Review

HIGHLIGHTED TOPIC | *Central CO$_2$ Chemoreception in Cardiorespiratory Control*

Hypercapnia causes cellular oxidation and nitrosation in addition to acidosis: implications for CO$_2$ chemoreceptor function and dysfunction

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Dean JB. Hypercapnia causes cellular oxidation and nitrosation in addition to acidosis: implications for CO$_2$ chemoreceptor function and dysfunction. *J Appl Physiol* 108: 1786–1795, 2010. First published February 11, 2010; doi:10.1152/japplphysiol.01337.2009.— Cellular mechanisms of CO$_2$ chemoreception are discussed and debated in terms of the stimuli produced during hypercapnic acidosis and their molecular targets: protons generated by the hydration of CO$_2$ and dissociation of carbonic acid, which target membrane-bound proteins and lipids in brain stem neurons. The CO$_2$ hydration reaction, however, is not the only reaction that CO$_2$ undergoes that generates molecules capable of modifying proteins and lipids. Molecular CO$_2$ also reacts with peroxynitrite (ONOO$^-$), a reactive nitrogen species (RNS), which is produced from nitric oxide ($^\bullet$NO) and superoxide ($^\bullet$O$_2^-$). The CO$_2$/ONOO$^-$ reaction, in turn, produces additional nitrosative and oxidative reactive intermediates. Furthermore, protons facilitate additional redox reactions that generate other reactive oxygen species (ROS). ROS/RNS generated by these redox reactions may act as additional stimuli of CO$_2$ chemoreceptors since neurons in chemosensitive areas produce both $^\bullet$NO and $^\bullet$O$_2^-$ and, therefore, ONOO$^-$. Perturbing $^\bullet$NO, $^\bullet$O$_2^-$, and ONOO$^-$ activities in chemosensitive areas modulates cardiorespiration. Moreover, neurons in at least one chemosensitive area, the solitary complex, are stimulated by cellular oxidation. Together, these data raise the following two questions: *1*) do pH and ROS/RNS work in tandem to stimulate CO$_2$ chemoreceptors during hypercapnic acidosis; and *2*) does nitrosative stress and oxidative stress contribute to CO$_2$ chemoreceptor dysfunction? To begin considering these two issues and their implications for central chemoreception, this minireview has the following three goals: *1*) summarize the nitrosative and oxidative reactions that occur during hypercapnic acidosis and isocapnic acidosis; *2*) review the evidence that redox signaling occurs in chemosensitive areas; and *3*) review the evidence that neurons in the solitary complex are stimulated by cellular oxidation.

reactive oxygen species; reactive nitrogen species; carbon dioxide; central chemoreception; hypercapnia

MOLECULAR O$_2$ AND MOLECULAR CO$_2$ GENERATE REACTIVE OXYGEN SPECIES/REACTIVE NITROGEN SPECIES

MOLECULAR OXYGEN is a strong oxidant that is reduced sequentially to yield oxygen free radicals and their highly reactive nonradical derivatives. Known as "reactive oxygen species" (ROS), these strong oxidants include superoxide anion ($^\bullet$O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals ($^\bullet$OH) among others (22). Molecular oxygen also reacts with L-arginine in the presence of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) and nitric oxide synthase (NOS) to yield the free radical nitric oxide ($^\bullet$NO) and citrulline (45,

52). Nitric oxide is an important signaling molecule in the central nervous system (CNS) and a "reactive nitrogen species" (RNS). Nitric oxide reacts with $^\bullet$O$_2^-$ to produce peroxynitrite (ONOO$^-$), which is yet another type of RNS. These various oxidation-reduction (i.e., redox) reactions occur under normal physiological conditions and contribute to normal mechanisms of cellular signaling (22, 26). Under adverse conditions, redox reactions can also produce excessive levels of ROS/RNS that exceed the body's endogenous antioxidant defenses resulting in "oxidative and nitrosative stress" and cellular dysfunction (22, 80). Not surprisingly, ROS and RNS production have been implicated in the brain stem's sensitivity to hypoxia (13, 36, 40, 47, 62) and hyperoxia (16–19, 57).

In this minireview, I propose that molecular CO$_2$ and H$^+$ are two additional sources of ROS/RNS that modulate brain stem neurons, including those in CO$_2$-chemosensitive areas. Molecular CO$_2$ also reacts with ONOO$^-$ to generate the RNS called

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nitrosoperoxocarboxylate ($ONO_2CO_2^-$)[1] under physiological conditions (21, 27, 78, 89, 91). Nitrosoperoxocarboxylate, in turn, produces reactive intermediates capable of oxidation as well as nitration and nitrosylation reactions, i.e., nitro-oxidative reactions (27, 31, 80, 86, 91). In addition, protons, independently of molecular CO_2, catalyze other redox reactions, most notably the Fenton reaction that produces the powerful oxidant, $^\bullet OH$, which nondiscriminately targets all components of the cell membrane (22, 70, 80). Consequently, the potential exists for increased CO_2 and/or H^+ during hypercapnic acidosis to facilitate various nitro-oxidative reactions in neurons that function in cardiorespiratory control (19).

The pervasiveness of CO_2/H^+-facilitated nitro-oxidative reactions in the bicarbonate (HCO_3^-)-buffered biological milieu raises two questions of potential importance for central CO_2 chemoreception. First, if hypercapnic acidosis (respiratory acidosis) and isocapnic acidosis (metabolic acidosis) enhance redox reactions under physiological conditions, then does redox signaling participate in the cellular mechanism of CO_2/H^+ chemoreception? Putnam et al. (72) have proposed a "multiple factors and multiple targets" theory of CO_2 chemoreception whereby neuronal chemosensitivity is explained by the combined effects of extracellular pH (pH_o), intracellular pH (pH_i), and molecular CO_2/HCO_3^- on multiple populations of K^+ and Ca^{2+} channels. For purposes of discussion here, I have proposed that CO_2-/H^+-dependent redox reactions and nitrosative reactions are additional "factors" that work in tandem with pH reactions to modulate the proteins and lipids forming the plasma membrane [the molecular "targets" (11)] of CO_2 chemoreceptors during hypercapnic acidosis and isocapnic acidosis. I also expect that CO_2-/H^+-dependent redox reactions will influence other populations of cardiorespiratory neurons in the brain stem; however, as presented below, the initial evidence indicates that putative central chemoreceptors have a lower threshold for redox stimulation than nonchemosensitive neurons (19, 57). If this hypothesis proves to be correct, then the clinically relevant second question is how does oxidative stress and nitrosative stress influence central CO_2 chemoreception and what role does it play in disorders of respiratory control that involve central chemoreceptors? Oxidative stress and nitrosative stress have been implicated in a number of neurological disorders and diseases (80) and may, likewise, contribute to onset of disorders of cardiorespiratory control and, in addition, result from O_2/CO_2/pH perturbations caused by disordered breathing patterns (43, 46, 51, 75, 79, 93).

I begin to address these issues and their possible implications for central chemoreception by 1) discussing nitro-oxidative reactions that are stimulated during hypercapnic acidosis and isocapnic acidosis in a HCO_3^--buffered system under physiological conditions; 2) reviewing the evidence that neurons in chemosensitive areas of the brain stem express the requisite oxidative enzymes and reactants that would enable these specific CO_2/H^+-driven redox and nitrosative reactions; and 3) summarizing the evidence that neuronal activity in the solitary complex (SC), including putative CO_2-chemoreceptor neurons, is stimulated by cellular oxidation that causes changes in mechanisms of excitability and pH_i regulation.

REDOX AND NITROSATIVE REACTIONS ACTIVATED DURING HYPERCAPNIC ACIDOSIS AND ISOCAPNIC ACIDOSIS

The reaction between carbon dioxide/bicarbonate and peroxynitrite is important for understanding mechanisms of oxidant-mediated toxicity. First, normal plasma bicarbonate and carbon dioxide concentrations are 25 and 1.3 mM, respectively, but higher levels could be achieved during tissue pathological events such as respiratory distress or ischemia-reperfusion. Second, excess production of peroxynitrite has been documented to occur during these and other instances of accelerated tissue superoxide and nitric oxide production, enhancing the potential for coordinated reaction mechanisms to occur between CO_2/HCO_3^-, peroxynitrite, and tissue target molecules.

—From Denicola et al.
(Ref. 21)

CO_2/HCO_3^- and peroxynitrite. Peroxynitrite ($ONOO^-$) is formed by the reaction of $^\bullet O_2^-$ and $^\bullet NO$ (20, 73, 86, 91) [Fig. 1, *reaction (Rxn) 3*]. The $^\bullet O_2^-$/$^\bullet NO$ reaction (Fig. 1, *Rxn 3*) has been called the "radical switch" that diverts $^\bullet NO$ away from its normal regulatory signaling actions toward potentially cytotoxic reactions that result in nitrosative and oxidative damage (27, 86). The $^\bullet NO$/$^\bullet O_2^-$ reaction is diffusion limited and occurs more rapidly than the catalyzed dismutation reaction of $^\bullet O_2^-$ (superoxide dismutase, SOD) to form H_2O_2 (4) (Fig. 1, *Rxn 4*). The $^\bullet NO$/$^\bullet O_2^-$ reaction that forms $ONOO^-$ occurs in both intracellular and extracellular compartments. Peroxynitrite readily crosses the plasma membrane in its protonated form (peroxynitrous acid, $ONOOH$) to react with intracellular substrates. Peroxynitrite anion also crosses the cell membrane via a 4,4′-diisothiocyano-2,2′-stilbene-disulfonic acid (DIDS)-sensitive anion exchanger that normally functions in Cl^-/HCO_3^- exchange (78); this latter mechanism predominates at physiological pH (89). Once formed, $ONOO^-$ undergoes several reactions to yield molecular products of varying reactivity (91): oxidation of sulfhydryl (thiol) groups to disulfides; nitration of aromatic compounds; *S*-nitrosylation of sulfur atoms in amino acid residues resulting in posttranslational modification of proteins (i.e., *S*-nitrosylated proteins, SNO); protonation to produce peroxynitrous acid that decomposes to form nitrate; and reaction with CO_2 to generate nitrosoperoxocarboxylate ($ONO_2CO_2^-$) (21, 26, 27, 31, 70, 71, 73, 78, 89–91). It is the last reaction between CO_2 and $ONOO^-$ that occurs ubiquitously in a CO_2/HCO_3^--buffered system (Fig. 1, *Rxn 5*) and, moreover, that is postulated here to be involved in nitrosative and oxidative modulation of brain stem neurons, including chemosensitive neurons, during hypercapnic acidosis and oxidative stress (Fig. 1, *Rxn 6*).

Peroxynitrite is unstable in CO_2/HCO_3^--buffered medium and is quickly consumed by its reaction with CO_2 to yield $ONO_2CO_2^-$ (21, 70, 71, 89) (Fig. 1, *Rxn 5*). Addition of the enzyme carbonic anhydrase (CA), which catalyzes the hydration/dehydration reactions of CO_2 and HCO_3^- (Fig. 1, *Rxn 7/Rxn 8*), also increases the rate of conversion of $ONOO^-$ to $ONO_2CO_2^-$ (89) (Fig. 1, *Rxn 5*). In the presence of CO_2/HCO_3^-, a limited number of molecular targets react directly with $ONOO^-$ (27, 70). Instead, biomolecules react with the various RNS intermediates that are produced from the $CO_2/ONOO^-$ reaction (3, 27, 70, 86, 91). The $CO_2/ONOO^-$ reaction is one

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[1] Nitrosoperoxocarboxylate ($ONO_2CO_2^-$) has also been called nitrosoperoxycarbonate anion (78), nitrosocarbonate anion (91), and by its IUPAC name, 1-carboxylateo-2-nitrosodioxidane (78).
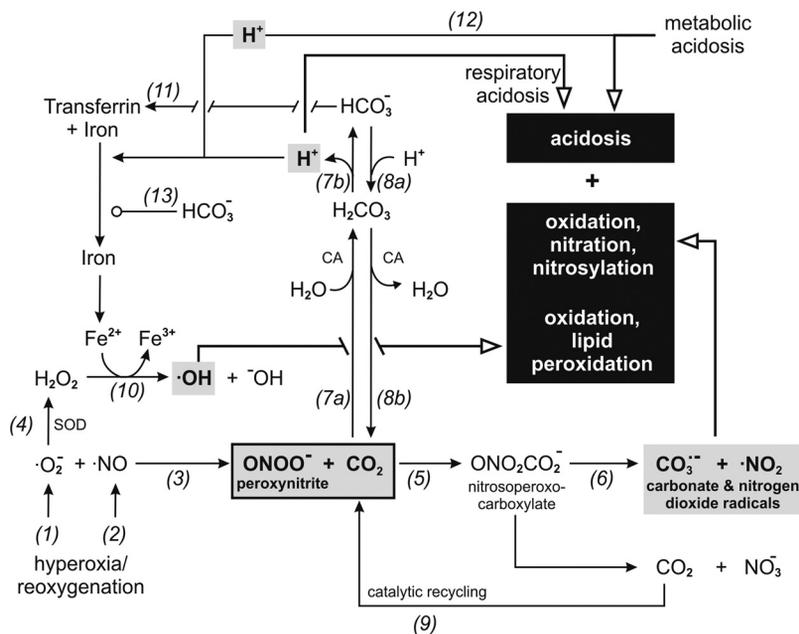
Fig. 1. Schematic of the relationships between hypercapnic acidosis [respiratory acidosis; *reactions* (*Rxns*) *7a* and *7b*] and isocapnic acidosis (metabolic acidosis; *Rxn 12*) and production of nitrosative and oxidative intermediates via peroxynitrite (ONOO$^-$; *Rxns 5* and *6*) and production of oxidative intermediates via the Fenton reaction (*Rxn 10*). Molecular oxygen produces both superoxide anion ($^\bullet$O$_2^-$) and nitric oxide ($^\bullet$NO) as described in the text (*Rxns 1* and *2*). These 2 ubiquitous radicals react to generate ONOO$^-$ (*Rxn 3*), which in a CO$_2$/HCO$_3^-$-buffered system (*Rxn 8*) immediately reacts with CO$_2$ to form nitrosoperoxo-carboxylate (ONO$_2$CO$_2^-$; *Rxn 5*) that is followed by several additional reactions summarized in the text; see also Vesela and Wilhelm (91). The most important reaction, in the context of the present discussion, is formation of carbonate radicals (CO$_3^{-\bullet}$) and nitrogen dioxide radicals ($^\bullet$NO$_2$) (*Rxn 6*). In the process, CO$_2$ is regenerated and recycled for production of more ONO$_2$CO$_2^-$ (*Rxn 9*). In addition, $^\bullet$O$_2^-$ (*Rxn 1*) is converted to hydrogen peroxide (H$_2$O$_2$) in a reaction catalyzed by superoxide dismutase (SOD; *Rxn 4*) and reacts with iron liberated from transferrin. Decreased pH increases dissociation of iron from transferrin (*Rxns 7b* and *12*) and accelerates the Fenton reaction rightward, producing hydroxyl radicals ($^\bullet$OH; *Rxn 10*). Metabolic acidosis (i.e., ↑H$^+$, ↓HCO$_3^-$, and no change PCO$_2$) is a more effective stimulus than respiratory acidosis (i.e., ↑H$^+$, ↑HCO$_3^-$, and ↑PCO$_2$) for liberating free iron since HCO$_3^-$ promotes binding of iron to transferrin (*Rxn 11*). CA, carbonic anhydrase; NO$_3^-$, nitrate anion; OH$^-$, hydroxyl anion; solid black arrowhead, acceleration of reaction; open circle, inhibition of reaction; open arrowhead with thick line, cellular consequence of reaction, include acidosis, nitro-oxidation, and lipid peroxidation.

of the fastest reactions that ONOO$^-$ undergoes (reaction rate, $k = 5.8 \times 10^4$ M$^{-1}\cdot$s^{-1} at 37°C), and it predominates in the extracellular space where the concentration of sulfhydryl groups is low and CO$_2$/HCO$_3^-$ concentration is high; that is, formation of ONO$_2$CO$_2^-$ is favored in the extracellular compartment over formation of disulfides (91). Radi (73) has proposed that over 90% of ONOO$^-$ initially reacts with CO$_2$ in the extracellular space and from 30–40% of ONOO$^-$ initially reacts with CO$_2$ in the intracellular space. As stated above, however, ONOO$^-$ has no problem crossing the cell membrane and gaining access to intracellular targets (78).

The CO$_2$/ONOO$^-$ reaction occurs over a broad range of pH, including physiologically relevant pH that stimulates breathing in vivo and CO$_2$-chemosensitive neurons under in vitro conditions, for example, arterial partial pressure of carbon dioxide (PCO$_2$) and pH ranges, respectively, from ∼35–45 Torr and ∼7.35–7.45. An increase in arterial PCO$_2$ of only a few Torr significantly increases minute ventilation (34, 59, 81). In rats, switching from air to 6% CO$_2$ in air decreases cerebrospinal fluid pH (pH$_{CSF}$) from 7.396 to 7.294 and neural pH$_i$ from 7.044 to 6.982 and stimulates minute ventilation. Breathing 11% CO$_2$ in air decreases pH$_{CSF}$ further to 7.190 and brain pH$_i$ to 6.910 and stimulates breathing (83). In rat brain stem tissue slices, acute exposures to moderate levels of hypercapnia decreases pH$_i$ by ≤0.2 pH units from a baseline pH$_i$ of ∼7.24 (28, 76, 77), decreases membrane conductance, and stimulates firing rate of neurons in CO$_2$-chemosensitive areas (14, 28, 42). Based on these studies, the physiologically relevant range of

pH$_{CSF}$/pH$_i$ for stimulation of chemoreceptors encompasses ∼7.4 down to 6.91. This range of pH overlaps with the range of pH (6.5–7.4) that the CO$_2$/ONOO$^-$ reaction occurs (21, 71, 89). At pH 7.4, the predominance of the CO$_2$/ONOO$^-$ reaction is demonstrated by the decrease in peroxynitrite-mediated oxidation products when CO$_2$/HCO$_3^-$ was added to the reaction vessel; that is, addition of CO$_2$/HCO$_3^-$ accelerated consumption of ONOO$^-$ to yield ONO$_2$CO$_2^-$ before ONOO$^-$ could react with other substrates and oxidize molecular targets such as glutathione and oxyhemoglobin (21). Increasing CO$_2$ further accelerates consumption of ONOO$^-$ (21, 71, 86, 89, 91).

The CO$_2$/ONOO$^-$ reaction redirects the primary reactivity of ONOO$^-$ in both intracellular and extracellular compartments to nitrosative and oxidative reactions through breakdown of ONO$_2$CO$_2^-$ into carbonate radicals (CO$_3^{-\bullet}$) and nitrogen dioxide radicals ($^\bullet$NO$_2$) and other species (70, 71, 91) (Fig. 1, *Rxn 6*). In the course of producing these derived nitro-oxidants, CO$_2$ is regenerated and recycled for further production of ONO$_2$CO$_2^-$ (71) (Fig. 1, *Rxn 9*). Thus molecular CO$_2$ acts as a catalyst for ONOO$^-$/ONO$_2$CO$_2^-$ reaction (Fig. 1, *Rxn 5*). The formation of CO$_3^{-\bullet}$ and $^\bullet$NO$_2$ is favored in the nonpolar environment of the plasma membrane (91). Carbonate radicals are more stable than $^\bullet$OH and can diffuse from their site of synthesis to induce oxidative and nitrosative stress with more species and molecular targets that are further removed. When CO$_3^{-\bullet}$ and $^\bullet$NO$_2$ react in concert, they constitute an efficient nitrating system that is pivotal to biological nitration of protein tyrosine residues (70). The majority of CO$_3^{-\bullet}$

reactions are protein oxidations via hydrogen atom abstraction and electron transfer (3). Important targets for $CO_3^-{}^\bullet$ include tyrosine, tryptophan, guanine, cysteine, sulfhydryl groups, and nucleic acids (27, 70, 86, 88, 91). Nitrogen dioxide is a RNS that can react with other radical species or oxidize biomolecules via electron transfer and hydrogen atom abstraction (3). Important targets include proteins, fatty acids, phenols, thiols, tyrosine, tryptophan, and cysteine residues (3, 70).

As stated above, $ONOO^-$ undergoes several other reactions to varying extent in the presence of CO_2/HCO_3^- (26, 91). The lack of comment in the literature on the effects of pH on these other reactions suggests that they are either unremarkable in their pH sensitivity or that their pH sensitivity is unstudied to date. Regardless, I mention one of these $ONOO^-$ reactions here—the S-nitrosylation (SNO) reactions—for the following two reasons: *1*) S-nitrosylation reactions have been implicated in cardiorespiratory control mechanisms in the nucleus tractus solitarius, which is the dorsal nucleus of the SC (see below) (47, 65); and *2*) S-nitrosylation reactions redirect $ONOO^-$ away from reacting with CO_2 and therefore influence formation of CO_2-dependent RNS (21, 26, 71, 86, 89, 91). Peroxynitrite induces SNO reactions (26, 90) that cause reversible nitrations at cysteine and methionine residues that are thought to be involved in normal signaling mechanisms (31, 87), including cardiorespiratory control (47, 65). Conversely, irreversible SNO reactions occur principally from nitration of tyrosine residues forming nitrosocarbonate and are believed to be involved in pathological processes (31, 35, 88).

H^+ and the Fenton reaction. Trace amounts of metal ions, such as iron, are important activators of redox reactions (86). The Fenton reaction (Fig. 1, *Rxn 10*) describes the iron-catalyzed reaction in which H_2O_2 is converted to $^\bullet OH$. Ferrous iron (Fe^{2+}) is oxidized by H_2O_2 to ferric iron (Fe^{3+}) plus hydroxyl radical and hydroxyl anion: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^\bullet OH + {}^-OH$. Hydroxyl radicals are more reactive and thus damaging than $CO_3^-{}^\bullet$ and $^\bullet NO_2$. They react with essentially all biomolecules that they collide with, which is why the effects of $^\bullet OH$ are more random and widespread compared to the targeted reactions initiated by $CO_3^-{}^\bullet$ and $^\bullet NO_2$ (70). Under normal conditions, oxidative stress from excessive production of $^\bullet OH$ is kept in check, in part, by the high-affinity iron-binding protein, transferrin (Tf), which is present in the CSF, plasma, extracellular fluid, and lymph (30). Binding of iron to Tf is strongly pH dependent (85), and decreasing pH_o increases dissociation of iron from Tf (2, 82) (Fig. 1, *Rxn 7b and Rxn 12*). At $pH_o = 7.4$, free Fe^{3+} binds to Tf and the transferrin receptor (TfR1) located on the plasma membrane. The Fe-Tf-TfR1 complex is internalized by endocytosis to form a vesicle (endosome) whose internal pH is maintained acidic (pH ~6.0) via proton pumps. Vesicular acidification causes dissociation of Fe^{3+} from Tf-TfR1, which is reduced to Fe^{2+} by a ferrireductase (63). Ferrous iron then goes to the mitochondrion, or, alternatively, it exits the endosome into the cytosol via the divalent metal transporter, DMT1 (30). Presumably, during acidosis, decreasing pH_o further acidifies vesicular pH causing enhanced release of Fe^{3+} from Tf-TfR1 and reduction to Fe^{2+}, which increases the pool of free iron catalyzing the Fenton reaction within the cell (30). Binding of iron to Tf, however, is also dependent on HCO_3^-. Transferrin binds one ferric ion and one anion, which is usually HCO_3^- (23) (Fig. 1, *Rxn 11*). Cellular acidosis caused by metabolic acidosis (Fig. 1, *Rxn 12*),

therefore, would favor dissociation of iron from Tf due to concurrent decreases in both pH and HCO_3^- (isocapnic acidosis), thereby providing Fe^{2+} for catalyzing the Fenton reaction and increasing production of $^\bullet OH$ (74, 91).

On the other hand, hypercapnic acidosis would stabilize the Fe-Tf-TfR1 complex in part by producing equimolar amounts of HCO_3^- and H^+ (Fig. 1, *Rxn 7b*), each species having opposing effects on the Fe-Tf-TfR1 complex (74, 91) (Fig. 1, *Rxn 13*). This is why hypercapnic acidosis is thought to provide neuroprotection during ischemia and hypercapnic hypoxia: increased levels of CO_2/HCO_3^- stabilize the Fe-Tf-TfR1 complex and decrease free iron availability for catalyzing redox reactions (74, 91). In addition, as discussed above, rapid conversion of $ONOO^-$ to $ONO_2CO_2^-$ during hypercapnic acidosis effectively decreases the oxidative damage caused by $ONOO^-$. At the same time, however, recall that the $CO_2/ONOO^-$ reaction has its own potentially adverse consequences on cellular function through production of reactive intermediates that cause cellular oxidation and nitrosative processes (3, 5, 27, 33, 70) (Fig. 1, *Rxn 5* and *Rxn 6*).

Acid-activated Fenton chemistry is an important mechanism in modulating neuronal function during pathological conditions such as ischemia, metabolic disorders, and chronically unstable breathing (22, 80, 93). Under more physiological conditions, the importance of the Fenton reaction in CO_2 chemoreception during acidosis remains to be determined since cells, in addition to the iron binding proteins (10, 30), have H_2O_2 disposing systems (catalase, peroxidase) that keep their levels of H_2O_2 low under normal conditions (22, 38). Having said that, redox signaling can occur in restricted cellular domains that do not reflect the overall redox state of the cell (68). It is conceivable that the Fenton reaction could likewise be restricted to a particular subcellular compartment that enables it to act as a physiological modulator during acidosis of chemosensitive neurons and other cardiorespiratory neurons. For example, possible restricted subcellular domains for targeted redox signaling include the plasma membrane (lipids, proteins, and/or cytoskeleton), cytosol, mitochondrial matrix, or mitochondrial intermembrane space (11, 37, 68). Compartmentalization within a particular nanostructural domain of the cell is thought to be a critical factor in determining if a given redox reaction functions in physiological regulation or as an agent of nitrosative and oxidative stress (68).

Other redox reactions influenced by H^+ and CO_2/HCO_3^-. Changes in pH modulate other redox reactions besides the Fenton reaction (70, 91). For instance, the protonated form of superoxide, $HO_2^\bullet$, which is favored by acidification, has greater reactivity than $^\bullet O_2^-$ and is thought to initiate membrane lipid peroxidation. Protonated superoxide is uncharged and more readily crosses the plasma membrane compared to the charged, impermeable superoxide anion (38). In addition, unconsumed H_2O_2 reacts with SOD, which is the catalytic enzyme that converts $^\bullet O_2^-$ to H_2O_2 (Fig. 1, *Rxn 4*). As a result, a reactive intermediate of SOD is produced that behaves as a hydroxyl radical (SOD-Cu^{2+}-$^\bullet OH$) and, in the process, inactivates SOD (91). In the presence of CO_2/HCO_3^-, however, HCO_3^- reacts with the SOD-Cu^{2+}-$^\bullet OH$ to produce active SOD and $CO_3^-{}^\bullet$ (91).

To summarize the first section, in a CO_2/HCO_3^--buffered system, increasing the level of CO_2 results in formation of carbonic acid and nitrosoperoxocarboxylate. Both reactions are

accelerated by carbonic anhydrase and occur in the extracellular and intracellular compartments. Carbonic acid immediately dissociates into H^+ and HCO_3^- causing cellular acidosis. At the same time, $ONO_2CO_2^-$ reacts to generate RNS intermediates that result in cellular oxidation, nitration, and nitrosylation. Likewise, acidosis accelerates other redox reactions, producing additional ROS, particularly during metabolic acidosis. The available pool of stimuli during hypercapnic acidosis and metabolic acidosis, therefore, includes decreased pH and various reactive intermediates that can potentially modulate membrane-bound proteins and lipids (11) through various nitrosative and oxidative reactions. The pool of reactive species produced during respiratory acidosis vs. metabolic acidosis is predicted to be different depending on the respective contributions from Fenton chemistry (proton activated) vs. peroxynitrite chemistry (CO_2/HCO_3^- activated). It is tempting to speculate that such differences in redox states could underlie differences in the brain stem chemoreceptor and ventilatory response to respiratory acidosis vs. metabolic acidosis (24).

EVIDENCE OF REDOX AND NITROSATIVE SIGNALING IN BRAIN STEM CHEMOSENSITIVE AREAS

What is the evidence, therefore, that neurons in chemosensitive areas produce the requisite enzymes (NOS, NADPH oxidase, and SOD) and reactants ($^\bullet O_2^-$, H_2O_2, $^\bullet NO$, and $ONOO^-$) to drive the $CO_2/ONOO^-$ reaction, Fenton reaction, and other pH-dependent redox reactions under physiological and pathological conditions of acidosis? There are multiple areas of CO_2-chemosensitivity dispersed throughout the brain stem (59), including the dorsal medulla [solitary complex, SC (15)] and pons [locus ceruleus (LC)], midline medullary raphe, pre-Bötzinger complex (PBC), and ventrolateral medulla (VLM), including the retrotrapezoid nucleus (RTN) and rostroventral medulla (RVLM). Of these, only the SC has been investigated to date in the context of how oxidative stress affects putative CO_2 chemoreceptors (19, 57, 58). The nucleus tractus solitarius (NTS) is recognized as a site of CO_2 chemosensitivity in the dorsocaudal medulla oblongata (59, 81). For reasons discussed elsewhere, I have proposed (15) that the boundaries of the dorsocaudal chemosensitive area in the medulla encompass the caudal NTS and dorsal motor nucleus of the vagus nerve (DMV). Hence, evidence for redox signaling in NTS and DMV, which together comprise the SC, is considered below. While few labs have studied redox signaling as it pertains to CO_2 chemoreception, several studies have investigated the mechanisms of ROS/RNS production in chemosensitive areas but from the perspective of understanding redox signaling mechanisms underlying cardiovascular control and dysfunction (8, 32, 41, 61) and the hypoxic ventilatory response (13, 39, 40, 47, 62).

Nitric oxide. Nitric oxide, which is required for $ONOO^-$ production, is produced with L-citrulline from L-arginine in a NOS-catalyzed reaction that uses molecular O_2 and NADPH (45, 52) (Fig. 1, *Rxn 2*). NOS immunoreactivity has been localized in the caudal NTS (40, 64, 67), DMV (94), PBC (48), RVLM (7, 13, 44, 64, 95), and dorsal raphe (66, 84). Microinjection of $^\bullet NO$ donor unilaterally into the NTS in awake and unrestrained rats stimulated ventilation in both normoxia and hypoxia, whereas microinjection of NOS inhibitor into the NTS blunted the hypoxic ventilatory response (62). Bilateral injection of NOS inhibitor into the caudal NTS of awake rats increased arterial blood pressure and decreased the ventilatory response to hypoxic activation of the peripheral chemoreceptors (36, 39). In the PBC, 53% of the neurons expressing neurokinin-1 receptor immunoreactivity—the marker for PBC neurons—were colocalized with NOS (48). Other investigations have focused on the larger structure, the RVLM. Sympathoexcitatory neurons in RVLM also express NOS (44, 95); these neurons are thought to be chemosensitive or to receive input from chemoreceptors in the RTN (53, 54). Nitric oxide has been implicated in RVLM control of the hypoxic ventilatory response (13), pressor response (9, 32, 41, 61), and heart failure (8). Serotonergic neurons in the dorsal raphe also express NOS (66, 84).

In addition, *S*-nitrosothiols are NO donors that produce $^\bullet NO$, nitrosonium cations (NO^+) and nitroxyl anions (NO^-) through enzymatic cleavage reactions (31, 90). *S*-nitrosothiols target neurons in the NTS in a stereoselective fashion for reflex control of blood pressure and the hypoxic ventilatory response. Microinjection of *S*-nitroso-L-cysteine into the NTS caused a dose-dependent increase in heart rate and arterial blood pressure (65) and mimicked the stimulatory effects of hypoxia on minute ventilation (47). Likewise, microinjection of *S*-nitrosocysteinyl glycine into the NTS also mimicked the hypoxic ventilatory response (47).

Superoxide anion. Superoxide anion, which is required for production of $ONOO^-$, is generated from enzymatically catalyzed reactions involving NADPH oxidase and xanthine oxidase (22) (Fig. 1, *Rxn 1*). NADPH oxidase was localized in caudal NTS neurons (32, 92), and NADPH oxidase-dependent $^\bullet O_2^-$ production was measured in NTS neurons (61). In addition, $^\bullet O_2^-$ is produced nonenzymatically by reduction of molecular O_2 at semiubiquinone at complexes I and III of the electron transport chain in mitochondria (22, 37) (Fig. 1, *Rxn 1*). Experiments in our laboratory indicate that $^\bullet O_2^-$ is produced in caudal NTS and DMV (i.e., SC) neurons during normobaric hyperoxia and, paradoxically, hypoxia (16, 17). The source of $^\bullet O_2^-$ during hypoxia is complex III in the mitochondria (17, 37). The source of $^\bullet O_2^-$ during hyperoxia is not complex III, however, and has not yet been identified (17). Increased production of ROS in RVLM has been implicated in the control of blood pressure, maintenance of hypertension, chronic heart failure, and cardiorespiratory responses to intermittent hypoxia (9, 29, 41). Specifically, $^\bullet O_2^-$ is produced in RVLM neurons from both NADPH oxidase (29) and mitochondrial electron transport (9, 60) and contributes to the pressor response. NADPH oxidase subunits ($p40^{phox}$, $p47^{phox}$ and $gp91^{phox}$) are expressed in RVLM neurons and their expression increases during chronic heart failure. Tempol, a $^\bullet O_2^-$ scavenger, inhibits development of experimental chronic heart failure and decreases blood pressure in control and hypertensive rats (29).

Peroxynitrite. The only chemosensitive region in which $ONOO^-$ activity has been measured is the RVLM; no other chemosensitive area has been investigated to my knowledge. In the RVLM, $ONOO^-$ has been implicated in fatal cardiovascular depression (7, 8) and endotoxin-induced apoptosis (25). That said, it is plausible that $ONOO^-$ is synthesized in other chemosensitive areas given reports that NOS/$^\bullet NO$ and NADPH oxidase/$^\bullet O_2^-$ and SOD are localized in a number of chemosensitive areas (see above). For example, formation of

$ONOO^-$ is thought to be regulated by SOD, which can lower the concentration of $^\bullet O_2^-$ (Fig. 1, *Rxn 4*) available for producing $ONOO^-$ (86). Superoxide dismutase has also been identified in the SC (61, 94). Likewise, $^\bullet NO$ and $^\bullet O_2^-$ activity have been identified in SC (16, 17, 32, 61, 92).

Fenton reaction. Cellular acidosis accelerates the Fenton reaction (Fig. 1, *Rxn 10*) by increasing dissociation of iron from Tf (Fig. 1, *Rxn 11*), making it available for reducing H_2O_2 to $^\bullet OH$. Transferrin is located throughout the medulla and pons (1, 55), with high concentrations noted in the LC (55), dorsal raphe (56), and SC (49, 55). Hydrogen peroxide, which reacts with iron in the Fenton reaction, is produced from $^\bullet O_2^-$ via the dismutase reaction catalyzed by SOD (22) (Fig. 1, *Rxn 4*). The presence of SOD, therefore, indicates ongoing production of H_2O_2 from $^\bullet O_2^-$ [and also possible control of $ONOO^-$ production, as discussed above (86)]. To date, $^\bullet O_2^-$ production has been demonstrated in the SC (16, 17, 32, 61, 92) and RVLM (9, 32, 60). Immunoreactivity for manganese-superoxide dismutase (Mn-SOD) and cooper/zinc-superoxide dismutase (Cu/Zn-SOD) was detected in SC neurons (61, 94). Transfection of adenovirus vectors encoding the Mn-SOD gene or Cu/Zn-SOD into the NTS reduced blood pressure in spontaneously hypertensive rats (41). Injection of H_2O_2 into the NTS induced a dose-dependent, transient bradycardia and hypotension in anesthetized rats. Likewise, injection of catalase inhibitor, which delays conversion of H_2O_2 to H_2O, thereby allowing accumulation of endogenously produced H_2O_2, caused a similar bradycardia and hypotension (6). The ANG II-induced pressor response elicited from RVLM neurons involves Ca^{2+}-dependent increase in mitochondrial $^\bullet O_2^-$ production. Overexpression of Mn-SOD, which accelerates conversion of $^\bullet O_2^-$ to $^\bullet OH$, decreases the magnitude of ANG II-induced ROS production and the pressor response (60).

To summarize the second section, the data, which come mostly from studies of the SC and RVLM, indicate that neurons in chemosensitive areas express NOS, NADPH oxidase, and SOD under baseline conditions and employ signaling mechanisms that use $^\bullet O_2^-$, $^\bullet NO$, $ONOO^-$, and SNO. Perturbing these redox signaling mechanisms through their activation or inhibition produces alterations in cardiorespiratory output. The relative contribution of these redox reactions to CO_2 chemoreception, however, is untested to date. It will be important to determine how perturbation of these redox reactions affects neuronal chemosensitivity in vitro as well as the ventilatory response to hypercapnia in intact and chemodenervated animals. For example, one could study mechanisms of whole animal and cellular CO_2 chemosensitivity against a background of acute and chronic oxidative and nitrosative conditioning to determine the role of redox signaling in CO_2 chemoreception (19). For example, the ventilatory response or cellular response to hypercapnic acidosis and metabolic acidosis could be studied by employing sustained hyperoxia to induce ROS/RNS production. These studies, of course, will need to redefine the control O_2 conditions, which are usually hyperoxic (see below)[2]. Alternatively, dietary manipulations that upregulate or downregulate endogenous antioxidant de-

fenses can be employed, or animal models lacking the requisite oxidative enzymes for producing ROS and RNS (e.g., SOD, NOS knockout mice, etc.).

OXIDATION AND ACIDOSIS STIMULATE SC NEURONS

If neurons in CO_2-chemosensitive areas of the brain stem employ redox signaling mechanisms, then oxidative stress is anticipated to perturb neuronal excitability, pH_i regulation, and alveolar ventilation. The evidence to date indicates that putative CO_2-chemoreceptor neurons in the SC are stimulated and acidified by oxidative stress (57, 58). Other chemosensitive areas have not yet been studied. In addition, hyperoxia, particularly isocapnic hyperoxia, is a powerful oxidant and stimulus of breathing, even following carotid body denervation, which indicates that hyperoxic stimuli—ROS/RNS—are acting centrally. The significance of this so called "hyperoxic hyperventilation" as evidence of redox modulation of respiratory networks was reviewed previously in *Journal of Applied Physiology* (19). To date, three sources of oxidative stress have been tested on neurons in the SC in rat brain stem slices: *1*) hyperoxia, which increases $^\bullet O_2^-$, $^\bullet NO$, and $ONOO^-$; *2*) oxidizing agents that target the amino acids cysteine and methionine to form disulfides; and *3*) chemical sources of ROS ($^\bullet O_2^-$ and H_2O_2) at constant hyperoxia (95%O_2).

Hyperoxia and chemical oxidants. Molecular oxygen has a positive redox potential ($E = +0.82$ V) making it an excellent oxidant that readily accepts electrons. Accordingly, hyperoxia is a reliable test stimulus for activating redox signaling mechanisms, especially those involving $^\bullet O_2^-$, $^\bullet NO$, and $ONOO^-$ (16–18, 20). Essentially all studies of neurons in reduced tissue preparations—such as rat brain stem slices—are done under hyperoxic control conditions (95%O_2 in CO_2/HCO_3^- medium). The only way to increase neural tissue partial pressure of oxygen (P_{O_2}) further is to increase barometric pressure and use of hyperbaric oxygen (HBO_2), which is the experimental approach we have used to investigate the effects of oxidative stress on CO_2-sensitive neurons in SC (18, 19, 57). Hyperbaric O_2 decreased membrane conductance and stimulated integrated firing rate (IFR) in 38% ($n = 43/113$) of the SC neurons tested. Of this group, 60 neurons, which included HBO_2-sensitive and -insensitive neurons, were also exposed to hypercapnic acidosis and/or chemical oxidants. Of these neurons, 48% ($n = 29/60$) were stimulated by HBO_2 and/or chemical oxidants; and of these, 90% ($n = 26/29$) were CO_2 excited. The excitatory effects of HBO_2 were blocked by the antioxidant, Trolox C, a membrane-permeable vitamin E analog. The excitatory effects of HBO_2 were mimicked by application of a chemical oxidant in 95% O_2 (57). Two chemical oxidants were tested, chloramine-T (CT) and N-chlorosuccinimide (NCS), neither of which produces ROS/RNS, but both are fairly specific oxidizers of cysteine and methionine. These chemical oxidants stimulated both CO_2-excited neurons and CO_2-insensitive neurons; altogether, 63% of the SC neurons tested were stimulated by CT/NCS. Whenever high CO_2 and HBO_2 were coadministered, the combined stimulation was greater than either stimulus alone. In half the neurons tested, the IFR response to hyperoxia and hypercapnia was additive, whereas in the other half of neurons tested the combined IFR response to hyperoxia and hypercapnia was greater than the sum of the individual responses.

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[2] For in vivo studies, it will be necessary to compare the ventilatory response to CO_2 in intact animals versus carotid body denervated animals. The caveats of using 100% O_2 to chemically denervate the peripheral CO_2 chemoreceptors have been reviewed elsewhere (19).

The unspoken caveat in brain slice studies is that equilibrating nutrient medium with 95% O_2 produces tissue hyperoxia throughout a 300- to 400-μm slice, including the core, that is equivalent to a rat breathing >2 atm of HBO_2 (18). Hyperoxic control medium induces ROS production in brain slices and increases cell death (12, 16, 17). Given that HBO_2 stimulates certain SC neurons maintained in 95% O_2 at room pressure (57), and that $\bullet O_2^-$ production increases significantly in brain slices maintained in 95% O_2 for 4 h (12, 16, 17), we have initiated experiments using a lower control level of O_2 (20–40%). Under these conditions, SC neurons in 300- to 400-μm-thick transverse slices, harvested from neonates and weaned rats, remained viable for many hours based on electrophysiology criteria (50) and capacity for cells to produce ROS during O_2 manipulation (16, 17). Under these new control conditions, acute exposure to normobaric hyperoxia (95% O_2) also stimulated IFR neurons in the SC, including CO_2-excited and CO_2-inhibited neurons (50).

Chemical oxidants, ROS, and intracellular pH. Acute exposure to chemical oxidants and generators of ROS in 95% O_2 caused significant decreases in pH_i in SC neurons. Chemical oxidants CT and NCS significantly inhibited Na^+/H^+ exchange leading to intracellular acidosis. Likewise, H_2O_2 and dihydroxyfumarate, which generates extracellular $\bullet O_2^-$, acidified SC neurons (58). The effect of normobaric hyperoxia on pH_i, by comparison, is more complicated than the other oxidative stimuli tested since O_2 availability influences a broader range of redox reactions through simultaneous production of $\bullet O_2^-$ and $\bullet NO$ and activation of multiple downstream redox reactions. In addition, hyperoxia will affect cellular respiration and neuronal activity. All of these effects, in turn, will influence pH_i. In SC neurons, pH_i is more alkaline during normobaric hyperoxia and decreases with decreasing tissue P_{O_2}. Specifically, average pH_i plateaus at 7.44–7.45 in superfusate equilibrated with 80–95% O_2. Decreasing steady-state O_2 to 60–40% decreases pH_i to another plateau averaging 7.34–7.35 (69). These data suggest that the excitatory effect of hyperoxia on the IFR of SC neurons (57) is mediated primarily by cellular oxidation rather than hyperoxia-induced acidification. At constant O_2, however, conditions that increase production of ROS and cause cellular oxidation would both acidify and oxidize SC neurons and IFR would be determined by the balance between oxidation and acidification (58), as illustrated next.

In the case of CT and NCS, one must consider the effects of both cellular oxidation and intracellular acidosis; that is, because chemical oxidants applied at constant O_2 resulted in decreased pH_i, were SC neurons stimulated by intracellular acidosis caused by oxidative stress or was the effect of cellular oxidation separate from that of cellular acidosis? To determine this, Mulkey et al. (58) developed a novel method for clamping pH_i at control level during exposure to CT using CO_2/HCO_3^- manipulations. These experiments showed that SC neurons increased IFR in the absence of intracellular acidosis indicating cellular oxidation alone stimulated SC neurons. Exposure to simultaneous cellular oxidation and acidification tended to cause a larger stimulation of IFR (58).

To summarize the final section, the foregoing discussion indicates that neurons in the dorsocaudal chemosensitive area of the medulla oblongata are stimulated by various sources of oxidative stimuli under in vitro conditions. The excitatory effects of hyperoxia are caused by decreased membrane conductance and are reversibly blocked by a membrane-permeable antioxidant. Putative CO_2-chemoreceptor neurons, that is, neurons stimulated by hypercapnic acidosis, are likewise stimulated by HBO_2 and chemical oxidants. Exposure to hypercapnic HBO_2 caused a greater stimulation of IFR than exposure to either stimulus alone. Chemical oxidants and generators of ROS at constant O_2 acidify SC neurons by inhibition of Na^+/H^+ exchange and stimulate IFR. The excitatory effects of oxidation and acidification occur independently of each other however. While the stimulatory effects of hyperoxia on IFR occur primarily in chemosensitive SC neurons, the effects of chemical oxidants (at constant O_2) on IFR and pH_i regulation indicate that redox stimulation influences the functions of a larger population of SC neurons. The functional significance of sensitivity to hyperoxia and chemical oxidants suggests that certain neurons in the SC, including CO_2-excited neurons (i.e., putative CO_2 chemoreceptors), use redox signaling mechanisms to carry out certain aspects of their normal functions (19). The excitability of SC neurons is determined, we propose, by the relative levels of pH_o/pH_i and ROS/RNS at any given time (58), both of which decrease membrane conductance. Presumably, different populations of pH-sensitive channels and redox-sensitive channels are involved, which include potassium channels (57, 58, 72). Redox- and nitrosative-sensitive components of the plasma membrane that are targeted by ROS and RNS during hyperoxia likewise are affected, we hypothesize, by ROS and RNS generated during hypercapnic acidosis (via $CO_2/ONOO^-$ reaction) or isocapnic acidosis at constant O_2 (via Fenton reaction) (Fig. 1). These same redox sensitive mechanisms would also render neurons in the SC vulnerable to oxidative and nitrosative stress during pathological conditions of ischemia, hyperoxia, and chronic respiratory acidosis and metabolic acidosis.

Perspectives

Carbon dioxide, like O_2 and several other gas species (70), is able to effect redox signaling mechanisms through production of oxidative and nitrosative derivatives. These effects of hypercapnia and acidosis on redox signaling have rarely been considered in studies of central CO_2 chemoreception (19, 57). During CO_2 retention, therefore, the scope of their importance in defining brain stem activity and, ultimately, alveolar ventilation, is unknown and requires further research. The ventilatory response in the conscious animal to changes in CO_2/pH is incredibly sensitive (34, 59, 81). I am postulating that these CO_2/H^+-sensitive redox reactions are poised, under physiological conditions, such that small perturbations in CO_2/pH produce changes in redox state that, in tandem with changes in cellular pH, affect central chemoreceptors (and other cardiorespiratory neurons) and thus alveolar ventilation. Clearly, the data indicate that neurons in multiple chemosensitive areas express the biochemical machinery for producing significant quantities of ROS/RNS during hypercapnic acidosis and isocapnic acidosis. Moreover, neurons in at least one chemosensitive area (SC) are stimulated directly by CO_2-induced acidification and oxidation, and indirectly through oxidation-induced acidification (57, 58). Thus chemoreceptor activity, at least in the SC, is going to be determined by the balance between the relative levels of pH_i and redox state during hypercapnic acidosis (58). Furthermore, the influence of any

CO_2/H^+-driven redox reaction will be amplified by conditions that enhance production of their reactants; that is, $^\bullet O_2^-$ and $^\bullet NO$ for the $CO_2/ONOO^-$ reaction, and $^\bullet O_2^-$, iron, and H_2O_2 for the Fenton reaction. Hence, hypoxia, posthypoxic reoxygenation, and hyperoxia (17), when combined with CO_2 retention, will result in increased ROS/RNS production through the combined redox and nitrosative chemistry of molecular O_2 and CO_2/H^+.

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