HIGHLIGHTED TOPIC | Reflexes from the Lungs and Airways

Mediator mechanisms involved in TRPV1 and P2X receptor-mediated, ROS-evoked bradypneic reflex in anesthetized rats

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Ruan, Ting, You Shuei Lin, Kae-Shin Lin, and Yu Ru Kou.

Mediator mechanisms involved in TRPV1 and P2X receptor-mediated, ROS-evoked bradypneic reflex in anesthetized rats. *J Appl Physiol* 101: 644–654, 2006. First published April 20, 2006; doi:10.1152/japplphysiol.00192.2006.—Inhalation of H₂O₂ is known to evoke bradypnea followed by tachypnea, which are reflexes resulting from stimulation by reactive oxygen species of vagal lung capsaicin-sensitive and myelinated afferents, respectively. This study investigated the pharmacological receptors and chemical mediators involved in triggering these responses. The ventilatory responses to 0.2% aerosolized H₂O₂ were studied before and after various pharmacological pretreatments in anesthetized rats. The initial bradypneic response was reduced by a transient receptor potential vanilloid 1 (TRPV1) receptor antagonist [capsazepine; change (Δ) = −53%] or a P₂X purinoceptor antagonist [iso-pyridoxal phosphate-6-azopyren-2',5'-disulphonate (PPADS); Δ = −47%] and was further reduced by capsazepine and iso-PPADS in combination (Δ = −78%). The initial bradypneic response was reduced by a cyclooxygenase inhibitor (indomethacin; Δ = −48%), ATP scavengers (apyrase and adenosine deaminase in combination; Δ = −50%), or capsazepine and indomethacin in combination (Δ = −47%), was further reduced by iso-PPADS and indomethacin in combination (Δ = −75%) or capsazepine and ATP scavengers in combination (Δ = −83%), but was not affected by a lipoxygenase inhibitor (nordihydroguaiaretic acid) or by any of the various vehicles. No pretreatment influenced delayed tachypnea. We concluded that 1) the initial bradypneic response to H₂O₂ results from activation of both TRPV1 and P₂X receptors, possibly located at terminals of vagal lung capsaicin-sensitive afferent fibers; 2) the functioning of the TRPV1 and P₂X receptors in triggering the initial bradypneia is, in part, mediated through the actions of cyclooxygenase metabolites and ATP, respectively; and 3) these mechanisms do not contribute to the H₂O₂-evoked delayed tachypnea.

VARIous LUNG DISEASES are manifested by increased production of pulmonary reactive oxygen species (ROS) (10, 13, 14, 44, 46, 48, 62). Excess pulmonary ROS have been suggested to stimulate vagal lung afferent fibers and trigger respiratory reflexes under pathological conditions, such as pulmonary air embolism or sepsis (8, 9, 30), or when the airways are insulted by inhaled irritants, such as cigarette or wood smoke (19, 26, 28, 29, 33, 36, 51, 53). These observations promote the concept that vagal lung afferent fibers may function as a sensor system that detects excess pulmonary ROS. However, the underlying mechanisms are still incompletely understood.

Our laboratory recently reported (51) that inhalation of aerosolized H₂O₂ acutely evokes initial reflex bradypnea followed by delayed reflex tachypnea and that these responses may result from stimulation of vagal lung capsaicin-sensitive and myelinated afferents, respectively. A subsequent electrophysiological study (53) revealed that delivery of aerosolized H₂O₂ indeed stimulates capsaicin-sensitive vagal lung afferent fibers, and this sensory transduction is mediated through transient receptor potential vanilloid 1 (TRPV1) receptors and P₂X purinoceptors. Both the reflex (51) and afferent responses (53) to H₂O₂ are largely or totally suppressed by antioxidants targeted at H₂O₂ or hydroxyl radicals, suggesting the involvement of ROS. Capsaicin-sensitive vagal lung afferent fibers are composed of mainly C fibers and some Aδ fibers, which are important to the regulation of respiratory functions under pathophysiological conditions (6, 35, 59). The TRPV1 and P₂X receptors are two ligand-gated nonselective cation channels (41, 56) that are located at terminals of these vagal lung afferent fibers (5, 15, 25, 34, 47, 58, 60). While the importance of TRPV1 and P₂X receptors in the sensory transduction of pulmonary ROS is recognized, their relative contributions to H₂O₂-evoked initial bradypnea and delayed tachypnea remain to be delineated.

TRPV1 receptors are activated by multiple stimuli, such as capsaicin, noxious heat, acid, and several products of arachidonic metabolism (5, 15, 16, 25, 37, 41, 56, 58). They can be viewed as an integrator of painful chemical and physical stimuli of the somatosensory system (56). On the other hand, P₂X receptors can be activated only by ATP and its congeners (49). ROS are known to increase the release of arachidonate metabolites in lung tissue (4, 7, 40). To synthesize these metabolites, arachidonic acid is converted to prostaglandins and thromboxane via cyclooxygenase or to leukotrienes via lipoxgenase (2). Additionally, ROS may cause rapid release of cytosolic ATP, which activates the P₂X receptors of pain nociceptors in the vicinity (11, 41). After its formation, ATP is sequentially degraded by enzymes to ADP, AMP, and adenosine 5'-triphosphate (45). Collectively, it is possible that arachidonate metabolites and ATP are part of a signaling cascade for the sensory

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transduction of pulmonary ROS, leading to elicitation of respiratory reflexes. Nevertheless, this possibility remains to be investigated.

The present study was undertaken in anesthetized rats to investigate the role of TRPV1 and P2X receptors in triggering initial bradypnea and delayed tachypnea evoked by inhalation of aerosolized H\textsubscript{2}O\textsubscript{2}. To assess the involvement of arachidonate metabolites and ATP in triggering these ventilatory responses, and to delineate the chemical mediator mediating the functioning of the TRPV1 or P2X receptors in triggering these ventilatory responses. To accomplish these objectives, the acute ventilatory responses to aerosolized H\textsubscript{2}O\textsubscript{2} were compared before and after pretreatment with antagonists of the TRPV1 and P2X receptors, inhibitors of cyclooxygenase and lipooxygenase, and scavengers of ATP.

**MATERIALS AND METHODS**

*Animal preparation.* Male Sprague-Dawley rats were anesthetized with an intraperitoneal injection of chloralose (100 mg/kg; Sigma Chemical, St. Louis, MO) and urethane (500 mg/kg; Sigma) dissolved in a borax solution (2%; Sigma). A polyethylene catheter was inserted into the jugular vein and advanced until the tip was close to the right atrium to allow intravenous administration of pharmacological agents. The right femoral artery was cannulated to allow measurement of the arterial blood pressure (ABP). During the course of the experiments, the right atrium was cannulated to allow measurement of the atrial to right atrium to allow intravenous administration of pharmacological agents. The right femoral artery was cannulated to allow measurement of the arterial blood pressure (ABP). During the course of the experiments, the right atrium was cannulated to allow measurement of the atrial to right atrium to allow intravenous administration of pharmacological agents.

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Table 1. Pharmacological agents used in this study

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug Function</th>
<th>Dose</th>
<th>Vehicle of Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsaicin</td>
<td>TRPV1 receptor agonist</td>
<td>0.75 μg/kg iv</td>
<td>Dimethyl sulfoxide, Tween 80, ethanol, saline</td>
</tr>
<tr>
<td>CPZ</td>
<td>TRPV1 receptor antagonist</td>
<td>3 mg/kg iv</td>
<td>Tween 80, ethanol, saline</td>
</tr>
<tr>
<td>αβ-meATP</td>
<td>TRPV1 receptor antagonist</td>
<td>15 μg/kg iv</td>
<td>Saline</td>
</tr>
<tr>
<td>iso-PPADS</td>
<td>P2X purinoreceptor antagonist</td>
<td>15 mg/kg iv</td>
<td>Saline</td>
</tr>
<tr>
<td>Phenylbiguanide</td>
<td>Serotonin-5-HT1 receptor agonist</td>
<td>6 μg/kg iv</td>
<td>Saline</td>
</tr>
<tr>
<td>Indo</td>
<td>Nonselective cyclooxygenase inhibitor</td>
<td>5 mg/kg iv</td>
<td>Dimethyl sulfoxide, Tween 80, ethanol, saline</td>
</tr>
<tr>
<td>NDGA</td>
<td>Nonselective lipooxygenase inhibitor</td>
<td>5 mg/kg iv</td>
<td>Dimethyl sulfoxide, Tween 80, ethanol, saline</td>
</tr>
<tr>
<td>Apyrase</td>
<td>Breakdown of ATP to AMP</td>
<td>20 U/rat it</td>
<td>PBS</td>
</tr>
<tr>
<td>ADA</td>
<td>Breakdown of adenosine</td>
<td>10 U/rat it</td>
<td>Glycerol, potassium phosphate</td>
</tr>
</tbody>
</table>

CPZ, capsazepine; αβ-meATP, αβ-methylene-ATP; iso-PPADS, iso-pyridoxal phosphate-6-azopropyl-2′,5′-disulphonate; Indo, indomethacin; NDGA, nordihydroguaiaretic acid; ADA, adenosine deaminase; TRPV1, transient receptor potential vanilloid 1; iv, intravenous injection; it, intratracheal instillation.

combination of iso-PPADS and Indo (iso-PPADS + Indo; group 12), a combination of CPZ, apyrase, and ADA (CPZ + apyrase + ADA; group 13), or their vehicles (vehicle 4; group 14). In the fourth study series, an investigation into the reflex nature of the observed responses was carried out, and the ventilatory response to H2O2 challenge was studied before and after bilateral cervical vagotomy (group 15). Based on the results of our previous study (51), at least 60 min were allowed to elapse between the two H2O2 challenges to avoid possible tachyphylaxis. To determine whether pretreatment with CPZ or iso-PPADS was effective, the reflex apneic responses induced by an intravenous injection of capsaicin or αβ-meATP were compared before and after pretreatment. To determine whether the functioning of the vagal lung afferents was affected, reflex apneic responses were induced by an intravenous injection of capsaicin or phenylbiguanide, and these were also compared before and after other pharmacological pretreatments.

**RESULTS**

Control ventilatory responses to H2O2. Inhalation of 0.2% aerosolized H2O2 consistently evoked an initial bradypnea followed by delayed tachypnea (Fig. 1, left) in all 150 rats studied. As a group (n = 150), the initial decrease in f began at 6.8 ± 0.2 s (range, 3–12 s) after onset of the challenge and lasted for 13.7 ± 0.4 s (range, 5–25 s). Subsequently, f quickly
increased and reached its peak at 36.2 ± 0.5 s (range, 25–50 s) after onset of the challenge. This delayed tachypnea lasted for 74.6 ± 3.6 s (range, 20–180 s). On average, both the lowest (68.4 ± 0.9 breaths/min) and peak f (98.3 ± 1.4 breaths/min) values during the initial and delayed periods, respectively, significantly differed from the baseline f (82.9 ± 0.6 breaths/min). Additionally, average VT values during these two periods were significantly smaller than the baseline VT (Table 2). Furthermore, at the onset of or during the delayed tachypneic period, augmented inspiration (3) was also evoked in 88 of the 150 rats studied (Fig. 1, left).

Role of TRPV1 and P2X receptors in the H2O2-evoked f responses. Pretreatment with CPZ alone (Fig. 2A), iso-PPADS alone (Fig. 2B), or CPZ+iso-PPADS (Figs. 1A and 2C) significantly suppressed the H2O2-evoked initial bradypnea, but had no effect on the H2O2-evoked delayed tachypnea. In contrast, pretreatment with vehicles (vehicle 1) failed to significantly affect these f responses (Figs. 1B and 2D). After pretreatment with CPZ, iso-PPADS, CPZ+iso-PPADS, and vehicle 1, the initial bradypneic responses were 46.7 ± 14.3, 53.1 ± 11.3, 21.5 ± 12.8, and 97.1 ± 16.5% of the control, respectively, and the delayed tachypneic responses were 99.3 ± 13.6, 109.8 ± 9.7, 96.9 ±
Table 3. Average apneic response to intravenous injections of receptor agonists before and after various experimental interventions in the 15 study groups

<table>
<thead>
<tr>
<th>Pretreatment (n = 10)</th>
<th>Agonist</th>
<th>Response to Agonist (Expiration Ratio, %) Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPZ</td>
<td>Capsaicin</td>
<td>560±53</td>
<td>100±2*</td>
</tr>
<tr>
<td>iso-PPADS</td>
<td>αβ-meATP</td>
<td>1.639±117</td>
<td>98±2*</td>
</tr>
<tr>
<td>CPZ+iso-PPADS</td>
<td>Phenylbiguanide</td>
<td>1.646±103</td>
<td>1.818±223</td>
</tr>
<tr>
<td>Vehicle 1</td>
<td>Capsaicin</td>
<td>582±64</td>
<td>595±74</td>
</tr>
<tr>
<td>Indo</td>
<td>Capsaicin</td>
<td>561±67</td>
<td>612±97</td>
</tr>
<tr>
<td>NGDA</td>
<td>Capsaicin</td>
<td>551±85</td>
<td>516±76</td>
</tr>
<tr>
<td>Vehicle 2</td>
<td>Capsaicin</td>
<td>472±42</td>
<td>511±65</td>
</tr>
<tr>
<td>Apyrase + ADA</td>
<td>Capsaicin</td>
<td>631±53</td>
<td>632±27</td>
</tr>
<tr>
<td>ADA</td>
<td>Capsaicin</td>
<td>529±37</td>
<td>511±56</td>
</tr>
<tr>
<td>Vehicle 3</td>
<td>Capsaicin</td>
<td>486±83</td>
<td>471±74</td>
</tr>
<tr>
<td>CPZ + Indo</td>
<td>Phenylbiguanide</td>
<td>1.715±195</td>
<td>1.775±176</td>
</tr>
<tr>
<td>iso-PPADS + Indo</td>
<td>Phenylbiguanide</td>
<td>2.398±110</td>
<td>2.417±104</td>
</tr>
<tr>
<td>CPZ + Apyrase + ADA</td>
<td>Phenylbiguanide</td>
<td>1.800±269</td>
<td>1.959±460</td>
</tr>
<tr>
<td>Vehicle 4</td>
<td>Phenylbiguanide</td>
<td>2.029±200</td>
<td>1.992±222</td>
</tr>
<tr>
<td>Vagotomy</td>
<td>Capsaicin</td>
<td>512±45</td>
<td>103±2*</td>
</tr>
</tbody>
</table>

Values in each group are the means ± SE of 10 animals. Capsaicin, αβ-meATP, and phenylbiguanide were separately injected into the vein as a bolus at doses of 0.75, 15, and 6 μg/kg, respectively. See the legend of Table 2 for details of the study groups. Expiration ratio = longest expiratory duration within 3 s after injection/baseline expiratory duration. *Significantly different from the value before intervention, P < 0.05.

19.2, and 105.4 ± 19.3% of the control, respectively. At the doses tested, pretreatment with CPZ alone and iso-PPADS alone effectively blocked the apneic response to intravenous injections of capsaicin and αβ-meATP, whereas pretreatments with vehicle 1 failed to do so (Table 3). In contrast, pretreatment with CPZ + iso-PPADS did not significantly affect the apneic responses to intravenous injection of phenylbiguanide (Table 3).

Involvement of arachidonate metabolites and ATP in the H₂O₂-evoked f responses. Pretreatment with Indo (Figs. 3A and 4A) significantly suppressed the H₂O₂-evoked initial bradypnea but had no effect on the H₂O₂-evoked delayed tachypnea. In contrast, pretreatment with NDGA (Fig. 4B) or the vehicle (vehicle 2) for Indo or NDGA (Figs. 3B and 4C) failed to significantly affect these f responses. After pretreatment with Indo, NDGA, and vehicle 2, the initial bradypneic responses were 51.6 ± 19.9, 98.4 ± 17.4, and 99.1 ± 19.1% of the control, respectively, and the delayed tachypneic responses were 97.1 ± 15.4, 95.5 ± 19.7, and 94.9 ± 14.4% of the control, respectively. On the other hand, pretreatment with apyrase + ADA (ATP scavengers) (Fig. 5A) significantly suppressed the H₂O₂-evoked initial bradypnea, but had no effect on the H₂O₂-evoked delayed tachypnea. In contrast, pretreatment with ADA alone (Fig. 5B) or the vehicles (vehicle 3) for apyrase + ADA (Fig. 5C) failed to significantly affect these f responses. After pretreatment with apyrase + ADA, ADA, and vehicle 3, the initial bradypneic responses were 50.4 ± 14.8, 108.3 ± 15.5, and 98.9 ± 12.8% of the control, respectively, and the delayed tachypneic responses were 94.9 ± 17.1, 105.8 ± 14.9, and 102.6 ± 19.5% of the control, respectively. At the doses tested, all pretreatments did not significantly affect the apneic responses to intravenous injection of capsaicin (Table 3).

Effects of chemical mediators of TRPV1 and P2X receptors. Pretreatment with CPZ + apyrase + ADA (Fig. 6C), CPZ + Indo (Fig. 6A), or iso-PPADS + Indo (Fig. 6B) significantly suppressed the H₂O₂-evoked initial bradypnea but had no effect on the H₂O₂-evoked delayed tachypnea. In contrast, pretreatment with their vehicles (vehicle 4) failed to significantly affect these f responses (Fig. 6D). After pretreatment with CPZ + Indo, iso-PPADS + Indo, CPZ + apyrase + ADA, and vehicle 4, the initial bradypneic responses were 53.3 ± 12.5, 25.3 ± 6.9, 17.0 ± 11.9, and 94.3 ± 9.4% of the control, respectively, and the delayed tachypneic responses were 93.1 ± 17.7, 99.7 ± 15.9, 98.8 ± 14.1, and 97.8 ± 18.5% of the control, respectively. At their doses tested, all pretreatments did not significantly affect the apneic responses to intravenous injection of phenylbiguanide (Table 3).

Fig. 3. Experimental records illustrating acute responses to inhalation of 0.2% aerosolized H₂O₂ in two anesthetized and spontaneously breathing rats. A: responses before and after pretreatment with indomethacin (Indo). B: responses before and after pretreatment with vehicle of Indo (vehicle 2). Horizontal bars indicate the duration (30 s) of H₂O₂ challenge. Note that the H₂O₂-evoked initial bradypnea was reduced after Indo. See legend to Fig. 1 for further explanations.
Effect of various pharmacological pretreatments on the 
H2O2-evoked VT responses. The H2O2-induced reduction in VT 
during both the initial and delayed periods was not significantly 
altered by any pharmacological intervention used in this study 
(Table 2; Figs. 1 and 3). Additionally, the number of animals 
displaying H2O2-evoked augmented breaths was not signifi-
cantly changed by any pharmacological intervention used in this 
study (Table 2; Figs. 1 and 3).

Reflex nature of the H2O2-evoked ventilatory responses. 
After vagotomy, both the initial bradypneic and delayed tachy-
pneic responses to H2O2 did not occur. On average, the initial 
lowest (44.3 ± 3.0 breaths/min) and delayed peak f values

![Graphs showing respiratory frequency responses to H2O2 inhalation before and after pharmacological pretreatments.](http://jap.physiology.org/)

Fig. 4. Mean responses of respiratory frequency to inhalation of 0.2% aerosolized H2O2 in three groups of rats. A–C: responses before and after pretreatment with Indo, nordihydroguaiaretic acid (NDGA), and vehicle of Indo and NDGA (vehicle 2). Data in each group are the means ± SE from 10 rats. *Significantly different from the corresponding baseline in the same group; significantly different from the corresponding control response: P < 0.05. See legend to Fig. 2 for further explanation.

![Graphs showing respiratory frequency responses to H2O2 inhalation with apyrase + ADA, ADA alone, and vehicle of apyrase + ADA.](http://jap.physiology.org/)

Fig. 5. Mean responses of respiratory frequency to inhalation of 0.2% aerosolized H2O2 in three groups of rats. A–C: responses before and after pretreatment, with a combination of apyrase and adenosine deaminase (apyrase + ADA), ADA alone, and vehicles of apyrase + ADA (vehicle 3). Data in each group are the means ± SE from 10 rats. *Significantly different from the corresponding baseline in the same group; significantly different from the corresponding control response: P < 0.05. See legend to Fig. 2 for further explanation.
Inhalation of 0.2% aerosolized H2O2 generally did not significantly differ from the baseline (43.0 ± 3.1 breaths/min). Furthermore, the H2O2-evoked reductions in Vr and augmented breaths observed during the initial or delayed periods were also abolished by vagotomy (Table 2). Additionally, the apneic responses to intravenous injection of capsaicin were eliminated by vagotomy (Table 3).

Response of ABP to H2O2 before and after experimental interventions. Inhalation of 0.2% aerosolized H2O2 generally produced an increase in ABP during the initial period and a decrease in ABP during the delayed period (Figs. 1 and 3). As a group, the ABP value changed from a baseline of 100.4 ± 0.7 mmHg (n = 150) to an initial peak value of 110.8 ± 0.9 mmHg and to a delayed lowest value of 87.6 ± 1.1 mmHg under control conditions. Additionally, the heart rate value changed from a baseline of 384.5 ± 3.3 beats/min (n = 150) to an initial lowest value of 342.9 ± 4.1 beats/min and to a delayed lowest value of 322.6 ± 4.0 beats/min under control conditions. The initial increase in ABP evoked by H2O2 challenge was not significantly altered by any of the pharmacological pretreatments but was abolished by vagotomy (Table 4). The delayed reduction in ABP evoked by H2O2 challenge was not significantly influenced by pretreatment with vehicles 1–4, NDGA, or ADA but was prevented by the rest of pharmacological pretreatments and vagotomy (Table 4).

Table 4. Average response of mean arterial blood pressure to H2O2 challenge before and after various experimental interventions in the 15 study groups

<table>
<thead>
<tr>
<th>Group (n = 10)</th>
<th>Before Intervention</th>
<th>After Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Initial response</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPZ</td>
<td>106.0±2.7</td>
<td>119.6±4.6*</td>
</tr>
<tr>
<td>iso-PPADS</td>
<td>105.3±3.1</td>
<td>113.8±4.6*</td>
</tr>
<tr>
<td>CPZ+iso-PPADS</td>
<td>105.0±2.2</td>
<td>115.2±3.1*</td>
</tr>
<tr>
<td>Vehicle 1</td>
<td>100.9±2.3</td>
<td>111.3±2.4*</td>
</tr>
<tr>
<td>Indo</td>
<td>101.1±1.7</td>
<td>109.5±2.8*</td>
</tr>
<tr>
<td>NDGA</td>
<td>99.1±1.6</td>
<td>110.2±2.2*</td>
</tr>
<tr>
<td>Vehicle 2</td>
<td>101.0±1.6</td>
<td>113.9±1.9*</td>
</tr>
<tr>
<td>Apyrase+ADA</td>
<td>97.7±2.3</td>
<td>110.4±2.1*</td>
</tr>
<tr>
<td>ADA</td>
<td>96.5±3.7</td>
<td>106.0±3.8*</td>
</tr>
<tr>
<td>Vehicle 3</td>
<td>98.5±2.4</td>
<td>108.9±3.2*</td>
</tr>
<tr>
<td>CPZ+Indo</td>
<td>104.6±1.8</td>
<td>114.8±3.4*</td>
</tr>
<tr>
<td>iso-PPADS+Indo</td>
<td>98.0±1.8</td>
<td>107.8±4.8*</td>
</tr>
<tr>
<td>CPZ+aprase+ADA</td>
<td>94.7±1.8</td>
<td>108.2±4.6*</td>
</tr>
<tr>
<td>Vehicle 4</td>
<td>97.8±4.8</td>
<td>109.0±4.7*</td>
</tr>
<tr>
<td>Vagotomy</td>
<td>99.5±2.1</td>
<td>107.8±2.5*</td>
</tr>
</tbody>
</table>

Values in each group are the means ± SE in mmHg of n = 10 animals. See the legend of Table 2 for details of the study groups. Peak values measured during 0–21 s and lowest values measured during 21–90 s after the onset of H2O2 challenge were defined as the initial and delayed responses, respectively. *Significantly different from the baseline value, P < 0.05.
MECHANISMS OF H₂O₂-EVOKED AIRWAY REFLEXES

DISCUSSION

Results of this study demonstrate that inhalation of 0.2% aerosolized H₂O₂ consistently evoked initial bradypnea followed by delayed tachypnea. Furthermore, during the tachypneic period, augmented breaths were also evoked in the majority of rats (59%) studied. The characteristics of these H₂O₂-evoked ventilatory responses were similar to those described previously (51). Bilateral cervical vagotomy totally prevented these ventilatory responses, confirming the previous finding (51) that they are airway reflexes mediated through vagal lung afferents. In our previous study (51), we suggested that the initial response and the delayed responses to H₂O₂ are airway reflexes mediated through vagal lung capsaicin-sensitive and myelinated afferents, respectively, because they can be prevented by perivagal capsaicin treatment and vagal cooling to 7°C. We also suggested that the reflex effects of H₂O₂ are due to the action of ROS, because the ventilatory responses to H₂O₂ are largely or totally suppressed by an antioxidant acting against H₂O₂ or hydroxyl radicals (51).

We further demonstrate that pretreatment with CPZ [change (Δ) = −53%] or iso-PPADS (Δ = −47%) reduced the initial bradypneic response to H₂O₂ by about one-half, while pretreatment with vehicles failed to do so. CPZ and iso-PPADS are antagonists of the TRPV1 and P2X receptors, respectively (22, 34), and the doses used in this study effectively prevented the reflex apneic response to intravenous injections of capsaicin and αβ-meATP, respectively. These observations suggest that both the TRPV1 and P2X receptors are important in eliciting the initial reflex bradypnea. The fact that pretreatment with CPZ and iso-PPADS in combination provided a more complete blockade of the initial bradypneic response (Δ = −78%) indicated that the functioning of the TRPV1 receptors in triggering the H₂O₂-evoked initial bradypneic response is, at least in part, independent from that of the P2X receptors. Furthermore, recent studies (31, 57) have demonstrated a positive interaction between TRPV1 and P2X receptors in response to ATP in isolated cell models. The functional interaction of TRPV1 and P2X receptors, however, has not been reported and remains to be elucidated. The suppressive effects of CPZ and iso-PPADS were unlikely to be due to the possible deleterious effects of these drugs on vagal lung capsaicin-sensitive afferents, because these drugs did not affect the reflex apneic response to intravenous injections of capsaicin. A serotonin 5-HT₃ receptor agonist, which serves as a stimulus sensitive afferents, because these drugs did not affect the reflex apneic response to intravenous injections of capsaicin. Accordingly, our observations suggest that both cyclooxygenase metabolites and ATP are involved in eliciting the initial reflex bradypnea. Although lipooxygenase metabolites have been demonstrated to activate TRPV1 receptors (5, 6, 20), they do not seem to play a role in the observed response.

We subsequently delineated the chemical mediator involved in the functioning of the TRPV1 and P2X receptors during the triggering of the reflex responses to H₂O₂. We found that, when TRPV1 receptors were blocked by CPZ, the additional removal of ATP by scavengers could further reduce the initial bradypneic response to H₂O₂ (Δ = −83%), whereas an additional inhibition of cyclooxygenase by Indo failed to do so (Δ = −47%). Similarly, when P2X receptors were blocked by iso-PPADS, an additional inhibition of cyclooxygenase was able to further reduce the initial bradypneic response to H₂O₂ (Δ = −75%). These results suggest that, in the event of the triggering of the initial reflex bradypnea, cyclooxygenase metabolites are linked to the functioning of the TRPV1 receptors, whereas ATP is associated with the functioning of the P2X receptors.

The exact mechanisms by which ROS activates the pathways of the cyclooxygenase metabolites/TRPV1 receptors and ATP/P2X receptors, leading to elicitation of the initial reflex bradypnea, remain to be elucidated. One possibility is that ROS causes the release of cyclooxygenase metabolites and ATP from lung cells, which subsequently activate the TRPV1 and P2X receptors, respectively, located at the terminals of vagal lung capsaicin-sensitive afferent fibers. In fact, ROS are known to increase the release of cyclooxygenase metabolites, such as prostaglandins in the lung tissue (7, 40). Certain prostaglandins have been shown to possess capsaicin-like actions on cardiac capsaicin-sensitive afferent fibers (16). Interestingly, cyclooxygenase also catalyzes the metabolism of anandamide (27, 64), an endocannabinoid, which is postulated to be an endogenous agonist of TRPV1 or P2X receptors has also been shown to cause inhibitory airway reflexes (34, 42).

The initial reflex bradypnea that we observed required an average latency of 6.8 s to be evoked following H₂O₂ challenges. This long latency caused us to doubt that the TRPV1 and P2X receptors located at the nerve terminals are directly stimulated by ROS themselves. As an alternative, ROS may indirectly activate the TRPV1 and P2X receptors through released chemical mediators. In this context, we have demonstrated that pretreatment with Indo (Δ = −48%) or apyrase+ADA (Δ = −50%) reduced the initial bradypneic response to H₂O₂ by about one-half, whereas pretreatment with NDGA, the vehicle of Indo, or the vehicles of apyrase+ADA failed to do so. Indo is a nonselective cyclooxygenase inhibitor, whereas NDGA is a nonselective lipooxygenase inhibitor (55). Apyrase is an enzyme that catalyses breakdown of ATP to AMP, whereas ADA is an enzyme that catalyses breakdown of adenosine. A combination of apyrase and ADA has been used to act as ATP scavengers (1, 54). The suppressive effects of apyrase+ADA could not be explained by its promotion of breakdown of adenosine only, since pretreatment with ADA alone failed to alter the initial bradypneic response to H₂O₂. Additionally, the suppressive effects of Indo and apyrase+ADA were unlikely to be due to the possible deleterious effects of these drugs on vagal lung capsaicin-sensitive afferents, because these drugs did not affect the reflex apneic response to intravenous injections of capsaicin. Accordingly, our observations suggest that both cyclooxygenase metabolites and ATP are involved in eliciting the initial reflex bradypnea.
ligns of TRPV1 receptors (37, 61). Prostaglandin ethanolamides are a novel class of the cyclooxygenase metabolites generated from this reaction (27, 64) and can bind or interact with TRPV1 receptors (39, 50). Furthermore, ROS have been shown to damage cells and cause a rapid release of cytosolic ATP, which activates P2X receptors of pain nociceptors in the vicinity (11, 41). Furthermore, ROS may have nondamaging effects, and, upon stimulation, nondamaged epithelial or endothelial cells may release ATP, exerting a paracrine effect on P2X receptors located on other cells (32). A second possibility is that baseline levels of cyclooxygenase metabolites and ATP are required to maintain the sensitivity of the TRPV1 and P2X receptors. Consequently, administration of Indo or ATP scavengers might make vagal lung capsaicin-sensitive afferents less responsive to ROS-related stimulus. However, this possibility is not likely, because pretreatment with Indo or ATP scavengers did not reduce the reflex apneic response to capsaicin or phenylbiguanide.

In contrast to their effects on initial bradypnea, all pharmacological pretreatments made in this study did not affect the H$_2$O$_2$-evoked delayed tachypnea and augmented breaths. These observations indicate that the pathways of the cyclooxygenase metabolite/TRPV1 receptors and ATP/P2X receptors do not contribute to these H$_2$O$_2$-evoked delayed responses. Thus it is conceivable that the vagal lung afferents responsible for triggering these delayed responses are myelinated afferents with a large diameter, whose activity arises from pulmonary rapidly adapting (irritant) receptors, as our laboratory has suggested previously (51). In fact, preliminary results from our electrophysiological study in anesthetized rats have revealed that delivery of aerosolized H$_2$O$_2$ by a respirator stimulated pulmonary rapidly adapting receptors (52). Likewise, the H$_2$O$_2$-evoked reduction in $V_r$ occurring during both the initial and delayed periods following H$_2$O$_2$ challenges was not significantly affected by any of the pharmacological pretreatments, suggesting that it is related to mechanisms other than the pathways of the cyclooxygenase metabolite/TRPV1 receptors and ATP/P2X receptors. This reduction in $V_r$ is not seen after vagotomy, indicating that it is mediated through vagal afferent and/or efferent pathways.

In accordance with our previous findings, inhalation of 0.2% aerosolized H$_2$O$_2$ initially produced an increase in ABP and subsequently caused a decrease in ABP. Accompanying these two periods of ABP changes, heart rate persistently dropped. Our laboratory previously (51) showed that the initial increase in ABP persisted after perivagal capsaicin treatment, but was prevented during vagal cooling to 7°C, which suggests that it may be a reflex resulting from stimulating pulmonary rapidly adapting receptors or myelinated vagal afferents that arise from other intrathoracic structures, such as the heart or large pulmonary vessels. Conversely, the delayed decrease in ABP persisted during vagal cooling to 7°C but was prevented after perivagal capsaicin treatment, indicating that it may be a reflex resulting from activation of vagal lung capsaicin-sensitive afferent fibers. These notions gain support from the observations made in this study that, when the initial bradypneic response to H$_2$O$_2$ was suppressed by pharmacological pretreatments relevant to the pathways of the cyclooxygenase metabolite/TRPV1 receptors and ATP/P2X receptors, the delayed decrease in ABP vanished. Conversely, when the delayed ventilatory responses to H$_2$O$_2$ were not altered by pharmacological pretreatments, the initial increase in ABP persisted. The cardiovascular reflexes originating from activation of vagal lung capsaicin-sensitive afferent fibers are quite clear (35), whereas that from activation of pulmonary rapidly adapting receptors has not been well defined (63). It is interesting to note that the reflex effect of pulmonary rapidly adapting receptors preceded the reflex effect of lung capsaicin-sensitive afferent fibers for ABP responses, whereas the reverse sequence was true for airway responses. It is possible that the depressor response mediated by lung capsaicin-sensitive afferent fibers was initially masked by an overriding increase in blood pressure mediated by a different set of afferent pathways. Furthermore, the depressor response seems to last for a much longer duration than bradypnea. It is possible that H$_2$O$_2$-induced releases of vasodilative mediators, such as histamine (38), are involved.

In summary, the initial bradypneic response to H$_2$O$_2$ results from the activation of both TRPV1 and P2X receptors, possibly located at terminals of vagal lung capsaicin-sensitive afferent fibers, and the functioning of the TRPV1 and P2X receptors in triggering the initial bradypnea is, in part, mediated through the actions of cyclooxygenase metabolites and ATP, respectively. These mechanisms, however, do not contribute to the H$_2$O$_2$-evoked delayed tachypnea and augmented breaths. While the functions of the TRPV1 and P2X receptors are relatively well established for somatosensory nociceptors and pain sensation (41, 56), their roles in the regulation of breathing, especially under pathological conditions, remain to be further explored. The results of this and our laboratory’s previous studies (51, 53) thus provide evidence to support the hypothesis that vagal lung capsaicin-sensitive afferent fibers can detect excess pulmonary ROS, thereby eliciting an inhibitory airway reflex, and that this sensory transduction is mediated through the pathways of the cyclooxygenase metabolite/TRPV1 receptors and ATP/P2X receptors. Vagal lung capsaicin-sensitive and myelinated afferents have been largely, up to now, implicated in various airway diseases, such as airway hyperreactivity, cough, and bronchoconstriction (35, 59), all of which may be related to excess production of ROS. The above implies that interfering with the above-mentioned pathways are possible target choices for potential therapeutic regimes to treat these ROS-related airway diseases.

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