardial ischemia-reperfusion, several strategies inhibiting NFkappaB reduced infarct size and post-infarction myocardial inflammation (100–102). NFkappaB inhibition has also been associated with an attenuated myocardial hypertrophy induced by chronic angiotensin II infusion or aortic banding in the mouse (103, 104). More importantly, in murine models of heart failure after myocardial infarction, p50 KO mice are protected from cardiac remodeling and left ventricular dysfunction (105, 106). Comparable results have been obtained using a strategy of myocardial overexpression of IkappaBalpha by adenovirus-mediated transfection in rats (107). In humans, myocardial tissue from patients with congestive heart failure exhibits a robust activation of IKK and NFkappaB DNA binding, as well as an increased expression of genes regulated by NFkappaB, such as cyclo-oxygenase II, iNOS, TNFalpha and adhesion molecules, supporting the involvement of NFkappaB in the process of cardiac remodeling in chronic heart failure (108–110).

Overall, reactive oxygen species (ROS) appear to favour NFkappaB activation (111), but the specific role of peroxynitrite in this regard is incompletely understood. A series of experiments performed in human polymorphonuclear cells (PMNCs) indicated that exposure to exogenous peroxynitrite, as well as induction of endogenous peroxynitrite generation by LPS, cytokines or Toll-like receptor (TLR)-9, activated NFkappaB, leading to an increase in IL-8 secretion (112–115), implying that peroxynitrite may amplify neutrophil-dependent responses during inflammation in an NFkappaB–dependent manner. Also, micromolar concentrations of peroxynitrite triggered NFkappaB activation, along with TNFalpha and IL-6 production in mononuclear cells, an effect proposed to depend on the nitration of IkappaB at Tyr^{42}, triggering its proteasomal degradation (116). By the same token, Yakovlev *et al.* recently reported that nitration of IkappaB on Tyr^{181} was sufficient to

provoke its dissociation from NFkappaB and to trigger its activation independently from serine phosphorylation (117). A similar mechanism has further been proposed by Bar-Shai and Reznick, investigating the effects of peroxynitrite on NFkappaB activation in skeletal muscle (118, 119).

At variance with these findings, two recent reports unveiled suppressive effects of peroxynitrite on NFkappaB activation in different experimental systems. Data by Park *et al* (120) showed that peroxynitrite inhibited both constitutive and induced NFkappaB activities in P19, SH-SY5Y and HEK293 cells via p65 nitration at Tyr^{66} and Tyr^{152}, leading to p65 destabilisation and nuclear export. In line with these findings, we have shown that a short exposure of cardiac H9c2 cells or endothelial EAHY-926 and HMEC-1 cells to peroxynitrite completely inhibited IKKbeta phosphorylation and NFkappaB activation in response to LPS or inflammatory cytokines (121). These effects were accompanied by an increase in IKKalpha phosphorylation, consistent with differential regulation of IKKalpha and IKKbeta by peroxynitrite. Together, these findings provide initial evidence for the unforeseen concept that peroxynitrite may downregulate inflammatory responses under certain conditions. The in vivo relevance of this mechanism needs now to be investigated.

3.2.5 Insulin signalling—As mentioned previously, insulin, in addition to its metabolic effects, plays a critical role in the maintenance of physiological endothelial function through its ability to stimulate NO release via a cascade of signalling, involving activation of the PI3K-Akt axis and the downstream serine phosphorylation of endothelial NO synthase. Conversely, peroxynitrite inhibits PKB/Akt signalling in endothelial cells (see above), and might thus disrupt this essential vasoprotective role of insulin, a hypothesis elegantly confirmed in a recent study by Song *et al.* (46). These authors showed that exposure of cultured human umbilical vein endothelial cells (HUVECs) to peroxynitrite or high glucose significantly inhibited both basal and insulin-stimulated serine phosphorylation of PKB/Akt and its activation. Using small interfering (si) RNA technologies, they could demonstrate that these changes were related to an increased phosphorylation and activity of Phosphatase and TENsin homologue deleted on chromosome 10 (PTEN), elicited by the tumor suppressor LKB1, eventually leading to apoptotic cell death. *In vivo*, the aortas of diabetic mice exhibited increased levels of nitrotyrosine, together with increased phosphorylation of PTEN and LKB1, but reduced PKB/Akt phosphorylation. Administration of PTEN-specific siRNA reversed PKB/Akt inhibition and mitigated apoptosis in diabetic mouse aortas. These findings therefore suggest that hyperglycaemia may promote apoptosis in endothelial cells through PKB/Akt downregulation, via a peroxynitrite-mediated, LKB1-dependent PTEN activation (46).

A further mechanism of peroxynitrite-mediated alteration of insulin signalling has been unveiled by Nomiyama *et al*, who reported that peroxynitrite inhibited insulin-stimulated glucose uptake in preadipocyte-derived 3T3-L1 cells by reducing insulin receptor substrate-1 (IRS-1) protein levels and associated PI3K activity, upstream of Akt/PKB (122). The mechanism of this effect was worked-up in rat fibroblasts expressing human insulin receptors, and exposed to the peroxynitite generator SIN-1. Using mass spectrometry, it was found that peroxynitrite induced at least four nitrated tyrosine residues in rat IRS-1, including Tyr^{939}, which is critical for the association of IRS-1 with the p85 subunit of PI3K. Thus, peroxynitrite reduces the level of IRS-1, while inducing at the same time its tyrosine nitration, thereby impairing insulin signalling on Akt/PKB.

2.2.6. Sympatho-adrenergic signalling—The catecholamines epinephrine (or adrenaline) and norepinephrine (or noradrenaline) are key modulators of cardiac and vascular function, whose biological effects are mediated by adrenergic receptors, which exist as nine distinct subtypes, termed $alpha_{1A}$, $alpha_{1B}$, $alpha_{1D}$, $alpha_{2A}$, $alpha_{2B}$, $alpha_{2C}$,

$beta_1$, $beta_2$ and $beta_3$. All these receptors are G-protein-coupled receptors which, upon activation, stimulate (Gs-proteins) or inhibit (Gi-proteins) the signalling cascade adenylyl cyclase-protein kinase A, or activate (Gq-proteins) another effector enzyme, phospholipase C (PLC) (123). Among the alpha receptors, type 1 receptors mainly mediate smooth muscle contraction and cellular hypertrophy, whereas type 2 receptors, located on presynaptic noradrenergic nerve terminals, inhibit the release of catecholamines, and thus are critical to regulate sympathetic output (124). Regarding the subtypes of beta receptors, $beta_1$ and $beta_2$ exert positive inotropic effects on the heart and relax vascular smooth muscle, while the $beta_3$ subtype is responsible for a negative inotropic effect in the heart (125).

Experimental studies have indicated that both alpha- and beta-adrenoceptors are biological targets of peroxynitrite. Benkusky *et al.* examined the effects of systemic administration of peroxynitrite on the changes in arterial pressure and vascular resistances produced by the alpha-adrenergic catecholamines epinephrine, norepinephrine, and phenylephrine in anesthetized rats, and found that it markedly inhibited both pressor and vaosonstrictor responses to these agonists (126). This observation was extended by Takakura *et al.*, who performed binding experiments of (3H) prazosin and norepinephrine in Chinese hamster ovary cells expressing recombinant human alpha adrenoceptors. Peroxynitrite significantly reduced the binding capacity of both $alpha_{1A}$ and $alpha_{1D}$-adrenoceptors in a concentration-dependent manner, and attenuated noradrenalin-stimulated increases in intracellular Ca^{2+} concentration in cells expressing $alpha_{1A}$, and $alpha_{1D}$-, but not $alpha_{1B}$-adrenoceptors. These effects translated into a markedly reduced contractile response to norepinephrine of isolated rat aortic strips (127). Although the underlying mechanism was not anticipated in these studies, the negative effect of peroxynitrite on alpha-adrenoceptors suggests that it may be involved in pathological states associated with reduced vascular contractility, e.g. septic shock. The latter hypothesis is further reinforced by the reported ability of peroxynitrite to directly inactivate catecholamines by oxidative modifications (128, 129).

With respect to beta-adrenoceptors, Lewis and co-workers recently showed that peroxynitrite could block the effects of the beta agonist isoproterenol on $beta_1$ and $beta_2$-, but not $beta_3$ adrenoceptors (130). Since $beta_1$ and $beta_2$, but not $beta_3$-adrenoceptors contain extracellular tyrosine residues susceptible to nitration reactions, these observations suggest that peroxynitrite impairs beta-adrenergic responses by nitrating tyrosine resides within $beta_1$ and $beta_2$ adrenoceptors (130), a mechanism which has been proposed to participate to the blunted response of the myocardium to beta-adrenoceptor stimulation in patients with chronic heart failure (131, 132).

4. CONCLUSIONS AND PERSPECTIVES

Emerging evidence presented in this review indicates that peroxynitrite exhibits the distinctive features of a signalling molecule *in vitro*, leading to either activation or inhibition of a wealth of cellular signal transduction pathways, whose dysregulation plays key roles in the development and perpetuation of cardiovascular pathologies. Whereas early studies were based on the concept that peroxynitrite, owing to its ability to nitrate tyrosine residues in proteins, would principally behave as an inhibitor of phosphotyrosine-dependent cell signalling, it rapidly turned out that, in many instances, peroxynitrite could upregulate multiple signalling cascades by inhibiting protein phosphatases and activating many different protein kinases. In spite of this experimental evidence, several limitations presently hinder the precise interpretation of these findings. First, given their conspicuous cell specificity, any generalization of these effects would be extremely speculative. Second, the likely cellular compartmentalization of the signalling response triggered by peroxynitrite remains essentially unexplored. Third, the scarcity of *in vivo* results that confirm (or disagree with) *in vitro* data indicate that the relevance of peroxynitrite-dependent signalling

in pathological states remains to be precised. Obviously, there is the need for further studies exploring signalling roles of peroxynitrite and its mechanisms both at the cell level and in the intact organism. Improving our understanding of such aspects of peroxynitrite biology in the pathogenesis of cardiovascular diseases will pave the way to the discovery of novel efficient drugs in the future.

Acknowledgments

Supported by grants from the Swiss National Fund for Scientific Research Nr 320000-118174/1 to LL and Nr 310000-118210 to GV and by the Intramural Research Program of the National Institutes of Health to PP.

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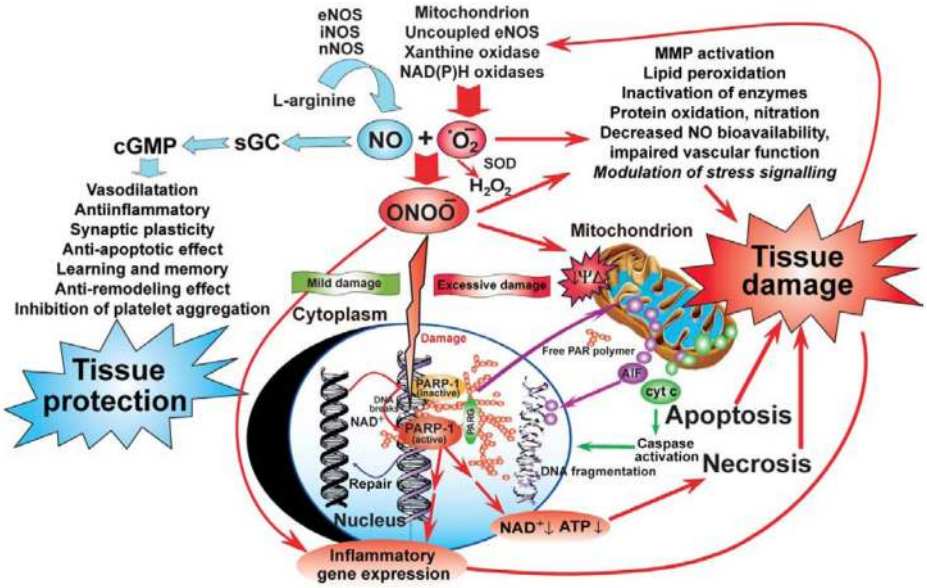

Figure 1.

Formation, chemical biology and major targets of peroxynitrite-induced cellular toxicity. Peroxynitrite is formed from the reaction between the free radicals nitric oxide (NO) and superoxide ($O_2^{\cdot -}$). NO arises from the oxidation of L-arginine by various NO synthases isoforms (endothelial-eNOS-, inducible-iNOS-, and neuronal-nNOS). In turn, NO activates soluble guanylyl-cyclase (sGC) to form the second messenger cyclic GMP (cGMP), which mediates many physiological and cytoprotective actions of NO, as indicated by the blue arrows. $O_2^{\cdot -}$ is formed in the mitochondrion, as well as by the enzymatic activities of xanthine oxidase, NADPH oxidase, and uncoupled eNOS. $O_2^{\cdot -}$ may be transformed by superoxide dismutase (SOD) into hydrogen peroxide (H_2O_2). When equimolar fluxes of NO and $O_2^{\cdot -}$ occur in close proximity, they react immediately to yield peroxynitrite ($ONOO^-$). In turn peroxynitrite triggers a myriad of cytotoxic effects including lipid peroxidation, protein nitration and oxidation, DNA oxidative damage, activation of matrix metalloproteinases (MMP), and inactivation of a series of enzymes (red arrows). If severe enough to overcome cellular antioxidant defenses, the biomolecular injuries initiated by peroxynitrite can damage mitochondria, leading to the collapse of the mitochondrial transmembrane potential ($\Delta\Psi$) followed by the release of proapoptotic factors (cytochrome C -cyt C- and apotosis inducing factor-AIF) and the induction of apoptosis, and can also result in the activation of the nuclear enzyme poly (ADP-ribose) polymearse (PARP). Activated PARP consumes nicotinamide (NAD) to build-up poly (ADP-ribose) polymers (PAR), which are themselves rapidly metabolized by the activity of poly (ADP-ribose) glycohydrolase (PARG). Some free PAR may exit the nucleus and travel to the mitochondria, where they amplify the mitochondrial efflux of AIF. Overactivation of PARP results in massive NAD consumption and collapse of cellular ATP, leading to cell necrosis.

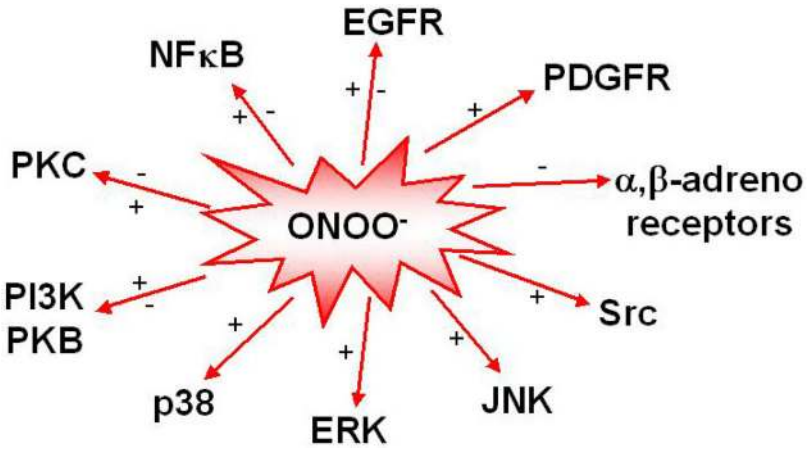

Figure 2.

Major signaling pathways influenced by peroxynitrite. The signs +/- indicate whether the particular signaling protein is inhibited or activated (or both) by peroxynitrite. EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; TrkB, receptor for brain-derived neurotrophic factor; JNK, c-jun-NH2-terminal kinase; ERK, extracellular-regulated kinase; p38, p38 MAP kinase; PI3K/PKB, phosphatidylinositol 3-kinase/protein kinase B; PKC, protein kinase C; NFκB, nuclear factor kappa B