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

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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).

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[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]).

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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).