Peroxynitrite Biology

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Abstract

Free radicals possess at least one unpaired electron in the outer electron orbit and usually, but not always, are highly chemically reactive. Molecular dioxygen (O_2) is stable; however, the oxygen-centered free radicals, superoxide ($O_2^{\bullet -}$) and hydroxyl ($^{\bullet}OH$), are not stable. In biological systems, *reactive oxygen species* (ROS), such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), play important signaling roles but may also contribute to cellular damage and disease development. Nitric oxide (also known as nitrogen oxide, or nitrogen monoxide, or simply NO) is also a free radical, and the existence of an unpaired electron may be reflected by the use of the abbreviation $NO^{\bullet}$ rather than NO. Unless discussing the three-redox forms of nitrogen monoxide (the nitrosonium ion, NO^+, the uncharged free radical, $NO^{\bullet}$, and the nitroxyl anion, NO^- or HNO), the abbreviation NO will be used throughout this chapter. *Reactive nitrogen species* (RNS) are produced in biological systems starting with the reaction of NO with $O_2^{\bullet -}$ to form the highly reactive RNS peroxynitrite ($ONOO^-$) that, unlike NO or $O_2^{\bullet -}$, is a very strong oxidant and nitrating agent. Thus, despite both NO and $O_2^{\bullet -}$ being free radicals, neither are as reactive as $ONOO^-$, and the toxicity of these two free radicals relates primarily to $ONOO^-$. Understanding how $ONOO^-$ modulates different intracellular biochemical pathways and how this may affect normal physiological processes and/or give rise to pathological conditions is an emerging area of great scientific interest. $ONOO^-$ exerts its adverse effects by direct interaction with CO_2, proteins that contain transition metal centers or thiols, or indirectly by aiding the generation of the highly potent hydroxyl radical. In this chapter, we outline the biochemistry and pathophysiology of $ONOO^-$ with a particular reference to cardiovascular disease and diabetes. We also address how scavenging strategies can attenuate the toxic effects of $ONOO^-$ and therefore may repress the pathophysiological effects of $ONOO^-$ and offer the potential for new therapeutic interventions.

Keywords

Free radicals • Hydroxyl radical, $^{\bullet}OH$ • Nitric oxide, NO • Peroxynitrite, $ONOO^-$ • Reactive nitrogen species, RNS • Reactive oxygen species, ROS • Superoxide, $O_2^{\bullet -}$

Abbreviations

($^{\bullet}NO_2$)	Nitrogen dioxide
($^{\bullet}OH$)	Hydroxyl radical
($CO_3^{\bullet -}$)	Carbonate radical
($O_2^{\bullet -}$)	Superoxide anion
ARE	Antioxidant response element
BH_2	Dihydrobiopterin
BH_4	Tetrahydrobiopterin

cGMP	Cyclic guanosine monophosphate
eNOS	Endothelial nitric oxide synthase
ERKs	Extracellular signal-regulated kinases
H_2O_2	Hydrogen peroxide
HClO	Hypochlorous acid
HONOO	Peroxynitrous acid
IκB	IκB kinase
JNK	c-Jun N-terminal kinases
NO	Nitrogen (mon)oxide
NO^+	Nitrosonium ion
NO^- (HNO)	Nitroxyl ion nitrosyl hydride, or hydrogen oxonitrate
$NO^\bullet$	Nitric oxide free radical
NRF2	Nuclear factor (erythroid-derived 2)-like 2
$ONOO^-$	Peroxynitrite
PARP	Poly(ADP-ribose) polymerase
PKC	Protein kinase C
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SERCA	Sarcoendoplasmic reticulum Ca^{2+}-ATPase
SOD	Superoxide dismutases
TNF-α	Tumor necrosis factor-alpha

Introduction

A reducing intracellular environment is essential for normal cellular function. Short-lived highly reactive molecules, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), that are capable of rapidly and drastically changing the normal reduced intracellular environment may therefore be detrimental to the normal function and survival of a cell. However, ROS and other small reactive molecules have been identified as important players in a wide array of physiologic and pathological conditions (Droge 2002). Thus, understanding that these molecules are endogenously produced under tightly controlled conditions suggests that ROS and RNS play significant roles in signal transduction events associated with various physiological processes (Droge 2002).

Oxygen is essential for respiration and energy production in aerobic organisms. However, ROS can turn out to be a serious threat to the cell, and oxidative stress is associated with disease, and thus the reduction of oxidative stress is a target for therapeutic intervention. In aerobic organisms, oxygen undergoes conversion to water in the mitochondria; however, an incomplete one-electron reduction results in the formation of a number of highly reactive (owing to their unpaired valence shell electrons) molecules commonly called ROS (Maulik and Das 2008). The ROS

produced in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), and free radicals such as the hydroxyl radical ($^{\bullet}OH$) and the superoxide anion ($O_2^{\bullet-}$). ROS, such as $O_2^{\bullet-}$ and H_2O_2, when produced in controlled amounts, have been found to be ideal signaling molecules since they are rapidly generated, easily degradable, and ubiquitously produced in all cell types (Droge 2002; Maulik and Das 2008).

Another gas, the short-lived nitric oxide (NO), which is also a free radical and may be designated as such by the use of the abbreviation $NO^{\bullet}$, is known to be an omnipresent intercellular messenger, modulating blood flow, neuronal activity, thrombosis, and a multitude of cellular events (Pacher et al. 2007). NO was initially identified in the 1980s as an endothelium-derived relaxation factor, EDRF, capable of causing vascular vasodilatation (Furchgott and Zawadzki 1980; Palmer et al. 1987; Furchgott and Vanhoutte 1989; Ignarro 1990; Thomas et al. 2008). Additionally, macrophages, participating in antitumor and anti-pathogen responses, were also found to produce NO (Stuehr and Nathan 1989; Granger and Hibbs 1996). It was not long before NO was identified to actively participate in pathophysiological responses as well. A number of studies have implied that endogenously generated NO can be toxic, while others have shown that it is protective. NO-associated toxicity was found to be associated with the generation of RNS (see review by Thomas et al. 2008).

The harmful effects of ROS and RNS on the cell are (1) damage to DNA (Wiseman and Halliwell 1996; Cooke et al. 2003; Pacher et al. 2007), (2) oxidation of lipids (Thomas et al. 1985; Radi et al. 1991a; Hall et al. 2004; Pacher et al. 2007), and (3) alteration of amino acids and proteins (Hall et al. 2004; Pacher et al. 2007; Grimsrud et al. 2008). However, during normal cellular activity, neither $O_2^{\bullet-}$ nor NO can be considered particularly toxic since there are normally efficient means to prevent their accumulation and limit their biological half-life (Beckman 1996; Pacher et al. 2007). Different forms of superoxide dismutase (SODs) located in the cytoplasm, SOD1, mitochondria, SOD2, or extracellular matrix, SOD3, rapidly remove $O_2^{\bullet-}$ and convert to H_2O, while NO rapidly diffuses through tissues into red blood cells where it is rapidly converted to nitrate (Beckman and Koppenol 1996; Butler et al. 1998; Joshi et al. 2002; Pacher et al. 2007). SOD, the major route for the elimination of $O_2^{\bullet-}$, removes $O_2^{\bullet-}$ at $2 \times 10^{9M-1.s-1}$ (Beckman and Koppenol 1996). NO can both inhibit and stimulate $O_2^{\bullet-}$-induced oxidation. Thus, when the sources of $O_2^{\bullet-}$ (NADPH oxidases in the plasma membrane, cytosolic xanthine oxidase, or mitochondrial respiratory complexes) and NO (nitric oxide synthases) are spatially associated and local NO concentrations reach micromolar levels, the superoxide and NO will yield the most potent and highly reactive RNS, $ONOO^-$, in a nonenzymatic reaction that has a rate constant of $6.7 \times 10^{9M-1.s-1}$ that is approximately four times faster than that for removal of $O_2^{\bullet-}$ by SOD (Beckman and Koppenol 1996; Jourd'heuil et al. 2001; see also Kissner et al. 1997). CO_2 is present in comparatively high concentrations in cells, and the reaction between $ONOO^-$ and the nucleophile CO_2 yields nitrogen dioxide ($^{\bullet}NO_2$) and carbonate radical ($CO_3^{\bullet-}$), which in turn initiates many of the

damaging reactions in the biological systems (Ferrer-Sueta and Radi 2009; Pacher et al. 2007). In addition, $ONOO^-$ is known to be a strong oxidant and nitrating agent interacting with electron-rich sulfhydryl groups, iron-sulfur centers, zinc thiolates, as well as sulfhydryl sites in tyrosine phosphatase enzymes (Castro et al. 1994; Crow et al. 1995; Radi et al. 1991b; Takakura et al. 1999). Thus, $ONOO^-$ can inhibit both phosphorylation and dephosphorylation processes in cell signaling pathways.

$ONOO^-$ directly, or indirectly, contributes to pathological conditions such as cardiac diseases, vascular diseases, stroke, cancer, neurodegenerative disorders, and diabetes (see review by Pacher et al. 2007). An accumulation of evidence suggests that $ONOO^-$ and its derivatives play critical roles in the progression of diabetes and diabetic-associated vascular complications. In the current chapter, we provide a brief overview of the pathophysiological actions of $ONOO^-$ with a particular focus on diabetes and how scavenging $ONOO^-$ might aid in alleviating the complications associated with diabetes.

The Biology of $ONOO^-$

A brief history of the chemistry and biochemistry of $ONOO^-$ has been comprehensively reviewed by Koppenol (2001). Co-localization of both NO and $O_2^{\bullet -}$ leads to the formation of $ONOO^-$. The oxidative chemistry is pH dependent and at pH 13 is stable for several days when stored at 4 °C (Bohle et al. 1994). Stability is also increased by hydrogen bonding in the presence of, for instance, mannitol and ethanol (Alvarez et al. 1998). In the cis-conformation, $ONOO^-$ is essentially stable; however, the protonated form, peroxynitrous acid (HONOO), has a pKa of 6.8 and decays to nitrate with a rate of 1.3 s−1 at 25 °C (Koppenol et al. 1992; Tsai et al. 1994). HONOO decomposes rapidly at physiological pH, and in the presence of bicarbonate, $ONOO^-$ reacts with CO_2 to produce NO_2^- and carbonate ion radicals (CO_3^-) [Reaction 1]. It has been assumed that $ONOO^-$ may be reduced to form hydroxide anion ($^{\bullet}OH$) and NO_2^- and that these are the oxidant species (Beckman et al. 1990; Goldstein and Merényi 2008) [Reaction 2]; however, $^{\bullet}OH$ is highly reactive, has a very short half-life of 10^{-9} s, and, except in organic solvents, would be subjected to extremely rapid scavenging (Sies 1963; Pryor and Squadrito 1995). Thus, most likely, it is an energetic and highly reactive transform of HONOO, termed HONOO*, that is the intermediate for the formation of nitrate, NO_3 (Koppenol et al. 1992; Goldstein et al. 1996) [Reaction 2]. For both $^{\bullet}OH$ and NO_2^- to be formed, it is argued that this must occur in a "cage" where the radicals are "protected" and held together by solvent molecules (Leffler and Grunwald 1963; Pryor and Squadrito 1995). This caged mechanism explains why it is not possible to completely scavenge the reactive molecule(s), assumed to be HONOO*, that is involved in $ONOO^-$-mediated reactions (Pryor 1966; Pryor and Squadrito 1995) (Fig. 9.1).

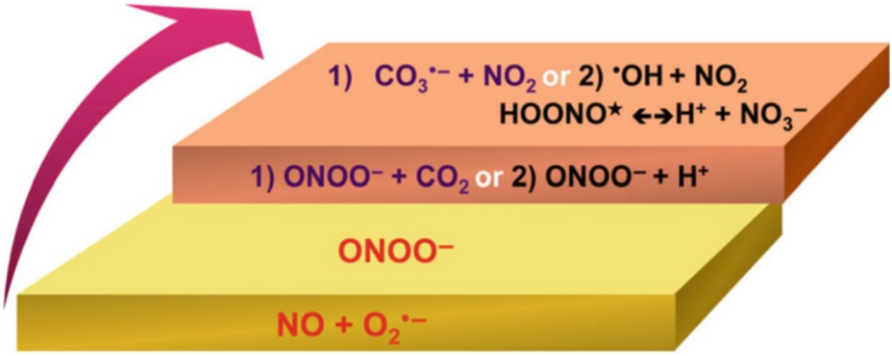

Fig. 9.1 Reactions leading to the formation of peroxynitrite and peroxynitrite-derived radicals. Co-localization of both nitric oxide (NO) and superoxide anion ($O_2^{\bullet-}$) leads to the formation of peroxynitrite ($ONOO^-$). Due to the availability of nucleophilic CO_2, $ONOO^-$ rapidly reacts with CO_2 to yield carbonate radical ($CO_3^{\bullet-}$) and nitrogen dioxide (NO_2)- [Reaction 1]. $ONOO^-$ may also be reduced to from hydroxide anion ($^{\bullet}OH$) and NO_2^-; however, in biological systems, it is an energetic form of peroxynitrous acid (HONOO), termed HONOO*, that is likely to be the intermediate for the formation of nitrate (NO_3^-) [Reaction 2]

The Biochemistry of $ONOO^-$

Formation

Numerous stimuli such as toxins, stress, ultraviolet light, ischemia/reperfusion injury, and inflammation can result in the production of $ONOO^-$ (Ahmad et al. 2009). $ONOO^-$ is produced mainly in macrophages, endothelial cells, platelets, leukocytes, neurons, etc., as a result of a rapid nonenzymatic combination reaction between NO and $O_2^{\bullet-}$ (Ahmad et al. 2009). Major cellular sources for the generation of $O_2^{\bullet-}$ are, for instance, mitochondria, NADPH oxidase, xanthine oxidase, and also from oxidation of catechols. Under normal physiological conditions, SODs rapidly remove the $O_2^{\bullet-}$ anion; however, under conditions when the generation of NO (at ~10 μM) outcompetes the capacity of SODs, NO reacts faster with $O_2^{\bullet-}$ than decomposition by SOD (Su and Groves 2010). It is assumed that the site of $ONOO^-$ formation is spatially associated with the sources of $O_2^{\bullet-}$ since $O_2^{\bullet-}$ has restricted diffusion due to its charged nature while NO is uncharged and easily diffusible (Ferrer-Sueta and Radi 2009; Su and Groves 2010).

Interactions of $ONOO^-$ with Biological Systems

In addition to local production, $ONOO^-$ can also act as a paracrine-like mediator and gain access to cells via anion channels (Denicola et al. 1998; Macfadyen et al. 1999). $ONOO^-$, in common with other oxidants, interacts with DNA, proteins/

amino acids, and lipids, leading to distinct cytotoxic effects (Ascenzi et al. 2010). The specifics of the $ONOO^-$ modification/biomolecular interactions pertaining to various pathological conditions, especially diabetes, will be detailed later in this chapter. This section briefly highlights the general aspects of $ONOO^-$ interactions:

(a) With CO_2: CO_2 being a nucleophile is a potent target for the strongly anionic $ONOO^-$ radical, especially since the concentration of CO_2 is particularly high in the biological system (Ferrer-Sueta and Radi 2009). The rapid reaction between CO_2 and $ONOO^-$ yields $CO_3^{\bullet -}$ and nitrogen dioxide ($^{\bullet}NO_2$), both of which are strong and short-lived oxidant radicals, which in turn target protein thiolates and aromatic residues participating in DNA base modifications (Augusto et al. 2002).

(b) With proteins: $ONOO^-$ interacts with various proteins at different levels, and these interactions are primarily responsible for the deleterious effects of $ONOO^-$ on biological systems. $ONOO^-$ promotes the nitration of tyrosine residues and the oxidation of methionine and cysteine residues (Beckman et al. 1992; Mohr et al. 1994; Pryor et al. 1994). The biological effects of $ONOO^-$-induced modifications of proteins include antioxidant enzyme inhibition/depletion, cytosolic enzyme inhibition, protein aggregation, activation of specific enzymes, membrane channel inhibition, modified/altered cellular signaling events, as well as the accelerated degradation of $ONOO^-$-modified proteins via the proteasome (Milstien and Katusic 1999; Szabo 2003; Szabo et al. 2007). $ONOO^-$ can also interact with and impair enzyme cofactors thereby indirectly affecting protein/enzyme function (Milstien and Katusic 1999; Szabo et al. 2007).

(i) *Reaction of* $ONOO^-$ *with Specific Amino Acid Residues in Proteins*: $ONOO^-$ alters protein structure and function by reacting with various amino acids in the polypeptide backbone of the various proteins. Cysteine oxidation (thiol oxidation) by $ONOO^-$ is one of the most prominent amino acid modifications in the biological system. $ONOO^-$-linked oxidation of cysteine residues is known to inactivate many enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mohr et al. 1994), creatine kinase (Konorev et al. 1998; Mihm et al. 2001), NADH dehydrogenase (complex I) (Radi et al. 2002a, b), succinate dehydrogenase (complex II) (Radi et al. 2002a, b), cytochrome c reductase (complex III) (Radi et al. 2002a, b), and ATP synthase (complex V) (Radi et al. 2002a, b). Phosphotyrosine-dependent signaling pathways may be enhanced by $ONOO^-$-dependent inactivation of tyrosine phosphatase enzymes (Takakura et al. 1999; Lopez et al. 2005). Conversely, $ONOO^-$ -associated cysteine oxidation has been shown to activate some enzymes such as matrix metalloproteinases (MMPs) and sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Okamoto et al. 2001; Adachi et al. 2004; Migita et al. 2005). $ONOO^-$ is also known to oxidize the endogenous antioxidant glutathione, making glutathione an efficient scavenger for $ONOO^-$ (Marshall et al. 1999; Bajt et al. 2003).

Tyrosine nitration is another prominent amino acid modification that can result from high levels of $ONOO^-$. Tyrosine nitration has been shown to

affect protein structure and function with resultant generation of antigenic epitopes, changes in catalytic efficiency, and impaired signal transduction (Kuhn et al. 1999; Knapp et al. 2001; Pacher et al. 2007). Enzymes such as glyceraldehyde-3-phosphate dehydrogenase, creatine kinase, and the mitochondrial respiratory chain complexes may, in addition to their susceptibility for cysteine oxidation, also be inactivated by tyrosine nitration making them seemingly vulnerable targets for $ONOO^-$ (Pacher et al. 2007). The tissue level of nitrotyrosine, resulting from $ONOO^-$-mediated tyrosine nitration, is often used as a marker for nitrative stress. $ONOO^-$-induced tyrosine nitration has been shown to be a key modification associated with altered protein structure and function responsible for clinical manifestations in diabetes-associated heart diseases (Xu et al. 2006), Alzheimer's disease, and Parkinson's disease (Aoyama et al. 2000). $ONOO^-$, to a lesser extent, may also oxidize methionine (Whiteman et al. 1996; Alvarez and Radi 2003; Stadtman and Levine 2003; Stadtman et al. 2003), tryptophan (Alvarez and Radi 2003), and histidine residues in certain proteins leading to inactivation of these affected proteins (Alvarez et al. 2004; Yamakura et al. 2005; Yamakura and Ikeda 2006).

(ii) *Reaction of* $ONOO^-$ *with Proteins Containing Transition Metal Centers*: Proteins with transition metal centers such as hemoglobin (Boccini and Herold 2004), myoglobin (Herold et al. 2003), and cytochrome c (Thomson et al. 1995; Herold et al. 2003) are vulnerable to $ONOO^-$-induced rapid oxidation of their ferrous heme into ferric forms (Ferrer-Sueta and Radi 2009; Su and Groves 2010). In addition, critical metabolic enzymes containing iron-sulfur centers (Djaman et al. 2004) such as mitochondrial aconitase (Castro et al. 1994; Hausladen and Fridovich 1994) and phosphogluconate dehydratase (Keyer and Imlay 1997) and zinc-sulfur motifs such as alcohol dehydrogenase (Crow et al. 1995) have been shown to be inactivated by $ONOO^-$ interaction. As previously noted, $ONOO^-$-modified proteins are also candidates for degradation via the proteasome (Szabo 2003).

(c) With lipids: $ONOO^-$ triggers lipid peroxidation in membranes, liposomes, and lipoproteins (Radi et al. 1991a; Rubbo et al. 1994; Rubbo and Freeman 1996; Rubbo 1998). The derivatives of $ONOO^-$-lipid interactions such as hydroperoxy radicals, conjugated dienes, and aldehydes in turn attack other lipid moieties thereby propagating the effects of free radical reactions followed by degeneration of membrane lipids and subsequently change membrane fluidity and permeability (Hogg and Kalyanaraman 1999). It has also been shown that $ONOO^-$-modified low-density lipoprotein (LDL) binds with high affinity to the scavenger receptors – a known risk for atherosclerotic plaque formation (Botti et al. 2005; Rubbo and O'Donnell 2005).

(d) With DNA: $ONOO^-$-induced oxidative modifications of nitrogen bases and the sugar phosphates of the nucleotide bases can result in mutagenesis (Ahmad et al. 2009). $ONOO^-$ potentiates nitration, nitrosation, and deamination of the purine bases (mainly guanine) in DNA (Douki and Cadet 1996; Douki et al. 1996).

8-nitro-guanine and 8-oxyguanine are prominent $ONOO^-$-induced modifications that occur, leading to single-strand breaks in the DNA (Niles et al. 2006). Additionally interactions of $ONOO^-$ with hydrogen atoms on sugar phosphates result in single-strand breaks which can trigger the activation of poly(ADP-ribose) polymerase (PARP), ultimately leading to cell death (Lorch et al. 2002; Ohshima et al. 2002; Szabo 1996; Szabo et al. 1996; Zingarelli et al. 1996).

Physiological Roles of RNS and $ONOO^-$

Substantial progress has been made to understand the routes of formation, mechanisms, cellular actions, and detoxification of RNS such as $NO^{\cdot}$, nitroxyl ion (NO^-, HNO), nitrosonium cation (NO^+), higher oxides of nitrogen, S-nitrosothiols (RSNOs), and $ONOO^-$. RNS molecules are well recognized for playing dual roles, both beneficial and deleterious, to living organisms (Patel et al. 1999; Ronson et al. 1999; Wolin 2000; Pacher et al. 2007; Triggle and Ding 2010). The primary cellular source of RNS is NO, an important intracellular messenger that exhibits a broad range of physiological properties that are dependent upon both concentration and duration of exposure. NO has both direct and indirect actions, with the indirect actions via RNS formation, which subsequently react with and dysregulate biological targets (Thomas et al. 2008). The physiological level of NO generated following endothelial nitric oxide synthase (eNOS) activation plays a vital role in the regulation of vascular tone, platelet function, angiogenesis, tissue oxygenation, vascular remodeling, and also anti-atherosclerotic actions (Naseem 2005; Pacher et al. 2007; Chen et al. 2008; Luiking et al. 2010). Disruption of NO production and/or bioavailability results in dysregulation of key physiological and cellular processes such as vasodilatation, platelet function, angiogenesis, apoptosis, and smooth muscle cell proliferation (see reviews by Pober et al. 2009; Triggle et al. 2012). The factors such as availability of the substrate L-arginine; elevated levels of circulating nitric oxide synthase (NOS) inhibitors such as asymmetrical dimethylarginine (ADMA); perturbed signal transduction reducing agonist-induced eNOS activation; reduced availability of tetrahydrobiopterin (BH_4), an essential cofactor; or the reaction of NO with other free radical species such as $O_2^{\bullet -}$ could potentially affect either the production of NO or the ability of NO to diffuse to its cellular targets (Scott-Burden 1995; Vallance and Leiper 2004; Naseem 2005; Leiper and Nandi 2011). The foremost mechanism thought to be responsible for the detrimental effects of NO is reaction with $O_2^{\bullet -}$ and formation of $ONOO^-$ in a diffusion-limited manner. Although most of the pathological effects of NO are mediated by $ONOO^-$, existing data also indicates that $ONOO^-$ may be a double-edged sword, as it exerts both cytotoxic as well as cytoprotective effects (Nossaman and Kadowitz 2008). The biological effects (beneficial/deleterious) of $ONOO^-$ exposure are greatly dependent on tissue concentration, biological environment in which it is present, and availability of detoxifying agents for conversion of $ONOO^-$ to another form. $ONOO^-$ exerts beneficial effect under

in vivo conditions when thiol-containing agents such as glutathione, albumin, cysteine (Radi et al. 1991b; Wu et al. 1994; Wolin 2000), and uric acid (Skinner et al. 1998) are available to facilitate $ONOO^-$ to donate NO (Ronson et al. 1999; Vinten-Johansen 2000; Ferdinandy and Schulz 2001). Putative important physiological roles for $ONOO^-$ include vasodilatation, inhibition of platelet aggregation, and inflammatory cell adhesion as well as protection against ischemia/reperfusion injury (Ronson et al. 1999; Vinten-Johansen 2000; Ferdinandy and Schulz 2001; Ferdinandy 2006; Uppu et al. 2007; Nossaman and Kadowitz 2008).

Vasodilatation Properties of $ONOO^-$

$ONOO^-$ produces a rapid concentration-dependent and endothelium-independent vasodilatation of aortic ring preparations (Liu et al. 1994; Villa et al. 1994; Wei et al. 1996; Ronson et al. 1999; Nossaman and Kadowitz 2008). Vasodilatation induced by $ONOO^-$ can be attributed to an increase in cGMP in vascular smooth muscle cells possibly involving the formation of S-nitrosothiols via the interaction between $ONOO^-$ and glutathione (Ronson et al. 1999; Nossaman and Kadowitz 2008). $ONOO^-$ can also hyperpolarize the vascular smooth muscle cell membrane via activation of ATP-sensitive potassium channels, myosin light chain phosphatase, and/or interference with calcium entry and release (Wei et al. 1996; Ademoglu et al. 2002; Li et al. 2004b; Graves et al. 2005). Furthermore, $ONOO^-$-mediated vasodilatation may subsequently result in the formation of a stable secondary intermediate(s) following reaction with glucose and/or other functional alcohol groups (Beckman et al. 1990; Moro et al. 1995; Dowell and Martin 1997).

Antithrombotic and Antiplatelet Aggregation Properties of $ONOO^-$

The actions of $ONOO^-$ on platelet function are concentration-dependent. $ONOO^-$ stimulates platelet aggregation at higher concentrations, whereas at lower concentrations inhibits platelet aggregation (Moro et al. 1994). Within the concentration range of 50–200 μM and in the presence of different platelet aggregation-inducing agents such as collagen, thrombin, and U46619 (thromboxane A2 mimetic), $ONOO^-$ inhibited platelet aggregation in a dose-dependent manner. Furthermore, addition of $ONOO^-$ could also reverse platelet aggregation previously induced by collagen, ADP, and thrombin. The cellular mechanism of the antiplatelet aggregation property of $ONOO^-$ appears to be related in part to a production of NO and/or a nitrosothiol compound. Thus, $ONOO^-$ nitrosylates thiols to form nitrosothiols, which then release NO and inhibit platelet aggregation; however, such a protective effect of $ONOO^-$ is offset when there is a persistent production of oxidants with the consequent depletion of thiols and a cytotoxic action (Moro et al. 1994; van der Vliet et al. 1995; Yin et al. 1995; Nowak and Wachowicz 2001).

Protective Effect of $ONOO^-$ on Ischemic Reperfusion Injury

Various studies have shown the cardio- and vasculoprotective effects of $ONOO^-$ in myocardial/reperfusion injury models (Lefer et al. 1997; Nossuli et al. 1997). In animals subjected to myocardial ischemia followed by reperfusion, administration of $ONOO^-$ (0.2, 2, or 20 μM) directly into the left ventricle beginning 10 min before and continued throughout the reperfusion period significantly protected the myocardium from developing substantial levels of necrosis. Furthermore, infusions of $ONOO^-$, in the low micromolar range, produced a significant reduction of ventricular fibrillation and ischemia/reperfusion-induced dysrhythmias (Hagar et al. 1991; Vegh et al. 1992; Nossuli et al. 1998; Altug et al. 1999). An attenuated ability of endothelial cells to inhibit adherence of neutrophils is a critical event in ischemic myocardial injury, and $ONOO^-$-mediated cardioprotection has been linked to attenuation and/or modulation of neutrophil-endothelial cell interaction, anti-inflammatory effects via its ability to act as a NO carrier or as an NO donor (Ronson et al. 1999). $ONOO^-$ modulates P-selectin expression, a cell adhesion molecule that plays an essential role in neutrophil adherence on the surface of activated endothelial cells (Burns et al. 1999). Pretreatment with $ONOO^-$ significantly reduces surface expression of P-selectin and inhibits leukocyte-endothelial cell interactions. Moreover, histological analysis demonstrates that $ONOO^-$ significantly attenuates polymorphonuclear leukocytes infiltration into heart tissue and protects the ischemic/reperfused rat heart by inhibiting polymorphonuclear leukocytes accumulation. These data show that $ONOO^-$, in nanomolar concentrations, can inhibit leukocyte-endothelial cell interactions and exert cytoprotective effects, and inhibition of P-selectin is a key mechanism in modulation of $ONOO^-$-mediated inhibition of leukocyte-endothelial cell interactions. It should, however, be stressed that, in these experiments, $ONOO^-$ demonstrated marked cardioprotection only when administrated via intraventricular route, whereas with an intravenous method of $ONOO^-$ infusion exerted cytotoxic effects (Lefer et al. 1997; Nossuli et al. 1998; Liu et al. 2000). Thus, the contribution of $ONOO^-$ to the cardioprotection reported in these studies may have been mediated by nitrosothiols as a result of the $ONOO^-$ being premixed with plasma prior to injecting into the animal, and therefore the $ONOO^-$ would have reacted with thiols and resulted in the formation of NO donor species (van der Vliet et al. 1998). In contrast to the conclusions reached by Lefer and coworkers (1997), Yasmin et al. (1997) reported that reperfusion of the ischemic rat heart results in the acute production of $ONOO^-$, and the inhibition of its biosynthesis with L-NMMA, or antagonizing its oxidant actions with the NO donor SNAP, may provide a strategy for the protection of the heart from ischemia/reperfusion injury.

Chemical modification of kinases and phosphatases by $ONOO^-$ can modify the cell signal transduction pathways and result in the up- or downregulation of signaling cascades in a concentration- and cell-dependent manner. For instance, $ONOO^-$-mediated S-glutathiolation of cysteine residues may result in activation of enzymes, including metalloproteinases, MMPs, which typically occurs at low concentrations

of $ONOO^-$ [<10 μM], and $ONOO^-$-mediated activation of MMP2 has been shown to be inhibited via the antioxidant effects of acetaminophen (see Klatt and Lamas 2000; Okamoto et al. 2001; Rork et al. 2006; Viappiani et al. 2009). SERCA can also be activated and calcium uptake enhanced by low concentrations of $ONOO^-$, and it has been shown that the key cysteine residue is Cys674 (Adachi et al. 2004). Interestingly S-glutathiolation of SERCA was reduced in atherosclerotic conditions when ROS were elevated, and this was argued to be a result of the irreversible oxidation of key thiols, such as Cys674, effects that were mimicked by mutation of Cys 674 (Adachi et al. 2004). Thus, S-glutathiolation via $ONOO^-$ plays an important role in the regulation of enzyme function suggesting that appropriate levels of $O_2^{\bullet -}$ are required to maintain at least some components of NO-mediated cell signaling (Adachi et al. 2004).

$ONOO^-$-induced phosphatidylinositol 3-kinase (PI3K) activation and Akt (protein kinase B) phosphorylation result in the activation of the nuclear factor (erythroid-derived 2)-like 2/antioxidant response element (Nrf2/ARE), transcription factor with subsequent upregulation of the antioxidant and cytoprotective glutathione-S-transferase, heme-oxygenase-1 in neural cells (Kang et al. 2002; Li et al. 2006). Studies with isolated fibroblasts, neutrophils, endothelial and vascular smooth muscle cells, neural cells, cardiomyocytes, and whole lung tissue show that $ONOO^-$ activates extracellular signal-regulated kinase (ERK), a critical regulator of myocardial hypertrophy as well as an important cardioprotective signaling molecule (Bapat et al. 2001; Liaudet et al. 2009).

In the vasculature, $ONOO^-$-dependent Janus kinase (JNK) activation, which plays an important prevent anti-atherosclerotic role, has been shown to occur in endothelial cells exposed to laminar shear stress, suggesting that $ONOO^-$ may represent a key molecular link between endothelial mechanical stress and endothelial function (Go et al. 1999). The cytoprotective or cytotoxic roles of protein kinase C (PKC) isoforms, PKC epsilon, and delta, respectively, in cardiovascular diseases have been extensively investigated (Churchill and and Mochly-Rosen 2007). NO-mediated PKC epsilon activation is thought to play a protective role during ischemic preconditioning (Ping et al. 1999), and $ONOO^-$ rather than NO may be responsible for this effect. Furthermore, $ONOO^-$ directly enhances the binding of recombinant PKC epsilon to anchoring protein, receptor for activated C kinase (RACK2), in an in vivo model of NO-mediated cardioprotection, thereby delineating a novel signaling mechanism by which NO activates PKC epsilon in the heart via the generation of $ONOO^-$ (Balafanova et al. 2002).

To summarize, $ONOO^-$, at low micromolar concentrations, upregulates multiple signaling cascades by acting as an NO carrier, or NO donor, thereby exhibiting distinctive physiological roles. However, there are several limitations to this generalization including cell specificity, $ONOO^-$ concentration within the tissue, the short half-life of $ONOO^-$, the biological environment, and the availability of thiol-containing agents. Concentration-dependent effects of $ONOO^-$ are discussed in more detail in the next section.

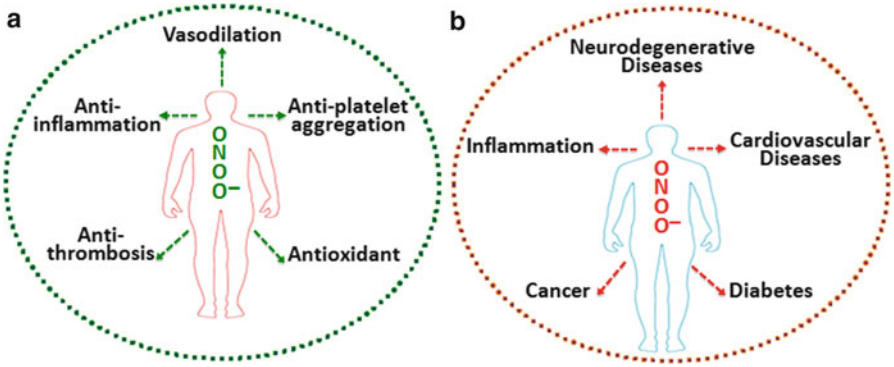

Fig. 9.2 Protective and pathological roles of peroxynitrite. At low concentration, $ONOO^-$ may play a protective role by acting as an NO carrier or NO donor as well as an activator, via S-glutathiolation, of enzymes such as SERCA (**a**). At higher concentrations, the effects of $ONOO^-$ shift from protective to pathological, and, by acting as pro-oxidant and causing irreversible oxidation/nitration of proteins, $ONOO^-$ has been implicated in many disease conditions (**b**)

Pathological Effects of $ONOO^-$

$ONOO^-$ toxicity is associated with many diseases (Fig. 9.2). The pathological effects of $ONOO^-$ are associated with the source of $O_2^{\bullet -}$ generation, length of formation time, and higher concentrations. Thus, moderate $ONOO^-$ generation over a long period can be detrimental to cell constituents, leading to reversible cell injury, irreversible cell injury, and even the induction of cell death through apoptosis/necrosis. Although it is difficult to assign a specific concentration range for the "physiological or enzyme activation" versus "pathophysiological/inactivation" effects of $ONOO^-$, in vitro studies, with MMP2, indicate that 0.3–1.0 μM of $ONOO^-$ activated the enzyme, whereas 30–100 μM attenuated enzyme activity (Viappiani et al. 2009). However, activation of MMP2 required the presence of glutathione, and thus the concentration-dependent effects of $ONOO^-$ will be very much dependent on the cellular milieu.

An important hallmark of many diseases, including cardiovascular, neurological disorders, cancer, and also aging, is that proteins, lipids, and nucleic acids are oxidized/nitrated by $ONOO^-$ (Pacher et al. 2007). Prolonged nitration of several important cardiac proteins, including creatine kinase, SERCA, and inactivation of voltage-gated ion channels, results in the acceleration of cardiac dysfunction. On the other hand, as already noted, the S-glutathiolation of cysteine residues may result in activation of enzymes such as MMPs and SERCA; however, this is concentration-dependent and inactivation occurs at higher concentrations most likely as a result of the irreversible oxidation of key protein thiols (Adachi et al. 2004; Viappiani et al. 2009).

Both nitration of cytoskeletal/structural proteins such as desmin, myosin, actinin, and tubulin by $ONOO^-$ as well as an increased accumulation of inflammatory cells in the vasculature have also been associated with endothelial and platelet dysfunction (Mihm et al. 2001; Mihm and Bauer 2002; Lee et al. 2003; Li et al. 2004a; Borbely et al. 2005; Lokuta et al. 2005). Moreover, as reviewed by Pacher et al. (2007), $ONOO^-$-mediated modification of amino acids, such as oxidation of tryptophan, methionine, and histidine, may also play an important role in the pathogenesis of neurodegenerative disorders.

The ability of $ONOO^-$ to trigger peroxidation in membrane lipids, liposomes, and lipoproteins is also an important aspect of $ONOO^-$-mediated cytotoxic effect of $ONOO^-$. Furthermore, $ONOO^-$-mediated oxidization of LDL and foam cell formation are suggested as critical events in the progression of vascular diseases including atherogenesis. $ONOO^-$-mediated damage to DNA, including oxidation of nucleotides of deoxyribose moieties, strand breaks, is critically involved in induction of cell death and inflammatory pathways and implicated in cancer, diabetes, and cardiovascular disease (see reviews by Pacher et al. 2007; Szabo et al. 2007).

$ONOO^-$ and Cardiovascular Disease

The cytotoxic effect of $ONOO^-$, as evidenced by data from both in vitro and in vivo studies, has been implicated in a variety of cardiovascular diseases including myocardial ischemic reperfusion injury, myocarditis, coronary artery disease, and congestive heart failure. $ONOO^-$ can activate matrix metalloproteins (MMPs) as well as PARP (Klatt and Lamas 2000; Okamoto et al. 2001; Virag and Szabo 2002). Inhibition of MMPs, notably MMP2, and PARP is a potential target for the treatment of heart failure (Gao et al. 2003; Cena et al. 2010; Pacher et al. 2005; Viappiani et al. 2009). The ability of $ONOO^-$ to damage vascular endothelium by increased lipid peroxidation, upregulation of adhesion molecule expression, altered endothelium-dependent vasodilatation, vascular smooth muscle cell proliferation, platelet aggregation, and thrombus formation have all been considered as causative factors of $ONOO^-$-induced vascular dysfunction (see review by Triggle et al. 2012). Furthermore, $ONOO^-$-mediated oxidation of BH_4 and uncoupling of eNOS accelerate the onset of endothelial dysfunction, vascular disease pathogenesis, and this aspect of the pathophysiological actions of $ONOO^-$ is discussed in more depth in the next section (see reviews by Pacher et al. 2007; Szabo et al. 2007; Triggle et al. 2012).

$ONOO^-$, Diabetes, and Vascular Disease

Growing evidence implicates that $ONOO^-$ formation is a major contributor to the initiation, progression, and pathogenesis of diabetes. The greatest contributor to the morbidity and mortality of diabetes is cardiovascular disease, and endothelial dysfunction is the earliest indicator of the onset of vascular dysfunction regardless

of whether it is secondary to diabetes or other pathophysiologies such as hypertension (Félétou and Vanhoutte 2006; Pober et al. 2009; Triggle and Ding 2010; Ghiadoni et al. 2011; Triggle et al. 2012).

Endothelial dysfunction can be defined as "Endothelial dysfunction reflects a reduced endothelium-dependent vasodilatation response to either an endothelium-dependent vasodilator, such as acetylcholine (or bradykinin), or to flow-mediated vasodilatation that is accompanied by elevated expression of adhesion molecules, enhanced vascular smooth muscle proliferation and the development of a hypercoagulatory state." Perhaps more simply endothelial dysfunction can be defined as "The failure of endothelial cells to perform their normal physiological functions" (see Pober et al. 2009; Triggle et al. 2012 for further discussion).

Hyperglycemia leads to an imbalance between the increased production of $O_2^{\bullet -}$ versus NO and results in the formation of $ONOO^-$ within the vascular wall. The vasoconstriction, proinflammatory, pro-thrombotic, and atherosclerotic effects of $ONOO^-$ have been suggested as critical contributors to diabetes-induced vascular disease (Zou et al. 2004). $ONOO^-$ upregulates the expression of proinflammatory cytokines interleukin-1 beta, IL-1β, and tumor necrosis factor-alpha, TNF-α, in pancreatic islet cells, thus promoting β cell destruction (Lakey et al. 2001).

Elevated plasma nitrite/nitrate, nitrotyrosine, and inducible nitric oxide synthase (iNOS) levels are considered as indices of excessive $ONOO^-$ formation in type 2 diabetes (Tannous et al. 1999; Ceriello 2002). It has been reported that $ONOO^-$-mediated elevated protein nitration, DNA damage, and oxidative stress eventually lead to β cell dysfunction and apoptosis (Fehsel et al. 1993; Szabo and Ohshima 1997; Hou et al. 2010; Kim et al. 2010; Liang et al. 2010; Stadler 2011). Accelerated $ONOO^-$ formation during hyperglycemic episodes results in increased nitrotyrosine formation; increased expression of cellular adhesion molecules, cytokines, and chemokines; PKC-dependent NADP(H) oxidase activation; altered vascular reactivity and diminished vasodilatation with altered activities of Na^+/K^+-ATPase and Ca^{2+}-ATPase in vascular endothelial cells; and ultimately diabetes-induced endothelial dysfunction (Cosentino et al. 2003a, b; Kossenjans et al. 2000; Soriano et al. 2001; Rabini et al. 2002; Szabo et al. 2002; Zou et al. 2002).

Data from both clinical and experimental studies indicates that enhanced $ONOO^-$ formation associated with diabetes results in nitrotyrosine formation in endothelial cells, cardiomyocytes, and fibroblasts and favors accelerated apoptosis and cell death (Frustaci et al. 2000; Kajstura et al. 2001; Ceriello et al. 2002). Exposure of human umbilical vein endothelial cells to $ONOO^-$ significantly inhibited both basal and insulin-stimulated Akt phosphorylation at Ser473 and Akt activity in parallel with increased apoptosis (Song et al. 2007). $ONOO^-$ can also trigger an impairment of endothelium-dependent vasodilatation, secondarily to a decrease in SERCA2A function (Kobayashi 2008). $ONOO^-$-mediated DNA strand breaks and consequent activation of nuclear enzyme poly(ADP-ribose) polymerase-1, PARP-1 have also been suggested as critical contributors to $ONOO^-$-mediated development of diabetic vascular complications (Garcia Soriano et al. 2001; Pacher et al. 2002; Pacher and Szabo 2005). Data from cell culture and animal studies suggest that diabetes is associated with increased

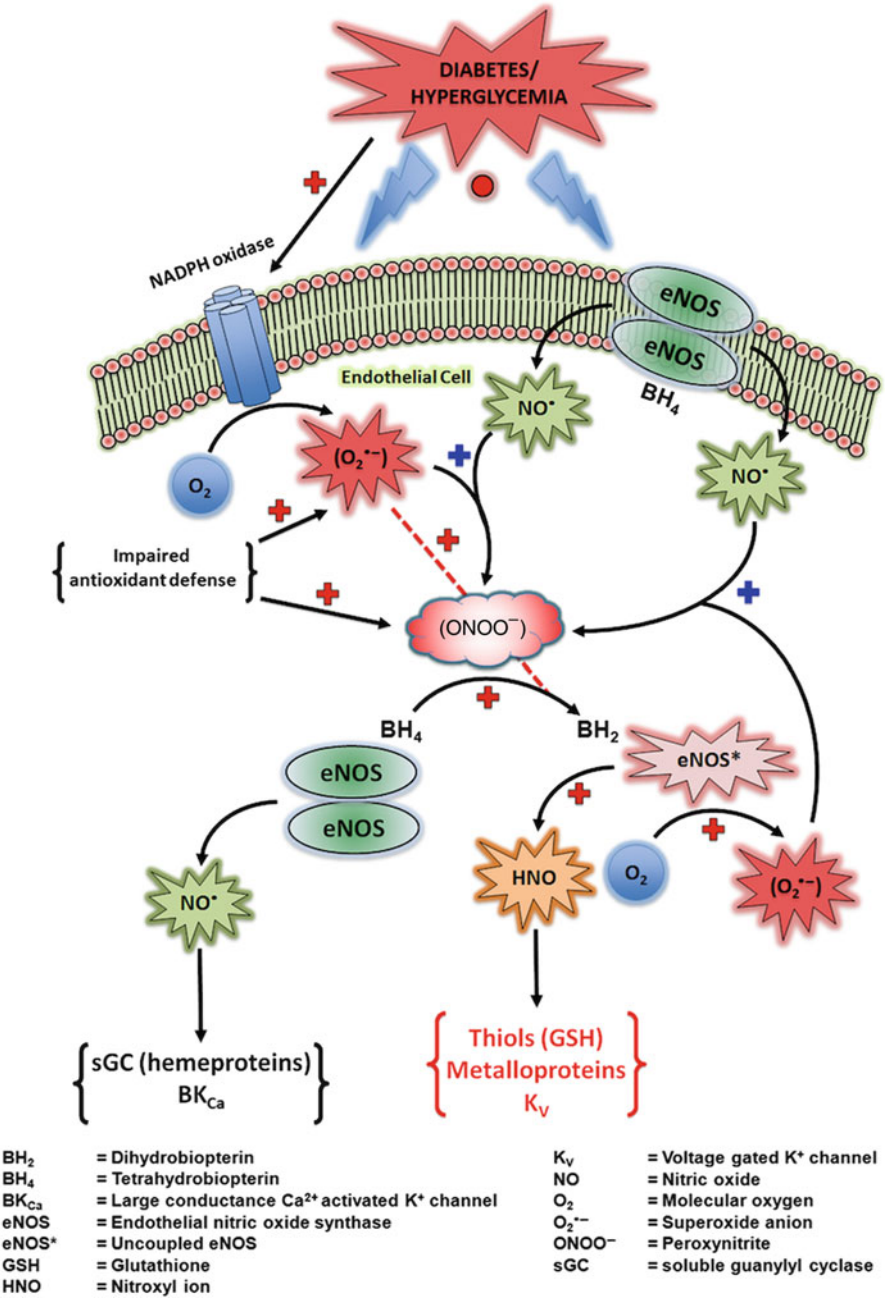

Fig. 9.3 Possible role of peroxynitrite in endothelial dysfunction in diabetes, eNOS uncoupling, and HNO production. In a diabetic milieu, the endothelial cells are constantly exposed to hyperglycemia, which is known to activate the NADPH oxidase leading to the formation of

tyrosine nitration of mitochondrial and vascular proteins, lipid peroxidation, activation of angiotensin II, and PARP1 expression in nerves, kidneys, and the retina that subsequently lead to apoptosis and necrosis in diabetes-associated vascular complications such as cardiomyopathy, neuropathy, and retinopathy (Thuraisingham et al. 2000; Ishii et al. 2001; Onozato et al. 2002; El-Remessy et al. 2003; Turko et al. 2003).

There is an accumulation of evidence indicating that both ROS and RNS contribute to endothelial and vascular dysfunction (Kvietys and Granger 2011). Thus, targeting ROS and RNS with the goal of improving endothelial function is a priority for the prevention and treatment of vascular-related diseases such as diabetes (Andrews et al. 2005; Ding and Triggle 2010; Triggle et al. 2012; Sharma et al. 2012). The infusion of a cofactor essential for eNOS function, BH_4, improves flow-mediated vasodilatation in forearm blood flow measurements in patients with type 2 diabetes (Heitzer et al. 2000). Furthermore, the direct addition of BH_4 to the organ bath in in vitro studies of human vascular tissue improves endothelium-dependent vasodilatation in blood vessels from patients undergoing coronary artery bypass grafting (Heitzer et al. 2000; Verma et al. 2000). Collectively these observations support the hypothesis that endothelial dysfunction is linked to a reduction in the bioavailability of BH_4. There is an extensive data set from studies with humans and rodents that supports this hypothesis (Pannirselvam et al. 2002, 2003; Cai et al. 2005; Kietadisorn et al. 2011; Triggle et al. 2012). BH_4 is recognized as a key regulator of eNOS and the dysregulation of BH_4 synthesis and/or bioavailability, particularly with respect to suboptimal levels of BH_4 versus oxidized products of BH_4, promotes production of $O_2^{\bullet-}$ from eNOS (and also neuronal NOS, nNOS) and leads to endothelial dysfunction (Vasquez-Vivar et al. 2002; Channon 2004; Aljofan and Ding 2010; Triggle and Ding 2010; Kietadisorn et al. 2011) (Fig. 9.3). In a diabetic milieu, the endothelial cells are exposed to hyperglycemia, which is known to activate the NADPH oxidase leading to the formation of excessive amounts of $O_2^{\bullet-}$. Since NO is locally produced in endothelial cells by eNOS, the NO and $O_2^{\bullet-}$ and rapidly combine to yield $ONOO^-$. $ONOO^-$ oxidizes BH_4, an essential cofactor for eNOS, to BH_2 (Milstien and Katusic 1999; Alp and Channon 2004; Sasaki et al. 2008). The reduction in the availability of BH_4 leads to eNOS uncoupling, shifting the function of eNOS from NO to $O_2^{\bullet-}$ production. Thus, low levels of BH_4, which is itself a weak antioxidant, or a reduced ratio of BH_4 relative to oxidized biopterins such as BH_2, is associated with eNOS functioning as an NADPH oxidase and as such molecular oxygen rather than L-arginine

Fig. 9.3 (continued) excessive amounts of superoxide anion ($O_2^{\bullet-}$). Since NO is locally produced in endothelial cells by eNOS, the NO and $O_2^{\bullet-}$ rapidly combine to yield $ONOO^-$. The $ONOO^-$ is known to cause the oxidation of tetrahydrobiopterin (BH_4, an essential cofactor for eNOS) to dihydrobiopterin (BH_2). The lack of availability of BH_4 leads to eNOS uncoupling, shifting the function of eNOS from NO to $O_2^{\bullet-}$ production. $O_2^{\bullet-}$ reacts with NO to yield $ONOO^-$. Additionally, uncoupled eNOS is known to catalyze the formation of nitroxyl ion (HNO) that may play a vasculoprotective role and enhance blood flow

becomes the electron recipient with the resultant production of superoxide, $O_2^{\bullet -}$ (Pannirselvam et al. 2002; Vasquez-Vivar et al. 2002; Cai et al. 2005).

As a result of a decrease in the BH_4/BH_2 ratio, BH_2 competes with BH_4 for binding to the oxygenase domain of eNOS, and this results in the "uncoupling" of eNOS from the dimeric and free radical $NO^{\bullet}$-generating enzyme to the $O_2^{\bullet -}$-producing monomeric enzyme (Kar and Kavdia 2011). As depicted in Fig. 9.3, it is the ratio of BH_4/BH_2 that is therefore of particular importance for determining whether it is NO or $O_2^{\bullet -}$ that is generated. The uncoupling of eNOS is most likely the key trigger for the acute reduction in flow-mediated vasodilatation that is seen postprandial, or following the ingestion of glucose, and also an important contributor to the reduction in flow-mediated vasodilatation that is seen in patients with diabetes (Kawano et al. 1999; Ihlemann et al. 2003; Crabtree et al. 2008; Aljofan and Ding 2010; Kar and Kavdia 2011).

Uncoupled eNOS catalyzes the formation of NO^- rather than $NO^{\bullet}$. Data in support of the critical importance of the BH_4/BH_2 ratio rather than the absolute level of BH_4 is provided by a study comparing the BH_4/BH_2 ratio in small mesenteric arteries from the type two diabetic db/db mouse versus nondiabetic control (Pannirselvam et al. 2002). Pannirselvam et al. (2002) reported that the BH_4/BH_2 ratio, but not the absolute level of BH_4, was significantly reduced and the small mesenteric arteries also exhibited profound endothelial dysfunction that, most likely, was due to a reduction in the bioavailability of NO (Pannirselvam et al. 2002, 2003). An uncoupled eNOS, wherein eNOS is in the monomeric rather than the dimeric form, is promoted by S-glutathionylation as well as thiyl radical formation and the oxidation of key cysteine residue(s) in the enzyme (Chen et al. 2010, 2011). BH_4 is highly sensitive to oxidation by $ONOO^-$ (Kohnen et al. 2001). Hyperglycemia and, paradoxically, hypoglycemia are associated with endothelial dysfunction, and, notably, hyperglycemia-induced endothelial dysfunction can be linked to oxidative stress, a downregulation of antioxidant enzymes, and an uncoupling of eNOS (Ding et al. 2007; Aljofan and Ding 2010; Giacco and Brownlee 2010; Wang et al. 2011; Triggle et al. 2012). It has been shown that increased levels of RNS, in particular $ONOO^-$, can suppress eNOS activity in streptozotocin-induced diabetic rats via the small GTPase, RhoA, in a RhoA/Rho-associated protein kinase (RhoA/ROCK)-dependent manner (El-Remessy et al. 2010; Sharma et al. 2012). Furthermore, treatment with the $ONOO^-$ decomposition catalyst FeTTPs improves vasodilatation in response to the endothelium-dependent vasodilator acetylcholine, lowers oxidative-stress and RhoA activity, upregulates eNOS expression, and enhances endothelial levels of NO (El-Remessy et al. 2010; Sharma et al. 2012).

In pathophysiological states, such as hypertension or type 1 and 2 diabetes, where blood glucose levels are raised, the production of ROS is also increased, and the expression and/or activity of enzymes such as NADPH oxidase are also elevated. Thus, such pathophysiological states promote the formation of $ONOO^-$ and enhance both oxidative and nitrosative stress (Forstermann and Munzel 2006; Ding et al. 2007; Aljofan and Ding 2010).

$ONOO^-$ plays an important role in the development of endothelial dysfunction and vascular disease; however, when BH_4 levels are reduced, NOS enzymes may also

generate, in addition to $O_2^{\bullet-}$, the nitroxyl form of NO, HNO/NO^-. In *Escherichia coli*, in the absence of BH_4, nNOS is overexpressed and generates NO^- from L-arginine, and these data suggest that the most important role for BH_4 (Pacher et al. 2007) is to enable nNOS to generate $NO^\bullet$ instead of NO^- (Rusche et al. 1998; Adak et al. 2000). NO^-, which preferably should be called nitrosyl hydride or hydrogen oxonitrate, has a unique and distinctive chemistry and biological profile when compared to $NO^\bullet$ (Fukuto et al. 2005; Flores-Santana et al. 2011; Kemp-Harper 2011).

A number of studies have demonstrated that NO^- contributes to the responses mediated by NO in the vasculature thus implying that both $NO^\bullet$ and NO^- must be produced physiologically. Furthermore, the cellular signaling pathways activated by these two chemical species of nitric oxide, notably the nature of the potassium channel subtype activated by $NO^\bullet$ versus NO^-, are distinct, perhaps suggesting differing, but overlapping, physiological functions and/or cellular compartmentalization (Ellis et al. 2000; Wanstall et al. 2001; Irvine et al. 2003; Andrews et al. 2005; Favaloro and Kemp-Harper 2009; Paolocci and Wink 2009; Kemp-Harper 2011; Yuill et al. 2011) – see Fig. 9.3.

Support for a different profile of physiological actions for NO^- versus $NO^\bullet$ is provided by studies that show that NO^- favors reaction with ferric heme while $NO^\bullet$ favors ferrous heme (Miranda et al. 2003a; Fukuto et al. 2005). For instance, the heme-containing soluble guanylyl cyclase would preferentially be activated by $NO^\bullet$, whereas peroxidases require the ferric state and would be preferentially regulated by NO^-. The unique chemistry of NO^- and its greater preference for thiols and metalloproteins indicate differing cellular roles for these two forms of NO (Liochev and Fridovich 2003; Miranda et al. 2003a, b; Donzelli et al. 2006). Predictably NO^- would have greater biological activity in cellular locations, such as membranes, or pathophysiological states when glutathione levels are low (Miranda et al. 2003b). Furthermore, it has been suggested that NO^- possesses vascular protective functions and NO^- is less susceptible to scavenging by superoxide and less likely to lead to tolerance and endothelial dysfunction (Bullen et al. 2011). In contrast to $NO^\bullet$, NO^- also has a positive inotropic action and protects against ischemia/reperfusion injury when applied before ischemia as well as exhibiting vascular antiproliferative actions (Paolocci et al. 2001; Irvine et al. 2003; Bullen et al. 2011; Kemp-Harper 2011; Tocchetti et al. 2011).

The interaction of NO^- with thiol proteins in the myocardium may provide the basis for NO^--mediated enhanced Ca^{2+} cycling and hence the positive inotropic action of NO^- (Fukuto and Carrington 2011). The unique chemistry of NO^- and differing physiological profile indicate that rather than reflecting a solely pathophysiological role for a BH_4-compromised milieu, the generation of NO^- is an important facet of the biology of NO that is regulated by $ONOO^-$. $ONOO^-$-mediated regulation of NOS and the generation of NO^- versus $NO^\bullet$ may therefore have profound effects on cell signaling and, under some circumstances, provide a protective influence on cardiovascular function. In addition, the cytotoxic actions of NO^- may also exert antiproliferative effects and reduce tumor progression (Norris et al. 2008).

Collectively, these data indicate that, like many signaling molecules, $ONOO^-$, through its regulation of the production of NO^- versus $NO^\bullet$, is also Janus faced.

$ONOO^-$ and Inflammation

Several lines of evidence indicate that overproduction of $ONOO^-$ critically contributes to pathogenesis of inflammatory disease such as chronic arthritis and inflammatory bowel disease (see review by Pacher et al. 2007). High levels of $ONOO^-$ are produced at sites of inflammation and have been implicated in promoting the sustained and/or prolonged development of various disease pathologies. The ability of $ONOO^-$ to trigger and enhance NFκB activity, as well as upregulate proinflammatory mediators, plays a critical role in inflammation-associated cell and tissue damage (Matata and Galinanes 2002; Bar-Shai and Reznick 2006). $ONOO^-$-mediated enhanced NFκB activity results in a greater expression of proinflammatory cytokines, chemokines, enzymes such as iNOS and cyclooxygenase 2 (COX2), and cell adhesion molecules (Cooke and Davidge 2002). Additionally, data from animal models of inflammatory disease indicate that $ONOO^-$-mediated PARP activation is a contributory factor to inflammatory diseases such as allergic encephalomyelitis, meningitis, and multiple sclerosis; ocular inflammation uveitis; asthma; and periodontal inflammation gingivitis (Landino et al. 1996; Zouki et al. 2001 and reviewed in Pacher et al. 2007; Szabo et al. 2007).

$ONOO^-$ and Cancer

$ONOO^-$ may contribute to carcinogenesis and tumor progression by weakening key cellular defense enzymes; increasing the production and infiltration of proinflammatory cytokines, matrix-degrading enzymes, and growth factors; inhibiting DNA repair enzymes; and enhancing cell proliferation, survival, migration, and angiogenesis. The ability of $ONOO^-$ to trigger DNA mutation by oxidative modification to, in particular, the nucleobase guanine enhances the potential for single-strand DNA breaks, mutagenesis, and carcinogenesis. Guanine is oxidized by $ONOO^-$ to cyanuric acid and oxazolone resulting in guanine fragmentation (Burney et al. 1999; Niles et al. 2006). In addition, studies have also provided evidence of a tumor promoting role of $ONOO^-$ resulting from its ability to nitrate and phosphorylate the NFκB inhibitory protein IκB, leading to the activation of NFκB-mediated proinflammatory signaling cascades and, therefore, promoting chronic inflammation and tumor development (Mabley et al. 2004; Gochman et al. 2011).

$ONOO^-$ and Neurological Disorders

Neurodegenerative disorders, such as amyotrophic lateral sclerosis, ALS, have also been associated with elevated generation/bioavailability of NO and accelerated formation of $ONOO^-$ (Verge et al. 1992; Solodkin et al. 1992; Beckman et al. 1993). Furthermore, $ONOO^-$-induced protein nitration, neuron degeneration, and DNA damage have all been implicated in neuronal cell death and necrosis

(Torreilles et al. 1999; Souza et al. 2000; Ischiropoulos and Beckman 2003; Reynolds et al. 2005; Pacher et al. 2007; Singh et al. 2007). $ONOO^-$-mediated protein nitration/oxidation and lipid peroxidation of neuronal synaptic and structural proteins such as α-synuclein, tubulin, and myelin have all been highlighted as critical steps in the pathogenesis of neurodegenerative diseases such as multiple sclerosis and Parkinson's and Alzheimer's diseases, and therefore $ONOO^-$ is a potential therapeutic target (see review by Tao et al. 2012).

Scavenging $ONOO^-$ and its Derivatives

The rate of disappearance of $ONOO^-$ follows first-order kinetics with a half-life of ~1 s at 37 °C and pH 7.4 although, as noted, the cis-conformation of $ONOO^-$ is quite stable (Koppenol et al. 1992; Tsai et al. 1994; Pryor and Squadrito 1995; Goldstein et al. 1996). Thus, the fate of the $ONOO^-$ radical will either be decomposition if no proximal oxidizable targets are available, or $ONOO^-$ will rapidly oxidize available proximal targets in most cases yielding secondary reactive species (Crow 2002). However, in a cellular environment, with an abundant supply of molecules that directly react with $ONOO^-$, then $ONOO^-$ will be consumed through direct reactions with these biomolecules.

In light of the multifaceted reaction capabilities of $ONOO^-$ that can cause adverse effects in biological systems, there is obvious therapeutic potential for the identification of potential $ONOO^-$ scavengers as protective agents. An ideal scavenger of $ONOO^-$ should (1) rapidly react with $ONOO^-$, (2) "contain" reactive intermediates, (3) be stable under in vivo conditions, (4) be able to traverse the plasma membrane and evenly distribute in the tissues, and (5) be nontoxic itself as well as when irreversibly modified upon its reaction with $ONOO^-$ (Crow 2002).

Natural and dietary products that contain antioxidants such as carotenoids (Panasenko et al. 2000), polyphenolic compounds (Arteel and Sies 1999), and epicatechin (Schroeder et al. 2003) have been shown to be protective against $ONOO^-$-induced toxicity (Arteel et al. 1999). However, the mechanisms by which these antioxidants render their protective effect are still unclear since they tend to react rather slowly with $ONOO^-$ and may also react with other reactive species including $ONOO^-$-derived radicals.

Various molecules have been identified that can react directly with $ONOO^-$ or with the $ONOO^-$-derived reactive radicals. In the biological system, peroxiredoxins are efficient scavengers of $ONOO^-$ (Bryk et al. 2000). The reactive thiol active site of peroxiredoxins is oxidized during the catalytic reduction of $ONOO^-$ to nitrite. The oxidized peroxiredoxins is then reduced back to its resting state by thioredoxin (Maulik and Das 2008). Owing to the capability of $ONOO^-$ to cause rapid cysteine oxidation, $ONOO^-$ also oxidizes the thiol group in glutathione, making glutathione an efficient scavenger of the $ONOO^-$ radical (Arteel et al. 1999; Bajt et al. 2003). Thiol-based antioxidants such as N-acetyl cysteine (Cuzzocrea et al. 2000) and dihydrolipoic acid have been shown to reduce $ONOO^-$-mediated toxicity (Szabo 2003).

Heme proteins (hemoglobin and methemoglobin) are also known to be efficient scavengers of $ONOO^-$ in biological systems, thereby protecting cells from the detrimental effects of $ONOO^-$ (Herold et al. 2002; Herold and Fago 2005; Romero and Radi 2005; Ascenzi et al. 2008, 2010; Ascenzi and Visca 2008). $ONOO^-$ isomerizes to form nitrate (NO_3^-) upon its reaction with ferrous deoxygenated heme proteins (Ascenzi et al. 2010).

Interest has grown in certain synthetic molecules (such as iron and manganese metalloporphyrins) that react directly with and are potent catalysts of $ONOO^-$ decomposition with potential as therapeutic agents (Salvemini et al. 1998; see also review by Szabo et al. 2007). These are commonly called $ONOO^-$ decomposition catalysts. Iron and manganese porphyrins are advantageous of being much effective at much lower concentrations and ability to be recycled.

$ONOO^-$ rapidly causes tyrosine nitration of proteins, and therefore tyrosine-containing peptides may also act as effective scavengers of $ONOO^-$ (Ye et al. 2007). Additionally, various studies have reported uric acid to be an efficient scavenger of $ONOO^-$ and its derived radicals such as $CO_3^{\bullet -}$ and $^{\bullet}NO_2$ (Kooy et al. 1994; Hooper et al. 1998; Squadrito et al. 2000). Seleno-compounds (Klotz and Sies 2003) and selenium-containing proteins such as glutathione peroxidase (Sies and Masumoto 1997; Sies et al. 1997; Arteel et al. 1998) have been reported to exhibit $ONOO^-$ scavenging properties. SOD mimetics have also exhibited $ONOO^-$ scavenging and protective effects (Batinic-Haberle et al. 2009).

Tempol, a well-studied compound used to protect cells and animals from oxidative stress, has also been shown to be an effective protective agent against $ONOO^-$-induced cytotoxicity (Fernandes et al. 2005a). Tempol is oxidized by $ONOO^-$-derived $CO_3^{\bullet -}$ yielding an oxoammonium cation, which then is reduced back to tempol by reacting with $ONOO^-$ and yielding oxygen and NO, thereby diverting the $ONOO^-/CO_2$ reactivity from protein tyrosine nitration to cysteine nitrosation (Fernandes et al. 2005a).

Finally, several commonly used drugs such as acetaminophen, propofol, simvastatin, pindolol, as well as herbal extracts have been demonstrated to possess $ONOO^-$ scavenging actions (Acquaviva et al. 2004; Choi et al. 2002; Fernandes et al. 2005b; Rork et al. 2006; Selley 2005; see also review by Szabo et al. 2007).

In general, the catalytic scavenger would be preferred over "sacrificial" scavengers (such as glutathione), which cannot be regenerated. In the future, as more knowledge is gained on the formation of $ONOO^-$ and its intermediates, more specific scavengers may be designed to combat the various pathological conditions where $ONOO^-$ and its derivatives play a significant role.

Conclusion and Future Prospects

The current chapter has focused on how $ONOO^-$ is formed from NO and $O_2^{\bullet -}$ and briefly summarized both the physiological and the pathological targets and effects of $ONOO^-$. However, as for any other ROS, it is difficult to interpret what level of

$ONOO^-$ formation is beneficial and where and what threshold levels need to be exceeded to initiate detrimental cellular actions. In order to develop a better therapeutic strategy to combat the adverse effects of excessive $ONOO^-$ levels in biological systems, it is necessary to also better understand the cellular processes that regulate the formation and degradation of $ONOO^-$. Clearly, however, studies on the protective effects of scavengers of $ONOO^-$ are warranted in disease models (diabetes, cardiovascular disease, cancer, etc.) to investigate questions related to dosage, formulation, and bioavailability.

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