行政院國家科學委員會專題研究計畫 成果報告

活性氧族群刺激大白鼠肺快適應性受器的機制
研究成果報告(精簡版)

計 畫 類 別 ：個別型
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執 行 期 間 ：95 年 08 月 01 日至 96 年 07 月 31 日
執 行 單 位 ：中山醫學大學醫學系生理學科

計 畫 主 持 人：阮婷
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行政院國家科學委員會補助專題研究計畫　成果報告

活性氧族群刺激大白鼠肺快適應性受器的機制

Mechanisms of pulmonary reactive oxygen species-induced stimulation of pulmonary rapidly adapting receptors in rats

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行政院國家科學委員會專題研究計畫執行成果報告

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中文摘要

肺發炎會造成肺中活性氧族群的過量產生以及出現咳嗽與呼吸道收縮等症狀。肺快適應受器可以偵測肺部生理與病理的改變並引發咳嗽與呼吸道收縮等呼吸反射。本實驗探討肺快適應受器是否可以偵測肺部活性氧族群的增加，如果可以，我們將進一步研究此感覺訊息傳遞的機制。學者認為快適應受器可區分成二類：平時幾乎不放電或呈不規則放電的安靜型快適應受器；以及規則的跟隨呼吸週期放電的相位型快適應受器。最近的研究發現，安靜型快適應受器對於化學性刺激比較敏感；相位型快適應受器則對機械性刺激反應較大。我們從麻醉、開胸的大白鼠記錄安靜型或相位型肺快適應受器的神經活性。實驗發現，動物吸入霧化的過氧化氫(0.4 %)的確會產生安靜型與相位型肺快適應受器神經活性增加的反應。然而此兩類肺快適應受器被興奮的型態截然不同。安靜型肺快適應受器的神經活性在吸入過氧化氫後隨即呈現不規則的放電增加；相反的，相位型肺快適應受器的放電頻率在較晚期才開始增大，而且其增加的神經反應與呼吸週期同步。推測過氧化氫引發這兩類快適應受器的興奮很可能是尋由不同的機制。不論是吸入 0.4% 過氧化氫引發安靜型或相位型肺快適應受器的神經興奮反應，都被前處理過氧化氫分解酶而完全預防。由本實驗結果推測，安靜型與相位型肺快適應受器可以感受到肺部活性氧族群的增加，此感覺訊息傳遞的機制我們正在探討中。

關鍵詞：氧自由基，藥理受器，肺機械學，感覺傳遞，肺迷走感覺神經

Introduction

Our airways and lungs are innervated by many vagal sensory receptors, which transmit the major afferent information to inform our central nervous system regarding the condition of the pulmonary system (7, 8). Owing to this function, vagal sensory receptors are known to play an important role in detecting the onset of pathophysiological conditions and are responsible for triggering defensive or protective airway reflexes (9-11). For example, cough and broncoconstriction are common reflex consequences elicited via vagal afferent pathways during lung inflammation (12). In this setting, activation of lung vagal sensory receptors leading to reflex consequences is believed to result from the action of inflammatory mediators on the sensory receptors (12-14). Although cough and bronchoconstriction are defensive airway reflexes, they apparently cause several adverse effects. As a result, antitussive drugs are administered to suppress cough and bronchospasm suppressants are given to suppress airway constrictrion. Evidently, interfering with the mechanisms involving in the vagal sensory activation is possible target choices for potential therapeutic regimes to treat cough and bronchoconstriction during lung inflammation. In this context, our understandings of mechanisms underlying lung vagal sensory transduction during lung inflammation have high clinical impact. Many inflammatory lung diseases are known to produce excess reactive oxygen species (ROS) (15, 16). Pulmonary rapidly adapting receptors (RARs) are a type of lung vagal sensory receptors that mediates reflex cough and bronchoconstriction (10, 17). This proposed study attempts to use a one-year period to investigate the stimulation of RARs by ROS and the sensory transduction mechanisms involving several pharmacological receptors and inflammatory mediators.

Lung Diseases and ROS

Lung diseases such as asthma, chronic obstructive pulmonary disease, endotoxin shock, and vascular microembolism, or inhalation of oxidant irritants such as toxic smoke, cigarette smoke, and ozone (15, 16, 18-21) may cause increased pulmonary production of reactive oxygen species from endogenous and/or exogenous sources. ROS are the most important free radicals in biological system (22-24). Reduction of oxygen by the transfer to it of a single electron will produce the superoxide anion (O_2^-). A two-electron reduction of oxygen would yield H_2O_2. H_2O_2 can rather easily break down, particularly in the presence of transition metal ions via the Fenton reaction (25), to produce the most reactive oxygen radicals, the hydroxyl radicals (·OH). Hence,

$O_2 + e^- \rightarrow \cdot O_2^-$

$O_2 + 2e^- \rightarrow H_2O_2 + O_2$

$H_2O_2 + Fe^{2+} \rightarrow \cdot OH + OH^- + Fe^{3+}$

$\cdot O_2^- + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$

The last reaction is catalyzed by free iron in the presence of both $\cdot O_2^-$ and H_2O_2, and has been termed as Haber-Weiss reaction. Therefore, the major ROS are$\cdot O_2^-$, H_2O_2, and ·OH (23). Under

normal condition, the ROS, once produced, are eliminated by the inherited antioxidant mechanisms in the airways and lungs (26). However, in many situations, the production of ROS is increased to a level that is beyond the handling capacity of the antioxidant mechanisms, which leads to tissue oxidative stress. Oxidative stress thus becomes the major factor contributing to many lung diseases (27, 28). Our knowledge regarding the impacts of ROS on lungs and airways is limited to their damage effects leading to oxidative lung injury.

Pulmonary Rapidly Adapting Receptors

RARs occur throughout the respiratory tract from the nose to the bronchi. They have thin myelinated nerve fibres (Aδ fibers) and adapt rapidly to a maintained mechanical stimulus, but often slowly to a chemical stimulus (7, 8, 10). These afferent fibers play a major role in airway physiology and pathophysiology, as their stimulation has been shown to elicit a variety of defensive reflexes, such as cough, bronchoconstriction, hyperventilation, augmented breaths, mucus secretion, and laryngeal closure (7, 8, 10). RARs are also called "irritant receptors" or "cough receptors" that are polymodal and response to a wide range of mechanical and chemical stimuli. They can be activiated by lung deflation, change of lung volume, bronchoconstriction, decrease of lung compliance, inhaled irritants, hyperosmolality, and inflammatory mediators (10, 29-38), as well as in various lung pathophysiological conditions such as, pulmonary edema, lung inflammation, and pulmonary embolism (13, 39, 40). Due to their chemical sensitivity to various chemicals or mediators, it is suggested that various pharmacological receptors may locate on their membrane of nerve terminals (5, 34-41). However, some investigators also proposed that these chemicals or mediators may activiate RARs indirectly by their effects on changes the lung mechanics (10, 42). Regardless direct or indirect effects, it is accepted that RARs function transducers of chemical stimuli. Recently, several studies have revealed that RARs can be further differentiated into two types. These new findings probably can solve the discrepancy of the previous results. Using *in vivo* single fiber recordings, investigators found that RARs have no or irregular impulse discharge (silent RARs) are much more chemosensitive than fire in phase with deflation one (phasic RARs) (5, 43). Studies using *in vitro* airway preparations demonstrated that pulmonary Aδ afferents whose cell bodies reside in the nodose ganglia appear to be mechanosensitive and do not respond to chemical stimuli. In contrast, pulmonary Aδ afferents whose cell bodies are present in the jugular ganglia appear to be chemosensitive and relatively insensitive to mechanical stimuli (34, 44, 45). However, different physiological properties of these two sub-types of RARs are still largely unknown.

Our Previous Study Relevant to This Proposal

The concept that vagal lung sensory receptors may play a vital role in the sensory transduction of ROS is considerably new. This concept is indirectly supported from the findings that activations of vagal lung sensory receptors by inhaled wood smoke or endotoxin shock are greatly attenuated by pretreatment with ·OH scavengers (29, 46, 47). These findings suggesting that ROS are part of a signaling cascade leading to stimulation of vagal lung

sensory receptors. However, questions still remain as to whether the suppressive effects of ·OH scavengers in these studies result from the prevention of stimulation of airway sensory receptors by increased ·OH and/or from the removal of the basal function of ·OH leading to diminished receptor sensitivity to these airway insults. We recently reported (3) that inhalation of aerosolized H_2O_2 evokes initial bradypnea followed by delayed tachypnea. The initial response was abolished after perivagal capsaicin treatment, but was prolonged during vagal cooling to 7 °C **(Figures 1 and 2)**; perivagal capsaicin treatment and vagal cooling are known to differentially block the conduction of unmyelinated C and myelinated fibers, respectively. The delayed responses were eliminated during vagal cooling, but emerged earlier after perivagal capsaicin treatment **(Figures 1 and 2)**. Vagotomy and catalase (an antioxidant for H_2O_2) totally suppressed this reflexive response, while sham nerve treatment and heat-inactivated catalase failed to do so. These results suggest that the H_2O_2-evoked reflex bradypnea and tachypnea are results from stimulation of vagal lung unmyelinated C afferent fibers and vagal lung myelinated afferent fibers, respectively. The follow-up electrophysiological studies demonstrated that H_2O_2 stimulates vagal lung C fibers via the actings of H_2O_2 and ·OH **(Figures 3 and 4)** and this afferent response is partly mediated through transient receptor potential vanilloid 1 (TRPV1) receptors and P2X purinoceptors **(Figure 5)**. However, the mechanisms underlying the H_2O_2 stimulates vagal lung myelinated afferent fibers are still unknown. There are two major types of vagal lung myelinated afferent fibers have been classified: pulmonary slowly adapting receptors (SARs) and RARs. Our preliminary results show 0.4 % H_2O_2 failed to significantly change the activity of SARs **(Figures 6 and 7)**. This finding suggests that RARs play a crucial role in detecting the excess production of ROS. However, direct electrophysiological evidence to support this notion is still lacking and its sensory transduction mechanisms are still unknown.

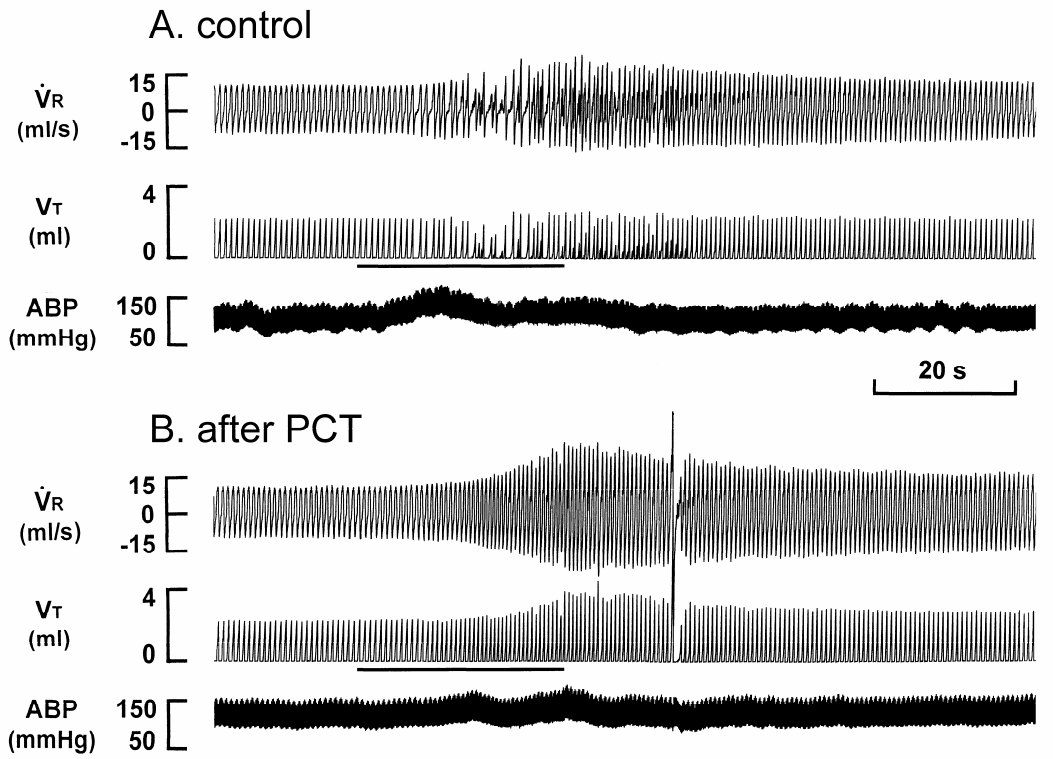

Figure 1. Experimental records illustrating acute responses to inhalation of 0.2% aerosolized H_2O_2 in an anesthetized and spontaneously breathing rat. A: control responses; B: responses after perivagal capsaicin treatment (PCT). $\dot{V}_R$, respiratory flow; V_T, tidal volume; ABP, arterial blood pressure. Horizontal bars indicate the duration (30 s) of H_2O_2 challenge. Between the two H_2O_2 challenges, 60 min was allowed to elapse. Note that the H_2O_2 challenge evoked bradypnea followed by tachypnea during the control, and that the former response was prevented after perivagal capsaicin treatment (Result from reference 3).

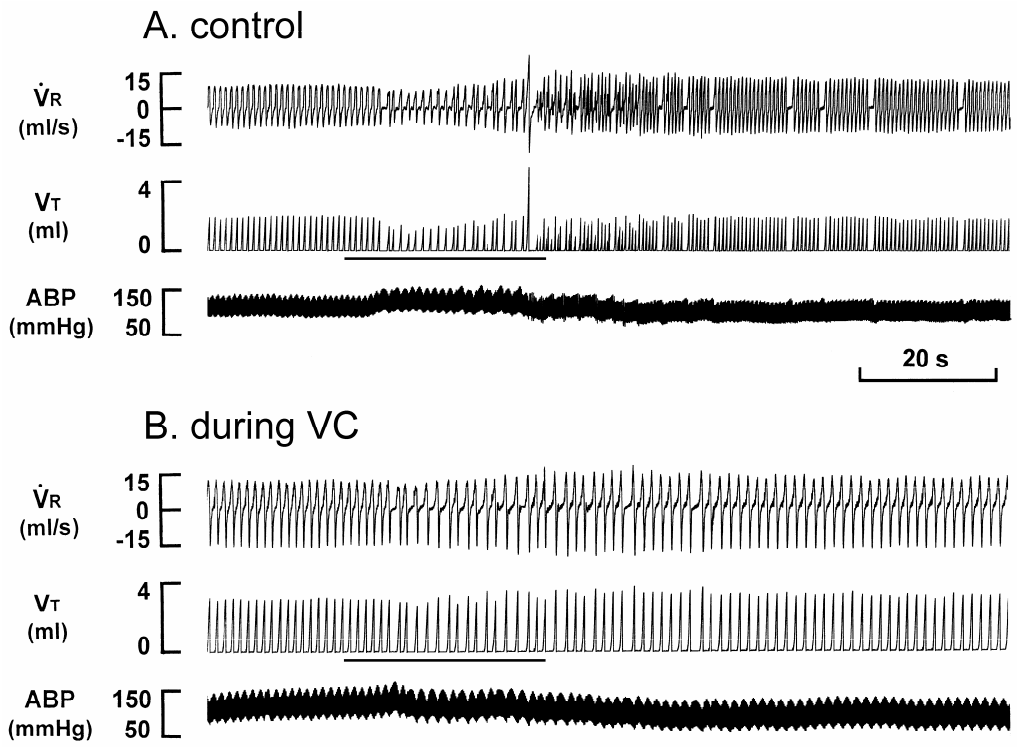

Figure 2. Experimental records illustrating acute responses to inhalation of 0.2 % aerosolized H_2O_2 in an anesthetized and spontaneously breathing rat. A: control responses; B: responses during vagal cooling (VC). $\dot{V}_R$, respiratory flow; V_T, tidal volume; ABP, arterial blood pressure. Horizontal bars indicate the duration (30 s) of H_2O_2 challenge. Between the two H_2O_2 challenges, 60 min was allowed to elapse. Note that the H_2O_2 challenge evoked bradypnea followed by tachypnea and augmented inspiration during the control, and that the latter two responses were prevented during vagal cooling (Result from reference 3).

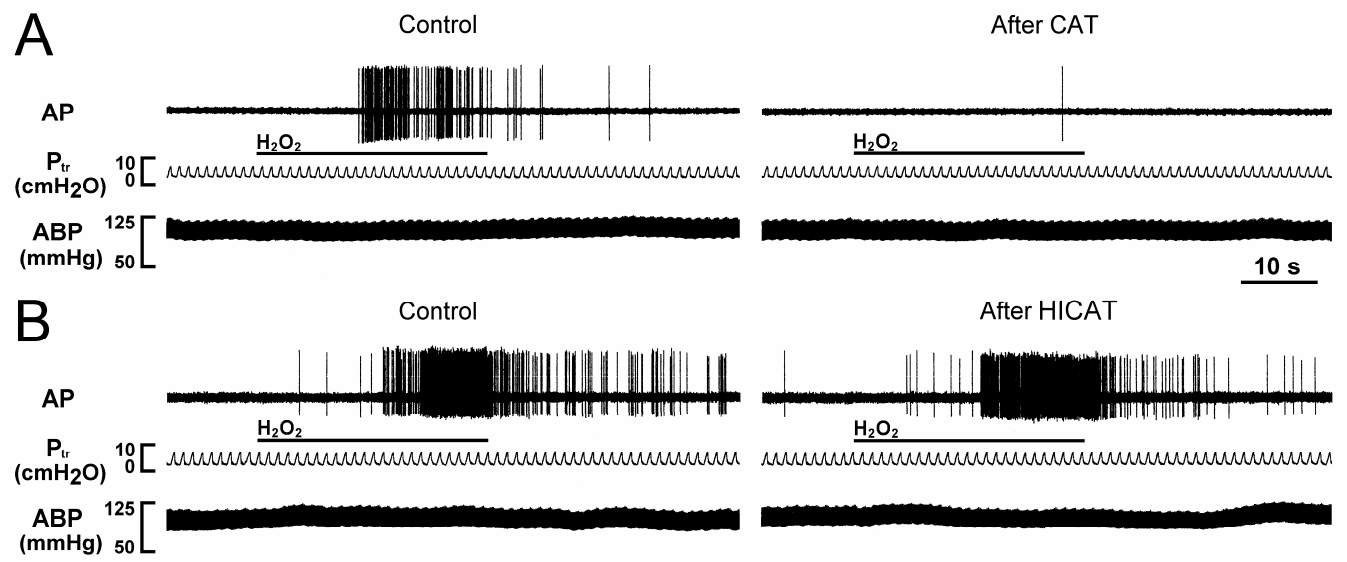

Figure 3. Responses of two rat vagal lung C fibers to 0.4 % aerosolized H_2O_2 before and after antioxidant pretreatment. A and B: pretreatment with catalase (CAT) and heat-inactivated catalase (HICAT), respectively. Pretreatments were made 10 min prior to the subsequent challenge by delivery of aerosolized with CAT (750,000 IU/ml) or HICAT (750,000 IU/ml) into lower airways for a period of 5 min using the nebulizer and circuit for delivery of H_2O_2 aerosol. The duration of H_2O_2 challenge is indicated by horizontal bars. The elapsed time intervals between two H_2O_2 challenges were 60 min. AP, action potential; P_{tr}, tracheal pressure; ABP, arterial blood pressure (Result from reference 2).

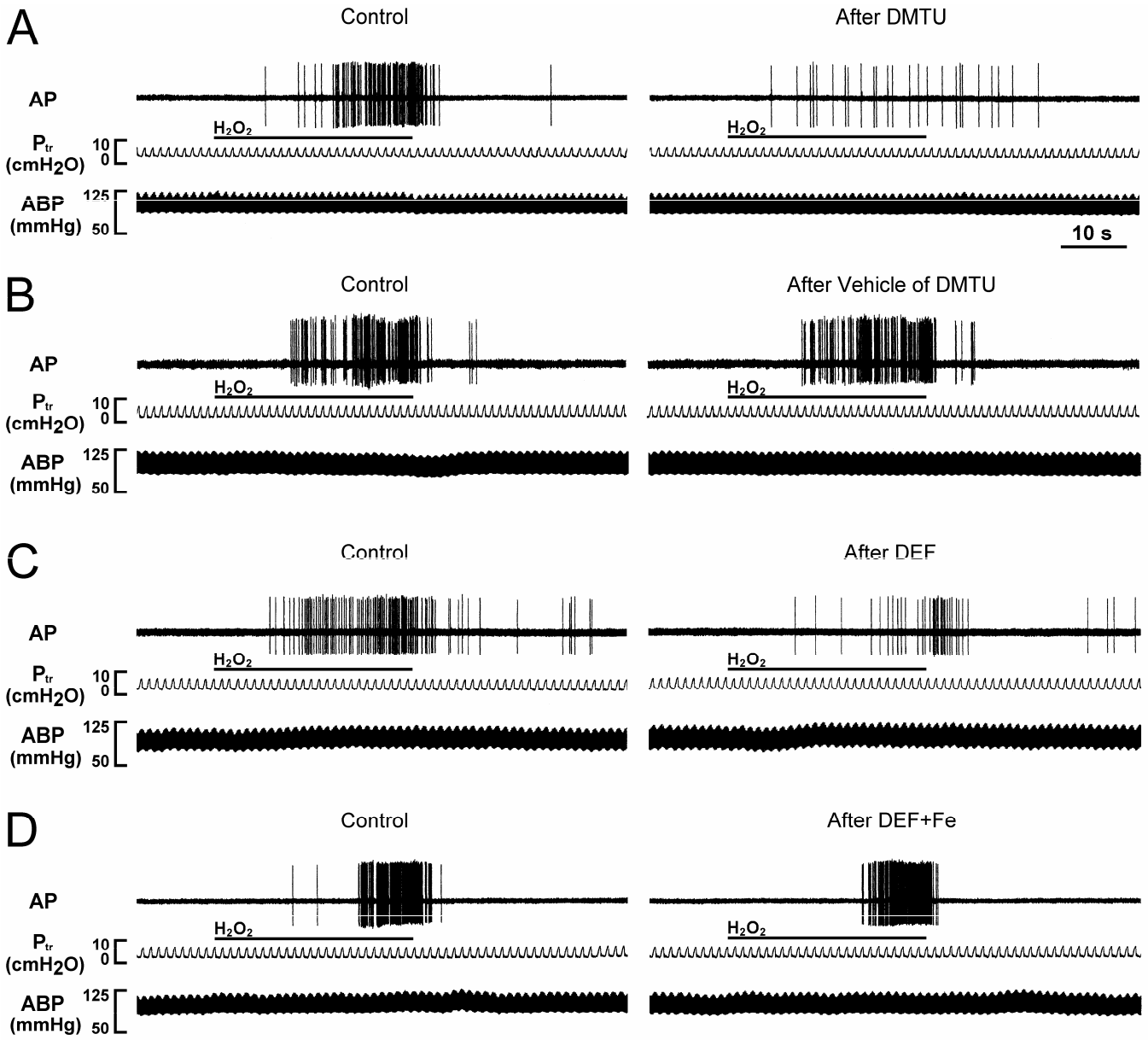

Figure 4. Responses of four rat vagal lung C fibers to 0.4 % aerosolized H_2O_2 before and after various antioxidant pretreatments. A-D: pretreatment with dimethylthiourea (DMTU; a scavenger for ·OH), vehicle of DMTU, deferoxamine (DEF; a iron-chelator) and iron-saturated DEF (DEF+Fe), respectively. Pretreatments were made 30 min prior to the subsequent challenge by slow injection of DMTU (1.5 g/kg), vehicle of DMTU, DEF (15 mg/kg) or iron-DEF+Fe (15 mg/kg) into the vein for 30 s. The duration of H_2O_2 challenge is indicated by horizontal bars. The elapsed time intervals between two H_2O_2 challenges were 60 min. AP, action potential; P_{tr}, tracheal pressure; ABP, arterial blood pressure (Result from reference 2).

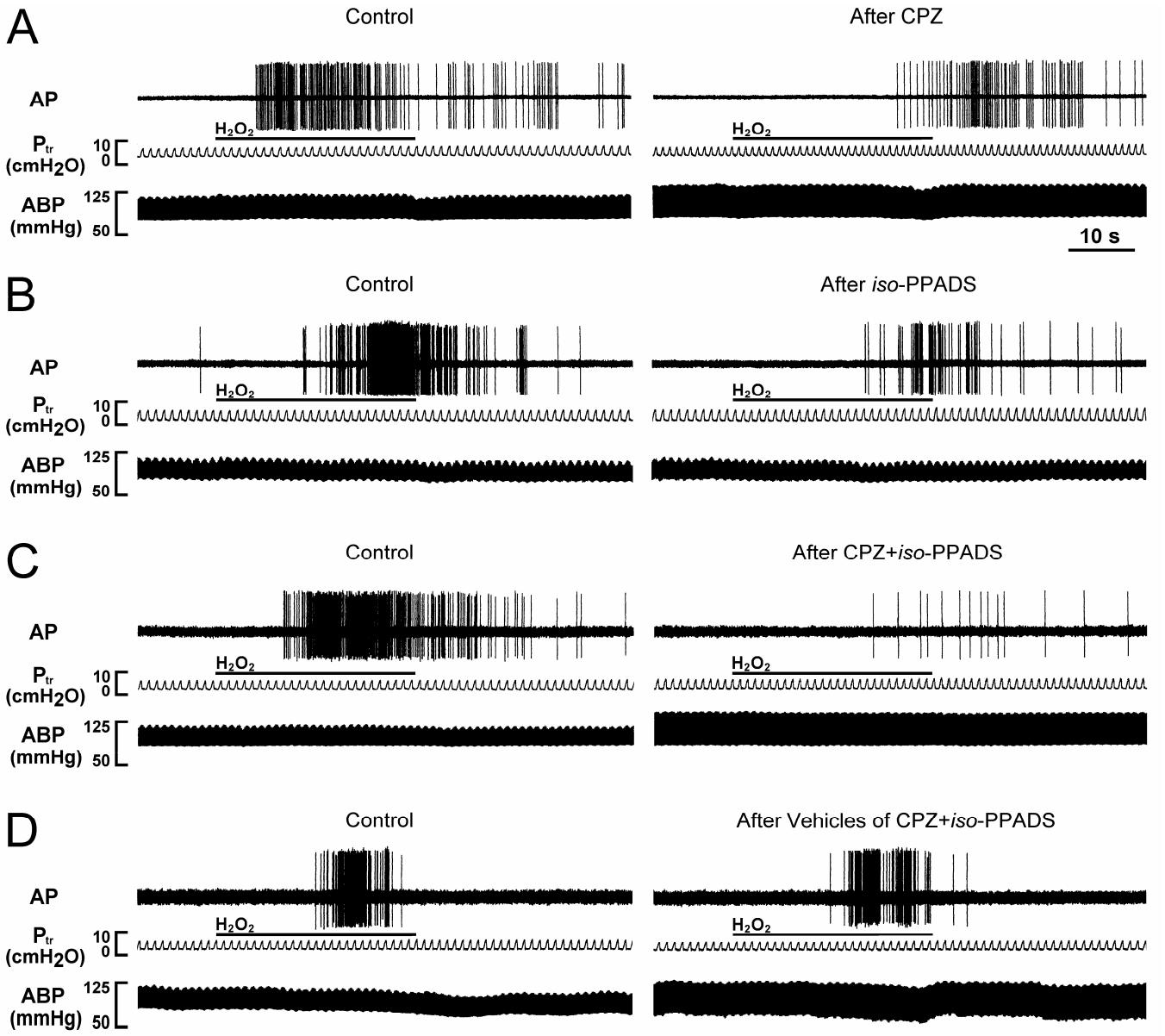

Figure 5. Responses of four rat vagal lung C fibers to 0.4 % aerosolized H_2O_2 before and after various antagonist pretreatments. A-D: pretreatment with capsazepine (CPZ; a TRPV1 receptor antagonist), *iso*-pyridoxalphosphate-6-azophenyl-2',5'-disulphonate (*iso*-PPADS; a P2X receptor antagonist), a combination of CPZ and *iso*-PPADS (CPZ+*iso*-PPADS), and vehicles of CPZ and *iso*-PPADS (vehicles of CPZ+*iso*-PPADS). The pretreatments were made 2, 2, 30 and 30 min prior to the subsequent challenge by slow injection of CPZ (3 mg/kg), vehicle of CPZ, *iso*-PPADS (20 mg/kg) and vehicle of *iso*-PPADS, respectively, into the vein for 30 s. The duration of H_2O_2 challenge is indicated by horizontal bars. The elapsed time intervals between two H_2O_2 challenges were 60 min. AP, action potential; P_{tr}, tracheal pressure; ABP, arterial blood pressure (Result from reference 2).

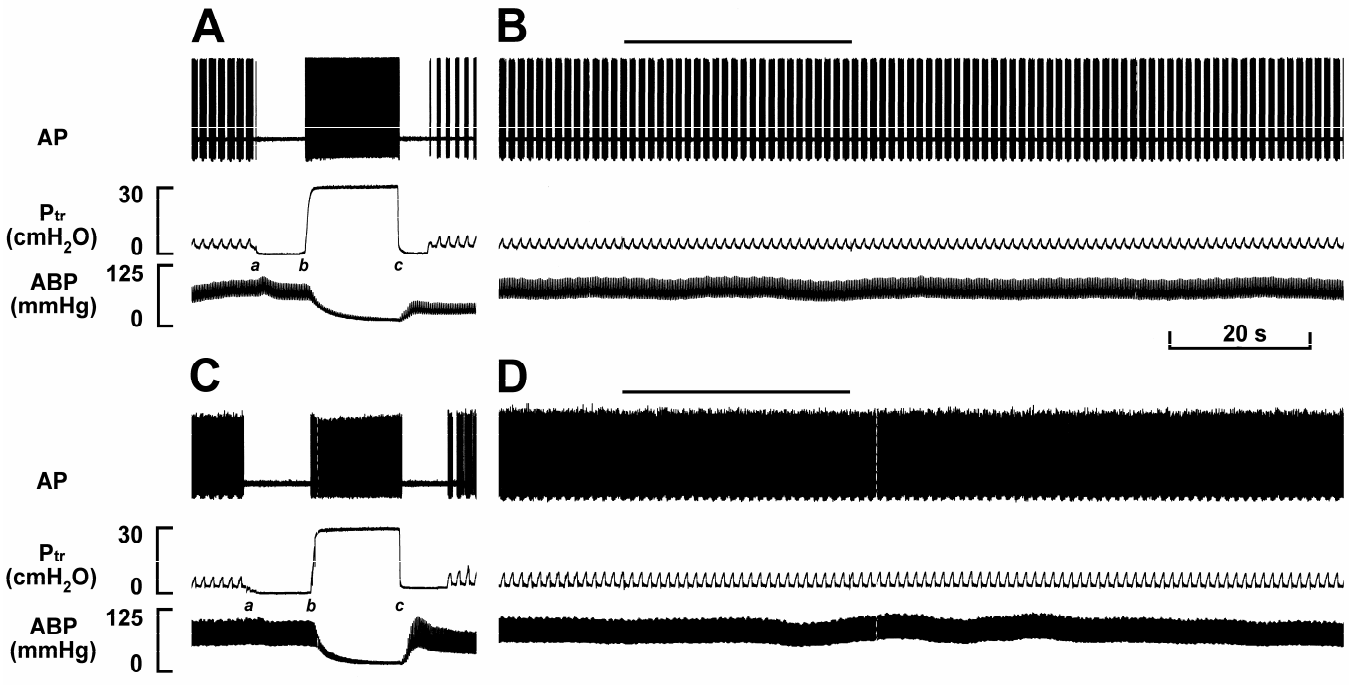

Figure 6. Afferent responses of a pulmonary phasic (A and B) and tonic (C and D) SARs to lung inflation and to aerosolized H_2O_2. A and C: lung was deflated to atmospheric pressure at *a* and *c*, and lung hyperinflated to 30 cmH_2O at *b*. B and D: 25 tidal breaths of aerosolized H_2O_2 (0.4 %) were delivered into lungs as indicated by horizontal bars. Ten minutes elapsed between lung inflation and H_2O_2 challenge. AP, action potential; P_{tr}, tracheal pressure; ABP, arterial blood pressure.

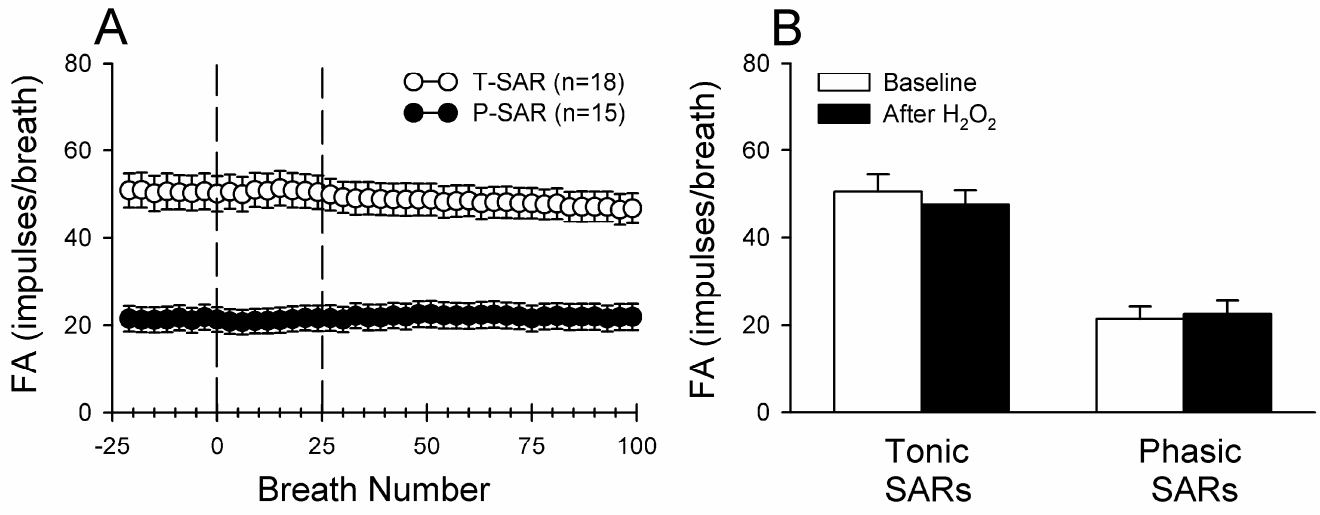

Figure 7. Average responses of pulmonary phasic (P-PSR) and tonic slowing adapting receptors (T-PSR) to aerosolized H_2O_2. A: aerosolized H_2O_2 (0.4 %) was delivered into lungs during the time period between two dashed lines. B: no significance could be detected between baseline activity and peak activity measured after H_2O_2 challenge. Data represent means ± SEM.

Possible Mechanisms of Stimulation of RARs by ROS

It is generally believed that chemicals stimulate RARs directly through the activation of their specific pharmacological receptors or indirectly through their effects on changes of lung mechanics. Therefore, one plausible notion that can easily proposed is that ROS act on "ROS receptors" to initiate the discharge of RARs or airway smooth muscle contraction. However, the notion would lack of support for the reason that no such "ROS receptors" have been postulated. The other plausible notion is that ROS stimulate release of chemical mediators (48-52), which subsequently activate RARs by activation pharmacological receptors located on their nerve endings or by change the bronchomotor tone. ROS may cause the release of chemical mediators, such as cyclooxygenase metabolites, ATP, histamine, serotnin, and bradykinin, into the extracellular space, which subsequently activate pharmacological receptors exist on the nerve endings of RARs (10, 29-38). There are five well-known pharmacological receptors is relevant to the chemical stimulation of RARs. Several electrophysiological and pharmacological studies have characterized TRPV1, P2X, H_1 histamine receptors, 5-HT_3 serotonin, and B_2 bradykinin receptors may locate on RARs membrane of nerve terminals (5, 34-41). Most of them are ligand-gated non-selective cation channels and can be activated by cyclooxygenase metabolites, ATP, histamine, serotnin, and bradykinin (1, 5, 34-41). Furthermore, these chemical mediators may cause bronchomotor tone change which subsequently activates RARs (10, 42). Since silent and phasic RARs have varied characteristics, ROS may possibly activiate these two subtypes of RARs by different mechanisms.

Existing Knowledge and Major Research Questions

Existing Knowledge

1. Various lung diseases produce excess pulmonary ROS.
2. RARs are important in detecting the onset of pulmonary pathological condition.
3. RARs are sensitive to both chemical and mechanical stimuli.
4. ROS may stimulate RARs.
5. There are various pharmacological receptors may on the RARs fiber terminals.
6. ROS may cause the release of verious chemical mediators, which possibly activate RARs via the direct action of mediators on the pharmacological receptors located at receptor terminals or indirectly through their effects on changes of lung mechanics.

Research questions

1. The mechanisms of sensory transduction of ROS by RARs are unknown.
2. The pharmacological receptors that participate in the sensory transduction of ROS by RARs are unclear.

3. The changes of lung mechanics involves in the sensory transduction of ROS by RARs remain to be investigated.
4. The differences in mechanisms of the sensory transduction of ROS by silent and phasic RARs remain to be explored.

Objectives

Using an *in vivo* rat model, the objectives of this study are:

1. To study the dose-response relationship of silent and phasic RARs response to H_2O_2.
2. To investigate the ROS mechanisms underlying H_2O_2-induced stimulation of RARs.
3. To test the involvements of pharmacological receptors including TRPV1, P2X, B_2 bradykinin, H_1 histamine, and 5-HT_3 serotonin receptors in the stimulation of RARs by ROS.
4. To determine whether changes of bronchomotor tone are part of the mechanisms underlying the stimulation of RARs by ROS.

To accomplish these objectives, aerosolized H_2O_2 will be delivered into the lower airway of anesthetized rats. The single fiber recording technique will be performed to measure the afferent responses of silent and phasic RARs to H_2O_2. Studies will be repeated after pretreatment with various antioxidant, various receptor antagonists, or bronchodilators.

Methods

Animal preparations

Male adult Sprague-Dawley rats (weight 400-450 g) will be anesthetized with intraperitoneal injection of chloralose (100 mg/kg) and urethane (500 mg/kg) dissolved in a borax solution (2 %). The femoral artery will be cannulated for recording arterial blood pressure. A polyethylene catheter (PE-50) will be inserted in the jugular vein with the tip placed close to the right atrium for intravenous (i.v.) administration of pharmacological agents. During the course of the experiments, supplemental doses of α-chloralose (20 mg/kg/hr, i.v.) and urethane (100 mg/kg/hr, i.v.) will be administered to maintain the abolition of pain reflexes induced by pinching the animal's tail. Body temperature will be maintained at 37 °C throughout the experiment by means of a servo-controlled heating blanket. At the end of the experiment, the animal will be killed by overdose KCl injection.

Assessment of respiratory signals

The animal's neck will be opened along the midline. A short tracheal cannula (PE-260) will be inserted just below the larynx via a tracheostomy and connected to a pneumotachograph (Fleisch, 4/0). A mid-line thoracotomy will be performed, and the edges of the rib cage were retracted. The lungs will then be ventilated using a rodent respirator (Harvard, 683) at a constant tidal volume of 9 ml/kg. The frequency of the respirator will be set at 50

breaths/min and kept constant throughout the experiment. The opening of the thorax will be covered by a sheet of polyethylene film to keep the lung moist and the expiratory outlet of the respirator will be placed under 3-4 cm of water to maintain a near-normal functional residual capacity. After a midline thoracotomy, both vagus nerves will be ligated just above the diaphragm to eliminate afferent signals arising from lower visceral organs. During the recording of vagal action potentials, the rats will be paralyzed with pancuronium bromide (0.5 mg/kg, i.v.). Periodically, the effect of pancuronium was allowed to wear off so that the depth of anesthesia could be checked. Respiratory flow will be measured with the pneumotachograph coupled with a differential pressure transducer (Validyne, MP45-14). The flow signal will be integrated to give tidal volume. Tracheal pressure (P_{tr}; transpulmonary pressure in an open-chest preparation) will be monitored by another differential pressure transducer (Validyne, MP45-28) via a side tap of the tracheal cannula. Total lung resistance (R_L) and dynamic lung compliance (C_{dyn}) will be determined using the subtraction method (53). All physiological signals will be recorded on a chart recorder (Gould, TA11) and a tape recorder (Neurocorder, DR-890) for later analysis.

Recording of afferent activity of RARs

Afferent activity arising from RARs will be recorded using conventional techniques. Briefly, a fine afferent filament will be split from the desheathed nerve trunk of the right vagus and placed on a platinum-iridium recording electrode. Action potentials will be amplified (P511K, Grass), monitored by an audio monitor (AM8, Grass), and displayed on an oscilloscope (model 420, Gould). The fine nerve filament will be subdivided until activity from only 1 unit is obtained. RARs are identified initially by their distinct phasic discharge synchronous with the respirator cycles, then by the hyperinflation of the lung (3-4 $\times$ V_T) or by lung deflation (P_{tr} = 0 cmH_2O, 10 sec). The single units of these receptors are further classified by their adaptation indexes (AIs) in response to constant-pressure lung inflation (P_{tr} = 30 cmH_2O, 10 sec). Also, the conduction velocity of these receptors will be measured by the method described previously (54). Two criteria will be used to identify the RARs in this study: (1) an AI to maintained lung inflation is >80% (55), and (2) conduction velocity is within the range of myelinated fibres. RARs will be further divided into two subgroups: phasic and silent; the latter has no or irregular baseline discharge. Prior to the end of each experiment, the general locations of the receptors studied will be identified within the lung structure by gently probing the tissues with a polyethylene rod (diameter = 2 mm).

Generation and delivery of aerosolized H_2O_2

An H_2O_2 solution (0.2 % or 0.4 %) will be prepared just prior to each generation by mixing 35% H_2O_2 (Shimakyu) with phosphate buffer solution (PBS) to the desired concentration with an adjusted pH value of 7.4. H_2O_2 aerosol wiil be generated by an active ultrasonic nebulizer (DeVilbiss, ULTRA-NEB 99) containing the H_2O_2 solution. The particle sizes of the aerosol generated by this nebulizer ranged from 0.5 to 5 μm. The air delivered by the respirator is then directed into a nebulizer cup containing no solution, PBS or an H_2O_2

solution, as controlled by turning a 3-way stopcock **(Figure 8)**. These two cups are sealed to prevent any leakageof air. The outlets to these two cups merged into one piece of tubing (i.d. = 8 mm) via another 3-way stopcock, which is connected to the distal end of the pneumotachograph **(Figure 8)**. Airway exposure to aerosolized H_2O_2 will be achieved by adjusting these two 3-way stopcocks for a 30-s period. Using a dye tracer, the time lag between the onset of challenge and the arrival of the aerosolized tracer in the airways is estimated to be 1–2 s. This estimation is based upon post-mortem checks of the presence of the dye tracer in the airways in 10 animals whose tracheal tubeswere quickly disconnected fromthe circuit delivering aerosolized dye tracer 1-2 s after the onset of challenge.

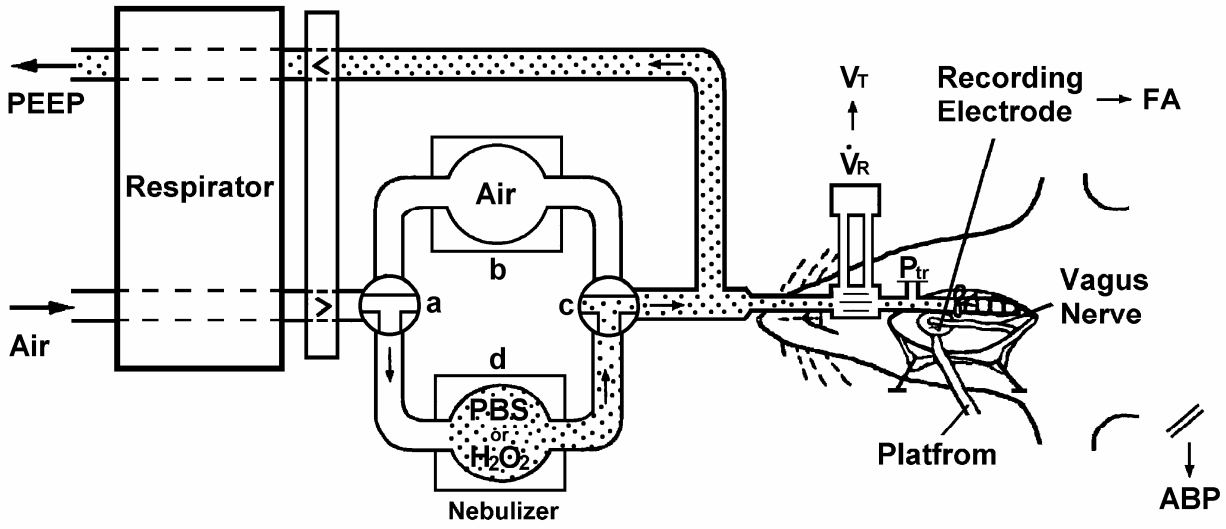

Figure 8. Schematic illustration showing the experimental setup. During the control period, the respirator delivered room air to the lungs (via a–b–c). To initial the challenge, two 3-way stopcocks were turned quickly during the expiratory phase, so that aerosolized PBS or H_2O_2 was delivered to the lungs (via a–d–c). To record fibre activity, a fine afferent filament was split from the de-sheathed right nerve trunk lying on a platform and placed on a recording electrode. $\dot{V}_R$, respiratory flow; V_T, tidal volume; P_{tr}, tracheal pressure; ABP, arterial blood pressure; FA, fiber activity; PEEP, positive end-expiratory pressure.

Pharmacological Agents

The following pharmacological agents in the table 1 will be used in this proposed study. The table describes the drug names, drug functions, their vehicle, and the references cited. These drugs can be classified into 4 groups for different purposes:

(1) Drugs to stimulate RARs: H_2O_2
H_2O_2 will be the source of ROS. Afferent responses to the challenge of 0 % (PBS), 0.2 %, and 0.4 % H_2O_2 will be studied.

(2) Drugs to study the role of H_2O_2 and ·OH: catalase, dimethylthiourea, and deferoxamine
Catalase and dimethylthiourea are scavengers for H_2O_2 and ·OH, respectively. Deferoxamine is a iron-chelator that prevents the formation of ·OH.

(3) Drugs to study the role of TRPV1, P2X, B_2 bradykinin, H_1 histamine, and 5-HT_3 serotonin receptors: capsazepine, *iso*-PPADS, HOE 140, pyrilamine, and tropisetron

Capsazepine, *iso*-PPADS, HOE 140, pyrilamine, and tropisetron are selective TRPV1, P2X, B_2, H_1 and 5-HT_3 receptor antagonists, respectively.

(4) Drugs to study the effects of bronchoconstriction: salbutamol

Salbutamol is a selective β_2 adrenergic receptor agonist and a potent bronchodilator.

Experimental Protocols

For conduct 4 series of experiments, a total of 240 animals will be evenly divided into 15 groups, which will be further subdivided into 30 subgroups with each 8 rats. Half of the subgroups are for the recording of silent RARs (groups 1-15), while the other half are for the recording of phasic RARs (groups 16-30). In the **Study 1** for investigating the dose-response relationship **(2 groups)**, silent and phasic RARs response to a challenge of PBS, 0.2 % or 0.4 % H_2O_2 will be studied. In the **Study 2** for assessing the role of H_2O_2 and ·OH **(12 groups)**, silent and phasic RARs response to 0.4 % H_2O_2 will be studied before and after pretreatment with catalase, heat-inactivated catalase, dimethylthiourea, vehicle of dimethylthiourea, deferoxamine, or iron-saturated deferoxamine. In the **Study 3** for assessing the role of TRPV1, P2X, B_2 bradykinin, H_1 histamine, and 5-HT_3 serotonin receptors **(12 groups)**, silent and phasic RARs response to 0.4 % H_2O_2 will be studied before and after pretreatment with capsazepine, *iso*-PPADS, HOE 140, pyrilamine, tropisetron , or vehicles. In the **Study 4** for assessing the effects of bronchoconstriction **(4 groups)**, silent and phasic RARs response to 0.4 % H_2O_2 will be studied before and after pretreatment with salbutamol or saline vehicle.

Data Analysis and Statistics

RARs neural activity and arterial blood pressure will be measured at 1-s intervals or on a breath-by-breath basis. An interval of at least 2 min before and 5 min after the challenge will be measured. Baseline data for each parameter will be calculated as the mean over the reading 2 min immediately before the challenge. The peak response will be defined as the value averaged over 3 breaths or over 3 seconds after the challenge. These physiological parameters will be analyzed using a computer equipped with an A/D converter (Gould, DASA 4600) and software (BioCybernatics, 1.0; Taipei, Taiwan). Results obtained from the computer analysis will be routinely checked with those obtained by manual calculations for accuracy. Data will be compared by paired *t*-test or two-way repeated-measures ANOVA followed by Fisher's least significant difference procedure when appropriate. A value of $P < 0.05$ was considered significant. All data are presented as the mean ± SEM.

Table 1. Pharmacological agents used in this proposed study

Drug	Drug function	Dose (nerve recording)	Vehicle for drug	Ref. #
H_2O_2	a ROS	0.4 %, aerosol, 30 s	PBS	2
catalase	a scavenger for H_2O_2	750,000 units/ml, aerosol, 5 min	PBS	2
dimethylthiourea	a scavenger for ·OH	i.v. 1.5 g/kg	saline	2
deferoxamine	a iron-chelator	i.v. 250 mg/kg	saline	2
capsazepine	a TRPV1 receptor antagonist	i.v. 3 mg/kg	8 % DMSO, 9 % ethanol , 9 % Tween 80, 74 % saline	2
iso-PPADS	a P2X purinoceptor antagonist	i.v. 20 mg/kg	saline	2
HOE 140	a B_2 bradykinin receptor antagonist	i.v. 200 μg/kg	saline	56
pyrilamine	a H_1 histamine receptor antagonist	i.v. 5 mg/kg	saline	57, 58
tropisetron	a 5-HT_3 receptor antagonist	i.v. 15 μg/kg	saline	5
salbutamol	a β_2 adrenergic receptor agonist	i.v. 0.5 mg/kg	saline	5

Definition of abbreviations:

TRPV1 receptor = transient receptor potential vanilloid 1 receptors

DMSO = dimethyl sulfoxide

iso-PPADS = iso-pyridoxalphosphate-6-azophenyl-2',5'-disulphonate

HOE 140 = D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin

Results

We found the afferent responses of a silent RAR to lung deflation, lung inflation, and to inhalation of aerosolized H_2O_2 (0.4 %, for 30 s) before and after pretreatment with catalase (a scavenger for H_2O_2) in anesthetized, open-chest rat (**Figure 9**). As shown, this receptor was activated by lung deflation and also by lung inflation (Panels A and D). However, the discharge evoked by lung deflation persisted thoughout the period, whereas the discharge evoked by lung inflation adopted rapidly. These characteristics of the responses indicated that the receptor is typically a RAR. This RAR was also vigrously activated by aerosolized H_2O_2 (Panel B) and the stimulation was totally prevnted by catalase (Panel C). **Figure 10** shows the group responses of the silent and phasic RARs to inhalation of aerosolized H_2O_2. As shown, discharge patterns of silent and phasic RARs after H_2O_2 challenges are totally different. The response of silent RARs to H_2O_2 appears to be immediately, whereas the response of phasic RARs to H_2O_2 appears to be after a delay. **Figure 11** and **figure 12** shows average responses of silent and phasic RARs to inhalation of aerosolized H_2O_2 before and after pretreatment with catalase. As shown, catalase completely abolished afferent responses of silent and phasic RARs to H_2O_2 challenge. Our prelminary results suggest that both silent and phasic RARs may function as an important afferent system during pulmonary insult by ROS. The results also suggest that the transduction mechanisms of silent and phasic RARs in detecting pulmonary ROS are through the different pathways. The results also indicate the feasibility of this proposed study.

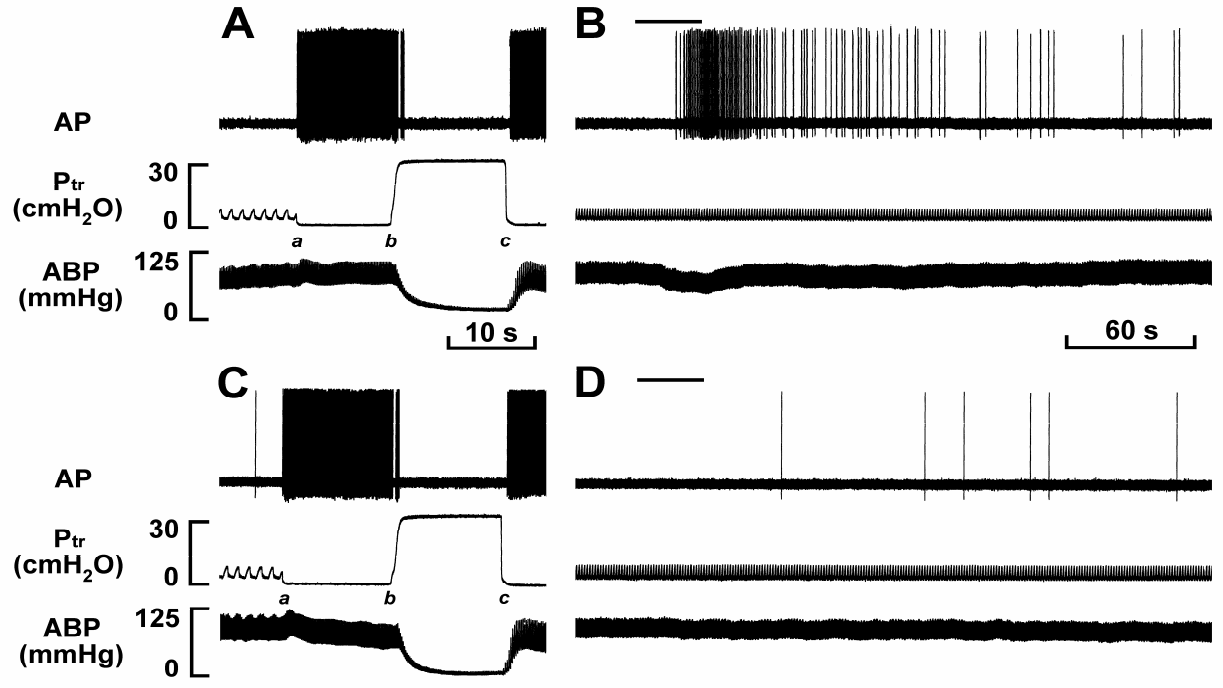

Figure 9. Afferent responses of a silent RARs to lung inflation and to aerosolized H_2O_2 before (A and B) and after pretreatment with catalase (C and D). A and C: lung was deflated to atmospheric pressure at *a* and *c*, and lung hyperinflated to 30 cmH_2O at *b*. B and D: 25 tidal breaths of aerosolized H_2O_2 (0.4 %) were delivered into lungs as indicated by horizontal bars. Sequence of interventions was A→B→D→C. Ten minutes elapsed between lung inflation and H_2O_2 challenge, and 60 minutes elapsed between two H_2O_2 challenges. AP, action potential; Ptr, tracheal pressure; ABP, arterial blood pressure. Note that time scales of A and C differ from those of B and D.

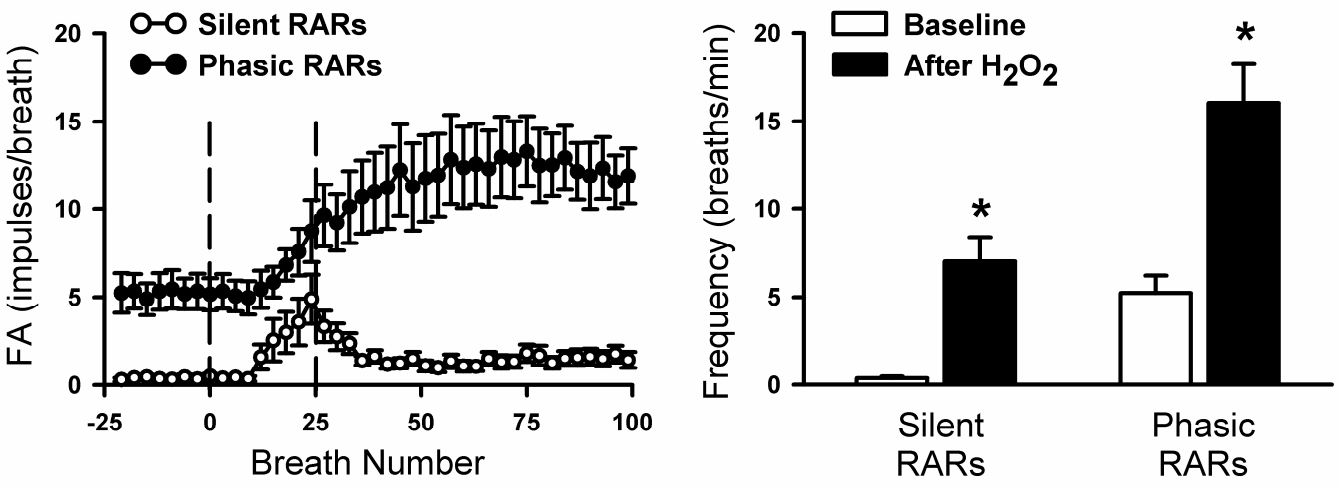

Figure 10. Average responses of silent RARs (n=7) and phasic RARs (n=6) to aerosolized H_2O_2. A: aerosolized H_2O_2 (0.4 %) was delivered into lungs during the time period between two dashed lines (30 s). B: *, significantly different from baseline activity. Data represent means ± SEM.

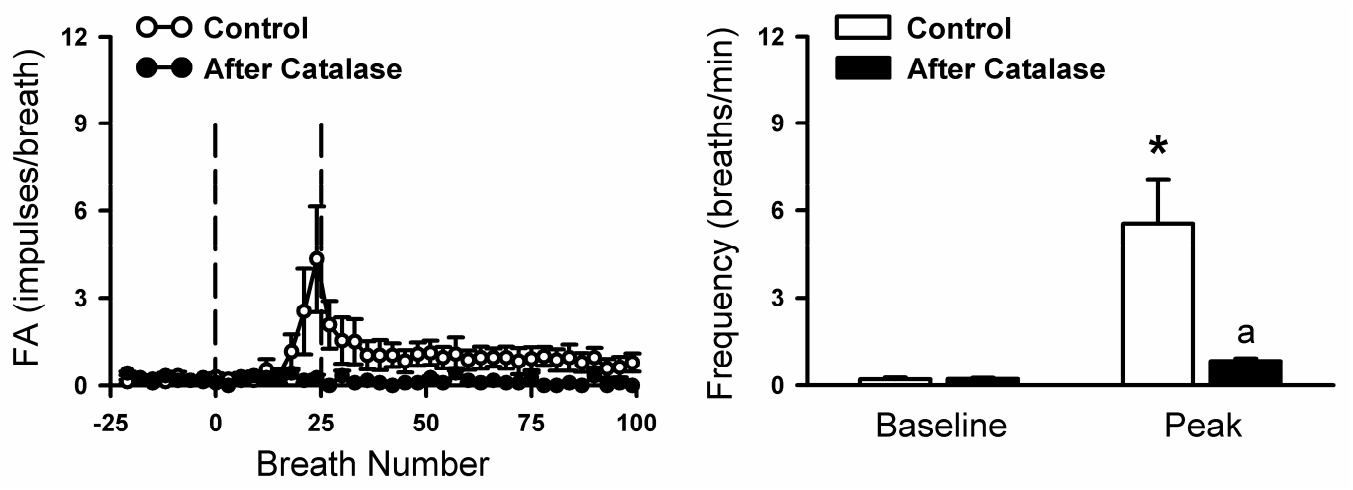

Figure 11. Average responses of silent RARs to aerosolized H_2O_2 (0.4 %) before and after catalase. A: aerosolized H_2O_2 was delivered into lungs during the time period between two dashed lines (30 s). B: *, significantly different from baseline activity; a, significantly different from response before catalase. Data are means ± SE of 4 silent RARs recorded from 4 rats.

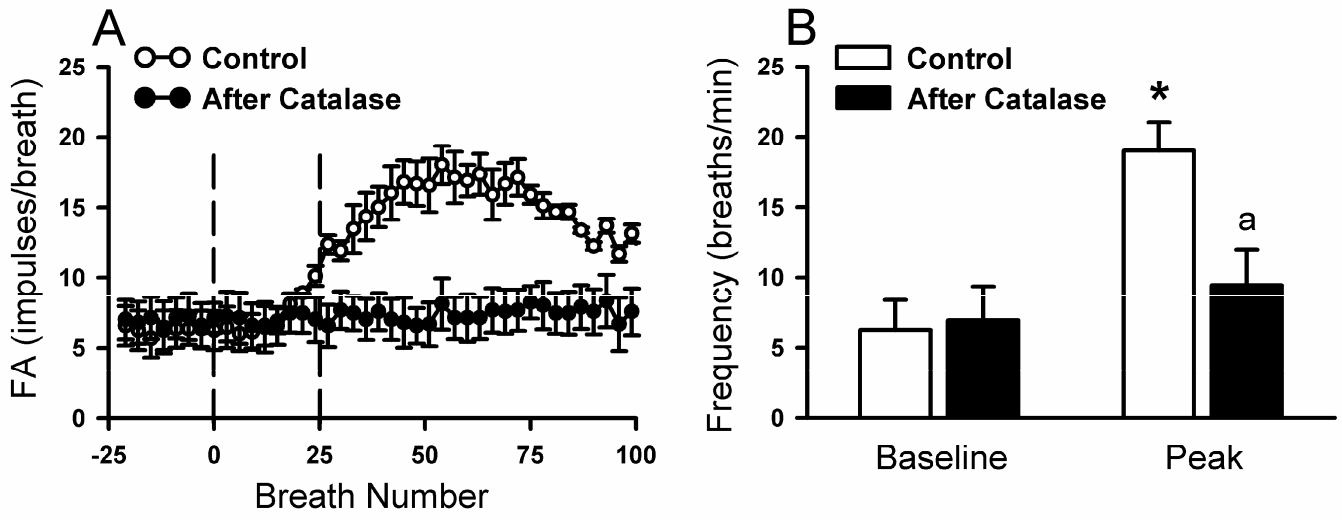

Figure 12. Average responses of phasic RARs to aerosolized H_2O_2 (0.4 %) before and after catalase. A: aerosolized H_2O_2 was delivered into lungs during the time period between two dashed lines (30 s). B: *, significantly different from baseline activity; a, significantly different from response before catalase. Data are means ± SE of 3 phasic RARs recorded from 3 rats.

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