Hemorrhagic hypotension-induced hypersensitivity of vagal pulmonary C-fibers to chemical stimulation and lung inflation in anesthetized rats

Lin RL, Lin YJ, Xu F, Lee LY. Hemorrhagic hypotension-induced hypersensitivity of vagal pulmonary C-fibers to chemical stimulation and lung inflation in anesthetized rats. Am J Physiol Regul Integr Comp Physiol 308: R605–R613, 2015. First published January 14, 2015; doi:10.1152/ajpregu.00424.2014.—This study was carried out to investigate whether hemorrhagic hypotension (HH) altered the sensitivity of vagal pulmonary C-fibers. The fiber activity (FA) of single vagal pulmonary C-fiber was continuously recorded in anesthetized rats before, during, and after HH was induced by bleeding from the femoral arterial catheter into a blood reservoir and lowering the mean systemic arterial pressure (MSAP) to ~40 mmHg for 20 min. Our results showed the following. First, after MSAP reached a steady state of HH, the peak FA response to intravenous injection of capsaicin was elevated by approximately fivefold. The enhanced C-fiber sensitivity continued to increase during HH and sustained even after MSAP returned to baseline during the recovery, but slowly returned to control ~20 min later. Second, responses of FA to intravenous injections of other chemical stimulants of pulmonary C-fibers (phenylbiguanide, lactic acid, and adenosine) and a constant-pressure lung hyperinflation were all significantly potentiated by HH. Third, infusion of sodium bicarbonate alleviated the systemic acidosis during HH, and it also attenuated, but did not completely prevent, the HH-induced C-fiber hypersensitivity. In conclusion, the pulmonary C-fiber sensitivity was elevated during HH, probably caused by the endogenous release of chemical substances (e.g., lactic acid) that were produced by tissue ischemia during HH. This enhanced C-fiber sensitivity may heighten the pulmonary protective reflexes mediated through these afferents (e.g., cough, J reflex) during hemorrhage when the body is more susceptible to other hazardous insults and pathophysiological stresses.

VAGAL BRONCHOPULMONARY C-fibers represent ~75% of the vagal afferents arising from the lung and airways (17). These unmyelinated afferents innervate the entire respiratory tract and play an important role in regulating the respiratory functions under various physiological and pathophysiological conditions (5, 21). Activation of these afferents elicits centrally mediated protective reflex responses via the cholinergic pathway, which include laryngeal closure, bronchoconstriction and airway hypersecretion, accompanied by cough, airway irritation, dyspnic sensation, and J reflex (5, 21). Activation of these sensory nerves can also trigger release of tachykinins and calcitonin gene-related peptide from the sensory terminals, which can act on a number of effector cells in the respiratory tract (e.g., smooth muscles, cholinergic ganglia, mucous glands, immune cells), and elicit the local “axon reflexes” such as bronchoconstriction, protein extravasation, and inflammatory cell chemotaxis (5, 18, 21).

Tissue ischemia is known to activate C-fiber nociceptors in various organ systems including heart, gastrointestinal tract, and skeletal muscles (2, 9, 20). However, its action on C-fibers in the lung is not known. Severe hemorrhage, a medical condition frequently encountered in patients who suffer from traumatic injury, can lead to tissue ischemia, hypoxia, and acidosis (8), accompanied by elevated levels of adenosine (32), lactate (23), pro-inflammatory cytokines (7), and reactive oxygen species (ROS) (27) in the circulating blood. Many of these chemical substances and mediators are known to exert potent stimulatory and sensitizing effects on pulmonary C-fiber afferents (21). In particular, acidosis can both activate (15) and sensitize vagal pulmonary C-fibers (10) to chemical stimuli. Hypoxia has also been shown to enhance the pulmonary C-fiber sensitivity to capsaicin (30).

In view of the background information described above, this study was carried out 1) to determine the effects of hemorrhagic hypotension (HH) on the baseline activity and sensitivity of vagal pulmonary C-fiber afferents to chemical and mechanical stimuli; and 2) to identify the possible contributing factors to the effects of HH on these afferents.

In most of the experimental models of hemorrhage, a mean systemic arterial pressure (MSAP) of 35–40 mmHg maintained for various durations was used to study the pathophysiology and therapeutic interventions (3). To regulate the extent and duration of HH in this study, we bled the animal to lower the MSAP to ~40 mmHg and then maintained it at a constant level for 20 min before infusing the blood back to the animal for recovery.

METHODS

All protocols were performed in accordance with Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and were also approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animal preparation. Male Sprague-Dawley rats (n = 56; body weight, 396 ± 6 g) were initially anesthetized with α-chloralose (0.1 mg/g ip) and urethane (0.5 mg/g ip) (Sigma-Aldrich, St. Louis, MO), and supplemental doses (one-tenth of the initial doses) of the same anesthetics were injected intravenously (iv) to maintain abolition of pain reflexes induced by paw pinch. A tracheal tube was inserted after a tracheotomy. The left jugular vein was cannulated by a PE-50 tubing for administering anesthetics and chemical agents. Both femoral arteries were cannulated: one was connected to a pressure transducer (Statham P23AC, Hato Rey, Puerto Rico) for measurements of systemic arterial pressure (SAP) and heart rate (HR), and the other one was connected to a blood reservoir for the HH challenge. Body temperature was maintained at ~36°C by a heating pad connected to a warm-water circulator (Baxter K-MOD 100, Deerfield, IL). At the
end of experiment, the animals were euthanized by intravenous injections of KCl.

Hemorrhagic hypotension. During the HH challenge, arterial blood was bled from the femoral arterial catheter into a reservoir containing 0.1 ml of 1,000 U/ml heparin (APP Pharmaceuticals, Schaumburg, IL) to avoid blood clotting. The height of the reservoir was lowered until the MSAP reached ~40 mmHg and maintained for 20 min. After the HH challenge, the blood in the reservoir was returned in its entirety to the rat via the venous catheter by gravity initially and completed by infusion with a sterile syringe. The MSAP changed progressively and slowly after the change in reservoir height. No measurement was made until after the MSAP reached a steady-state level, which was considered as the onset (0 min) of the HH challenge or the recovery period.

Recording of pulmonary C-fiber activity. Afferent activities of single-unit vagal pulmonary C-fibers were recorded, as described in details previously (13). Briefly, the lung was artificially ventilated with room air by a constant-volume respirator (Ugo Basile model 7025, Comerio, Italy), and a midline thoracotomy was performed; the expiratory outlet of the respirator was placed under 3 cmH2O pressure to maintain a near-normal functional residual capacity (FRC). Tidal volume (Vt) and frequency (f) were set at 8 ml/kg and 60 breaths/min, respectively. The caudal end of the cut right vagus nerve was placed on a small dissecting platform and immersed in mineral oil. A thin filament was teased away from the desheathed nerve trunk and further split until the afferent activity arising from a single unit was electrically isolated. The nerve trunk was ligated just above the diaphragm to eliminate afferent signals arising from lower visceral organs.

Pulmonary C-fiber activity was searched initially by their mild responses to lung inflation that was performed by delivery of three or four consecutive Vt via the respirator after occluding the expiratory flow at FRC and by an immediate (delay < 1 s) response to bolus injection of capsaicin (Cap; 0.50–1.00 μg/kg) into the right atrium (13). The general locations of C-fiber terminals were identified by their responses to gentle pressing of the lungs with a blunt-end glass rod at the end of the experiment, which could not, however, exclude the possibility that some of these endings might innervate the small intrapulmonary airways receiving blood supply from pulmonary circulation (13). Action potential (AP), tracheal pressure (Ptr), and SAP were recorded on a thermal writer (Gould TW11, Cleveland, OH) and analyzed by a data acquisition system (Biocybernetics TS-100, Taipei, Taiwan) at a sampling rate of 3,000 Hz in this study.

Measurement of oxidative stress. To determine whether oxidative stress was generated by HH, protein carbonyls, the products of oxidized protein side chain (especially of Pro, Arg, Lys, and Thr), were chosen as the markers of oxidative modification of proteins. These moieties are chemically stable for both their detection and storage (6). The concentration of protein carbonyl in the plasma was measured by a commercial colormetric kit (Cayman Chemicals, Ann Arbor, MI). Briefly, venous blood samples (1.0 ml) were drawn from anesthetized rats (artificially ventilated) by syringes coated with heparin (Innovative Med Tech, Leawood, KS) during control, at the end of HH, and at the end of recovery. The blood samples were centrifuged at 1,000 g for 10 min at 4°C to collect the plasma samples. Each sample was loaded in two 2-ml vials (200 μl each) and mixed with 800 μl of 2,4-dinitrophenylhydrazine (as sample) or 800 μl of 2.5 M HCl (as control). After 1 h of incubation (brief vortex every 15 min during incubation) in the dark, both vials were placed on ice and washed by 20% trichloroacetic acid (TCA), 10% TCA, and then three times of ethanol-ethyl acetate (1:1), followed by a centrifugation (10,000 g for 10 min at 4°C). After the final wash, protein pellets were resuspended by 500 μl of guanidine hydrochloride, and vials were centrifuged again to remove leftover debris. A spectrophotometer was then used to measure the absorbance of each vial at 385 nm.

Experimental design and protocol. Four series of experiments were carried out. Study 1 was carried out to determine the effects of the HH challenge on MSAP, mean pulmonary arterial pressure (MPAP), and HR and to measure the changes in blood gases in the systemic and pulmonary blood. To measure the MPAP, a catheter was inserted via a jugular venous catheter into the pulmonary artery in 4 rats surgically prepared in the same manner as in study 2; the tip position of the catheter was verified by monitoring the pressure signal as it was advanced from right ventricle into pulmonary artery. In a separate series, blood samples (~0.1 ml) were drawn from both the aorta (via a femoral arterial catheter) and right ventricle (via a jugular venous catheter) before and at 20 min after both onset and termination of the HH challenge and placed into test cartridges (Abaxis CG4+, Union City, CA) for blood gas analysis (Abaxis i-STAT 1). In study 2, we investigated the effects of HH on pulmonary C-fiber responses to chemical and mechanical stimulations. Chemical stimulations were applied by intravenous bolus injections of several known chemical stimulants of rat vagal pulmonary C-fibers: Cap (0.25–1.00 μg/kg), a...
selective activator of the transient receptor potential vanilloid type 1 receptor; adenosine (Ado; 0.1–0.3 mg/kg), an activator of the A1 adenosine receptor (14); lactic acid (LA; 5.0–10.0 mg/kg), an activator of acid-sensing ion channel (15); and phenylbiguanide (PBG; 1.0–8.0 μg/kg), an activator of the 5-hydroxytryptamine type 3 receptor (5). The mechanical stimulation was applied by hyperinflation of the lung (HI) with a constant \( P_r \) of 30 cmH\(_2\)O (lung volume reaching \( \sim 3 \times V_T \) above FRC) and maintained for 10 s. Study 3 was carried out to determine whether oxidative stress was induced by the HH challenge. Venous blood samples were collected and their plasma concentrations of protein carbonyl were measured as described above. In study 4, we tested whether the HH-induced pulmonary C-fiber hypersensitivity was caused by production of lactic acid resulting from tissue hypoxia. To alleviate systemic acidosis, solution of sodium bicarbonate (NaHCO\(_3\)) was infused intravenously at a rate of 86 μmol·kg\(^{-1}\)·min\(^{-1}\) (range: 70–200) in a total volume of 3 ml for 30 min; the infusion was initiated as soon as the blood reservoir was lowered. To determine whether the HH-induced C-fiber hypersensitivity could be attenuated by increasing the oxygen intake in the lung, we continuously delivered 100% oxygen to the lung via the ventilator before, during, and after the HH challenge in another group of rats.

**Preparation of chemical solutions.** The stock solution of Cap (250 μg/ml) was prepared in 10% Tween 80, 10% ethanol, and 80% saline and that of PBG (1 mg/ml), LA (300 mg/ml), and NaHCO\(_3\) (10 mM/ml) were prepared in isotonic saline (0.9% NaCl). These stock solutions were stored at \(-20^\circ\text{C}\) and prepared daily at the desired concentrations for injection by dilution with isotonic saline based on the animal’s body weight. Ado was dissolved in sterile water and prepared daily before the experiment. All these chemicals were purchased from Sigma-Aldrich.

**Data analysis.** The baseline fiber activity (FA) was averaged over a 20-s interval. \( \Delta \text{FA} \) was calculated as the difference between peak FA (averaged over 2-s and 10-s intervals for chemical stimulations and lung inflation, respectively) and the averaged baseline FA in each fiber. Data were analyzed statistically with either one-way or two-way repeated measures ANOVA, and pair-wise comparisons were made with a post hoc analysis (Fisher’s least significant difference). A \( P \) value of <0.05 was considered significant. Data are reported as means ± SE.

### RESULTS

After the reservoir was lowered to initiate bleeding, the MSAP declined progressively and slowly; the time required to reach a new steady-state level (\( \sim 40 \) mmHg) varied among animals and its average was 7.8 ± 0.5 min. To normalize the time of data calculation between animals, the time after the MSAP reached steady state was considered as the onset (0 min) of the HH challenge. During the recovery when the blood in the reservoir was returned in its entirety to the rat, the MSAP also increased slowly and reached a steady state after 8.2 ± 0.3 min, which was considered as the beginning (0 min) of the recovery period.

![Fig. 2. Changes in blood gases in the systemic and pulmonary blood induced by the HH challenge in anesthetized, open-chest and artificially ventilated rats. Top: partial pressures of O\(_2\) (PrO\(_2\)) and CO\(_2\) (PrCO\(_2\)), pH, and lactate concentration in the mixed venous blood drawn from a jugular venous catheter placed in the right ventricle. Bottom: partial pressures of O\(_2\) (PaO\(_2\)) and CO\(_2\) (PaCO\(_2\)), pH, and lactate concentration in the systemic arterial blood drawn from a femoral arterial catheter with its tip placed in the aorta. Control, before the HH challenge; HH (20 min), after the MSAP reached a steady state of HH (\( \sim 40 \) mmHg) for 20 min; Rec (20 min), after the MSAP was returned to the control level for 20 min during recovery. *Significantly different from the corresponding control (\( P < 0.05; n = 5 \) in each group). Data are means ± SE.](http://ajpregu.physiology.org/doi/abs/10.1152/ajpregu.00424.2014)
**Study 1: systemic effects of HH challenge.** At the onset of HH, when MSAP was dropped from 84.9 ± 2.6 to 42.3 ± 0.6 mmHg (P < 0.05, n = 46; Fig. 1), the MPAP decreased from 13.5 ± 3.7 to 7.3 ± 2.0 mmHg (P < 0.05, n = 4), and HR decreased slightly but significantly from 331 ± 6 to 320 ± 7 beats/min (P < 0.05, n = 46); data of MSAP and HR also included those collected in studies 2 and 4, in which the same surgical preparations as in this study series were performed. When HH was maintained for 20 min, the MSAP, MPAP, and HR remained relatively stable at 39.3 ± 0.9 mmHg, 8.1 ± 2.2 mmHg, and 316 ± 6 beats/min, respectively. MSAP, MPAP, and HR all returned to near the control levels during the recovery period (Fig. 1).

Blood gases in arterial and venous blood were measured in 5 rats. After HH was maintained for 20 min, blood samples drawn from the right ventricle (mixed venous blood) showed a decrease of the partial pressure of O₂ (PvO₂) by ~32.7% (P < 0.05, n = 5), an increase of PvCO₂ by ~14.5% (P < 0.05, n = 5), and a reduction of pH (an increase of H⁺ concentration by 32.16%; P < 0.05, n = 5), accompanied by a large increase of lactate concentration by ~353.8% (P < 0.05, n = 5; Fig. 2). All these changes except venous pH showed full recovery 20 min after the termination of HH (Fig. 2, top). In contrast, at the same time points, the systemic arterial blood showed no change in PaO₂ from the baseline (P > 0.05, n = 5), a decrease of PaCO₂ by ~24.9% (P < 0.05, n = 5), a reduction of pH (an increase of H⁺ concentration by 15.55%; P < 0.05, n = 5), and a pronounced increase in the lactate concentration by ~882.1% (P < 0.05, n = 5). All these changes showed full recovery 20 min after the termination of HH (Fig. 2, bottom).

**Study 2: effect of HH on pulmonary C-fiber sensitivity.** Pulmonary C-fibers were generally quiescent during the baseline at control (Figs. 3 and 4).
beginning of the HH challenge to a peak of 0.064 ± 0.039 imp/s at the end of 20 min HH (P < 0.05, n = 11; Fig. 5, left). During the recovery period, baseline FA gradually decreased and returned to control after the termination of HH for 20 min.

At control (before HH), the bolus injection (iv) of a low dose of Cap triggered a short and mild burst of discharge of pulmonary C-fibers (e.g., Fig. 3). After MSAP reached the steady state of HH, ΔFA triggered by the same dose of Cap increased from 2.18 ± 0.56 imp/s at control to 10.82 ± 1.72 imp/s at 0 min of HH (P < 0.05, n = 11); the increased ΔFA continued at 10 min and reached the peak at 20 min of the HH challenge (15.02 ± 1.94 imp/s, P < 0.05, n = 11; Fig. 5, middle). This pulmonary C-fiber hypersensitivity to Cap was accompanied by intensified bradycardia (Figs. 3 and 4). The increased sensitivity remained significantly higher than control immediately after the MSAP returned to control (8.26 ± 1.98 imp/s, P < 0.05, n = 11; Fig. 5, middle) and slowly returned to near the control level 20 min later (Fig. 4, top, and Fig. 5, middle).

A pronounced potentiating effect of the HH challenge was also found consistently in pulmonary C-fiber responses to all other chemical stimulants of pulmonary C-fibers tested in this study: Ado (n = 9), LA (n = 8), and PBG (n = 8) (Fig. 6). It should be noted that there was a difference in the magnitude and pattern of the HH-induced potentiation between the C-fiber responses to these chemical stimulants; for example, at 0 min of HH, the responses to Ado, LA, and PBG were elevated to 123%, 177%, and 688% of their control responses, respectively (Fig. 6). The ΔFA evoked by bolus injection of vehicle (isotonic saline) was 0.17 ± 0.11 imp/s at control and 0.08 ± 0.08 imp/s after the HH challenge was maintained for 20 min (P > 0.05, n = 6).

Pulmonary C-fibers exhibited low sensitivity to HI at control (before HH). However, the FA response to HI (P<sub>r</sub> = 30 cmH<sub>2</sub>O; 10-s duration) increased progressively during the HH challenge: the HI-evoked ΔFA increased from 0.35 ± 0.12 imp/s at control to 0.98 ± 0.32 imp/s (P < 0.05, n = 8) at 10 min, and 1.08 ± 0.33 imp/s (P < 0.05, n = 8) at 20 min of HH. This elevated sensitivity to HI persisted even after the MSAP returned to control after HH and gradually returned to the control level 20 min later (Fig. 5, right).

**Study 3: effect of HH on oxidative stress.** The plasma concentrations of protein carbonyl were 26.55 ± 0.89, 21.13 ± 0.72, and 23.26 ± 0.90 nmol/ml for control, after 20 min of HH, and after 20 min of recovery, respectively (P < 0.05, n = 6). However, total protein also showed a similar trend of decrease: 54.54 ± 2.79, 43.00 ± 1.76, and 47.74 ± 0.76 mg/ml at the corresponding time points (P < 0.05, n = 6). Thus the plasma protein carbonyl content per unit of total protein did not change during or after the HH challenge: 0.49 ± 0.03, 0.49 ± 0.02, and 0.49 ± 0.02 nmol/mg for control, at 20 min after the onset of HH, and at 20 min after the termination of HH, respectively (P > 0.05, n = 6).

**Study 4: possible influences of systemic acidosis and tissue hypoxia induced by HH.** Infusion of NaHCO<sub>3</sub> (86 μmol·kg<sup>-1</sup>·min iv) for 30 min during the HH challenge significantly attenuated the systemic acidosis, measured by the reduction in venous blood pH at the end of the HH challenge (Fig. 7, middle), but it did not change the HH-induced lactate production (Fig. 7, right). Accompanying the change in venous blood pH, infusion of NaHCO<sub>3</sub> significantly attenuated the HH-induced C-fiber hypersensitivity to Cap at the end of the HH challenge (P < 0.05, n = 8; Fig. 7, left).

Ventilation with 100% O<sub>2</sub> did not significantly change either PO<sub>2</sub> or lactate concentration in the mixed venous blood at the end of the HH challenge (P > 0.05, n = 5; Fig. 8, middle and right), and it also failed to alter the C-fibers hypersensitivity to Cap induced by the HH challenge (P > 0.05, n = 7; Fig. 8, left).

**DISCUSSION**

This study clearly demonstrated that vagal pulmonary C-fiber sensitivity was markedly elevated during HH. The C-fiber hypersensitivity developed at the onset of the HH challenge increased progressively during HH and persisted even after the arterial blood pressure returned to the control level during recovery. The increased sensitivity was found in their responses to both Cap injection and HI; the latter is of particular
interest because lung expansion is a natural physiological action, and pulmonary C-fibers normally have low sensitivity to HI (13, 19). In addition, the increased chemical sensitivity induced by HH in these afferents was not limited to Cap but was also evident in their responses to other chemical stimulants of C-fibers tested in this study: Ado, PBG, and LA; each of them represents a selective activator of a specific type of receptor expressed in the pulmonary C-fiber sensory endings. The hypersensitivity of pulmonary C-fiber afferents to these chemical stimulants was accompanied by intensified bradycardia (e.g., Figs. 3 and 4), probably resulting from the reflexes elicited by stimulation of C-fibers in the contralateral lung. Although the mechanism(s) underlying this effect generated by HH could not be determined in this study, several potential contributing factors should be considered.

It has been shown that acute severe hypoxia can enhance the C-fiber sensitivity to Cap (30). The HH challenge was expected to induce systemic tissue hypoxia, which was confirmed by a substantial decrease in PO2 in the mixed venous blood, despite no change in the arterial PO2 (Fig. 2); the tissue hypoxia presumably resulted from a reduction of the blood flow and oxygen delivery to peripheral tissues. The C-fiber endings are located in the lung parenchyma that receives blood supply from pulmonary circulation (5) and, therefore, responsive to the changes in chemical compositions of the mixed venous blood (from pulmonary artery) and not that in the systemic arterial blood. Ventilation with 100% O2 did not significantly attenuate the tissue hypoxia and lactic acid production (Fig. 8). The lack of effect is not unexpected because the increase in arterial O2 content by oxygen ventilation is limited to the minute increase in dissolved O2, which cannot offset the deficit in O2 delivery to the tissue resulting from the reduced blood supply during HH. The fact that ventilation with O2 did not significantly alter the C-fiber hypersensitivity induced by the HH challenge (Fig. 8, left) further suggests a possible contribution of tissue hypoxia to the increased C-fiber sensitivity during HH. Indeed, both lactic acid and adenosine are produced by anaerobic metabolism during tissue hypoxia, and both have been shown to exert a potent stimulatory and sensitizing effects on rat pulmonary C-fiber afferents in our previous studies (12, 14, 15). The fact that a distinct increase in lactate concentration and PO2 in the mixed venous blood (Fig. 2) clearly indicate that HH induces tissue hypoxia in this study. Furthermore, HH is known to stimulate sympathetic activity and increase the levels of sympathetic nerves; therefore, HH-generated tissue hypoxia may increase peripheral sympathetic nerve activity and consequently contribute to the increased C-fiber responses.

Fig. 5. Effects of HH on the pulmonary C-fiber baseline activity and on the pulmonary C-fiber responses to Cap (0.25–1.00 μg/kg) and HI (tracheal pressure maintained at 30 cmH2O for 10 s) in anesthetized, open-chest and artificially ventilated rats. FA, averaged baseline fiber activity over 20-s interval. ΔFA, the difference between peak fiber activity (averaged over 2-s and 10-s intervals for Cap and HI, respectively) and averaged baseline FA (20-s interval) in each fiber. Control, before the HH challenge; HH (0, 10, or 20 min), responses obtained after the MSAP reached a steady state of HH (~40 mmHg) for 0, 10, or 20 min; Rec (0 or 20 min), responses obtained after the MSAP was returned to control for 0 or 20 min during recovery. *Significantly different from control baseline or response (P < 0.05). Data are means ± SE.

Fig. 6. Effects of HH on the pulmonary C-fiber responses to adenosine (Ado; 0.1–0.3 mg/kg), lactic acid (LA; 5.0–10.0 mg/kg), and phenylbiguanide (PBG; 1.0–8.0 mg/kg) in anesthetized, open-chest and artificially ventilated rats. For the explanations of calculation of ΔFA and time points of measurements, see legend of Fig. 5. *Significantly different from the corresponding control response (P < 0.05). Data are means ± SE.
of circulatory catecholamines, and epinephrine has been shown to enhance the sensitivity of pulmonary C-fibers to Cap and HI via an activation of β3-adrenoceptor (11).

The sensitivity of pulmonary C-fiber afferents can be also elevated by an increase in CO2 in the alveolar air and pulmonary blood, and the action can be prevented by a pretreatment with NaHCO3 to reverse the acidosis, indicating the involvement of hydrogen ion (10). In this study, both acidosis and hypercapnia were found in the pulmonary arterial blood (systemic mixed venous blood) drawn from the right ventricle during HH, and these changes were reversible after MSAP returned to control (Fig. 2, top). In addition, the blood lactate concentration was also markedly elevated during HH (Fig. 2, top), suggesting an excessive production of lactic acid probably caused by tissue hypoxia and anaerobic metabolism. Interestingly, when infusion of NaHCO3 alleviated the HH-induced systemic acidosis, it also significantly attenuated the C-fiber hypersensitivity at the end of the HH challenge (Fig. 7, left). These results seem to suggest a possible involvement of hydrogen ion in the augmented C-fiber sensitivity induced by the HH challenge.

MPAP was substantially reduced during HH (Fig. 1). Although the change in pulmonary blood flow caused by the hemorrhage was not measured in this study, presumably it was also reduced during HH due to the loss of total blood volume. If so, for the same injected chemical solution, its concentration in the pulmonary circulation may have been higher during HH than control, which could have contributed, in part, to the enhanced sensitivity. However, we do not believe this to be a major contributing factor for the following reasons. First, the hypersensitivity of pulmonary C-fiber induced by HH was not limited to intravenous injections of chemical stimulants; a similar potentiation was also observed in their response to HI during HH (Figs. 3 and 5). Second, the C-fiber responses to chemical challenges showed a trend to increase progressively during the 20 min of HH. In fact, immediately after the MSAP was lowered to 40 mmHg (at the beginning of the HH challenge), there was no significant increase in C-fiber responses to some of the chemical stimulants (e.g., Ado, LA; Fig. 6). Third, the hypersensitivity of pulmonary C-fibers persisted even after the MSAP had already returned completely to the control level during recovery. Finally, in our pilot study of the effect of HH...
on pulmonary chemoreflex responses elicited by C-fiber stimulation, the potentiating effect of HH was also clearly observed when Cap was delivered by aerosol inhalation (instead of intravenous injection; data not shown).

It is well documented that severe hemorrhage can induce systemic oxidative stress and trigger local release of ROS resulting from tissue ischemia-reperfusion (26). Some of these ROS, such as hydrogen peroxide and hydroxyl radicals, can activate pulmonary C-fibers (24, 25). To test a possible involvement of ROS in the HH-induced C-fiber hypersensitivity, we measured the plasma protein carbonyl content, one of the most widely used markers of oxidative stress (4, 6), and compared before between, during, and after the HH challenge. However, we did not detect any increase in the plasma level of protein carbonyl during either HH or the recovery phase. Although we cannot completely rule out a possible involvement of a lower level of ROS undetected by protein carbonyl in our study, it is unlikely that oxidative stress plays a major part in the elevated C-fiber sensitivity in this study.

Another mechanism possibly involved in the HH-induced C-fiber hypersensitivity is the systemic inflammation caused by hemorrhage. It is well documented that acute hemorrhage causes a surging production of pro-inflammatory cytokines from macrophages and other cells of innate immune system (3). For example, pronounced increases in the plasma levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, and IL-10 have been reported in the studies when hemorrhage of similar intensity and duration of hypotension as that in our study was induced (1). Some of these cytokines such as TNF-α and IL-1β are known to exert potent stimulatory and/or sensitizing effects on vagal pulmonary C-fiber afferents (16, 22, 31).

These C-fiber afferents are known to play an important role in the pulmonary defense function. Activation of these afferents by inhaled irritants (e.g., cigarette smoke, sulfur dioxide, ozone, etc.) or endogenous chemical mediators (e.g., hydrogen ion, adenosine 5′-triphosphate, bradykinin, eosinophil granule-derived cationic proteins, etc.) can elicit powerful protective reflex responses, such as cough, mucus secretion, bronchoconstriction, dyspneic sensation, and J reflex (5, 21). Furthermore, these sensory nerves have been recognized to play an important role in the neural-immune interaction in response to various airway assaults (e.g., allergens, irritant chemicals) (21, 28); for example, neuropeptides (e.g., substance P) released from pulmonary C-fiber endings upon activation can interact with a number of immune cells (e.g., macrophage, mast cell) and modulate their activity and cytokine release (21, 28). Hence, their sensitivity is not only important in protecting the lung and body against potential health hazardous actions caused by air-borne chemical irritants, but also in regulating airway function under various pathophysiological conditions (5, 21). Our results obtained in this study would suggest that these protective functions of the lung are heightened during hemorrhage when the body is more susceptible to other hazardous insults and pathophysiological stresses.

**Perspectives and Significance**

This study demonstrated that HH induced an elevated sensitivity of pulmonary C-fiber afferents to various chemical stimuli and lung inflation, which persisted even when the MSAP had returned to the control level after the termination of HH. We believe that the hypersensitivity was probably generated by the endogenous release of inflammatory mediators and chemical substances including hydrogen ion that are produced by tissue ischemia/hypoxia during HH. The elevated C-fiber sensitivity may be important in enhancing the pulmonary defense function during this pathophysiological condition. In addition, it may also play a part in the vagal anti-inflammatory mechanism (28, 29) that inhibits the synthesis and release of pro-inflammatory cytokines, thereby preventing further tissue damage caused by hemorrhage and tissue ischemia.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: R.-L.L., Y.-J.L., F.X., and L.-Y.L. conception and design of research; R.-L.L. and Y.-J.L. performed experiments; R.-L.L., Y.-J.L., and L.-Y.L. analyzed data; R.-L.L., Y.-J.L., F.X., and L.-Y.L. interpreted results of experiments; R.-L.L., Y.-J.L., and L.-Y.L. prepared figures; R.-L.L., Y.-J.L., F.X., and L.-Y.L. drafted manuscript; R.-L.L., F.X., and L.-Y.L. edited and revised manuscript; R.-L.L., Y.-J.L., F.X., and L.-Y.L. approved final version of manuscript.

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