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Review

Pro-oxidative effect of peroxynitrite regarding biological systems: a special focus on high-molar-mass hyaluronan degradation

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Dedicated to Professor Radomír Nosáľ, MD, DSc on the occasion of his 70th birth anniversary

Abstract. Current understanding on the role of peroxynitrite in etiology and pathogenesis of some human diseases, such as cardio-vascular diseases, stroke, cancer, inflammation, neurodegenerative disorders, diabetes mellitus and diabetic complications has recently led to intensive investigation of peroxynitrite involvement in physiology and pathophysiology.

Mechanism of cytotoxic effects of peroxynitrite involve its reactions with lipids, DNA/RNA, proteins, and polysaccharides, thus triggering cellular responses ranging from subtle changes of cell functioning to severe oxidative damage of the affected macromolecules leading to necrosis or apoptosis. The present work is aimed at providing a brief overview of i) peroxynitrite biosynthesis and reaction pathways *in vivo*, ii) its synthetic preparation *in vitro*, and iii) to reveal its potential damaging role *in vivo*, on actions studied *via* monitoring *in vitro* hyaluronan degradation. The complex biochemical behavior of peroxynitrite is determined by a number of variables, such as chemistry of the reaction itself, depending mostly on the involvement of conformational structures of different energy states, concentration of the species involved, content of reactive intermediates and trace transition metal ions, contribution of carbon dioxide, presence of trace organics, and by the reaction kinetics. Recently, *in vitro* studies of oxidative cleavage of hyaluronan have, in fact, been the subject of growing interest. Here we also describe our experimental set-up for studying peroxynitrite-mediated degradation of hyaluronan, a system, which may be suitable for testing prospective pharmacological substances.

Key words: Antioxidants — Hyaluronan degradation — 3-*N*-Morpholinosydnonimine — Peroxynitrite — Reactive oxygen and nitrogen species

Abbreviations: ASC, ascorbate; $CO_3^{\bullet-}$, trioxocarbonate anion radical; SOD, superoxide dismutase; ECM, extracellular matrix; ecNOS, endothelial-constitutive form of NOS; EDRF, endothelium-derived relaxing factor; EPR, electron paramagnetic resonance; GAGs, glycosaminoglycans; GSH, l-glutathione; GS$^{\bullet}$, glutathionyl radical; GSNO, *S*-nitrosoglutathione; $^{\bullet}NO$, nitric oxide radical; $^{\bullet}NO_2$, nitrogen dioxide radical; NOS, nitric oxide synthase; $^1O_2$, singlet oxygen; $^{\bullet}OH$, hydroxyl radical; $ONOO^-$, peroxynitrite anion; ONOOH, peroxynitrous acid; $O_2^{\bullet-}$, superoxide anion radical; RONS, reactive oxygen and nitrogen species; SIN-1,3-*N*-morpholinosydnonimine.

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Introduction

Under conditions of increased production of superoxide anion radical ($O_2^{\bullet -}$) and nitric oxide radical ($^{\bullet}NO$), these two radicals may react together yielding a more potent prooxidant, peroxynitrite anion ($ONOO^-$). These reactive species differ in their half lives and thus their distances they can diffuse from their original sites are different. Half life of $O_2^{\bullet -}$ anion radical determines its potential distance of diffusion to 0.4 μm thus limiting its action to an intracrine one, i.e. within the cells of their origin or in a very close vicinity of them. On the other hand, half life of $^{\bullet}NO$ radical enables it to diffuse through the tissue up to 100 μm thus allowing it to exert a paracrine action on neighboring cells. And, the half life of $ONOO^-$ determines its diffusion distance to ~5 μm, so it can act rather locally (Pacher et al. 2007).

Peroxynitrite is capable of nitrating proteins at their tyrosine residues thus producing nitrotyrosine (Radi 2004). The nitration and covalent modification of proteins and DNA by $ONOO^-$ is termed nitrative stress, which has been first observed as accumulation of nitrotyrosine residues in atherosclerotic plaques (Beckman et al. 1994a), and also in the placenta (Myatt 2010). Later, it has been found in many other pathologies, particularly those associated with inflammation (Myatt et al. 1996; Lyall et al. 1998).

Indeed, peroxynitrite, one of the most important endogenous oxidizing and nitrating species, is a mediator of cellular and tissue injury in various pathophysiological situations. Biochemical pathways of this metabolite, producing free radicals, are responsible for the oxidative and nitrosative stress in biological systems (Pacher et al. 2003, 2005; Pérez-De La Cruz et al. 2005).

Peroxynitrite formation *in vivo* and its behavior in the organism have been reported (for review, see Murphy et al. 1998; Pacher et al. 2007). $ONOO^-$ interactions with lipids, DNA, and proteins *via* direct oxidative reactions or indirect, radical-mediated mechanisms have been implicated in various pathological events, such as stroke, myocardial infarction, diabetes, chronic heart failure, circulatory shock, chronic inflammatory diseases, and cancer (Pacher et al. 2007).

Peroxynitrite biosynthesis, synthesis, its oxidation and nitration reactions as well as reactions with various antioxidants have been summarized (for review, see Ducrocq et al. 1999; Hrabárová et al. 2007). Other papers have emphasized that $ONOO^-$ is actually one of the most reactive among the other reactive oxygen and nitrogen species having a potential to induce tissue damage during systemic inflammatory response and circulatory shock (Evgenov and Liaudet 2005). Also, biological significance of peroxynitrite, its precursors and reactions have been reported, including involvement of its cytotoxic action in various pathologies. The biomarker of the $ONOO^-$ reaction with protein tyrosines – 3-nitrotyrosine formation has been identified e.g. in Lou Gehrig's and Parkinson's diseases, cancer, atherosclerosis, and in aging process (Kamat 2006).

The role which peroxynitrite plays in neurodegenerative inflammatory disorders including Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, and the Parkinsonism dementia complex of Guam, has been reflected (Torreilles et al. 1999). Peroxynitrite has also been indicated to play an important role in regulating brain mitochondria function (Brookes et al. 1998). In fact, extra- or intra-mitochondrial $ONOO^-$ generation, its diffusion through mitochondrial compartments causing alteration in mitochondrial energy and calcium homeostasis, its fast, direct and free radical-dependent reactions with target biomolecule resulting in oxidation, nitration, and nitrosation of inner and outer membrane and intermembrane space, its fate, as well as signaling of cell death have been demonstrated (Radi et al. 2002).

Overview on the preferential protein targets of peroxynitrite and the role of proteins in its detoxifying pathways has been offered (Alvarez and Radi 2003). Possible mechanisms of $ONOO^-$ reaction pathways conferring to the proteins (hemoglobin, myoglobin, cytochrome *c*) and, especially, their scavenging role have been discussed (Pietraforte et al. 2003). Peroxynitrite as a potent cytotoxin plays a key role in the pathogenesis of cardiovascular dysfunction leading to progression of diabetic retinopathy, neuropathy, and nephropathy by attacking various biomolecules in vascular endothelium, vascular smooth muscle, and myocardium (Pacher and Szabó 2006).

Free radicals in biological systems

Though traditionally understood as deleterious prooxidants in biological environment, free radicals, due to their various redox actions, may play a key role in preventing biological systems from a variety of stress factors. They are formed and subsequently used to maintain homeostasis under physiological conditions. *In vivo* redox pathways mediated by reactive oxygen-, nitrogen- and thiol- species play pivotal roles in many physiological and pathological events, regarding intra- and inter-cellular signaling. Several free radicals, e.g. thiyl radical and nitrogen dioxide radical ($^{\bullet}NO_2$) are known to promote *cis–trans* isomerization of double bonds of poly-unsaturated fatty acids. A trioxocarbonate anion radical ($CO_3^{\bullet -}$) is a potential oxidant of nucleic acids, e.g. guanine bases of DNA (Luc and Vergely 2008).

These highly reactive species are either oxygen-derived such as $O_2^{\bullet -}$, hydroxyl ($^{\bullet}OH$), $HOO^{\bullet}$, and $CO_3^{\bullet -}$ radicals or nitrogen-derived such as $^{\bullet}NO$ and $^{\bullet}NO_2$ radicals. The non-radical reactive metabolites are potential radical precursors, such as H_2O_2, H_2S, HOCl, singlet oxygen ($^1O_2$), O_3, and $ONOO^-$ (Ďuračková and Knasmüller 2007).

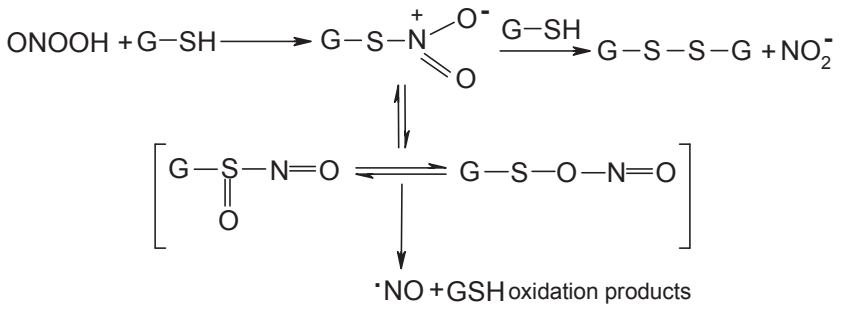

Figure 1. Proposed mechanism of nitric oxide radical formation *via* reaction of peroxynitrous acid and L-glutathione (Balazy et al. 1998).

Reactive oxygen and nitrogen species (RONS), the $ONOO^-$ precursors, have their origin either in endogenous sources (mitochondria, peroxisomes, NADPH oxidase, cytochrome P450, and xantine oxidoreductase system) or in exogenous sources (ultraviolet light, ionizing radiation, photochemical smog, xenobiotics, and environmental toxins) (Mangialasche et al. 2009).

Anti-oxidative systems

Thiol-based species, such as L-glutathione (GSH) and thioredoxin(s), functioning together with ascorbate (ASC) and α-tocopherol (vitamin E), play a crucial role in *in vivo* preventing biological systems from oxidative and nitrosative damage (Fig. 1 and 2). Biological antioxidants can be classified according to their molecular size as either high-molar-mass: superoxide dismutase (SOD), catalase, GSH peroxidase, transferrin, albumin, polyphenols or low-molar-mass compounds: ASC, GSH, uric acid, vitamin E, coenzyme Q (Ďuračková and Knasmüller 2007).

A natural flavonoid, quercetin, has been found as an efficient $ONOO^-$ scavenger mediating the reduction of 3-nitrotyrosine formation. Under biological conditions, $ONOO^-$ can be converted to nitrosothiols *via* the detoxification reaction *in vivo* thus regenerating $^{\bullet}NO$ (Hayashi et al. 2004). *In vivo*, $ONOO^-$ is mostly trapped by substrates, such as thiols or metaloproteins through a bimolecular pathway, and, only its minor fraction is quickly converted into "radical-like" reactive species (Balavoine and Geletii 1999). Biomolecular pathways of $ONOO^-$-mediated cell death and the role of $^{\bullet}NO$ and $ONOO^-$ in cardiovascular pathophysiology have been outlined (Pacher et al. 2007).

Hemoglobin and myoglobin are involved in reversible O_2 binding and transport in the blood or O_2 storage in the muscle, respectively. They undergo several redox reactions to either scavenge toxic free radicals and RONS at oxidative stress, or in the case of brain-expressed neuroglobin, against pathogenic pathways connected with tissue hypoxia and ischemia, thus protecting neurons. The major contribution to the cellular damage is herein ascribed to the $ONOO^-$ formation (Herold and Fago 2005; Shivashankar 2006).

Among others, thiourea was reported to be one of the most effective $ONOO^-$ scavengers (Table 1) (Li et al. 1997). Inhibitory effects of various antioxidants (thiourea, mannitol, vinpocetine, propofol) on the hyaluronan depolymerization induced by $^{\bullet}OH$ radicals, generated either by the system H_2O_2 *plus* Cu(II) or $ONOO^-$, were demonstrated (Mendoza et al. 2007).

When extracellular $^{\bullet}NO$ radical scavenging by quercetin was prevented by the action of SOD $^{\bullet}NO$ bioactivity was increased (López-López et al. 2004). The $^{\bullet}NO$ radical scavenging and suppressing effects by catechins, the main constituents of green tea, were evaluated (Tsai et al. 2007).

Nitric oxide radical, a peroxynitrite precursor

Nitric oxide radical is a biologically ubiquitous lipophilic species. It was classified as a signaling, reactive free radical species, acting as a specific "messenger" in numerous biological processes (Symons 2000). Since it has been found out that

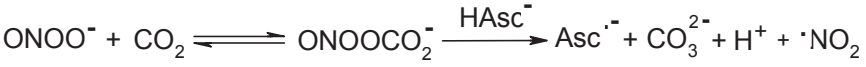

Figure 2. Formation of ascorbyl anion radical and nitric dioxide radical *via* reaction of peroxynitrite anion and carbon dioxide in the presence of ascorbate anion (Kurz 2004).

Table 1. Pathophysiological effects of peroxynitrite on the hierarchy of biomolecular organization in the cell

Substrate	Product	$ONOO^-$-mediated cytotoxic effects	Potential disease development	Commentary	Application of antioxidants	References
Low-molar-mass biomolecules (aminoacids, vitamins, mononucleotides, monosaccharides, peptides...)						
α-Tocopherol in rat brain mitochondria and synaptosomes	Tocopherolquinone (Mitochondrial α-tocopherol more susceptible to oxidation than synaptosomal one)	*In vitro* rapid and selective $ONOO^-$-induced oxidation	Neurodegenerative disorders due to membrane peroxidation	$ONOO^-$ reactivity testing with natural scavengers in biological environment	α-Tocopherol, Vitamin C, Thiols	(Vatassery et al. 1998)
Tyrosine	3-Nitrotyrosine	Direct $ONOO^-$ nitrative pathway ($^{\bullet}OH + {}^{\bullet}NO_2$)	Post-bypass systemic inflammatory response induced by cardiopulmonary bypass	Elevated levels of 3-nitrotyrosine *in vivo*	Quercetin (flavonoid)	(Ischiropoulos et al. 1992; Beckman et al. 1994b; Hayashi et al. 2004)
High-molar-mass biomacromolecules (carbohydrates, lipids, proteins, hormones, nucleic acids...)						
High-molar-mass HA	Low-molar-mass HA fragments: alkyl-, alkoxy- and peroxy-radicals	*In vitro* HA rapid degradation *via* nucleophilic $ONOO^-$-mediated attack on a HA-glycosidic bond	Rheumatoid arthritis	*In vivo* elevated production of $ONOO^-$ under inflammatory conditions; Reduced limiting viscosity number of HA	Thiourea; DMSO; Mannitol; Sodium benzoate	(Li et al. 1997; Corsaro et al. 2004)
Viable rat thymocytes DNA	Deamination of DNA bases; DNA nicks formation due to H-atom abstraction from ribose	Opening the sugar ring leading to DNA strand breakage; Oxidation of cellular thiols	Irreversible oxidative damage leading to the inactivation of mitochondrial electron carriers	Trolox exhibited DNA protection by $^{*}ONOOH$ scavenging	DMSO; Mannitol; Sodium benzoate; Trolox	(Salgo et al. 1995)
Supramolecular complexes (ribosomes, enzymes...)						
Brain neocortical synaptosomal membrane proteins, oxidation-sensitive GS	Inactivation of GS, protein conformational changes, protein carbonyls formation	Neuronal cell death; Oxidation of membranous and cytosolic proteins	Alzheimer's disease	Potential involvement of $ONOO^-$ in neurodegenerative processes	Thiol-containing tripeptide glutathione	(Koppal et al. 1999)
Ribosomes; Various biomolecules	Biomarkers of lipid peroxidation, protein oxidation/nitrosylation, and DNA/rRNA strand breaks	Oxidative and nitrosative stress due to the dysregulation of redox balance; Proteosynthesis decrease	Alzheimer's disease; Mild cognitive impairment	ONS biomarkers study to clarify pathogenesis, diagnosis and prognosis of Alzheimer's disease	Denitrase enzyme; SOD; Glutathion reductase; Thioredoxin/thioredoxin reductase; Heme oxygenase; Methionine sulphoxide reductase	(Mangialasche et al. 2009)
Subcellular organelles (nucleus, membrane, mitochondria, rough/smooth endoplasmatic reticulum...)						
Rat brain mitochondria	Lipid peroxides	Irreversible inhibition of mitochondrial respiration	Parkinson's and Alzheimer's disease; Multiple and amyotrophic lateral sclerosis; Ageing	Cytotoxic effect of $ONOO^-$ leading to an increased membrane bilayer proton permeability, and decreased mitochondrial membrane potential	Glutathione; Lipid-soluble antioxidants (α-Tocopherol)	(Brookes et al. 1998)

AF, ammonium formiate; AGE, agarose gel electrophoresis; $^{\bullet}C(O)R$, acyl radical of hyaluronan; CV, capillary viscometry; DMSO, dimethylsulfoxide; DTPA, diethylenetriaminepentaacetate; ESI-MS, electrospray ionisation mass spectrometry; HA, hyaluronan; ^1H NMR, proton nuclear magnetic resonance; M_w, weight-average of molar masses; PAGE, polyacrylamide gel electrophoresis; PB, phosphate buffer; P, T, M, polymer, tetrasaccharide, monomer of hyaluronan (monomeric units: GlcNAc and GlcU); $RO^{\bullet}$, alkoxyl radical; $ROO^{\bullet}$, peroxyl radical; SB/ML, sodium benzoate/mannitol; SEC, size-exclusion chromatography; SF, synovial fluid; TMIs, transition metal ions; TRAP, total peroxyl radical-trapping antioxidant parameter.

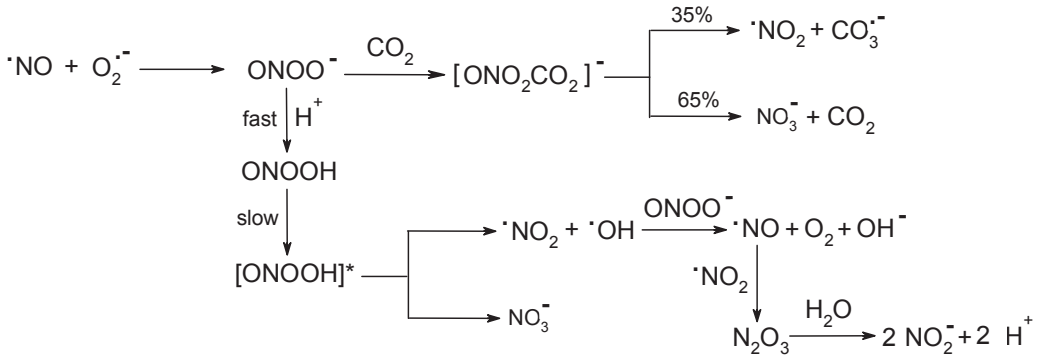

Figure 3. Hypothetical mechanism of various biomolecular pathways of peroxynitrite anion (Pfeiffer et al. 1997).

•NO serves as one of the key modulators of vascular tone in a role of endothelium-derived relaxing factor (EDRF), many studies were performed covering often unlike functions of •NO. Activity of nitric oxide synthases (NOSs), a family of enzymes catalyzing production of •NO, has been found in most cell types (Pacher et al. 2007). In addition to the direct interactions of •NO with signal transduction pathways of the cell, the specific reactions of •NO-derived products, such as $ONOO^-$, with intra- and extracellular components may be responsible for damaging effects of •NO. Direct and indirect actions of •NO and its derivatives are quite diverse and depend primarily on concentration and cell type. Diffusion conditions of cellular oxidants ($O_2^{\bullet-}$, •NO, $ONOO^-$, and •OH) have been demonstrated in detail (Pacher et al. 2007).

The most significant *in vivo* reaction of •NO is its combination with $O_2^{\bullet-}$ (Fig. 3), released from several cellular sources, to form $ONOO^-$. This species is relatively stable, however, its protonized form, peroxynitrous acid (ONOOH) undergoes rapid decay forming harmful •OH and $^{\bullet}NO_2$ radicals.

•NO radical is synthesized *in vivo* by a variety of cell types, including macrophages, vascular endothelial cells, neutrophils, hepatocytes, phagocytes, and neurons. It is a major participant in several physiological functions such as blood pressure regulation (Hogg et al. 1992; Czapski and Goldstein 1995). Neutrophils and macrophages generate •NO *via* an L-arginine-dependent pathway (Fig. 4) (Jourd´heuil et al. 1997). Large quantities of •NO are produced by the endothelial-constitutive form of NOS (ecNOS) and inducible form of NOS in neutrophils and monocytes upon inflammatory stimulation (Dawson and Dawson 1995; Conner and Grisham 1996; Hassan et al. 1998).

Evidence for the reaction of $ONOO^-$ with GSH giving an important biologically active metabolite, *S*-nitroglutathione, promoting a time-dependent production and oxidation of •NO (Fig. 1) has been reported (Balazy et al. 1998). *S*-nitrosothiols are obtained *via S*-nitrosation of primary and secondary or tertiary thiols (Feelisch 1998). Hofstetter et al. (2007) claim that *S*-nitrosoglutathione (GSNO), generated *in vivo*, undergoes homolysis forming •NO and glutathionyl radical (GS•). However, no GSNO is produced reacting •NO

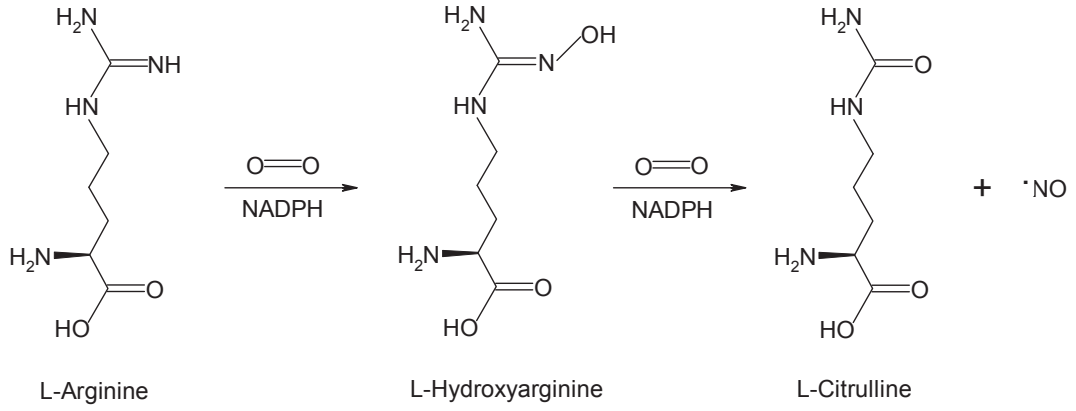

Figure 4. Nitric oxide radical production from amino acid L-arginine (Schönafinger 1999).

with GS$^{\bullet}$ for a very rapid intramolecular radical rearrangement of GS$^{\bullet}$ to $^{\bullet}$GS, and, since the reaction of GS$^{\bullet}$ with $^{\bullet}$NO is very slow.

Superoxide anion radical

Superoxide anion radical is formed in neutrophils, monocytes, macrophages, and eosinophils due to the action of NADPH oxidase during a so-called "respiratory burst". NADPH oxidase, a highly regulated enzyme complex composed of a number of proteins, reduces oxygen to $O_2^{\bullet-}$ according to the reaction (1):

$$2O_2 + NADPH \rightarrow 2O_2^{\bullet-} + NADP^+ + H^+ \quad (1)$$

Another $O_2^{\bullet-}$ source is xanthine oxido-reductase, also called xanthinoxidase. This molybdenum and iron-containing flavoprotein catalyzes oxidation of hypoxanthine to xanthine and then to uric acid. Molecular oxygen is the substrate and the products include $O_2^{\bullet-}$. Other cellular sources for $O_2^{\bullet-}$ are mitochondria in stressed cells, the formation of met-hemoglobin, and the reduction of oxygen by quinone radicals or by GSSG$^{\bullet-}$ (Stern et al. 2007).

In situ generated $O_2^{\bullet-}$ undergoes a rapid spontaneous dismutation reaction (2) forming hydrogen peroxide, catalyzed by the SOD anti-oxidative enzyme family (Zhao et al. 2007):

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

$O_2^{\bullet-}$ anion radical is a one-electron oxido-reductant. It does not have direct toxic effects on living targets; however, upon penetrating to important physiological sites, it is converted to hydrogen peroxide, $^1O_2$, and possibly to $^{\bullet}$OH radicals. Although $O_2^{\bullet-}$ is unable to degrade hyaluronan on its own, it can participate in reactions with transition metal ions (3) to produce highly reactive and damaging species – $^{\bullet}$OH radicals (4). $O_2^{\bullet-}$ anion radicals play also a decisive role by converting $^{\bullet}$NO to the powerful oxidant $ONOO^-$ (Stern et al. 2007).

The iron-catalyzed Haber-Weiss reaction (5), which makes use of the Fenton chemistry (4), has been considered to be the major mechanism by which the highly reactive $^{\bullet}$OH radicals are generated in biological systems (Kehrer 2000).

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2 \quad (3)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^-OH + {}^{\bullet}OH \quad (4)$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + {}^-OH + {}^{\bullet}OH \quad (5)$$

Biosynthesis, fate and pathophysiological effects of peroxynitrite

Peroxynitrite – product of nitric oxide and superoxide reaction

Peroxynitrite anion is generated *in vivo* by a fast radical-radical, diffusion-controlled, recombination reaction of $O_2^{\bullet-}$ and $^{\bullet}$NO. It exists in equilibrium with its protonized form – ONOOH ($pK_a = 6.8$) at physiological pH (Fig. 3). $ONOO^-$ is known to decay spontaneously with a short half-life of 1 s. Therefore, it is rather difficult to assign its active concentration, which takes part in biochemical oxidations (Vatassery et al. 1998).

Various $ONOO^-$-like conformational forms of sulfur-based analogues such as $SNSS^-$ (Bojes et al. 1982), might be expected (Fig. 5). There have been reported only two possible planar conformations of $ONOO^-$, ´cyclic´*cis*- and *trans*-form, both practically equal in energy (Symons 2000).

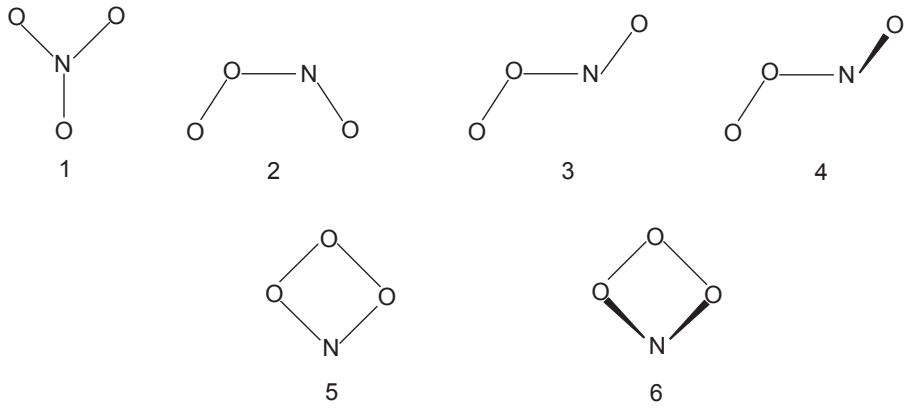

Figure 5. Various conformational alternatives for the peroxynitrite anion: 1, branched chain; 2, *cis* (planar open-chain); 3, *trans* (planar open-chain); 4, helical (nonplanar open-chain); 5, cyclic (planar chain); 6, bent (nonplanar chain) (Bojes et al. 1982).

The role of transition metals on peroxynitrite functioning

The reactivity of peroxynitrite is highly pH-dependent and is influenced by the presence of metals, thiols, and bicarbonate anion (Ischiropoulos et al. 1995). Reactions of peroxynitrite with thiols, transition metal (Fe, Cu, Mn) ions, carbon dioxide, tyrosine residues represent major pathways accounting for biological effects of this species (Ischiropoulos et al. 1992; Radi et al. 2001; Rodionov 2003). Fe(III) and Cu(II) catalyze heterolysis and react with peroxynitrite to form a potent nitrating agent with the reactivity of the nitryl cation, even when the metals are bound in proteins (Koppenol et al. 1992). Trace metals, particularly copper, in alkaline solutions, catalytically increase the rate of loss of peroxynitrite, at least 75%, to nitrite and oxygen. EDTA addition inactivates the metal function by forming chelates (Edwards and Plumb 1994). As reported (Ducrocq et al. 1999), transition metals including Fe(III)-EDTA and Cu/Zn-SOD can catalyze peroxynitrite-mediated nitration of phenolic compounds. The kinetic study of Fe(III)-EDTA-$ONOO^-$ and Fe(III)-EDTA-H_2O_2 model systems formation, as a function of pH, was used with the aim to elucidate the iron speciation in natural waters as well as to understand the mechanism of oxygen activation by iron containing proteins (Sharma et al. 2010).

Pathophysiology of biological structures

Peroxynitrite is involved in pathogenesis of many diseases. A short overview on $ONOO^-$-mediated pathophysiological processes is given in Table 1.

As to either direct cytotoxic oxidative/nitrosative or indirect peroxynitrite-derived radical-mediated interactions with cellular components and important target biomacromolecules, which may occur *in vivo*, protein oxidation and nitration are important features of $ONOO^-$ cytotoxicity. Peroxynitrite can cause chain scission of DNA promoting oxidation and nitration of DNA fragments finally resulting in the formation of 8-nitroguanine (Jourd´heuil et al. 1997).

Peroxynitrite damages *via* several distinctive mechanisms. First, it has direct toxic effects on all biomolecules and causes lipid peroxidation, protein oxidation, and DNA damage. The second mechanism involves the induction of several transcription factors leading to cytokine-induced chronic inflammation. Finally, it causes epigenetic perturbations that exaggerate nuclear factor κ-B mediated inflammatory gene expression. Lessons-learned from the treatment of several chronic disorders including pulmonary diseases suggest that chronic inflammation and glucocorticoid resistance are regulated by prolonged $ONOO^-$ production. The combination of elevated $^{\bullet}NO$ plus excess $O_2^{\bullet-}$ with the formation of high levels of $ONOO^-$ is the proverbial intracellular "devil's triangle". The "devil's triangle" changes the nature of oxidative stress. Basically, every time $^{\bullet}NO$ and $O_2^{\bullet-}$ collide, they form $ONOO^-$. $^{\bullet}NO$ is the only known biological molecule that reacts faster with $O_2^{\bullet-}$ and is produced in such high concentrations that it out-competes endogenous SOD; hence, the creation of the "devil's triangle" (for review, see Korkmaz et al. 2009).

Peroxynitrite is believed to act as a cytotoxic agent in the development of post-bypass systemic inflammatory response. $ONOO^-$ plays as well an important role in the pathogenesis of diabetic retinopathy, neuropathy, and nephropathy by attacking various biomolecules in vascular endothelium and vascular smooth muscle (Pacher and Szabó 2006).

Peroxynitrite functions in a manner of $^{\bullet}OH$ radical-like reactivity (Fig. 6). It directly oxidizes proteins yielding carbonyl species resulting in/due to the side-chain and peptide-bond cleavage. Formation of free or protein-bound 3-nitrotyrosine – an $ONOO^-$ biomarker *in vivo* – by tyrosine residues nitration (Crow and Beckman 1995; Shivashankar 2006) has proven alterations in protein functioning resulting in a changed catalytic activity, cell signaling and cytoskeletal organization (Schopfer et al. 2003). In addition, such modified proteins cannot be phosphorylated by tyrosine kinases (Martin et al. 1990).

If taken from a more specific viewpoint, a fundamental $ONOO^-$ reaction in biological systems is a rapid binding of carbon dioxide in equilibrium with bicarbonate anion under physiological conditions forming effective one-electron radical oxidants – $CO_3^{\bullet-}$ and $^{\bullet}NO_2$. They can readily oxidize amino acids (cysteine, tyrosine) yielding corresponding cysteinyl- or tyrosinyl radical species. Finally, nitrated products can be formed due to the diffusion-controlled termination reaction of $^{\bullet}NO_2$ with biomolecule-derived radicals. Peroxynitrous acid can easily cross lipid bilayers and the products of its decay – $^{\bullet}OH$ and $^{\bullet}NO_2$ radicals – trigger lipid peroxidation as well as lipid and protein nitration at hydrophobic regions (Szabó et al. 2007).

The $^{\bullet}NO/O_2^{\bullet-}$ couple released from 3-*N*-morpholinosydnonimine (SIN-1) mediates a CO_2-insensitive conversion of GSH to GSNO that activates soluble guanylyl cyclase – the major physiological target of sydnonimine-based vasodilators such as molsidomine – *via* trace metal-catalyzed release of $^{\bullet}NO$. Molsidomine is bioactivated in the liver *via* catalytic action of hepatic esterases resulting in the formation of SIN-1 (Schrammel et al. 1998). Monitoring the kinetics of NADH fluorescence quenching, using different buffer systems, showed that SIN-1 decomposition releasing $ONOO^-$ is carried out at various half-times. The $ONOO^-$ concentration maximum reached was 1.2–3.6% of applied sequential SIN-1 pulses. The method was used to simulate chronic exposure of cells or subcellular components to peroxynitrite (Martin-Romero et al. 2004).

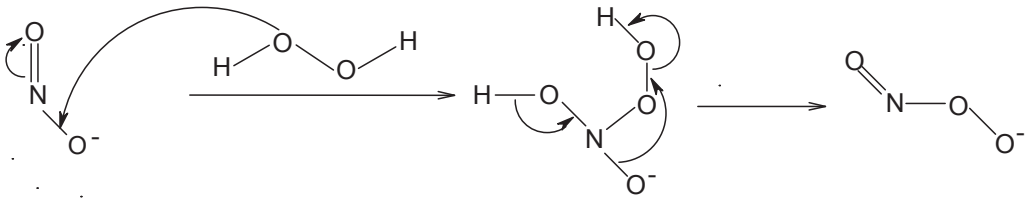

Figure 6. Baeyer-Villiger-like reaction scheme of peroxynitrite synthesis from sodium nitrite and acidified hydrogen peroxide.

Peroxynitrite synthesis

Reaction of sodium nitrite and acidified hydrogen peroxide

Peroxynitrite can be prepared by the most convenient, simple and possibly cleanest way (Saha et al. 1998) from sodium nitrite and acidified hydrogen peroxide according to the reaction (6) and (7). $ONOO^-$ can be synthesized at room temperature (20°C) obtaining 170–185 mmol/l concentration with a yield of ~ 85–95%, with residual nitrite of ~0.001% and residual H_2O_2 of 10–50%. The $ONOO^-$ presence is indicated by a strong yellow color. The $ONOO^-$ concentration, as a function of its absorbance at 302 nm, is assayed by absorption spectrophotometry using a standard value of molar extinction coefficient: $\varepsilon_{302} = 1670\ (mol/l)^{-1} \cdot cm^{-1}$.

$$H_2O_2 + HNO_2 \rightarrow ONOOH + H_2O \quad (6)$$

$$ONOOH + OH^- \rightarrow ONOO^- + H_2O \quad (7)$$

Baeyer-Villiger-like reaction mechanism of $ONOO^-$ synthesis has been proposed (Pfeiffer et al. 1997) yielding 80–100 mmol/l alkaline $ONOO^-$ solutions.

Peroxynitrite was prepared using a quenched flow mixing reactor/stopped flow apparatus (Corsaro et al. 2004). A final $ONOO^-$ concentration (170–220 mmol/l), at a flow rate of 26 ml/min, was obtained; NO_2^-, NO_3^- and H_2O_2 are contaminants after reaction (Radi et al. 1991). Excess of H_2O_2 was removed by addition of solid MnO_2. The treatment of the final solution with MnO_2, however, leads to a 10–15% loss of the product.

Peroxynitrite generation by SIN-1

SIN-1, an *in vivo* $^{\bullet}NO$ donor, is a mesoionic (mesomeric *plus* ionic), five-membered heterocyclic sydnone having a biological significance due to its planar aromatic structure and specific physico-chemical properties. It is a highly polarized compound with high dipole moment (Yelamaggad et al. 2005). SIN-1, a potent nitrovasodilator, simultaneously generates, in the presence of oxygen, *via* the non-enzymatic self-decomposition *in situ* $^{\bullet}NO$ and $O_2^{\bullet -}$ (Andrekopoulos et al. 2004), in a 1 : 1 stoichiometry, which rapidly recombine to give $ONOO^-$ (8):

$$^{\bullet}NO + O_2^{\bullet -} \rightarrow ONOO^- \quad (8)$$

It is a three step mechanism (Fig. 7). As shown by several authors (Schrammel et al. 1998; Singh et al. 1999; Rojas Wahl 2004), in the first step, SIN-1 isomerises to an open ring at alkaline pH forming SIN-1A, in the second step SIN-1A undergoes one-electron oxidation to SIN-$1^{\bullet +}$ (SIN-1B)

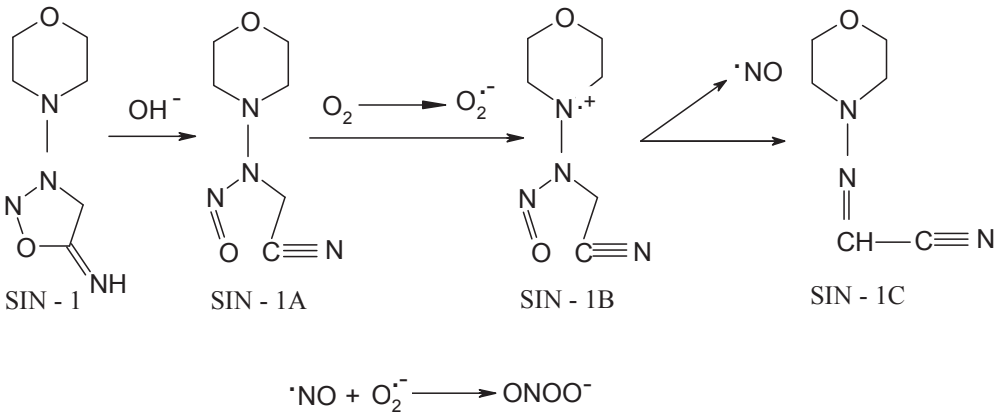

Figure 7. Peroxynitrite formation from SIN-1 (Hrabárová et al. 2007).

generating $O_2^{\bullet-}$ from O_2. In the third step, SIN-1$^{\bullet+}$ releases $^{\bullet}$NO and becomes inactive (SIN-1C). $^{\bullet}$NO recombines in an equimolar ratio with $O_2^{\bullet-}$ to form $ONOO^-$ (Espey et al. 2002). SIN-1 probably behaves like an $^{\bullet}$NO donor *in vivo* and like an $ONOO^-$ donor in oxygenated buffer (Singh et al. 1999).

Other methods used on peroxynitrite synthesis

In Table 2, there are briefly summarized several methods used on $ONOO^-$ preparation. They are, in more detail, outlined (for review, see Hrabárová et al. 2007).

Scission of glycosaminoglycans mediated by peroxynitrite

Complex polysaccharides, glycosaminoglycans (GAGs), are widely distributed in the endothelial extracellular matrix (ECM) of tissues among which hyaluronan as well as heparin, heparan/keratan/dermatan/chondroitin sulfates have been the subject of an intensive study. They differ in their sugar composition, and, except for hyaluronan, in the position of the sulfate group as well as the degree of sulfation. They are typically heterogeneous in chain length and negatively charged. They also differ in the type of uronic acid, e.g. glucuronic or iduronic acid. A relatively anaerobic cartilage matrix is mainly composed of the two major protein groups, collagens and proteoglycans. Regulation of breakdown *versus* synthesis of these complex biopolymers consisting of a core protein with one or more covalently bound GAG chains specifies the cartilage matrix integral function. They contain large quantities of water *via* multiple hydrogen bonds and thus expanding their three-dimensional space (Stefanovic-Racic et al. 1993; Hassan et al. 1998).

Rheumatoid arthritis and osteoarthritis exist both as a consequence of the degradation of GAGs mostly *via* $^{\bullet}$OH radical action, the product of $ONOO^-$ decomposition. The $^{\bullet}$NO-mediated degradation of GAGs has two pathways. The first one is initiated by the conversion of nitric oxide to nitrous acid, while the second one includes $ONOO^-$. Heparin and heparan sulfate are susceptible to degradation *via* nitrous acid, hyaluronan *via* $ONOO^-$, and chondroitin sulfates partially *via* both reagents (Hassan et al. 1998). Decomposition of heparan sulfate and other GAGs of the extracellular matrix by $^{\bullet}$NO may be significant in pathophysiological situations (apoptosis, atherosclerotic plaque release, metastatic, inflammatory conditions) (Ghael et al. 1997). Endothelial-cell-derived $^{\bullet}$NO is capable of degrading heparin and heparan sulfate *via* HNO_2 rather than $ONOO^-$. Along with cleavage of the glycosidic bond, the amino and

Table 2. Representative pattern of various methods applied on peroxynitrite synthesis

Precursors	Reaction type	Reaction mechanism	c_{ONOO^-} (mol/l)	References
O_3 and NaN_3	Reaction between ozone and sodium azide (H_2O_2 free ozonation)	$N_3^- + 2O_3 \rightarrow ONOO^- + N_2O + O_2$	$3.2–3.4 \times 10^{-2}$	(Kurz 2004; Al-Assaf et al. 2003)
$NH_2OH/NH_2Cl/Na_2N_2O_3$	Autoxidation of hydroxylamine	$NH_2OH + O_2 + OH^- \rightarrow H_2O_2 + NO^- + H_2O$ $NO^- + O_2 \rightarrow ONOO^-$	3.3×10^{-3}	(Hughes and Nicklin 1971)
Aqueous solution of sodium/potassium nitrate	UV irradiation/photolysis/γ-radiolysis of alkali metal nitrates	Photons (λ = 254–280 nm) $Na^+/K^+NO_3^- \rightarrow ONOO^-$	Not mentioned	(Edwards and Plumb 1994)
H_2O_2 and $(CH_3)_2CH(CH_2)_2NO_2$	Reaction of hydrogen peroxide and isoamyl nitrite (Two-phase nitrosation)	H_2O_2 and $(CH_3)_2CH(CH_2)_2NO_2 + OH^- \rightarrow ONOO^- + (CH_3)_2CH(CH_2)_2OH + H_2O$	1.0	(Kurz 2004; Uppu and Pryor 1996)
KO_2 and $^{\bullet}$NO	Reaction of potassium superoxide and nitric oxide aq. soln.	$KO_2 + {}^{\bullet}NO + 2\ OH^- \rightarrow ONOO^- + H_2O_2 + K^+$	$7–11 \times 10^{-2}$	(Khan et al. 2000)
$[N(CH_3)_4]^+[O_2^{\bullet}]^-$ and $^{\bullet}$NO	$^{\bullet}$NO preparation from sodium nitrite and ferrous sulfate Synthesis in liquid ammonia	$2\ NaNO_2 + FeSO_4 + 2\ H_2O \rightarrow 2\ {}^{\bullet}NO + Fe^{3+} + SO_4^{2-} + 2\ Na^+ + 4\ OH^-$ $[N(CH_3)_4]^+[O_2^{\bullet}]^- + {}^{\bullet}NO(g) + NH_3(l) \rightarrow [N(CH_3)_4]^+[ONOO]^-$	0.162*	(Kurz 2004; Bohle et al. 2004)
H_2O_2 and $CH_3CH_2O(CH_2)_2NO_2$	Reaction of nucleophiles with alkyl (2-ethoxyethyl) nitrites	$CH_3\text{-}CH_2\text{-}O\text{-}CH_2\text{-}CH_2\text{-}ONO + HOO^- \rightarrow CH_3\text{-}CH_2\text{-}O\text{-}CH_2\text{-}CH_2\text{-}OH + ONOO^-$	$1.5–\ 6 \times 10^{-2}$	(Leis et al. 1993)
$^{\bullet}$NO and alkaline H_2O_2	$^{\bullet}$NO preparation from nitrosyl-sulfuric acid Alkaline nitrosation of hydrogen peroxide	$2HNOSO_4 + H_2O \rightarrow 2H_2SO_4 + {}^{\bullet}NO + NO_2$ $2HOO^- + 2\ OH^- + {}^{\bullet}NO \rightarrow ONOO^- + 2H_2O + O_2$	$5–60 \times 10^{-4}$	(Petriconi and Papee 1966)

*the value expressed in grams.

sulfate groups are both eliminated. Inflammatory processes may be responsible for release of excess $^{\bullet}NO$ and $O_2^{\bullet-}$, forming $ONOO^-$ capable to degrade hyaluronan but not heparan sulfate. The balance between $^{\bullet}NO$ and $O_2^{\bullet-}$ determines which GAG component of the ECM is destroyed and it may be important in regulating the disease processes (Darley-Usmar et al. 1995; Vilar et al. 1997).

Hyaluronan susceptibility to peroxynitrite oxidative action

Hyaluronan is a linear/non-branched glycosaminoglycan bearing no sulfate groups, composed of alternating disaccharide units: d-glucuronic acid (GlcU) and *N*-acetyl-d-glucosamine (GlcNAc). It is a naturally occurring biopolymer widely distributed in vertebrate tissues. Aqueous hyaluronan solutions ($pK_a = 3.2$) are represented by negatively charged macromolecules exhibiting a three-dimensional structure with extensive intramolecular hydrogen bonding. Highly viscous hyaluronan solutions – with average molar mass (10^6–10^7 Da) – have gel-like properties at the concentration range of 2–4 mg/ml in the synovial fluid, in which it serves also as the joint-lubricating agent (Šoltés et al. 2006).

At neutral pH, decomposition products of the probably more reactive *trans*-$ONOO^-$, released from a complex radical pair [$^{\bullet}OH$ – $ONO^{\bullet}$], $^{\bullet}NO_2$ and $^{\bullet}OH$ radicals, degrade hyaluronan (Li et al. 1997; Vilar et al. 1997; Hassan et al. 1998), as depicted in Fig. 8. The polymeric chains are predominantly cleaved by $^{\bullet}OH$ radicals. The concentration of hyaluronan fragments has a progressive tendency, which is linear with $ONOO^-$ concentration up to 0.15 mmol/l. It reaches 4 μmol/l at high $ONOO^-$ concentration. Each $ONOO^-$ molecule generates 1.12×10^{-2} hyaluronan chain breaks resulting in reduced solution viscosity (Al-Assaf et al. 2003). Protective scavengers against deleterious action of RONS on this biopolymer might be e.g. thiourea (extremely effective), dimethyl sulfoxide (moderately effective), sodium benzoate and mannitol (slightly effective).

$ONOO^-$ exhibits $^{\bullet}OH$ radical-like reactivity derived from the vibrationally exciting state of probably less stable *trans*-ONOOH (Tsai et al. 1994). Hyaluronan does not undergo the degradation by $^{\bullet}NO$ and HNO_2 due to a protective effect of its *N*-acetyl groups (Vilar et al. 1997). At acidic pH, $ONOO^-$-dependent *C*-centered carbon radicals are formed in monomers, in the tetrasaccharide as well as in the hyaluronan polymer, as revealed by spin-trapping electron paramagnetic resonance (EPR) spectroscopic experiments. This fact supports the hypothesis of oxidative pathway involved in the degradation of hyaluronan playing a key role in the development and progression of rheumatoid arthritis (Corsaro et al. 2004). $CO_3^{\bullet-}$ anion radical, the product of the biologically relevant reaction of $ONOO^-$ with carbon dioxide, causes ~20% yields of hyaluronan chain scission *via* not well-known mechanism (Al-Assaf et al. 2006).

It was shown that hyaluronan ameliorates osteoarthritis chondrocyte protection against oxidative stress caused by exposure to a genotoxic $ONOO^-$-induced mitochondrial DNA damage. The biopolymer enhances the DNA repair, chondrocyte viability, preserves ATP levels in primary chondrocytes, and protects them from cytokine-induced mitochondrial DNA damage as well as from mitochondria-driven apoptosis (Grishko et al. 2009).

Monitoring the kinetics of oxidative degradation of hyaluronan applying a synthetically prepared $ONOO^-$ solution containing residual H_2O_2 or H_2O_2-free $ONOO^-$ solution, respectively, *via* rotational viscometry was reported (Stankovská et al. 2006). Various investigations on $ONOO^-$ pro-oxidative action resulting in hyaluronan fragmentation are summarized in Table 3.

As to the investigation on oxidative degradation of high-molar-mass hyaluronan by reactive free radicals, an overview (Šoltés et al. 2006) has been provided on currently used analytical methods to evaluate the impact of different

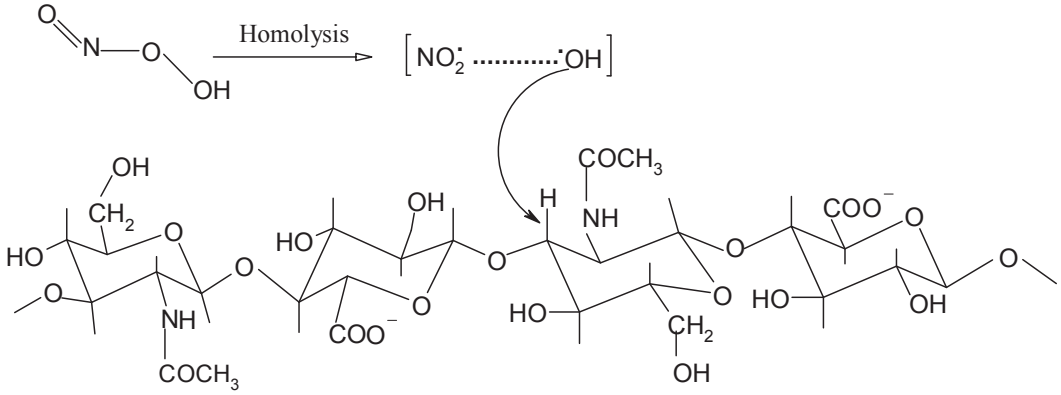

Figure 8. Hyaluronan attack mediated by peroxynitrite.

Table 3. High-molar-mass hyaluronan degradation by peroxynitrite-mediated free radical species

The aim of the work	Na-HA-M_w (MDa)	$c_{Na\text{-}HA}$ (mg/ml or * mmol/l)	c_{ONOO^-} (mmol/l)	$ONOO^-$-mediated free radical species	Effects of TMIs on HA oxidative degradation	Methodology used on experimental degradative study	Scavenging conditions for RONS	Pathophysiological consequences and therapeutical aspects	References
Comparison of the kinetics of HA oxidative degradation by H_2O_2-$ONOO^-$/H_2O_2-free-$ONOO^-$ system	1.378	2.50 (0.15 mol/l NaCl)	1.0; 0.10	$^{\bullet}OH$; $RO^{\bullet}$; $ROO^{\bullet}$	$ONOO^-$ and HA-radicals decomposition accelerated by Mn(II)	Brookfield rotational viscometer	Not mentioned	Lowered HA-viscosity leading to a joint inflammation	(Stankovská et al. 2006)
Study of HA chain breaks in context with potential $ONOO^-$ pathways to $^{\bullet}OH$ formation	0.44	* 5.30 (0.01 mol/l PB/ DTPA)	3.06	$^{\bullet}OH$; $CO_3^{\bullet-}$; $RO^{\bullet}$; $ROO^{\bullet}$	Fenton reaction due to trace TMI ($^{\bullet}OH$ formation)	Stopped-flow system (Applied Photophysics Reactor)	$^{\bullet}OH$ scavenging by NO_2^- ions at high $ONOO^-$ concentrations	$ONOO^-$-mediated chronic inflammation (HA fragmentation induced by NOS in murine macrophages)	(Al-Assaf et al. 2003)
Study of the degradation and anti-oxidative effect of Na-, Zn-, Co-, Cu-, and Mn-HA associates	1.20	* 6.30 (Mops; glycine)	0.0011–0.0085 mmol $ONOO^-$/mmol T	$^{\bullet}OH$; $ROO^{\bullet}$; $O_2^{\bullet-}$	TMI-HA associates resistance against $ONOO^-$-induced HA oxidative degradation	Spectrophotometry	The $^{\bullet}OH$ and $O_2^{\bullet-}$ scavenging capacity and TRAP of HA associates assay; $ONOO^-$-scavenging effects: Co^{2+} > Cu^{2+}/Mn^{2+} > Zn^{2+}	The correlation between RONS-mediated TMI-HA-degradation and the scavenging capacity ⇒ TMI-HA associates effective in therapy (SF protection)	(Balogh et al. 2003)
Study of anti-oxidative effects and degradation of Zn-HA complex against RONS in comparison with Na-HA	1.43–3.30 × 10^{-4}	* 4.50 (1.0 mmol/l $ZnSO_4$)	Not mentioned	$O_2^{\bullet-}$; $ONOO^-$-derived radicals	$ONOO^-$ inhibition by Zn-HA	Pyrogallol red bleaching assay; SEC	$ONOO^-$-induced HA oxidative degradation limited by scavenging effect of Zn-HA complex	Enhancement of therapeutical benefits of Na-HA by Zn anti-oxidative effects	(Illés et al. 2004)
Comparison of the effects of $ONOO^-$ on HA (P, T, M) degradation	Not mentioned	10.0 P (PB/ DTPA); 2.0 mol/l (150 mmol/l PB); 4.0 T (25 mmol/l AF)	5.0; 20	$^{\bullet}OH$; *C*-centered carbon radicals; $RO^{\bullet}$; $ROO^{\bullet}$	Metal-catalyzed nitration by $ONOO^-$ avoided	^{1}H-NMR; ESI-MS; EPR	Not discussed	Involvement of $^{\bullet}NO$ pathway in development and progression of RA ⇒ $^{\bullet}OH$ progression at low pH in inflamed SF	(Corsaro et al. 2004)
Comparison of the effect of TMs on HA oxidative degradation induced by $ONOO^-$ and TMI/H_2O_2 system	2.0–3.0 (apparent)	3.60 (0.15 mol/l NaCl/ 0.10 mol/l PB)	0.04–0.67	$^{\bullet}OH$; $^{\bullet}NO_2$	H_2O_2-Cu^{2+}/ Fe^{3+}/EDTA-mediated $^{\bullet}OH$ formation	AGE; CV	HA protection from $ONOO^-$ and $^{\bullet}OH$ attack by TM addition (TH > DMSO > SB/ML)	Biological impact on HA cleavage may differ *via* either free $^{\bullet}OH$ or $ONOO^-$/ONOOH degradative action	(Li et al. 1997)
Comparison of HA and other GAGs degradation by $ONOO^-$/ONOOH or SIN-1 system	0.12	2.0 (Chelex-treated 0.10 mol/l PB)	10	$^{\bullet}OH$; $^{\bullet}C(O)R$; *C*-centered carbon radicals; $ROO^{\bullet}$	Contaminating TMIs removal *via* Chelex resin	PAGE; EPR	Not discussed	Time-, concentration-, and pH-dependent fragmentation of extracellular matrix GAGs into disaccharides at sites of inflammation	(Kennett and Davies 2007)

c, concentration; DMSO, dimethylsulfoxide; GS, glutamine synthetase; Na-HA-M_w, Na–hyaluronan average-molar-mass; *ONOOH, conjugated peroxynitrous acid; ONS, oxidative and nitrosative stress. Other abbrevations see in the text.

effects of RONS on oxidative degradation of high-molar-mass hyaluronan resulting in the alterations of its structure (chain size, molar mass and solution viscosity reduction). Hyaluronans are well-characterized by their molar mass distribution *via* viscometry and light scattering.

Rheological parameters – markers of hyaluronan oxidative degradation can be detected *via* capillary or rotational viscometry. Capillary viscometry-based Mark-Houwink equation was used (Gura et al. 1998) to characterize hyaluronan fragments after being undergone to ultrasonical degradation.

Chemical modification of hyaluronan monosaccharide units after their oxidative degradation due to the action of free radicals is best characterized by EPR method. Indeed, identification of the generation of free radicals as a consequence of anti- or pro-oxidative action of d-penicillamine in the degradative system {hyaluronan *plus* ASC *plus* Cu(II)} was recently monitored *via* EPR method (Valachová et al. 2009). Al-Assaf et al. (1999) used EPR method to study the formation of various radicals in the reaction of the Ti(III) *plus* H_2O_2 system-generating $^{\bullet}OH$ radicals with hyaluronan. Their results support the thesis that each $^{\bullet}OH$ radical action may result in a single hyaluronan chain scission. Thus, hyaluronan radicals were identified at the C_5 (GlcU) and C_6 (GlcNAc) moieties.

Pro- or anti-oxidative effects of potential low-molar-mass thiol-based scavengers of $^{\bullet}OH$ radicals, d-penicillamine and GSH, were studied monitoring the kinetics of hyaluronan oxidative degradation by the Weissberger´s system {ASC *plus* Cu(II)} using rotational viscometry. Fragmented hyaluronan samples were also tested by non-isothermal chemiluminescence method revealing effects of these thiols on decomposition of hydroperoxides and removal of oxygenated structures. Fourier-transformed infrared spectroscopy indicated possible thiol incorporation into hyaluronan biopolymer (Hrabárová et al. 2009).

Biomedical and therapeutical importance

The research on ubiquitous endobiotics, playing important role in biological systems, such as nitric oxide radical and peroxynitrite, has recently led to a numerous publication activity meeting all aspects of physiological and pathological areas of interest.

Because oxidative stress plays a key role in the pathogenesis of cancer and many metabolic diseases, an effective antioxidant therapy would be of great importance in these circumstances. Nevertheless, convincing randomized clinical trials revealed that antioxidant supplementations were not associated with significant reduction in incidence of cancer, chronic diseases, and all-cause mortality. As oxidation of essential molecules continues, it turns to nitro-oxidative stress because of the involvement of nitric oxide in pathogenesis processes (for review, see Korkmaz et al. 2009).

A possible explanation for the potential negative effect of antioxidant supplements might be that RONS in moderate concentrations are essential mediators of reactions by which the body gets rid of unwanted cells. Thus, if administration of antioxidant supplements decreases free radicals, it may interfere with essential defensive mechanisms for ridding the organism of damaged cells, including those that are injured, precancerous and cancerous (Linnane et al. 2007).

Detrimental $ONOO^-$ effects, during its over-production, exacerbated *via* its reaction with carbon dioxide may result even in a redirection of $ONOO^-$ specificity. Surprisingly, besides this, $ONOO^-$ exhibits significant biomedical effects – protective effect i) at high concentrations – it behaves as anti-viral, anti-microbial, and anti-parasitic agent, and ii) at low concentrations, it stimulates protective mechanisms in the cardiovascular, nervous, and respiratory systems (Ascenzi et al. 2010).

There are several commercial drugs with a direct $ONOO^-$-scavenging potential. Despite this, most of them preferentially exhibit an indirect $ONOO^-$-derived secondary radical-detoxification activity *in vitro*. Many classical therapeuticals have, however, less effectivity in the $ONOO^-$ deactivation *in vivo* for their quite low rate constants (Szabó et al. 2007). It is worth of mentioning that e.g. acetaminophen has been proven to attenuate $ONOO^-$-induced matrix metalloproteinase activity and related cardiotoxicity (Rork et al. 2006). A typical inhibitor/chelator of iron-dependent formation of $^{\bullet}OH$ radicals – desferoxamine – has been also confirmed as an effective inhibitor of the $ONOO^-$-mediated oxidation and nitration *via* reaction with the $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals (Bartesaghi et al. 2004). What is speculative/intriguing, tyrosine-containing peptides can also play a role of $ONOO^-$-derived radical scavengers at the cellular level. They may function as good neutralizers of the cytotoxic effects of exogenous and endogenous $ONOO^-$, inhibiting thus its pro-apoptotic effect on neurons (Ye et al. 2007). Pharmacological strategies to attenuate the toxic effects of peroxynitrite have been, in present, focused on the catalytic reduction of this species to nitrite and its isomerization to nitrate by metalloporphyrins – exogenous $ONOO^-$ scavengers – potential candidates for drug development for cardiovascular, inflammatory, and neurodegenerative diseases. These decomposition catalysts can be effectively applied as therapeutics for $ONOO^-$-induced pathology (Szabó et al. 2007).

Enormous challenge still remains to reduce a disease derived-human suffering if a biological specificity against deleterious actions of oxidants in living systems is better understood. A special interest has to be focused on experimental investigations elucidating specific cellular mechanisms such as the effects of oxidative and nitrosative stress in the respiratory systems, signaling pathways of reactive radical species as

well as the role of anti-oxidative systems. The management of the pathogenesis of various diseases may eventually lead to discovery of novel therapeutical and clinical strategies.

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