Hydrogen Peroxide in the Lung Parenchyma Stimulates Vagally Mediated Phrenic Activity*

Galia K. Soukhova, PhD;† Muhammad Ahmed, MD; Eugene C. Fletcher, MD, FCCP; and Jerry Yu, MD, PhD

Study objective: To elucidate the mechanism of hyperpnea and tachypnea, which are the common findings in cardiopulmonary patients.

Rational: Recently, it was found that activating pulmonary afferents by directly injecting hypertonic saline solution into the lung periphery causes a vagally mediated neural hyperpnea and tachypnea, ie, the excitatory lung reflex. Since reactive oxygen species are released during a variety of pulmonary diseases, we examined whether hydrogen peroxide (H₂O₂), a common mediator in cardiopulmonary diseases, can initiate the same excitatory lung reflex.

Measurements and results: We recorded phrenic efferent activity in anesthetized, open chest, artificially ventilated rabbits as an index of respiratory drive and examined the respiratory responses to injections of H₂O₂ (10 μmol in 0.1 mL). The responses were compared with those to hypertonic saline solution (8.1%, 0.1 mL). H₂O₂ and hypertonic saline solution increased both the rate (mean [+ SEM], 43 ± 8% and 61 ± 10%, respectively; n = 30; p = 0.001) and the amplitude of phrenic bursts (12 ± 2% and 20 ± 4%, respectively; n = 30; p = 0.033). These responses were abolished by bilateral vagotomy.

Conclusion: H₂O₂ can initiate the excitatory lung reflex. Therefore, mediator(s) released in pulmonary diseases could be one of the mechanisms causing hyperpnea and tachypnea.

Key words: breathing control; pulmonary receptors; reflex; vagus nerve

Abbreviation: DMSO = dimethyl sulfoxide

Hyperpnea and tachypnea in patients with cardiopulmonary diseases involving the lung parenchyma. Despite vigorous efforts to investigate the responsible factors, the mechanisms that produce hyperpnea and tachypnea have not been completely identified. In an animal model of lung diseases,1,2 vagal afferents are found to mediate an alteration of a breathing pattern.3 Recently, we observed an excitatory lung reflex during activation of pulmonary afferents by directly injecting 0.1 mL hypertonic saline solution into the lung periphery of a rabbit.4 The reflex response included neural hyperpnea and tachypnea. Therefore, this reflex may have an important role in cardiopulmonary diseases. On the other hand, increased levels of reactive oxygen species, such as superoxide, hydrogen peroxide, and hydroxyl free radicals, have been associated with a number of pulmonary diseases,5,6,7 especially when the diseases involve the lung parenchyma. For example, oxygen radicals are thought to be involved in hyperoxia, emphysema, bronchopulmonary dysplasia, ARDS, and acute lung injury (such as bleomycin toxicity). We hypothesize that reactive oxygen species may activate vagal afferents in the lung periphery, thereby evoking hyperpnea and tachypnea. In the present study, H₂O₂ was locally injected into the lung parenchyma while whole phrenic nerve discharge was measured as an index of respiratory drive. Activation of the excitatory lung reflex would suggest the possibility that H₂O₂ may be responsible for hyperpnea and tachypnea in cardiopulmonary diseases involving lung parenchyma.

Materials and Methods

General

Experiments were conducted on 35 male New Zealand white rabbits (body weight, 2.0 to 2.6 kg). The detailed procedures...
were described in a previous report. In short, the rabbits were initially anaesthetized with 1M ketamine, 37.5 mg/kg, and 1M xylazine, 5 mg/kg, and surgical anesthesia was maintained by additional doses of IV sodium pentobarbital (10 mg). During the experiment, anesthesia was maintained by IV infusion of α-chloralose (1%) and urethane (10%) at 1.1 to 2.0 mL/h. The trachea was cannulated low in the neck, the chest was opened, and the lungs were ventilated with room air by a small animal ventilator (model 683; Harvard Apparatus; South Natick MA) in which the expiratory outlet was connected to 3 to 4 cm H₂O of positive end-expiratory pressure. Airway pressure was monitored by a pressure transducer attached to a side arm of the tracheal tube. Tidal volume was set at 10 mL/kg body weight. Ventilatory frequency was adjusted to maintain a constant peak airway PCO₂ of about 40 mm Hg (which may have been an underestimate of alveolar PCO₂). CO₂ level was periodically monitored by an infrared analyzer (LB-2; SensorMedics; Yorba Linda, CA). The machine was calibrated by known CO₂ concentrations. The femoral artery was cannulated for BP monitoring. Phrenic activity, its time-averaged signals, airway pressure, and BP were recorded by a thermorecorder (Dash IV; Astro-Med; West Warwick, RI).

To determine whether H₂O₂ can activate pulmonary receptors and reflexly stimulate breathing, we monitored phrenic activity as an index of respiratory drive and injected 0.1 mL H₂O₂ in 0.9% NaCl directly into the lung parenchyma (5 to 7 mm under the surface) through a 30-gauge needle. H₂O₂ was diluted to the desired concentration with 0.9% NaCl. In 30 rabbits, the response was compared with that to 0.1 mL injection of 8.1% NaCl. The response to 0.1 mL 0.9% NaCl was also examined as a vehicle control.

To determine whether H₂O₂-stimulated breathing is mediated through hydroxyl free radicals, we measured the respiratory responses to H₂O₂, 10 μmol in 0.1 mL, in 11 rabbits before and after local injection of the hydroxyl free radical scavenger, dimethyl sulfoxide (DMSO; 5% in 0.2 mL).

**Phrenic Nerve Recordings**

The phrenic nerve from C6 (right or left) was separated from the surrounding tissue and transected. The central end of the nerve was de-sheathed and placed on a bipolar silver electrode, which was connected to a high impedance probe (HIP5; Grass Instrument Division; West Warwick, RI) and then to an amplifier (P511; Grass Instrument Division). Nerve activity was monitored by a loudspeaker. Both the raw nerve signal and its "integrated signal," ie, its moving time-averaged signals obtained by a leaky integrator (7P3D; Grass Instrument Division; time constant, 50 ms), were recorded. The amplitude and rate of phrenic bursts were examined in response to the local injection of H₂O₂.

**Data Analysis**

Data are presented as mean (± SEM). A paired Student’s t test was used to compare two groups of data from the same animals. A p value < 0.05 was considered as statistically significant.

**RESULTS**

Injection of H₂O₂ (in 0.1 mL 0.9% NaCl) caused neural hyperpnea and tachypnea (Fig 1), which were exhibited by increases in the amplitude and burst rate of the phrenic neurogram. The response pattern to H₂O₂ is similar to that evoked by a local injection of 8.1% NaCl. The most prominent response is neural tachypnea (Figs 1, 2); in addition, the mean arterial BP increased by 5.5 ± 0.7 and 6.7 ± 0.6 mm Hg, respectively in response to the injections of H₂O₂ and 8.1% NaCl (n = 20; p < 0.01). There was no difference between the increases in BP. On the other hand, as we reported in a previous study, injecting 0.1 mL 0.9% NaCl did not alter the respiratory pattern (Fig 3).

**Figure 1.** Similar respiratory responses to injection of 0.1 mL 8.1% NaCl (top panel) or 1 μmol H₂O₂ (bottom panel) into the periphery of the right lung in an anesthetized, open-chest rabbit receiving artificial ventilation. Traces from top to bottom: ENG, the electroneurogram of the left phrenic nerve; ENG (Integ.), the “integrated” (time-averaged) electroneurogram; Paw, airway pressure; and BP. Three event marks on the top denote the insertion of the needle, and the start and end of the injection. Note that the phrenic activity increased immediately. The breathing rate was still linked to the ventilator cycle. The phrenic nerve gave two bursts, although fused, per ventilator cycle in some cases. Also note that there was an increase in BP after the injections.

**Figure 2.** A comparison of respiratory responses to local injections of hydrogen peroxide, 10 μmol (open bar) and hypertonic saline solution (crossed bar; n = 30). The responses are expressed as the percent increase in phrenic amplitude or frequency (burst rate) from the baseline after the initiation of the excitatory lung reflex. *Denotes the difference (p < 0.05) in responses between the two agents.
In order to determine whether the excitatory reflex can be repeatedly evoked at the same injection point, we measured the respiratory responses to injection of \( \text{H}_2\text{O}_2, 10 \mu\text{mol in 0.1 mL 0.9\% NaCl} \), at an interval of every 30 min for 2.5 h (\( n = 9 \)). It was found that the response can be repeated for six injections without a change in rate and amplitude of phrenic response (Fig 4).

In 11 rabbits, we measured the respiratory responses to \( \text{H}_2\text{O}_2, 10 \mu\text{mol in 0.1 mL} \), before and after a local injection of 5\% DMSO (0.2 mL) and found that there was no significant difference between DMSO-treated and nontreated responses to \( \text{H}_2\text{O}_2 \). It is worth a notice that DMSO itself did not cause any changes in phrenic activity. \( \text{H}_2\text{O}_2 \) injection increased the rate of phrenic bursts by \( 41.7 \pm 10\% \) before DMSO and by \( 38.6 \pm 10\% \) after DMSO (\( p > 0.05 \)). To further ensure that DMSO and \( \text{H}_2\text{O}_2 \) distribute to the same extent, we compared the responses of four rabbits to \( \text{H}_2\text{O}_2 (10 \mu\text{mol}) \) and to a mixture containing \( \text{H}_2\text{O}_2 (10 \mu\text{mol}) \) and 5\% DMSO. Again, we found no difference between the two responses. In five separate rabbits, the respiratory responses to \( \text{H}_2\text{O}_2 \) were abolished after bilateral cervical vagotomy.

**Discussion**

Hyperpnea, tachypnea, and dyspnea are common findings in many cardiopulmonary diseases. Recently, an excitatory, vagally mediated reflex in the lung (excitatory lung reflex) was identified in the rabbit. Injection of hypertonic saline solution (8.1\%) directly into the lung parenchyma, in a volume (0.1 mL) that is a fraction of the deflated lung volume (40 mL), initiates the excitatory lung reflex. It increased phrenic activity, which was exhibited by an increased amplitude and rate of bursts without substantial effects on the cardiovascular system. This reflex may be important in the pathophysiologic process of several pulmonary diseases. It is interesting that a local injection of phenylbiguanide, a C fiber stimulant, did not evoke this reflex.

Reactive oxygen species are known as mediators for many cardiopulmonary pathophysiologic processes. Therefore, they could be responsible for the common symptoms and signs of cardiopulmonary diseases. The aim of the present study was to determine whether reactive oxygen species, specifically \( \text{H}_2\text{O}_2 \), can initiate the excitatory lung reflex. Indeed, our results show that an injection of 10 \( \mu\text{mol} \) \( \text{H}_2\text{O}_2 \) stimulated breathing. Thus, the present results provide the first evidence to demonstrate that \( \text{H}_2\text{O}_2 \) can initiate the excitatory lung reflex, thereby causing neural hyperpnea and tachypnea. Since \( \text{H}_2\text{O}_2 \) is a common mediator released during many cardiopulmonary diseases, the present study lends support for the hypothesis that the excitatory lung reflex is responsible for hyperpnea and tachypnea in cardiopulmonary diseases.

Reactive oxygen species are believed to be responsible for many diseases in different organ systems. They are recognized as playing an important role in many pulmonary diseases. It has been suggested that hydroxyl free radicals activate pulmonary C fibers in dogs. Directly applying \( \text{H}_2\text{O}_2 \) to the surface of the heart initiates a cardiac reflex through the vagal afferents. \( \text{H}_2\text{O}_2 \) has also been shown to evoke a vagally mediated reflex when applied topically to the GI tract. Until now, there
was no direct evidence to show that H$_2$O$_2$ could stimulate pulmonary afferents to cause reflex effects on breathing. By employing a local injection technique, we were able to directly deliver H$_2$O$_2$ to the vicinity of pulmonary receptors. Our data provide convincing evidence that H$_2$O$_2$ can evoke the excitatory lung reflex because 0.9% NaCl containing H$_2$O$_2$ produced neural hyperpnea and tachypnea, but pure 0.9% NaCl did not. The amount of H$_2$O$_2$ (10 μmol, and in some cases, even 1 μmol) used to evoke the excitatory lung reflex is comparable with the amount used in activating vagal afferents in the GI tract (44 μmol)$^{15}$ or in the heart (3 μmol)$^{14}$. It is likely that the concentration at the immediate vicinity of the receptor field is much lower than the concentration injected because there should be a concentration gradient from the injection point to the nerve endings. In addition, H$_2$O$_2$ is readily metabolized by catalase and glutathione enzymes, which are found in the lung.$^{11,16}$ Therefore, the concentration of H$_2$O$_2$ diffused to the receptor field should be far less than that applied.

Our experiments were not designed to determine by which mechanism H$_2$O$_2$ activates pulmonary receptors. However, our data could be used to exclude some possibilities. H$_2$O$_2$ can cause cell death by lysis in several cell lines.$^{12}$ However, our data do not prove this mechanism to be responsible. If this were the case, repeated injections of H$_2$O$_2$ at the same place would abolish or at least attenuate the response. It is possible that the activation of the excitatory lung reflex is due to the production of an hydroxyl free radical. However, some arguments can be used against it. First, H$_2$O$_2$ evoked the response within a few seconds. It is known that H$_2$O$_2$ takes time to convert to a hydroxyl free radical, especially under a circumstance in which there are no abundant transitional ions.$^{17}$ Second, pretreatment with DMSO or mixing H$_2$O$_2$ with DMSO did not prevent the response to H$_2$O$_2$. However, it still could be argued that DMSO failed to protect the initiation of the excitatory lung reflex because H$_2$O$_2$ diffused to the area of the nerve ending where DMSO did not reach. On the other hand, DMSO is a small molecule, is lipid soluble, and therefore, should access the area where H$_2$O$_2$ diffused. However, at this point, we do not have direct evidence to accept or refute this argument.

**Conclusion**

Our study provides the first evidence that H$_2$O$_2$, a common mediator released during many cardopulmonary diseases, can initiate the excitatory lung reflex, thereby causing neural hyperpnea and tachypnea by activating pulmonary afferents.

**References**

2. Guz A, Trenchard DW. The role of non-myelinated vagal afferent fibers from the lungs in the genesis of tachypnoea in the rabbit. J Physiol 1971; 213:345–347