Hydrogen peroxide differentially affects activity in the pre-Bötzing- er complex and hippocampus

Alfredo J. Garcia III,1 Shakil A. Khan,3 Ganesh K. Kumar,3 Nanduri R. Prabhakar,3 and Jan-Marino Ramirez1,2
1Center for Integrative Brain Research, Seattle Children’s Research Institute and 2Departments of Neurological Surgery and Pediatrics, University of Washington, Seattle, Washington; and 3Institute for Integrative Physiology and Center for Systems Biology of Oxygen Sensing, Department of Medicine, University of Chicago, Chicago, Illinois

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Garcia AJ 3rd, Khan SA, Kumar GK, Prabhakar NR, Ramirez JM. Hydrogen peroxide differentially affects activity in the pre-Bötzing- er complex and hippocampus. J Neurophysiol 106: 3045–3055, 2011. First published August 17, 2011; doi:10.1152/jn.00550.2010.—Reactive oxygen species (ROS) modulate neuronal excitability. In the present study we examined the effects of hydrogen peroxide (H2O2), a well established ROS, on neuronal activity from two neonatal mouse brain regions, i.e., the pre-Bötzing complex (preBötC) within the ventral respiratory column (VRC) and the CA1 area of the hippocampus. In the preBötC, 2.2 mM H2O2 evoked a transient depression followed by augmentation of neuronal activity. The iron chelator defereroxamine (500 μM) did not prevent H2O2-mediated neuronal augmentation but prevented the initial depression. Combined application of Fe²⁺ and H2O2 only caused depression of the preBötC rhythm. In contrast, H2O2 suppressed neuronal activity in the CA1 region, and this effect was accentuated by coapplication of Fe²⁺ and H2O2, suggesting that hydroxyl radical generated by Fenton reaction mediates the effects of H2O2 on CA1 neuronal activity. Malondialdehyde (MDA) levels were unaltered in H2O2-treated preBötC, whereas MDA levels were markedly elevated in the CA1 region. These findings suggest that 1) exogenous administration of H2O2 exerts differential effects on neuronal activities of preBötC versus CA1 neuronal populations and 2) H2O2 is a potent modulator of respiratory rhythmogenesis from the preBötC without affecting global oxidative status.

A GROWING NUMBER OF STUDIES indicate that superoxide anion (O2⁻) and hydrogen peroxide (H2O2), well-characterized reactive oxygen species (ROS), function as neuromodulators. H2O2 affects presynaptic Ca²⁺ entry at the neuromuscular junction (Giniatullin and Giniatullin 2003), and O2⁻ has been hypothesized to contribute to the long-term facilitation of phrenic and hypoglossal motor outputs (Macfarlane and Mitchell 2008). H2O2 generated from O2⁻ sensitizes the carotid body response to hypoxia and induces sensory plasticity manifested as sensory long-term facilitation (Peng et al. 2003, 2009). In midbrain dopaminergic neurons, H2O2 reduces neuronal excitability by affecting ATP-sensitive potassium (KATP) channels (Avshulomov et al. 2005). In adult CA1 neurons of the hippocampus, O2⁻ (Knapp and Klann 2002) and low concentrations of H2O2 (Kamsler and Segal 2003) induce long-term potentiation, an established form of neuronal plasticity.

Neonates experience higher levels of ROS because of transition from the low-oxygen environment in utero to air (Perrone et al. 2010), and the resulting oxidative stress has been implicated in several neuropathological conditions including reperfusion injury following resuscitation (Saugstad 2001a; Varma et al. 2003) as well as oxygen toxicity resulting from the use of supplemental oxygen (Poets 2010; Saugstad 2001b). Additionally, oxidative stress may contribute to apnea of prematurity (Pawar et al. 2009). Although the effects of ROS are well documented in the adult nervous system, relatively little is known on the effects of ROS on the neonatal nervous system. In the present study, we tested the effects of H2O2 on neuronal activity of the pre-Bötzing complex (preBötC) in neonates, an area that is critical for breathing and hence survival (McKay et al. 2005; Ramirez and Garcia 2007). To test whether the effects of H2O2 are region selective, parallel experiments were performed on CA1, an area that is critical for learning and memory. Our results demonstrate that H2O2 causes an initial depression followed by excitation of neuronal activity in preBötC without inducing oxidative stress. In striking contrast, H2O2 depressed CA1 neuronal activity, and this effect was associated with increased oxidative stress.

METHODS

Solutions and pharmacology. Artificial cerebrospinal fluid (aCSF; in mM: 118 NaCl, 24 NaHCO3, 1.0 NaH2PO4, 3.0 KCl, 1 MgCl2, 1.5 CaCl2, and 30 d-glucose, pH 7.4) equilibrated with carbogen (95% O2, 5% CO2) was used for control O2 conditions. Intracellular patch electrode solution contained (in mM) 140 K-gluconic acid, 1 CaCl2, 10 EGTA, 2 MgCl2, 4 Na ATP, and 10 HEPES (pH 7.2). The NMDA receptor antagonist 3-((R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) and the AMPA/kainate receptor antagonist 6-cyano-7-nitroquininoxaline-2,3-dione (CNQX) were obtained from Tocris Cookson (Ellisville, MO). The glycine receptor antagonist strychnine (STR), the GABA_A-receptor antagonist picrotoxin (PTX), FeSO4, and defereroxamine were purchased from Sigma Aldrich (St. Louis, MO). H2O2 solution (unstabilized; 6%) was from VRW International (Radnor, PA).

Slice preparation. In accordance with National Institutes of Health guidelines, all experiments were performed either at The University of Chicago or at Seattle Children’s Research Institute with protocols approved by the Animal Care and Use Committees at the respective institutions. Transverse medullary brain stem slices and coronal cortical-hippocampal brain slices were prepared from neonatal CD1 mice (P7–P12). Mice were anesthetized (via either ether or isoflurane inhalation) and rapidly decapitated, and the brain and brain stem were...
quickly removed. Either the cerebrum or the brain stem was mounted to an agar block with cyanoacrylate glue. The mounted tissues were submerged in ice-cold aCSF equilibrated with carbogen, and slices of the region of interest were cut with a vibratome.

Transverse medullary brain stem slices (600 μm thick) were prepared with protocols described previously (Ramírez et al. 1996). The transverse medullary slice contained a portion of the ventral respiratory column (VRC) that included the preBötC and all anatomical landmarks previously described (Fig. 1A; Ramírez et al. 1996). In well-oxygenated conditions, two integrated waveforms are readily identified—fictive eupnea and fictive sighs. Fictive eupnea is characterized by an augmenting or bell-shaped population burst, while fictive sighs occur with a much lower frequency and are large amplitude with a biphasic burst shape (Lieske et al. 2000; Fig. 1B).

Fictive sighs are also followed by a prolonged interevent interval (i.e., postsigh apnea). Only one slice per brain stem was taken and transferred immediately to the recording chamber, where it was allowed to recover for 30–60 min prior to experimentation. In a subset of experiments, either one or both VRC regions were microdissected from the brain stem slice. In these VRC islands, the intrinsic rhythmic activity from the preBötC neuron is preserved, whereas the circuitry contributing to rhythmogenesis is greatly reduced. Cortical cortico-hippocampal brain slices (400–500 μm thick) were cut from a cerebrum mounted on agar in an orientation in which the frontal lobe was parallel to the cutting blade of the vibratome. The cortico-hippocampal slices were transferred into aCSF equilibrated with carbogen at ~21°C and bisected at the central fissure, yielding two usable slices. Experiments on the cortico-hippocampal slices were performed within 2 h after the slice preparation.

Electrophysiological recordings. After the transfer of hippocampal or medullary slices or VRC islands into the recording chamber (6-mL volume), carbogen-equilibrated aCSF (33 ± 2°C) was circulated and recycled (18–20 mL/min; a total volume of 100 mL) via a peristaltic pump. To initiate the fictive respiratory rhythm from the neuronal population of the VRC or spontaneous bursting from the CA1 neuronal population, the extracellular KCl concentration was elevated from 3 to 8 mM over a 20-min period. Experiments were initiated after 10-min equilibration of the slices with 8 mM KCl.

Extracellular recordings were made from either the CA1 neuronal population or the preBötC located in the VRC, using the medullary brain stem slices or VRC islands. The population rhythm from the preBötC generates distinct burst patterns similar to and implicated with in vivo patterns of breathing (Lieske et al. 2000; Pena et al. 2004). Under well-oxygenated conditions, two population burst patterns of preBötC can be discriminated: 1) fictive inspiratory bursts (i.e., fictive eupnea) and 2) fictive sighs (Fig. 1B). Using the integrated waveforms of the population bursts, we determined how H 2O2 affected the burst patterns and altered the frequency of fictive inspiratory bursts as well as fictive sighs. Extracellular recordings were made with glass electrodes filled with aCSF (~2 MΩ). Population activity was amplified, filtered (low pass 1.5 kHz; high pass 250 Hz), rectified, and integrated (time constant 60 ms) in real time. In a subset of extracellular recordings, continuous unilateral microinjection of H 2O2 (1.67–2.34 μL/s; 600-s duration) over one VRC region of the brain slice was performed. The injection pressure ranged between 30 and 60 Torr.

Patch-clamp recordings were made in brain stem slices from putative inspiratory neurons of the preBötC with a multiclamp amplifier (Molecular Devices, Sunnyvale, CA). For recordings, borosilicate glass electrodes (4–6 MΩ) prepared with a P-97 Flaming/Brown micropipette puller (Sutter Instrument, Novato, CA) and filled with intracellular patch electrode solution were used. A single recording from one neuron per brain stem slice was made. Inspiratory neurons were identified by their in-phase discharge pattern with the population rhythm. Experiments were performed in whole cell current-clamp configuration. Upon establishing a stable intracellular recording, the putative inspiratory neuron was isolated from the network with the AMPA receptor antagonist CNQX (10 μM), the NDMA receptor antagonist CCP (10 μM), the GABA_A receptor antagonist PTX (50 μM), and the glycine receptor antagonist STR (1 μM). When neurons exhibited a small (~2 mV) depolarization, a constant holding current (~−35 pA) was applied to return the membrane potential (V_m) back to the original steady-state V_m and the current was maintained for the entire duration of the experiment. Once a neuron was isolated, a current injection step protocol consisting of the injection of hyperpolarizing currents (~120 to 0 pA with a 20-pA step) and one depolarizing current (0 to 80 pA with a 80-pA step) was used. Measurements with this protocol were made in triplicate during 1) control conditions, 2) the early exposure to H 2O2 (eH 2O2; 60–180 s), and 3) the late exposure to H 2O2 (lH 2O2; 480–600 s). The mean value of the triplicate measurements was considered as the response of a given neuron during the aforementioned conditions. The calculated junction potential of ~12 mV was subtracted post hoc from V_m. Input resistance (R_i) of each neuron was calculated using the changes in V_m that resulted from the hyperpolarizing current injections. The number

Fig. 1. The pre-Bötzinger complex (preBötC) in the transverse medullary brain stem slice generates a population rhythm activity similar to and implicated with respiratory patterns found in vivo. A: cartoon schematic of the transverse brain stem slice containing the preBötC. This preparation also contains other neuronal regions related and unrelated to respiration. Anatomical landmarks include the nucleus ambiguus (NA), the hypoglossal nucleus (XII), fiber bundles of the hypoglossal nerve (CNXII), and the inferior olive (IO). Placing an extracellular recording electrode (RE) over the ventral respiratory column allows for the recording of the population rhythm from the preBötC. B: representative trace of the integrated (∫) and raw (preBötC) population activity originating from the preBötC. Inset: expanded timescale identifying the fictive sigh, postsigh apnea, and fictive normal inspiratory bursts (i.e., fictive eupnea).
of action potentials generated by injecting +80 pA was also determined.

Both extracellular and intracellular electrophysiological recordings were made with Clampex 9.0 or 10.0 (Molecular Devices). Extracellular recordings were sampled at 1.67 kHz, while intracellular recordings were sampled at 20 kHz. Typical experiments had a 600 s recording of baseline activity in control condition followed by a 600 s exposure to either experimental agent or washout. The time of switching between two different solutions was 60 ± 8 s.

Lipid peroxidation. The degree of lipid peroxidation was determined by measuring malondialdehyde (MDA) levels in slices treated with H$_2$O$_2$ (2.2 mM), Fe$^{2+}$-H$_2$O$_2$ (100 µM, 2.2 mM), or vehicle (control). The conditions and protocols used for the exposure of slices to H$_2$O$_2$ or Fe$^{2+}$-H$_2$O$_2$ were identical to those used in the respective extracellular recording experiments in which the agents were bath applied. For controls, slices were incubated with aCSF containing 8 mM KCl for 1,200 s. The slices were homogenized in 20 mM phosphate buffer, pH 7.4, and the homogenates were centrifuged in an Eppendorf microcentrifuge at 500 g for 10 min at 4°C. MDA levels were determined with procedures described previously (Raghuraman et al. 2011) and expressed as nanomoles per milligram of protein.

Statistics. Analyses of the integrated burst area, instantaneous frequency ($f_{inst}$), and burst count were conducted with Clampfit 9.0 (Molecular Devices). The irregularity score of $f_{inst}$ was used as a measure of burst-to-burst variability. It was calculated with the following formula from consecutive cycle length values:

$$ S_N = \frac{100 \times \text{ABS}(f_{inst}^{t+1} - f_{inst}^{t-1})}{f_{inst}^{t+1} - f_{inst}^{t-1}} $$

with $S_N =$ score of the $n$th cycle, $f_{inst}^{t+1}$, its $f_{inst}^{t+1}$, $f_{inst}^{t-1}$ the $f_{inst}$ of the preceding burst, and ABS the absolute value (see Telgkamp et al. 2002). With this formula, a low irregularity score represents a rhythm with low burst-to-burst variability in $f_{inst}$.

In dose-response experiments, plots of mean $f_{inst}$ as a function of elapsed time were made to readily identify H$_2$O$_2$-mediated suppression of frequency. Individual points of the mean $f_{inst}$ represented the moving average of $f_{inst}$ from four consecutive bursts. In some experiments, data bins were taken both prior to ($t = -120$ s) and during ($t = 180, 300$, and 600 s) experimental manipulations. In H$_2$O$_2$ washout and Fe$^{2+}$-H$_2$O$_2$ experiments, additional bins were taken during experimental manipulation at $t = 780, 900$, and 1,200 s. Each data bin was 120 s in duration to accurately capture the transient depression caused by H$_2$O$_2$ in the preBötC. Integrated burst frequency (i.e., burst count) and area were normalized to control conditions (i.e., $t = -120$ s).

Unless stated otherwise, $n$ values represent the number of individual slices from which the quantification was conducted. Numerical data are presented as means ± SE. Differences between two means were analyzed with Student’s t-test and were considered significant at $P < 0.05$. Differences between three or more means were determined with one-way repeated-measures ANOVA followed by multiple comparisons testing (Dunnett’s comparison). Statistics were calculated with either the KyPlot Data and Visualization software package (Kyenslab, Tokyo, Japan) or Graphpad InStat (Graphpad Software, La Jolla, CA).

RESULTS

Effects of H$_2$O$_2$ on inspiration-related neuronal activity in preBötC. Neuronal activity in the preBötC was characterized as bursts of activity in well-oxygenated conditions, i.e., fictive inspiration. An example illustrating the effect of H$_2$O$_2$ (2.2 mM for 600 s) on bursting activity of preBötC is shown in Fig. 2A. Superfusion of H$_2$O$_2$ over the slice caused an initial depression followed by augmentation of neuronal activity, and this effect was seen in 17 of 20 preparations. On average, neuronal activity ($f_{inst}$) decreased from 0.21 ± 0.03 Hz (control) to 0.17 ± 0.03 Hz, resulting in a decrease in burst frequency (54 ± 7% of control). At the end of H$_2$O$_2$ exposure, $f_{inst}$ increased to 0.28 ± 0.02 Hz with concomitant increase in

![Fig. 2. H$_2$O$_2$ biphasically affects the frequency of fictive normal inspiratory bursts. A: representative trace of the integrated population activity originating from the preBötC (jpreBötC) illustrating the biphasic effect that 2.2 mM H$_2$O$_2$ has on burst frequency of fictive inspiratory bursts. This biphasic effect was characterized initially by suppression followed by augmentation in burst frequency. Inset: expanded traces of single fictive normal inspiratory bursts taken during control (CON) or during H$_2$O$_2$ exposure (time bins 180, 300, and 600). B: effects of H$_2$O$_2$ on burst frequency; in a subset of experiments ($n = 5$) H$_2$O$_2$ was washed out and followed for an additional 600 s (gray bars) to demonstrate the reversibility of the effects of H$_2$O$_2$. C and D: irregularity score of $f_{inst}$ [C: arbitrary units (A.U.)] and integrated burst area (D) were assessed by comparing average metric values (120-s bins) during control (CON) to those at 180, 300, and 600 s (black bars) during the 600-s exposure to H$_2$O$_2$ ($n = 17$ of 20 demonstrated a biphasic frequency response to H$_2$O$_2$: **$P < 0.01$). Washout bins were taken at 780, 900 and 1,200 s. †No significant differences were found between washout values and respective control values.](https://www.jn.org/content/jn/106/6/3047.full.pdf)
burst frequency (150 ± 16% of control; Fig. 2B). During the early phase, H$_2$O$_2$ transiently increased the irregularity score of $f_{\text{inst}}$ from 0.32 ± 0.03 to 0.54 ± 0.08 (Fig. 2C), which returned to the control level in the late phase of H$_2$O$_2$ exposure. On the other hand, H$_2$O$_2$ had no significant effect on the integrated burst area of fictive inspiration (Fig. 2D).

To assess whether the effects of H$_2$O$_2$ are reversible, neuronal activity was recorded for an additional 600 s after washout of H$_2$O$_2$ ($n = 5$). Inspiratory burst frequency returned toward baseline with a tendency to undershoot (74 ± 11%), which was not statistically significant (Fig. 2B). At the end of the washout period, burst area and irregularity score were 105 ± 19% and 116 ± 14% of control, respectively.

H$_2$O$_2$ exposure increased the number of fictive sighs from 1.63 ± 0.38 per 600 s to 3.95 ± 0.72 per 600 s (Fig. 3A; $n = 20$). The integrated burst area of fictive sighs was unaffected by H$_2$O$_2$. Unlike the biphasic effect of H$_2$O$_2$ on $f_{\text{inst}}$ of fictive inspiration, the $f_{\text{inst}}$ of the postsigh apnea during the early (0–300 s) and late (>300 s) phases of H$_2$O$_2$ exposures were comparable to that of untreated control animals (Fig. 3B).

Analysis of the dose response revealed that the duration of suppressed neuronal activity depended on H$_2$O$_2$ concentration (Fig. 4, A and B, left). The increase in neuronal activity was evident with as low as 440 μM H$_2$O$_2$, and the magnitude of excitation further increased with 2.2 mM. The magnitude of excitation elicited with 4.4 mM was the same as that evoked by 2.2 mM, indicating that the response reached plateau (Fig. 4B, right).

**Effect of H$_2$O$_2$ application on neuronal activity from the VRC.** To determine whether a local administration of H$_2$O$_2$ rather than superfusion produces a similar biphasic change in burst frequency of VRC, effects of unilateral microinjections of H$_2$O$_2$ into the VRC within the brain slice were tested. H$_2$O$_2$ microinjections suppressed the frequency of the rhythm by
In another series, VRC islands were micro-dissected and then exposed to H$_2$O$_2$ (2.2 mM) via superfusion. This further reduced preparation preserved stable rhythmogenesis from the preBötC when left untreated (Fig. 5B; Johnson et al. 2001). In this preparation, H$_2$O$_2$ did not evoke predictable changes in frequency (Fig. 5B;). However, a comparison of the magnitude of change (independent of directionality) demonstrated that H$_2$O$_2$ exposure affects burst frequency of the rhythm (Fig. 5Bi).

Effects of H$_2$O$_2$ on individual preBötC neurons. To assess the effects of H$_2$O$_2$ on membrane properties of preBötC neurons, whole cell current-clamp recordings were made from putative inspiratory neurons. These neurons were isolated from the network with bath application of CNQX (10 μM), CCP (10 μM), PTX (50 μM), and STR (1 μM). In isolation, all neurons ($n=7$) became silent, having a steady-state $V_m$ of $-61.67 \pm 2.33$ mV and a $R_n$ of $155.07 \pm 20.81$ MΩ. Exposure to H$_2$O$_2$ did not lead to significant changes in $V_m$ (eH$_2$O$_2$: $61.25 \pm 2.45$ mV; lH$_2$O$_2$: $61.01 \pm 2.43$ mV). However, a majority of neurons ($n=5$) showed decreased $R_n$ during eH$_2$O$_2$ (range of $\Delta R_n$ = $-1.20$ to $-16.59$ MΩ) (Fig. 6A) followed by a rebound in the late phase i.e., lH$_2$O$_2$ (Fig. 6A, inset). In 71% of neurons ($n=5$), the number of evoked action potentials during the early phase of H$_2$O$_2$, i.e., eH$_2$O$_2$, decreased to 65 ± 18% of control (Fig. 6B) but was not statistically significant ($P = 0.11$). The depressed action potential frequency was transient in four of the five neurons tested (Fig. 6B).

Fe$^{2+}$-H$_2$O$_2$ does not cause a biphasic change in the frequency of respiratory activity from the preBötC and deferoxamine does not prevent augmentation caused by H$_2$O$_2$. The following experiments were performed to assess the potential contribution of hydroxyl radical ($^\bullet$OH) generation by Fenton reaction to H$_2$O$_2$-induced neuronal changes in preBötC . To this end, brain slices containing preBötC were superfused with aCSF containing FeSO$_4$ (100 μM; Fe$^{2+}$). By itself, Fe$^{2+}$ had no effect on the integrated burst area, pattern of regularity, or...
frequency of rhythmogenesis originating from the preBöC neurons. Although exposure to H2O2 (2.2 mM, 600 s) did not have a significantly measurable effect on the mean input resistance (Rm) of network isolated preBöC neurons (n = 7), during early exposure to H2O2 (eH2O2, t = 60–180 s of exposure) 5 of 7 neurons exhibited a decrease in Rm, while 2 of 7 showed an increase in Rm (grey symbols). Inset: plot of the delta Rm of the 5 neurons showing a decrease in Rm during eH2O2 and their delta Rm during the last 120 s of H2O2 exposure (iH2O2). B: representative trace of an individual neuron using the step protocol to determine Rm and relative changes in excitability. In this example a decreased Rm occurs during eH2O2 that rebounds during iH2O2. This change in Rm is accompanied by an initial drop in excitability that rebounds during iH2O2, as determined by the number of evoked action potentials during each phase. The decrease in evoked excitability during eH2O2 was observed in 5 of 7 neurons; 4 of these 5 neurons exhibited a rebound in iH2O2.

H2O2 suppresses spontaneous bursting in the CA1 neuronal population. To determine whether the effects of H2O2 were regionally specific to the preBöC, the following studies were performed on CA1 neurons of the hippocampus exposed to either H2O2 or Fe2+-H2O2. H2O2 (2.2 mM bath application) suppressed spontaneous bursting activity of the CA1 neurons (Fig. 8A; n = 6). Integrated burst frequency decreased within the first bin during H2O2 exposure to 25 ± 13% (Fig. 8B), corresponding to the decrease in finst from 0.83 ± 0.08 Hz (during control) to 0.32 ± 0.14 Hz (in H2O2). By the end of the exposure, H2O2 increased irregularity score of finst from 0.20 ± 0.04 during control to 1.02 ± 0.32 in exposure (Fig. 8C) and reduced integrated burst area to 43 ± 7% (Fig. 8D). Although Fe2+ (100 μM) had no effect on burst frequency (Fig. 9B) or irregularity score of finst (Fig. 9C), it reduced the integrated burst area by 56 ± 7% of control (Fig. 9D). Addition of H2O2 to the Fe2+-supplemented aCSF suppressed burst frequency (4.03 ± 2.46% of control; Fig. 9B) and further suppressed integrated burst area (16 ± 19% of control; Fig. 9D). In a subset of experiments (n = 4), preexposure to deferoxamine (500 μM, 600 s) prevented the suppressive effects of H2O2 on the burst frequency from the CA1 neuronal population (data not shown).

Effects of H2O2 on lipid peroxidation in CA1 and preBöC neurons. MDA levels were monitored as an index of lipid peroxidation (Peng et al. 2006; Ramanathan et al. 2005) in untreated (control), H2O2-treated (2.2 mM for 600 s), and Fe2+ (100 μM)-H2O2 (2.2 mM)-treated preBöC and cortico-hippocampal slices. Basal MDA levels were comparable between cortico-hippocampal brain slices and medullary brain slices (Fig. 10; n = 7 slices). MDA levels significantly increased in the cortico-hippocampal brain slices after exposure to either H2O2 (6.92 ± 0.12 nmol/mg protein, n = 6 slices) or Fe2+-H2O2 (7.78 ± 0.18 nmol/mg of protein, n = 7 slices; Fig. 10A). On the other hand, MDA levels in the brain stem slices treated with H2O2 (5.68 ± 0.25 nmol/mg protein, n = 6 slices; P value = 0.12) were similar to untreated control (4.71 ± 0.38 nmol/mg protein, n = 6 slices; Fig. 10B), whereas combined treatment with Fe2+-H2O2 caused a significant increase in
DISCUSSION

We tested the hypothesis that the neonatal central nervous system (CNS) responds to acute ROS exposure in differential and region-specific manners. We used experimental paradigms with various oxidants and juxtaposed the response to these paradigms in two different neonatal neuronal populations. The oxidants used here powerfully suppress population bursting in the CA1 region of the hippocampus. By contrast, H$_2$O$_2$ causes a biphasic change in the frequency of spontaneous bursting originating from the preBöC. Together, these novel findings lead to the general conclusion that the net action of ROS on neuronal function is dependent on the brain region and the type of ROS generated. The implications of this conclusion and these findings are discussed in further detail below.

Study considerations. Simply defined, the increase in the concentration of H$_2$O$_2$ with either H$_2$O$_2$ alone or Fe$^{2+}$-H$_2$O$_2$ could be considered a form of oxidative stress, as H$_2$O$_2$ in both paradigms shifts the redox state within the system to a more oxidized environment. Because of the abundance of oxidizable lipids in brain slices, lipid peroxidation, reflected by MDA levels, was the chosen metric for determining whether H$_2$O$_2$ or Fe$^{2+}$-H$_2$O$_2$ significantly increased global oxidative stress (Peng et al. 2006; Ramanathan et al. 2005). Fe$^{2+}$-H$_2$O$_2$ caused a significant shift in oxidative state and created measurable oxidative stress in both brain stem and cortico-hippocampal slices. H$_2$O$_2$ significantly increased lipid peroxidation in cortico-hippocampal slices. However, similar to observations reported in adult striatal slices (Chen et al. 2001), H$_2$O$_2$ did not significantly increase lipid peroxidation in brain stem slices. Thus H$_2$O$_2$ and Fe$^{2+}$-H$_2$O$_2$ do not appear to produce the same type of oxidative state within the brain stem slice, and, furthermore, an increase in global oxidative stress does not appear to be requisite for exogenous H$_2$O$_2$ to profoundly impact on neuronal activity from the preBöC.
Modulation of rhythmogenesis from the neonatal preBötC.

Despite the minimal effect of H₂O₂ on lipid peroxidation in the brain stem slice, H₂O₂ caused a profound biphasic frequency effect on rhythmogenesis from the preBötC. However, H₂O₂ neither caused a sustained destabilization of burst to burst variability nor significantly affected integrated burst area, and upon washout the H₂O₂-mediated effects were reversible. Although iron chelation prevented the initial depression of frequency in the majority of experiments, the steady-state augmentation was attenuated but not blocked. Moreover, Fe²⁺/H₁₁₀₀₁·H₂O₂ could not mimic the effect of H₂O₂. Together these observations suggest that the effects of H₂O₂ on rhythmogenesis do not represent a generalized response to a shift in redox state, but rather H₂O₂ specifically modulates the frequency of respiratory activity from the preBötC.

We do, however, recognize that the observed effects of H₂O₂ on rhythmogenesis likely occur during and after non-physiological conditions, such as that during and after apneas. In such conditions, H₂O₂ availability is presumably increased upon reoxygemanation from hypoxia and coupled with minimal free Fe²⁺ availability. Thus the steady-state effect of exogenous H₂O₂ on the preBötC may be a functionally relevant response to an acute sudden increase in H₂O₂. Moreover, ROS are endogenously produced in local neuronal environments not only during upswings in oxygen but also during well-oxygenated conditions (Bao et al. 2009; D’Agostino et al. 2007), making it possible for baseline production of endogenous H₂O₂ to influence rhythmogenesis under more physiologically relevant conditions, but this remains to be determined.

Importance of local circuitry to effects of H₂O₂ on the preBötC and H₂O₂ sensitivity of preBötC neurons.

Rhythmicity originating from the preBötC is influenced by several presynaptic connections that are presumably still active in the brain stem slice. Bath application of H₂O₂ coupled with population level recordings limited the ability to discriminate between H₂O₂ sensitivity of presynaptic elements outside the preBötC and the direct intrinsic H₂O₂ sensitivity of preBötC neurons. To address this limitation, experiments using unilateral microinjection of H₂O₂ over the VRC respiratory islands and intracellular patch-clamp recordings were conducted. Microinjection of H₂O₂ over the VRC was capable of transiently suppressing burst frequency but unable to augment frequency. This suggests that the H₂O₂-induced augmentation may represent a more integrated response potentially involving medullary regions outside the preBötC. Indeed, experiments involving the much-reduced VRC islands demonstrated that H₂O₂ causes unpredictable changes in the respiratory frequency generated within the preBötC. In aggregate, these experiments show that 1) the preBötC-containing VRC region possesses a specific sensitivity to H₂O₂ but 2) the biphasic response is an integrated network response that potentially involves also presynaptic elements located throughout the respiratory circuit of the brain stem slice. Such areas may include input from not only the contralateral preBötC but also other regions such as...
the raphe, A1C1, and nucleus tractus solitarii. These other neuronal populations are potentially also sensitive to \( \text{H}_2\text{O}_2 \) and may indirectly influence the \( \text{H}_2\text{O}_2 \) response of rhythmogenesis from the preBötC.

The hypothesis that the \( \text{H}_2\text{O}_2 \) response emerges through an integrated network response is further corroborated by our observation that, on average, \( \text{H}_2\text{O}_2 \) did not significantly affect intrinsic membrane properties or excitability of the sampled preBötC neurons. However, there was a tendency for \( \text{H}_2\text{O}_2 \) to decrease \( R_n \) and alter excitability in the majority of these neurons. In this group, \( R_n \) and excitability initially decreased with \( \text{H}_2\text{O}_2 \), suggesting that \( \text{H}_2\text{O}_2 \) may be increasing the \( \text{K}^+ / \text{H}_11001 \) conductance in these neurons. A potential candidate for \( \text{H}_2\text{O}_2 \) to increase \( \text{K}^+ \) conductance in these neurons may be the \( \text{K}_\text{ATP} \) channel. The \( \text{K}_\text{ATP} \) channel has been shown to be influenced by \( \text{H}_2\text{O}_2 \) regulating excitability of midbrain neurons (Avshalumov et al. 2005). It is likely, however, that \( \text{H}_2\text{O}_2 \) has action on more than just one effector that influences neuronal excitability, since the influence of \( \text{H}_2\text{O}_2 \) on membrane properties of preBötC neurons and population rhythms from VRC islands is not uniform. The notion that \( \text{H}_2\text{O}_2 \) results in an integrated network response is reminiscent of the hypoxic response of the preBötC, which also involves a complex network reconfiguration during which the discharge pattern of respiratory neurons is differentially altered (Pena et al. 2004)—nonpacemaking and pacemaking neurons. Pacemaking neurons can be further subdivided into cadmium-sensitive and cadmium-insensitive pacemaker neurons (Thoby-Brisson and Ramirez 2001; Pena et al. 2004). These different types of neurons are characterized not only by their heterogeneous membrane properties but also by their differential responsiveness to hypoxia. Thus it may not be surprising that \( \text{H}_2\text{O}_2 \) did not uniformly affect intrinsic membrane properties or excitability of individual preBötC neurons. Because our sampling of preBötC neurons was limited to nonpacemaking inspiratory neurons, it is currently unknown to what extent \( \text{H}_2\text{O}_2 \) affects the intrinsic membrane properties and excitability of other types of respiratory neurons including cadmium-sensitive and cadmium-insensitive pacemaker neurons. Future studies will need to characterize all types of respiratory neurons to gain insights into the network reconfiguration that governs the integrated \( \text{H}_2\text{O}_2 \) response.


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exposure to H$_2$O$_2$ (2.2 mM 600 s; n = homogenates from 7 slices) significantly increased lipid peroxidation, while exposure to Fe$^{2+}$-H$_2$O$_2$ (100 µM-2.2 mM 600 s; n = homogenates from 5 slices) increased lipid peroxidation in cortico-hippocampal brain slices compared with time-matched untreated controls (n = homogenates from 6 slices). While under normal conditions Fe$^{2+}$ is tightly regulated in the CNS (Moos 2002; Moos et al. 2007), a compromised blood-brain barrier potentially increases Fe$^{2+}$ availability where the transition metal becomes readily available to catalyze H$_2$O$_2$ into the more deleterious species, •OH, leading to oxidative injury and damage (Halliwell 2006). For example, severe traumatic brain injury or stroke can damage the blood-brain barrier and often correlates with oxidative injury. The effect of H$_2$O$_2$ on the preBötC rhythm in such cases may be better reflected by the effects observed with Fe$^{2+}$-H$_2$O$_2$, as opposed to the H$_2$O$_2$ paradigm. Fe$^{2+}$-H$_2$O$_2$ suppressed frequency and integrated burst area but did not prevent the generation of rhythmogenesis from the preBötC. Compared with activity from the CA1 neuronal population, rhythmogenesis from preBötC appears to be substantially more resistant to oxidative environments. This relative resistance of the preBötC to oxidative modifications may involve differences in lipid composition, intrinsic antioxidant protection, or activity of repair enzymes between the regions studied here. While the basis of this relative resistance of rhythmogenesis from the preBötC to redox alterations is unknown, our observations emphasize the importance of continued network output from the preBötC in oxidative environments, as loss of activity would ultimately result in the cessation of breathing in vivo (McKay et al. 2005; Ramirez and Garcia 2007).

Neurophysiological implications of the action of H$_2$O$_2$. Here we demonstrate that regional differences in response to acute changes in H$_2$O$_2$ exist between two areas in neonatal brain slices. While the activity from the neonatal CA1 neuronal population appears to share sensitivity to oxidative environments similar to its adult counterpart, rhythmogenesis from the preBötC appears to differentially respond to various oxidative environments. Whether this differential sensitivity is preserved in the adult is currently unknown. On an acute timescale, the sudden rise in ROS during reoxygenation may not always lead to immediate oxidative stress and injury. In the adult, ROS appear to be involved with long-term facilitation of the sensory and motor components of respiration (Macfarlane and Mitchell 2008; Peng et al. 2003). In these cases, ROS modulate respiratory control that may or may not lead to immediate oxidative stress. However, it is important to recognize that chronic states of intermittent hypoxia, such as that occurring in untreated sleep apnea and apneas of prematurity, often correlate to increased oxidative stress. Thus, in chronic conditions in which oxygen homeostasis oscillates even without significant changes in transition metal availability, ROS, like H$_2$O$_2$, can also overwhelm antioxidant defenses. In such chronic cases, oxidative stress can influence gene expression and alter physiology (Kumar et al. 2006; Peng et al. 2006; Yuan et al. 2004). These changes may ultimately redefine how a given area of the brain responds to oxidative stress.

![Cortico-hippocampal Brain Slice](image1)

**Fig. 10.** While acute exposure to H$_2$O$_2$ or Fe$^{2+}$-H$_2$O$_2$ increases lipid peroxidation in cortico-hippocampal brain slices, only Fe$^{2+}$-H$_2$O$_2$ increases lipid peroxidation in brain stem slices. A: exposure to H$_2$O$_2$ (2.2 mM 600 s; n = homogenates from 6 slices) or Fe$^{2+}$-H$_2$O$_2$ (100 µM-2.2 mM 600 s; n = homogenates from 7 slices) increased lipid peroxidation in cortico-hippocampal brain slices compared with time-matched untreated controls (n = homogenates from 7 slices). B: while exposure to Fe$^{2+}$-H$_2$O$_2$ (100 µM-2.2 mM 600 s; n = homogenates from 5 slices) significantly increased lipid peroxidation, no significant increase (N.S.) in lipid peroxidation was observed during exposure to H$_2$O$_2$ (2.2 mM 600 s; n = homogenates from 6 slices) compared with time-matched untreated controls (n = homogenates from 6 slices). **P < 0.01, ***P < 0.001.

At least in part, to a generalized sensitivity of neonatal CA1 neurons to oxidative stress, as presynaptic bursting from the CA3 region was still observed during the H$_2$O$_2$-mediated depression of bursting from the CA1 region. This would be in agreement with a previous study in which the presynaptic volley from the adult CA3 neuronal population was unaffected by Fe$^{2+}$-H$_2$O$_2$ (Pellmar 1987). However, while presynaptic action potential generation appears to be unaffected, it is possible that the amount of presynaptic neurotransmitter release decreases in oxidizing conditions (Pellmar 1986). Hence, the site of action of H$_2$O$_2$ in this neonatal neuronal circuit may involve presynaptic, postsynaptic, or some combination of both elements. Although our experiments did not discriminate the precise location of action, these experiments demonstrate that, as in the adult, activity originating from the neonatal CA1 neuronal population is suppressed by H$_2$O$_2$, which could be prevented with chelation of Fe$^{2+}$ with deferoxamine. Because the effects of H$_2$O$_2$ could be mimicked by Fe$^{2+}$-H$_2$O$_2$ and blocked by deferoxamine, it is likely that H$_2$O$_2$ and •OH (produced from Fe$^{2+}$-H$_2$O$_2$) overlap in the mechanism(s) of action suppressing activity. Similarly, lipid peroxidation caused by either H$_2$O$_2$ alone or H$_2$O$_2$-Fe$^{2+}$ was increased in neonatal cortico-hippocampal brain slices. In agreement with our findings, experiments from adult hippocampal brain slices showed that iron chelation prevents H$_2$O$_2$-mediated suppression of evoked activity from the adult CA1 neuronal population (Avshalumov et al. 2000). Hence, the neonatal CA1 neuronal population appears to have an acute sensitivity to a general shift in oxidative environments, responding in a manner consistent with that found in the adult population.

Rhythmogenesis from the preBötC in the presence of Fe$^{2+}$-H$_2$O$_2$. While under normal conditions Fe$^{2+}$ is tightly regulated in the CNS (Moos 2002; Moos et al. 2007), a compromised blood-brain barrier potentially increases Fe$^{2+}$ availability where the transition metal becomes readily available to catalyze H$_2$O$_2$ into the more deleterious species, •OH, leading to oxidative injury and damage (Halliwell 2006). For example, severe traumatic brain injury or stroke can damage the blood-brain barrier and often correlates with oxidative injury. The effect of H$_2$O$_2$ on the preBötC rhythm in such cases may be better reflected by the effects observed with Fe$^{2+}$-H$_2$O$_2$, as opposed to the H$_2$O$_2$ paradigm. Fe$^{2+}$-H$_2$O$_2$ suppressed frequency and integrated burst area but did not prevent the generation of rhythmogenesis from the preBötC. Compared with activity from the CA1 neuronal population, rhythmogenesis from preBötC appears to be substantially more resistant to oxidative environments. This relative resistance of the preBötC to oxidative modifications may involve differences in lipid composition, intrinsic antioxidant protection, or activity of repair enzymes between the regions studied here. While the basis of this relative resistance of rhythmogenesis from the preBötC to redox alterations is unknown, our observations emphasize the importance of continued network output from the preBötC in oxidative environments, as loss of activity would ultimately result in the cessation of breathing in vivo (McKay et al. 2005; Ramirez and Garcia 2007).
responds to fluctuations in ROS like H$_2$O$_2$. Hence, the neuro-
physiological response to ROS in a given brain region may be
dynamically influenced by several factors including the time-
scale at which changes in ROS status are studied.

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DISCLOSURES

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