Interplay of carbon dioxide and peroxide metabolism in mammalian cells

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The carbon dioxide/bicarbonate (CO_2/HCO_3^-) molecular pair is ubiquitous in mammalian cells and tissues, mainly as a result of oxidative decarboxylation reactions that occur during intermediary metabolism. CO_2 is in rapid equilibrium with HCO_3^- *via* the hydration reaction catalyzed by carbonic anhydrases. Far from being an inert compound in redox biology, CO_2 enhances or redirects the reactivity of peroxides, modulating the velocity, extent, and type of one- and two-electron oxidation reactions mediated by hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$/ONOOH). Herein, we review the biochemical mechanisms by which CO_2 engages in peroxide-dependent reactions, free radical production, redox signaling, and oxidative damage. First, we cover the metabolic formation of CO_2 and its connection to peroxide formation and decomposition. Next, the reaction mechanisms, kinetics, and processes by which the CO_2/peroxide interplay modulates mammalian cell redox biology are scrutinized in-depth. Importantly, CO_2 also regulates gene expression related to redox and nitric oxide metabolism and as such influences oxidative and inflammatory processes. Accumulated biochemical evidence *in vitro*, *in cellula*, and *in vivo* unambiguously show that the CO_2 and peroxide metabolic pathways are intertwined and together participate in key redox events in mammalian cells.

The carbon dioxide/bicarbonate (CO_2/HCO_3^-) molecular pair is ubiquitous in mammalian cells and tissues, and its roles in key physicochemical properties, metabolic processes, and gene expression are increasingly recognized. Herein, we will specifically analyze how CO_2 levels modulate peroxide-dependent reactions and as such influences redox signaling and oxidative damage (1–8). Far from being an inert compound in redox biology, CO_2 has been progressively shown to enhance or redirect the reactivity of peroxides, modulating the velocity, extent, and type of one- and two-electron oxidation reactions mediated by hydrogen peroxide (H_2O_2) and peroxynitrite[1] (5, 9–13). In this sense, key oxidative posttranslational modifications in proteins such as thiol oxidation and tyrosine nitration are strongly influenced by cellular CO_2 levels (8, 12, 14–18). Mitochondria represent central sites of CO_2 formation in mammalian cells *via* the oxidative decarboxylation reactions associated to the Krebs cycle (19). In the cytosol, the oxidative phase of the pentose phosphate pathway (PPP) contributes to substantial CO_2 formation, many times coupled to the cellular need of NADPH for peroxide metabolism (20, 21). Once formed, CO_2 in large part converts to and is in equilibrium with bicarbonate anion (HCO_3^-) *via* the (reversible) action of carbonic anhydrases (CAs) (22). CO_2 levels and gradients across cellular compartments in mammalian cells can connect energy and peroxide metabolism and participate in the regulation of various intertwined cellular processes.

Herein, the biochemical mechanisms by which CO_2 engages on peroxide-dependent reactions and impacts on redox signaling and oxidative damage will be analyzed and summarized. The interactions of CO_2 with biologically relevant peroxides produce a collection of reactive and short-lived one- and two-electron oxidants. For instance, the reaction of H_2O_2 with CO_2 yields peroxymonocarbonate (HCO_4^-), a strong two-electron oxidant that accelerates H_2O_2 reactivity with key biotargets such as protein thiols. The reaction of peroxynitrite anion ($ONOO^-$) with CO_2 yields nitrosoperoxocarboxylate ($ONOOCO_2^-$) that rapidly decays into carbonate radical ($CO_3^{\bullet-}$) and nitrogen dioxide ($^{\bullet}NO_2$), promoting one-electron oxidations and nitrations. The review will examine reaction mechanisms, kinetics, and processes by which the CO_2/peroxide interplay controls mammalian cell redox biology. Moreover, the analysis will integrate the CO_2-dependent regulation of gene expression related to redox and nitric oxide ($^{\bullet}NO$) metabolism, which further influences oxidative and inflammatory processes.

Detailed biochemical analysis of the CO_2/peroxide interplay at the cellular and subcellular levels assists on data interpretation and refinement of experimental designs and methodologies to dissect molecular mechanisms of redox-dependent cell signaling and injury.

CO_2 at the crossroads of the energy-redox axis

CO_2 is constantly produced in aerobic mammalian cell metabolism as part of oxidation processes in biomolecules connected to cellular respiration and energy generation. For

[1] Peroxynitrite refers to the sum of the anionic and acid forms, namely peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH), p*K*a = 6.8.

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instance, mammalian mitochondria, which are central loci of aerobic hydrocarbon catabolism, are the main cellular sources of CO_2 through the oxidative decarboxylation of α- and β-ketoacids. These metabolic intermediates include pyruvate, isocitrate, and α-ketoglutarate and are substrates for the enzymatic action of specific dehydrogenases. Indeed, the action of pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase results in the formation of CO_2 in parallel with the two-electron reduction of NAD^+ to NADH, with the latter serving as electron donor to complex I of the respiratory electron transport chain. In this way, oxidative decarboxylation reactions in mitochondria couple energy release from the catabolism of metabolic intermediates with respiratory activity, oxygen consumption, and ATP synthesis (Fig. 1).

Metabolic CO_2 generation connected to redox processes can also occur in the cytosol by the activation of the PPP; in fact, the oxidative decarboxylation of 6-phosphogluconate in the presence of $NADP^+$ by the reaction catalyzed by 6-phosphogluconate dehydrogenase leads to the formation of ribulose 5-phosphate, CO_2, and NADPH. The NADPH in turn can be used for a variety of metabolic process, most notably in the context of this review, providing the reducing equivalents needed to catabolize peroxides *via* the action of redox proteins and enzymes. In fact, the PPP (and therefore CO_2 production) is largely accelerated under enhanced cellular oxidant formation or oxidative stress conditions (20, 23), representing an adaptive mechanism to cope with the excess amounts of, for example, H_2O_2 or peroxynitrite (14, 24). Notably, NADPH is also required to generate (1) superoxide radical ($O_2^{\bullet-}$) and H_2O_2 by the NADPH oxidase protein family (NOX 1–5, DUOX 1–2) (21) and (2) $^{\bullet}NO$, a precursor of peroxynitrite, by the nitric oxide synthases (NOS 1–3) (25). Thus, the simultaneous formation of CO_2 and NADPH parallels peroxide metabolism (Fig. 1).

CO_2 metabolism also involves non-oxidative decarboxylation and carboxylation reactions; indeed, the action of decarboxylases that lead to CO_2 release and carboxylases that

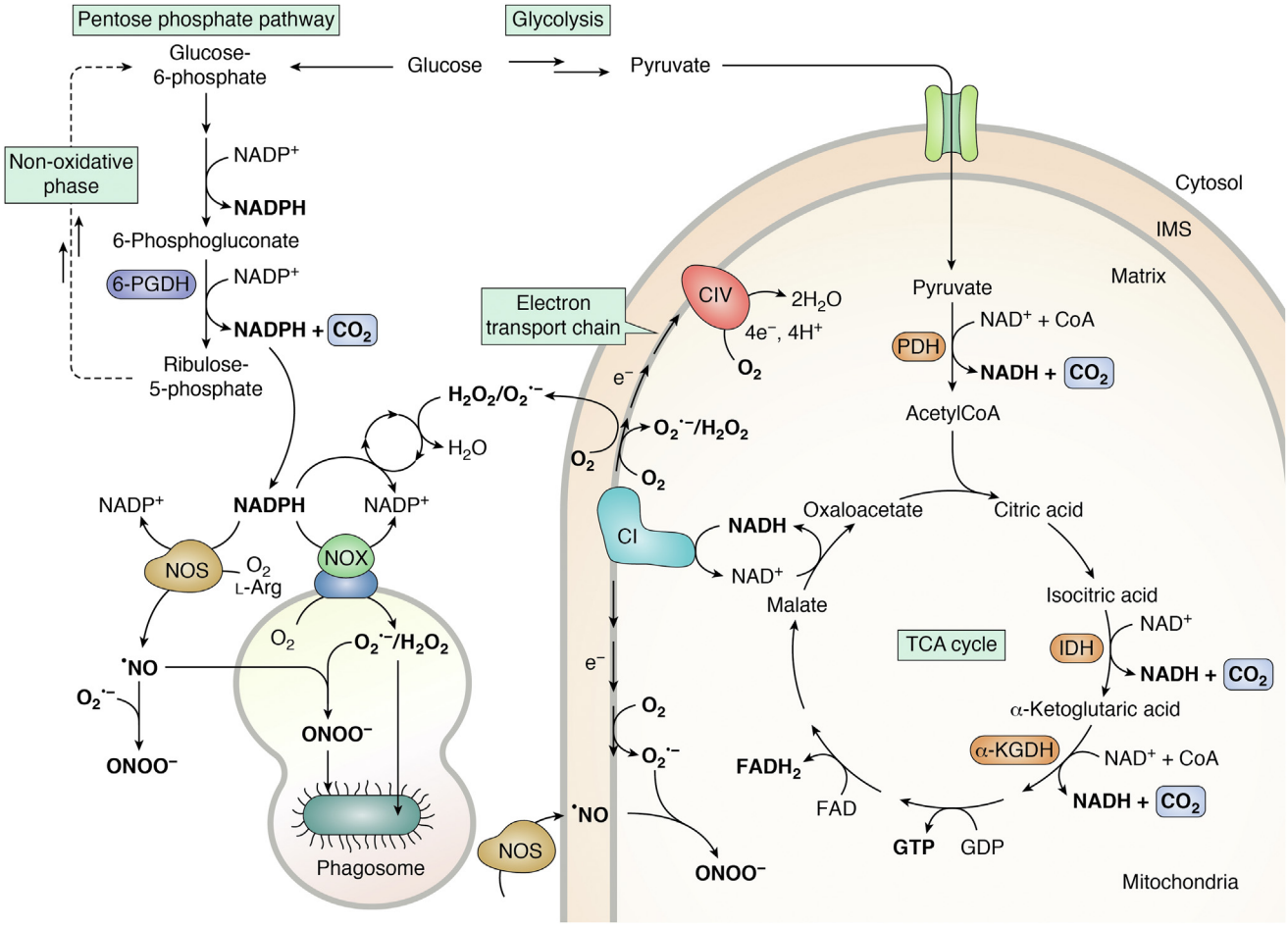

Figure 1. Decarboxylation reactions in redox pathways and relation with peroxide metabolism. Glycolysis yields pyruvate in the cytosol and enters mitochondria; in mitochondria pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase catalyze oxidative decarboxylation reactions that convert NAD^+ to NADH and generate CO_2. The reducing equivalents of NADH and $FADH_2$ feed the mitochondrial electron transport chain in a process that finalizes with the four-electron reduction of molecular oxygen to water. In the course of electron transport there is small percentage leakage for the monovalent reduction of oxygen to $O_2^{\bullet-}$ that in turn dismutates enzymatically to H_2O_2. Mitochondria can emit H_2O_2 to extramitochondrial compartments. In the cytosol, glucose can also follow the pentose phosphate pathway, which in its oxidative phase yields NADPH and CO_2, the latter by the action of 6-phosphogluconate dehydrogenase. The reducing equivalents of NADPH can be used for a number of redox reactions, most notably in the context of this review for the formation of $O_2^{\bullet-}$ (and subsequently H_2O_2) *via* the membrane bound NADPH oxidases (*e.g.*, toward the phagosome), $^{\bullet}NO$ synthesis by NOS, and peroxide detoxification (in GSH- or thioredoxin-based peroxidatic systems). TCA cycle, tricarboxylic acid cycle.

incorporate CO_2 (or bicarbonate, HCO_3^-) into organic molecules play central roles interconnecting catabolism, anabolism, and energy metabolism in mammalian cells (1, 26). However, in mammalian cells, the levels of metabolic CO_2 production normally largely exceed CO_2 consumption and, as a result, there is a net and significant CO_2 evolution and release. Approximately, 1 kg CO_2 per day is produced by one person (26).

It is well known that CO_2 can promote modifications in proteins under physiological conditions by its combination with neutral amines to form carbamates (27). This post-translational carbamylation reaction involves the nucleophilic attack by CO_2 on *N*-terminal amino or lysine ε-amino groups (28). It is now also established that CO_2 also participates in oxidative posttranslational modifications reactions mediated by H_2O_2 and peroxynitrite; these processes require the intermediate formation of CO_2-derived species such as HCO_4^- and $ONOOCO_2^-$, respectively, that modulate amino acid oxidation and nitration (4, 8, 13).

CO_2-dependent formation of bicarbonate and connection with acid-base homeostasis

Once formed, CO_2 can be slowly hydrated to carbonic acid (H_2CO_3) and in turn H_2CO_3, a weak acid, is deprotonated to HCO_3^- (reviewed in (8) and references therein). The hydration reaction is reversible and therefore CO_2 in solution is in equilibrium with bicarbonate:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \qquad k_f = 0.03\ s^{-1};\ k_r = 20\ s^{-1} \qquad [1]$$

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^- (fast) \quad K_a = 5.7 \times 10^{-4} \qquad [2]$$

This route of equilibration is rather slow at neutral pH, but CA, which is widely distributed in mammalian tissues and microorganisms and extremely efficient enzymes (22, 29–31), catalyze reaction [1] and helps the system approach equilibrium *in vivo*. To note, while CA is mostly cytosolic, some tissues such as liver contain mitochondrial isoforms (19, 29, 32).

The CO_2/HCO_3^- molecular pair influences cell and tissue pH, and conversely, pH influences the CO_2/HCO_3^- equilibrium. Indeed, the following relationship among these parameters applies according to the Henderson–Hasselbalch equation (33):

$$\mathrm{pH} = pK_a + log\frac{[HCO_3^-]}{[CO_2\ (aq)]} \qquad (3)$$

CO_2 effectively acts as the weak acid in this system, and HCO_3^- is its conjugate base. Importantly, dissolved CO_2 is in equilibrium with gaseous CO_2:

$$CO_2\ (aq) \rightleftharpoons CO_2\ (g) \qquad [4]$$

The equilibrium constant for this reaction is defined by Henry's law (*i.e.*, the amount of dissolved CO_2 in a tissue or fluid is proportional to its partial pressure, PCO_2).

Reactions 1 and 2 can be combined for the CO_2/HCO_3^- equilibrium in solution:

$$H^+ + HCO_3^- \rightleftharpoons CO_2\ (aq) + H_2O \qquad [5]$$

$$K_{app} = \frac{[H^+][HCO_3^-]}{[CO_2(aq)]} \qquad ([6])$$

The apparent p*K*a for this acid-base system applicable in human physiology to be used in Equation 3 is *ca.* 6.1 to 6.4 (34, 35) and is a result of the various participating equilibria (Fig. 2, panel *A*).

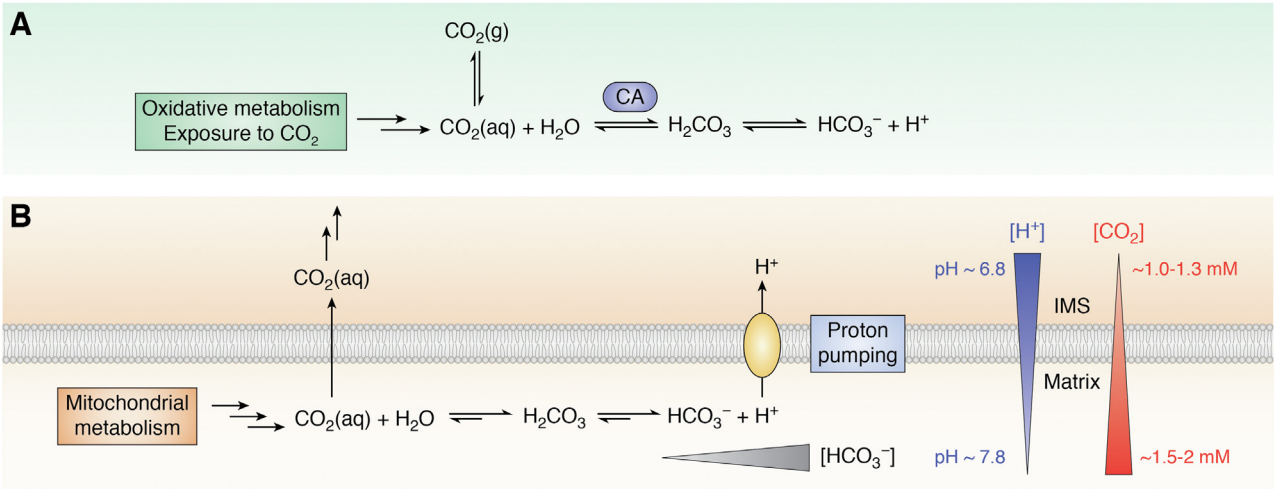

Figure 2. The carbon dioxide–bicarbonate equilibria in mammalian tissues. *A*, metabolic or environmental CO_2 exposure results in dissolved CO_2, which *via* CA-catalyzed hydration yields H_2CO_3, in equilibrium with HCO_3^-. CO_2 in solution equilibrates with CO_2 gas. The apparent p*K*a of the overall CO_2/HCO_3^- equilibria is 6.1 to 6.4. All the indicated processes are readily reversible. Modified from (34). *B*, mitochondrial metabolism produces large levels of CO_2 than can either hydrate to H_2CO_3 (nonenzymatically or enzymatically depending on tissue) or diffuse out as a function of concentration gradient. Importantly, the pH of the matrix in active mitochondria is basic due to the pumping of H^+ to the intermembrane space, which generates an electrochemical gradient across the inner membrane. This basicity facilitates more dissociation of H_2CO_3 to HCO_3^- than in other cellular and extracellular compartments under physiological conditions. CA, carbonic anhydrase.

However, the actual p*K*a for H_2CO_3 has been recently reported as 3.5 (35).

$$pK_a = pK_{app} - \log K_D \quad [7]$$

where $K_D=[CO_2]/[H_2CO_3]$ [8]

The value of K_D is not known exactly and has been a major reason for the difficulties in obtaining the exact value of K_a for Equation 2.

Under physiologically relevant conditions, $[CO_2]$ in tissues ranges in the order of 1 to 2 mM in equilibrium with $[HCO_3^-]$ (22, 33, 34, 36–39); the concentration of latter will ultimately depend on the local pH. For instance, in plasma at pH 7.4 and 37 °C the $[CO_2]$ and $[HCO_3^-]$ are *ca.* 1.3 mM and 24 mM, respectively. While the $[CO_2]/[HCO_3^-]$ ratio is close to 1/20 at pH 7.4; this value changes in cell/tissue compartments having different pH values (*e.g.*, cytosol *ca.* 7.0, mitochondrial matrix *ca.* 7.8–8.0, Golgi apparatus *ca.* 6.6) (8, 40). The high concentration of HCO_3^- in equilibrium with its conjugated acid (H_2CO_3/CO_2 (aq)) plays a central role as a physiological buffer system in human biology (33, 34). Thus, acting as a homeostatic pH control mechanism, changes in tissue CO_2 or H^+ levels influences the equilibrium in Equation 5. Acid-base disorders (acidosis or alkalosis) result in a primary change in the arterial PCO_2 ("respiratory" origin) or HCO_3^- concentration ("metabolic" origin) (38, 39). For example, excess metabolic formation of organic acids (*e.g.*, lactate *via* anaerobic glycolysis) drives the equation to the right, consuming HCO_3^- and generating CO_2 (34, 38, 39). Deviations from physiological human arterial plasma CO_2 and HCO_3^- concentrations in clinical conditions range from 1 mM to 3 mM for CO_2 and 10 to 30 mM HCO_3^- (38, 39, 41, 42) and require medical intervention (43). The relationship among CO_2/HCO_3^- levels, cell/tissue pH, physiological acid-base regulatory mechanisms, and their disruption in disease conditions has been reviewed elsewhere (34, 38, 39).

Importantly, CO_2 permeates across membranes as a function of concentration gradients and associated to the dynamics of formation and consumption in different compartments (34). In the case of mitochondria, they are usually the main sources of CO_2 in mammalian cells under most metabolic conditions, with a net outflux of CO_2 through membrane permeation (19, 22); thus, the mitochondrial $[CO_2]$ is usually higher than cytosolic $[CO_2]$, establishing a CO_2 concentration gradient (Fig. 2*B*).

CO_2 dynamics in mitochondria and beyond

In a metabolically active mitochondria with a matrix pH of 7.8 to 8.0 (40, 44), $[CO_2]$ can reach values ≥2 mM (8, 19); the levels of mitochondrial $[HCO_3^-]$ could be well above 100 mM (calculated from Equation 3), especially on those cell types where CO_2 hydration is rapidly catalyzed by mitochondrial isoforms of CA (*i.e.*, liver) (Fig. 2*B*). Otherwise, CO_2 permeation from mitochondria to the cytosol outcompetes the nonenzymatic hydration (19). In contrast to CO_2, HCO_3^- is not permeable across lipid bilayers, and therefore in mammalian cells, its transport across membranes relies on the existence of bicarbonate transporters (34).

Overall, the mitochondrial $[CO_2]$ could have fluctuations depending on the metabolic commitment to oxidative decarboxylation reactions, local pH changes[2], mitochondrial CA activity, and CO_2 consumption *via* HCO_3^--dependent carboxylation reactions of the urea cycle (*i.e.*, catalyzed by carbamoyl phosphate synthetase I) and gluconeogenesis (*i.e.*, catalyzed by pyruvate carboxylase) (Fig. 3).

As mitochondria also constitute main intracellular sources of H_2O_2 in redox-dependent processes (45), its formation and emission in parallel with that of CO_2 lays the ground for their synergistic interactions. In addition, it has been recently shown that enhanced mitochondrial-derived H_2O_2 release to the cytosol leads to activation of the PPP in mammalian cells (46) (Fig. 1).

Early indications of CO_2 in the modulation of redox processes

While CO_2 has been classically considered as an almost unreactive product of mammalian cell redox metabolism, evidence laboriously accumulated over several decades substantiates that both CO_2 and HCO_3^- participate in the modulation of free radical and peroxide-mediated reactions. Thus, a recapitulation of key early discoveries connecting CO_2/HCO_3^- with oxygen free radicals and peroxide biochemistry will be provided first.

Radiation chemistry data: Kinetics of the reaction of $^{\bullet}OH$ with HCO_3^- and the detection of carbonate radicals

The modulatory action of the CO_2/HCO_3- in free radical and redox processes was originally hinted in the 1960s by radiation chemistry experiments. Indeed, the first observation of the carbonate radical by pulse radiolysis of aqueous solutions was in 1962, by Hart and Boag, who observed a composite spectrum of $CO_3^{\bullet-}$ and the hydrated electron (e^-_{aq}) after pulse radiolysis of deaerated 0.5 M sodium carbonate, with only the carbonate radical spectrum seen in aerated solution since oxygen removes the hydrated electron (47).The extinction coefficient of the carbonate radical at 600 nm is 1860 M^{-1} cm^{-1} (48), and the reactions can be monitored using ultrafast kinetics spectrophotometry. In 1965, using pulse radiolysis of water, the rate constants of the reaction of hydroxyl radical ($^{\bullet}OH$, $E^{ó\bullet}_{OH/H_2O}$ = +2.32 V) with HCO_3^- were published (49–51).

$CO_3^{\bullet-}$ can be conveniently produced by radiolysis of water containing HCO_3^-/CO_3^{2-} and saturated with N_2O (to scavenge e_{aq}^-, producing $^{\bullet}OH$). The reactions are:

$$^{\bullet}OH + HCO_3^- \rightarrow H_2O + CO_3^{\bullet-} \quad [9]$$

$$^{\bullet}OH + CO_3^{2-} (+H^+) \rightarrow H_2O + CO_3^{\bullet-} \quad [10]$$

[2] In state 3 mitochondria, the pH value of the intermembrane space and matrix are *ca.* 6.8 and 7.8, respectively, due to proton pumping associated to the electron transport chain.

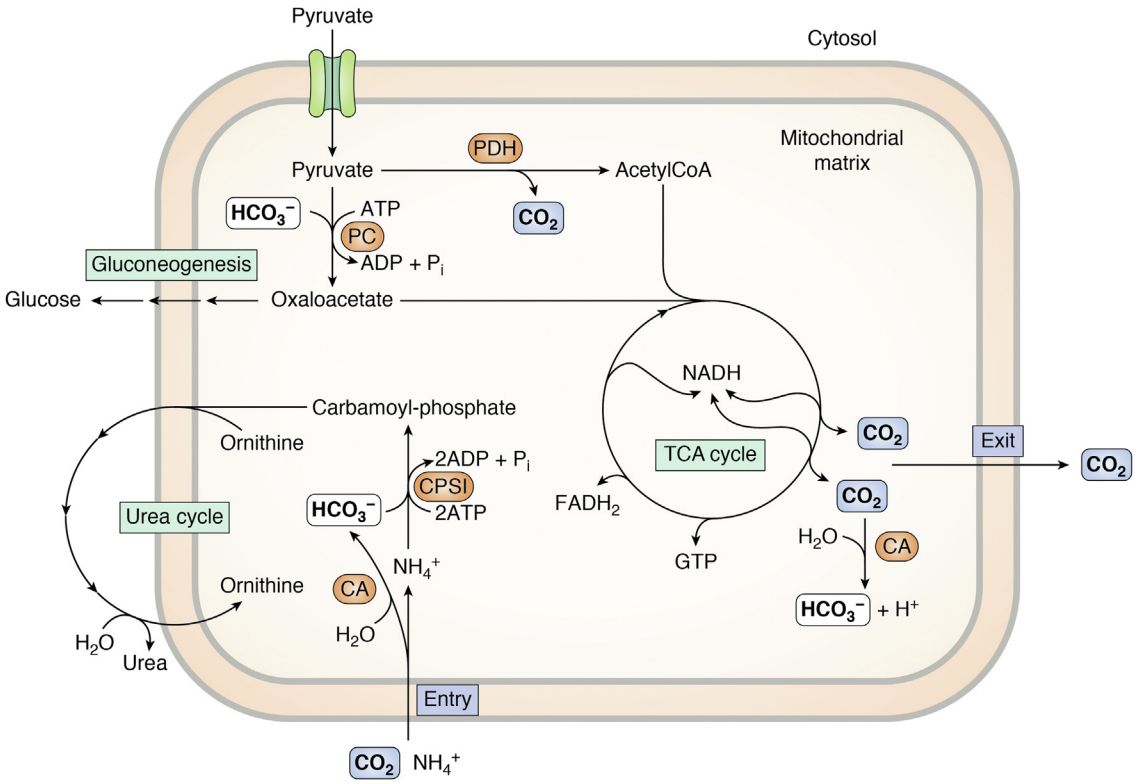

Figure 3. Carbon dioxide and bicarbonate dynamics in mitochondria. Mitochondria are key cellular sources of CO_2 *via* oxidative decarboxylation reactions linked to aerobic energy metabolism. In the case of mammalian liver mitochondria, they can be major consumers of HCO_3^- during ureogenesis or gluconeogenesis. Indeed, the ATP-dependent reactions catalyzed by carbamoyl phosphate synthase (CPSI) and pyruvate carboxylase (PC), respectively, use as substrate HCO_3^-. Under appropriate metabolic requirement liver mitochondria are equipped with considerable CA activity to convert CO_2 to HCO_3^-, which otherwise diffuses out of mitochondria. Moreover, if needed, cytosolic CO_2 can diffuse into the mitochondria (19). CA, carbonic anhydrase.

($k_9 = 8.5 \times 10^6$ M^{-1} s^{-1}, $k_{10} = 3.9 \times 10^8$ M^{-1} s^{-1}, that is, $k_{10}/k_9 \sim 46$ at ambient temperature). These rate constants have been obtained over a wide temperature range (52). About 10% of radicals are $H^\bullet$ atoms, which react much more slowly compared to $^\bullet OH$ ($k_{11} = 4.4 \times 10^4$ M^{-1} s^{-1}) (53):

$$H^\bullet + HCO_3^- \rightarrow H_2 + CO_3^{\bullet-} \quad [11]$$

Under most biological conditions, the reaction of $^\bullet OH$ with HCO_3^- is not a predominant one[3] since the rate constant (k_9) is much lower than the rate constants of $^\bullet OH$ with most biological targets that are close to the diffusion-control limit, *ca.* 10^9 M^{-1} s^{-1}; on the other hand, the reaction of $^\bullet OH$ with CO_3^{2-}, although with a higher rate constant, is also of little biological relevance because of the marginal amounts of CO_3^{2-} existing within the pH range in mammalian cells.

$$HCO_3^- \rightleftharpoons H^+ + CO_3^{2-} \quad pK_{12} = 10.3 \quad [12]$$

A compilation of rate constants for reactions of $CO_3^{\bullet-}$ with 181 substances has been published (48), but this is now outdated and incomplete. Some additional rate constants can be obtained in more recent works, which include the reaction of $CO_3^{\bullet-}$ with lipoic acid, desferrioxamine, and 5,5-dimethyl–1–pyrroline-N-oxide, among other target molecules (54–57). $CO_3^{\bullet-}$ can promote both protein and DNA oxidation (8, 58).

The actual existence of $CO_3^{\bullet-}$ in the anionic form at physiological pH was for some time a subject of debate and depends on the pK_a value for:

$$HCO_3^\bullet \rightleftharpoons H^+ + CO_3^{\bullet-} \quad [13]$$

While pK_{13} values of 9.6 or 7.9 were reported in some early studies (summarized in (48)), a later study, using a flow system to irradiate mixtures of H_2CO_3 and HCO_3^- within 50 ms of their formation, has demonstrated that the $HCO_3^\bullet$ is a strong acid, p$K_{13} < 0$, contrary to the earlier reports (the rate constant for reaction of $^\bullet OH$ with H_2CO_3 is 7×10^4 M^{-1} s^{-1} at about 5 °C) (59).

Although less oxidizing than $^\bullet OH$, $CO_3^{\bullet-}$ ($E^{\circ}_{CO_3^{\bullet-}/HCO_3^-} = +1.78$ V) is a strong one-electron oxidant that acts by both electron transfer and hydrogen abstraction mechanisms to produce radicals from the oxidized targets (8, 58). The one-electron oxidation of HCO_3^- by $^\bullet OH$ is thermodynamically favored with the net value for the reaction 9 of + 0.54 V (58, 60).

Production of carbonate radical secondary to xanthine oxidase–dependent reactions

In the biochemical literature, work by Hodgson and Fridovich in 1976 postulated the formation of $CO_3^{\bullet-}$ during the

[3] However, conditions that result in $[HCO_3^-] > 100$ mM favor that a fraction of $^\bullet OH$ produced in tissue radiation exposure could evolve to $CO_3^{\bullet-}$.

turnover of xanthine oxidase in the presence of CO_2/HCO_3^- (61). Utilizing acetaldehyde and molecular oxygen as substrates, xanthine oxidase catalyzes the oxidation of acetaldehyde to acetic acid and the concomitant formation of $O_2^{\bullet-}$ and H_2O_2 as follows[4]:

$$\text{Acetaldehyde} + O_2 \xrightarrow{\text{xanthine oxidase}} \text{acetic acid} + H_2O_2 + O_2^{\bullet-} \quad (14)$$

In the presence of carbonated solutions at pH = 10, the xanthine oxidase turnover resulted in spontaneous chemiluminescence, which was dependent on carbonate in a concentration-dependent manner (61). As in the presence of transition metal traces such as iron, $O_2^{\bullet-}$ and H_2O_2 evolve to $^{\bullet}OH$ *via* the Haber–Weiss mechanism (Equation 15) (62); light emission was attributed to the following reaction steps:

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Fe} OH^- + {}^{\bullet}OH + O_2 \quad (15)$$

$$^{\bullet}OH + CO_3^{2-} \rightarrow OH^- + CO_3^{\bullet-} \quad [16]$$

$$2\ CO_3^{\bullet-} \rightarrow \rightarrow h\nu + \text{products} \quad [17]$$

Indeed, the $^{\bullet}OH$-dependent formation of $CO_3^{\bullet-}$ (Equation 16) is followed by its recombination reaction (Equation 17) to yield excited species that decay with light emission in the blue/green region (400–550 nm) (61, 63, 64). The rate constant of reaction 17 determined at 0.1 M Na_2CO_3 is $2.0 \times 10^7\ M^{-1}\ s^{-1}$ (65).

The presence of carbonated solutions also increases luminol chemiluminescence induced during xanthine oxidase turnover (66), likely by enhancing luminol oxidation by $CO_3^{\bullet-}$ (67). A similar stimulatory effect of bicarbonate was later observed during peroxynitrite-dependent luminol chemiexcitation (*vide infra*) (10)

Peroxynitrite, an unstable peroxide in carbonated solutions

Peroxynitrite is the product of the diffusion-controlled reaction between $O_2^{\bullet-}$ and $^{\bullet}NO$.

$$O_2^{\bullet-} + {}^{\bullet}NO \rightarrow ONOO^- \quad [18]$$

$$ONOO^- + H^+ \rightleftharpoons ONOOH \quad [19]$$

Peroxynitrite anion is in equilibrium with peroxynitrous acid (ONOOH) with a p*K*a = 6.8, meaning that both species coexist under biologically relevant conditions. The biological chemistry of peroxynitrite has been reviewed recently (8). One of the key reactions of peroxynitrite is that with CO_2, this reaction was first hinted in 1969 by Keith and Powell (68),who observed the instability and rapid decay of peroxynitrite in carbonated buffers, which the authors referred to as experimentally "intolerable." This observation was later substantiated in specific studies that revealed the change in reactivity that bicarbonate buffers imposed on peroxynitrite-mediated reactions, starting with a study in which the intermediate formation of a $ONOOCO_2^-$ adduct was proposed (10). Mechanistic and fast kinetic studies revealed the reaction to occur strictly between $ONOO^-$ and CO_2 and not with other acid-base species (69) (recently reviewed in (5), *vide infra*).

The formation of peroxymonocarbonate

In 1986, Flanagan *et al.* established for the first time that HCO_3^--containing solutions in the pH range 7.0 to 9.5 under excess H_2O_2 lead to the formation of peroxymonocarbonate (HCO_4^-) as inferred by Raman and ^{13}C-NMR spectroscopy (70). The possibility that HCO_4^- could participate in biochemical reactions was postulated as early as in 1978 (71). Later on, the actual reactions, kinetics, and equilibria involving the CO_2/HCO_3^- molecular pair and H_2O_2 that lead to HCO_4^- formation was disclosed (72, 73) and will be analyzed in detail later in the text.

Interactions of bicarbonate with transition metal centers: Oxidation and disproportionation reactions

Bicarbonate may also modulate transition metal–dependent oxidation processes. On one hand, HCO_3^- may promote transition metal–dependent site-specific oxidation of biotargets; in particular, HCO_3^- facilitates Fenton-type reactions during amino acid oxidation by H_2O_2 (74–76). On the other hand, HCO_3^-–Mn complexes catalyze the disproportionation of H_2O_2 in a catalase-like manner (77). Likewise, it is possible that HCO_3^-–Mn complexes may favor $O_2^{\bullet-}$ dismutation (78).

The participation of HCO_3^- in transition metal–dependent redox reactions triggered by H_2O_2 and $O_2^{\bullet-}$ in biology remains largely unexplored. Thus, while the formation of redox active complexes of HCO_3^- with transition metals is possible and opens possibilities for their participation in the modulation of oxidative reactions *in vitro*, their role *in vivo* remains only speculative.

Chemical aspects of the reaction of CO_2 with peroxides

Herein, we will analyze how the reactions of CO_2 with peroxynitrite and/or H_2O_2 lead to CO_2-derived reactive intermediates that promote one- and two-electron oxidations.

Peroxynitrite and carbonate radical

The nucleophilic character of peroxynitrite anion (8, 79) enables its fast reaction with CO_2. Indeed, the pH-independent rate constant for the reaction between $ONOO^-$ and CO_2 has been determined as $k_{20} = 3 \times 10^4\ M^{-1}\ s^{-1}$ (25 °C) (69) or $5.8 \times 10^4\ M^{-1}\ s^{-1}$ (37 °C) (64); the product of the reaction is a transient adduct (eq.[20]), nitrosoperoxocarboxylate ($ONOOCO_2^-$) that readily undergoes homolysis to yield $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ in 35% yields (Equation 21), with the rest isomerizing to nitrate (NO_3^-) (recently reviewed in (5, 8)).

[4] The reduction of molecular oxygen at the xanthine oxidase active site can occur *via* one- or two-electron mechanisms to yield $O_2^{\bullet-}$ and H_2O_2, respectively. The relative formation of $O_2^{\bullet-}$ as a function of molecular oxygen consumption is commonly known as percent univalent flux; this value varies depending on substrate type and concentration, oxygen tension, and general reaction conditions. For instance, low turnover substrates increase percent univalent flux (176, 177).

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \quad [20]$$

$$ONOOCO_2^- \rightarrow {}^{\bullet}NO_2 + CO_3^{\bullet-} \quad [21]$$

Because of the velocity of the reaction 20 which is a function of the product of k_{20} times $[CO_2]$, the biological chemistry of peroxynitrite is highly influenced by the existing levels of CO_2 in cells and tissues; CO_2 represents a key biological target of peroxynitrite (8). For a comparative analysis of the relative weight of the CO_2 reaction on the fate of peroxynitrite *versus* that of other biotargets, see (80, 81). For example, the reaction of peroxynitrite with cytosolic concentrations of CO_2 (*ca.* 1.3 mM) yields a pseudo–first order rate constant in the order of 60 s^{-1}, a reference value that is utilized to assess the relevance of alternative routes of peroxynitrite consumption (80). For example, this value is much larger than that of the reaction of peroxynitrite with cytosolic GSH (*ca.* 10 s^{-1}) and usually smaller than that of the reactions with peroxiredoxins[5] (*ca.* > 100 s^{-1}) (80, 82).

$CO_3^{\bullet-}$ and ${}^{\bullet}NO_2$ are good one-electron oxidants ($E^{ó}_{NO2/NO2-}$ = 0.9 V); in addition, ${}^{\bullet}NO_2$ can participate in nitration reactions (58, 83). In this respect, the presence of CO_2 usually promotes peroxynitrite-dependent protein tyrosine nitration, as $CO_3^{\bullet-}$ readily oxidizes tyrosine to tyrosyl radical, which then undergoes a fast recombination reaction with ${}^{\bullet}NO_2$ to yield protein 3-nitrotyrosine (8, 84). Also, thiol oxidation by peroxynitrite in the presence of CO_2 shifts from the direct two-electron process (*i.e.*, to sulfenic acid) towards $CO_3^{\bullet-}$- and ${}^{\bullet}NO_2$-mediated one-electron oxidations to thiyl radical (56, 85, 86) (Fig. 4)

Hydrogen peroxide and peroxymonocarbonate

CO_2 reacts slowly with H_2O_2 to yield HCO_4^- (p*K*a = 3.4, corresponding to the dissociation of the carboxylate group) in two different reactions (73). On one hand, CO_2 can undergo perhydration with H_2O_2 (Equation 22), in a reaction analogous to that of hydration (addition of water) described previously (Equation 1). The process can be decomposed in two separate reactions, namely the perhydration reaction *per se* 22 to yield H_2CO_4, followed by deprotonation to reach an acid-base equilibrium 23.

$$CO_2\ (aq) + H_2O_2 \rightleftharpoons H_2CO_4\ k_f = 0.02\ M^{-1}\ s^{-1};\ k_r = 22\ s^{-1} \quad [22]$$

$$H_2CO_4 \rightleftharpoons H^+ + HCO_4^-\ (fast)\ K_a = 2.9 \times 10^{-4}\ M \quad [23]$$

H_2O_2 reacts more rapidly than water with CO_2 (0.02 $M^{-1}s^{-1}$ *versus* ~8 × 10^{-5} $M^{-1}s^{-1}$).

Alternatively, HO_2^-, the conjugated base of H_2O_2 (p*K*a = 11.7), adds as a nucleophile to CO_2 (the carbon atom is an electrophilic Lewis acid center in CO_2) (Equation 24), in a reaction analogous to that of $ONOCO_2^-$ formation (Equation 20).

[5] Concentration values of different peroxiredoxins largely vary depending on cell type, with over a 100-fold change from values in the range of 2 to 240 μM (80, 82, 178); therefore, its competition with CO_2 for peroxynitrite goes from modest to substantial.

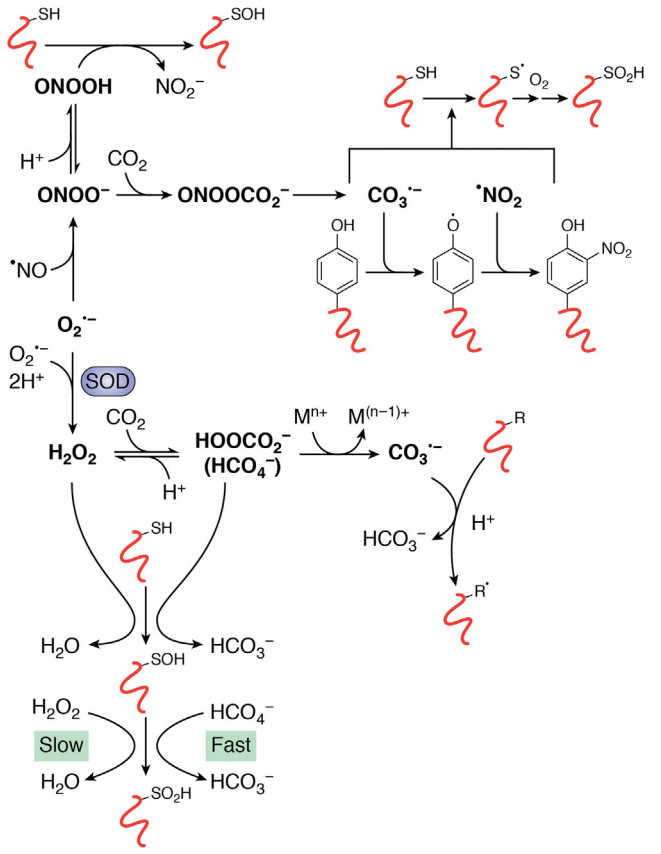

Figure 4. Carbon dioxide in peroxide-dependent oxidation reactions. Superoxide radical can yield peroxynitrite anion ($ONOO^-$) or H_2O_2 by its reactions with ${}^{\bullet}NO$ or another $O_2^{\bullet-}$ molecule (catalyzed by SOD), respectively. Peroxynitrite anion protonates to peroxynitrous acid (ONOOH) which reacts in a two-electron oxidation process with thiols to yield the corresponding sulfenic acid (RSOH). Alternatively, $ONOO^-$ reacts with CO_2 to yield a transient species, $ONOOCO_2^-$, that undergoes homolysis to the free radicals $CO_3^{\bullet-}$ and ${}^{\bullet}NO_2$. Peroxynitrite-derived radicals promote one-electron oxidations in biomolecules to yield, for example, tyrosyl and thiyl radicals which subsequently evolve to stable products such as 3-nitrotyrosine (a specific biomarker of ${}^{\bullet}NO$-derived oxidants) or sulfinic acid (RSO_2H). H_2O_2 and HCO_4^- (formed in the presence of CO_2, see (Equations 22–24)) can also directly oxidize thiols to sulfenic acid. Moreover, they can also promote thiol hyperoxidation to sulfinic acid, a process that is typically faster for HCO_4^- than H_2O_2. Finally, in the presence of transition metal centers HCO_4^- can evolve to $CO_3^{\bullet-}$ and promote one-electron oxidations. SOD, superoxide dismutase.

$$CO_2\ (aq) + HO_2^- \rightleftharpoons HCO_4^-\ k_f = 280\ M^{-1}\ s^{-1};\ k_r = 1.8 \times 10^{-3}\ s^{-1} \quad [24]$$

The estimated rate constant for the reaction of HO_2^- with CO_2 (280 $M^{-1}s^{-1}$) at 25 °C is substantially smaller than that for OH^- (8500 $M^{-1}s^{-1}$) (73) and $ONOO^-$ (3×10^4 $M^{-1}s^{-1}$) (69).

Both reactions 22 and 24 are rather slow and contribute to HCO_4^- formation under physiologically relevant conditions; indeed, while the rate constant of reaction 24 is higher than that of reaction 22, the opposite occurs is terms of concentration of peroxide (*i.e.*, H_2O_2 *versus* HO_2^-) at physiologically relevant pH. Thus, at pH 7.4, it is estimated that reactions 22 and 24 contribute in 59% and 41% to HCO_4^- formation, respectively (87). Recognizably, pH changes will affect both the CO_2/HCO_3^- and H_2O_2/HO_2^- ratios and will be reflected on the relative contribution and velocity of reactions 22 and 24. In this regard, the contribution of HO_2^- to initial HCO_4^-

formation increases with increasing pH, dominating above pH 8. The elementary reactions and their equilibrium and rate constants for peroxymonocarbonate formation have been comprehensively reported in (73).

For practical and experimental purposes, the following overall equilibrium applies:

$$H_2O_2 + HCO_3^- \rightleftharpoons HCO_4^- + H_2O \quad [25]$$

$$\frac{[HCO_4^-]}{[H_2O_2] \times [HCO_3^-]} = K_{eq} = 0.31\ M^{-1} \text{at } 25°C. \quad (26)$$

Thus, at equilibrium, HCO_4^- concentration can be calculated as follows:

$$[HCO_4^-] = 0.31 \times [H_2O_2] \times [HCO_3^-] \quad [27]$$

which for 25 mM HCO_3^- (0.025 M) typically represents *ca*. 1% of initial H_2O_2 (87).

HCO_4^- (E^6= +1.8 V) is a strong two-electron oxidant, with a redox potential similar to that of H_2O_2 (E^6= +1.77 V). However, HCO_4^- typically reacts with target molecules at rates 100 to 1000 times faster than those of H_2O_2 (86–88). For instance, the second order rate constants of H_2O_2 and HCO_4^- with GSH [Equations 28a and 28b] are 1.9 and 1.6 × 10^2 $M^{-1}s^{-1}$, respectively (reviewed in (87)). This reactivity inversely correlates with the p*K*a of the conjugated acid of the peroxide leaving group[6].

$$H_2O_2 + GS^- \rightarrow HO^- + GSOH \quad [28a]$$

$$HCO_4^- + GS^- \rightarrow CO_3^{2-} + GSOH \quad [28b]$$

HCO_4^- also reacts faster with both free and protein-bound methionine (*e.g.*, in α1-proteinase inhibitor) than H_2O_2, to yield the corresponding two-electron oxidation product methionine sulfoxide (88). As methionine oxidation represents a reaction that can regulate protein function *in vitro* and *in vivo* (*e.g.*, pyruvate kinase M2) (89, 90), direct assessment of the role of CO_2 on peroxide-dependent methionine oxidation (88, 91–94) becomes necessary[7].

On the other hand, HCO_4^- can be reduced by one electron *via* transition metal centers to yield $CO_3^{\bullet-}$, likely

$$HCO_4^- + Me^{n+} \rightarrow CO_3^{\bullet-} + Me^{(n-1)+} + OH^- \quad [29]$$

Thus, the CO_2-dependent formation of HCO_4^- leads to the activation of H_2O_2 for both two electron 28 and radical 29 chemistry, the latter in combination with transition metals (73, 87)

Peroxymonocarbonate can be also generated at the active site of redox enzymes and promote CO_2-dependent oxidations and peroxidations (87, 95–100). Two well-known examples of these processes have been described for Cu/Zn superoxide dismutase (96, 98, 99) and xanthine oxidase (100). Once formed, the reduction of HCO_4^- at the enzyme active sites yields $CO_3^{\bullet-}$, which is the proximal oxidant responsible for the CO_2-dependent one-electron oxidations. Formation of ternary complexes at the enzyme active site with HO_2^- and CO_2 to yield metal-bound HCO_4^- has been invoked to explain how bicarbonate buffers accelerate H_2O_2-dependent oxidations (reviewed in (87)).

CO_2 in the modulation of peroxynitrite-dependent and hydrogen peroxide–dependent oxidations in biology

Kinetic considerations

CO_2 is a relevant biological target of peroxynitrite (Equation 20) and determines part of its fate and half-life in different biological compartments (8, 80, 81). The "CO_2 pathway" of peroxynitrite decomposition is in competition with other relevant reactions including its catabolism by peroxiredoxins (8, 24). Indeed, kinetic analysis taking into consideration existing concentrations of CO_2 in intracellular compartments, pH, and the second order rate constant of reaction 20 indicates a pseudo–first order rate constant of peroxynitrite decay by CO_2 in the order of 60 to 100 s^{-1}. This k' value translates into a half-life ($t_{1/2} = \ln 2/k'$) in the order of 10 ms, a time scale that in extracellular compartments allows the diffusion of peroxynitrite a mean distance >5 μm (8, 101, 102). Obviously, the half-life of peroxynitrite significantly shortens intracellularly where other fast reacting targets such as peroxiredoxins ($k' >$ 100 s^{-1}) react (for a detailed kinetic analysis see (8, 80, 81)). Obviously, changing levels of CO_2 will affect the route and the extent by which peroxynitrite decomposes into $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals (and nitrate, NO_3^-). It is important to note that in extracellular environments, the CO_2 pathway exerts significant control on the reactivity and diffusion of peroxynitrite due to the scarcity of other biotargets that can compete at significant rates (12, 80). Indeed, as the half-lives of $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ are very short (< ms to μs time scale), in extracellular compartments, the CO_2 reaction with peroxynitrite usually represents a major "decay" pathway that partially limits peroxynitrite diffusion to cells and focusses its oxidative chemistry in extracellular or plasma membrane targets (102–107) (Fig. 5). In the case of mitochondria, the high levels of CO_2 promote the organelle-specific oxidation and nitration of mitochondrial proteins by peroxynitrite, even under basal conditions (80, 82, 108, 109).

In the case of H_2O_2, due to the kinetic and equilibria properties of its reactions with CO_2 (either with H_2O_2 22 or HO_2^- 24), at any given time, only a small fraction would be present as HCO_4^-. The H_2O_2/CO_2 pathway will be in competition with other H_2O_2-consuming processes, in particular, those with peroxiredoxins, catalase and GSH peroxidase that occur at very fast rates (14, 24, 87, 110) and with H_2O_2 diffusion and transport across membranes (Fig. 5). The slow velocity of formation of HCO_4^- and the fractional amount found at equilibrium questions, at first glance, the possible role of HCO_4^- in biological oxidations. However, additional factors must be taken into consideration. First, consumption of HCO_4^- by reactions with target molecules will continuously shift the reactions 22 and 24 to the right. Second,

[6] For the reactions of H_2O_2 and HCO_4^-, the p*Ka* of the corresponding leaving groups (*i.e.*, OH^-, CO_3^{2-}) are 15.7 and 10.3, respectively.

[7] In the case of the reaction of methionine with peroxynitrite, the presence of CO_2 would divert the two-electron oxidation of the amino acid toward one-electron oxidations that yield methional and ethylene (91, 92). $CO_3^{\bullet-}$ reacts very fast with methionine 1.2 × 10^8 $M^{-1}s^{-1}$ (48, 179).

the formation of HCO_4^- can be accelerated by CA (73). Also, HCO_4^- formation is favored in the presence of lipids and proteins, shifting the equilibrium of reaction 25 to the right and increasing K_{eq} values (16). Thus, it is kinetically possible for the reaction of H_2O_2 with CO_2 to generate a biologically relevant flux of HCO_4^-.

In cellular compartments such as the macrophage phagosome, the drop of pH values and increase of CO_2 levels observed during the activation of cytotoxic processes (111–113) are expected to be influential on the biological chemistry of both peroxynitrite and H_2O_2 (Fig. 6).

Effect of CO_2 on peroxynitrite-dependent processes

Initial work assessing the effect of CO_2 in the microbicidal effect of extracellularly added peroxynitrite on bacteria and parasites showed a protective effect in cell killing (12, 114, 115). These observations are due to the fact that at low microbial suspension densities (intercellular distances of several micrometer among them), the reaction of extracellularly added peroxynitrite with CO_2 (*i.e.*, bicarbonate-containing solutions) and its decay by isomerization and radical (self-coupling and crosscoupling) recombination reactions outcompetes peroxynitrite diffusion to cells (5, 116) (Fig. 5). However, when considering the interactions of macrophage-derived peroxynitrite with pathogens located inside phagosomes (diffusion distances $<<1$ μm), extracellular CO_2 only partially competes with peroxynitrite permeation to cells and leads to enhanced nitration of the microbial membrane by the localized action of peroxynitrite-derived $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ (111, 117, 118). Moreover, once peroxynitrite has reached inside the pathogen, intracellular CO_2 is in competition with enzymatic systems that catabolize peroxynitrite (*e.g.*, microbial peroxiredoxins), and therefore, $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ promote microbicidal effects *via* oxidation and nitration reactions bypassing the peroxiredoxin detoxification pathway (111, 117–119). Thus, the "peroxynitrite-CO_2" toxicity experiment in diluted cell suspensions, if not analyzed within the actual biological context, may lead to erroneous interpretations: the fact that CO_2 may be a "protectant" from cytotoxic or microbicidal effects of peroxynitrite *in vitro* does not usually extrapolates to biologically relevant

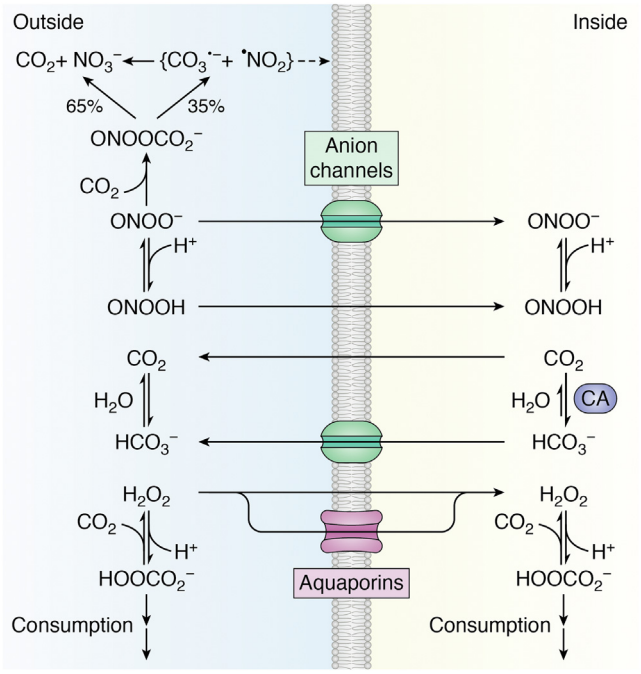

Figure 5. Peroxide reactions with CO_2 in competition with diffusion and transport across the plasma membrane. Carbon dioxide is a strong contender for the diffusion of peroxynitrite and its transport across membranes *via* anion channels or passive diffusion (102). The fast reaction of peroxynitrite with CO_2 limits peroxynitrite diffusion and focusses its reactivity and decay. The rapid extracellular homolysis of $ONOOCO_2^-$ results in the formation of radicals that may recombine to NO_3^- before reaching target molecules or a membrane. Hydrogen peroxide can cross membranes by passive diffusion or facilitated by aquaporins (174, 175). Events related to the H_2O_2 plus CO_2-dependent formation and consumption of HCO_4^- are shown. Peroxynitrite and H_2O_2 kinetics, equilibria, and transport will be influenced by the levels of CO_2/HCO_3^-, which in turn is dictated by metabolic CO_2 formation (or CO_2 exposure) and dynamic aspects that involve CA-catalyzed reactions, diffusion, and transport. The figure exemplifies extracellular peroxides diffusing toward an intracellular compartment. CA, carbonic anhydrase.

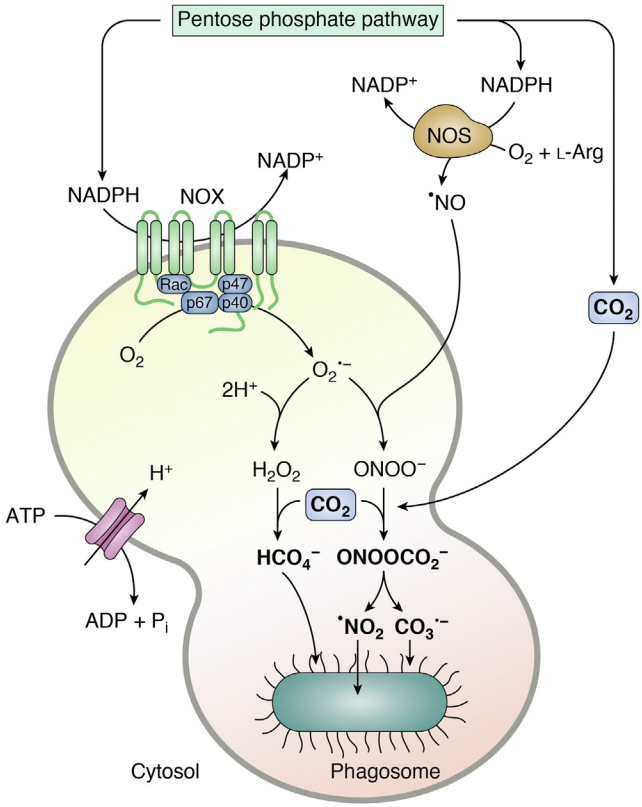

Figure 6. Carbon dioxide–derived oxidants in the macrophage phagosome. Engulfment of pathogens in the phagosome leads to a series of metabolic events directed to cause oxidative killing of the invader microbial cell. In this regard, activation of NOX leads to the formation of $O_2^{\bullet-}$ and H_2O_2 and in the case of immunostimulated cells, the concomitant generation of $^{\bullet}NO$ and peroxynitrite. These processes are coupled with the activation of the PPP that generates NADPH for the catalytic action of NOX and NOS and CO_2 that can diffuse inside the phagosomal lumen and contribute to the formation of HCO_4^- and $ONOOCO_2^-$. At the same time, membrane-bound ATP-dependent pumps release H^+ toward the phagosome causing a drop in pH. NOS, nitric oxide synthase; NOX, NADPH oxidase; PPP, pentose phosphate pathway.

situations *in vivo* involving close cell-to-cell interactions and in microcompartments such as phagosomes and mitochondria (116, 120, 121)[8]. In essence, CO_2 *in vivo* focusses the reactivity of peroxynitrite to a very narrow region within the micrometer distance scale.

On the other hand, there has been a recent debate on whether urease-dependent CO_2 formation in *Helicobacter pylori* serves to neutralize the cytotoxic effects of peroxynitrite released by inflammatory cells in the stomach (122–124). *H. pylori* is usually considered an extracellular pathogen but it can be also found intracellularly, which has been associated to the persistence of the bacteria in the stomach. The discussion is quite interesting because, in effect, urease activity blunts peroxynitrite-dependent cytotoxicity in *H. pylori in vitro*. Close inspection to the data also shows that (1) addition of HCO_3^- (but not NH_4^+) and (2) urease-dependent CO_2 formation decrease bacterial protein tyrosine nitration and peroxynitrite-dependent toxicity. This latter result indicates that the protective effects are occurring extracellularly; indeed, the effects of CO_2 were intracellular, protein tyrosine nitration should have increased and peroxynitrite partially spared from its detoxification by bacterial peroxiredoxins. *H. pylori* contains CA that acts synergistically with urease for pH acclimation and colonization in the gastric mucosa. Thus, *H. pylori* CO_2 emission toward the extracellular milieu and in the context of intercellular distances in the micrometer range promotes extracellular peroxynitrite decay and spares the bacterium from oxidative toxicity. However, the overall relevance of these elegant findings to the pathophysiology of *H. pylori* infection to the stomach remains, in this author's opinion, undefined. It is important to note, however, that *H. pylori* CA and urease participate in the control of bacterial CO_2 levels and counteract macrophage-derived oxidative killing constituting key factors for the establishment, progression, and/or control of infection (31, 123, 124).

In turn, CO_2 is a substantial target of peroxynitrite inside mitochondria and influenced by changes in the rates of the Krebs cycle and oxidative stress conditions where the concentrations of reduced mitochondrial peroxiredoxins (Prx3 and Prx5) (*i.e.*, peroxide catabolism) fall (82, 109, 125). The reaction of mitochondrial peroxynitrite with CO_2 leads to protein oxidation and nitration, even under basal physiological conditions (108). Accordingly, several nitrated proteins were detected in control mitochondria isolated from rat liver as well as in mitochondria from the heart of mice suffering diabetes, a disease known to be associated with increase nitro-oxidative stress (reviewed in (8, 126). In the latter case, Prx3[9] was among the nitrated proteins detected (127). Also, recent work *in vivo* in a model of vascular dysfunction shows that mitochondrial peroxiredoxins become overoxidized under conditions of excess peroxynitrite formation (109). In an *in vitro* experiment using isolated Prx3, enzyme nitration and hyperoxidation is only observed when exposed to excess peroxynitrite; this observation is consistent with the fast and dominant reaction of peroxynitrite with the peroxidatic thiol of Prx3 and that hyperoxidation rates are usually $\sim 10^3$ slower than oxidation rates (82). However, in the presence of CO_2, a fraction of peroxynitrite bypasses the Prx3 and Prx5 detoxification routes and promote oxidative posttranslational modifications *in vivo* (82, 109).

Effect of CO_2 on hydrogen peroxide–mediated processes

The reaction of CO_2 with H_2O_2 results on HCO_4^- that oxidizes biomolecules such as thiols, typically more readily than H_2O_2 alone. In fact, while the second order rate constants for the reaction of H_2O_2 with the single protein thiol (Cys34) of human serum albumin and GSH are in the order of 1 to 2 $M^{-1}s^{-1}$, respectively, these values increase *ca.* 100-fold for their reaction with HCO_4^- (87). The accelerating effect of CO_2 on H_2O_2-dependent thiol oxidation was proposed to explain the oxidative inactivation of protein tyrosine phosphatases (PTPs) in cells (13). PTPs are known to be important molecular targets in redox signal transduction processes with their inactivation leading to increased intracellular phosphorylation (110). Indeed, one of the most enigmatic problems in redox biology has been how H_2O_2 inactivates PTPs, as the direct reaction is quite slow (*ca.* 10–40 $M^{-1}s^{-1}$ for PTP1B). Elegant biochemical and crystallographic work showed that HCO_4^- was capable of promoting the two-electron oxidation of the catalytic cysteine in PTP1B with an apparent rate constant one order of magnitude higher than that of H_2O_2 (*i.e.*, 396 $M^{-1}s^{-1}$ at 25 mM HCO_3^-, pH 7.0 and 37 °C (13)). Considering that at 25 mM HCO_3^-, only *ca.* 1% of H_2O_2 would be present as HCO_4^-, the actual rate constant of the reaction would be in the range of 4×10^4 $M^{-1}s^{-1}$.

While the disparate reactivity of HCO_4^- *versus* H_2O_2 over thiols is seen for low molecular weight thiols and some protein thiols[10], this characteristic is lost for peroxidatic protein thiols (*e.g.*, peroxiredoxins), in which rate constants for both H_2O_2 and HCO_4^- (and also for ONOOH) converge in upper values in the order of 10^7 to 10^8 $M^{-1}s^{-1}$ (24). Interestingly, peroxide-mediated thiol hyperoxidation[11] (*i.e.*, by the reaction with sulfenic acid intermediates, Equations 30a and 30b) is faster for HCO_4^- than H_2O_2, representing a mechanism of peroxiredoxin oxidative inactivation.

[8] The extracellular half-life of peroxynitrite with or without CO_2 is very different. It is about one 1 s without *versus* 10 ms with physiological [CO_2]. The much shorter half-life is the reason for why the presence of CO_2 may decrease toxicity when cells are in suspension. These considerations help explain some discrepancies in the literature.

[9] Peroxiredoxin 3 is exclusively located in mitochondria. Its nitration underscores that idea that part of mitochondrial peroxynitrite escapes Prx3 and Prx5-dependent catabolism by reaction with CO_2, which, in turn, results in the formation of nitrating and oxidizing species.

[10] The peroxidatic thiol in AhpE from *M. tuberculosis* reacts with H_2O_2 and HCO_4^- with *k* of 8.2×10^4 $M^{-1}s^{-1}$ and 1.1×10^7 M^{-1} s^{-1} (180), respectively. In the case of the AhpE sulfenic acid, the *k* values for the hyperoxidation reaction with H_2O_2 and HCO_4^- are 40 M^{-1} s^{-1} and 2.1×10^3 M^{-1} s^{-1} (181) at pH 7.4 and 25 °C, respectively

[11] The terms thiol "hyperoxidation" or "overoxidation" are used interchangeably in the literature to denote the oxidation of thiols over the sulfenic acid redox state toward sulfinic or sulfonic acid derivatives.

ASBMB

$$RSOH + H_2O_2 \rightarrow RSO_2H + H_2O \quad [30a]$$

$$RSOH + HCO_4^- \rightarrow RSO_2H + HCO_3^- \quad [30b]$$

For instance, HCO_4^--mediated human cytosolic (Prx1 and 2) and mitochondrial (Prx3) peroxiredoxin hyperoxidation occurs significantly faster than the corresponding reaction with H_2O_2 (15, 17). For instance, the reaction of Prx1 with HCO_4^- and H_2O_2 occur with estimated k of 1.5×10^5 and 2.9×10^3 $M^{-1}s^{-1}$, respectively (17).

Studies on the relative toxicity of H_2O_2 *versus* HCO_4^- to microorganisms are almost lacking. One report indicates enhanced H_2O_2-dependent cytoxicity in the presence of CO_2 to *Pseudomona aeruginosa* (128). This result points to a potential increased microbicidal action of HCO_4^- over H_2O_2 in preventing bacterial growth and removing biofilms for disinfection purposes. The contribution of these reaction chemistries to the control of invading pathogens at the immune cell phagosomes was somehow hinted in early work on radiolytic inactivation of bacteria (116, 119) and remains to be elucidated (Fig. 6). It is relevant to appreciate that the respiratory burst accompanying foreign body recognition by phagocytic cells promotes CO_2 generation by the PPP (129), with a quotient respect to O_2 consumption close to one (112) (Fig. 6).

In addition to peroxide reactivity: CO_2 and gene expression during inflammatory oxidative stress

While this review underscores reaction mechanisms by which CO_2 directly influences rates, fate, and yields of peroxide-mediated oxidations, it is important to note that CO_2 can also modulate gene expression of proteins and enzymes linked to redox metabolism. Thus, the *in cellula* and *in vivo* effects of CO_2 in oxidative modifications that can take place in the period of hours or days must take into consideration the fact that CO_2 can provoke a specific repertoire of transcriptional events in a dose-dependent manner. In particular, genes associated with inflammation, immunity, and metabolism are CO_2 sensitive and the process evolutionarily conserved (3, 130–134).

Early indications of intertwined events between the effects of CO_2 on redox reaction chemistry and gene expression originated from studies in immunostimulated alveolar macrophages that generate significant levels of $^{\bullet}NO$ and peroxynitrite; this cellular system caused surfactant protein A tyrosine nitration in a process enhanced by the presence of CO_2 (135). In addition to the CO_2-catalyzed formation of nitrating species from peroxynitrite (*i.e.*, $CO_3^{\bullet-}$ and $^{\bullet}NO_2$), CO_2 may induce or upregulate NOS activity, which can additionally contribute to the enhanced protein tyrosine nitration. In fact, a 30 to 60 min exposure to 1.2 mM CO_2 (*i.e.*, cells incubated in a buffered media supplemented with 25 mM $NaHCO_3$, under 5% CO_2, 95% air; PCO_2 ~40 Torr, pH = 7.4) led to a significantly higher NOS activity in lipopolysaccharide-stimulated alveolar macrophages (135). In line with this observation, cytokine plus lipopolysaccharide stimulated alveolar epithelial cells exposed to high levels of CO_2 (*e.g.*, 5%–15%, hypercapnia) for 3 to 48 h revealed an increased production of $^{\bullet}NO$ and NOS expression and activity; this process was associated to cell injury and protein tyrosine nitration, underscoring that the interplay of $^{\bullet}NO$-derived species with CO_2 participate in inflammatory processes (136).

The mechanisms by which CO_2 may regulate gene expression and transcriptional responses involving NOS are yet to be fully disclosed. A complex interaction exists between CO_2 signaling, NF-κB, IKKα, and NOS expression (3, 134, 137–140). CO_2-dependent regulation of inflammatory signaling is in part dependent of the CO_2 sensitivity of the NF-κB pathway and usually associated to the suppression of proinflammatory cytokines (137); however, CO_2-induced inflammation has been also reported (141). In turn, the relationship between the NF-κB pathway and NOS is intricate: while the regulation of NOS expression is governed predominantly by the transcription factor NF-κB, $^{\bullet}NO$ exerts a biphasic regulation of the NF-κB pathway (138). Thus, in the context of inflammatory oxidative stress, CO_2 can directly modulate peroxide-mediated oxidations and also influence both redox and $^{\bullet}NO$ metabolism *via* the regulation of gene expression.

Implications of the CO_2 and peroxide interplay in redox signaling and metabolism

Mechanistic analysis on H_2O_2 reactivity and specificity in thiol-based cell signaling has been presented recently (14). Redox signaling typically involves the reversible H_2O_2-dependent oxidation of proteins (*e.g.*, thiol-disulfide transitions) that are not themselves particularly H_2O_2 reactive in isolated systems. Thus, efforts are underway to reveal how the "H_2O_2 signal" in cells can specifically result in target protein oxidation and subsequent downstream effects. Recent evidence points to H_2O_2 plus CO_2-derived HCO_4^- as a feasible contributory redox signaling intermediate. In addition, CO_2 modulates peroxynitrite-mediated thiol oxidation and tyrosine nitration processes in a way that may also impact cell signaling. Thus, we will analyze proposed mechanisms of peroxide-dependent signaling in which CO_2 can play an important role.

CO_2 increases the reactivity of H_2O_2 toward PTP1B

Phosphorylation cascades represent central processes in cellular redox signaling, with the phosphorylation state usually reflecting the relative activity of kinases and phosphatases. In this regard, the reversible regulation of PTP1B activity *via* transient oxidative inactivation at the active site cysteine represents one of the hallmarks of redox events in cell signaling (14, 142, 143). In cells, the formation of H_2O_2 is a central requisite for PTP1B inactivation, which in turn results in increase of the phosphorylation state induced by receptor tyrosine kinase (RTK) activation. Notably, RTK activation triggers transient H_2O_2 production from plasma membrane–bound NADPH oxidases (NOXs) (21, 144). Indeed, RTK activation facilitates NOX assembly to generate extracellular $O_2^{\bullet-}$, which in turn is readily converted enzymatically to H_2O_2 (*i.e.*, by EC-SOD) and enters the cells to promote redox signaling. This way, the tyrosine kinase–dependent phosphorylation events are synergistically coupled to H_2O_2-dependent inactivation of PTP1B. However, one of the most mysterious issues in the redox

signaling field has been how the rather sluggish reaction of H_2O_2 with PTP1B (k = 24 $M^{-1}s^{-1}$, reviewed in (87)) would lead to cellular responses in the time range of minutes and outcompeting the much faster reactions of H_2O_2 with abundant peroxidatic systems such as those of peroxiredoxins and GSH peroxidases (k *ca.* 10^7–10^8 $M^{-1}s^{-1}$) (24, 110). Indeed, the thioredoxin reductase/thioredoxin/peroxiredoxin system can completely inhibit H_2O_2-dependent PTP1B oxidation *in vitro* in phosphate buffer systems (145, 146). However, upon addition of HCO_3^-/CO_2, PTP1B is more readily oxidized by H_2O_2 and peroxiredoxins are not capable of completely preventing PTP1B inactivation (11). These observations are compatible with reaction of HCO_4^- with PTP1 (13), where peroxiredoxins cannot fully neutralize the reaction of H_2O_2 with CO_2. In a recent elegant work, studies in an adenocarcinoma cell line stimulated with epidermal growth factor (EGF), known to act through RTK, have shown that increasing intracellular HCO_3^- concentrations enhanced total protein phosphotyrosine levels in parallel with the occurrence of PTP1B oxidation/inactivation. In fact, the presence of HCO_3^- was an absolute requirement for EGF-induced cellular oxidation of PTP1B[12], allowing physiological steady-state levels of H_2O_2 to inactivate PTPs within a time scale of minutes (11, 13). Notably, NOX activation is typically coupled with enhanced CO_2 formation by the PPP (20, 112) These data reconcile mechanisms of PTP1B-mediated H_2O_2 oxidation/inactivation *in vitro* and *in vivo* and points to CO_2 and HCO_4^- as relevant intermediates in redox signaling.

CO_2 favors H_2O_2-dependent peroxiredoxin hyperoxidation and affects redox relays

H_2O_2-dependent thiol hyperoxidation of peroxiredoxins is significantly accelerated in HCO_3^--containing buffers (15, 17), with CO_2 typically increasing the *k* value by two orders of magnitude. Thus, by this mechanism, a fraction of the oxidized peroxidatic protein thiol in the sulfenic acid state can react with HCO_4^- and evolve to hyperoxidized (and inactive) forms (*i.e.*, sulfinic acid) in kinetic competition with its reaction with the resolving protein cysteine residue leading to the formation of an intermolecular disulfide in the "typical" peroxiredoxins (such as peroxiredoxins 2 and 3, (24)). Peroxiredoxin hyperoxidation jeopardizes the reversibility of redox signaling in the context of "redox relays" mediated by the peroxiredoxin sulfenic acid intermediate interacting with other thiol-containing proteins (147, 148). Also, peroxiredoxin hyperoxidation may further increase H_2O_2 levels due to the inability of these inactivated peroxiredoxins to decompose H_2O_2, which may secondarily favor the oxidation of less H_2O_2-reactive proteins. Thus, CO_2 provides a feasible mechanism for the "floodgate" hypothesis of redox signaling (149, 150), under which excess H_2O_2 levels disrupts signaling by sequestering a fraction of peroxiredoxin in an inactive state[13].

[12] The thioredoxin reductase-thioredoxin system can reduce back the thiol-oxidized PTP1 to the active state (146).

[13] This hyperoxidized proteoform may, in the case of peroxiredoxins, return to the resting state by the action of ATP-dependent reducing enzymatic mechanisms such as sulfiredoxins (182).

CO_2, tyrosine nitration, and redox signaling

Protein tyrosine nitration may affect signaling pathways in a variety of ways (8). Both tyrosine nitration yields and regioselectivity are affected by the presence of CO_2 (151–153). As biologically relevant examples, protein tyrosine nitration in HSP90 (heat shock protein 90) (154, 155) and NGF (nerve growth factor) (156) triggers cell death pathways, causes PP2A (protein serine phosphatase) inactivation and enhanced cellular phosphorylation (157, 158), affects the ability of the cytokine CCL2 (C–C motif chemokine ligand 2) to exert its normal chemoattractant activity for immune cells (159), and disrupts insulin receptor substrate–1 (IRS–1)–dependent signaling (160, 161). In these contexts, information on how CO_2 levels modulate tyrosine nitration and signaling events is basically lacking.

On the other hand, CO_2 may also affect tyrosine nitration and signaling cascades by changes in gene expression patterns. Early work showed that hypercapnic exposure to lung cells lead to enhanced protein nitrotyrosine levels, which were associated to increased NOS expression and activity (135, 136). Thus, cellular tyrosine nitration stimulated by high CO_2 levels may be a combination of increased $^{\bullet}NO$/peroxynitrite formation together with the favored formation of $ONOOCO_2^-$, although precise disclosure is lacking.

The effects of hypercapnia *in vivo* in terms of protein tyrosine nitration and inflammatory oxidative stress are yet to be defined. For example, while it was indicated that long term exposure to hypercapnia may exert anti-inflammatory actions and potentially decrease extents of protein tyrosine nitration (162), other works show that CO_2 can promote $^{\bullet}NO$ and peroxynitrite production, protein nitrotyrosine formation, and oxidative injury (163). A complex relationship between cell/animal CO_2 exposure and inflammatory mediators exists with an overall anti-inflammatory effect of CO_2, in part through downregulation of the NF-κB signaling pathways (131). Nonetheless, recent works indicated that selective tyrosine nitration in protein serine phosphatase PP2A regulatory subunits leads to enzyme inactivation and enhanced cellular phosphorylation events that result in upregulation of the NF-κB pathway (157, 158); these processes may promote proinflammatory phenotypes. No information is available as to what extent CO_2 regulates tyrosine nitration (and activity) in PP2A. Thus, as inflammation, cytokines and NF-κB are intertwined with the $^{\bullet}NO$ and redox pathways; the effect of different CO_2 exposure regimes on peroxynitrite-dependent signaling cascades involving tyrosine nitration require specific future studies.

Other possible effects of CO_2 on peroxynitrite-dependent redox signaling

In addition to the effects of peroxynitrite on signaling pathways through protein tyrosine nitration, PTPs can be inactivated by peroxynitrite *via* thiol oxidation (164, 165). In this regard, low concentrations of peroxynitrite promote a cellular tyrosine hyperphosphorylated state (8, 166), while high concentrations impair phosphorylation and trigger apoptotic cell death. The thiol oxidation process is also expected to be influenced by

changing CO_2 levels. Indeed, the presence of CO_2 shifts the mechanism of peroxynitrite-mediated thiol oxidation and favors the initiation of oxygen-dependent radical chain reactions (8). Moreover, enhanced levels of CO_2 partially outcompete peroxynitrite detoxification pathways and promote free radical–dependent oxidation and hyperoxidation of thiol-containing proteins, which may end up influencing signaling pathways. In this regard, it is interesting to consider that low levels of peroxynitrite facilitate mitochondrial biogenesis (167) and, potentially, mitophagy (168). Albeit speculative, it would be worth to explore how the CO_2 and peroxynitrite interplay in mitochondria may influence redox-dependent cascades related to mitochondrial turnover and cell signaling. The potential connection among redox homeostasis, mitochondrial oxidant formation, and mitochondrial biogenesis have been reviewed recently (168) and opens possibilities to understand how peroxide-mediated signaling (*e.g.*, *via* thiol oxidation) in the presence of CO_2 contribute to the process.

Conclusions

The elements presented in this review lead to the conclusion that metabolically derived CO_2 participates in the modulation of redox reactions that range from signaling to toxicity. In this regard, CO_2 assists shaping the cellular redox landscape. Indeed, CO_2 is well suited to couple intermediary metabolism with redox signaling both in mitochondrial and extramitochondrial sites (Fig. 1). Moreover, the concomitant metabolic formation of reactive species such as $O_2^{\bullet-}$, H_2O_2, and peroxynitrite during the oxidative burst of activated phagocytes together with CO_2 (formed by the PPP) may play important roles on oxidative killing of invading pathogens *via* formation of HCO_4^- and/or $ONOOCO_2^-$ (Fig. 6). Obviously, changes in CO_2 levels also lead to pH changes and specific efforts are required to dissect their relative influence in cellular responses. But, it has become clear that CO_2 *per se* participates in the modulation of cell physiology and pathology by a series of mechanisms that include (1) direct posttranslational protein modifications (*e.g.*, carbamylation, (27, 169)), (2) influence in gene expression (131), and (3) participation in peroxide-dependent reactions (reviewed herein). Thus, tight control and measurements of CO_2 levels in cell/tissue experiments become a clear necessity when analyzing redox processes and inflammatory oxidative stress. To this end, development and validation of CO_2-sensing methodologies such as electrodes and chemical probes that could be applied to cell systems will open new research opportunities (19, 170). Moreover, better translation of the impact of CO_2 to redox biology will inevitably require cell culture incubations with relevant oxygen levels[14] (171).

An important aspect to consider in relation to the role of CO_2 in redox processes is that while many reactions involving reactive oxidizing intermediates in signaling events occur at very fast rates in localized cellular areas (*e.g.*, H_2O_2 reactions with peroxiredoxins, time scale of seconds, (172)), CO_2 facilitates H_2O_2 to oxidatively modify targets such PTPs within a more extended time frame (*e.g.*, several minutes, (13)) supporting the idea of various speed layers for the time course of redox signaling (*i.e.*, fast and slow redox signals, (173)). Another evolving story in relation to redox signaling and CO_2 relates to the regulation of phosphatases by thiol oxidation or tyrosine nitration. Intriguingly, CO_2 facilitates PTP inactivation by HCO_4^--mediated thiol oxidation (13), while, on other hand, protein serine phosphatase (PP2A) inactivation is mediated by tyrosine nitration (158), a process typically catalyzed by the CO_2-dependent decay of peroxynitrite. Thus, CO_2 can participate in the redox control of cellular phosphorylation events by inactivation mechanisms that involve thiol oxidation or tyrosine nitration in tyrosine (PTP1B) or serine (PP2A) phosphatases, respectively. Finally, the impact of CO_2 sensing in gene expression associated to redox and $^{\bullet}NO$ metabolism requires a much in-depth analysis in future studies.

While it is critically important to define the influence of the CO_2/HCO_3^- pair on peroxide-dependent processes under physiological conditions, pathophysiological events (*e.g.*, metabolic, respiratory or renal disorders) leading to alterations of its equilibria and concentrations (38, 39) are expected to affect the formation and fate of CO_2-derived oxidants; this latter aspect has been scarcely hinted in the literature (6, 135, 136) and deserves specific future attention.

In summary, accumulated biochemical evidence *in vitro*, *in cellula*, and *in vivo* unambiguously show that CO_2 and peroxide metabolism are intertwined: this interplay must be explicitly considered to judiciously study, analyze, and interpret biological processes in the context of redox signaling and toxicity.

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Abbreviations—The abbreviations used are: CA, carbonic anhydrase; NOS, nitric oxide synthase; NOX, NADPH oxidase; PPP, pentose phosphate pathway; PTP, protein tyrosine phosphatase.

[14] In this context, cell culture conditions must also consider the actual concentration of redox active compounds such as ascorbate, which *in vitro* is normally present at much lower levels than *in vivo*, and readily reacts with CO_2-derived reactive species such as $CO_3^{\bullet-}$ ($k = 1.1 \times 10^9\ M^{-1}s^{-1}$, (183)).

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