Chemical oxidants acidify solitary complex (SC) neurons in the rat.

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INTRODUCTION

Breathing high levels of oxygen (*i.e*. hyperoxia) causes hyperventilation and unstable breathing in normal and carotid-deafferented animals (1,2). Prolonged exposure to hyperoxia can disrupt central nervous system (CNS) function and result in a condition termed CNS O_2 toxicity, the primary sign of which is convulsion (3,4). The mechanism by which hyperoxia stimulates ventilation and disrupts CNS function is unknown; however, these effects are thought to result from increased production of reactive oxygen species (ROS) during hyperoxia and subsequent oxidation of cellular components vital to normal function (4,5). At moderate levels, ROS, including superoxide and nitric oxide, as well as their reactive nonradical derivatives (*e.g.* peroxide, S-nitrosothiols), modulate many physiological processes (4,6), including the hypoxic ventilatory response (7). However, at higher concentrations, ROS can result in oxidative stress that damages cellular components and therefore, are toxic to most cells (4,6). For example, ROS have been implicated in central respiratory control disorders, such as central alveolar hypoventilation syndrome (*e.g.* sudden infant death syndrome) (8).

We have studied the electrophysiological effects of oxidative stress imposed by hyperoxia or by chemical oxidants on neurons from the dorsal medulla oblongata (9-11). In particular, we have studied the nucleus tractus solitarius and dorsal motor nucleus (*i.e*. solitary complex, SC). We showed that acute exposure to hyperoxia or chemical oxidants selectively stimulated firing rate of CO_2/H^+-chemosensitive SC neurons (4,11). The SC is an important cardio-respiratory control center and CO_2/H^+-chemosensitive neurons are thought to provide the primary stimulus for breathing (12). Therefore, our results may explain how oxidative stress causes hyperventilation, and with chronic exposure, possibly contributes to central cardio-respiratory control dysfunction. In addition, we showed that the antioxidant Trolox-C blocked the effects of hyperoxia, but not of hypercapnia, on neuronal excitability, thus suggesting that hyperoxia effects neuronal excitability by a ROS-dependent mechanism (11). We also showed

that high CO_2 (hypercapnia) stimulates CO_2/H^+-chemosensitive brainstem neurons by a decrease in intracellular pH (pH_i) (13). Together, these results indicate that CO_2/H^+ and redox signaling mechanisms are both present in the CO_2/H^+-chemosensitive population of SC neurons. These results, then, beg the following question: Is there an interaction between the CO_2/H^+ and redox signaling mechanisms at the level of the single neuron? The goal of this study was to test the hypothesis that oxidative stress in the form of chemical oxidants causes a decrease in pH_i.

METHODS

To test this possibility, we measured pH_i using ratiometric fluorescence imaging microscopy, utilizing the pH-sensitive fluorescent dye, 2'-7'-bis(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF), in SC neurons in rat brainstem slices. BCECF is in a state that cannot be further oxidized. Therefore, we assumed that chemical oxidants do not directly affect the fluorescence of the dye. Details regarding the preparation of brain slices (9) and use of BCECF for pH imaging in brain slices have been previously described (14). Briefly, the brainstems from rat pups ranging in age from 2 to 15 days after birth were isolated and cut into 300 μm thick transverse slices. Slices were loaded in the dark with 20 μM BCECF (in the membrane permeable acetoxy methylester form) for 30-60 minutes at 37°C and washed at room temperature in artificial cerebral spinal fluid (aCSF) of the following composition (mM): 125 NaCl, 5.0 KCl, 1.3 $MgSO_4$, 26 $NaHCO_3$, 1.24 KH_2PO_4, 2.4 $CaCl_2$, 10 glucose and equilibrated with 95% O_2-5% CO_2, pH = 7.45. Experiments were performed on individual slices transferred to a superfusion chamber positioned on the stage of an inverted Nikon Diaphot microscope. The dye was excited every 60 seconds by brief alternating pulses of light with wavelengths of 500 nm (pH sensitive) and 440 nm (pH insensitive). pH_i is proportional to the ratio of emitted fluorescence (535 nm) at these two excitation wavelengths (F_{500}/F_{440}). This fluorescence ratio was normalized to the fluorescence ratio value at pH 7.2. Normalized fluorescence was converted to pH_i using the equation of Ritucci, *et al* (15). While measuring pH_i, oxidative stress was imposed by exposing a slice to the chemical oxidants chloramine-T (CT; Sigma-Aldrich) or N-chlorosuccinimide (NCS; Sigma-Aldrich) at concentrations previously shown to stimulate firing rates in 67% of SC neurons (500μM for CT and 1.0 mM for NCS) (11). pH_i responses to CT and NCS were compared to hypercapnic acidosis (15% CO_2), which is also known to stimulate firing rates of SC neurons (11-13). Paired-sample t-tests ($p \leq 0.05$) were used to determine when the mean population difference in pH_i at control and during oxidative stress differed significantly from zero.

RESULTS

Intracellular pH measured under control conditions was 7.42 ± 0.005 (N=31) and was similar to values previously reported for SC neurons (15). Exposure of SC neurons to CT and NCS caused a decrease in pH_i of -0.25 ± 0.02 pH units (N=29) and -0.24 ± 0.02 pH units (N=18), respectively (Figure 1). Interestingly, both CT- and NCS-induced acidification typically reached stable plateaus and showed no signs of pH_i regulation during oxidative stress. On return to control aCSF, pH_i typically showed some return to control levels; however, in most cases, pH_i, did not return to control levels even thirty minutes after removal of CT or NCS (not shown).

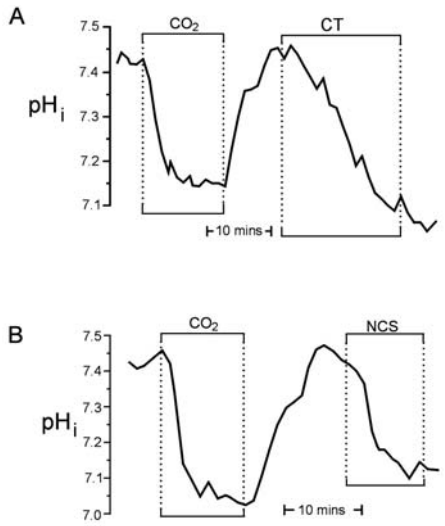

Fig. 1. Exposure to chemical oxidants decreased pH_i in 56% of SC neurons tested. A, representative pH_i trace from an individual SC neuron showed that 15% CO_2 decreased pH_i (~0.25 pH units) in a fashion typical for SC neurons. That is, pH_i remained acidified for the duration of the CO_2 exposure (*i.e.* no pH_i regulation; 13,15). Subsequent exposure to Chloramine T (CT, 500μM) decreased pH_i by an amount comparable to that of 15% CO_2 (~0.25 pH units). However, in most cases, pH_i failed to return to near-control levels when CT was removed (not shown). B, representative pH_i trace from one SC neuron showed that N-chlorosuccinimide (NCS, 1 mM) decreased pH_i by ~0.3 pH for the duration of the exposure (*i.e.* no pH_i regulation; 15). pH_i did not typically return to control levels when NCS was removed (not shown).

DISCUSSION

The observation that chemical oxidants decreased pH_i similarly to hypercapnic acidosis suggests that the response of CO_2/H^+-chemsensitive neurons to oxidative stress is partially mediated by decreased pH_i. We have previously shown that decreased pH_i plays a major role in the response of CO_2/H^+-chemosensitive brainstem neurons to hypercapnia (13). Also, we have shown that oxidative stress and hypercapnia have similar effects on the excitability of CO_2/H^+-chemosensitive neurons. That is, oxidative stress (hyperoxia, CT or NCS) and hypercapnia (15% CO_2) increase firing rate in conjunction with decreased membrane conductance (g_m), possibly by decreasing potassium channel conductance (11,13). Together, these results indicate that oxidative stress and CO_2 signaling mechanisms share a point of interaction (*i.e.* decreased pH_i) that may contribute to the response of CO_2/H^+-chemsensitive neurons to oxidative stress.

The mechanism by which chemical oxidants cause an intracellular acidification is unknown. However, there are several ways oxidative stress may decrease pH_i: i) oxidative stress may disrupt the citric acid cycle (16), resulting in increased lactic acid production (17); ii) oxidative stress may increase ATP hydrolysis, thereby releasing H^+ (19); and iii) oxidative stress may disrupt pH_i regulation by oxidizing the Na^+/H^+ exchanger (15). With regard to the first possibility, the buffering power of cells in this region is high, at 45 mM/pH unit (20,21). Therefore, to change pH_i by 0.25 pH units would require ~11 mM lactic acid (one H^+/lactic acid) to be produced during oxidative stress. This value is similar to transient increases in lactate levels reported to occur during focal brain activation, ~6 mM (22). It has further been shown that oxidative stress can increase lactate production in the CNS (17). Concerning the second possibility, for ATP breakdown to decrease pH_i by 0.25 pH units, hydrolysis of ~11 mM ATP (H^+/ATP) would be required. This value is well in excess of intracellular ATP levels in the brain, ~4.6 to 6.4 mM (23,24). With regard to the third possibility, our findings that pH_i did not recover during exposure to chemical oxidants suggests that the Na^+/H^+ exchanger, which is the major pH_i recovery mechanism in SC neurons (15), was inhibited by oxidative stress. However, Chambers-Kersh, *et al* (21) showed that the inhibition of the Na^+/H^+ exchanger under control conditions with amiloride did not acidify neurons in the nucleus tractus solitarius (*i.e.* dorsal SC). The possibilities listed above (i-iii) are not mutually exclusive and it is likely that the effects of oxidative stress involve a combination of these effects.

Oxidative stress-induced acidification may also feed back to exacerbate the effects of oxidative stress by increasing ROS production. For example, decreased pH_i may dissociate iron

from transferrin (5, 25) and possibly, ferritin (26), to facilitate iron catalysis of superoxide and hydrogen peroxide to the very reactive hydroxyl radical (*i.e.* Fenton reaction) (4) or increase the formation of reactive nitrogen species (25). Our current working model of the effects of oxidative stress and pH_i on the excitability of CO_2/H^+-chemosensitive neurons from the brainstem is summarized in Figure 2. This model illustrates that oxidative stress and CO_2 may both increase ventilation by stimulating CO_2/H^+-chemosensitive SC neurons. Possible interactions between oxidative stress, decreased pH_i and firing rate are also indicated.

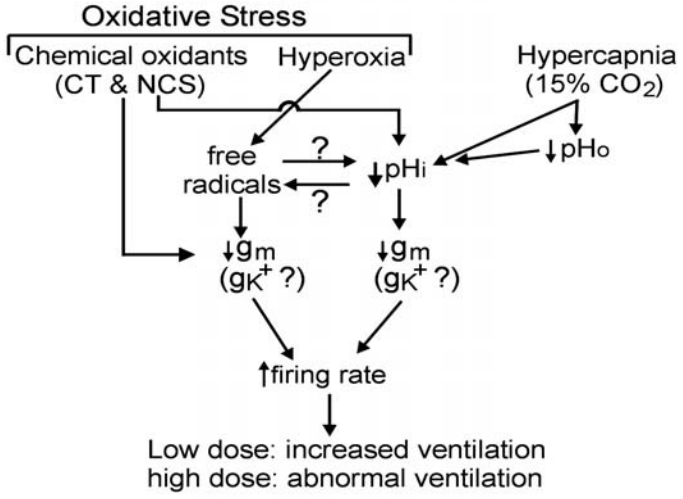

Fig. 2. Current model of the effects of oxidative stress on SC neurons. Oxidative stress in the form of chemical oxidants (CT & NCS) or hyperoxia via free radicals decreases membrane conductance (g_m), by possibly decreasing conductance of a K^+ channel, and increases the excitability of CO_2/H^+-chemosensitive neurons (11). Hypercapnia decreases pH_i, which causes decreased membrane conductance, possibly by decreasing K^+ channel conductance, and increases excitability of CO_2/H^+-chemosensitive neurons (13). Chemical oxidants also decrease pH_i (this study), suggesting that decreased pH_i plays a central role in the selective sensitivity of CO_2/H^+-chemosensitive neurons to oxidative stress. Previous research by others indicates that there is an interaction between free radicals and pH_i (18,24). This, however, has yet to be determined for neurons in the SC.

The observation that chemical oxidants decrease pH_i explains, in part, why acid-sensitive neurons (*i.e.* CO_2/H^+-chemoreceptors) are also highly sensitive to oxidative stress (11) and could thus help explain how oxidative stress causes hyperventilation (1,2). Likewise, it may explain how severe or chronic oxidative stress contributes to central cardio-respiratory control dysfunction (8).

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