Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 349 (2006) 359–363
BBRC
www.elsevier.com/locate/ybbrc

CO_2 impairs peroxynitrite-mediated inhibition of human caspase-3 ☆

Paolo Ascenzi [a,b,*], Maria Marino [a], Enea Menegatti [c]

[a] Department of Biology, University 'Roma Tre', Viale Guglielmo Marconi 446, I-00146 Roma, Italy
[b] National Institute for Infectious Diseases I.R.C.C.S. 'Lazzaro Spallanzani', Via Portuense 292, I-00149 Roma, Italy
[c] Department of Pharmaceutical Sciences, University of Ferrara, Via Fossato di Mortara 17-19, I-44100 Ferrara, Italy

Received 31 July 2006
Available online 18 August 2006

Abstract

Peroxynitrite ($ONOO^-$) is a transient powerful oxidant produced *in vivo* as the reaction of nitrogen monoxide (•NO) with superoxide ($O_2^{\bullet -}$). The peroxynitrite reactivity is modulated by carbon dioxide (CO_2) which enhances the peroxynitrite-mediated nitration of aromatics and partially impairs the oxidation of thiols. Here, the effect of CO_2 on the peroxynitrite-mediated inhibition of human caspase-3, the execution enzyme of the apoptotic cascade, is reported. Peroxynitrite inhibits the catalytic activity of human caspase-3 by oxidizing the Sγ atom of the Cys catalytic residue. In the absence of CO_2, 1.0 equivalent of peroxynitrite inactivates 1.0 equivalent of human caspase-3. In the presence of the physiological concentration of CO_2 ($=1.3\times10^{-3}$ M), 1.0 equivalent of peroxynitrite inactivates only 0.38 equivalents of human caspase-3. Peroxynitrite affects the k_{cat} value of the human caspase-3 catalyzed hydrolysis of *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin, without altering K_m. Both in the absence and presence of CO_2, the reducing agent dithiothreitol does not prevent human caspase-3 inhibition by peroxynitrite and does not reverse the peroxynitrite-induced inactivation of human caspase-3. These results represent the first evidence for modulation of peroxynitrite-mediated inhibition of cysteine proteinase action by CO_2, supporting the role of CO_2 in fine tuning of cell processes (*e.g.*, apoptosis).

Keywords: Human caspase-3; Cysteine proteinase; Peroxynitrite; Carbon dioxide; Enzyme inhibition

The free radical nitrogen monoxide (•NO), generally known as 'nitric oxide', was first prepared by the action of nitric acid on metals like copper and called 'nitrous air' [1]. More than two centuries later, •NO was found to be pivotal in many biological functions [2–5].

In 1986, superoxide ($O_2^{\bullet -}$) was reported to be a scavenger of •NO which at that time was defined as endothelial-derived relaxing factor [6]. Soon thereafter peroxynitrite ($ONOO^-$)[1] was identified as the product of the reaction of $O_2^{\bullet -}$ with •NO [7]. Peroxynitrite is more reactive than its precursors $O_2^{\bullet -}$ and •NO [7]. The peroxynitrite ability to oxidize biomolecules (*e.g.*, proteins, lipids, and DNA) is at the root of atherosclerosis, inflammation, and neurodegenerative disorders [3–5,8,9].

Recently, bicarbonate (HCO_3^-) was reported to decrease the microbicidal effect of peroxynitrite [10,11] and carbon dioxide (CO_2) was shown to react with various free radical species, including peroxynitrite [12–14]. Given that the concentration of CO_2 *in vivo* is relatively high due to high levels of HCO_3^- ($=1.3\times10^{-3}$ M and 2.5×10^{-2} M, respectively, in plasma), most of the peroxynitrite produced would rapidly form a very short-living adduct, believed to be 1-carboxylato-2-nitrosodioxidane ($ONOOC(O)O^-$). This oxidant, stronger than peroxynitrite, decays by homolysis of the O–O bond yielding the reactive species trioxocarbonate and nitrite radicals $CO_3^{\bullet -}$ and $^{\bullet}NO_2$, respectively), which then proceed towards

☆ *Abbreviations:* DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp-al; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid).

* Corresponding author. Fax: +39 06 5517 6321.
E-mail address: ascenzi@uniroma3.it (P. Ascenzi).

[1] The recommended IUPAC nomenclature for peroxynitrite is oxoperoxonitrate(1^-); for peroxynitrous acid, it is hydrogen oxoperoxonitrate. The pK_a value for the $ONOOH \leftrightarrow ONOO^- + H^+$ equilibrium is 6.8 (see [19,54]). The term peroxynitrite is used in the text to refer generically to both $ONOO^-$ and its conjugate acid ONOOH (see [27]).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.08.050

nitrate (NO_3^-) and CO_2, or by directly yielding NO_3^- and CO_2 [12–21].

CO_2 facilitates peroxynitrite-mediated oxidation of aromatics. Indeed, most reactions of $CO_3^{\cdot-}$ are one-electron oxidations with preference for tyrosine and tryptophan, while $^{\cdot}NO_2$ can undergo recombination with other radical species, addition to double bonds, and one-electron oxidations (but its reducing potential is much lower than that of $CO_3^{\cdot-}$) [13–28]. In contrast, CO_2 decreases peroxynitrite-mediated oxidations such as of methionine and cysteine [13]. Indeed, CO_2 outcompetes the thiols for the direct reaction with peroxynitrite as the second order rate constant for reaction of peroxynitrite with cysteine ($\sim 4 \times 10^3$ M^{-1} s^{-1}) is lower than that for reaction with CO_2 ($\sim 3 \times 10^4$ M^{-1} s^{-1}) [12,13,29–31]. However, thiol oxidation is only partially decreased because of the oxidation mediated by $CO_3^{\cdot-}$ and $^{\cdot}NO_2$ radicals [31]. Thus, CO_2, generally considered to be inactive, redirects the specificity of peroxynitrite and reduces the lifetime of peroxynitrite (from the second to the millisecond range) [19,20].

Here, the effect of CO_2 on the peroxynitrite-mediated inhibition of the catalytic activity of human caspase-3, a cysteine proteinase displaying a pivotal role in apoptosis [32,33], is reported. CO_2 impairs the peroxynitrite-mediated inhibition of human caspase-3, representing an unexpected modulator of cysteine proteinase action. This supports the role of CO_2 in fine tuning of cell processes (*e.g.*, apoptosis).

Materials and methods

Recombinant human caspase-3, *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (DEVD-AMC), *N*-acetyl-Asp-Glu-Val-Asp-al (DEVD-CHO), dithiothreitol (DTT), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes), and leupeptin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human caspase-3 was reductively activated with DTT ($=1.0 \times 10^{-3}$ M) [34], DTT and by-products were removed by gel-filtration on a Sephadex G-25 column (from Pharmacia, Uppsala, Sweden) [35]. The human caspase-3 concentration was determined by active site titration using the inhibitor DEVD-CHO [34]. Peroxynitrite was prepared from KO_2 and $^{\cdot}NO$ (from Aldrich Chemical Company, Inc., Milwaukee, WI, USA) and from HNO_2 and H_2O_2 [36]. The peroxynitrite stock solution was diluted with degassed 1.0×10^{-2} M NaOH to reach the desired concentration [27]. For the experiments carried out in the absence of CO_2, the 1.0×10^{-1} M Hepes buffer (pH = 7.5) and the 1.0×10^{-2} M NaOH solutions were prepared fresh daily and thoroughly degassed. Experiments in the presence of CO_2 ($=1.3 \times 10^{-3}$ M) were carried out by adding to the human caspase-3 solution the required amount from a freshly prepared 5.0×10^{-1} M sodium bicarbonate solution. The CO_2 concentration is always expressed as the true concentration in equilibrium with HCO_3^- [27]. All the other chemicals were obtained from Merck AG (Darmstadt, Germany). All products were of analytical or reagent grade and used without purification.

The catalytic activity of human caspase-3 was measured in continuous assays using the fluorogenic substrate DEVD-AMC, as previously reported [34]. Briefly, DEVD-AMC (final concentration, 1.0×10^{-6} M–1.0×10^{-4} M range) was added to the human caspase-3 solution (final concentration, 2.0×10^{-7} M–1.0×10^{-5} M range) and fluorescence (380 nm excitation wavelength, and 460 nm absorption wavelength) was measured continuously over 1 min, at pH 7.5 (1.0×10^{-1} M Hepes buffer) and 25.0 °C. Under all the experimental conditions, no gaseous phase was present.

The effect of peroxynitrite on the catalytic activity of human caspase-3 was determined by incubation of the enzyme (final concentration, 2.0×10^{-7} M–1.0×10^{-5} M range) with peroxynitrite (final concentration, 1.0×10^{-7} M and 1.0×10^{-4} M), for 30 min, at pH 7.5 (1.0×10^{-1} M Hepes buffer) and 25.0 °C. Then, DEVD-AMC (final concentration, 1.0×10^{-6} M–1.0×10^{-4} M range) was added to the reaction mixture and the human caspase-3 activity assayed [34,37].

The effect of CO_2 on the peroxynitrite-mediated inhibition of human caspase-3 was investigated by incubation of the enzyme (final concentration, 2.0×10^{-7} M–1.0×10^{-5} M range) with peroxynitrite (final concentration, 1.0×10^{-7} M and 1.0×10^{-4} M) and CO_2 ($=1.3 \times 10^{-3}$ M) for 30 min, at pH 7.5 (1.0×10^{-1} M Hepes buffer) and 25.0 °C. Then, DEVD-AMC (final concentration, 1.0×10^{-6} M–1.0×10^{-4} M range) was added to the reaction mixture and the caspase-3 activity assayed [34,37].

The effect of DTT on the peroxynitrite-mediated inhibition of caspase-3, in the absence and presence of CO_2 ($=1.3 \times 10^{-3}$ M), was investigated by the simultaneous incubation of the active enzyme (final concentration, 2.0×10^{-7} M–1.0×10^{-5} M range) with DTT (final concentration, 1.0×10^{-3} M) and peroxynitrite (final concentration, 1.0×10^{-4} M) for 30 min, at pH 7.5 (1.0×10^{-1} M Hepes buffer) and 25.0 °C. Then, the catalytic activity of caspase-3 was assayed using DEVD-AMC (final concentration, 1.0×10^{-4} M) [34]. Furthermore, the inactivated enzyme, obtained by 1.0×10^{-4} M peroxynitrite-pre-treatment in the absence and presence of CO_2, was incubated with DTT (final concentration, 1.0×10^{-3} M) for 30 min, at pH 7.5 (1.0×10^{-1} M Hepes buffer) and 25.0 °C. Then, the enzyme catalytic activity was assayed using DEVD-AMC (final concentration, 1.0×10^{-4} M) [34].

The steady-state data for the human caspase-3 catalyzed hydrolysis of DEVD-AMC, both in the absence and presence of peroxynitrite and CO_2, were analyzed in the framework of the classical minimum two-step mechanism (Scheme 1):

$$E + S \overset{K_m}{\leftrightarrow} X \overset{k_{cat}}{\rightarrow} E + P, \qquad \text{(Scheme 1)}$$

where E is human caspase-3, S is the substrate (*i.e.*, DEVD-AMC), X represents the enzyme-substrate and enzyme-product intermediates, P indicates the hydrolysis products (*i.e.*, DEVD and AMC), k_{cat} ($=V_{max}/[E]$) is the catalytic constant, and K_m is the Michaelis constant [34]. Values of k_{cat} and K_m have been determined from data analysis according the classical Michaelis-Menten equation (Eq. (1)):

$$v_i = k_{cat} \times [E] \times [S]/(K_m + [S]), \qquad (1)$$

where v_i is the initial velocity [38].

Results

The hydrolysis of DEVD-AMC catalyzed by human caspase-3 follows simple Michaelis-Menten kinetics, in the absence and presence of peroxynitrite and CO_2 (Fig. 1). Under all the experimental conditions, the initial velocity (*i.e.*, v_i) for the hydrolysis of DEVD-AMC catalyzed by human caspase-3 is strictly linear on the active enzyme concentration (Fig. 2 and Table 1).

Values of k_{cat} and K_m for the human caspase-3 catalyzed hydrolysis of DEVD-AMC obtained in the absence of peroxynitrite and CO_2 ($=12.9$ s^{-1} and 8.6×10^{-6} M, respectively, at pH 7.5 and 25.0 °C; Fig. 1) are in excellent agreement with those previously reported ($k_{cat} = 14.0$ s^{-1} and $K_m = 1.0 \times 10^{-5}$ M, at pH 7.5 and room temperature) [34]. In the absence of peroxynitrite, values of steady-state parameters for the hydrolysis of DEVD-AMC catalyzed by human caspase-3 are unaffected by CO_2 ($=1.3 \times 10^{-3}$ M), values of k_{cat} and K_m being 13.4 s^{-1} and 8.8×10^{-6} M, respectively, at pH 7.5 and 25.0 °C (Fig. 1).

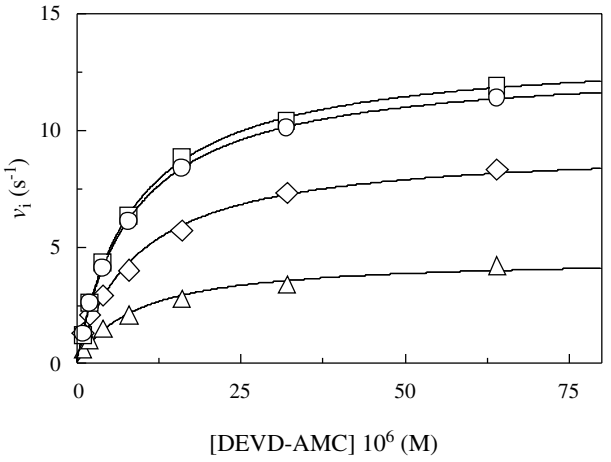

Fig. 1. Effect of peroxynitrite and CO_2 on the human caspase-3 catalyzed hydrolysis of DEVD-AMC. The dependence of the initial velocity (*i.e.*, v_i) on the substrate concentration (*i.e.*, [DEVD-AMC]) for the human caspase-3 catalyzed hydrolysis of DEVD-AMC was obtained in the absence of both peroxynitrite and CO_2 (circles), in the presence of CO_2 ($=1.3\times10^{-3}$ M; squares), in the presence of peroxynitrite ($=6.5\times10^{-7}$ M; triangles), and in the presence of both peroxynitrite and CO_2 ($=6.5\times10^{-7}$ M and 1.3×10^{-3} M, respectively; diamonds). The human caspase-3 concentration was 1.0×10^{-6} M. The continuous lines, representing data best fit, were calculated according to the Michaelis-Menten equation (Eq. (1)) with sets of parameters given in the text. The average standard deviation for values of v_i was ±7%. The average standard deviation for values of k_{cat} and K_m was ±11%. All data were obtained at pH 7.5 (1.0×10^{-1} M Hepes buffer) and 25.0 °C. For experimental details, see text.

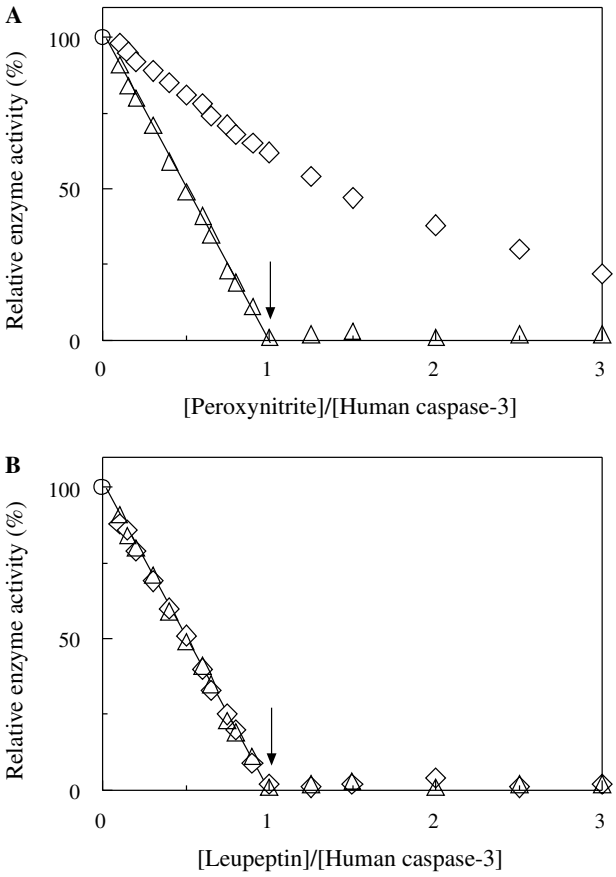

Fig. 2. Dependence of the relative human caspase-3 activity on the [peroxynitrite]/[human caspase-3] (panel A) and [leupeptin]/[human caspase-3] (panel B) molar ratio. The relative enzyme activity was determined in the absence of peroxynitrite, leupeptin, and CO_2 (circles), in the presence of peroxynitrite or leupeptin (triangles), and in the presence of peroxynitrite or leupeptin and CO_2 (diamonds). The arrows indicate that 1 equivalent of peroxynitrite (panel A) or leupeptin (panel B) inactivates 1 equivalent of human caspase-3. The human caspase-3 concentration was 1.0×10^{-6} M. The peroxynitrite concentration ranged between 1.0×10^{-7} M and 3.0×10^{-6} M. The CO_2 concentration was 1.3×10^{-3} M. The average standard deviation for values of the relative enzyme activity was ±8%. All data were obtained at pH 7.5 (1.0×10^{-1} M Hepes buffer) and 25.0 °C. For further experimental details, see text.

Peroxynitrite inhibits the hydrolysis of DEVD-AMC catalyzed by human caspase-3 (Figs. 1 and 2). The enzyme activity (expressed by v_i and k_{cat}) decreases on increasing peroxynitrite concentration, the K_m value being independent of the peroxynitrite concentration. At the [peroxynitrite]/[human caspase-3] molar ratio = 0.65, k_{cat} is 4.5 s^{-1} and K_m is 8.7×10^{-6} M (Fig. 1). As shown in Fig. 2, 1.0 equivalent of peroxynitrite inactivates 1.0 equivalent of human caspase-3.

CO_2 impairs peroxynitrite-mediated inhibition of human caspase-3 (Figs. 1 and 2). In the presence of CO_2, the enzyme activity (expressed by v_i and k_{cat}) is higher than that obtained in the absence of CO_2 at fixed peroxynitrite concentration, the K_m value being unaffected by peroxynitrite and CO_2. In the presence of CO_2 ($=1.3\times10^{-3}$ M), k_{cat} is 9.3 s^{-1} and K_m is 9.2×10^{-6} M, at the [peroxynitrite]/[human caspase-3] molar ratio = 0.65 (Fig. 1). As shown in Fig. 2, 1.0 equivalent of peroxynitrite inactivates 0.38 equivalents of human caspase-3. The complete human caspase-3 inactivation is achieved at the [peroxynitrite]/[human caspase-3] molar ratio >4.

Both in the absence and presence of CO_2 ($=1.3\times10^{-3}$ M), leupeptin, a typical cysteine proteinase inhibitor [39], induces the complete suppression of human caspase-3 action at the [leupeptin]/[human caspase-3] molar ratio $\geqslant 1$ (Fig. 2). This indicates that CO_2 modulates selectively peroxynitrite-mediated activity of human caspase-3; whereas CO_2 does not affect cysteine protease inhibition by leupeptin.

To verify that human caspase-3 inactivation occurs *via* a DTT-dependent or DTT-independent mechanism, the prevention and the reversibility of peroxynitrite-mediated cysteine proteinase inactivation by DTT was investigated. As shown in Table 1, the addition of DTT to inactivated human caspase-3, as obtained by pre-incubation with peroxynitrite in the absence and presence of CO_2, does not restore the cysteine proteinase activity. Moreover, DTT does not prevent the peroxynitrite-mediated inhibition of human caspase-3, both in the absence and presence of CO_2 (Table 1). Therefore, the peroxynitrite-mediated oxidation of the Sγ atom of the Cys catalytic residue of human caspase-3 may result in sulfenic, sulfinic or sulfonic acid formation which represent DTT-irreversible oxidation states of protein-bound thiol groups (see [29,37]).

Table 1
Effect of CO_2, DTT, and peroxynitrite on the hydrolysis of DEVD-AMC catalyzed by human caspase-3, at pH 7.5 (1.0×10^{-1} M Hepes buffer) and 25.0 °C

Inhibitor/activator	v_i (s^{-1})	Relative enzyme activity (%)	[E] (M)	[S] (M)	[Peroxynitrite] (M)	[CO_2] (M)	[DTT] (M)
None[a]	11.4	100	1.0×10^{-6}	6.4×10^{-5}	—	—	—
CO_2[a]	11.9	104	1.0×10^{-6}	6.4×10^{-5}	—	1.3×10^{-3}	—
DTT[a]	11.3	99	1.0×10^{-6}	6.4×10^{-5}	—	—	1.0×10^{-3}
Peroxynitrite[a]	0.23	2.0	1.0×10^{-6}	6.4×10^{-5}	3.0×10^{-6}	—	—
Peroxynitrite + DTT[a,b]	0.21	1.8	1.0×10^{-6}	6.4×10^{-5}	3.0×10^{-6}	—	1.0×10^{-3}
Peroxynitrite + DTT[a,c]	0.13	1.1	1.0×10^{-6}	6.4×10^{-5}	3.0×10^{-6}	—	1.0×10^{-3}
Peroxynitrite + CO_2[a]	2.5	22	1.0×10^{-6}	6.4×10^{-5}	3.0×10^{-6}	1.3×10^{-3}	—
Peroxynitrite + CO_2 + DTT[a,b]	2.6	23	1.0×10^{-6}	6.4×10^{-5}	3.0×10^{-6}	1.3×10^{-3}	1.0×10^{-3}
Peroxynitrite + CO_2 + DTT[a,c]	2.2	19	1.0×10^{-6}	6.4×10^{-5}	3.0×10^{-6}	1.3×10^{-3}	1.0×10^{-3}

[a] The human caspase-3/CO_2/DTT/peroxynitrite incubation time was 30 min. The human caspase-3/DEVD-AMC reaction time was 1 min. For experimental details, see text.
[b] Addition of DTT to the inactivated human caspase-3, as obtained by pre-incubation with peroxynitrite in the absence and presence of CO_2, did not restore the enzyme activity. For experimental details, see text.
[c] Simultaneous incubation of DTT with human caspase-3 and peroxynitrite, in the absence and presence of CO_2, did not prevent enzyme inhibition. For experimental details, see text.

Discussion

·NO and related compounds (*e.g.*, peroxynitrite) are signaling molecules which initiate intercellular and intracellular signals [2–5]. The most characterized downstream ·NO signaling pathway relates to the soluble guanylate cyclase pathway, with downstream phosphorylation cascades leading to effector functions [40,41]. Moreover, ·NO-mediated signaling occurs through direct modification of target proteins (*e.g.*, oxidation of thiols and aromatics) [3,8,20,42–50].

Peroxynitrite modulates enzyme activity through chemical modifications of reactive residues (*e.g.*, Cys) (see [8,20,46–50]) as well as by oxidizing metal centers (*e.g.*, heme) (see [51–53]). The peroxynitrite-mediated chemical modifications of the Cys catalytic residue of cysteine proteinases block the enzyme activity *in vitro* and *in vivo* (see [46,49]). Among others, peroxynitrite inhibits human caspase-3 activity, a cysteine proteinase displaying a pivotal role in apoptosis [32,33], by a DTT-independent mechanism (see Table 1). This supports the role of ·NO and related compounds (*e.g.*, peroxynitrite) in fine tuning of apoptosis [37,46]. The processing of human pro-caspase-3 to its active form is considered to be a point of no return in the death signaling cascade [32,33]. In fact, human caspase-3 represents the execution enzyme of the caspase cascade that cleaves the inhibitor of caspase-activated DNase, to activate DNA degrading DNases [33]. Therefore, *S*-nitrosylation of human caspase-3 provides a mechanism to abort the apoptotic program initiated by extracellular and intracellular signaling [33].

The present data represent the first evidence for the interplay between peroxynitrite and CO_2 in modulating the human caspase-3 action. Indeed, peroxynitrite inhibits completely human caspase-3 activity (1:1 molar ratio); whereas, at the CO_2 concentration achievable *in vivo* ($=1.3 \times 10^{-3}$ M), only a partial inhibition of human caspase-3 activity by peroxynitrite (=38%) is observed (see Figs. 1 and 2, and Table 1). This suggests a role of CO_2 in the modulation of the apoptotic program initiated by free radicals ·NO and $O_2^{\cdot -}$. CO_2 redirects the specificity of peroxynitrite from thiols towards aromatics [19,20], in line with the protective role of hypercapnia against free radical damage [14]. In conclusion, the regulatory effects here reported could represent a general mechanism by which cell processes are fine tuned by CO_2 levels.

Acknowledgments

This work was supported by grants from University 'Roma Tre' (CLAR 2006 to P.A.) and from the Ministry of Health of Italy (National Institute for Infectious Diseases I.R.C.C.S. 'Lazzaro Spallanzani', Ricerca corrente 2006 to P.A.).

References

[1] J. Priestley, Observations on different kinds of air, Phil. Trans. 62 (1772) 147–264.
[2] S. Moncada, R.M.J. Palmer, E.A. Higgs, Nitric oxide: physiology, pathophysiology, and pharmacology, Pharmacol. Rev. 43 (1991) 109–142.
[3] J.S. Beckman, W.H. Koppenol, Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly, Am. J. Physiol. 271 (1996) C1424–C1437.
[4] L.J. Ignarro, Nitric oxide as a unique signaling molecule in the vascular system: a historical overview, J. Physiol. Pharmacol. 53 (2002) 503–514.
[5] E. Clementi, E. Nisoli, Nitric oxide and mitochondrial biogenesis: a key to long-term regulation of cellular metabolism, Comp. Biochem. Physiol. 142 (2005) 102–110.
[6] R.J. Gryglewski, R.M. Palmer, S. Moncada, Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor, Nature 320 (1986) 454–456.
[7] J.S. Beckman, T.W. Beckman, J. Chen, P.A. Marshall, B.A. Freeman, Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide, Proc. Natl. Acad. Sci. USA 87 (1990) 1620–1624.
[8] C. Ducrocq, B. Blanchard, B. Pignatelli, H. Oshima, Peroxynitrite: an endogenous oxidizing and nitrating agent, Cell. Mol. Life Sci. 55 (1999) 1068–1077.

[9] A. Denicola, R. Radi, Peroxynitrite and drug-dependent toxicity, Toxicology 208 (2005) 273–288.
[10] L. Zhu, C. Gunn, J.S. Beckman, Bactericidal activity of peroxynitrite, Arch. Biochem. Biophys. 298 (1992) 452–457.
[11] A. Denicola, H. Rubbo, D. Rodriguez, R. Radi, Peroxynitrite-mediated cytotoxicity to *Trypanosoma cruzi*, Arch. Biochem. Biophys. 304 (1993) 279–286.
[12] S.V. Lymar, J.K. Hurst, Rapid reaction between peroxynitrite ion and carbon dioxide: implications for biological activity, J. Am. Chem. Soc. 117 (1995) 8867–8868.
[13] A. Denicola, B.A. Freeman, M. Trujillo, R. Radi, Peroxynitrite reaction with carbon dioxide/bicarbonate: kinetics and influence on peroxynitrite-mediated oxidations, Arch. Biochem. Biophys. 333 (1996) 49–58.
[14] A. Veselá, J. Wilhelm, The role of carbon dioxide in free radical reactions of the organism, Physiol. Res. 51 (2002) 335–339.
[15] M.G. Bonini, R. Radi, G. Ferrer-Sueta, A.M. Da C. Ferreira, O. Augusto, Direct EPR detection of the carbonate radical anion produced from peroxynitrite and carbon dioxide, J. Biol. Chem. 274 (1999) 10802–10806.
[16] R. Radi, A. Denicola, B.A. Freeman, Peroxynitrite reactions with carbon dioxide-bicarbonate, Methods Enzymol. 301 (1999) 353–367.
[17] R. Meli, T. Nauser, P. Latal, W.H. Koppenol, Reaction of peroxynitrite with carbon dioxide: intermediates and determination of the yield of $CO_3^{\cdot -}$ and NO_2, J. Biol. Inorg. Chem. 7 (2002) 31–36.
[18] O. Augusto, M.G. Bonini, A.M. Amanso, E. Linares, C.C.X. Santos, S.L. De Menezes, Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology, Free Radic. Biol. Med. 32 (2002) 841–859.
[19] S. Goldstein, J. Lind, G. Merényi, Chemistry of peroxynitrites and peroxynitrates, Chem. Rev. 105 (2005) 2457–2470.
[20] P. Ascenzi, A. Bocedi, P. Visca, M. Minetti, E. Clementi, Does CO_2 modulate peroxynitrite specificity? IUBMB Life (2006) in press.
[21] W.A. Pryor, K.N. Houk, C.S. Foote, J.M. Fukuto, L.J. Ignarro, G.L. Squadrito, K.J. Davies, Free radical biology and medicine: it's a gas, man! Am. J. Physiol. Regul. Integr. Comp. Physiol. (2006) in press.
[22] A. Gow, D. Duran, S.R. Thom, H. Ischiropoulos, Carbon dioxide enhancement of peroxynitrite mediated protein tyrosine nitration, Arch. Biochem. Biophys. 333 (1996) 42–48.
[23] S.V. Lymar, Q. Jiang, J.K. Hurst, Mechanism of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite, Biochemistry 35 (1996) 7855–7861.
[24] D. Pietraforte, M. Minetti, One electron oxidation pathway of peroxynitrite decomposition in human blood plasma: evidence for the formation of protein tryptophan-centred radicals, Biochem. J. 321 (1997) 743–750.
[25] D. Pietraforte, M. Minetti, Direct ESR detection of peroxynitrite-induced tyrosine-centred protein radicals in human blood plasma, Biochem. J. 325 (1997) 675–684.
[26] M. Minetti, G. Scorza, D. Pietraforte, Peroxynitrite induces long-lived tyrosyl radical(s) in oxyhemoglobin of red blood cells through a reaction involving CO_2 and a ferryl species, Biochemistry 38 (1999) 2078–2087.
[27] S. Herold, K. Shivashankar, M. Mehl, Myoglobin scavenges peroxynitrite without being significantly nitrated, Biochemistry 41 (2002) 13460–13472.
[28] H. Zhang, C. Andrekopoulos, J. Joseph, J. Crow, B. Kalyanaraman, The carbonate radical anion-induced covalent aggregation of human copper, zinc superoxide dismutase, and α-synuclein: intermediacy of tryptophan- and tyrosine-derived oxidation products, Free Radic. Biol. Med. 36 (2004) 1355–1365.
[29] R. Radi, J.S. Beckman, K.M. Bush, B.A. Freeman, Peroxynitrite oxidation of sulfhydryls, J. Biol. Chem. 266 (1991) 4244–4250.
[30] G. Scorza, M. Minetti, One electron oxidation pathway of thiols by peroxynitrite in biological fluids: bicarbonate and ascorbate promote the formation of albumin disulphide dimers in human blood plasma, Biochem. J. 329 (1998) 405–413.
[31] M.G. Bonini, O. Augusto, Carbon dioxide stimulates the production of thiyl, sulfinyl, and disulfide radical anion from thiol oxidation by peroxynitrite, J. Biol. Chem. 276 (2001) 9749–9754.
[32] D.R. Green, Apoptotic pathways: ten minutes to dead, Cell 121 (2005) 671–674.
[33] Z.B. Wang, Y.Q. Liu, Y.F. Cui, Pathways to caspase activation, Cell Biol. Int. 29 (2005) 489–496.
[34] M. Garcia-Calvo, E.P. Peterson, D.M. Rasper, J.P. Vaillancourt, R. Zamboni, D.W. Nicholson, N.A. Thornberry, Purification and catalytic properties of human caspase family members, Cell Death Differ. 6 (1999) 362–369.
[35] M. Xian, X. Chen, Z. Liu, K. Wang, P.G. Wang, Inhibition of papain by *S*-nitrosothiols. Formation of mixed disulfides, J. Biol. Chem. 275 (2000) 20467–20473.
[36] W.H. Koppenol, R. Kissner, J.S. Beckman, Syntheses of peroxynitrite: to go with the flow or on solid grounds? Methods Enzymol. 269 (1996) 296–302.
[37] S. Mohr, B. Zech, E.G. Lapetina, B. Brune, Inhibition of caspase-3 by *S*-nitrosation and oxidation caused by nitric oxide, Biochem. Biophys. Res. Commun. 238 (1997) 387–391.
[38] L. Michaelis, M. Menten, Die Kinetik der Invertinwirkung, Biochem. Z. 49 (1913) 333–369.
[39] A.J. Barrett, N.D. Rawlings, J.F. Woessner (Eds.), Handbook of Proteolytic Enzymes, 2nd ed., Academic Press, London, 2004.
[40] J.W. Denninger, M.A. Marletta, Guanylate cyclase and the NO/cGMP signaling pathway, Biochim. Biophys. Acta 1411 (1999) 334–350.
[41] S.P.L. Cary, J.A. Winger, M.A. Marletta, Tonic and acute nitric oxide signaling through soluble guanylate cyclase is mediated by nonheme nitric oxide, ATP, and GTP, Proc. Natl. Acad. Sci. USA 102 (2005) 13064–13069.
[42] S.R. Jaffrey, H. Erdjument-Bromage, C.D. Ferris, P. Tempst, S.H. Snyder, Protein *S*-nitrosylation: a physiological signal for neuronal nitric oxide, Nat. Cell Biol. 3 (2001) 193–197.
[43] J.S. Stamler, S. Lamas, F.C. Fang, Nitrosylation: the prototypic redox based signaling mechanism, Cell 106 (2001) 675–683.
[44] C. Nathan, The moving frontier in nitric oxide-dependent signaling, Sci. STKE 257 (2004) pe52.
[45] R. Radi, Nitric oxide, oxidants, and protein tyrosine nitration, Proc. Natl. Acad. Sci. USA 101 (2004) 4003–4008.
[46] P. Ascenzi, A. Bocedi, F. Polticelli, M. Bolognesi, Structural consensus rules for cysteine proteinase inhibition by NO(-donors), Curr. Enz. Inhib. 1 (2005) 231–238.
[47] J.S. Stamler, Redox signaling: nitrosylation and related target interactions of nitric oxide, Cell 78 (1994) 931–936.
[48] J.S. Stamler, A. Hausladen, Oxidative modifications in nitrosative stress, Nature Struct. Biol. 5 (1998) 247–249.
[49] P. Ascenzi, L. Salvati, M. Bolognesi, M. Colasanti, F. Ponticelli, G. Venturini, Inhibition of cysteine protease activity by NO-donors, Curr. Protein. Pept. Sci. 2 (2001) 137–153.
[50] B. Alvarez, R. Radi, Peroxynitrite reactivity with amino acids and proteins, Amino Acids 25 (2003) 295–311.
[51] C. Bouton, Nitrosative and oxidative modulation of iron regulatory proteins, Cell. Mol. Life Sci. 55 (1999) 1043–1053.
[52] C.E. Cooper, Nitric oxide and iron proteins, Biochim. Biophys. Acta 1411 (1999) 290–309.
[53] S. Herold, A. Fago, Reactions of peroxynitrite with globin proteins and their possible physiological role, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 142 (2005) 124–129.
[54] W.H. Koppenol, The basic chemistry of nitrogen monoxide and peroxynitrite, Free Radic. Biol. Med. 25 (1998) 385–391.