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Peroxynitrite and nitrosoperoxycarbonate, a tightly connected oxidizing–nitrating couple in the reactive nitrogen–oxygen species family: new perspectives for protection from radical-promoted injury by flavonoids

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Abstract

Peroxynitrite is the product of the reaction of nitric oxide with superoxide radical and is implicated in the pathogenesis of a wide variety of human diseases, being responsible for in-vivo oxidation/nitration events. Nitrosoperoxycarbonate anion, formed by the interaction of peroxynitrite with CO_2/bicarbonate at physiological concentrations, provides a new interpretation of oxidative/nitrative processes formerly attributed to peroxynitrite. The aim of this review is to summarize the chemistry and biology of peroxynitrite and radical species related to nitrosoperoxycarbonate anion, as well as the information available regarding the molecular mechanisms that determine and regulate radical-promoted injury by the two tightly connected species at physiological concentrations. Interception of carbonate and nitro radicals produced by interaction of peroxynitrite with CO_2/bicarbonate, as in-vivo prevention of pathological events, creates new perspectives for the evaluation of safe scavengers of oxidative/nitrative stress at the physiological level. In this respect, natural products such as flavonoids hold a preeminent position among the vast array of compounds endowed with such properties.

Introduction

Nitric oxide (nitrogen monoxide, $^{\bullet}NO$), first described as the principal endothelium-derived relaxing factor, is involved in a variety of physiological and pathophysiological events (Ozben & Tomasi 2003). $^{\bullet}NO$ is a free radical with an unpaired electron that is physiologically generated from L-arginine under the catalytic control of three different nitric oxide synthase (NOS) isoforms (Alderton et al 2001). Nitric oxide can directly interact with biological targets only at low concentrations and/or if exposure of the biological system occurs for short periods of time. However, indirect $^{\bullet}NO$ effects are provoked by the action of reactive nitrogen–oxygen species (RNOS) formed by the reaction of $^{\bullet}NO$ either with O_2 or superoxide anion. Principally, RNOS are formed under high local concentrations of $^{\bullet}NO$ or owing to long-term exposure to nitric oxide (Wink & Mitchell 1998; Eberhardt 2000; Espey et al 2002; Dedon & Tannenbaum 2004).

As a free radical, $^{\bullet}NO$ can donate or accept an electron to become a nitrosonium cation (NO^+) or a nitroxyl anion (NO^-) (Hughes 1999). All NO-related redox forms are responsible for charge-transfer reactions, forming nitroxyl complexes or nitrogen species that are ultimately responsible for oxidation, nitration or nitrosation reactions. When such a complex array of reactions occurs under biological conditions, oxidative, nitrative and nitrosative stress are the result. For instance, NO^+ exists in a pool of nitrosating species that include N_2O_3 and that can nitrosate amine and thiols to corresponding *N*-nitrosoamines and *S*-nitrosothiols. *S*-Nitrosothiols themselves can serve as $^{\bullet}NO$ donors at pH values of biological relevance (Hogg 2002; Giustarini et al 2003).

Peroxynitrite $ONOO^-$: chemistry and biology

Reaction of $^{\bullet}NO$ with superoxide anion $O_2^{\bullet-}$ yields peroxynitrite anion (oxoperoxonitrate(1$^-$), $ONOO^-$) and this process can be considered physiologically as a mechanism for

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regulating •NO activity (Beckman & Koppenol 1996; Ferdinandy 2006). It is generally assumed that it can be formed in-vivo from the reaction between superoxide ($O_2^{\bullet-}$) and nitric oxide and that a rapid diffusion-controlled reaction ($k = 3.9–6.7 \times 10^9$ $M^{-1}s^{-1}$) takes place under conditions of oxidative stress and inflammation (Huie & Padmaja 1993; Kobayashi et al 1995; Pryor & Squadrito 1995; Radi et al 2001). In normal conditions, nitric oxide and superoxide radicals are produced at different rates in the same cellular or extracellular compartment and this prevents a massive production of peroxynitrite. An alternative pathway to peroxynitrite formation may involve reaction of oxygen with nitroxyl anion (NO^-), in turn formed by •NO reduction (Hughes 1999; Kirsch & de Groot 2002).

Peroxynitrite $ONOO^-$ is protonated to its conjugated acid ONOOH (hydrogen oxoperoxonitrate(1−), peroxynitrous acid) and the two chemical species are collectively often referred to as peroxynitrite. The weak acid ONOOH ($pK_A = 7.49 \pm 0.06$ at 37°C) rapidly decomposes, with a short half-life, at physiological pH (1.9 s at pH 7.4) (Beckman et al 1990). The main product from $ONOO^-$ decay in the absence of targets is the stable nitrate anion (Koppenol 1998).

The mechanism of peroxynitrite reactivity is still under discussion, since peroxynitrite chemistry strongly depends on the conditions of in-vitro studies and on the technology used (Goldstein et al 1998, 1999; Richeson et al 1998). For instance, it has been proposed that hyaluronic acid and related saccharide oxidation by peroxynitrite is due to hydroxyl radical (Corsaro et al 2004). Peroxynitrite itself is a relatively strong oxidant that is able to participate either in one-electron oxidation of transition metal ion complexes or in the two-electron oxidation of thiols (Alvarez & Radi 2003). Peroxynitrous acid can generate hydroxyl radical (•OH) and nitro radical ($NO_2^{\bullet}$) by homolysis and these radicals become part of the cascade of oxidation/nitration agents (Merenyi et al 1998). In-vivo, however, these species become relevant only at acid pH (e.g. ischaemic tissue) because at physiological pH the proton-catalysed decay is too slow to compete with biotargets that react directly with peroxynitrite.

Under pathophysiological conditions, location and relative rates of production of •NO and superoxide radicals are constantly changing and this may be critical in determining the amount and, ultimately, the reactivity of peroxynitrite. For example, recombination of nitrogen dioxide arising from homolysis of peroxynitrite with an excess of •NO could produce species such as N_2O_3, the principal nitrosating RNOS (Jourd'heuil et al 2001). The cellular damage will be marginal or rapidly repaired for short and low fluxes of peroxynitrite, which are expected to be scavenged by endogenous antioxidants. However, prolonged or large fluxes of peroxynitrite will result in oxidation/nitration of critical cellular targets, ranging from inactivation of enzymes and ion channels to inhibition of mitochondrial respiration. This cannot be handled by repair mechanisms and cells undergo basic cell death pathways, apoptosis or necrosis. The exact mechanism of peroxynitrite-induced cell death initiation remains to be established. Low concentrations of peroxynitrite may trigger apoptotic death, while higher concentrations induce necrosis, with cellular energetics (ATP level) serving as the switch between these two modes of cell death (Virág et al 2003).

At the molecular level, the main events that have been attributed to peroxynitrite or its equivalent radicals include oxidation of a variety of biomolecules such as proteins (Alvarez & Radi 2003), lipids (Carr et al 2000), carbohydrates (Moro et al 1995), DNA (Yu et al 2005) and low molecular mass antioxidants (Hogg et al 1994; Bartlett et al 1995; Botti et al 2004). In circulation, peroxynitrite is able to lower the total peroxyl-trapping capacity, deplete low molecular mass antioxidants, oxidize thiol groups or induce lipid peroxidation and tyrosine nitration (van der Vliet et al 1994; Gow et al 1996; Kocic et al 2004).

Peroxynitrite does not react at appreciable rates with tyrosine and nitration occurs through a free radical mechanism involving one-electron oxidation of tyrosine leading to tyrosyl radical, which then rapidly reacts with $NO_2^{\bullet}$ to yield 3-nitrotyrosine. On the other hand, the nitro radical ($NO_2^{\bullet}$) is a strong oxidant and a potent nitrating agent able to oxidize and nitrate free tyrosine in-vitro (Huie 1994; Goldstein et al 2000a). The complete picture of peroxynitrite-derived reactions with tyrosine include the formation of 3,3′-dityrosine and smaller amounts of 3-hydroxytyrosine (Radi et al 2001). Formation of 3-nitrotyrosine is the evidence generally presented for peroxynitrite participation as a contributor to tissue injury in several human diseases (Radi 2004), although other species are involved in the oxidization/nitration of tyrosyl residues in-vivo, for example myeloperoxidase, an enzyme capable of catalysing the above reactions in the presence of H_2O_2 and nitrite anion (the oxidation product of •NO) (Sampson et al 1998).

The involvement of peroxynitrite in protein nitrative modifications and its related role in pathological events has been recently reviewed (Salvemini et al 2006). It has been observed that the advent of recent methodologies such as proteomics has revealed some specificity in protein site nitration and consequent modification or loss of function that is especially relevant if this mechanism involves enzymes of pathophysiological significance (Cassina et al 2000; Balafanova et al 2002; Vadseth et al 2004).

Above considerations on the biological effects of peroxynitrite suggest a definite role for this RNOS in a variety of pathological events. These aspects are included in an exhaustive review recently published by Olmos et al (2007), which also covers a wide range of drugs capable of modulating the biological and pathological effects of peroxynitrite. In a few pathological events, the involvement of peroxynitrite has been clearly evidenced, for example in the case of the pathogenesis of renal ischaemia–reperfusion injury (Walker et al 2000; Rhyu et al 2002). Peroxynitrite has also been recognized to play a considerable role in dopaminergic neurotoxicity (Imam et al 2001), glaucoma (El-Remessy et al 2003), and diabetes (Szabó et al 2002).

Effect of CO_2 on peroxynitrite reactivity

There is increasing evidence that the mechanism and reactivity of peroxynitrite in-vitro, and probably in-vivo, can be altered by bicarbonate/carbon dioxide, which are present at significant concentrations in biological systems (Gow et al 1996; Lymar & Hurst 1996; Uppu et al 1998; Berlett et al

1998; Squadrito & Pryor 1998; Jourd'heuil et al 1999; Tien et al 1999; Santos et al 2000; Vasela & Wilhelm 2002).

At physiological concentration of CO_2, peroxynitrite-dependent nitration is enhanced (Gow et al 1996), whereas oxidation is lowered (Berlett et al 1998; Ascenzi et al 2006). The importance of the concentration of CO_2 on peroxynitrite-promoted reactions has been demonstrated for nitration/nitrosation (Uppu et al 2000) or production of thiyl, sulfinyl and disulfide radicals (Bonini & Augusto 2001). The free radical mechanism of tyrosine modification by peroxynitrite in the presence and in the absence of the bicarbonate–carbon dioxide pair has been confirmed and the tyrosyl radical detected by continuous-flow and spin-trapping electron paramagnetic resonance (Pietraforte & Minetti 1997; Santos et al 2000).

Nitrosoperoxycarbonate $ONO_2CO_2^-$: chemistry and biology

The reaction between peroxynitrite anion and CO_2 presents a rate constant of 3.0×10^4 M^{-1} s^{-1}, leading to the formation of a postulated highly reactive short-lived secondary oxidant, the nitrosoperoxycarbonate anion (1-carboxylato-2-nitrosodioxidane anion, $ONO_2CO_2^-$) (Figure 1). Nitrosoperoxycarbonate anion can be considered as the biologically active form of peroxynitrite that does not terminate the action of peroxynitrite but rather redirects its reactivity (Squadrito & Pryor 1998; Goldstein et al 2000b). The homolysis of $ONO_2CO_2^-$, in fact, opens the route to other radical pathways, related to the formation of carbonate and nitro radicals ($CO_3^{\bullet-}$ and $NO_2^{\bullet}$) (Goldstein & Czapski 1998; Hodges & Ingold 1999; Goldstein et al 2001; Meli et al 2002). The carbonate radical $CO_3^{\bullet-}$ (trioxidocarbonate(1$^-$)) has been detected by electron paramagnetic resonance spectroscopy at physiological pH (Bonini et al 1999) and is a strong one-electron oxidant that rapidly abstracts one electron, preferably from tyrosine, to yield tyrosyl radical, which recombines with $NO_2^{\bullet}$ affording 3-nitrotyrosine. Nitration yields increase significantly due to the formation of tyrosyl radical by $CO_3^{\bullet-}$, a process more efficient than the one related to another oxidating species, $^{\bullet}$OH. In these conditions, dimerization of tyrosine also occurs, whereas hydroxylation is inhibited (Santos et al 2000). Formation and localization of 3-nitrotyrosine and 3-hydroxytyrosine residues in proteins may be useful to distinguish between events mediated by peroxynitrite/nitrosoperoxycarbonate and those induced by other RNOS. The role of carbonate radical anion is becoming increasingly important in biological oxidative stress (Squadrito & Pryor 1998; Augusto et al 2002). For instance, the CO_2 effect on peroxynitrite-mediated inhibition of human caspase reveals a new role for related radicals in the tuning of cell processes such as apoptosis (Ascenzi et al 2006). Finally, the reported selective oxidization of guanine in double-stranded oligonucleotides by carbonate radical (Shafirovich et al 2001) has been recently confirmed as being responsible for the paradoxical selection of guanines in high ionization potential guanine–cytosine sequences (Margolin et al 2006).

Peroxynitrite scavengers: a general outlook

In connection with increasing evidence on the pathophysiological role of peroxynitrite, targeting peroxynitrite-induced cytotoxic pathways might be proposed as a strategy to alleviate adverse symptoms of a diverse variety of metabolic disorders and diseases, especially during infection and inflammation (Arteel et al 1999). Defence strategies against peroxynitrite-mediated deleterious effects should be based on prevention of formation of this RNOS and for this purpose inhibitors of NOS or SOD could be considered, as well. A more viable approach can rely upon scavengers able to intercept peroxynitrite and absorb its total or maximum oxidative capacity. Alternatively, repair of damage induced by the action of peroxynitrite should be based on quenching generated radicals or on reaction with any secondary reactive species produced (Klotz & Sies 2003). Furthermore, it should be taken into account that stress signalling mechanisms affected by peroxynitrite may interact with all above defence mechanisms (Arteel et al 1999; Klotz & Sies 2003). Research on natural and synthetic antioxidants capable of interfering with peroxynitrite-mediated damage is, at present, actively pursued and a vast array of compounds has been recently reviewed (Olmos et al 2007).

Among synthetic compounds, special attention should be directed towards substituted metalloporphyrins, which are promising therapeutics as peroxynitrite decomposition catalysts

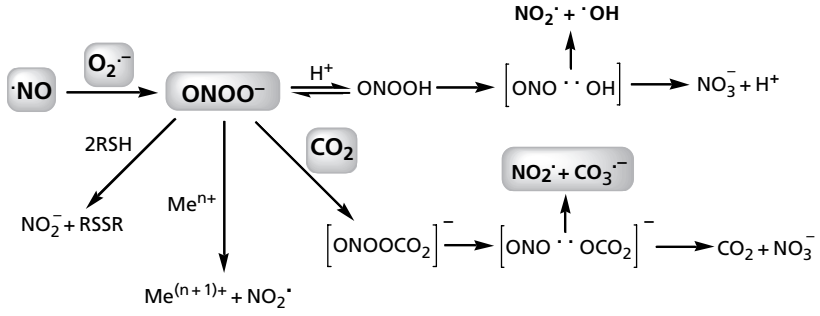

Figure 1 Peroxynitrite and nitrosoperoxycarbonate anion formation and reactivity: direct oxidation by peroxynitrite could be impaired by carbon dioxide, and some oxidation/nitration processes could be attributed to carbonate radical anion and nitric radical reactivity.

(Crow 2000; Cuzzocrea et al 2001). Natural products that are available in the human diet or that could be introduced into human nutrition as nutraceuticals (Lee et al 2004) could constitute a class of safe scavengers.

Natural products from diet as peroxynitrite scavengers

The human diet is rich in a great variety of micronutrients with antioxidant properties (Halliwell 1996), fruits and vegetables being especially indicated for the prevention of cellular oxidative damage (Prior 2003). This is particularly important if protection from reactive oxygen species and RNOS is considered, in light of the recognized role of oxidative stress in carcinogenesis (Halliwell 1999; Klaunig & Kamendulis 2004). Dietary factors may exert an important role in this respect, going from regulation of $^{\bullet}NO$ synthesis (Wu & Meininger 2002) to protection from RNOS-mediated damage such as tyrosine nitration (Verhagen et al 1996; Kato et al 1997; Pannala et al 1997; Chung et al 1998). Polyphenols are aromatic polyhydroxylated compounds widely distributed in fruits, vegetables and beverages such as tea, beer and wine (Manach et al 2004), and their antioxidant properties are well recognized (Valdez et al 2004). Flavonoids constitute a broad class of phenolic compounds ubiquitously present in fruits and vegetables (Harborne & Williams 2000), and their health benefits are well recognized (Yao et al 2004). The general flavonoid structure consists of a flavan nucleus arranged in three rings (A, B, C) with different levels of oxidation and pattern of substitution. Flavonoids are active antioxidants in-vitro against a wide array of radicals (Pietta 2000) and the most important structural features in this respect are summarized for quercetin (Figure 2A) as follows: (i) hydroxyl groups at positions 3′ and 4′ in the B ring; (ii) unsaturation between atoms 2 and 3 in conjugation with a 4-oxo function; and (iii) hydroxyl groups at positions 3 and 5 (Cotelle 2001). The antioxidant efficacy is less documented in-vivo, mainly because of limited knowledge about their uptake in humans (Ross & Kasum 2002). In fact, bioavailability and bioefficacy of flavonoids and of polyphenols, in general, is far from being elucidated and it is only recently that epidemiological studies have been undertaken (Manach et al 2005).

Flavonoid–peroxynitrite interaction

Protection against the effects of peroxynitrite may be exhibited by polyphenols as crude extracts in beverages (Bixby et al 2005) or wine (Valdez et al 2004), and this has been investigated in some detail with regard to flavonoids (Ohshima et al 1998; Pannala et al 1999; Yokozawa et al 2003; Kim et al 2004; Shin et al 2005).

Flavonoid scavenging activity against $ONOO^-$ has been monitored through the oxidation of dihydrorhodamine 123 (Santos & Mira 2004) or NADH (Boveris et al 2002), nitration of tyrosine (Schroeder et al 2001a, b), and 8-nitro-guanine formation (Ohshima et al 1998). In addition to chemical and biochemical assays, the use of more sensitive biological systems such as aortic ring chemiluminescence has been proposed in order to elucidate the ability of flavonoids to interfere in $ONOO^-$ mediated damage (Valdez et al 2004).

In general, the mechanisms by which polyphenols are able to scavenge peroxynitrite have been extensively studied

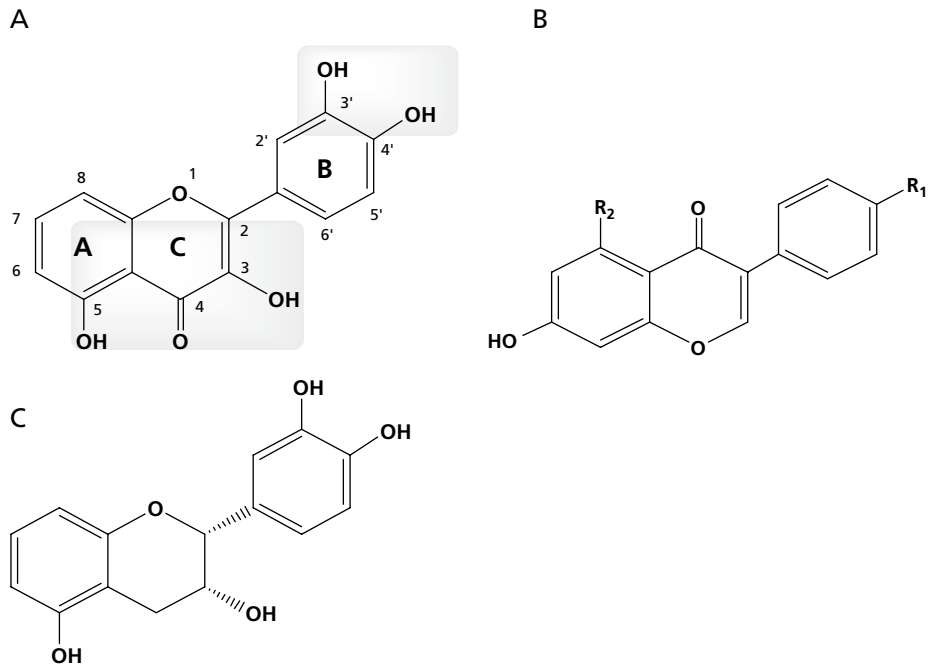

Figure 2 A. Quercetin structural features responsible for antioxidant activity. B. Structurally related isoflavones from soybeans (genistein ($R_1 = R_2 = OH$), daidzein ($R_1 = OH$, $R_2 = H$) and biochanin-A ($R_1 = OCH_3$, $R_2 = OH$)) investigated as peroxynitrite scavengers. C. Structure of epicatechin.

in-vitro and a the bulk of the investigation has concerned flavonoids, due to the richness of structural frameworks present in this class of natural compounds.

By studying structurally related flavonoids it has been proved that the presence, number and position of hydroxyl groups are very important in peroxynitrite scavenging activity. The ortho-hydroxyl structure, especially the catechol group in the B ring, seems essential for peroxynitrite scavenging activity (Haenen et al 1997; Heijnen et al 2001b; Choi et al 2002), and the 2,3-double bond (Santos & Mira 2004) also plays an important role. Furthermore, the activity related to the OH group at position 3 depends on substitutents at positions 5 and 7 (Heijnen et al 2001a), while the presence of the carbonyl moiety is not essential for the scavenger activity (Choi et al 2002). By studying three isoflavones occurring in soybeans (Figure 2B), it has been observed that, in-vitro, peroxynitrite-mediated nitration occurs on the tyrosine-like B-ring of genistein and daidzein. No nitration product of biochanin-A was found and this confirms that nitration is the preferential mechanism of monohydroxylated structures (Boersma et al 1999).

It would be useful to establish which flavonoid, if any, can be expected to preferably scavenge peroxynitrite and peroxynitrite-deriving free radicals, but controversial information has been obtained even when studying a single flavonoid. For instance, epicatechin (Figure 2C), a flavonoid particularly abundant in green tea and red wine, has been identified as a selective inhibitor of $^{\bullet}$NO-related oxidation and nitration reactions (Wippel et al 2004). However, Schroeder et al (2001a) have shown that nitration is effectively suppressed by this polyphenol and only marginal protective effects were observed against oxidative reactions.

Two possible mechanisms for polyphenol-mediated peroxynitrite scavenging, that is nitration and electron donation, have been proposed (Pannala et al 1998). Studies on structure–antioxidant activity relationship of polyphenols (Rice-Evans et al 1996) or hydroxycinnamates (Kerry & Rice-Evans 1998) have been extended to peroxynitrite-mediated scavenging by flavonoids (Heijnen et al 2001b). It was proposed that nitration of tyrosine can be inhibited by different mechanisms and monohydroxylated structures act as alternative substrates for nitration, whereas catechol structures are oxidized to *o*-quinones.

A limiting factor for determining the scavenger mechanism of free radical damage in-vivo by flavonoids is their relatively low plasma concentrations. Therefore, it is relevant to mention structure–scavenging activity studies of some flavonoid metabolites such as 3-*O*-methyl and 3-*O*-glucuronate (Pollard et al 2006). These metabolites are particularly important because flavonoids are almost exclusively present in the human diet as glycosylated derivates and it has been shown that *O*-glucuronidation reduces flavonoid reactivity towards $ONOO^-$. It has also been revealed that *O*-methylation of the B-ring catechol containing epicatechin reduces its electron donation ability, but at the same time favours the formation of nitrated and even nitrosylated derivates. Catechol-containing flavonoids, epicatechin and quercetin, yielded oxidation products that are trapped by glutathione with the formation of related conjugates. All these products have been identified and may constitute novel circulating flavonoid metabolites with unrevealed bioactivity.

Effect of CO_2 on peroxynitrite scavengers

It is now well established that peroxynitrite reacts faster with CO_2 than with most biological molecules and this event can take place in any cellular compartment where peroxynitrite and carbon dioxide are present (Squadrito & Pryor 2002). Therefore, a new view of oxidative/nitrative stress related to peroxynitrite has to be considered. Interesting reconsiderations on the activity of peroxynitrite scavengers have appeared in the literature and new perspectives merge from published results. For instance, it has been reported that in the presence of 25 mM bicarbonate, the ability of uric acid, ascorbate, Trolox and glutathione to inhibit peroxynitrite mediated tyrosine and guanine nitration is decreased (Whiteman et al 2002). The recently disclosed lipoic acid protection by peroxynitrite induced damage (Rezk et al 2004) has been integrated by pulse radiolysis studies, showing that the carbonate radical is responsible for the formation of a one-electron oxidant lipoic acid radical cation (Trujillo et al 2005). Another study considered the ability of thiols, nitric oxide donors and purine derivatives to inhibit peroxynitrite-induced dityrosine formation in a physiological buffer containing bicarbonate/CO_2 (Ferdinandy & Schulz 2001). It was shown that both reduced and oxidized thiols, nitric oxide donors and urate, but not other purine derivatives, reduce peroxynitrite-induced dityrosine formation.

It has been reported that the inhibition of peroxynitrite-induced nitration of tyrosine by glutathione becomes highly effective in the presence of carbon dioxide (Kirsch et al 2001). The reactivity of peroxynitrite with melatonin at various pH values and different carbon dioxide concentrations has been studied (Peyrot et al 2003), as well as the protection of low density lipoprotein by phenolic compounds from peroxynitrite at physiological CO_2 concentrations (Ferroni et al 2004). The reactivity of peroxynitrite with respect to dietary phenolic antioxidants is significantly reduced in the presence of CO_2 (Ketsawatsakul et al 2000) and no scavenging activity was observed with a synthetic flavonoid, 7-substituted rutin (Tibi & Koppenol 2000). However, $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ radicals arising from the nitrosoperoxycarbonate anion hydrolysis have been efficiently scavenged by epicatechin (Miau et al 2001), indicating that inhibition of tyrosine nitration is due to quenching of those radicals. Furthermore, it has been demonstrated that epicatechin blocks peroxynitrite-induced tyrosine nitration and dimerization by interfering with the tyrosyl radical, rather than with peroxynitrite itself (Schroeder et al 2001b). In any event, it can be concluded that physiological concentrations of bicarbonate and formation of nitrosoperoxycarbonate anion or derived radicals can modify the ability of polyphenols to prevent peroxynitrite-mediated reactions.

Conclusion

Peroxynitrite reactivity, which is well established in-vitro, is responsible for in-vivo oxidation/nitration reactions and this RNOS is generally considered one of the major causative agents of the nitrative stress. Peroxynitrite reaction with physiological concentrations of CO_2/bicarbonate through the intermediate formation of nitrosoperoxycarbonate $ONO_2CO_2^-$ and

related radical species casts a new light on the role of peroxynitrite in cell damage. Scavenging of carbonate and nitro radicals occurring from peroxynitrite/CO_2 interaction could constitute a new target for in-vivo prevention of oxidative/nitrative stress. Understanding the molecular mechanisms that determine and regulate the above radical-promoted injury and elucidation of structure–activity relationships can constitute the basis for the selection of new scavengers. In this respect, the high physiological concentrations of CO_2/bicarbonate should require the assumption of high quantities of compounds, in order to contrast related oxidative/nitrative events. This creates an important perspective for natural and safe scavengers of oxidation/nitration processes. Phenolic compounds, and flavonoids in particular, are well suited for this purpose, since their ability to counteract peroxynitrite-mediated damages at non-toxic concentrations is already well documented.

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