



Short communication

Peroxynitrite-induced cytotoxicity: mechanism and opportunities for intervention

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Abstract

Peroxynitrite is formed in biological systems when superoxide and nitric oxide are produced at near equimolar ratio. Although not a free radical by chemical nature (as it has no unpaired electron), peroxynitrite is a powerful oxidant exhibiting a wide array of tissue damaging effects ranging from lipid peroxidation, inactivation of enzymes and ion channels via protein oxidation and nitration to inhibition of mitochondrial respiration. Low concentrations of peroxynitrite trigger apoptotic death, whereas higher concentrations induce necrosis with cellular energetics (ATP and NAD) serving as switch between the two modes of cell death. Peroxynitrite also damages DNA and thus triggers the activation of DNA repair systems. A DNA nick sensor enzyme, poly(ADP-ribose) polymerase-1 (PARP-1) also becomes activated upon sensing DNA breakage. Activated PARP-1 cleaves NAD⁺ into nicotinamide and ADP-ribose and polymerizes the latter on nuclear acceptor proteins. Peroxynitrite-induced overactivation of PARP consumes NAD⁺ and consequently ATP culminating in cell dysfunction, apoptosis or necrosis. This cellular suicide mechanism has been implicated among others in the pathomechanism of stroke, myocardial ischemia, diabetes and diabetes-associated cardiovascular dysfunction. Here, we review the cytotoxic effects (apoptosis and necrosis) of peroxynitrite focusing on the role of accelerated ADP-ribose turnover. Regulatory mechanisms of peroxynitrite-induced cytotoxicity such as antioxidant status, calcium signalling, NFκB activation, protein phosphorylation, cellular adaptation are also discussed.

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1. Peroxynitrite production in biological systems

Nitric oxide (NO[•]) is a unique diffusible molecular messenger in the vascular and nervous system. NO[•] is produced by a family of enzymes called nitric oxide synthetases (NOS) through enzymatic oxidation of the guanidino group of L-

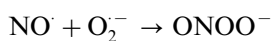
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arginine (Marletta et al., 1998). This occurs in two sequential monooxygenase reactions utilizing NADPH as cosubstrate and involving the utilization of molecular oxygen (Marletta et al., 1998). Constitutive expression of two NOS isoforms is responsible for a low basal level of NO[•] synthesis in neural cells (nNOS or NOS1) and endothelial cells (eNOS or NOS3). These constitutively expressed NOS isoforms require calcium for their enzymatic activity. Induction of the inducible isoform (iNOS or NOS2) by cytokines and/or bacterial products (endotoxin/LPS) has been observed in virtually all cell types tested including macrophages, dendritic cells, fibroblasts, chondrocytes, osteoclasts, astrocytes, epithelial cells and results in the production of large amounts of NO (Nathan, 1997). Mitochondria also contain a unique NO producing enzyme (mtNOS) (Ghafourifar and Richter, 1997). Although enzymatic NO[•] production accounts for the bulk of NO[•] formed in biological systems, alternative pathways also exist. In the skin, for example, non-enzymatic reduction of sweat nitrite has been shown to give rise to NO[•] production (Weller et al., 1996).

Whereas the physiological effects (e.g. vasorelaxation, neuronal signalling) of NO[•] are mostly mediated by the activation of guanylate cyclase (Arnold et al., 1977), the mechanism of pathophysiological effects is much more complex. A heavily debated feature of NO[•] is its cytotoxic effect. The controversy arises from observations reporting both cytotoxic and cytoprotective effects of NO[•] depending on variables of the assay systems used. In cases where NO[•] was found cytotoxic, it was questioned whether NO[•] directly or indirectly, through the formation of more reactive oxidative species such as peroxynitrite exerted its cytotoxic effects (Beckman and Koppenol, 1996). Peroxynitrite (ONOO⁻) is formed when NO[•] and superoxide anion react in a near diffusion-limited reaction (Beckman and Koppenol, 1996). Sources of superoxide include the mitochondrial respiratory chain where there is a constant leak of superoxide, NADPH oxidases, xanthine oxidase and autoxidation of several biomolecules such as catecholamines or myoglobin. The most powerful cellular antioxidant system protecting against the harmful effects of superoxide is embodied by

superoxide dismutases (SOD) (CuZnSOD in the cytosol and MnSOD in the mitochondria). However, it was shown that NO[•] efficiently competes with SOD for superoxide (Beckman and Koppenol, 1996). Joseph Beckman has therefore proposed (Beckman and Koppenol, 1996) that under conditions of increased NO[•] production, NO[•] can outcompete SOD for superoxide resulting in peroxynitrite (ONOO⁻) formation.



As both excess NO[•] or excess superoxide decreases the bioavailability of peroxynitrite, equimolar concentrations of the radicals are ideal for peroxynitrite formation (Radi et al., 2001). Peroxynitrite anion (ONOO⁻) is in a pH-dependent protonation equilibrium with peroxynitrous acid (ONOOH). Homolysis of ONOOH gives rise to formation of the highly reactive hydroxyl radical ([•]OH) mediating molecular and tissue damage associated with peroxynitrite production (Radi et al., 2001).

2. Peroxynitrite-induced apoptosis

Despite of its non-radical nature, peroxynitrite is more reactive than its parent molecules. Peroxynitrite initiates lipid peroxidation (Radi et al., 1991b), causes DNA breakage (Salgo et al., 1995) and reacts with thiols (Radi et al., 1991a). Peroxynitrite-induced protein modifications include protein oxidation (on methionine, cysteine, tryptophane or tyrosine residues) and nitration (of tyrosine or tryptophane residues). However, enzymes containing a redox active transition metal center are the prime targets of the oxidant (Beckman and Koppenol, 1996). Reactions of peroxynitrite are affected by the local pH and the microenvironment with hydrophobic membrane compartments favoring nitration and aqueous environments favoring oxidation. Moreover, carbon dioxide reacts with peroxynitrite resulting in the formation of nitroso-peroxocarbonates (Radi et al., 2001). The ubiquitous presence of CO₂ at high concentration may favor this reaction route. As nitroso-peroxocarbonates divert peroxynitrite-induced protein modifications toward nitration,

CO₂ is now considered as key determinant of peroxynitrite chemistry.

When peroxynitrite-induced cellular damage reaches a level where it cannot be handled by the repair mechanisms, cells undergo one of the basic cell death pathways, apoptosis or necrosis. Apoptosis is the 'default' death pathway characterized, among other parameters, by a compact morphology, maintenance of plasma membrane integrity, mitochondrial depolarization, secondary oxidant production, activation of caspases (cysteinyll aspartate specific proteases) and oligonucleosomal DNA fragmentation (Green and Kroemer, 1998). The first report indicating that peroxynitrite can trigger apoptotic death came from Pryor's laboratory. They have detected DNA fragmentation in peroxynitrite treated thymocytes (Salgo et al., 1995). Later, activation of caspase-3, a key player in the caspase cascade has also been detected in thymocytes (Virág et al., 1998b) and HL-60 cells (Virág and Szabó, 1998c; Lin et al., 1998). These early reports have later been followed by a series of publications on the cytotoxic effects of peroxynitrite in lymphoblastoid cells (Li et al., 2002), dopaminergic SH-SY5Y cells (Saeki et al., 2000; Yamamoto et al., 2002), human aortic endothelial cells (Foresti et al., 1999), osteoblasts (Reiff et al., 2001), HaCaT keratinocytes (Szabó et al., 2001), cardiac myocytes (Arstall et al., 1999) human and rat islet cells (Hadjivassiliou et al., 1998). Prototypical apoptosis models utilize apoptosis inducers such as tumor necrosis factor or FAS ligand acting upon cell surface death receptors. Channeling the death signal from these receptors to apoptotic effector machineries is well described (Green and Kroemer, 1998). However, it is not quite clear, how peroxynitrite triggers the apoptotic machinery. Mitochondria are likely sites for peroxynitrite-induced apoptosis initiation. Mitochondria are now recognized as central organizers of apoptosis (Green and Kroemer, 1998). A characteristic sequence of events including opening of mitochondrial permeability transition pore, mitochondrial depolarization, secondary superoxide production, release of apoptotic mediators from the intermembrane space to the cytoplasm, takes place in apoptosing cells (Green and Kroemer, 1998). Some of the mitochondria-derived apoptogenic

factors act as nucleases (e.g. endonuclease G), nuclease activators (e.g. apoptosis-inducing factor, cytochrome *c*), or serine proteases (e.g. Omi/HtrA2) (Fig. 1). Peroxynitrite was found to inhibit the mitochondrial respiratory chain by inactivating complexes I–III (Lizasoain et al., 1996). Furthermore, adenosine nucleotide translocator, a member of the permeability pore is also targeted by peroxynitrite (Vieira et al., 2001). The characteristic mitochondrial perturbations have also been described in peroxynitrite treated cells (Virág et al., 1998b). The role of mitochondria in peroxynitrite-induced apoptosis is also supported by findings that bcl-2, a mitochondrial antiapoptotic protein inhibits peroxynitrite-induced apoptosis (Virág and Szabó, 2000; Spear et al., 1998). The cellular energetics may become compromised by peroxynitrite also via alternative mechanisms (e.g. inactivation of creatine kinase in cardiomyocytes) which may also contribute to peroxynitrite cytotoxicity (Mihm et al., 2001a).

Recent reports from Bauer's laboratory indicate a possible role for free 3-nitrotyrosine, in peroxynitrite-induced apoptosis (Mihm et al., 2000). They found that preincubation of rat thoracic aorta segments with 3-nitrotyrosine resulted in selective, concentration-dependent impairment of acetylcholine-induced vasorelaxation indicative of endothelial dysfunction. Moreover, nitrotyrosine triggered DNA damage in the endothelial cells (Mihm et al., 2000) and proved to be neurotoxic in vivo (Mihm et al., 2001b). These data suggest that nitrotyrosine, released from proteins nitrated by peroxynitrite, is more than a benign biomarker in vivo, and may contribute to vascular endothelial dysfunction and neurotoxicity through promotion of DNA damage and/or apoptosis (Mihm et al., 2000). Nonetheless, the exact mechanism of peroxynitrite-induced apoptosis initiation remains to be investigated. The executional phase of apoptosis is carried out by caspases which are likely to be activated by mitochondria-derived apoptogenic factors in peroxynitrite treated cells (Green and Kroemer, 1998). Caspase activation has also been reported to occur during the course of peroxynitrite-induced apoptosis (Virág et al., 1998b; Virág and Szabó, 1998c; Lin et al., 1998). A detailed analysis of caspase activation has revealed that

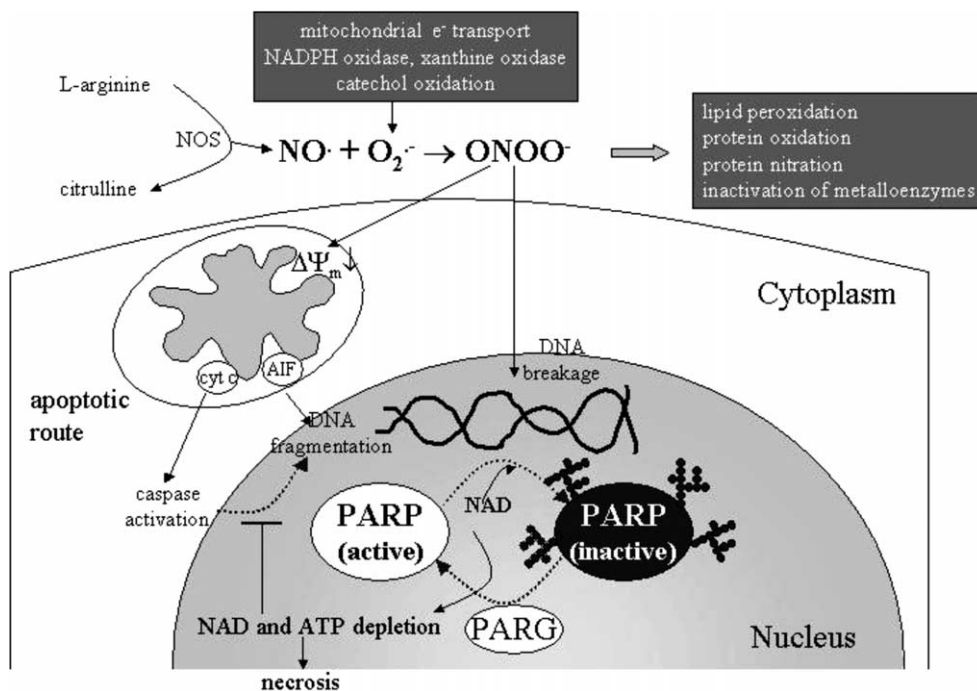


Fig. 1. Peroxynitrite-induced cytotoxic pathways. Nitric oxide and superoxide react to form peroxynitrite which damages cells via various damaging effects such as lipid peroxidation, inactivation of metalloenzymes and other proteins by oxidation and nitration. Peroxynitrite also acts on mitochondria triggering the release of proapoptotic factors such as apoptosis-inducing factor (AIF) and cytochrome *c*. These factors mediate caspase dependent and independent apoptotic death pathways. Moreover, peroxynitrite-induced DNA breakage activates PARP leading to NAD and ATP depletion and consequently to necrosis.

caspase 3-like proteases and (to a lesser extent) caspase 2, but not caspase 1 or caspase 6, are required for peroxynitrite-induced apoptosis (Zhuang and Simon, 2000).

3. Peroxynitrite-induced necrosis: role of poly(ADP-ribose) polymerase-1

Whilst low concentrations of peroxynitrite trigger apoptosis, higher concentrations of the oxidant compromise the apoptotic machinery forcing the cells to die by necrosis (Virág et al., 1998a,b; Bonfoco et al., 1995). For a long time, necrosis was thought to be a passive process resulting from the inability of the cells to cope with high degree of oxidative stress. Recently, a new paradigm has

emerged identifying an active element in oxidative stress-induced necrosis. According to this concept, degree of the activation of poly(ADP-ribose) polymerase-1 (PARP-1) determines the fate of the oxidatively-injured cells (Virág and Szabo, 2002). PARP-1 is activated by DNA strand break. Activated PARP-1 catalyzes the cleavage of NAD⁺ into nicotinamide and ADP-ribose and uses the latter to synthesize branched nucleic acid-like polymers poly(ADP-ribose) covalently attached to nuclear acceptor proteins. The branched polymer, the size of which varies from a few to 200 ADP-ribose units, may facilitate recruitment of DNA repair enzymes to the sites of DNA injury (Virág and Szabo, 2002). In vivo the most abundantly poly-ADP-ribosylated protein is PARP-1 itself and auto-poly(ADP-ribosylation) represents

a major regulatory mechanism for PARP-1 resulting in the downregulation of the enzyme activity. The polymer is degraded by poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase with the latter enzyme removing the protein proximal ADP-ribose residue (Virag and Szabo, 2002). The concerted action of PARP-1 and PARG maintains a highly accelerated ADP-ribose turnover in peroxynitrite treated cells. As a result, NAD becomes depleted in the cells leading to malfunctioning glycolysis, Krebs cycle, mitochondrial electron transport and eventually to ATP depletion (Berger et al., 1986). Moreover, shortage on ATP is exaggerated by attempts of the cells to resynthesize NAD from ATP and nicotinamide. The net result of this pathway is a dramatic drop in cellular ATP (Berger et al., 1983). As the apoptotic machinery is known to depend on ATP (Volbracht et al., 1999; Nicotera et al., 1998, 2000), apoptosis is incapacitated and necrosis takes predominance. This cellular suicide hypothesis described by Nathan Berger (Berger et al., 1983) has been applied by our group to peroxynitrite cytotoxicity. We have used mouse thymocytes (and later several other cell types, too) to validate the role of PARP-1 in peroxynitrite-induced cytotoxicity. Low concentrations (<20 μM) of peroxynitrite caused apoptotic thymocyte death characterized by phosphatidylserine exposure, caspase activation and DNA fragmentation (Virag et al., 1998b). At higher concentrations of peroxynitrite, however, PARP activation and ATP depletion occurred, apoptotic parameters (DNA fragmentation, caspase activation) declined and necrotic death occurred as indicated by the breakdown of plasma membrane integrity (Virag et al., 1998b). Inhibition of PARP-1 by 3-aminobenzamide or the absence of PARP-1 in PARP-1 deficient thymocytes resulted in dramatic protection against the loss of plasma membrane integrity (necrosis). In the same time, output of apoptotic parameters (DNA fragmentation and caspase activation) increased in cells treated with PARP inhibitors or in PARP-1 deficient cells (Virag et al., 1998b). These findings indicate that PARP-1 activation diverts the default apoptotic process toward necrosis.

An interesting new finding of our work was to establish that similarly to apoptosis, peroxynitrite-induced, PARP-1 mediated necrotic death is also accompanied by mitochondrial alterations (collapse of mitochondrial membrane potential, overproduction of superoxide and mitochondrial membrane damage) and calcium mobilization (Virag et al., 1998a). Inhibition or the absence of PARP-1 provided remarkable protection from derailment of mitochondrial functions indicating the central role of PARP-1 in peroxynitrite-induced mitochondrial perturbation (Virag et al., 1998a).

The deterioration of cellular energetic status may play a central role in the 'cell death switch role' of PARP-1. This hypothesis is supported by findings that cellular ATP levels determine the mode of cell death (apoptosis versus necrosis) (Bonfoco et al., 1995; Leist et al., 1997; Nicotera et al., 1998, 2000). Moreover, a recent report from Swanson's laboratory supports a central role of cellular energy homeostasis in PARP-1 mediated cell death (Ying et al., 2002). In these experiments, mouse cortical astrocyte and astrocyte-neuron cocultures were treated with the DNA alkylating agent, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in order to activate PARP-1. Studies using the 2-deoxyglucose method confirmed that glycolytic flux was reduced by more than 90% in MNNG-treated cultures. The addition of 5 mmol/l of alpha-ketoglutarate, 5 mmol/l pyruvate, or other mitochondrial substrates to the cultures after MNNG treatment reduced cell death from approximately 70% to near basal levels (Ying et al., 2002).

4. Regulation of peroxynitrite-induced cytotoxicity

The mechanism of peroxynitrite-induced cytotoxicity is cell type dependent. The ratio of PARP dependent and PARP independent pathways varies between different cell types with thymocytes and other primary lymphoid cells representing one end of the spectrum (mostly PARP independent) and HL-60 myeloid cell line standing at the other end (no PARP dependence). Of note, out of the many cell lines tested in our laboratory, HL-60

cells were the only ones not protected by PARP inhibitors from peroxynitrite-induced cytotoxicity. The question arises, what the underlying principle behind the differential peroxynitrite sensitivity of the various cell types may be? These factors may or maynot be linked to PARP activation.

4.1. PARP dependent factors

Thymocytes represent an ideal cellular model for the analysis of PARP dependent resistance factors as in this cell type, peroxynitrite-induced cell death is mainly PARP dependent (Virag et al., 1998b). We have reported that TPEN, a zinc chelator, inhibits peroxynitrite-induced PARP activation and necrosis (Virág et al., 1999a). The mechanism of cytoprotection is not known. However, given that two zinc finger motives are responsible for DNA binding of PARP-1, TPEN may interfere with this process. Furthermore, peroxynitrite induces calcium mobilization both from intra- and from extracellular sources and intracellular calcium chelation protects from peroxynitrite-induced PARP activation and necrosis (Virag et al., 1999b). In these experiments, calcium chelators abolished peroxynitrite-induced DNA breakage indicating that they may act upstream to PARP activation. In a cell-free system, calcium chelators did not inhibit peroxynitrite induced DNA breakage (Virag et al., 1999b). These findings support the hypothesis that calcium signalling triggers secondary events leading to DNA damage and subsequently PARP activation. These secondary events may include production of secondary ROI (reactive oxygen intermediates) in the mitochondria. This novel concept implies that although peroxynitrite can directly break DNA in cell-free system, the mechanism of DNA breakage may be fundamentally different in a cellular environment. A calcium-dependent, mitochondrial production of secondary oxygen radicals has been reported in other cellular models of oxidative stress (Guidarelli et al., 2000a,c).

We have also identified purines (hypoxanthine > inosine > adenosine) as potential endogenous PARP inhibitors (Virag and Szabo, 2001). This observation may have implication for ischemia reperfusion injury where these substances

reach high enough concentrations during ischemia to modulate PARP activation by ROI overproduced during the reperfusion phase. Recently, we have identified cell density signalling as a new factor regulating peroxynitrite sensitivity of HaCaT keratinocytes. We have showed that subconfluent (10–95%) cultures are more sensitive to peroxynitrite or hydrogen peroxide-induced cell death than confluent monolayers. The resistance to oxidative stress provided by high cell density involved both inhibition of caspase activation and PARP activation but not protein kinase C signalling. Our data may explain the resistance to oxidative stress of superficial, highly differentiated keratinocytes and may indicate that basal proliferative keratinocytes are possible sensitive in vivo targets of oxidative stress injury. By virtue of the epidermal calcium gradient (increasing calcium concentration in baso-superficial direction), our data also raise the possibility that calcium signalling and density-dependent signalling are interrelated.

Recently a new regulatory element of PARP activation has been identified. It has long been known that PARP-1 auto-poly-ADP-ribosylates itself leading to downregulation of enzyme activity. By removing inhibitory ADP-ribose residues from PARP-1, PARG may reactivate PARP-1 and thus may help maintain a high NAD/ADP-ribose turnover. This hypothesis has recently been tested by Swanson's group reporting that PARG inhibition by gallotannin and nobotanin B protected astrocytes and neuronal cells from oxidative stress (Ying and Swanson, 2000; Ying et al., 2001). We have confirmed these data in HaCaT keratinocytes and A549 pulmonary epithelial cells and found similar cytoprotection by gallotannin (submitted for publication).

Two studies have reported that peroxynitrite induces the expression of heat shock proteins. One of the studies has shown that the cytoprotective effect of the heat shock response is related to inhibition of PARP-1 activation (Szabo et al., 1996b). It is worthwhile that nitric oxide and peroxynitrite were found to have different effect on heat shock protein 70 expression in human monocytes with peroxynitrite inducing and nitric oxide not affecting HSP 70 expression (Adrie et

al., 2000). However, the mechanism by which heat shock inhibits PARP activation and other cytotoxic pathways remains to be elucidated.

4.2. PARP independent factors

The overall antioxidant status obviously determines the sensitivity of cells toward peroxynitrite toxicity. The importance of glutathione is supported by several observations. Increased glutathione levels, as achieved by administration of gamma-glu-cys-ethyl ester, has been shown to protect cortical synaptosomes from peroxynitrite-induced damage (Drake et al., 2002). Furthermore, depletion of cellular glutathione pools by buthionine sulfoximide sensitizes cells and animals to peroxynitrite toxicity or peroxynitrite-mediated inflammatory tissue injury, respectively (Cuzzocrea et al., 1998). In an in vivo model of myocardial ischemia, coadministration of ascorbic acid with glutathione methyl ester (GSHme) markedly enhanced the protective effects of GSHme, although ascorbic acid alone had no effect (Gao et al., 2002). The protection exerted by the combination of GSHme and ascorbic acid was significantly greater than that observed with 1 mM GSHme alone. Moreover, treatment with GSHme alone or GSHme plus ascorbic acid markedly reduced myocardial nitrotyrosine levels, suggesting that these treatments attenuated myocardial peroxynitrite formation (Gao et al., 2002). Manganese- (Szabo et al., 1996a; Ferrer-Sueta et al., 1999) or ferrous porphyrin compounds (Shimanovich and Groves, 2001; Salvemini et al., 1998) often sold as superoxide dismutase mimetics or peroxynitrite decomposition catalysts also protect from peroxynitrite. Several other antioxidants such as ebselen (Roussyn et al., 1996; Masumoto and Sies, 1996; Sata et al., 1997) or melatonin (Gilad et al., 1997; Cuzzocrea et al., 1997) as well as phytopharmacoins (plant-derived antioxidants) (Choi et al., 2002a,b; Valdez et al., 2002) have also been shown to protect from the deleterious effects of peroxynitrite.

Peroxyntirite-induced apoptotic death can be prevented by classical apoptosis inhibitors such as caspase inhibitors (Virág et al., 1998b; Zhuang and Simon, 2000) or bcl-2 overexpression (Virág and

Szabó, 2000; Spear et al., 1998). Although bcl-2 has been reported to provide protection against both apoptotic and necrotic stimuli, we have shown that it protected thymocytes from peroxynitrite-induced apoptotic but not against PARP-1 mediated necrotic death (Virág and Szabó, 2000).

In addition to antioxidants and direct antiapoptotic interventions other alternatives may also exist to modulate peroxynitrite-triggered cytotoxic pathways. The most intensively studied pathway is the inhibition of tyrosine kinase cascades by peroxynitrite-mediated tyrosine nitration (Beckman, 1996). Nitration of critical tyrosine residues by peroxynitrite in tyrosine kinase substrates interferes with the phosphorylation of the proteins and may inhibit downstream signalling events (Gow et al., 1996; Kong et al., 1996). Peroxynitrite has been shown to activate several signal transduction pathways. For example, peroxynitrite triggers the activation of various types of kinases, the G protein-phosphatidylinositol 3 kinase (PI3 kinase) pathway and phospholipase A2 (Klotz et al., 2000; Kaji et al., 2002; Guidarelli et al., 2000b). Many of these signalling pathways have been implicated in the regulation of cell death (Cross et al., 2000; Holmstrom and Eriksson, 2000; Sarmay, 2002). The G receptor-coupled PI3 kinase activation pathway for example was proposed to counteract peroxynitrite toxicity in primary rat astrocytes, a cell type expressing opioid receptors. Treatment of cells with morphine significantly protected astrocytes from apoptosis mediated by the peroxynitrite donor SIN-1, whereas it did not in other types of cells including C6 glioma, RAW 264.7, and HL-60 cells (Kim et al., 2001). The effects of morphine on SIN-1-induced cytotoxicity were inhibited by pretreatment with the G(i) protein inhibitor, pertussis toxin, and the PI3 kinase inhibitors, wortmannin and LY294002 (Kim et al., 2001). These results suggest that morphine may protect primary rat astrocytes from peroxynitrite-induced cytotoxicity via the signalling cascades that involve both G protein and PI3 kinase.

Maeda's group has investigated the activation of mitogen-activated protein kinase (MAP kinase) in relation to cell death induced by peroxynitrite in human neuroblastoma SH-SY5Y cells (Saeki et al., 2000). Exposure of the cells to peroxynitrite

caused transient increase in MAP kinase activity, and resulted in cell death. PD98059, a selective inhibitor of MAP kinase kinase, reduced peroxynitrite-induced cell death suggesting that activation of MAP kinase may be involved in cell death induced by peroxynitrite (Saeki et al., 2000). Furthermore, stimulation of several growth factor receptors for example by insulin like growth factor (Saeki et al., 2002), acidic fibroblast growth factor (Reiff et al., 2001), fibroblast growth factor-1 (Spear et al., 1998) or nerve growth factor (Spear et al., 1997, 1998) has also been found to modulate (in most cases to protect from) peroxynitrite toxicity. In the case of epidermal growth factor receptor (EGFR), peroxynitrite was found to crosslink the receptors in A431 epidermoid carcinoma cells resulting in dimer formation. Covalent EGFR dimerization by peroxynitrite probably involved intermolecular dityrosine cross-linking and was enhanced after receptor activation with epidermal growth factor. Furthermore, irreversibly cross-linked EGFR was more extensively tyrosine-phosphorylated compared with the monomeric form. However, exposure of A431 cells to peroxynitrite markedly reduced the kinetics of tyrosine phosphorylation of a downstream EGFR substrate, phospholipase C- γ 1. This study indicates that peroxynitrite may also interfere with tyrosine kinase pathways with a mechanism different from tyrosine nitration.

An interesting issue is the potential regulatory role of NF κ B in peroxynitrite cytotoxicity. NF κ B is a redox-sensitive transcription factor regulating the expression of various inflammatory mediators (Janssen-Heininger et al., 2000). The antiapoptotic effects of NF κ B activation are also well documented (Aggarwal, 2000; Mattson et al., 1997), however, the outcome (death promotion or prevention) is cell type and stimulus dependent (Bours et al., 2000). The parent molecules of peroxynitrite appear to regulate NF κ B activation in an opposing manner with superoxide (likely via hydrogen peroxide formation) activating and nitric oxide inhibiting NF κ B activation (Schmidt et al., 1995; von Knethen et al., 1999; Marshall and Stamler, 2002). Inhibition of NF κ B activation by NO-mediated nitrosylation has—at least in part—been made responsible for the proapoptotic

effect of NO (Marshall and Stamler, 2002). Furthermore, immunoprecipitation studies showed that NO stabilized the NF-kappa B inhibitor, I kappa B alpha, by preventing its degradation from NF-kappa B (Peng et al., 1995). NO also increased the mRNA expression of I kappa B alpha (Peng et al., 1995). The contrasting roles of superoxide and NO on NF κ B activation point toward the importance of understanding conditions regulating peroxynitrite formation. Peroxynitrite itself has also been found to activate NF κ B (Matata and Galinanes, 2002). However, the role of NF κ B in the regulation of peroxynitrite-induced cell death has not yet been investigated in detail. Only one study addressed the issue and found that peroxynitrite treatment did not activate NF κ B in IEC-6 enterocytes but inhibition of NF κ B by transfection with AdI κ B, a mutated I κ B functioning as a superrepressor of NF κ B activation, significantly enhanced peroxynitrite-induced apoptosis in IEC-6 cells (Potoka et al., 2002).

Chronic exposure of cells to sublethal peroxynitrite concentrations (e.g. in inflammation) may also allow cells to develop adaptive responses to oxidative stress. This kind of regulation may involve upregulation of defense proteins/antioxidant enzymes. In A549 cells, peroxynitrite has been shown to trigger expression of mRNA for MnSOD, a key antioxidant enzyme (Jackson et al., 1998). However, MnSOD protein and enzyme activity were not changed. In Dr. Ischiropoulos's lab, a peroxynitrite resistant cell line has been generated by repeated exposure of the cells to sublethal concentrations of peroxynitrite. DNA chip technology will permit large-scale analysis of changes in gene expression pattern of peroxynitrite resistant cultures and may provide insight into the mechanism of cellular adaptation to oxidative stress situations.

5. Conclusions

Peroxynitrite has been implicated in the pathomechanism of various diseases. However, the lack of specific tools to unequivocally verify whether peroxynitrite is indeed produced in all of these

conditions and is responsible for at least part of the tissue injury has put peroxynitrite in the center of heated debates. Nonetheless, as evidence supporting the pathophysiological role of peroxynitrite is constantly increasing, targeting peroxynitrite-induced cytotoxic pathways is now accepted as a viable strategy to alleviate disease signs in numerous diseases. Which one of the above listed interventions or combinations of them provides the highest therapeutic benefit with the least risk remains to be seen.

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References

- Adrie, C., Richter, C., Bachelet, M., Banzet, N., Francois, D., Dinh-Xuan, A.T., Dhainaut, J.F., Polla, B.S., Richard, M.J., 2000. Contrasting effects of NO and peroxynitrites on HSP70 expression and apoptosis in human monocytes. *Am. J. Physiol. Cell Physiol.* 279, C452–C460.
- Aggarwal, B.B., 2000. Apoptosis and nuclear factor-kappa B: a tale of association and dissociation. *Biochem. Pharmacol.* 60, 1033–1039.
- Arnold, W.P., Mittal, C.K., Katsuki, S., Murad, F., 1977. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. *Proc. Natl. Acad. Sci. USA* 74, 3203–3207.
- Arstall, M.A., Sawyer, D.B., Fukazawa, R., Kelly, R.A., 1999. Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. *Circ. Res.* 85, 829–840.
- Beckman, J.S., 1996. Oxidative damage and tyrosine nitration from peroxynitrite. *Chem. Res. Toxicol.* 9, 836–844.
- Beckman, J.S., Koppenol, W.H., 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol.* 271, C1424–C1437.
- Berger, N.A., Sims, J.L., Catino, D.M., Berger, S.J., 1983. Poly(ADP-ribose) polymerase mediates the suicide response to massive DNA damage: studies in normal and DNA-repair defective cells. *Princess Takamatsu Symp.* 13, 219–226.
- Berger, S.J., Sudar, D.C., Berger, N.A., 1986. Metabolic consequences of DNA damage: DNA damage induces alterations in glucose metabolism by activation of poly (ADP-Ribose) polymerase. *Biochem. Biophys. Res. Commun.* 134, 227–232.
- Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., Lipton, S.A., 1995. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. USA* 92, 7162–7166.
- Bours, V., Bentires-Alj, M., Hellin, A.C., Viatour, P., Robe, P., Delhalle, S., Benoit, V., Merville, M.P., 2000. Nuclear factor-kappa B, cancer, and apoptosis. *Biochem. Pharmacol.* 60, 1085–1089.
- Choi, H.R., Choi, J.S., Han, Y.N., Bae, S.J., Chung, H.Y., 2002a. Peroxynitrite scavenging activity of herb extracts. *Phytother. Res.* 16, 364–367.
- Choi, J.J., Oh, Y.K., Kim, H.S., Kim, H.C., Ko, K.H., Kim, W.K., 2002b. Mimosine prevents the death of glucose-deprived immunostimulated astrocytes by scavenging peroxynitrite. *Glia* 39, 37–46.
- Cross, T.G., Scheel-Toellner, D., Henriquez, N.V., Deacon, E., Salmon, M., Lord, J.M., 2000. Serine/threonine protein kinases and apoptosis. *Exp. Cell Res.* 256, 34–41.
- Cuzzocrea, S., Zingarelli, B., Gilad, E., Hake, P., Salzman, A.L., Szabo, C., 1997. Protective effect of melatonin in carrageenan-induced models of local inflammation: relationship to its inhibitory effect on nitric oxide production and its peroxynitrite scavenging activity. *J. Pineal Res.* 23, 106–116.
- Cuzzocrea, S., Zingarelli, B., O'Connor, M., Salzman, A.L., Szabo, C., 1998. Effect of L-buthionine-(S,R)-sulphoximine, an inhibitor of gamma-glutamylcysteine synthetase on peroxynitrite- and endotoxin shock-induced vascular failure. *Br. J. Pharmacol.* 123, 525–537.
- Drake, J., Kanski, J., Varadarajan, S., Tsoras, M., Butterfield, D.A., 2002. Elevation of brain glutathione by gamma-glutamylcysteine ethyl ester protects against peroxynitrite-induced oxidative stress. *J. Neurosci. Res.* 68, 776–784.
- Ferrer-Sueta, G., Batinić-Haberle, I., Spasojević, I., Fridovich, I., Radi, R., 1999. Catalytic scavenging of peroxynitrite by isomeric Mn(III) N-methylpyridylporphyrins in the presence of reductants. *Chem. Res. Toxicol.* 12, 442–449.
- Foresti, R., Sarathchandra, P., Clark, J.E., Green, C.J., Motterlini, R., 1999. Peroxynitrite induces haem oxygenase-1 in vascular endothelial cells: a link to apoptosis. *Biochem. J.* 339 (Pt 3), 729–736.
- Gao, F., Yao, C.L., Gao, E., Mo, Q.Z., Yan, W.L., McLaughlin, R., Lopez, B.L., Christopher, T.A., Ma, X.L., 2002. Enhancement of glutathione cardioprotection by ascorbic acid in myocardial reperfusion injury. *J. Pharmacol. Exp. Ther.* 301, 543–550.
- Ghafourifar, P., Richter, C., 1997. Nitric oxide synthase activity in mitochondria. *FEBS Lett.* 418, 291–296.

- Gilad, E., Cuzzocrea, S., Zingarelli, B., Salzman, A.L., Szabo, C., 1997. Melatonin is a scavenger of peroxynitrite. *Life Sci.* 60, PL169–PL174.
- Gow, A.J., Duran, D., Malcolm, S., Ischiropoulos, H., 1996. Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. *FEBS Lett.* 385, 63–66.
- Green, D., Kroemer, G., 1998. The central executioners of apoptosis: caspases or mitochondria? *Trends Cell Biol.* 8, 267–271.
- Guidarelli, A., Fiorani, M., Cantoni, O., 2000a. Calcium-dependent mitochondrial formation of species promoting strand scission of genomic DNA in U937 cells exposed to tert-butylhydroperoxide: the role of arachidonic acid. *Free Radical Res.* 33, 477–487.
- Guidarelli, A., Palomba, L., Cantoni, O., 2000b. Peroxynitrite-mediated release of arachidonic acid from PC12 cells. *Br. J. Pharmacol.* 129, 1539–1541.
- Guidarelli, A., Tommasini, I., Fiorani, M., Cantoni, O., 2000c. Essential role of the mitochondrial respiratory chain in peroxynitrite-induced strand scission of genomic DNA. *IUBMB Life* 50, 195–201.
- Hadjivassiliou, V., Green, M.H., James, R.F., Swift, S.M., Clayton, H.A., Green, I.C., 1998. Insulin secretion, DNA damage, and apoptosis in human and rat islets of Langerhans following exposure to nitric oxide, peroxynitrite, and cytokines. *Nitric Oxide* 2, 429–441.
- Holmstrom, T.H., Eriksson, J.E., 2000. Phosphorylation-based signaling in Fas receptor-mediated apoptosis. *Crit. Rev. Immunol.* 20, 121–152.
- Jackson, R.M., Parish, G., Helton, E.S., 1998. Peroxynitrite modulates MnSOD gene expression in lung epithelial cells. *Free Radical Biol. Med.* 25, 463–472.
- Janssen-Heininger, Y.M., Poynter, M.E., Baeuerle, P.A., 2000. Recent advances towards understanding redox mechanisms in the activation of nuclear factor kappaB. *Free Radical Biol. Med.* 28, 1317–1327.
- Kaji, T., Kaieda, I., Hisatsune, T., Kaminogawa, S., 2002. 3-Morpholinopyridone hydrochloride induces p53-dependent apoptosis in murine primary neural cells: a critical role for p21(ras)-MAPK-p19(ARF) pathway. *Nitric Oxide* 6, 125–134.
- Kim, M.S., Cheong, Y.P., So, H.S., Lee, K.M., Kim, T.Y., Oh, J., Chung, Y.T., Son, Y., Kim, B.R., Park, R., 2001. Protective effects of morphine in peroxynitrite-induced apoptosis of primary rat neonatal astrocytes: potential involvement of G protein and phosphatidylinositol 3-kinase (PI3 kinase). *Biochem. Pharmacol.* 61, 779–786.
- Klotz, L.O., Schiege, S.M., Sies, H., Holbrook, N.J., 2000. Peroxynitrite activates the phosphoinositide 3-kinase/Akt pathway in human skin primary fibroblasts. *Biochem. J.* 352 (Pt 1), 219–225.
- Kong, S.K., Yim, M.B., Stadtman, E.R., Chock, P.B., 1996. Peroxynitrite disables the tyrosine phosphorylation regulatory mechanism: lymphocyte-specific tyrosine kinase fails to phosphorylate nitrated cdc2(6-20)NH2 peptide. *Proc. Natl. Acad. Sci. USA* 93, 3377–3382.
- Leist, M., Single, B., Castoldi, A.F., Kuhnle, S., Nicotera, P., 1997. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J. Exp. Med.* 185, 1481–1486.
- Li, C.Q., Trudel, L.J., Wogan, G.N., 2002. Genotoxicity, mitochondrial damage, and apoptosis in human lymphoblastoid cells exposed to peroxynitrite generated from SIN-1. *Chem. Res. Toxicol.* 15, 527–535.
- Lin, K.T., Xue, J.Y., Lin, M.C., Spokas, E.G., Sun, F.F., Wong, P.Y., 1998. Peroxynitrite induces apoptosis of HL-60 cells by activation of a caspase-3 family protease. *Am. J. Physiol.* 274, C855–C860.
- Lizasoain, I., Moro, M.A., Knowles, R.G., Darley-Usmar, V., Moncada, S., 1996. Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose. *Biochem. J.* 314, 877–880.
- Marletta, M.A., Hurshman, A.R., Rusche, K.M., 1998. Catalysis by nitric oxide synthase. *Curr. Opin. Chem. Biol.* 2, 656–663.
- Marshall, H.E., Stamler, J.S., 2002. Nitrosative stress-induced apoptosis through inhibition of NF-kappa B. *J. Biol. Chem.* 277, 34233–34238.
- Masumoto, H., Sies, H., 1996. The reaction of ebselen with peroxynitrite. *Chem. Res. Toxicol.* 9, 262–267.
- Matata, B.M., Galinanes, M., 2002. Peroxynitrite is an essential component of cytokines production mechanism in human monocytes through modulation of nuclear factor-kappa B DNA binding activity. *J. Biol. Chem.* 277, 2330–2335.
- Mattson, M.P., Goodman, Y., Luo, H., Fu, W., Furukawa, K., 1997. Activation of NF-kappaB protects hippocampal neurons against oxidative stress-induced apoptosis: evidence for induction of manganese superoxide dismutase and suppression of peroxynitrite production and protein tyrosine nitration. *J. Neurosci. Res.* 49, 681–697.
- Mihm, M.J., Coyle, C.M., Schanbacher, B.L., Weinstein, D.M., Bauer, J.A., 2001a. Peroxynitrite induced nitration and inactivation of myofibrillar creatine kinase in experimental heart failure. *Cardiovasc. Res.* 49, 798–807.
- Mihm, M.J., Jing, L., Bauer, J.A., 2000. Nitrotyrosine causes selective vascular endothelial dysfunction and DNA damage. *J. Cardiovasc. Pharmacol.* 36, 182–187.
- Mihm, M.J., Schanbacher, B.L., Wallace, B.L., Wallace, L.J., Uretsky, N.J., Bauer, J.A., 2001b. Free 3-nitrotyrosine causes striatal neurodegeneration in vivo. *J. Neurosci.* 21, RC149.
- Nathan, C., 1997. Inducible nitric oxide synthase: what difference does it make? *J. Clin. Invest.* 100, 2417–2423.
- Nicotera, P., Leist, M., Ferrando-May, E., 1998. Intracellular ATP, a switch in the decision between apoptosis and necrosis. *Toxicol. Lett.* 102–103, 139–142.
- Nicotera, P., Leist, M., Fava, E., Berliocchi, L., Volbracht, C., 2000. Energy requirement for caspase activation and neuronal cell death. *Brain Pathol.* 10, 276–282.
- Peng, H.B., Libby, P., Liao, J.K., 1995. Induction and stabilization of I kappa B alpha by nitric oxide mediates

- inhibition of NF-kappa B. *J. Biol. Chem.* 270, 14214–14219.
- Potoka, D.A., Upperman, J.S., Nadler, E.P., Wong, C.T., Zhou, X., Zhang, X.R., Ford, H.R., 2002. NF-kappaB inhibition enhances peroxynitrite-induced enterocyte apoptosis. *J. Surg. Res.* 106, 7–14.
- Radi, R., Beckman, J.S., Bush, K.M., Freeman, B.A., 1991a. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* 266, 4244–4250.
- Radi, R., Beckman, J.S., Bush, K.M., Freeman, B.A., 1991b. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* 288, 481–487.
- Radi, R., Peluffo, G., Alvarez, M.N., Naviliat, M., Cayota, A., 2001. Unraveling peroxynitrite formation in biological systems. *Free Radical Biol. Med.* 30, 463–488.
- Reiff, D.A., Kelpke, S., Rue, L., III, Thompson, J.A., 2001. Acidic fibroblast growth factor attenuates the cytotoxic effects of peroxynitrite in primary human osteoblast precursors. *J. Trauma* 50, 433–438.
- Roussyn, I., Briviba, K., Masumoto, H., Sies, H., 1996. Selenium-containing compounds protect DNA from single-strand breaks caused by peroxynitrite. *Arch. Biochem. Biophys.* 330, 216–218.
- Saeki, M., Kamisaki, Y., Maeda, S., 2000. Involvement of mitogen-activated protein kinase in peroxynitrite-induced cell death of human neuroblastoma SH-SY5Y cells. *Neurosci. Res.* 38, 213–216.
- Saeki, M., Maeda, S., Wada, K., Kamisaki, Y., 2002. Insulin-like growth factor-1 protects peroxynitrite-induced cell death by preventing cytochrome *c*-induced caspase-3 activation. *J. Cell Biochem.* 84, 708–716.
- Salgo, M.G., Squadrito, G.L., Pryor, W.A., 1995. Peroxynitrite causes apoptosis in rat thymocytes. *Biochem. Biophys. Res. Commun.* 215, 1111–1118.
- Salvemini, D., Wang, Z.Q., Stern, M.K., Currie, M.G., Misko, T.P., 1998. Peroxynitrite decomposition catalysts: therapeutics for peroxynitrite-mediated pathology. *Proc. Natl. Acad. Sci. USA* 95, 2659–2663.
- Sarmay, G., 2002. Phosphatidylinositol 3-kinase, phosphoinositides and apoptosis. *Polyphosphoinositol phosphatase and apoptosis.* *Subcell. Biochem.* 36, 309–333.
- Sata, N., Klonowski-Stumpe, H., Han, B., Haussinger, D., Niederau, C., 1997. Cytotoxicity of peroxynitrite in rat pancreatic acinar AR4-2J cells. *Pancreas* 15, 278–284.
- Schmidt, K.N., Amstad, P., Cerutti, P., Baeuerle, P.A., 1995. The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-kappa B. *Chem. Biol.* 2, 13–22.
- Shimanovich, R., Groves, J.T., 2001. Mechanisms of peroxynitrite decomposition catalyzed by FeTMPS, a bioactive sulfonated iron porphyrin. *Arch. Biochem. Biophys.* 387, 307–317.
- Spear, N., Estevez, A.G., Barbeito, L., Beckman, J.S., Johnson, G.V., 1997. Nerve growth factor protects PC12 cells against peroxynitrite-induced apoptosis via a mechanism dependent on phosphatidylinositol 3-kinase. *J. Neurochem.* 69, 53–59.
- Spear, N., Estevez, A.G., Johnson, G.V., Bredesen, D.E., Thompson, J.A., Beckman, J.S., 1998. Enhancement of peroxynitrite-induced apoptosis in PC12 cells by fibroblast growth factor-1 and nerve growth factor requires p21Ras activation and is suppressed by Bcl-2. *Arch. Biochem. Biophys.* 356, 41–45.
- Szabo, C., Day, B.J., Salzman, A.L., 1996a. Evaluation of the relative contribution of nitric oxide and peroxynitrite to the suppression of mitochondrial respiration in immunostimulated macrophages using a manganese mesoporphyrin superoxide dismutase mimetic and peroxynitrite scavenger. *FEBS Lett.* 381, 82–86.
- Szabo, C., Wong, H.R., Salzman, A.L., 1996b. Pre-exposure to heat shock inhibits peroxynitrite-induced activation of poly(ADP) ribosyltransferase and protects against peroxynitrite cytotoxicity in J774 macrophages. *Eur. J. Pharmacol.* 315, 221–226.
- Szabó, É., Virág, L., Bakondi, E., Gyüre, L., Haskó, G., Bai, P., Hunyadi, J., Gergely, P., Szabó, C., 2001. Peroxynitrite production, DNA breakage and poly(ADP-ribose) polymerase activation in a mouse model of oxazolone-induced contact hypersensitivity. *J. Invest. Dermatol.* 117, 74–80.
- Valdez, L.B., Actis-Goretta, L., Boveris, A., 2002. Polyphenols in red wines prevent NADH oxidation induced by peroxynitrite. *Ann. NY Acad. Sci.* 957, 274–278.
- Vieira, H.L., Belzacq, A.S., Haouzi, D., Bernassola, F., Cohen, I., Jacotot, E., Ferri, K.F., El Hamel, C., Bartle, L.M., Melino, G., Brenner, C., Goldmacher, V., Kroemer, G., 2001. The adenine nucleotide translocator: a target of nitric oxide, peroxynitrite, and 4-hydroxynonenal. *Oncogene* 20, 4305–4316.
- Virág, L., Salzman, A.L., Szabo, C., 1998a. Poly(ADP-ribose) synthetase activation mediates mitochondrial injury during oxidant-induced cell death. *J. Immunol.* 161, 3753–3759.
- Virág, L., Scott, G.S., Cuzzocrea, S., Marmer, D., Salzman, A.L., Szabo, C., 1998b. Peroxynitrite-induced thymocyte apoptosis: the role of caspases and poly (ADP-ribose) synthetase (PARS) activation. *Immunology* 94, 345–355.
- Virág, L., Szabó, C., 1998c. The crucial role of apopain in the peroxynitrite-induced apoptotic DNA fragmentation. *Free Radical Biol. Med.* 25, 1075–1082.
- Virág, L., Salzman, A.L., Szabó, C., 1999a. Inhibition of poly(ADP-ribose) synthetase (PARS) and protection against peroxynitrite-induced cytotoxicity by zinc chelation. *Br. J. Pharmacol.* 126, 769–777.
- Virág, L., Scott, G.S., Antal-Szalmas, P., O'Connor, M., Ohshima, H., Szabo, C., 1999b. Requirement of intracellular calcium mobilization for peroxynitrite-induced poly(ADP-ribose) synthetase activation and cytotoxicity. *Mol. Pharmacol.* 56, 824–833.
- Virág, L., Szabó, C., 2000. Bcl-2 protects peroxynitrite-treated thymocytes from poly(ADP-ribose) synthase (PARS) independent apoptotic but not from PARS-mediated necrotic cell death. *Free Radical Biol. Med.* 29, 704–713.

- Virag, L., Szabo, C., 2001. Purines inhibit poly(ADP-ribose) polymerase activation and modulate oxidant-induced cell death. *FASEB J.* 15, 99–107.
- Virag, L., Szabo, C., 2002. The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. *Pharm. Rev.* 54, 375–429.
- Volbracht, C., Leist, M., Nicotera, P., 1999. ATP controls neuronal apoptosis triggered by microtubule breakdown or potassium deprivation. *Mol. Med.* 5, 477–489.
- von Knethen, A., Callsen, D., Brune, B., 1999. Superoxide attenuates macrophage apoptosis by NF-kappa B and AP-1 activation that promotes cyclooxygenase-2 expression. *J. Immunol.* 163, 2858–2866.
- Weller, R., Pattullo, S., Smith, L., Golden, M., Ormerod, A., Benjamin, N., 1996. Nitric oxide is generated on the skin surface by reduction of sweat nitrate. *J. Invest. Dermatol.* 107, 327–331.
- Yamamoto, T., Maruyama, W., Kato, Y., Yi, H., Shamoto-Nagai, M., Tanaka, M., Sato, Y., Naoi, M., 2002. Selective nitration of mitochondrial complex I by peroxynitrite: involvement in mitochondria dysfunction and cell death of dopaminergic SH-SY5Y cells. *J. Neural Transm.* 109, 1–13.
- Ying, W., Swanson, R.A., 2000. The poly(ADP-ribose) glycohydrolase inhibitor gallotannin blocks oxidative astrocyte death. *Neuroreport* 11, 1385–1388.
- Ying, W., Seigny, M.B., Chen, Y., Swanson, R.A., 2001. Poly(ADP-ribose) glycohydrolase mediates oxidative and excitotoxic neuronal death. *Proc. Natl. Acad. Sci. USA* 98, 12227–12232.
- Ying, W., Chen, Y., Alano, C.C., Swanson, R.A., 2002. Tricarboxylic acid cycle substrates prevent PARP-mediated death of neurons and astrocytes. *J. Cereb. Blood Flow Metab.* 22, 774–779.
- Zhuang, S., Simon, G., 2000. Peroxynitrite-induced apoptosis involves activation of multiple caspases in HL-60 cells. *Am. J. Physiol. Cell Physiol.* 279, C341–C351.