

# Sensory transduction of pulmonary reactive oxygen species by capsaicin-sensitive vagal lung afferent fibres in rats

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The mechanisms of sensory transduction of pulmonary reactive oxygen species (ROS) by capsaicin-sensitive vagal lung afferent fibres are unclear. To investigate the role of transient receptor potential vanilloid 1 (TRPV1) receptors and P2X purinoceptors in this sensory transduction, we recorded fibre activity (FA) from 132 fibres of this type in 132 anaesthetized and ventilated rats. Airway challenge of aerosolized H<sub>2</sub>O<sub>2</sub> (0, 0.2 and 0.4%) produced a concentration-dependant fibre stimulation. The fibre responses to 0.4% H<sub>2</sub>O<sub>2</sub> were attenuated by dimethylthiourea (a hydroxyl radical ( $\cdot$ OH) scavenger; change in fibre activity ( $\Delta$ FA),  $-55 \pm 9\%$ ) or deferoxamine (an iron-chelator that prevents formation of  $\cdot$ OH;  $\Delta$ FA,  $-59 \pm 9\%$ ), were prevented by catalase (an enzyme catalysing H<sub>2</sub>O<sub>2</sub>;  $\Delta$ FA,  $-96 \pm 3\%$ ) and were unaffected by the vehicle for dimethylthiourea, iron-saturated deferoxamine or heat-inactivated catalase. The fibre responses to 0.4% H<sub>2</sub>O<sub>2</sub> were attenuated by capsazepine (a TRPV1 receptor antagonist;  $\Delta$ FA,  $-39 \pm 9\%$ ) or *iso*-pyridoxalphosphate-6-azophenyl-2',5'-disulphonate (*iso*-PPADS, a P2X receptor antagonist;  $\Delta$ FA,  $-51 \pm 9\%$ ), were further reduced by capsazepine and *iso*-PPADS in combination ( $\Delta$ FA,  $-70 \pm 13\%$ ), and were unaltered by their vehicles. The fibre responses to cigarette smoke (20 ml), an irritant that generates ROS, were attenuated by dimethylthiourea ( $\Delta$ FA,  $-61 \pm 9\%$ ) or capsazepine and *iso*-PPADS in combination ( $\Delta$ FA,  $-67 \pm 9\%$ ). These results suggest that both the TRPV1 and P2X receptors mediate the sensory transduction of ROS, especially H<sub>2</sub>O<sub>2</sub> and  $\cdot$ OH, by capsaicin-sensitive vagal lung afferent fibres.

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Lung pathological conditions such as endotoxin shock (Minamiya *et al.* 1995), vascular microembolism (Wang *et al.* 1992) and inhalation of oxidant irritants such as toxic or cigarette smoke (Pryor, 1992), cause increased pulmonary production of reactive oxygen species (ROS). The major ROS are the superoxide anion radical, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radical ( $\cdot$ OH) (Comhair & Erzurum, 2002). The superoxide anion radical dismutates to form H<sub>2</sub>O<sub>2</sub>, which in the presence of iron can further react to form  $\cdot$ OH via the Fenton reaction (Comhair & Erzurum, 2002). Several animal studies have shown that airway reflexes (Lee, 1990; Kou *et al.* 1997; Lin & Kou, 1997; Chen & Kou, 2000; Ho & Kou, 2000) and lung afferent responses (Chen *et al.* 1997; Lai & Kou, 1998*a,b*; Lai *et al.* 2005) evoked under these pathological conditions are abolished or attenuated by antioxidant treatments. These observations lead to the notion that ROS are part of a signalling cascade leading to stimulation of lung afferent fibres (Lee & Pisarri, 2001). The findings (Soukhova *et al.*

1999; Ruan *et al.* 2003) that exogenous H<sub>2</sub>O<sub>2</sub> challenge evokes airway reflexes, which are mediated through lung vagal afferents, support this notion. However, there is no direct electrophysiological evidence demonstrating the stimulatory effect of ROS on lung vagal afferent fibres, and the mechanisms underlying this sensory transduction remain to be explored.

Capsaicin, a pungent active ingredient of hot pepper, mainly stimulates lung vagal afferent C fibres and some A- $\delta$  fibres, which are important to the regulation of respiratory and cardiovascular functions under both normal and pathophysiological conditions (Coleridge & Coleridge, 1986; Lee & Pisarri, 2001; Carr & Udem, 2003). These capsaicin-sensitive vagal lung afferent fibres are sensitive to a variety of chemicals or irritants and are considered as nociceptive-like free nerve endings (Coleridge & Coleridge, 1986; Lee & Pisarri, 2001; Carr & Udem, 2003). Investigations of afferent responses of these vagal fibres to certain agonists reveal that

various pharmacological receptors may be present at the membrane of nerve terminals (Udem & Carr, 2001). For example, studies using whole animals (Lee & Lundberg, 1994; Pelleg & Hurt, 1996; Udem & Carr, 2001) or *ex vivo* airway and/or lung preparations (Fox *et al.* 1995; Udem & Carr, 2001; Kollarik & Udem, 2002; Carr *et al.* 2003; Udem *et al.* 2004) suggested that the transient receptor potential vanilloid 1 (TRPV1) receptors and the P2X purinoceptors play a role in sensory transduction functions of these afferent fibres. Indeed, *in vitro* electrophysiological and pharmacological studies have characterized TRPV1 and P2X receptors located on the membrane of jugular or nodose vagal neurones (Khakh *et al.* 1995; Vulchanova *et al.* 1997; Szallasi & Blumberg, 1999; Dunn *et al.* 2001; Gu *et al.* 2003; Ichikawa & Sugimoto, 2003; Udem *et al.* 2004). The importance of TRPV1 and P2X receptors to pain sensation has also been well documented in somatosensory nociceptors (McCleskey & Gold, 1999). It has been demonstrated that ROS can activate visceral afferent C fibres (Stahl *et al.* 1993; Ustinova & Schultz, 1994*a,b*; Huang *et al.* 1995). Previous investigations in rats suggested that the activation of vagal cardiac C fibres by ROS is mediated through the TRPV1 receptors (Schultz & Ustinova, 1998) and that ROS activate purinergic P2 receptors in aortic smooth muscle cells (Shen *et al.* 2000). Additionally, ROS may damage cells and cause a rapid release of cytosolic ATP, which activates the P2X receptors resulting in the stimulation of pain nociceptors (Cook & McCleskey, 2002). However, the role of TRPV1 and P2X receptors in the sensory transduction of ROS by capsaicin-sensitive vagal lung afferent fibres is still unclear.

Based upon the abovementioned existing knowledge, we hypothesized that both TRPV1 and P2X receptors located at fibre terminals mediate sensory transduction of ROS by capsaicin-sensitive vagal lung afferent fibres. To test this hypothesis, the present study was undertaken in anaesthetized rats to investigate: (1) the stimulatory effects of airway challenge of aerosolized H<sub>2</sub>O<sub>2</sub> on these afferent fibres; (2) ROS mechanisms underlying H<sub>2</sub>O<sub>2</sub>-induced stimulation of these afferent fibres using various antioxidant pretreatments; (3) the involvements of TRPV1 and P2X receptors in H<sub>2</sub>O<sub>2</sub>-induced stimulation of these afferent fibres using selective receptor antagonist

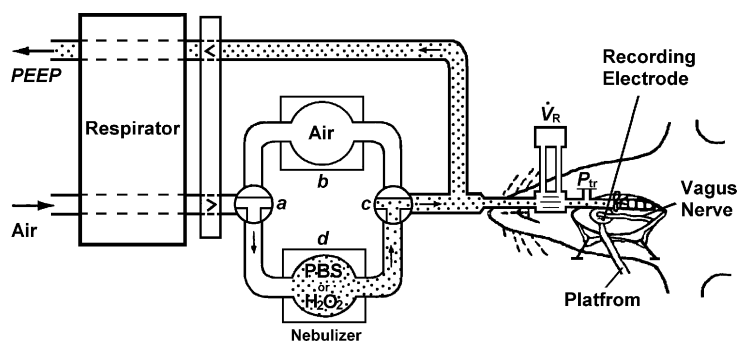
pretreatments; and (4) the importance of TRPV1 and P2X receptors in the afferent responses of these fibres to airway challenge of cigarette smoke, an irritant that is known to generate ROS (Pryor, 1992).

## Methods

### Animal preparation

All protocols were in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health, USA and were approved by the Institutional Animal Care and Use Committee of the National Yang-Ming University, Taiwan. Male Sprague-Dawley rats were anaesthetized with an intraperitoneal injection of  $\alpha$ -chloralose (100 mg kg<sup>-1</sup>; Sigma Chemical Co., St Louis, MO, USA) and urethane (500 mg kg<sup>-1</sup>; Sigma) dissolved in a borax solution (2%; Sigma). A polyethylene catheter was inserted into the jugular vein and advanced until the tip was close to the right atrium for intravenous administration of pharmacological agents. The right femoral artery was cannulated to measure arterial blood pressure. During the course of the experiments, supplemental doses of  $\alpha$ -chloralose (20 mg kg<sup>-1</sup> h<sup>-1</sup>) and urethane (100 mg kg<sup>-1</sup> h<sup>-1</sup>) were administered to maintain the abolition of pain reflexes induced by pinching the animal's tail. During the recording of vagal action potentials, the rats were paralysed with pancuronium bromide (0.5 mg kg<sup>-1</sup>, intravenous; Orgnon Teknika, Boxtel, Holland). Periodically, the effect of pancuronium was allowed to wear off so that the depth of anaesthesia could be checked. Body temperature was maintained at 37°C throughout the experiment by means of a servo-controlled heating blanket. At the end of the experiment, animals were killed by intravenous injection of overdose of the anaesthetics.

Methodologies for other animal preparations and measurements of physiological parameters used in this study have been previously described (Lai & Kou, 1998*a*; Lai *et al.* 2005). In brief, the trachea was cannulated and a midline thoracotomy was performed. The lungs were ventilated at a constant tidal volume of 9 ml kg<sup>-1</sup> and a constant frequency of 50 breaths min<sup>-1</sup> (Fig. 1).



**Figure 1. Schematic illustration showing the experimental setup**

During the control period, the respirator delivered room air to the lungs (via *a–b–c*). To initiate the challenge, two 3-way stopcocks were turned quickly during the expiratory phase, so that aerosolized PBS or H<sub>2</sub>O<sub>2</sub> 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;  $P_r$ , tracheal pressure; PEEP, positive end expiratory pressure.

Arterial blood pressure, respiratory flow, tidal volume ( $V_T$ ) and tracheal pressure ( $P_{tr}$ ; transpulmonary pressure in an open-chest preparation) were monitored. Total lung resistance ( $R_L$ ) and dynamic lung compliance ( $C_{dyn}$ ) were determined using the subtraction method (Mead & Whittenberger, 1953).

### Recording of afferent activity

Afferent activity arising from capsaicin-sensitive vagal lung afferent fibres was recorded using techniques described elsewhere (Lai & Kou, 1998a; Lai *et al.* 2005). Briefly, a fine afferent filament of the right vagus nerve was split from the de-sheathed right nerve trunk lying on a platform and placed on a platinum-iridium unipolar recording electrode to record afferent nerve activity (Fig. 1). To search for these afferent fibres, the lungs were hyperinflated in a step-like manner to four or five times  $V_T$ . Capsaicin-sensitive vagal lung afferent fibres are activated by lung hyperinflation to a high volume (e.g. 3 or 4  $\times V_T$ ) (Lee & Pisarri, 2001; Carr & Undem, 2003). Once the presence of a suspected single unit was detected, capsaicin (0.75 g kg<sup>-1</sup>; Sigma) was injected as a bolus into the vein. Only afferent fibres that showed stimulation within 2 s after the injection were studied. The recording of a single fibre was confirmed by matching spike templates. Prior to the end of each experiment, the general locations of the receptors studied were identified within the lung structure by gently probing the tissues with a polyethylene rod (diameter, 2 mm). The conduction velocities of these afferent fibres were not measured due to the limitation of the technique with respect to the experimental set-up (Fig. 1).

### Generation and delivery of aerosolized H<sub>2</sub>O<sub>2</sub>

Various concentrations (0% (PBS), 0.2% and 0.4%) of a H<sub>2</sub>O<sub>2</sub> solution were prepared just prior to each set of experiments by mixing 35% H<sub>2</sub>O<sub>2</sub> (Shimakyu, Osaka, Japan) with PBS to the desired concentration with the pH adjusted to 7.4. H<sub>2</sub>O<sub>2</sub> aerosol was generated by an active ultrasonic nebulizer (ULTRA-NEB 99, DeVilbiss) containing the H<sub>2</sub>O<sub>2</sub> solution. The particle sizes of the aerosol generated by this nebulizer ranged from 0.5 to 5  $\mu$ m. The air delivered by the respirator was then directed into a nebulizer cup containing no solution, PBS or an H<sub>2</sub>O<sub>2</sub> solution, as controlled by turning a 3-way stopcock (Fig. 1). These two cups were well sealed to prevent any leakage of air. The outlets to these two cups merged into one piece of tubing (i.d., 8 mm) via another 3-way stopcock, which was connected to the distal end of the trachea cannula (Fig. 1). Airway exposure to aerosolized H<sub>2</sub>O<sub>2</sub> was 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 was estimated to be 1–2 s. This estimation was based upon post-mortem checks of the presence of the dye tracer in the airways in 10 animals whose tracheal tubes were quickly disconnected from the circuit delivering aerosolized dye tracer 1 s after the onset of challenge.

### Generation and delivery of cigarette smoke

Cigarettes (Long Life, Taiwan Tobacco and Liquor Production, Taipei, Taiwan) without filters were combusted with a syringe-driven apparatus. A puff of smoke (20 ml) was drawn into a syringe at a constant rate of 2 ml s<sup>-1</sup>, injected into a delivery circuit, as previously described (Lee *et al.* 1989), and delivered directly into the lungs via the inspiratory line during five to six ventilatory cycles (~6–7 s).

### Administration of antioxidant

Catalase (CAT; Sigma) was dissolved in PBS to a concentration of 750 000 IU ml<sup>-1</sup>. Heat-inactivated catalase (HICAT) was prepared by heating the CAT solution to 100°C for 15 min. Aerosol of CAT or HICAT were generated and delivered into the lower airways for a period of 5 min using the nebulizer and the circuit for delivery of the H<sub>2</sub>O<sub>2</sub> aerosol. Both dimethylthiourea (DMTU; Sigma) and deferoxamine (DEF; Sigma) were dissolved in saline. Iron-saturated DEF (DEF + Fe) was prepared by adding 98 mg FeCl<sub>3</sub>·6H<sub>2</sub>O (Fluka) to 1 ml DEF (250 mg ml<sup>-1</sup>) for 1 h at room temperature. DMTU (1.5 g kg<sup>-1</sup>), saline, DEF (15 mg kg<sup>-1</sup>) or DEF + Fe was slowly injected into the vein for 30 s. CAT is an enzyme that catalyses the breakdown of H<sub>2</sub>O<sub>2</sub> into O<sub>2</sub> and H<sub>2</sub>O (Comhair & Erzurum, 2002). DEF is an iron-chelator, which prevents the formation of ·OH derived from H<sub>2</sub>O<sub>2</sub> via the Fenton reaction (Halliwell, 1989), whereas DMTU is a ·OH scavenger (Fox, 1984). The doses of CAT, DMTU and DEF have been used previously in the study of H<sub>2</sub>O<sub>2</sub>-evoked airway reflexes (Ruan *et al.* 2003).

### Administration of receptor agonist or antagonist

The stock solution of capsaicin (250  $\mu$ g ml<sup>-1</sup>), a TRPV1 receptor agonist, was prepared by dissolving it in a solution containing 1% Tween 80 (Sigma), 1% ethanol and 98% saline. The stock solutions of  $\alpha,\beta$ -methylene-ATP ( $\alpha,\beta$ -meATP, 2 mg ml<sup>-1</sup>, Sigma), a P2X receptor agonist, phenylbiguanide (2 mg ml<sup>-1</sup>, Sigma), a serotonin 5-HT<sub>3</sub> receptor agonist, or *iso*-pyridoxalphosphate-6-azophenyl-2',5'-disulphonate (*iso*-PPADS, 50 mg ml<sup>-1</sup>, Tocris Cookson, Ellisville, MO, USA), a P2X receptor antagonist (Ralevic & Burnstock, 1998; Irnich *et al.* 2001), were prepared by dissolving them in saline. The stock solution of capsazepine (CPZ, 10 mg ml<sup>-1</sup>, Sigma),

a TRPV1 receptor antagonist, was prepared by first dissolving in dimethyl sulfoxide (Sigma) at a concentration of 40 mg ml<sup>-1</sup> and further diluting with saline containing 10% Tween 80 and 10% ethanol. Except for the stock solution of CPZ, which was stored at 4°C, the others were stored at -20°C. Injected solutions of these chemical agents at desired concentrations were prepared daily by diluting with saline on the basis of the animal's body weight. Capsaicin,  $\alpha,\beta$ -meATP and phenylbiguanide, in a volume of 0.2 ml, were separately injected into the vein as a bolus at doses of 0.75, 200 and 4  $\mu$ g kg<sup>-1</sup>, respectively. CPZ and *iso*-PPADS, in a volume of 0.2 ml, were slowly injected into the vein over 30 s at doses of 3 and 20 mg kg<sup>-1</sup>, respectively. Each of these injections was then flushed into the right atrium by an injection of 0.4 ml saline. The doses and effective time of these agents were adopted or modified from studies reported previously (Lee & Lundberg, 1994; Pelleg & Hurt, 1996; Kirkup *et al.* 1999; Ho *et al.* 2001). The doses of CPZ and *iso*-PPADS were chosen also because they could block the afferent responses to capsaicin and  $\alpha,\beta$ -meATP, respectively, in our preliminary study.

### Experimental design and protocols

In this study, 132 capsaicin-sensitive vagal lung afferent fibres were recorded from 132 rats (weight, 432  $\pm$  5 g) and were divided into 16 groups to conduct four series of experiments. Group 1 contained 12 afferent fibres, while each of the Groups 2–16 contained eight afferent fibres. In the first study series, the afferent fibre responses to a challenge of PBS, 0.2% or 0.4% H<sub>2</sub>O<sub>2</sub> were studied (Group 1). In the second study series, afferent fibre responses to capsaicin or 0.4% H<sub>2</sub>O<sub>2</sub> were studied before and after pretreatment with CAT (Group 2), HICAT (Group 3), DMTU (Group 4), vehicle of DMTU (Group 5), DEF (Group 6) or DEF + Fe (Group 7). In the third study series, afferent fibre responses to  $\alpha,\beta$ -meATP or 0.4% H<sub>2</sub>O<sub>2</sub> were studied before and after pretreatment with *iso*-PPADS alone (Group 8). Additionally, afferent fibre responses to capsaicin or 0.4% H<sub>2</sub>O<sub>2</sub> were studied before and after pretreatment with CPZ alone (Group 9), a combination of CPZ and *iso*-PPADS (CPZ + *iso*-PPADS; Group 10) or a combination of vehicles of CPZ and *iso*-PPADS (Group 11). Finally, afferent fibre responses to  $\alpha,\beta$ -meATP or phenylbiguanide were studied before and after pretreatment with a combination of CPZ and *iso*-PPADS (Group 12). In the fourth study series, afferent fibre responses to capsaicin or cigarette smoke (20 ml) were studied before and after pretreatment with DMTU (Group 13), vehicle of DMTU (Group 14), a combination of CPZ and *iso*-PPADS (Group 15) or a combination of vehicles of CPZ and *iso*-PPADS (Group 16). The sequence of two or three challenges of afferent fibre stimulants was alternated to achieve a balanced design. Before each H<sub>2</sub>O<sub>2</sub> challenge, the animal's lungs were

hyperinflated ( $P_{tr} > 25$  cmH<sub>2</sub>O) to establish a constant volume history. Based upon the results of our preliminary study, at least 60 min was allowed to elapse between two H<sub>2</sub>O<sub>2</sub> or smoke challenges to avoid possible tachyphylaxis. The elapsed time between challenges of PBS and H<sub>2</sub>O<sub>2</sub> was 60 min. The elapsed time between capsaicin and H<sub>2</sub>O<sub>2</sub> challenges, between capsaicin and smoke challenges, between  $\alpha,\beta$ -meATP and H<sub>2</sub>O<sub>2</sub> challenges or between  $\alpha,\beta$ -meATP and phenylbiguanide challenges was 35 min. Pretreatments with CAT, HICAT, DMTU, vehicle of DMTU, DEF, DEF + Fe, CPZ, vehicle of CPZ, *iso*-PPADS and vehicle of *iso*-PPADS were made 10, 10, 30, 30, 30, 30, 2, 2, 30 and 30 min, respectively, prior to the challenge of capsaicin, H<sub>2</sub>O<sub>2</sub>, cigarette smoke,  $\alpha,\beta$ -meATP or phenylbiguanide. These pretreatment times were different because they have different effective times to reach their drug effects and maximal efficacy as reported by others (Lee & Lundberg, 1994; Ruan *et al.* 2003) or as indicated by our preliminary study.

### Data analysis and statistics

Neural activity of capsaicin-sensitive vagal lung afferent fibres and mean arterial blood pressure were continuously analysed at 1-s intervals, and  $R_L$  and  $C_{dyn}$  were continuously analysed on a breath-by-breath basis over an interval of at least 2 min before, and 3 min after, each challenge of afferent fibre stimulant. These data were then averaged over 3 s or over three breaths to give mean values for plotting responses over time. Baseline data of these physiological parameters were calculated as the average values over the 30-s or 30-breath period immediately preceding each challenge with afferent fibre stimulant. The peak response was defined as the maximal or minimal value averaged over 3 s or over three breaths after each challenge with the afferent fibre stimulant. As the mean values and variability of their baseline activity were quite small, afferent fibres were judged to be activated by stimulants when the peak response exceeded its baseline activity by at least 1 impulse s<sup>-1</sup>. Once afferent fibres were judged to be activated, the time of the first mean value of discharge averaged over 3-s period that exceeded the baseline activity by at least 1 impulse s<sup>-1</sup> was regarded as the commence time of stimulation. These physiological parameters were analysed using a computer equipped with an analog-digital converter (DASA 4600; Gould, Columbus, OH, USA) and appropriate software (BioCybernetics 1.0; Taipei, Taiwan). Results obtained from the computer analysis were routinely checked with those obtained by manual calculations for accuracy. Results were compared by paired *t*-test or two-way repeated-measures analysis of variance followed by Fisher's least significant difference procedure where appropriate. A value of  $P < 0.05$  was considered significant. All data are presented as the mean  $\pm$  s.e.m.

## Results

### Characteristics of the capsaicin-sensitive vagal lung afferent fibres studied

All 132 capsaicin-sensitive vagal lung afferent fibres studied had irregular and sparse baseline activities (Fig. 2) and were stimulated only when the lungs were hyperinflated to three or four tidal volumes (Fig. 2A). Intravenous injection of capsaicin stimulated each of these afferent fibres (Fig. 2B). Their mean fibre activity (FA) increased from a baseline of  $0.03 \pm 0.01$  impulses  $s^{-1}$  to peaks of  $0.37 \pm 0.03$  and  $8.04 \pm 0.34$  impulses  $s^{-1}$  ( $n = 132$ ) in response to hyperinflation and capsaicin, respectively. All these afferent fibres were localized within the lung structure.

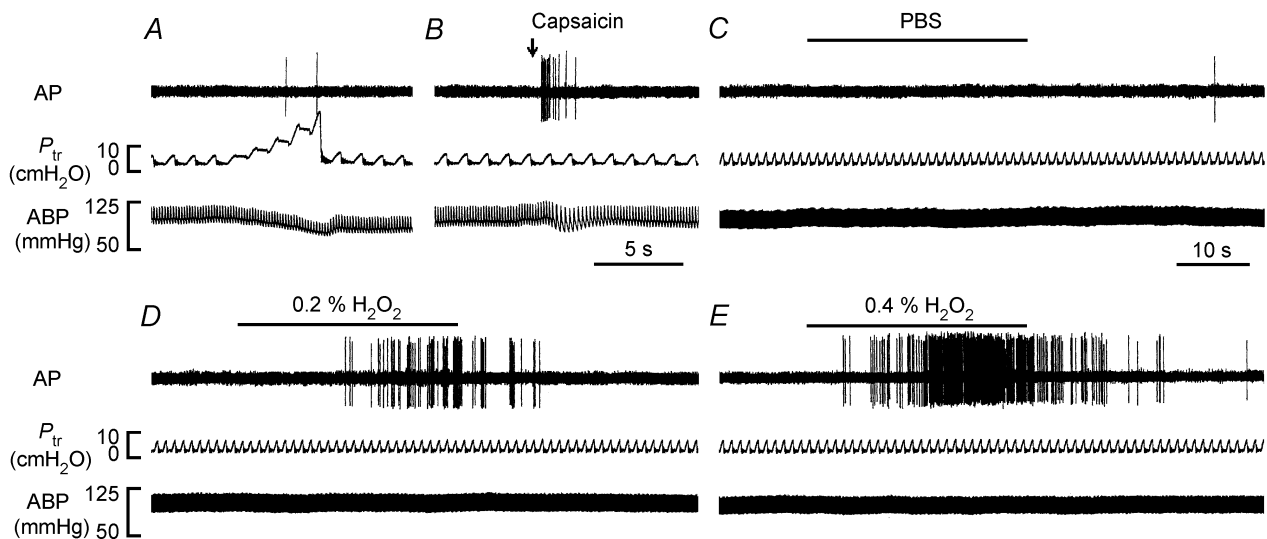
### Concentration-dependant afferent stimulation by H<sub>2</sub>O<sub>2</sub>

In the first study series, 12 capsaicin-sensitive vagal lung afferent fibres were randomly selected to receive challenges of aerosolized PBS or H<sub>2</sub>O<sub>2</sub>. Airway challenge of aerosolized PBS, the vehicle of H<sub>2</sub>O<sub>2</sub>, did not affect the activity of any of the 12 afferent fibres tested (Figs 2C and 3A). In contrast, an airway challenge of 0.4% aerosolized H<sub>2</sub>O<sub>2</sub> stimulated 10 of the 12 afferent fibres tested (Fig. 2E). The stimulation began  $10.7 \pm 6.6$  s (range, 5–15 s;  $n = 10$ ) after H<sub>2</sub>O<sub>2</sub> challenge and lasted

for a mean duration of  $50.8 \pm 13.3$  s (range, 39–69 s) (Fig. 3A). When stimulated, these afferent fibres fired irregularly, and the evoked discharge was not in phase with the ventilatory cycle (Fig. 2E). Airway challenge of 0.2% aerosolized H<sub>2</sub>O<sub>2</sub> stimulated only eight of the 12 afferent fibres tested and evoked a milder afferent stimulation with respect to the onset time, amplitude and duration of the afferent responses (Figs 2D and 3A). As a group ( $n = 12$ ), the peak change in fibre activity ( $\Delta$ FA) evoked by 0.4% H<sub>2</sub>O<sub>2</sub> ( $\Delta$ FA,  $5.49 \pm 1.23$  impulses  $s^{-1}$ ) was significantly greater than that evoked by 0.2% H<sub>2</sub>O<sub>2</sub> ( $\Delta$ FA,  $2.43 \pm 0.05$  impulses  $s^{-1}$ ); both were significantly greater than that evoked by PBS alone ( $\Delta$ FA,  $0.17 \pm 0.08$  impulses  $s^{-1}$ ) (Fig. 3A).

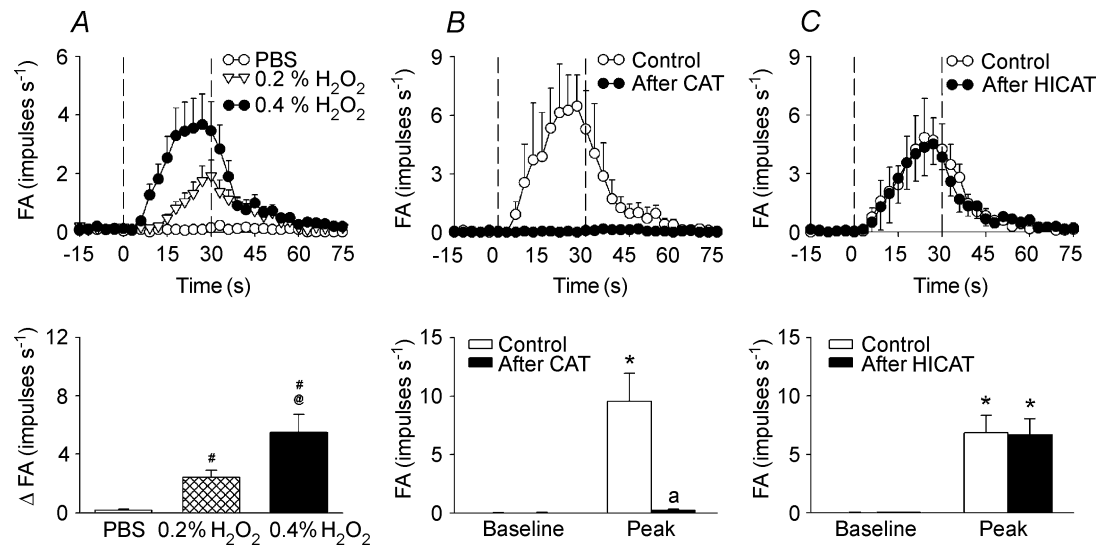
### ROS mechanisms underlying the afferent stimulation by H<sub>2</sub>O<sub>2</sub>

In the second study series, 48 capsaicin-sensitive vagal lung afferent fibres that responded to 0.4% aerosolized H<sub>2</sub>O<sub>2</sub> were divided into six groups (each  $n = 8$ ) to receive various pretreatments. All pretreatments did not significantly alter either their baseline FA (Figs 3–6) or their afferent responses to capsaicin (Table 1). The average fibre responses to 0.4% H<sub>2</sub>O<sub>2</sub> were entirely prevented by pretreatment with catalase ( $\Delta$ FA,  $-96 \pm 3\%$ ; control,  $9.52 \pm 2.39$ ; after treatment,  $0.18 \pm 0.11$  impulses  $s^{-1}$ ; Figs 3B and 4A). They were



**Figure 2.** Responses of a rat capsaicin-sensitive vagal lung afferent fibre to lung hyperinflation, intravenous capsaicin and airway challenges of aerosolized PBS or H<sub>2</sub>O<sub>2</sub>

A, the lungs were hyperinflated in a step-like manner to five times tidal volume; B, capsaicin ( $0.75 \mu\text{g kg}^{-1}$ , 0.2 ml) was injected into a catheter (dead space,  $\sim 0.3$  ml) with its tip close to the right atrium and flushed into the vein with saline (0.4 ml) as indicated by the arrow; C–E, airway delivery of aerosolized PBS, 0.2% H<sub>2</sub>O<sub>2</sub> or 0.4% H<sub>2</sub>O<sub>2</sub> was achieved by directing the air into an active nebulizer cup containing a solution for 30 s using the respirator. The duration of H<sub>2</sub>O<sub>2</sub> challenge is indicated by the horizontal bars. The elapsed time intervals between hyperinflation and capsaicin injection, capsaicin injection and PBS challenge, PBS and H<sub>2</sub>O<sub>2</sub> challenges, and two H<sub>2</sub>O<sub>2</sub> challenges were 5, 15, 60 and 60 min, respectively. AP, action potential;  $P_{\text{tr}}$ , tracheal pressure; ABP, arterial blood pressure.

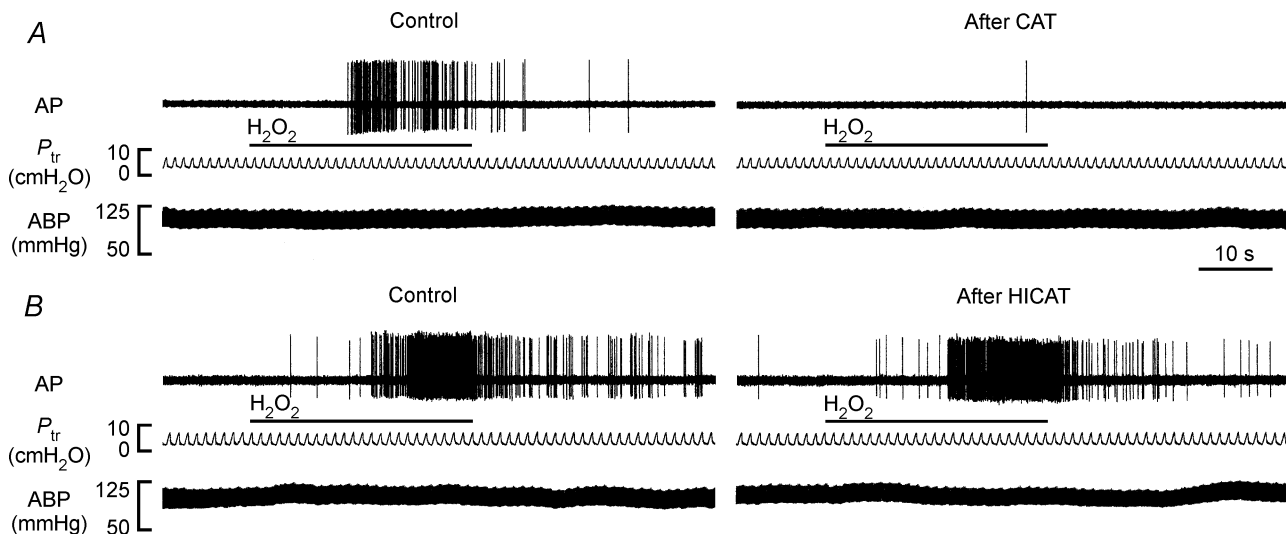


**Figure 3. Mean responses of capsaicin-sensitive vagal lung afferent fibres to various concentrations of aerosolized  $H_2O_2$  and to 0.4% aerosolized  $H_2O_2$  before and after antioxidant pretreatment**

A, responses to aerosolized PBS or  $H_2O_2$  in one group of fibres; B and C, responses to 0.4% aerosolized  $H_2O_2$  before and after pretreatment with catalase (CAT) or heat-inactivated catalase (HICAT) in the other two groups. Pretreatments were made 10 min prior to the subsequent challenge by delivery of aerosolized CAT or HICAT (both  $750\,000\text{ IU ml}^{-1}$ ) into lower airways for a period of 5 min using the nebulizer and circuit for delivery of  $H_2O_2$  aerosol. In the upper panels, the data were averaged over 3 s to give mean values to plot responses over time. The duration of  $H_2O_2$  challenge is indicated by the interval between dashed lines. #Significantly different from response to PBS; @significantly different from response to 0.2%  $H_2O_2$ ; \*significantly different from corresponding baseline; <sup>a</sup>significantly different from response before pretreatment (control). FA, fibre activity ( $\text{impulses s}^{-1}$ );  $\Delta$ FA, difference between peak FA after  $H_2O_2$  challenge and average baseline activity. Data are mean  $\pm$  S.E.M. of 12 fibres from 12 rats in A and eight fibres from eight rats in B and C.

also significantly attenuated by pretreatment with dimethylthiourea ( $\Delta$ FA,  $-55 \pm 9\%$ ; control,  $7.99 \pm 1.69$ ; after treatment,  $3.59 \pm 1.01\text{ impulses s}^{-1}$ ; Figs 5A and 6A) or deferoxamine ( $\Delta$ FA,  $-59 \pm 9\%$ ; control,  $7.64 \pm 1.46$ ;

after treatment,  $3.67 \pm 1.40\text{ impulses s}^{-1}$ ; Figs 5C and 6C). However, they were not significantly affected by pretreatment with either heat-inactivated catalase ( $\Delta$ FA,  $0 \pm 17\%$ ; control,  $6.78 \pm 1.79$ ; after treatment,



**Figure 4. Responses of two rat capsaicin-sensitive vagal lung afferent fibres to 0.4% aerosolized  $H_2O_2$  before and after antioxidant pre-treatment**

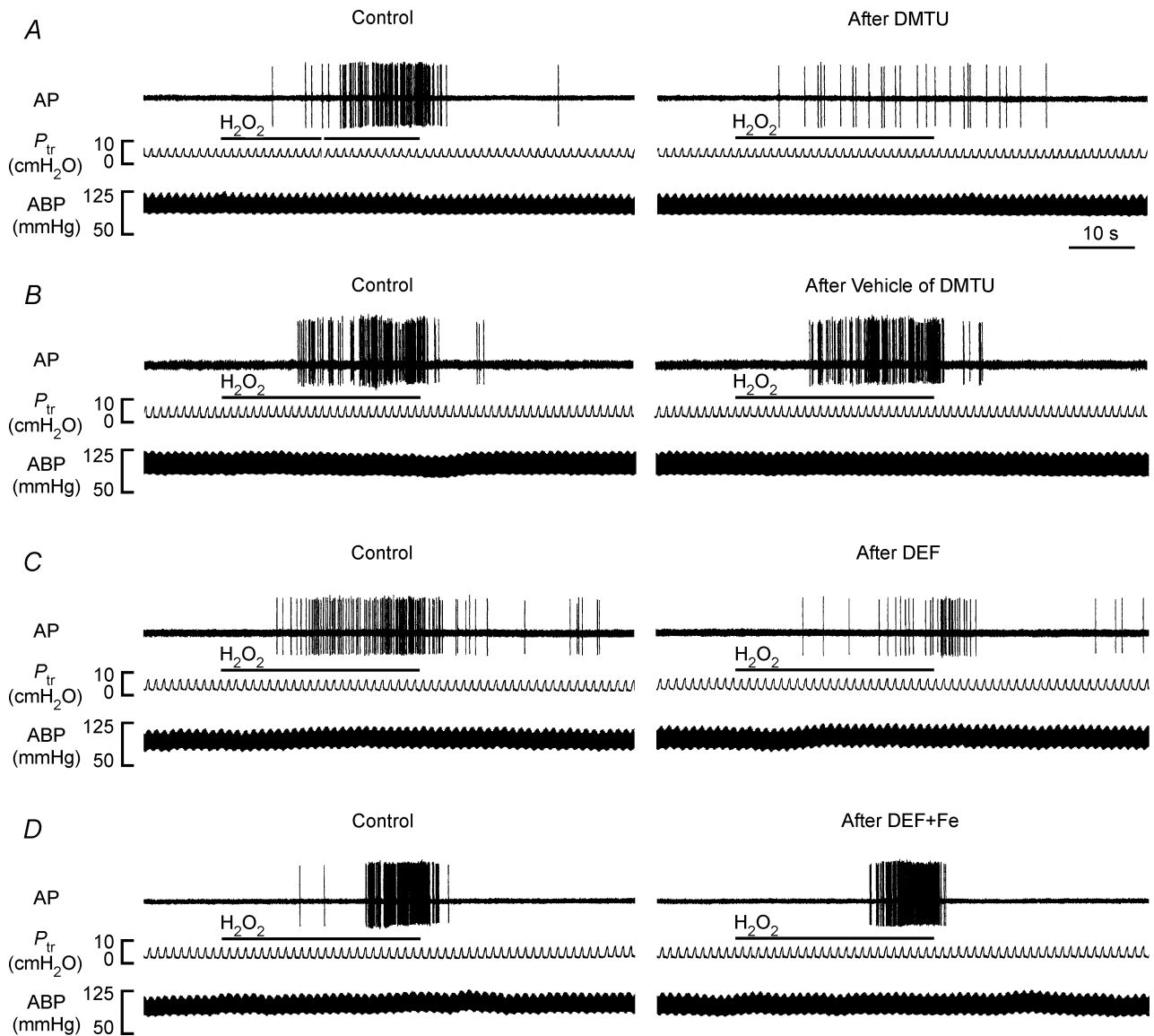
A and B, pretreatment with catalase (CAT) and heat-inactivated catalase (HICAT), respectively. The duration of  $H_2O_2$  challenge is indicated by horizontal bars. The elapsed time intervals between two  $H_2O_2$  challenges were 60 min. AP, action potential;  $P_{tr}$ , tracheal pressure; ABP, arterial blood pressure. See legend of Fig. 3 for detail.

$6.28 \pm 1.41$  impulses  $s^{-1}$ ; Figs 3C and 4B), the vehicle of dimethylthiourea ( $\Delta FA$ ,  $16 \pm 12\%$ ; control,  $7.89 \pm 1.64$ ; after treatment,  $8.13 \pm 1.45$  impulses  $s^{-1}$ ; Figs 5B and 6B) or iron-saturated deferoxamine ( $\Delta FA$ ,  $-7 \pm 24\%$ ; control,  $6.94 \pm 0.87$ ; after treatment,  $6.45 \pm 2.05$  impulses  $s^{-1}$ ; Figs 5D and 6D).

### Role of TRPV1 and P2X receptors in afferent stimulation by $H_2O_2$

In the third study series, 32 capsaicin-sensitive vagal lung afferent fibres that responded to 0.4% aerosolized

$H_2O_2$  were divided into four groups (each  $n = 8$ ) to receive various pretreatments. An additional group of eight capsaicin-sensitive vagal lung afferent fibres that did not receive  $H_2O_2$  challenge test was employed to receive pretreatment with a combination of capsazepine and *iso*-PPADS. Among them, intravenous injections of  $\alpha, \beta$ -meATP and phenylbiguanide were also performed to stimulate 16 and eight afferent fibres, respectively (Table 2). All pretreatments did not significantly alter baseline FA (Figs 7 and 8). Pretreatment with capsazepine alone or *iso*-PPADS alone successfully prevented fibre responses to capsaicin and  $\alpha, \beta$ -meATP, respectively (Table 2).



**Figure 5. Responses of four capsaicin-sensitive vagal lung afferent fibres to 0.4% aerosolized  $H_2O_2$  before and after various antioxidant pretreatments**

A–D, pretreatment with dimethylthiourea (DMTU), vehicle of DMTU, deferoxamine (DEF) and iron-saturated DEF (DEF + Fe), respectively. Pretreatments were made 30 min prior to the subsequent challenge by slow injection of DMTU ( $1.5 \text{ g kg}^{-1}$ ), vehicle of DMTU, DEF ( $15 \text{ mg kg}^{-1}$ ) or DEF + Fe ( $15 \text{ mg kg}^{-1}$ ) 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.

Pretreatment with a combination of capsazepine and *iso*-PPADS effectively blocked the response of fibres to either capsaicin or  $\alpha,\beta$ -meATP, but it did not significantly affect fibre responses to phenylbiguanide (Table 2). Under these circumstances, the average fibre responses to 0.4%  $\text{H}_2\text{O}_2$  were significantly attenuated by pretreatment with capsazepine alone ( $\Delta\text{FA}$ ,  $-39 \pm 9\%$ ; control,  $8.88 \pm 1.62$ ; after treatment,  $4.65 \pm 0.17$  impulses  $\text{s}^{-1}$ ; Figs 7A and 8A) or *iso*-PPADS alone ( $\Delta\text{FA}$ ,  $-51 \pm 9\%$ ; control,  $8.55 \pm 1.29$ ; after treatment,  $3.55 \pm 0.50$  impulses  $\text{s}^{-1}$ ; Figs 7B and 8B), but were not significantly affected by pretreatment with a combination of the vehicles of capsazepine and *iso*-PPADS ( $\Delta\text{FA}$ ,  $12 \pm 19\%$ ; control,  $8.44 \pm 0.78$ ; after treatment,  $8.80 \pm 1.16$  impulses  $\text{s}^{-1}$ ; Figs 7D and 8D). Pretreatment with a combination of capsazepine and *iso*-PPADS further reduced the average fibre responses to a level ( $\Delta\text{FA}$ ,  $-70 \pm 13\%$ ; control,  $7.91 \pm 1.07$ ; after treatment,  $1.95 \pm 0.75$  impulses  $\text{s}^{-1}$ ) where the average  $\text{H}_2\text{O}_2$ -evoked discharge did not significantly differ from the baseline discharge (Figs 7C and 8C).

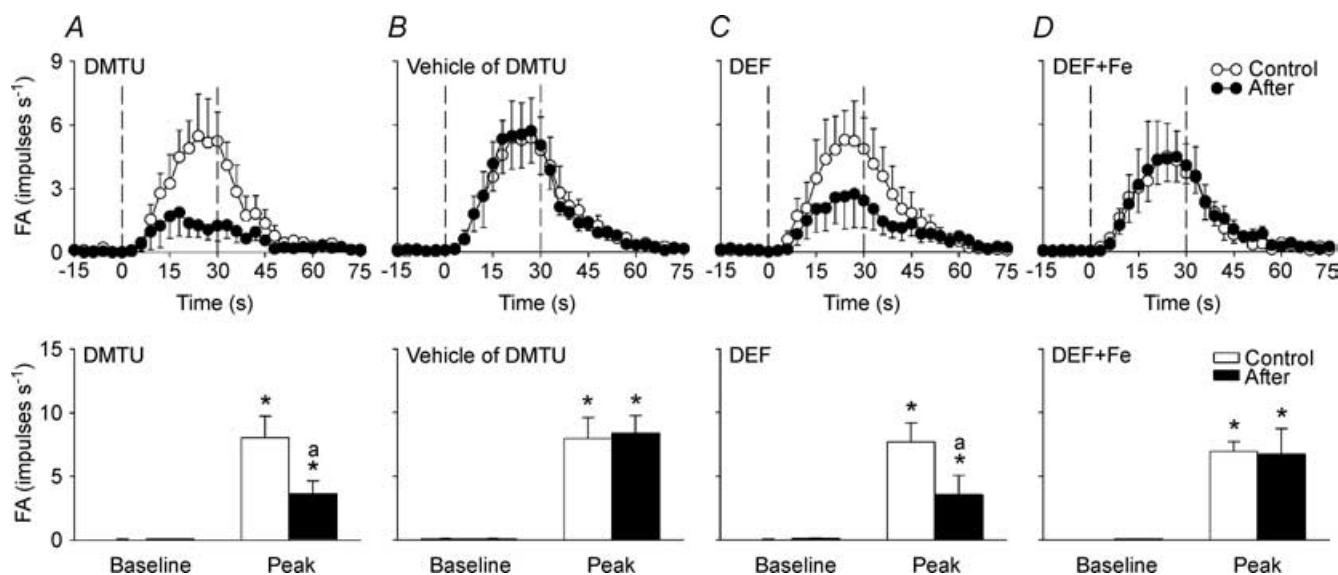
### Cardiopulmonary responses to $\text{H}_2\text{O}_2$

After airway challenge with aerosolized PBS, the mean arterial blood pressure, total lung resistance and dynamic lung compliance were  $88 \pm 4$  mmHg,  $0.13 \pm 0.01$   $\text{cmH}_2\text{O ml}^{-1} \text{s}^{-1}$  and  $0.41 \pm$

$0.02 \text{ ml}^{-1} \text{cmH}_2\text{O}^{-1}$ , respectively, which did not significantly differ from their corresponding baselines ( $88 \pm 4$  mmHg,  $0.13 \pm 0.01$   $\text{cmH}_2\text{O ml}^{-1} \text{s}^{-1}$  and  $0.42 \pm 0.02$   $\text{ml cmH}_2\text{O}^{-1}$ , respectively;  $n = 12$ ,  $P > 0.05$ ). After airway challenge with 0.4% aerosolized  $\text{H}_2\text{O}_2$ , the mean arterial blood pressure, total lung resistance and dynamic lung compliance were  $82 \pm 4$  mmHg,  $0.15 \pm 0.01$   $\text{cmH}_2\text{O ml}^{-1} \text{s}^{-1}$  and  $0.38 \pm 0.02$   $\text{ml cmH}_2\text{O}^{-1}$ , respectively, which differs slightly but significantly from their corresponding baselines ( $89 \pm 4$  mmHg,  $0.13 \pm 0.01$   $\text{cmH}_2\text{O ml}^{-1} \text{s}^{-1}$  and  $0.41 \pm 0.02$   $\text{ml cmH}_2\text{O}^{-1}$ , respectively;  $n = 12$ ,  $P < 0.05$ ).

### Role of TRPV1 and P2X receptors in afferent stimulation by cigarette smoke

In the fourth study series, 32 capsaicin-sensitive vagal lung afferent fibres that responded to challenge of cigarette smoke were divided into four groups (each  $n = 8$ ) to receive various pretreatments. Within 1–3 s after smoke delivery, their FA increased from a baseline of  $0.04 \pm 0.01$  impulses  $\text{s}^{-1}$  to a peak of  $7.96 \pm 0.65$  impulses  $\text{s}^{-1}$  ( $n = 32$ ). All pretreatments did not significantly alter baseline FA (Figs 9 and 10). Pretreatment with either dimethylthiourea ( $\Delta\text{FA}$ ,  $-61 \pm 9\%$ ; control,  $8.81 \pm 1.42$ ; after treatment,  $4.10 \pm 1.44$  impulses  $\text{s}^{-1}$ ; Fig. 9A and 10A) or a combination of capsazepine and *iso*-PPADS ( $\Delta\text{FA}$ ,  $-67 \pm 9\%$ ; control,



**Figure 6. Mean afferent responses to 0.4% aerosolized  $\text{H}_2\text{O}_2$  before and after various antioxidant pretreatments in four groups of capsaicin-sensitive vagal lung afferent fibres**

A–D, pretreatment with dimethylthiourea (DMTU), vehicle of DMTU, deferoxamine (DEF) and iron-saturated DEF (DEF + Fe), respectively. In the upper panels, data were averaged over 3 s to give mean values to plot responses over time. The duration of  $\text{H}_2\text{O}_2$  challenge is indicated by the interval between the dashed lines. \*Significantly different from corresponding baseline; <sup>a</sup>significantly different from response before pretreatment (control). FA, fibre activity (impulses  $\text{s}^{-1}$ ). Data are mean  $\pm$  s.e.m. of eight fibres from eight rats for each group. See legend of Fig. 5 for detail.



**Table 1. Average peak responses of capsaicin-sensitive vagal lung afferent fibres to intravenous injection of capsaicin before and after various antioxidant pretreatments**

Pretreatment	Control (impulses s <sup>-1</sup> )		After pretreatment (impulses s <sup>-1</sup> )	
	Baseline	Peak response	Baseline	Peak response
CAT	0.01 ± 0.01	7.56 ± 0.07	0.02 ± 0.02	7.44 ± 0.63
HICAT	0.01 ± 0.01	6.65 ± 0.75	0.02 ± 0.02	6.35 ± 1.07
DMTU	0.03 ± 0.02	8.29 ± 1.43	0.04 ± 0.02	8.04 ± 1.36
Vehicle of DMTU	0.01 ± 0.01	7.25 ± 0.63	0.01 ± 0.01	7.77 ± 1.56
DEF	0.01 ± 0.01	7.17 ± 1.33	0.00 ± 0.00	7.23 ± 1.53
DEF + Fe	0.01 ± 0.01	7.04 ± 0.67	0.04 ± 0.02	7.65 ± 0.45

Baseline values are data averaged over 30 s, whereas peak values are data averaged over 3 s. Data in each group are the mean ± s.e.m. of eight fibres from eight rats. No statistical significance was found between any two groups of mean. CAT, catalase; HICAT, heat-inactivated catalase; DMTU, dimethylthiourea; Vehicle of DMTU, vehicle of dimethylthiourea; DEF, deferoxamine; DEF + Fe, iron-saturated deferoxamine.

**Table 2. Average peak responses of capsaicin-sensitive vagal lung afferent fibres to intravenous injection of three receptor agonists before and after various antagonist pretreatments**

Receptor agonist	Pretreatment	Control (impulses s <sup>-1</sup> )	After pretreatment (impulses s <sup>-1</sup> )
Capsaicin	CPZ	8.14 ± 1.31	0.19 ± 0.13*
Capsaicin	CPZ + <i>iso</i> -PPADS	7.74 ± 1.38	0.22 ± 0.13*
Capsaicin	Vehicles of CPZ + <i>iso</i> -PPADS	7.52 ± 1.10	7.61 ± 1.41
$\alpha,\beta$ -meATP	<i>iso</i> -PPADS	8.82 ± 2.39	0.24 ± 0.24*
$\alpha,\beta$ -meATP	CPZ + <i>iso</i> -PPADS	10.83 ± 2.52	0.98 ± 0.76*
Phenylbiguanide	CPZ + <i>iso</i> -PPADS	11.79 ± 1.99	10.70 ± 1.82

Baseline values are data averaged over 30 s, whereas peak values are data averaged over 3 s. \*Significantly different from response before pretreatment (control). Data in each group are the mean ± s.e.m. of eight fibres from eight rats.  $\alpha,\beta$ -meATP,  $\alpha,\beta$ -methylene-ATP; CPZ, capsazepine; *iso*-PPADS, *iso*-pyridoxalphosphate-6-azophenyl-2',5'-disulphonate; CPZ + *iso*-PPADS, a combination of CPZ and *iso*-PPADS; vehicles of CPZ + *iso*-PPADS, a combination of vehicles of CPZ and *iso*-PPADS.

7.93 ± 1.60; after treatment, 3.25 ± 1.37 impulses s<sup>-1</sup>; Figs 9C and 10C) significantly reduced the average fibre responses to cigarette smoke, whereas pretreatment with either the vehicle of dimethylthiourea ( $\Delta$ FA, 7 ± 11%; control, 7.63 ± 1.08; after treatment, 7.79 ± 0.98 impulses s<sup>-1</sup>; Figs 9B and 10B) or a combination of the vehicles of capsazepine and *iso*-PPADS ( $\Delta$ FA, -2 ± 11%; control, 7.31 ± 1.26; after treatment, 7.19 ± 1.48 impulses s<sup>-1</sup>; Figs 9D and 10D) failed to do so. Furthermore, a combination of capsazepine and *iso*-PPADS totally prevented the afferent stimulation by capsaicin ( $\Delta$ FA, -99 ± 1%; control, 9.94 ± 1.84; after treatment, 0.20 ± 0.17 impulses s<sup>-1</sup>), whereas pretreatment with either dimethylthiourea ( $\Delta$ FA, -2 ± 7%; control, 7.78 ± 1.36; after treatment, 7.36 ± 1.23 impulses s<sup>-1</sup>), the vehicle of dimethylthiourea ( $\Delta$ FA, -1 ± 7%; control, 8.81 ± 0.85 after treatment, 8.73 ± 1.03 impulses s<sup>-1</sup>) or a combination of the vehicles of capsazepine and *iso*-PPADS ( $\Delta$ FA, -1 ± 9%; control, 9.82 ± 1.60; after treatment, 9.70 ± 1.57 impulses s<sup>-1</sup>)

did not significantly alter the average fibre response to capsaicin.

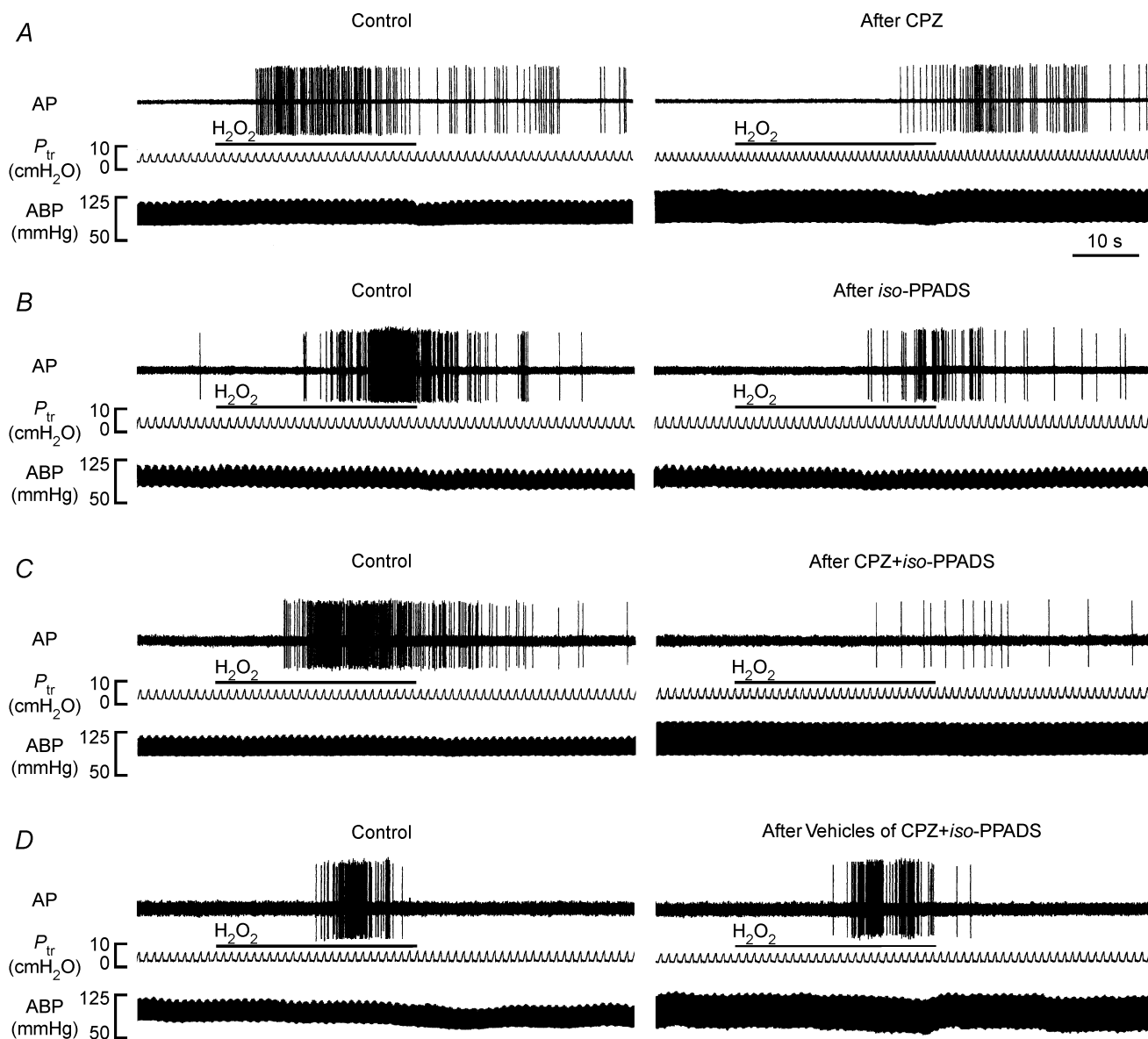
## Discussion

Results of the first part of this study demonstrate that airway challenge of aerosolized H<sub>2</sub>O<sub>2</sub> stimulated capsaicin-sensitive vagal lung afferent fibres in a concentration-dependent fashion. The afferent responses to H<sub>2</sub>O<sub>2</sub> were totally prevented by catalase and significantly reduced by either dimethylthiourea or deferoxamine. In contrast, the afferent responses to H<sub>2</sub>O<sub>2</sub> were not affected by heat-inactivated catalase, the vehicle of dimethylthiourea or iron-saturated deferoxamine. Catalase is an enzyme that catalyses the breakdown of H<sub>2</sub>O<sub>2</sub> into O<sub>2</sub> and H<sub>2</sub>O (Comhair & Erzurum, 2002). Dimethylthiourea is a ·OH scavenger (Fox, 1984), whereas deferoxamine is an iron-chelator, which prevents the formation of ·OH derived from H<sub>2</sub>O<sub>2</sub> via the Fenton reaction (Halliwell, 1989). The suppressive effects of these

antioxidants on afferent responses to  $\text{H}_2\text{O}_2$  were unlikely to be due to any anaesthetic or deleterious influence on these afferent fibres, because their afferent responses to capsaicin were unaffected by these pretreatments. It is conceivable that these antioxidants prevented or attenuated afferent responses by lowering the ROS burden. The afferent responses to two  $\text{H}_2\text{O}_2$  challenges were similar, suggesting that a prior capsaicin injection did not alter the fibre responsiveness to the subsequent  $\text{H}_2\text{O}_2$  challenge. Taken together, these results suggest that the

observed stimulation of capsaicin-sensitive vagal lung afferent fibres is a consequence resulting from the specific action of  $\text{H}_2\text{O}_2$  and this is in part mediated through the involvement of  $\cdot\text{OH}$  derived from  $\text{H}_2\text{O}_2$  and possibly the superoxide radical.

Capsaicin-sensitive vagal lung afferent fibres are a subpopulation of pulmonary sensory nerve endings whose afferent activity is conducted through mainly unmyelinated C fibres and some myelinated A- $\delta$  fibres (Coleridge & Coleridge, 1986; Lee & Pisarri, 2001; Carr



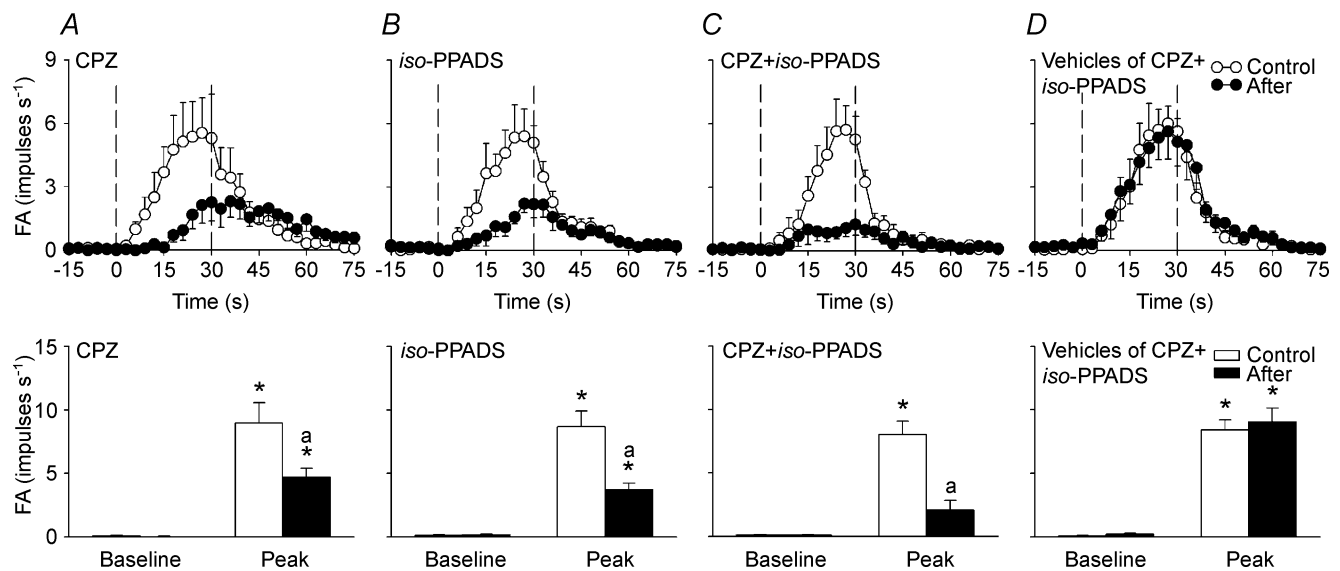
**Figure 7. Responses of four rat capsaicin-sensitive vagal lung afferent fibres to 0.4% aerosolized  $\text{H}_2\text{O}_2$  before and after various antagonist pretreatments**

A–D, pretreatment with capsazepine (CPZ), *iso*-pyridoxalphosphate-6-azophenyl-2',5'-disulphonate (*iso*-PPADS), 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 ( $3 \text{ mg kg}^{-1}$ ), vehicle of CPZ, *iso*-PPADS ( $20 \text{ mg kg}^{-1}$ ) and vehicle of *iso*-PPADS, respectively, into the vein for 30 s. The duration of  $\text{H}_2\text{O}_2$  challenge is indicated by the horizontal bars. The elapsed time intervals between two  $\text{H}_2\text{O}_2$  challenges were 60 min. AP, action potential;  $P_{\text{tr}}$ , tracheal pressure; ABP, arterial blood pressure.

& Undem, 2003). In rats, a right-atrial injection of capsaicin evokes an intense stimulatory effect in 89% of the pulmonary C fibres tested, but only a mild stimulation in 6% of the rapidly adapting pulmonary receptors and none in the slowly adapting pulmonary stretch receptors; the latter two groups are myelinated afferent fibres (Ho *et al.* 2001). Measurements of conduction velocities of this type of lung vagal afferent fibres in our previous studies (Lai & Kou, 1998*a*, 1998*b*; Lai *et al.* 2005) reveal that they are mainly in the category of C fibres. These 'nociceptive-like' afferent fibres are known to play an important role in detecting the onset of pulmonary pathophysiological conditions and triggering various airway reflexes (Coleridge & Coleridge, 1986; Lee & Pissarri, 2001; Carr & Undem, 2003). Excess production of ROS is a common feature of various pulmonary pathophysiological conditions including asthma (Emelyanov *et al.* 2001), chronic obstructive pulmonary disease (Ferreira *et al.* 2001), endotoxin shock (Minamiya *et al.* 1995), vascular microembolism (Wang *et al.* 1992) and oxidant lung injury (Pryor, 1992). The concept that capsaicin-sensitive vagal lung afferent fibres may detect the existence of excess ROS in the lungs is relatively new (Soukhova *et al.* 1999). This concept is indirectly supported by the findings that C fibre-mediated airway reflexes are evoked by cigarette smoke (Lee, 1990), inhaled wood smoke (Kou *et al.* 1997) or pulmonary air embolism (Chen & Kou, 2000) and that the afferent responses of pulmonary C fibres to the latter

two insults (Chen *et al.* 1997; Lai & Kou, 1998*a*) or endotoxin (Lai *et al.* 2005) are greatly attenuated by  $\cdot\text{OH}$  scavengers. The results of the present study provide the first direct electrophysiological evidence to support the notion that capsaicin-sensitive vagal lung afferent fibres are important in the sensory transduction of ROS in the lungs. Our observations are also in agreement with recent findings that inhalation of aerosolized  $\text{H}_2\text{O}_2$  evokes a reflex bradypnoea that is mediated through the action of ROS on vagal lung unmyelinated afferents (Ruan *et al.* 2003).

That ROS can activate visceral afferent nerve fibres is not unique to the airways and lungs. Topical application of  $\text{H}_2\text{O}_2$  to the gastrointestinal tract stimulates abdominal sympathetic afferent C fibres in cats or rats (Stahl *et al.* 1993; Adelson *et al.* 1996). Topical application of  $\text{H}_2\text{O}_2$  to the heart activates cardiac sympathetic afferent C fibres in cats or rats (Huang *et al.* 1995; Abe *et al.* 1998; Schultz & Ustinova, 1998), cardiac vagal afferent C fibres in rats (Ustinova & Schultz, 1994*a,b*; Schultz & Ustinova, 1996, 1998) and cardiac vagal chemosensitive afferent fibres in guinea pigs (Thompson *et al.* 2000). In agreement with our findings, the  $\text{H}_2\text{O}_2$ -induced stimulation of abdominal or cardiac sympathetic (Stahl *et al.* 1993; Huang *et al.* 1995) and vagal (Schultz & Ustinova, 1996) afferent C fibres can be attenuated or abolished by either dimethylthiourea or deferoxamine, suggesting the involvement of  $\cdot\text{OH}$ . The excitatory effect of ROS has also been demonstrated in other neural tissues.

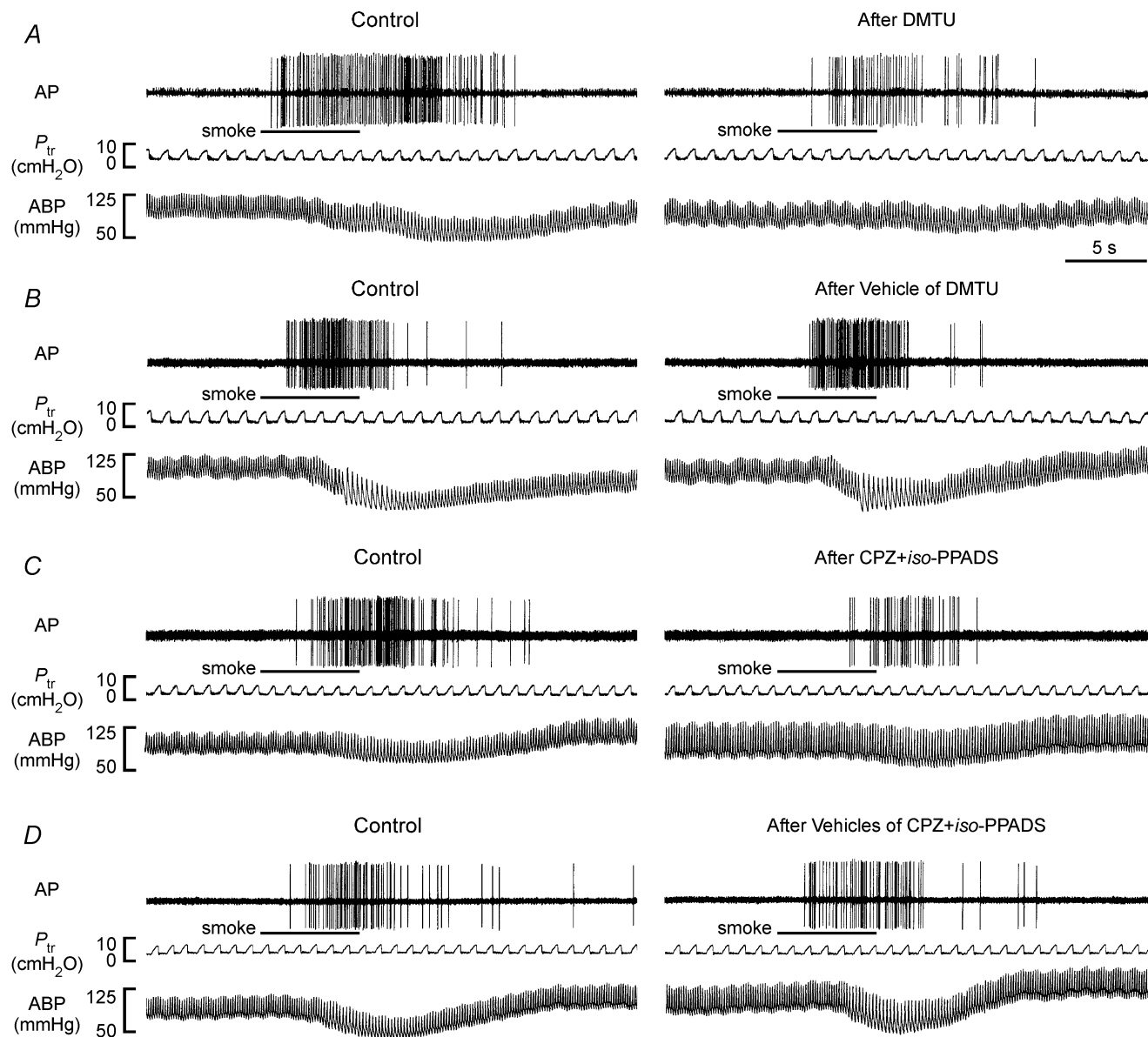


**Figure 8. Mean afferent responses to 0.4% aerosolized  $\text{H}_2\text{O}_2$  before and after various antagonist pretreatments in four groups of capsaicin-sensitive vagal lung afferent fibres**

A–D, pretreatment with capsazepine (CPZ), *iso*-pyridoxalphosphate-6-azophenyl-2',5'-disulphonate (*iso*-PPADS), a combination of CPZ and *iso*-PPADS (CPZ + *iso*-PPADS), and vehicles of CPZ and *iso*-PPADS (vehicles of CPZ + *iso*-PPADS). In the upper panels, data were averaged over 3 s to give mean values to plot responses over time. The duration of  $\text{H}_2\text{O}_2$  challenge is indicated by the interval between the dashed lines. \*Significantly different from corresponding baseline; <sup>a</sup>significantly different from response before pretreatment (control). FA, fibre activity (impulses  $\text{s}^{-1}$ ). Data are mean  $\pm$  S.E.M. of eight fibres from eight rats for each group. See legend of Fig. 7 for detail.

For example, direct application of  $H_2O_2$  depolarizes brain nerve terminals (Tretter & Adam-Vizi, 1996) and myenteric neurones (Wada-Takahashi & Tamura, 2000) isolated from guinea pigs. Bath application of  $H_2O_2$  increases the activity of sympathetic preganglionic neurones isolated from rats (Lin *et al.* 2003). Thus, it appears that ROS has stimulatory effects on a variety of neural tissues.

Results of the second part of this study demonstrate that either capsazepine alone ( $\Delta FA$ ,  $-39 \pm 9\%$ ) or *iso*-PPADS alone ( $\Delta FA$ ,  $-51 \pm 9\%$ ) is able to partially attenuate the  $H_2O_2$ -evoked responses of capsaicin-sensitive vagal lung afferent fibres. The specific blocking effects of capsazepine and *iso*-PPADS on TRPV1 and P2X receptors, respectively, were confirmed by complete blocking of afferent responses to their corresponding receptor agonists,



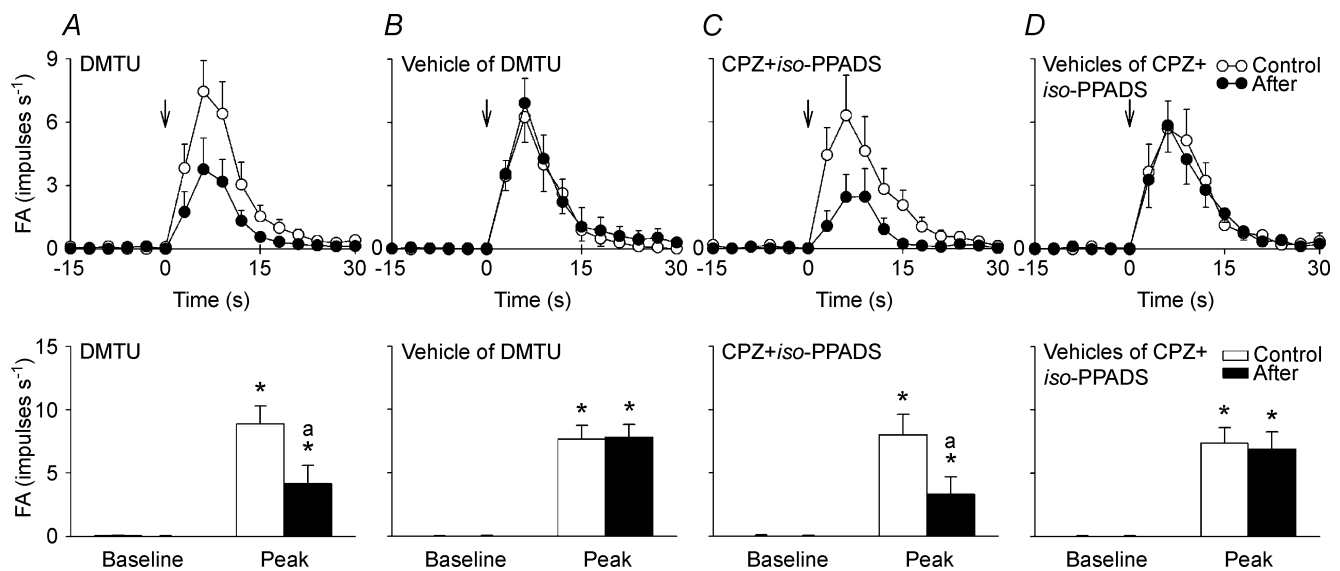
**Figure 9. Responses of four capsaicin-sensitive vagal lung afferent fibres to cigarette smoke before and after various pretreatments**

A–D, pretreatment with dimethylthiourea (DMTU), vehicle of DMTU, a combination of capsazepine and *iso*-pyridoxalphosphate-6-azophenyl-2',5'-disulphonate (CPZ + *iso*-PPADS), and vehicles of CPZ and *iso*-PPADS (vehicles of CPZ + *iso*-PPADS), respectively. Cigarette smoke (20 ml) was delivered directly into the lungs via the inspiratory line during five to six ventilatory cycles as indicated by the horizontal bars. Pretreatments were made 30, 30, 2, 2, 30 and 30 min prior to the subsequent challenge by slow injection of DMTU ( $1.5 \text{ g kg}^{-1}$ ), vehicle of DMTU, CPZ ( $3 \text{ mg kg}^{-1}$ ), vehicle of CPZ, *iso*-PPADS ( $20 \text{ mg kg}^{-1}$ ) and vehicle of *iso*-PPADS, respectively, into the vein for 30 s. The elapsed time intervals between two smoke challenges were 60 min. AP, action potential;  $P_{tr}$ , tracheal pressure; ABP, arterial blood pressure.

capsaicin and  $\alpha, \beta$ -meATP. Their specific blocking effects were also indicated by the fact that a combination of capsazepine and *iso*-PPADS did not affect afferent responses to phenylbiguanide (an agonist of 5-HT<sub>3</sub> receptors). Capsazepine has been shown to have no scavenger effect on ROS (Schultz & Ustinova, 1998), while *iso*-PPADS, to our knowledge, also has no such effect and is most probably acting through P2X receptors (Ralevic & Burnstock, 1998; Irnich *et al.* 2001). Collectively, our observations indicate that both TRPV1 and P2X receptors mediate the stimulation of capsaicin-sensitive vagal lung afferent fibres by ROS. TRPV1 and P2X receptors are two ligand-gated, non-selective cation channels that have been cloned (Valera *et al.* 1994; Caterina *et al.* 1999). TRPV1 receptors are activated by stimuli such as capsaicin, noxious heat, acid, anandamide (a cannabinoid receptor agonist) and several products of lipoxygenases (Fox *et al.* 1995; McCleskey & Gold, 1999; Szallasi & Blumberg, 1999; Hwang *et al.* 2000; Undem & Carr, 2001; Kollarik & Undem, 2002; Lin & Lee, 2002; Carr *et al.* 2003) and can be viewed as an integrator of painful chemical and physical stimuli in the somatosensory system (Szallasi & Blumberg, 1999). P2X receptors can be activated by ATP released from damaged cells and their activation causes a pain sensation (McCleskey & Gold, 1999; Dunn *et al.* 2001; Cook & McCleskey, 2002). It has been suggested that both TRPV1 and P2X receptors are located at the terminals of capsaicin-sensitive vagal lung afferent fibres. Administration of capsaicin or ATP has been shown to

stimulate these afferent fibres in whole animals (Lee & Lundberg, 1994; Pelleg & Hurt, 1996; Undem & Carr, 2001) and in *ex vivo*, vagally innervated, airway and/or lung preparations (Fox *et al.* 1995; Undem & Carr, 2001; Kollarik & Undem, 2002; Carr *et al.* 2003; Undem *et al.* 2004). While the functions of TRPV1 and P2X receptors are relatively established in somatosensory nociceptors (McCleskey & Gold, 1999; Szallasi & Blumberg, 1999; Dunn *et al.* 2001), their roles in the transduction function of capsaicin-sensitive vagal lung afferent fibres remain to be explored. Our results thus provide evidence to support the notion that the sensory transduction of ROS by these lung afferent fibres is mediated through both the TRPV1 and the P2X receptors.

We further demonstrated that a combination of capsazepine and *iso*-PPADS provided a more complete blockade of H<sub>2</sub>O<sub>2</sub>-evoked responses of capsaicin-sensitive vagal lung afferent fibres ( $\Delta$ FA,  $-70 \pm 13\%$ ), while pretreatment with their vehicles failed to alter the responses. These results suggest that the functional significance of TRPV1 receptors in the ROS-induced afferent stimulation is, at least in part, independent from the P2X receptors. To that end, the stimulation of cardiac vagal or sympathetic afferent fibres by ROS seems to be totally mediated by TRPV1 receptors because capsazepine completely prevents their afferent response to topical application of H<sub>2</sub>O<sub>2</sub> (Schultz & Ustinova, 1998). However, we cannot exclude the possibility that there is a functional interaction between TRPV1 and P2X receptors



**Figure 10. Mean afferent responses to cigarette smoke before and after various pretreatments in four groups of capsaicin-sensitive vagal lung afferent fibres**

A–D, pretreatment with dimethylthiourea (DMTU), vehicle of DMTU, a combination of capsazepine and *iso*-pyridoxal phosphate-6-azophenyl-2',5'-disulphonate (CPZ + *iso*-PPADS), and vehicles of CPZ and *iso*-PPADS (vehicles of CPZ + *iso*-PPADS), respectively. In the upper panels, data were averaged over 3 s to give mean values to plot responses over time. The onset of smoke challenge is indicated by the arrows. \*Significantly different from corresponding baseline; <sup>a</sup>significantly different from response before pretreatment (control). FA, fibre activity (impulses s<sup>-1</sup>). Data are mean  $\pm$  s.e.m. of eight fibres from eight rats for each group. See legend of Fig. 9 for detail.

in ROS-induced afferent stimulation. TRPV1 receptors have been shown to colocalize with P2X<sub>3</sub> receptor in rat dorsal root ganglion neurones and the terminals of their axons (Guo *et al.* 1999). It has been demonstrated that activation of purinoceptors by ATP augments the ionic currents evoked by activation of TRPV1 receptors in rat dorsal root ganglion neurones (Tominaga *et al.* 2001). Conversely, it has been reported that TRPV1 receptors are important in the regulation of ATP release in mice urinary bladder (Birder *et al.* 2002).

The mechanisms by which ROS activate TRPV1 and P2X receptors resulting in stimulation of capsaicin-sensitive vagal lung afferent fibres remain unclear. A direct activation of TRPV1 and P2X receptors located on the C-fibre nerve terminals by ROS has been postulated (Schultz & Ustinova, 1998; Shen *et al.* 2000). Furthermore, H<sub>2</sub>O<sub>2</sub> may act on cardiac sarcolemmal P2 receptors by changing ATP binding (Musat & Dhalla, 1996). As an alternative, ROS may indirectly activate TRPV1 and P2X receptors located at the nerve terminals by the actions of released chemical mediators or receptor ligands. For example, ROS may cause a release of lipoxygenase products in the lung tissues (Matyas *et al.* 2002), which are activators of TRPV1 receptors (Hwang *et al.* 2000; Udem & Carr, 2001; Carr *et al.* 2003). Additionally, ROS may damage cells and can rapidly release cytosolic ATP, which activates P2X receptors of pain nociceptors in the vicinity (McCleskey & Gold, 1999; Cook & McCleskey, 2002). Furthermore, ROS may have non-damaging effects and, upon stimulation, non-damaged epithelial or endothelial cells may release ATP and in this way may exert its paracrine effects on P2X receptors located on other cells (Lazarowski *et al.* 2003). It is unlikely that the observed ROS-induced sensory stimulation was due to changes in lung mechanics because these afferent fibres are high-threshold mechanoreceptors (Coleridge & Coleridge, 1986; Lee & Pisarri, 2001; Carr & Udem, 2003) and because the airway challenge of 0.4% aerosolized H<sub>2</sub>O<sub>2</sub> only slightly affected total lung resistance and dynamic lung compliance.

In this study, a combination of capsazepine and *iso*-PPADS did not completely block H<sub>2</sub>O<sub>2</sub>-evoked responses of capsaicin-sensitive vagal lung afferent fibres. This incomplete blockade does not seem to be related to the doses of antagonists because, in our preliminary study, doubling their doses did not improve the suppressive effects. The small but residual afferent response observed after pretreatment presumably arises from mechanisms involving ROS, but other than activation of TRPV1 and P2X receptors. To that end, it is known that the nerve terminals of these afferent fibres possess several other pharmacological receptors (Udem & Carr, 2001). Furthermore, although the afferent responses to capsaicin were not influenced with repeated challenges, this does not mean that afferent responses to other stimuli were not altered. In spontaneously breathing rats, inhalation

of aerosolized H<sub>2</sub>O<sub>2</sub> produces an early increase and a subsequent decrease in arterial blood pressure (Ruan *et al.* 2003). In this study, airway challenge of 0.4% aerosolized H<sub>2</sub>O<sub>2</sub> only caused a small drop in arterial blood pressure. Differences in the method of H<sub>2</sub>O<sub>2</sub> challenge, ventilatory mode and animal preparations may account for the discrepancy between the blood pressure responses in our study and in that of Ruan *et al.* (2003).

Instead of H<sub>2</sub>O<sub>2</sub>, we further employed cigarette smoke as the challenge to test the hypothesis that TRPV1 and P2X receptors are important in the sensory transduction of ROS by capsaicin-sensitive vagal lung afferent fibres. Cigarette smoke was chosen as the stimulus because it can generate ROS (Pryor, 1992) and because it triggers vagal C fibre-mediated bradypnoea via a ·OH mechanism (Lee, 1990). Indeed, we found that the afferent responses of these fibres were attenuated by pretreatment with dimethylthiourea suggesting the involvement of ·OH in the sensory activation by cigarette smoke. Furthermore, the afferent responses to cigarette smoke were suppressed by pretreatment with a combination of capsazepine and *iso*-PPADS, supporting the notion that TRPV1 and P2X receptors play a role in this sensory activation.

In conclusion, our results suggest that ROS, especially H<sub>2</sub>O<sub>2</sub> and ·OH, may stimulate capsaicin-sensitive vagal lung afferent fibres in rats and that this sensory transduction is mediated through both TRPV1 and P2X receptors. The present findings thus enhance our knowledge regarding the functional significance of TRPV1 and P2X receptors in airway afferent physiology and pharmacology. Capsaicin-sensitive vagal lung afferent fibres have been largely implicated in various airway diseases, such as airway hyper-reactivity, cough and bronchoconstriction (Lee & Pisarri, 2001), all of which may be related to excess production of ROS. The potential therapeutic effects of TRPV1 and P2X receptor antagonists in treating these ROS-related airway diseases are mostly unknown and thus require further investigation.

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