Role of ATP in the ROS-mediated laryngeal airway hyperreactivity induced by laryngeal acid-pepsin insult in anesthetized rats

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GASTROESOPHAGEAL REFLUX DISEASE (GERD) is a common disorder of the gastrointestinal tract caused by the backflow of gastric contents into the upper digestive tract (32, 39). When the effects of refluxed gastric contents extend beyond the esophagus, it is referred to as extraesophageal reflux (EER) (32, 39). EER is associated with several respiratory manifestations including laryngeal airway hyperreactivity (LAH), which is characterized by increased sensitivity of laryngeal vagally mediated reflexes such as cough, glottic stop, or laryngeal adductor responses (5, 13, 34, 40, 47). LAH is thus defined as a sensory hyperresponsiveness of the laryngeal mucosa leading to exaggerated reflex responses to stimuli. LAH may contribute to chronic cough and paroxysmal laryngospasm seen in EER patients (5, 13, 29, 32, 34, 39, 41). Since EER patients who display LAH have a high incidence of laryngeal inflammation (5, 40, 41) and because inflammatory mediators can increase the excitability of airway afferent fibers (8, 18), it has been postulated that sensitization of laryngeal sensory receptors by inflammatory mediators may be the cause of LAH. However, the hypothesis remains unproven, and the mediator mechanisms are still unclear.

We recently (49) established a rat model of EER with both LAH and laryngeal inflammation induced by laryngeal insult with acid (pH 5) and pepsin, two major components of refluxed gastric contents that cause laryngeal injury (2, 25). This LAH is mediated through sensitization of the capsaicin-sensitive laryngeal afferent fibers (49), a type of nociceptive-like free nerve endings (7, 8). This LAH is prevented by scavengers of hydroxyl radical (·OH), suggesting a critical role of reactive oxygen species (ROS) (49), an important category of inflammatory mediators in GERD (19, 33).

Although the role of ROS seems to be clear, they may not be the sole mediator involved in the LAH. For example, the ROS-induced activation of capsaicin-sensitive lung vagal afferent fibers is partly mediated through the action of the released adenosine 5′-triphosphate (ATP) on P2X purinoceptors located on fiber terminals (42, 43). In addition, ROS may damage cells, causing a subsequent release of cytosolic ATP into their surroundings, which activates the P2X receptors located on nearby nociceptors (10, 12, 35). Furthermore, ATP can sensitize capsaicin-sensitive ion channels in the primary sensory neurons, leading to hyperalgesia (26, 30, 48). These findings support our hypothesis that laryngeal acid-pepsin insult may sequentially increase ROS and then increase the sensitivity of capsaicin-sensitive laryngeal afferent fibers in LAH.

To test our hypothesis, we undertook the present study in anesthetized rats to investigate, first, whether laryngeal insults with acid-pepsin and hydrogen peroxide (H2O2, a major type of ROS) produce LAH with similar characteristics; second, whether activation of P2X receptors by ATP is important to the LAH induced by laryngeal insult with acid-pepsin or H2O2; third, whether laryngeal application of an ATP analog also induces an LAH that is mediated through the capsaicin-sensitive laryngeal afferent fibers; and finally, whether both laryngeal insults with acid-pepsin and H2O2 promote increases in levels of ATP, lipid peroxidation (an index of oxidative stress), and inflammation of the larynx. To accomplish these objectives, we measured the reflex apneic response to laryngeal ammonia provocation to reflect laryngeal reflex reactivity. The
effects of laryngeal acid-pepsin or H$_2$O$_2$ insult on this apneic response were determined to evaluate the LAH.

**MATERIALS AND METHODS**

*Animal preparation.* All protocols were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats were initially anesthetized with an intraperitoneal injection of α-chloralose (100 mg/kg; Sigma Chemical, St. Louis, MO) and urethane (500 mg/kg; Sigma) dissolved in a borax solution (2%). The depth of anesthesia was maintained by supplemental doses of chloralose (20 mg·kg$^{-1}$·h$^{-1}$) and urethane (100 mg·kg$^{-1}$·h$^{-1}$). The right femoral artery and jugular vein were cannulated to record arterial blood pressure and for administration of pharmacological agents, respectively. The animal’s neck was opened, and the superior laryngeal nerves (SLNs) were isolated. Body temperature was maintained at about 37°C.

*Functionally isolated laryngeal preparation.* The methods used for the preparation of a functionally isolated larynx have been reported previously (28, 49). In brief, a lower tracheal catheter (PE-260) was inserted caudally just above the thoracic inlet, whereas an upper tracheal catheter (PE-200) was inserted cranially with its tip placed slightly below the cricoid cartilage. An oral tube was introduced through the mouth with its tip placed at the vallecula. During the experiment, rats breathed spontaneously via the lower tracheal catheter. Respiratory flow and tidal volume were measured.

*Preparation of the pharmacological agents.* The acid-pepsin solution was prepared by adding pepsin to normal saline (0.9% NaCl) (0.0025–2.5 mg/ml) and adjusted to pH 5 with 1 N NaOH. The H$_2$O$_2$ solution (35%; Shimakyu, Osaka, Japan) was diluted with saline to a concentration of 1% (pH 7.4). The solutions of α,β-methylene-ATP (α,β-meATP; a P2X receptor agonist; 5 mM; Sigma), iso-pyridoxalphosphate-6-azophenyl-2′,5′-disulfonate (iso-PPADS, a P2X receptor antagonist; 2 μM; Tocris Cookson, Ellvise, MO) (17, 37) and dimethylthiourea (DMTU, a •OH scavenger; 4.8 M; Sigma) (15) were prepared by dissolving the chemicals in saline. The solution of apyrase (an enzyme that catalyzes breakdown of ATP to AMP; 800 U/ml; Sigma) was prepared by dissolving the chemical in PBS. The solution of adenosine deaminase (ADA, an enzyme that catalyzes breakdown of adenosine; 417 U/ml; Sigma) was prepared by dissolving the chemical in 50% glycerol and 50 mM potassium phosphate and was further diluted to a concentration of 400 U/ml with PBS. A combination of apyrase and ADA (apyrase + ADA) has previously been used as an ATP scavenger (1).

*Laryngeal provocation with ammonia or α,β-meATP.* The methods used for laryngeal ammonia provocation have been described previously (46, 49). In brief, 5 ml of ammonia vapor (0.2% in air) was continuously delivered into the isolated larynx and flowed out via the oral tube. Laryngeal provocation with α,β-meATP (5 mM, 30 μl) were carried out by spraying the solution into the larynx via a spinal needle.

*Laryngeal insult with acid-pepsin or H$_2$O$_2$.* For acid-pepsin insult, a small piece of filter paper (1 × 10 mm) was presoaked with the acid-pepsin solution, carefully inserted into the larynx via the oral tube, and then removed 40 s after insertion. For H$_2$O$_2$ insult, 30 μl of H$_2$O$_2$ solution (1%) was sprayed into the larynx via a spinal needle. After these insults, an elapsed time of at least 60 min was allowed before ammonia provocation.

*Laryngeal application of pharmacological agents.* Solutions containing apyrase + ADA (apyrase, 800 U/ml; ADA, 400 U/ml; iso-PPADS (2 μM, 2 μl), α,β-meATP (5 mM, 30 μl), DMTU (4.8 M, 2 μl), or their vehicles were sprayed into the laryngeal segment via a spinal needle; these applications were made 30 min before the laryngeal insult. In addition, applications of iso-PPADS or its vehicle was made 30 min before each ammonia provocation. The doses and treatment times of these agents were determined based on our preliminary investigations, which indicated that the drug effects of

![Fig. 1. Schematic illustrations showing the experimental protocols of this study. Timelines in A–F depict protocols for studies 1–6 described in MATERIALS AND METHODS. Acid-pepsin, H$_2$O$_2$, or α,β-methylene-ATP (α,β-meATP) represent laryngeal treatment for induction of laryngeal airway hyperreactivity. NH$_3$ represents laryngeal ammonia provocation. Apyrase and adenosine deaminase (apyrase + ADA), dimethylthiourea (DMTU), iso-pyridoxalphosphate-6-azophenyl-2′,5′-disulfonate (iso-PPADS), and their vehicles were given as pretreatments 30 min before laryngeal insult with acid-pepsin or H$_2$O$_2$. Two additional pretreatments with iso-PPADS and its vehicle were made 30 min before laryngeal ammonia provocations due to the short effective time of iso-PPADS. SLN cut represents denervation of superior laryngeal nerves. See text for further explanations.](http://jap.physiology.org/content/106/12/1585.full.pdf)
iso-PPADS, apyrase + ADA, DMTU, and α,β-meATP were able to last ~40, 140, 180, and >360 min, respectively.

Measurements of lipid peroxidation in laryngeal tissues and ATP concentrations in laryngeal fluid. Levels of lipid peroxidation in the laryngeal tissue (20 mg) were determined by measuring malondialdehyde and its dihydropyridine polymers at 356-nm excitation and 426-nm emission by using a fluorescence spectrophotometer (F-4500; Hitachi, Tokyo, Japan) (14). ATP concentrations in laryngeal fluids were determined immediately (<30 s) after sample collection by using an ATP assay kit (ATPLite; PerkinElmer, Waltham, MA) and a luminescence assay system (Multilabel Readers; PerkinElmer) with the firefly luciferin-luciferase reaction (20).

Histological preparation and examination. After the animals were killed by intravenous injection of an overdose of the anesthetics, their larynxes were excised and fixed by immersion in a buffered neutral formalin solution for 48 h. Tissue specimens were embedded in paraffin and were cut transversely into 5-μm-thick sections, which were subsequently stained with hematoxylin and eosin. These sections were examined by a qualified pathologist in a blind fashion. The histological assessment was quantified using a scoring system (49). For each section, structures at three different areas were randomly selected, and their injury scores were averaged.

Experimental design and protocol. In this study, 208 rats (weight 377 ± 23 g) were randomly divided into 25 groups (groups 1–18 and 23–25, each n = 8; groups 19–22, each n = 10) of animals. Figure 1 depicts the experimental protocols. Study 1 (Fig. 1A) was performed to obtain the control response. The reflex apneic responses to four laryngeal ammonia provocations were studied. The first provocation was made 40 min before laryngeal insult with 0.0025–2.5 mg/ml pepsin (groups 1–4) in pH 5 solution. The other provocations were made at 1, 2, and 3 h after insult. Study 2 (Fig. 1B) was performed to test the possibility that ATP might be involved in the acid-pepsin-induced LAH. The reflex apneic responses to four laryngeal ammonia provocations were studied. The first provocation was made 40 min before laryngeal application of apyrase + ADA (group 5), vehicle of apyrase + ADA (group 6), iso-PPADS (group 7), or vehicle of iso-PPADS (group 8). Subsequently, 30 min elapsed before laryngeal acid-pepsin insult. The other provocations were made at 1, 2, and 3 h after insult. Study 3 (Fig. 1C) was performed to investigate the H2O2-induced LAH. The reflex apneic responses to five laryngeal ammonia provocations were studied. The first provocation was made at 40 min before laryngeal insult with H2O2 (group 9) or its vehicle (group 10). The other provocations were made at 1, 2, 3, and 4 h after insult. SLN denervation was performed at 30 min before the last provocation. The effect of SLN denervation on the acid-pepsin-induced LAH was investigated previously (49) and thus was not evaluated in this study. Study 4 (Fig. 1D) was performed to test the possibility that ROS and ATP might be involved in the H2O2-induced LAH. The reflex apneic responses to four laryngeal ammonia provocations were studied. The first provocation was made 40 min before laryngeal application of DMTU (group 11), vehicle of DMTU (group 12), apyrase + ADA (group 13), vehicle of apyrase + ADA (group 14), iso-PPADS (group 15), or vehicle of iso-PPADS (group 16). Subsequently, 30 min elapsed before laryngeal H2O2 insult. The other provocations were made at 1, 2, and 3 h after insult. Study 5 (Fig. 1E)
was performed to test the possibility that laryngeal ATP might induce LAH. The reflex aperine responses to three laryngeal ammonia provocations were studied. The first provocation was made 40 min before laryngeal application with α,β-meATP (group 17) or its vehicle (group 18). The other provocations were made at 1 and 2 h after α,β-meATP application. Study 6 (Fig. 1F) was performed to investigate whether laryngeal insult with acid-pepsin or H2O2 might induce an increase in levels of inflammation, lipid peroxidation, and ATP in the larynx. Laryngeal insult with acid-pepsin (group 19), H2O2 (group 20), or their respective vehicles (groups 21 and 22) were performed. Two hours later, the laryngeal fluids of five animals in each group were collected, and their laryngeal tissues were sampled for measurements of ATP and lipid peroxidation, whereas the larynxes of the other five animals were excised for the pathohistological study. To collect laryngeal fluids, a suction catheter (PE-50) was inserted into the upper tracheal catheter with its tip close to the laryngeal segment. The laryngeal fluids were sucked by a syringe via this suction catheter. Study 7 was performed to investigate the potency and duration of blocking effect of iso-PPADS. The reflex aperine responses to four laryngeal provocations with α,β-meATP were investigated. One hour was allowed to elapse between any two α,β-meATP provocations. Laryngeal pretreatments with iso-PPADS (group 23) or its vehicle (group 24) were made 30 min before the second and third α,β-meATP provocations. Study 8 was performed to investigate the possible type of laryngeal afferents that responded to α,β-meATP. The reflex aperine responses to laryngeal α,β-meATP provocations and mechanical provocations were investigated before and during perineural capsaicin treatment (PCT) of superior laryngeal nerves (group 25) with a procedure described previously (49). Laryngeal mechanical provocations were carried out by probing the laryngeal segment with a nylon thread.

Data analysis and statistics. Respiratory flow, tidal volume, and expiratory duration (Ti) were analyzed on a breath-by-breath basis. Mean arterial blood pressure and heart rate were measured at 1-s intervals. These physiological parameters were analyzed using a computer system (BioCybernatics, 1.0; Taipei, Taiwan). The longest Ti occurring during the first 5 s after laryngeal provocation was divided by the baseline Ti, and the value was then multiplied by 100 to give a percentage aperine index. The data for cardiopulmonary parameters were compared using one-way repeated-measures ANOVA followed by Fisher’s least significant difference procedure where appropriate. The injury scores were compared using the Mann-Whitney U-test. The data for lipid peroxidation and ATP concentrations were compared using Student’s t-test. A value of $P < 0.05$ was considered significant. All data are means ± SE.

RESULTS

Laryngeal acid-pepsin insult produces LAH. In the control animals, an aperine response was elicited within 1 s after laryngeal ammonia provocation (Fig. 2). After acid (pH 5)-pepsin (2.5 mg/ml) insult, the same ammonia provocation elicited a similar magnitude of aperine response after the first hour but evoked a significantly augmented aperine response after the second and third hour compared with the control response (Figs. 2A and 3). When the concentration of pepsin was decreased from 2.5 mg/ml to 2.5 μg/ml, the time of occurrence of LAH was delayed and the number of animals displaying LAH was reduced (Table 1). As a group, the mean aperine responses in animals treated with pH 5-pepsin at concentrations of 2.5 and 25 μg/ml were not significantly different from control values at any time point (Table 1). Insult with pH 5-pepsin (2.5 mg/ml) was then chosen as the standard insult for the subsequent studies.

Activation of P2X receptors by ATP is important in acid-pepsin-induced LAH. In the groups with laryngeal pretreatment with apyrase + ADA (ATP scavengers) or iso-PPADS (a P2X receptor antagonist), ammonia provocation evoked an aperine response that did not significantly differ from the control response at the second hour after insult with acid-pepsin (Figs. 2B and 3), a time during which LAH was supposed to occur. The blocking effect of iso-PPADS on the aperine response to laryngeal α,β-meATP provocation was proven to vanish at 90 min after its pretreatment (Table 2). Hence, after the effects of apyrase + ADA and iso-PPADS had worn off, the same ammonia provocation elicited a significantly greater aperine response at the third hour after insult with acid-pepsin (Figs. 2B and 3) compared with the responses under baseline condition. In contrast, in the groups pretreated with vehicles of apyrase + ADA or iso-PPADS, the LAH induced by acid-pepsin insult was unaffected (Figs. 2A and 3).
but was unaffected by pretreatment with their vehicles (Fig. 5). LAH was prevented by laryngeal pretreatment with these drugs induced by laryngeal acid-pepsin insult. The H2O2-induced in animals with laryngeal insult with vehicle of H2O2, this acid-pepsin-induced LAH reported previously (49). In contrast, through the SLNs. This neural mechanism is similar to that of pepsin-induced LAH (Fig. 4). A subsequent denervation of the larynx was insulted with H2O2. Laryngeal H2O2 insult produced an LAH with characteristics similar to those of acid-pepsin-induced LAH (Fig. 4). A subsequent denervation of the SLNs abolished the apneic response to ammonia provocation (Fig. 4A), indicating that the H2O2-induced LAH was mediated through the SLNs. This neural mechanism is similar to that of acid-pepsin-induced LAH reported previously (49). In contrast, in animals with laryngeal insult with vehicle of H2O2, this LAH was not observed over the same time period (Fig. 4A). In addition, the H2O2-induced LAH was prevented by laryngeal pretreatment with DMTU but not with its vehicle, suggesting a critical role for 'OH (Fig. 4B).

Activation of P2X receptors by ATP is important in H2O2-induced LAH. The effects of laryngeal pretreatment with apyrase + ADA or iso-PPADS on the LAH induced by laryngeal H2O2 insult (Fig. 5) were similar to those on the LAH induced by laryngeal acid-pepsin insult. The H2O2-induced LAH was prevented by laryngeal pretreatment with these drugs but was unaffected by pretreatment with their vehicles (Fig. 5).

![Image](http://jap.physiology.org/)

**Table 1. Apneic response to laryngeal ammonia provocation before and after laryngeal insults with 4 concentrations of pepsin in pH 5 solution in 4 study groups**

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Control</th>
<th>1 h after insult</th>
<th>2 h after insult</th>
<th>3 h after insult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin, mg/ml</td>
<td>2.5</td>
<td>0.25</td>
<td>0.025</td>
<td>0.0025</td>
</tr>
<tr>
<td>Apneic index, %</td>
<td>504±30</td>
<td>517±30</td>
<td>510±10</td>
<td>504±15</td>
</tr>
<tr>
<td>Animals with LAH, %</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Apneic index, %</td>
<td>537±34</td>
<td>534±32</td>
<td>503±27</td>
<td>496±27</td>
</tr>
<tr>
<td>Animals with LAH, %</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Apneic index, %</td>
<td>980±115*</td>
<td>609±125</td>
<td>533±47</td>
<td>503±28</td>
</tr>
<tr>
<td>Animals with LAH, %</td>
<td>100</td>
<td>25</td>
<td>12.5</td>
<td>0</td>
</tr>
<tr>
<td>Apneic index, %</td>
<td>1038±98*</td>
<td>808±98*</td>
<td>607±75</td>
<td>536±48</td>
</tr>
<tr>
<td>Animals with LAH, %</td>
<td>100</td>
<td>50</td>
<td>25</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Data in each group are means ± SE from 8 rats. The longest expiratory duration (Ti) occurring during the first 5 s after laryngeal provocation was divided by the baseline Ti, and the value was then multiplied by 100 to give a percentage apneic index. When the ammonia-evoked apneic index exceeded the baseline by at least 20%, the animal was defined as displaying laryngeal airway hyperreactivity (LAH). *P < 0.05, significantly different from baseline in the same group.

**Laryngeal H2O2 insult also produces LAH.** To examine whether a direct increase in ROS level might produce LAH, the larynx was insulted with H2O2. Laryngeal H2O2 insult produced an LAH with characteristics similar to those of acid-pepsin-induced LAH (Fig. 4). A subsequent denervation of the SLNs abolished the apneic response to ammonia provocation (Fig. 4A), indicating that the H2O2-induced LAH was mediated through the SLNs. This neural mechanism is similar to that of acid-pepsin-induced LAH reported previously (49). In contrast, in animals with laryngeal application of the vehicle of H2O2, this LAH was not observed over the same time period (Fig. 4A). In addition, the H2O2-induced LAH was prevented by laryngeal pretreatment with DMTU but not with its vehicle, suggesting a critical role for 'OH (Fig. 4B).

Activation of P2X receptors by ATP is important in H2O2-induced LAH. The effects of laryngeal pretreatment with apyrase + ADA or iso-PPADS on the LAH induced by laryngeal H2O2 insult (Fig. 5) were similar to those on the LAH induced by laryngeal acid-pepsin insult. The H2O2-induced LAH was prevented by laryngeal pretreatment with these drugs but was unaffected by pretreatment with their vehicles (Fig. 5).

**Table 2. Effect of pretreatment with iso-PPADS or its vehicle on the apneic response to 4 laryngeal α,β-meATP provocations in 2 study groups**

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Control</th>
<th>Response to 2nd Provocation</th>
<th>Response to 3rd Provocation</th>
<th>Response to 4th Provocation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apneic index, %</td>
<td>504±30</td>
<td>102±1*</td>
<td>101±1*</td>
<td>783±210</td>
</tr>
<tr>
<td>Apneic index, %</td>
<td>537±34</td>
<td>534±32</td>
<td>503±27</td>
<td>496±27</td>
</tr>
<tr>
<td>Apneic index, %</td>
<td>980±115*</td>
<td>609±125</td>
<td>533±47</td>
<td>503±28</td>
</tr>
<tr>
<td>Apneic index, %</td>
<td>1038±98*</td>
<td>808±98*</td>
<td>607±75</td>
<td>536±48</td>
</tr>
</tbody>
</table>

Data in each group are means ± SE from 8 rats. One hour was allowed to elapse between any two β-methylene-ATP (α,β-meATP) provocations. Laryngeal pretreatment with iso-pyridoxalphosphate-6-azophenyl-2,2′-disulfonate (iso-PPADS) or its vehicle were made 30 min before the 2nd and 3rd α,β-meATP provocations. Apneic response is expressed as apneic index (see Table 1 legend for definition). *P < 0.05, significantly different from vehicle.

![Image](http://jap.physiology.org/)

**Fig. 4. Mean apneic responses to laryngeal ammonia provocations before and after laryngeal insult with H2O2 or its vehicle in 4 study groups. A: responses before and after laryngeal insult with H2O2 or its vehicle. Denervation of SLNs was performed at the 4th hour after insult. B: responses before and after laryngeal insult with H2O2 in animals pretreated with laryngeal application of DMTU or vehicle. Recovery of the response was obtained after the drug effect had worn off. Baseline Ti was calculated as the mean over 10 breaths immediately before provocation. *P < 0.05, significantly different from the response before H2O2 or vehicle insult in the same group. #P < 0.05, significantly different from the response of the vehicle group at the same time point. Data in each group are means ± SE from 8 rats.**

**Activation of P2X receptors by ATP analog alone also produces LAH.** In animals with laryngeal application of α,β-meATP, ammonia provocation evoked a significantly augmented apneic response during the first and second hours after application compared with the control response (Fig. 6). In contrast, in animals with laryngeal application of the vehicle for α,β-meATP, the LAH was not observed over the same time period (Fig. 6). To identify the type of laryngeal afferents that responded to α,β-meATP, we investigated the reflex apneic responses to laryngeal provocation by this agonist before and during PCT of SLNs, a procedure that selectively blocks the neural conduction of capsaicin-sensitive afferent fibers (49). It was found that PCT nearly abolished the apneic response to α,β-meATP provocation (apneic index before vs. during PCT, 565 ± 171 vs. 109 ± 3%), whereas it did not significantly affect the apneic response to mechanical provocation in the same animals (apneic index before vs. during...
PCT, 630 ± 103 vs. 559 ± 77%), indicating a selective blocking effect of PCT.

Laryngeal insults with acid-pepsin or H2O2 promote increases in lipid peroxidation, ATP, and inflammation in the larynx. The levels of lipid peroxidation in laryngeal tissues and ATP in the laryngeal fluid sampled at 2 h after laryngeal insults with acid-pepsin (Fig. 7, A and B) and H2O2 (Fig. 7, C and D) were both significantly higher than those sampled at 2 h after vehicle insult. Histological examination revealed that laryngeal insults with acid-pepsin (Fig. 8B) or H2O2 (Fig. 8D) produced a mild epithelial damage, as evidenced by epithelial shedding and/or observable infiltration of inflammatory cells, compared with insults with their vehicles (Fig. 8, A and C). Overall, the total injury score in the acid-pepsin group (2.6 ± 0.2) was not significantly different from that in the H2O2 group (2.2 ± 0.2), and both scores were significantly greater than scores for their corresponding controls (vehicle of acid-pepsin, 1.1 ± 0.1; vehicle of H2O2, 1.4 ± 0.2).

Hemodynamics. In all animals, the arterial blood pressure and heart rate were stable until the end of each experiment. Before and at the end of experiment, the mean values for arterial blood pressure were 101.2 ± 14.1 and 99.9 ± 11.3 mmHg, respectively, and the mean values for heart rate were 367.2 ± 40.0 and 360.3 ± 34.3 beats/min, respectively.

DISCUSSION

We (49) recently reported that a laryngeal acid-pepsin insult produces an LAH that is mediated through sensitization of the capsaicin-sensitive laryngeal afferent fibers by ROS. In the current study, we further demonstrated that this LAH was prevented by laryngeal pretreatment with either ATP scavengers or a P2X receptor antagonist, indicating the importance of activation of P2X receptors by endogenous ATP. A plausible hypothesis is that ATP release following acid-pepsin insult activates P2X receptors located on the nerve endings and that this induces sensitization of these afferent fibers, resulting in LAH. This notion is strongly supported by the observations that direct application of αβ-meATP alone could also produce LAH and that the acid-pepsin insult indeed promoted the release of endogenous ATP as shown by the elevated level in the laryngeal fluid. The αβ-meATP-induced LAH also appears to be mediated through capsaicin-sensitive afferent fibers, because the laryngeal reflex response to this agonist can be eliminated by a procedure that selectively blocks the neural conduction of these afferent fibers. The onset of LAH induced by laryngeal αβ-meATP was sooner than that induced by acid-pepsin insult. This may possibly be due to the fact that exogenous αβ-meATP has a direct action, whereas endogenous ATP requires a certain period of time to be released following acid-pepsin insult. Taken together, in addition to ROS, the present findings suggest the crucial role of activation of P2X receptors by ATP in the acid-pepsin-induced LAH.
Since scavenging either *OH or ATP completely abrogated
the acid-pepsin-induced LAH, their functional contributions
ought to be interrelated in series. In this study, we found that
laryngeal H2O2 insult induced an LAH that was abrogated by
a *OH scavenger, ATP scavengers, or a P2X receptor antago-
nist. In addition, laryngeal H2O2 insult caused an increase in
the levels ATP, lipid peroxidation, and inflammation in the
larynx. These findings suggest that an increase in laryngeal
ROS promotes the release of ATP in the inflamed larynx,
which may subsequently activate P2X receptors, serving as a
downstream mechanism responsible for the development of the
observed LAH. Since these H2O2-induced pathophysiological
Fig. 7. Increases in levels of lipid peroxidation in laryngeal
tissues (A and C) and ATP in the laryngeal fluid (B and D)
sampled at 2 h after laryngeal insult with acid-pepsin (A and B)
or H2O2 (C and D). RFU, relative fluorescent unit. *P < 0.05,
significantly different from the vehicle. Data in each group are
means ± SE from 5 rats.
Fig. 8. Light micrographs of laryngeal tissues from 4 anesthe-
tized rats excised at 2 h after laryngeal insult with acid-pepsin
(B), H2O2 (D) or their respective vehicles (A and C). All
magnifications, ×100. e, Epiglottis; vc, vocal cord. Arrows
indicate epithelium damage; arrowheads indicate subepithelial
inflammatory cell infiltration.
consequences were similar to those induced by acid-pepsin insult, it is possible that a comparable mechanism with this hierarchy of ROS and ATP is involved in the development of the acid-pepsin-induced LAH. Alternatively, H$_2$O$_2$ may act nonspecifically as an irritant leading to LAH in the same manner as acid-pepsin insult.

Our results fully support the hypothesis that inflammatory mediators can sensitize laryngeal afferent fibers, resulting in LAH in EER patients, as postulated by other investigators (5, 40, 41). The hierarchy of ROS and ATP as the sensitizing mediators is not surprising because, in other organs, inflamed tissues usually generate excess ROS that may damage or stimulate cells, resulting in release of their cytotoxic ATP to act on other cells in the vicinity (10, 12, 35). The source of ATP could not be determined in this study. However, because increased levels of ATP could be detected in the laryngeal fluid, airway epithelial cells are a plausible candidate (24).

Capsaicin-sensitive afferent fibers are present in the larynx (23, 31). Functional studies in the stimulation of capsaicin-sensitive airway afferent fibers by receptor agonist suggest that P2X receptors may be located at the terminals of these afferent fibers (37, 42). The possibility that activation of these P2X receptors by ATP may sensitize capsaicin-sensitive laryngeal afferent fibers, resulting in LAH, however, has not been previously reported. In another visceral organ, activation of P2X receptor by α,β-meATP induces sensitization of vagal mechanoreceptors during esophageal inflammation in ferrets (36). In addition, in vivo studies have demonstrated that activation of P2X receptor by α,β-meATP produces hyperalgesia in mice or rats, a response that is mediated through capsaicin-sensitive nociceptive afferent fibers (50, 51, 52). In vitro studies have shown that activation of P2X receptors by α,β-meATP sensitizes rat cutaneous nerve terminals of the nociceptive C- and A-$\delta$ fibers to mechanical or heat stimulation (26, 52, 53). Thus it appears that capsaicin-sensitive afferent fibers in both somatic and visceral organs are vulnerable to sensitization following activation of P2X receptors by ATP.

The pepsin concentrations in the sputum samples collected from EER patients range from 0.003 to 22 mg/ml (22). Thus one potential limitation of our experimental model is that the effective concentration of pepsin (2.5 mg/ml) for induction of LAH is higher than physiological concentrations measured in patients. However, the laryngeal insults by gastric contents in EER patients usually are repeated and recurrent during reflux episodes. This scenario is obviously different from that with only one laryngeal insult by acid-pepsin or H$_2$O$_2$ in our experimental model. The other limitation of this study is that our findings were based on the measurement of reflex apneic responses elicited by laryngeal irritation. We did not measure the responses mediated by other efferent arms, such as laryngeal adduction or cough. We assumed that our findings could be extrapolated to these two responses, which are commonly observed in EER patients.

Our results may have several clinical implications. First, we found that a single laryngeal exposure to pepsin at a weakly acidic pH (pH 5) for only 40 s can sensitize capsaicin-sensitive laryngeal afferent fibers, resulting in an LAH that persists for at least 1 h. Since stimulation of these afferent fibers can trigger cough reflex in human subjects (11, 16), our findings are consistent with the report that EER patients show a long-lasting increase in cough reflex sensitivity to inhaled capsaicin aerosol (5, 13, 34, 40). Furthermore, our observations are in agreement with the emerging concept that a new category of weakly acidic reflux (pH 4-7) should be defined and that GERD patients in this category are associated with chronic cough (6, 44, 45). Second, capsaicin-sensitive laryngeal afferent fibers are polymodal fibers that also can be stimulated by various mediators such as acid, ATP, ROS, histamine, and arachidonate metabolites or by many inhaled irritants (7, 8, 27, 42, 43). The accessibility of laryngeal stimuli to these afferent fibers may be increased in inflamed larynx when laryngeal mucosal permeability is elevated. Thus the unexplained chronic cough found in patients with GERD or EER could be due to the fact that the cough is more vulnerable to being triggered when sensitized capsaicin-sensitive laryngeal afferent fibers are stimulated by acidic gastric juices during reflux episodes, or by inflammatory mediators and inhaled irritants when there is no reflux (4, 5, 6, 32, 41). These two scenarios probably can explain the clinical observations that the temporal pattern of reflux may or may not correlate well with that of the cough (3, 4, 11) and that proton pump inhibitor treatment may or may not relieve chronic cough in these patients (5, 9, 21, 38).

In conclusion, our findings suggest that laryngeal insult with acid-pepsin or H$_2$O$_2$ induces inflammation and produces excess ROS in the rat’s larynx. The latter may in turn promote the release of ATP to activate P2X receptors, resulting in sensitization of capsaicin-sensitive laryngeal afferent fibers and LAH. Although the optimal therapy to treat chronic cough in these patients is still evolving, the functional significance of ROS, ATP, and P2X receptors in the development of LAH induced by laryngeal acid-pepsin or H$_2$O$_2$ insult may provide a basis for possible target choices when investigating potential therapeutic regimes.

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