Nitric Oxide and Peroxynitrite: Effects on Cardiovascular Function

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Summary

Cardiovascular diseases represent a major cuase of death in the western world. Both nitric oxide and peroxynitrite have been implicated in different cardiovascular processes. Nitric oxide is an endogenous vasodilator and mediator of several important physiological processes and it is involved in a number of protective mechanisms in cells. However, overproduction of nitric oxide can occur and this can lead to cytotoxicity. On the other hand, peroxynitrite is a potent biological oxidant formed from the near-diffusion limited reaction between superoxide anion and nitric oxide. It has been associated with mechanism of cell death such as necrosis and more recently with apoptois. In this review, were discussed some of the various roles of both, and their implicance in physiological and pathophysiological processes, which take place in cardiovascular system.
Key words: Nitric oxide, peroxynitrite, cardiovascular function

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Introduction

Nitric oxide ($NO^{\cdot}$) is an endogenous mediator of several important physiological processes and it is involved in a number of protective mechanisms in cells [1]. However, overproduction of $NO^{\cdot}$ can occur and this can lead to cytotoxicity [2]. $NO^{\cdot}$ is produced by a family of enzymes called nitric oxide synthases (NOS) through enzymatic oxidation of the guanidino group of L-arginine to form citruline and $NO^{\cdot}$. This occurs in two sequential monooxygenase reactions utilizing NADPH and tetrahydrobiopterin (BH_4) as cosubstrates and involving the utilization of oxygen [3]. Whereas the physiological effects of $NO^{\cdot}$ (e.g. vasorelaxation, neuronal signaling) are mostly mediated by the activation of the soluble isoform of guanylyl cyclase (GC) [4], the mechanism of pathophysiological effects is much more complex [5]. Initially, it was thought that all the biologic activity of $NO^{\cdot}$ was readily attributable to its diffusion and reaction with a single target, enzyme GC (reviewed in [6]). Contemporary understanding of $NO^{\cdot}$ biology lies in stark contrast to this concept. It is now know that $NO^{\cdot}$ has a number of relevant biologic targets that are dictated, in part, by its site of synthesis, its relative concentration, and the availability of coreactants. At physiologicaly relevant low nanomolar concentrations, the predominant reactions of $NO^{\cdot}$ are with heme and heme-copper centers [6].

Perhaps the best-characterized heme target for $NO^{\cdot}$ is the soluble isoform of GC. This heme protein binds $NO^{\cdot}$ with a biomolecular rate constant of ~ 10^8 M^{-1} s^{-1} [7], permiting its effective competition with other $NO^{\cdot}$ targets and facilitating the well-characterized increase in cyclic guanosine 3´,5´-monophosphate (cGMP) in response to $NO^{\cdot}$ [8]. Binding of $NO^{\cdot}$ to the heme results in an initial six-coordinate NO-Fe^{2+} - histidine complex. Subsequent breakage of the histidine-to-iron bond leads to formation of five-coordinated nitrosyl-heme complex that initiates a conformational change resulting in activation of the enzyme (reviewed in [6]). The resulting increase in cGMP is largely responsible for vasodilatation and the inhibition of platelet aggregation and proliferation of smooth muscle cells [9,10]. Another important target for $NO^{\cdot}$ at physiological concentration is the heme-copper protein cytochrome oxidase [11]. Binding of $NO^{\cdot}$ inhibits the oxidase, and this associated with an improvement in the efficiency of energy metabolism in the mitochondria. As a consequence, endogenously produced $NO^{\cdot}$ from the endothelium has considerable influence over tissue oxygen consumption [12].

On the other hand, peroxynitrite ($ONOO^-$) is a potent biological oxidant formed from the near-diffusion limited reaction between superoxide anion ($O_2^{\cdot-}$) and $NO^{\cdot}$ [13]. This radical-radical combination reaction undergoes with a second order rate constant of 10^{10} M^{-1} s^{-1} [14-16]. $ONOO^-$ exists in protonation equilibrium with peroxynitrous acid (ONOOH, pKa = 6.8) [17]. Thus, under biological conditions both $ONOO^-$ and ONOOH will be present, the ratio depending on local pH [18]. Since both precursor radical species, $NO^{\cdot}$ and $O_2^{\cdot-}$, are transient in nature, the biological formation of $ONOO^-$ requires the simultaneous generation of both radicals which, in addition, must approach and react within the same compartment. However, while $NO^{\cdot}$ has a biological half-life in the range of a seconds and readily diffuses across membrane, $O_2^{\cdot-}$ lasts less than milliseconds and permeates membranes only via anion channels. Thus, due to both the greater half-life and facile diffusion of $NO^{\cdot}$ compared to $O_2^{\cdot-}$, $ONOO^-$ formation will predominantly occur nearer to the $O_2^{\cdot-}$ formation sites (reviewed in [19]).

$ONOO^-$ promotes biological effects via different types of reactions, which could be classified in three main groups; 1: direct redox reactions, 2: reaction with carbon dioxide, and 3: homolytic cleavage of $ONOO^-$ (reviewed in [19]). Due to target molecule reactions, the biological half-life of $ONOO^-$ is estimated to be less than 100 ms [20,21]. This half-life is long enough for $ONOO^-$ to potentially travel some distances (5-20 μm) across extra- and/or intracellular compartments. However in addition to the estimated difussion in aqueous enviroments, the biological effects and detection of this oxidant agent will be influenced by its ability to permeate cell membranes, via anion channels (reviewed in [19]).

The aim of the current review, therefore, is to discuss the roles of $NO^{\cdot}$ and its secondary oxidant $ONOO^-$ in the pathophysiology of cardiovascular diseases, especially in myocardial ischemia-reperfusion (IR) injury, as a potential site for intervention to limit the damage.

Biological actions of vascular nitric oxide

In order to understand how decreased $NO^{\cdot}$ impacts vascular disease, it is necessary to understand its many roles in vascular homeostasis. $NO^{\cdot}$ has three major properties in the vascular system: anti-ischemia/antihypertension, antiatherosclerosis, and antithrombosis [22]. The anti-ischemia/antihypertension property of $NO^{\cdot}$ follows from its actions to stimulate the production of vascular smooth muscle cGMP and from its action to promote angiogenesis [23].

Elevations in cGMP within vascular smooth muscle cell through the direct nitrosylation of soluble GC leads to processes, which prevent the entry and promote the extrusion of calcium thereby leading to vasodilation. This vasodilator activity has many important clinical consequences including maintenance and enhancement of coronary and peripheral blood flow as well as maintenance of blood presure and attenuation of hypertension in both systemic and pulmonary vascular beds [24,25]. Anti-ischemia is also achieved through $NO^{\cdot}$-dependent angiogenesis. $NO^{\cdot}$ acts in concert with vascular endothelial growth factor (VEGF) to enhance endothelial cell proliferation as well as migration by stimulating podokinesis and by enhancing expression of urokinase-type plasminogen activator [26,27]. $NO^{\cdot}$ also prevents apoptosis of newly formed vascular cells. Also, by virtue of being vasodilator, $NO^{\cdot}$ decreases shear stress in the newly formed vessels which can potentially disrupt endothelial cell interaction with the surrounding extracellular matrix [28]. Other roles of $NO^{\cdot}$ during IR injury will be analyzed latter in this paper.

The anti-atherosclerosis property of $NO^{\cdot}$ comes from its ability to reduce intracellular oxidative stress as well as inhibit key early atherogenesis-signaling processes. Inhibition of these signaling processes leads to down-regulation of oxidative enzymes, the reduction of leukocyte accumulation, and the inhibition of vascular smooth muscle cells proliferation and migration (reviewed in [22]). A reduction of intracellular oxidative stress by $NO^{\cdot}$ reduces the presence of damaging reactive oxygen species (ROS) and is accomplished by several mechanisms [29]. $NO^{\cdot}$ can scavenge directly $O_2^{\cdot-}$, although the product of this reaction, $ONOO^{-}$, is itself a highly reactive specie. However, $ONOO^{-}$ may subsequently nitrosylate sulfhydryl groups to form S-nitrosothiols which can themselves participate in vasodilation, platelet antiaggregation, and monocyte adhesion inhibition (reviewed in [22]). $NO^{\cdot}$ may also terminates the autocatalytic chain of lipid peroxidation that is initiated by oxidized low density lipoprotein (oxLDL) and/or intracellular ROS generation [30]. $NO^{\cdot}$ may directly suppress ROS generation by nitrosylating oxidative enzimes to inactive forms. For example, nitrosylation of NADPH oxidase, prevents the association of its components rendering it inactive [31].

Finally, $NO^{\cdot}$ can inhibit the gene expression. Modulation of various atherogenesis-signaling processes by $NO^{\cdot}$ occurs partly through the inactivation of especific transcriptional proteins such as nuclear factor κB (NFκB) [32]. This effect of $NO^{\cdot}$ appears to be due in part to direct stabilization and/or increased expression of IκBa, which complexes to NFκB to inhibit its transcriptional activity [33]. Stabilization of the inactive NFκB/IκBa complex prevents the gene transcription of oxidative enzymes, as well as protein involved in leukocyte accumulation. Specifically, of glycoproteins adhesion molecules such as vascular cell adhesion molecule (VCAM) and chemokines such as monocyte chemotactic protein 1 (MCP-1). Expression of this proteins by endothelial cells, is inhibited within minutes and in a dose-dependent fashion upon exposure to $NO^{\cdot}$ [34,35].

Alterations in endothelial oxidant-nitric oxide signaling

The vascular endothelium appears to have multiple potential sources of ROS production, including mitochondrial respiratory chain, uncoupled NOS, NADPH oxidases, and xanthine oxidase (XO) [36,37]. The most dominant initial effect of increased ROS production by endothelium appears to be the attenuating action of $O_2^{\cdot-}$ on $NO^{\cdot}$ signaling [38]. Enhancement of this interaction seems to occur in multiple vascular diseases because of increased $O_2^{\cdot-}$ production [39]. In the absence of adequate levels of $NO^{\cdot}$, the pathophysiological effects of ROS are likely to dominate the siganling and oxidative stress responses that are observed [38].

The alterations in endothelial signaling caused by increased $O_2^{\cdot-}$ production contribute to important processes, such as the promotion of vasoconstriction or vasospasm, attenuation of the inhibition of platelet aggregation, and promotion of neutrophil adhesion. The effects of a simultaneous elevation of $NO^{\cdot}$ and $O_2^{\cdot-}$ will probably be dominated by the actions of $ONOO^-$, which will change as tissue antioxidant systems such as glutathione (GSH) become stressed and antioxidant enzymes become inactivated [38]. One of the first pathophysiological conditions observed to activate the production of increased levels of endothelium-derived ROS and ROS-mediated signaling responses was IR injury [40]. Also, ROS appear to have prominent roles in other chronically activated signaling processes associated with the evolution of key cardiovascular disease, including hypertension and atherosclerosis [41,42]. However, under more severe conditions, the responses observed are likely to be dominated by the pathological actions of oxidant agents, such as a loss of the protective effects $NO^{\cdot}$ as a result of damage caused by the metabolites that are produced, and the activation of inflammatory responses and thrombosis [38].

Biological action of vascular peroxynitrite

While generation of $ONOO^-$ may be beneficial in terms of host defense against invading microorganisms, excess $ONOO^-$ may be detrimental and entails damage to biomolecules. The mitogen-activated protein kinase (MAPK) pathways are among the signaling pathways that appear to be activated by a great variety of stressful stimuli, including oxidative stress [43]. $ONOO^-$ was demostrated to activate all three MAP kinase family members, p38 and c-Jun-N-terminal kinases (JNK) as well as the extracellular-signal-regulated kinases (ERK 1/2), in a wide variety of cell types, including bovine endothelial cells [44] and human neutrophils [45,46].

Consequences of exposure of cells to $ONOO^-$, in addition to the activation of these pathways, are the induced expression of stress genes such as c-fos, heme oxygenase-1, or the growth arrest and DNA damage-inducible (Gadd) proteins 34, 45, 153, and the induction of apoptosis. Apoptosis has been linked with MAPK activation since Xia *et al*. [47] proposed a crucial role of p38 and JNK as proapoptotic stimuli in PC12 cells, whereas activation of ERK seemed to be antiapoptotic (reviewed in [43]).

Although, evidence indicates that $ONOO^-$ formation effectivelly limits $NO^{\cdot}$ bioavailability by quenching $NO^{\cdot}$, other properties of $ONOO^-$ appear to limit endothelial function as well. This secondary oxidant readly oxidizes BH_4, thereby limiting the activity of the endothelial isoform of NOS (eNOS) and facilitating $O_2^{\cdot-}$ production [48]. Atherosclerosis is relacionated with reduced vascular levels of BH_4 and its $ONOO^-$ -mediated oxidation has been proposed as a physiologically relevant mechanism of impaired $NO^{\cdot}$ bioactivity [49]. Another pathway of eNOS uncoupling involves $ONOO^-$ -mediated oxidation of the Zn-thiolate center, resulting in the conversion of active eNOS dimer to inactive eNOS monomers. Such uncoupling, mediated by $ONOO^-$, involves the oxidation of one (or several) of the four cysteine residues coordinated to the Zn-atom present in the eNOS dimer [50]. Thus $ONOO^-$ have multiple biologic activities that could lead to impaired $NO^{\cdot}$ bioactivity by its limiting production [48]. This contributes with redox homeostasis disruption in vasculature and can leads to pathophysiological conditions.

Peroxynitrite-induced apoptosis

Apoptosis cell death 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 and oligonucleosomal

DNA fragmentation [51]. The first report indicating that $ONOO^-$ can trigger apoptotic death detected DNA fragmentation in $ONOO^-$ treated thymocytes [52]. Later, activation of caspase-3, a key player in the caspase cascade has also been detected in thymocytes [53].
Prototypical apoptosis models utilize apoptosis inducers such as tumor necrosis alfa (TNF-α) or FAS ligand acting upon cell surface death receptors. Channeling the death signal from this receptor to apoptotic effector machineries is well described. A characteristic sequence of events including opening of mitochondrial permeability transition pore, mitochondrial depolarization, secondary $O_2^{\cdot-}$ production, release of apoptotic mediators from the intermembrane space to the cytoplasm, takes place in apoptosing cells [51]. The role of mitochondria in apoptosis induced by $ONOO^-$ is also supported by findings that bcl-2, a mitochondrial antiapoptotic protein inhibits this cell death mechanism [54].
Other reports indicate a possible role for free 3-nitrotyrosine, in apoptosis induced by $ONOO^-$. 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. These data suggest that nitrotyrosine, released from proteins nitrated by $ONOO^-$, may contributes to vascular endothelial dysfunction through promotion of DNA damage and/or apoptosis [55].

Peroxynitrite-induced necrosis

Whilst low concentrations of $ONOO^-$ trigger apoptosis, higher concentrations of the oxidant compromise the apoptotic machinery forcing the cells to die by necrosis (reviewed in [5]). 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. In 2002, 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 (PARP) determines the fate of the oxidatively-injured cells [56]. PARP is activated by DNA strand break; activated PARP 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 (reviewed in [5]). 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 [56]. The concerted action of PARP and PARG maintains a highly accelerated ADP-ribose turnover in $ONOO^-$ 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 [57]. Moreover, shortage on ATP is exaggerated by attempts of the cells to resynthezise NAD from ATP and nicotinamide. The net result of this pathway is a dramatic drop in cellular ATP. As the apoptotic machinery is known to depend on ATP, apoptosis is incapacitated and necrosis takes predominance [58].
In summary, it is important to note that apoptosis has been recently proven to represent a dominant form of cardiomyocyte death in IR, and myocardial apoptosis has been suggested as the initiating factor of postinfarction left-ventricular remodeling [59,60]. Although oxidants and free radicals are considered important triggers of myocardial apoptosis in such conditions, the exact apoptotic stimulus still remains elusive [60].

Myocardial ischemia-reperfusion injury

Coronary artery disease remains a major cause of death in the western world. The primary pathological manifestation of coronary artery disease is myocardial damage due to IR injury [61]. Heart tisuue is remarkably sensitive to oxygen deprivation [2]. The level of IR-induced myocardial injury can range from a small insult resulting in limited myocardial damage to a large injury culminating in myocyte death. Importantly, major IR injury to the heart can result in permanent disability or death [62]. Like many cells, when deprived of oxygen (anoxia), cardiac cells can maintaining ATP levels by glycolytic ATP production, and can then revert smoothly to oxidative metabolism on reperfusion [63]. However, if blood flow is restricted, as in myocardial infarct, the cells accumulate glycolytic by-products (lactate, H^+) in addition to suffering from oxygen deprivation [64]. This is a condition known as ischemia and can damage cardiac cells irreversibly. Paradoxically, however, the major damage to ischemic cells comes on the reoxygenation (reperfusion). During reperfusion, the cells tipically undergo further contraction (hypercontracture) and membrane damage, and concluded in cell death [65,66]. Cardiac muscle is a highly aerobic tissue. As noted, under normal conditions, it obtains virtually all its energy from oxidative metabolism. Cosequently, restriction of the blood supply to cardiac muscle has serious pathological consequences, leading to cell death in the oxygen-depleted region (infarcted) [2]. During hypoxia or ischemia, the supply of oxygen to the respiratory chain fails. Non-esterified fatty acid levels rise, although probably as a result of lipid breakdown rather than the concomitant cesation of fatty acid oxidation [67,68]. The tricarboxylic acid cycle is blocked, and no energy is available from oxidative phosphorylation. This leads to an accumulation of cytoplasmatic NADH, with the $NADH/NAD^+$ ratio increasing severalfold. In anoxia, ATP levels can still be maintaining by glycolisis [63], but in ischemia this is accompained by accumulation of lactate and a decrease in cytoplasmatic pH (5.5-6.0 after 30 min of ischemia) [69-71], and glycolysis is also inhibited [2].

Other researchers have emphasized the overproduction of ROS on reperfusion as a source of cell damage [72], and it is notable that approximatelly 50 % of free protein sulfhydrilic groups disappear, presumably owing to interference with the glutathione redox system [73]. Although cytosolic NADPH can be involved in maintaining GSH, the balance may shift towards the production of ROS by cytosolic NADPH-oxidase; blocking NADPH production by inhibiting glucosa-6-phosphate dehydrogenase, as well as inhibiting its re-oxidation (by NADPH-oxidase or nitric oxide synthase) is, unexpectedly, protective against reperfusion injury [74].

Despite the complexity in the mechanisms responsible for the IR-induced myocardial damage, essential factors leading to cellular injury have been delineated [62]. Evidence indicates that several interrelated factors, including a decrease in cellular ATP levels, accumulation of hydrogen ions, calcium overload, calpain activation, leukocyte activation, and production of ROS contribute to IR injury [75-80]. A substantial body of evidence implicates ROS in the cellular injury induced by IR in the heart (reviewed in [81]). The precise mechanism of cell injury by these oxidants is not fully known, but DNA, lipids, and proteins are likely targets. Based on studies in which exogenous oxidants are applied to cells, it is apparent that dose-dependent injury is observed, with lower levels of oxidative stress associated with lower levels of cell death [82,83].

Nitric oxide and myocardial IR injury

$NO^{\cdot}$ has been associated with protection against ischemic cell death in a large number of studies (reviewed in [81]), although the mechanism and site of action are not known. $NO^{\cdot}$ is required for the cytoprotective effect induced by ischemic preconditioning in the heart

[84-86], but it is not clear whether $NO^{\cdot}$ acts before the ischemia, during the ischemia, or during reperfusion [81]. The balance between protective and deleterious effects of $NO^{\cdot}$ has led to difficulties in assessing its role(s) in IR. Its levels do rise during IR and, under these conditions, $NO^{\cdot}$ can interfere with mitochondrial functions [88]. Thus $NO^{\cdot}$ can induces cell death by necrosis through inhibition of mitochondrial respiration [89,90], or trigger apoptosis mediated by the mitochondrial permeability transition and by citochrome *c* release [91]. These disparate effects may be explained by the wide range of potential biochemical targets of $NO^{\cdot}$ in the cell, including tyrosine, and methionine residues in proteins; metal-containing prosthetic groups such as heme moieties; and other reactive molecules including $O_2^{\cdot-}$. In addition, the abundance of potential targets and the dose dependence of the responses may explain the diversity of effects that have been observed under different experimental conditions [81].

Given that a majority of studies indicate that $NO^{\cdot}$ functions in a cardioprotective role during myocardial ischemia and preconditioning [92], it becomes important to identify the mechanism(s) responsible for these protective effects. Putative mechanisms include the beneficial effects resulting from $NO^{\cdot}$-mediated increases in cGMP [93,94], an attenuation of calcium accumulation in myocytes [95], a decrease in myocardial oxygen consumption [96], an opening of the mitochondrial ATP-dependent potassium channels [97-99], or an inhibition of mitochondrial permeability transition during IR [100], and also a protective role of $NO^{\cdot}$ against loss of mitochondrial membrane potential ($\Delta\psi$) and apoptosis was shown [101].

ROS and oxidative stress contribute to the cell injury associated with IR, based on the observations that overexpression of antioxidant enzymes confers protection [102,103] and that chemical antioxidants administrated throughout the course of IR experiments decrease cell death [13]. $NO^{\cdot}$ is capable of inducing immediate protection when administered to the heart which suggests that it could acts by attenuating oxidative stress in the cell [103].

A heavily debated features of $NO^{\cdot}$ is its cytotoxic effect. The controversy arises from observations reporting both cytotoxic and cytoprotective effects of $NO^{\cdot}$ depending on variables of the assay systems used. In cases where $NO^{\cdot}$ was found cytotoxic, it was questioned whether $NO^{\cdot}$ directly or indirectly, through the formation of more reactive oxidative species such as $ONOO^-$ exerted its cytotoxic effects [5,103].

Peroxynitrite and myocardial IR injury

Increasing evidence supports the role of $ONOO^-$ generation as a pivotal mechanism of cell dysfunction and cell death in a number of pathological conditions, including circulatory shock [104], atherosclerosis [105], and IR injury [106]. $ONOO^-$ can produce considerable damage to most cellular components either directly, via one-or two-electron oxidations, or indirectly, via the generation of free radicals formed during $ONOO^-$ homolysis (yielding $^{\cdot}OH$ and $NO^{\cdot}_2$ radicals) or from its reaction with carbon dioxide (yielding $CO_3^{\cdot-}$ and $NO^{\cdot}_2$) [13,107]. The myocardial cytotoxicity of $ONOO^-$ involves oxidation of proteins (primarily on cysteine-bound thiols), lipids, DNA and nitration of protein tyrosine residues represent the major toxic consequences of $ONOO^-$ in biological systems [108,109]. This oxidant, acts as a potent signaling molecule in cardiomyocytes, activating metalloproteinases [110], all members of the MAP kinase family [111], inhibiting key myocardial enzymes such as reticulum sarcoplasmic Ca^{2+} ATPase [112], and creatine kinase [113], and modulating of nuclear factor NFκB signaling [114]. Furthermore, a major pathway of $ONOO^-$ dependent myocardial cytotoxicity relies on oxidative DNA damage and activation of the nuclear enzyme PARP (reviewed in [115]). Activated PARP cleaves its substrate nicotinamide adenine dinucleotide (NAD^+) into nicotinamide and ADP-ribose.

ADP-ribose polymers become attached to a variety of proteins, whose function becomes thereby modulated [116]. The major collateral damage related to PARP activation is the severe depletion of cellular NAD^+ stores, translating into reduced glycolytic activity and depressed mitochondrial electron transport, which eventually culminate in bioenergetic collapse and cell necrosis [117]. In contrast to its role in cell necrosis, the role of $ONOO^-$ in triggering cardiomyocyte apoptosis has been poorly investigated [116]. Apoptosis is orchestrated by the proteolytic activation of cysteine proteases known as caspases and regulated by proteins belonging to the bcl-2 family. Distinct pathways of apoptosis converge to the activation of executioner caspase-3, which cleaves multiple downstream cellular targets [117].

In summary, detecting and defining the participation of $NO^{\cdot}$ and $ONOO^-$ during cell and tissue damage in cardiovascualr diseases is an active and rapidly evolving area of research. More extensive and judicious application of current methodologies and development of more specific ones, will further contribute to a major understanding of the processes in which are implicate and will propitiate an appropriate pharmacological interventions.

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