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NORMOBARIC HYPEROXIA (95% O_2) STIMULATES CO_2-SENSITIVE AND CO_2-INSENSITIVE NEURONS IN THE CAUDAL SOLITARY COMPLEX OF RAT MEDULLARY TISSUE SLICES MAINTAINED IN 40% O_2

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Abstract—We tested the hypothesis that decreasing the control level of O_2 from 95% to 40% reduces tissue partial pressure of oxygen (pO_2), decreases extracellular nitric oxide ($^{\cdot}NO$) and decreases intracellular superoxide ($^{\cdot}O_2^-$) while maintaining viability in caudal solitary complex (cSC) neurons in slices (~300–400 μm; neonatal rat P2–22; 34–37 °C). We also tested the hypothesis that normobaric hyperoxia is a general stimulant of cSC neurons, including CO_2-excited neurons. Whole-cell recordings of cSC neurons maintained in 40% O_2 were comparable to recordings made in 95% O_2 in duration and quality. In 40% O_2, cSC neurons had a significantly lower spontaneous firing rate but similar membrane potentials and input resistances as cSC neurons maintained in 95% O_2. Tissue pO_2 was threefold lower in 40% O_2 versus 95% O_2. Likewise, extracellular $^{\cdot}NO$ and intracellular $^{\cdot}O_2^-$ were lower in 40% versus 95% O_2. 67% of neurons maintained in 40% O_2 control were stimulated by hyperoxia (95% O_2) compared to 81% of neurons maintained in 95% O_2 that were stimulated during hyperoxic reoxygenation following acute exposure to 0–40% O_2. cSC slices maintained in 40% O_2 exhibited CO_2-chemosensitive neurons, including CO_2-excited (31.5%) and a higher incidence of CO_2-inhibited (31.5%) neurons than previously reported. Likewise, a higher incidence of CO_2-inhibited and lower incidence of CO_2-excited neurons were observed in 85–95% O_2. 82% of O_2-excited neurons were also CO_2-chemosensitive; CO_2-excited (86%) and CO_2-inhibited neurons (84%) were equally stimulated by hyperoxia. Our findings demonstrate that chronic (hours) and acute (minutes) exposure to hyperoxia stimulates firing rate in the majority of cSC neurons, most of which are also CO_2 chemosensitive. Our findings support the hypothesis that recurring exposures to acute hyperoxia and hyperoxic reoxygenation—a repeating surge in tissue pO_2—activate redox and nitrosative signaling mechanisms in CO_2-chemosensitive neurons that alter expression of CO_2 chemosensitivity (e.g., increased expression of CO_2-inhibition) compared to sustained hyperoxia (85–95% O_2).

Key words: chemosensitivity, hypercapnia, nitric oxide, superoxide, nucleus tractus solitarius, dorsal motor nucleus of the vagus.

INTRODUCTION

The caudal solitary complex (cSC), consisting of the caudal nucleus tractus solitarius (cNTS) and the caudal dorsal motor nucleus of the vagus (cDMV), is one of at least eight sites of central CO_2-chemosensitivity residing in the mammalian CNS that functions in CO_2-chemoreception and pH homeostasis (Nattie, 1999; Nattie and Li, 2009; Dean and Putnam, 2010). Although the cNTS and cDMV are distinct structures within the brain stem, there is considerable overlap in the distribution of afferent fibers, such that many previous authors have found it difficult to determine if afferent projections went to one or both nuclei and in some cases refer to it instead as the dorsal motor complex (Leslie, 1985; Onai et al., 1987; Yamamoto et al., 2010) or SC (Ruggiero et al., 1994; Dean and Putnam, 2010). Additionally, neurons within the cDMV have dendritic branches that penetrate into the ventral NTS (Shapiro and Miselis, 1985; Browning et al., 1999) and NTS neurons project into the DMV (Davis et al., 2004) reinforcing the notion that these structures are functionally interconnected. Dean, Putnam and colleagues (Dean et al., 2001; Dean and Putnam, 2010) propose that CO_2 chemosensitivity resides in both nuclei of the cSC and both nuclei are stimulated by redox and nitrosative stimuli generated

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Abbreviations: ACSF, artificial cerebrospinal fluid; cDMV, cadual dorsal motor nucleus of the vagus; cNTS, caudal nucleus tractus solitarius; cSC, caudal solitary complex; DHE, dihydroethidium; DIC, differential interference contrast; EGTA, ethylene glycol tetraacetic acid; FI, fluorescence intensity (arbitrary units); H_2O_2, hydrogen peroxide; HEPES, hydroxyethyl piperazineethanesulfonic acid; NADPH, nicotinamide adenine dinucleotide phosphate; $^{\cdot}NO$, nitric oxide; NOS, nitric oxide synthase; $^{\cdot}O_2^-$, superoxide anion; $ONOO^-$, peroxynitrite; pH_i, intracellular pH; pO_2, partial pressure of oxygen; R_{in}, input resistance (MΩ); RMP, resting membrane potential (mV); RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; V_m, membrane potential (mV).

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during hyperoxia (Dean et al., 2004) *and* hypercapnic acidosis (Dean, 2010).

Neurons in the cSC of medullary tissue slices (rat), including CO_2-chemosensitive neurons, are stimulated by an increase in tissue oxygen tension at hyperbaric pressure. For example, hyperbaric hyperoxia decreases membrane conductance and has a predominantly stimulatory effect on the firing rate of CO_2-excited neurons in the cSC. The underlying membrane conductance changes and excitatory firing rate response during hyperbaric hyperoxia are blocked by an antioxidant (Mulkey et al., 2003a). Exposure to chemical oxidants, applied at a constant level of control tissue oxygenation (95% O_2 at room pressure), mimics the stimulatory effects of hyperbaric hyperoxia (Mulkey et al., 2003a). In addition, exposure to chemical oxidants or a chemical generator of reactive oxygen species (ROS), applied at a constant level of control tissue oxygenation (95% O_2 at room pressure), decreases intracellular pH (pH_i) (Mulkey et al., 2004b). If pH_i is clamped by raising extracellular bicarbonate or lowering CO_2 while co-applying chemical oxidant, cSC neurons are still stimulated by oxidation alone. Thus, cSC neurons respond to *both stimuli:* oxidation and cellular acidification. In the intact animal, chronic hypoxia induces a form of neural plasticity that up-regulates the proportion of CO_2-inhibited neurons in slices harvested from these animals without significantly affecting the population of CO_2-excited neurons (Kline et al., 2007; Nichols et al., 2009b). Together, these results suggest that neurons in the cSC, including CO_2-chemosensitive neurons, are stimulated and modulated by O_2 perturbations over a broad range of oxygenation. These changes in partial pressure of oxygen (pO_2) activate intrinsic redox and nitrosative signaling mechanisms that reside in the cSC and other cardio-respiratory centers of the mammalian brain stem (Haxhiu et al., 1995; Wang et al., 2004; Dean, 2010). We have postulated that the excitatory effects of hyperoxia and other oxidative conditions on firing rates of cSC neurons may partially underlie the paradoxical phenomenon in respiratory physiology known as hyperoxic hyperventilation (Dean et al., 2004) and hyperoxic hyperpnea (Pilla et al., 2013).

Typically, brain slice experiments are performed by aerating a bicarbonate-buffered artificial cerebrospinal fluid (ACSF) medium with a gas mixture of 95% O_2 and 5% CO_2. Alternatively, a HEPES-buffered medium is equilibrated with 100% O_2. Because of our interest in hyperoxia as a neurostimulant of respiration (Mulkey et al., 2003a; Dean et al., 2004; Pilla et al., 2013) and reliable source of redox and nitrosative stress (Dean et al., 2003; Dean, 2010), we have been reevaluating the typical control O_2 conditions used for rat brain slice experiments; for example see (Mulkey et al., 2001, 2003a; Dean et al., 2004; D'Agostino et al., 2007; Ciarlone et al., 2013). Previous experiments published by this laboratory have shown that the oxygen tension throughout all depths of a 300–400-μm-thick rat brain tissue slice (34–37 °C, ACSF equilibrated with 95% O_2) is significantly above physiological norms (*in vivo*). In fact, the pO_2 is equivalent to the range of brain tissue pO_2s that occur when an intact mammal breathes over 2 atmospheres (203 kPa) absolute of hyperbaric oxygen (Mulkey et al., 2001; Garcia et al., 2010a). Additionally, experiments in CA1 hippocampal slices harvested from adult rats showed a dose-dependent relationship between the level of oxygenation of the superfusion medium and rate of production of superoxide anion ($^{\cdot}O_2^-$) and nitric oxide ($^{\cdot}NO$) at ~37 °C (Hwang, 2004; D'Agostino et al., 2007) and the level of isoprostane production (Fessel et al., 2002). Superoxide and other free radicals and their highly reactive derivatives have been implicated in normal cellular redox and nitrosative signaling mechanisms as well as abnormal redox and nitrosative stresses that can lead to cell damage or even cell death (Dröge, 2002; Dean, 2010). Lowering the control level of oxygenation should lower the amount of reactive species produced, as previously reported (Fessel et al., 2002; Hwang, 2004; D'Agostino et al., 2007), thereby lowering the amount of redox and nitrosative modulation of neurons studied in brain tissue slices.

Therefore, we hypothesize that neurons in the cSC will remain viable according to electrophysiological criteria after incubation and maintenance in a lower level of control oxygenation using 40% O_2. We predict that lowering the ACSF oxygen level from 95% to 40% will have no negative impact on the viability of cSC neurons, as assessed by their membrane potential (V_m), input resistance (R_{in}), and action potential properties (overshoot >0 mV, repetitively firing spontaneously or when depolarized by current injection) since the slice preparation maintains sufficient oxygenation, even at the core, in a 400-μm-thick brain slice (Mulkey et al., 2001; Garcia et al., 2010a). Secondly, we hypothesize that acute exposure to normobaric hyperoxia (95% O_2 at room pressure; i.e., the typical control level of O_2 used in brain slice experiments) will act as a neurostimulant, depolarizing V_m, increasing firing rate and increasing R_{in} (i.e., decreasing membrane conductance) of many cSC neurons, including CO_2-excited neurons as previously demonstrated using hyperbaric oxygen (Mulkey et al., 2003a). If true, then cSC neurons exhibit sensitivity to a broad range of oxygenation and, presumably, redox and nitrosative neuromodulation. Finally, we hypothesize that cSC neurons will retain CO_2-chemosensitivity while maintained in 40% O_2 control ACSF as demonstrated previously using higher levels of control and test oxygenation (Mulkey et al., 2003a).

The present study addresses the following four questions: (1) can whole-cell recordings be established and maintained sufficiently long in a lower level of control oxygen (40% O_2) to study the O_2- and CO_2-chemosensitivities of neurons in the cSC? (2) How does neuronal activity in slices maintained in ACSF equilibrated with 40% O_2 compare to slices maintained in 95% O_2? (3) What is the effect of acute exposure to (a) hyperoxia (i.e., 40% O_2 control→95% O_2 hyperoxia→40% O_2 recovery) and (b) hyperoxic reoxygenation (i.e., 95% O_2 control→deoxygenation using ⩽ 40% O_2→reoxygenation using 95% O_2 for recovery) on V_m, firing rate, and R_{in} of cSC neurons? (4) Is CO_2-chemosensitvity maintained in control ACSF