

Respiratory tract inflammation during the induction of chronic bronchitis in rats: role of C-fibres

N.C. Long, J. Abraham, L. Kobzik, E.A. Weller, G.G. Krishna Murthy, S.A. Shore

Respiratory tract inflammation during the induction of chronic bronchitis in rats: role of C-fibres. N.C. Long, J. Abraham, L. Kobzik, E.A. Weller, G.G. Krishna Murthy, S.A. Shore. ©ERS Journals Ltd 1999.

ABSTRACT: The hypothesis that chronic stimulation of C-fibres by inhaled irritants contributes to the inflammatory changes that occur during the development of chronic bronchitis was tested.

The effect of neonatal capsaicin pretreatment on the development of respiratory tract inflammation was examined in a rat model of chronic bronchitis induced by SO₂ exposure. Adult capsaicin- and vehicle-treated rats were exposed to SO₂ (250 parts per million (ppm) 5 h·day⁻¹) for one day, 2 weeks or 4 weeks. Nasal (NL), airway (AL) and bronchoalveolar (BAL) lavages were performed and the number and types of cells in the lavage fluids measured. SO₂-induced changes in ventilation were also measured on day 1 of SO₂ exposure and in the 3rd and 5th week of exposure.

In the vehicle-treated rats, neutrophils became elevated in NL after just one day of SO₂ exposure, in AL after 2 weeks, and in the BAL after 4 weeks. In comparison to vehicle animals, more neutrophils were recovered in the AL of capsaicin-treated rats after one day of SO₂ (p=0.012), and in the BAL after 2 or 4 weeks (p=0.004 and p=0.01, respectively). On day 1, SO₂ caused a transient increase in tidal volume and a sustained decrease in frequency that was not different in capsaicin- and vehicle-treated rats. With continued exposure, these ventilatory responses to SO₂ were attenuated in both groups of rats.

These data support the hypothesis that the presence of C-fibres limits or delays the inflammation that occurs during the development of chronic bronchitis induced by SO₂ exposure. The protective effect of C-fibres is not the result of ventilatory responses to stimulation of these afferents.

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Prolonged exposure of humans to respiratory irritants such as cigarette smoke can lead to the development of chronic bronchitis, a syndrome characterized by cough and mucous hypersecretion. Chronic bronchitis is also characterized by airway inflammation [1]. Models of chronic bronchitis, involving long-term exposure to high concentrations of SO₂ gas, have been developed in rats and dogs [2–8]. In these models, animals develop many of the histological changes associated with human chronic bronchitis: epithelial damage, mucous hypersecretion, and influx of inflammatory cells into the airway tissue and lumen. In rats, airway obstruction and increased airway responsiveness to inhaled aerosolized methacholine are also observed [8].

Inhaled irritants such as SO₂, cigarette smoke, acrolein, and formaldehyde, stimulate C-fibres, a class of unmyelinated sensory nerve fibres, which contain a variety of neuropeptides, including the tachykinins substance P (SP) and neurokinin A (NKA), as well as calcitonin gene-related peptide (CGRP) and nitric oxide. Stimulation of C-fibres causes responses that resemble many aspects of chronic bronchitis, including cough [9], mucous secretion [10] and airway narrowing [11]. Stimulation of these nerves also causes an inflammatory response known as

neurogenic inflammation, which involves increased airway blood flow [12], increased capillary permeability [13], and increased adherence of neutrophils to the airway vascular endothelium, the first step in the migration of these cells into the airways and lungs [14].

Based on the ability of agents known to induce chronic bronchitis to stimulate C-fibres, as well as the observed responses to C-fibre stimulation, it was hypothesized that these nerve fibres play a role in the pathogenesis of chronic bronchitis. To test this hypothesis, bronchitis was induced by chronic SO₂ exposure in normal rats and in rats treated with capsaicin neonatally. Neonatal treatment of rats with capsaicin destroys C-fibres and causes irreversible depletion of tachykinins in the tissues that receive C-fibre innervation [15].

In order to examine the role of C-fibres in the inflammation that occurs during the induction of bronchitis, capsaicin- and vehicle-treated rats were exposed either to air or SO₂ for 5 h·day⁻¹ for 1 day, 2 weeks or 4 weeks. Lavages (nasal, airway, and bronchoalveolar) were then performed and the numbers and types of cells in the lavage fluids measured to determine the magnitude and nature of the inflammatory changes due to SO₂ exposure. The results show that inflammation, as indicated by the presence of

Physiology Program, Harvard School of Public Health, Boston, MA, USA.

Correspondence: S.A. Shore
Physiology Program
Harvard School of Public Health
665 Huntington Ave.
Boston
MA 02115
USA
Fax: 1617 4323468

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lavage neutrophils, is greater, and progresses more rapidly down the respiratory tract in capsaicin- than in vehicle-treated rats. These results suggest that rather than contributing to the pathogenesis of chronic bronchitis, C-fibres actually protect the airways during induction of chronic bronchitis by SO₂ exposure.

High concentrations of SO₂ have been demonstrated to stimulate C-fibres, and stimulation of C-fibres results in rapid shallow breathing [16]. Such a breathing pattern could be expected to limit SO₂ penetration to the deep lung. Consequently, animals treated with neonatal capsaicin and lacking such C-fibre-mediated ventilatory reflexes might actually have had greater SO₂ exposure in the deep lung and concomitant increased injury and inflammation resulting in greater physiological changes. To examine this hypothesis, minute ventilation and its components, tidal volume and breathing frequency were measured, during air or SO₂ exposure in capsaicin and vehicle-treated rats. Because the development of bronchitis results in increased amounts of airway mucus, which may limit the penetration of SO₂ to nerve endings, ventilatory responses to SO₂ were examined on the first day of exposure and during the 3rd and 5th week of SO₂ exposure.

Methods

Animals

The methods used in this study were approved by the Harvard Medical Area Standing Committee on Animals. Pregnant female Sprague-Dawley rats were purchased from Charles River (Wilmington, MA, USA) and housed individually. The litters from these rats were treated with either capsaicin or vehicle 2 or 3 days after birth. The capsaicin-treated litters were injected subcutaneously with a mixture of 50 mg·kg⁻¹ capsaicin (Spectrum Chemical Mfg. Co., Gardena, CA, USA) dissolved in 10% ethanol, 10% Tween-80 and 80% saline. After the injection, the rat pups were placed in an open box into which 100% oxygen was flowing for 30 min before being returned to their mothers in their original cages. The vehicle-treated rats were injected with a mixture of 10% Tween-80 and 10% ethanol in saline, but otherwise treated identically. At the time the experiments were performed, the rats ranged in age from 3.5–8 months. Only the female rats were used in this study to minimize problems of interpretation consequent to animal growth over the 4–5 week period during which measurements were obtained. The mean body weight of the capsaicin and vehicle rats on the day that the lavages were performed was 259±13 g and 279±11 g (mean±SEM), respectively.

Assessment of tachykinin depletion by capsaicin

To confirm the efficacy of the capsaicin pretreatment, the trachea and lungs of one cohort of capsaicin and vehicle rats not exposed to SO₂ were assayed for SP and NKA. Rats were killed with an overdose of sodium pentobarbital. The trachea and lungs of each rat were immediately removed, frozen in liquid nitrogen and stored at -70°C until tachykinins were extracted and assayed by

solid phase enzyme-linked immunosorbent assay (ELISA), as previously described [17, 18]. The antibodies for both SP and NKA were purchased from Peninsula Labs (Belmont, CA, USA). The lower limit of detection of the SP and NKA assays were 3 fmol and 1 fmol respectively. The NKA antibody had a <3% cross-reactivity with SP, and Student's t-tests were used to compare levels of SP and NKA in the lungs and trachea of capsaicin and vehicle rats.

SO₂ exposure

Rats were placed in individual wire mesh cells in a 76 × 46 × 46 cm exposure chamber. The SO₂ concentration in the air of the chamber was 250 parts per million (ppm), and was monitored continuously by a pulsed fluorescent SO₂ analyser (Thermo Electron Series 43; Franklin, MA, USA). The condition of the rats and the SO₂ levels in the chamber were checked hourly throughout the exposure period. Capsaicin and vehicle rats were exposed to SO₂ at the same time and in the same chamber. The air-exposed controls were exposed in an identical chamber to filtered room air. When the animals were not being exposed to SO₂ or air, they were housed in group cages (4–6 rats·cage⁻¹) under a laminar flow hood.

Protocol

Rats were exposed either to air or 250 ppm SO₂ for 5 h·day⁻¹ for 1 day, 2 weeks or 4 weeks (5 days·week⁻¹). The concentration and duration of SO₂ exposure were chosen based on previous reports which indicate that this exposure protocol is sufficient to result in a syndrome similar to human chronic bronchitis over a period of 4–5 weeks [4, 5, 8, 19, 20]. Separate cohorts of rats were used for each of the exposure periods. The rats were lavaged either later the same day or on the day after exposure to air or SO₂. For measurements of ventilatory response, the animals were exposed to the same concentrations of SO₂ gas for 5 weeks.

Lavages

To assess the effect of SO₂ exposure on airway inflammation, and to assess its distribution along the airway tree, nasal (NL), airway (AL), and bronchoalveolar (BAL) lavages were performed on capsaicin and vehicle rats exposed to air or SO₂ for each of the three exposure periods. The AL, which consists of the first few millilitres of lavage fluid collected from the lungs, is considered to be a sample of cells from the central airways. RENNARD *et al.* [21] demonstrated that the first lavage, which they termed the bronchial lavage, contained a greater concentration of columnar epithelial cells than the subsequent lavages, suggesting that the first lavage samples the airways to a greater extent than the distal lung. A greater proportion of epithelial cells was observed in the present study in the AL than in the BAL of SO₂-exposed rats. In the AL of SO₂ exposed rats, epithelial cells ranged 22–56% of total cells depending on the animal treatment and the week of exposure. In BAL, epithelial cells were never >2.8% of

total cells. Both AL and BAL were performed because a study of patients with chronic bronchitis by THOMPSON *et al.* [22] showed a more pronounced increase in the number of neutrophils in bronchial lavage samples, in comparison to distal lavage samples.

Just before the lavages were performed, the rats were killed by injection of a lethal dose of sodium pentobarbital and a tracheostomy was created. To perform the AL, 1 mL of sterile saline was instilled into the trachea by a syringe, and immediately aspirated. To perform the BAL, 5 mL of fluid were instilled and then aspirated while the chest was massaged. This was repeated 10 times to make a final volume of 55 mL of BAL fluid. Finally, to perform the NL, the trachea was clamped off with a haemostat, then 3 mL of sterile saline were instilled into one nostril, while the liquid was simultaneously withdrawn from the other nostril.

The lavage fluids were centrifuged at $400 \times g$ at 4°C for 10 min. The pelleted cells were resuspended in saline, and the number and type of cells were determined as follows. A well-mixed sample from each lavage return was cytocentrifuged onto microscope slides (Cytospin 2; Shandon Southern Instruments, Sewickley, PA, USA), air dried, and stained with Wright-Giemsa stain (VWB Stat Stain, Brisbane, CA, USA). From these slides, a differential count of 600 cells was performed. The number and size of cells found in a separate aliquot (100 μL for NL and AL, and 50 μL for BAL) was analysed by a cell counter (Elzone - Particle Data, Elmhurst, IL, USA). The number of cells $>6 \mu\text{m}$ in diameter, along with the differential counts were used to calculate the total number of neutrophils, epithelial cells and macrophages in the lavage fluids.

Statistical analysis of lavage data

In this study, animals that were pretreated with either capsaicin or vehicle were exposed to either air or SO_2 . It was of interest to determine whether capsaicin pretreatment altered the response to SO_2 , as characterized by changes in NL, AL, and BAL numbers of neutrophils and AL numbers of epithelial cells. The analysis was focused on these four primary outcomes, based on previously reported data in a canine model of SO_2 -induced bronchitis [7] that indicated marked increases in neutrophils and epithelial cells with SO_2 exposure. Increased numbers of neutrophils are also the predominant cellular change observed in airway and lung lavage fluid from human chronic bronchitic patients [22, 23]. To determine if capsaicin pretreatment alone caused any changes in these parameters, lavage fluid was also analysed from air exposed animals.

Because the present data set included animals that were not expected to have significant changes in their lavage parameters (*i.e.* the air-exposed capsaicin and vehicle rats), an analysis was devised to evaluate the results without the findings being diluted by the presence of these values, as would have occurred with a two-way analysis of variance (ANOVA). Specifically, an ANOVA was performed to assess, in the primary outcomes, 1) whether SO_2 exposure caused inflammatory changes in the lavage fluids of capsaicin and vehicle rats, 2) whether there were baseline differences in these inflammatory parameters in capsaicin and vehicle rats exposed to air, and 3) whether there was a

difference between the response of capsaicin and vehicle rats to SO_2 exposure. A multivariate analysis of variance (MANOVA) model was also suitable to evaluate these three objectives across all of the primary outcomes. In order to control for differences in the total number of cells recovered in the AL and BAL, which may have occurred by the trapping of cells in the mucus, neutrophils were also measured and compared as a fraction of total cells. To normalize the distribution of the data, the base 10 log of the number or percentage of cells was used for all of the statistical analyses described above. Data reported in the figures and tables are the mean \pm SEM of the base 10 log of the number of cells. Multiple comparisons were not corrected for.

In addition to the primary outcomes listed above, epithelial cell numbers were also measured in the NL and BAL, and macrophage numbers in all of the lavage fluids. However, the numbers of these cells were not included in the MANOVA, since, based on previous studies [7, 22, 24], the numbers of these cells were not expected to change. These data are included because the relatively unchanging values show that the lavages were performed in a consistent manner. The p-values for these outcomes in the same comparisons as described above are reported.

Body weight

The body weights of the rats were measured before and after the four-week exposure period. The body weight data were analysed by ANOVA, and are reported as mean \pm SEM.

Monitoring ventilation

In order to determine whether differences in the ventilatory response to SO_2 between capsaicin and vehicle rats might have contributed to differences in the inflammatory response, ventilation was measured during SO_2 exposure. For these experiments, the animals were exposed chronically to SO_2 gas (250 ppm, $5 \text{ h}\cdot\text{day}^{-1}$, $5 \text{ days}\cdot\text{week}^{-1}$ for 5 weeks). Ventilation was monitored on the first day of exposure and once during the 3rd and the 5th week. When ventilation was not being monitored, the animals were placed in small wire mesh cages as described above. For measurements of ventilation, rats were placed in a plexiglass restraining tube that served as a nose-only exposure flow plethysmograph. The tube was fitted with a silicone rubber gasket designed to fit snugly around the animal's neck and seal the head from the rest of the body. Once the animal was in the tube, a large piston was moved into place behind the animal. The piston served to prevent the animal from moving and to seal the body chamber from the outside air. Air displaced at the body surface as the animal breathed, passed across a pneumotachograph (8 mm diameter fitted with a screen filter) attached to a differential pressure transducer (Model 163PC01D75; Omega Engineering Inc., CT, Stamford, USA). The resulting flow signal was analysed by a computer program (BUXCO, Troy, NY, USA) which computed minute ventilation (V'_E), tidal volume (V_T), respiratory frequency (f_R), inspiratory (I), and expiratory (E) time on a breath-by-breath basis and reported the average of each of these values every minute. The cranial end of the tube was inserted into the

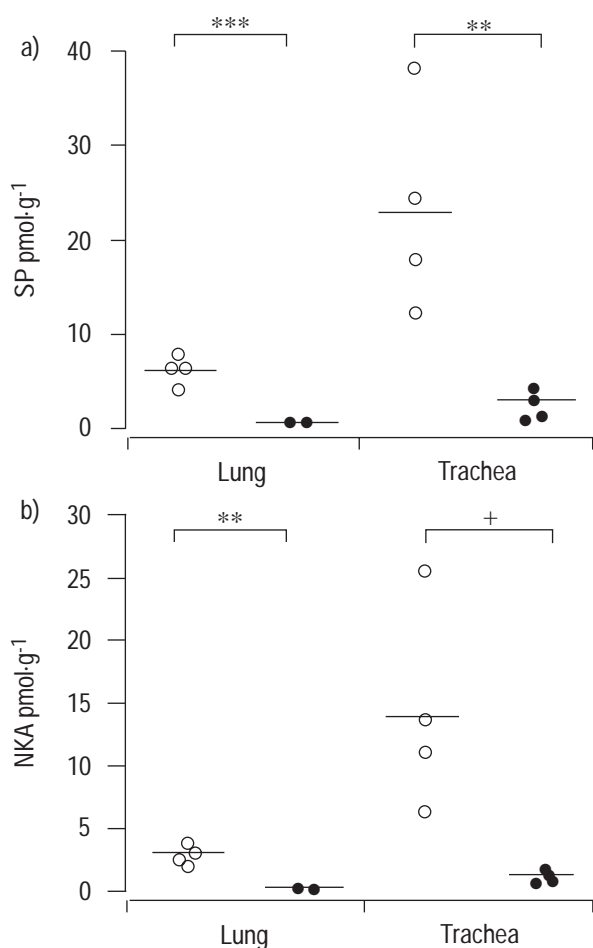


Fig. 1. – Concentration of substance P (SP; a) and neurokinin A (NKA; b) in the lung and trachea of capsaicin- (●) and vehicle- (○) treated rats, as analysed by enzyme-linked immunosorbent assays. Each point represents data from one rat. **, $p < 0.01$; ***, $p < 0.001$; +, $p < 0.02$, differences between vehicle and capsaicin for each tissue.

exposure chamber, and after a 45-min adaptation period, the animals were exposed either to filtered air or to SO₂ (250 ppm for 5 h). For each ventilatory parameter, mean values over the 20 min immediately prior to SO₂ exposure were determined for each animal, and data are reported as percentage changes from those values. In each animal, 5 min averages around the time point 10 min after initiation of SO₂, and at every 20 min thereafter were computed. The effect of SO₂ exposure or capsaicin treatment on these parameters was then assessed by repeated measures ANOVA.

Results

Assessment of tachykinin depletion by capsaicin

The concentration of SP and NKA extracted from the lungs and tracheas were significantly lower in capsaicin than vehicle rats (fig. 1), confirming the efficacy of the treatment.

Effect of SO₂ exposures

Tables 1–3 show the number of neutrophils, epithelial cells, and macrophages in the NL, AL and BAL of vehicle and capsaicin rats exposed to air or SO₂ for 1 day, 2 weeks, or 4 weeks. MANOVAs using neutrophil numbers in the NL, AL and BAL and epithelial cell numbers in the AL as outcome indicators, resulted in an overall significance of SO₂ exposure at each time point (1 day $p = 0.0005$; 2 weeks $p = 0.0001$; 4 weeks $p = 0.0001$). ANOVAs were used to assess the time course of SO₂-induced changes in each of the parameters ("SO₂ effect" in tables 1–3). The results of this analysis are discussed separately below for the vehicle and capsaicin rats.

Table 1. – The number of cells in the nasal lavage fluid of capsaicin- and vehicle-treated rats

	Air		SO ₂		p-values			
	Vehicle	Capsaicin	Vehicle	Capsaicin	SO ₂ effect		Capsaicin effect	
					Vehicle	Capsaicin	Air	SO ₂
1 Day								
n	7	7	13	12				
Neutrophils	4.46±0.36	4.68±0.31	6.02±0.10	6.27±0.12	0.0001	0.0001	0.54	0.32
Epithelial	5.38±0.20	4.68±0.31	5.46±0.15	5.41±0.02	0.80	0.95	0.99	0.84
Macrophages	4.31±0.23	3.69±0.27	5.10±0.15	5.18±0.14	0.007	0.0001	0.06	0.75
2 weeks								
n	5	6	11	11				
Neutrophils	5.22±0.14	5.44±0.19	6.31±0.13	6.99±0.15	0.0001	0.0001	0.43	0.0009
Epithelial	6.13±0.14	5.86±0.06	5.92±0.12	6.10±0.12	0.27	0.19	0.21	0.23
Macrophages	5.60±0.30	5.07±0.34	5.20±0.15	5.45±0.26	0.30	0.30	0.23	0.42
4 weeks								
n	5	4	11	7				
Neutrophils	4.35±0.36	4.31±0.37	6.51±0.11	6.84±0.21	0.0001	0.0001	0.35	0.25
Epithelial	5.42±0.33	5.87±0.19	5.32±0.09	5.71±0.15	0.69	0.56	0.14	0.08
Macrophages	4.45±0.38	4.72±0.28	4.93±0.14	5.29±0.19	0.13	0.12	0.48	0.20

Data are the log (base 10) of the number of neutrophils, epithelial cells and macrophages in the lavage fluids of capsaicin- or vehicle-treated rats exposed to air or SO₂ for 1 day, 2 weeks or 4 weeks. Values given are mean±SEM. The fluids were analysed to determine whether SO₂ exposure altered the number of cells recovered from vehicle- and capsaicin-treated rats ("SO₂ effect"), and whether capsaicin treatment altered the numbers of cells recovered from air and SO₂ exposed rats ("Capsaicin effect").

Table 2. – Number of cells in the airway lavage fluid of capsaicin- and vehicle-treated rats

	Air		SO ₂		p-values			
	Vehicle	Capsaicin	Vehicle	Capsaicin	SO ₂ effect		Capsaicin effect	
					Vehicle	Capsaicin	Air	SO ₂
1 Day								
n	7	7	14	14				
Neutrophils	3.83±0.25	3.89±0.25	4.23±0.20	4.87±0.14	0.86	0.002	0.86	0.001
Epithelial	4.25±0.11	4.53±0.27	5.08±0.19	5.30±0.23	0.02	0.03	0.47	0.33
Macrophages	5.74±0.07	5.65±0.9	5.34±0.08	5.24±0.08	0.003	0.002	0.56	0.32
2 weeks								
n	5	6	12	12				
Neutrophils	2.94±0.19	2.91±0.23	4.92±0.16	5.03±0.15	0.0001	0.0001	0.92	0.61
Epithelial	4.84±0.06	4.71±0.16	5.62±0.14	5.39±0.14	0.002	0.004	0.64	0.20
Macrophages	4.96±0.16	5.19±0.14	5.39±0.11	5.25±0.10	0.03	0.74	0.28	0.35
4 weeks								
n	6	7	14	10				
Neutrophils	3.09±0.24	3.58±0.29	5.30±0.13	4.71±0.11	0.0001	0.0003	0.12	0.17
Epithelial	4.28±0.20	4.55±0.22	5.03±0.14	4.72±0.23	0.006	0.62	0.48	0.07
Macrophages	5.54±0.07	5.59±0.12	5.27±0.21	4.96±0.19	0.27	0.008	0.083	0.08

Data and statistical analysis as for table 1.

Vehicle rats. In comparison to air-exposed animals, the number of neutrophils in the NL (table 1) was significantly elevated after exposure to SO₂ for just one day and remained elevated at 2 and 4 weeks of SO₂ exposure ($p \leq 0.0001$ for each time point). No significant changes were seen in NL epithelial cell counts. A significant increase in NL macrophage counts was seen at 1 day, but not at 2 or 4 weeks. In the AL (table 2), neutrophil numbers were not affected by a single day of SO₂ exposure, but were significantly increased after 2 or 4 weeks of SO₂ exposure ($p \leq 0.0001$ at both time points). The number of epithelial cells in the AL fluid of SO₂-exposed rats was significantly increased compared to the air-exposed rats at all time points examined (1 day, $p=0.02$; 2 weeks, $p=0.002$; 4 weeks, $p=0.006$). The number of macrophages in the AL was decreased after 1 day of SO₂, increased after 2 weeks, perhaps as a result of the trapping of these cells in mucus, and unchanged at 4 weeks. Vehicle rats showed sig-

nificant changes in BAL levels of neutrophils only at 4 weeks of SO₂ exposure ($p=0.007$) and did not show any significant changes in epithelial cells or macrophages.

Capsaicin rats. As in the vehicle rats, the NL from animals exposed to SO₂ for a single day contained significantly more neutrophils than did NL from air-exposed animals, and NL neutrophils remained elevated at 2 and 4 weeks ($p \leq 0.0001$ for each time point). Likewise, NL macrophages were elevated at day 1, but not at any other timepoint. Unlike the vehicle rats, in the capsaicin rats, a single day of SO₂ resulted in a significant increase in neutrophils in the AL ($p=0.0002$). This increase in AL neutrophil numbers persisted after 2 and 4 weeks of SO₂ exposure ($p=0.0001$ and $p=0.0003$, respectively). The number of epithelial cells in the AL fluid was also elevated after 1 day and 2 weeks of SO₂ exposure ($p=0.03$ and $p=0.004$, respectively). Macrophage numbers were lower after 1

Table 3. – The number of cells in the bronchoalveolar lavage fluid of capsaicin- and vehicle treated rats

	Air		SO ₂		p-values			
	Vehicle	Capsaicin	Vehicle	Capsaicin	SO ₂ effect		Capsaicin effect	
					Vehicle	Capsaicin	Air	SO ₂
1 Day								
n	7	7	14	13				
Neutrophils	4.38±0.09	5.12±0.21	4.80±0.08	5.02±0.17	0.07	0.66	0.009	0.23
Epithelial	4.81±0.22	5.01±0.20	4.96±0.15	5.14±0.19	0.63	0.67	0.58	0.45
Macrophages	7.1±0.08	7.17±0.32	7.19±0.08	7.08±0.06	0.43	0.45	0.63	0.22
2 weeks								
n	5	6	12	12				
Neutrophils	4.42±0.09	4.91±0.23	4.92±0.12	5.53±0.17	0.06	0.015	0.10	0.004
Epithelial	5.30±0.10	5.77±0.12	5.18±0.13	5.38±0.18	0.63	0.11	0.12	0.31
Macrophages	7.07±0.09	7.23±0.10	7.13±0.04	7.17±0.05	0.53	0.45	0.13	0.57
4 weeks								
n	6	7	14	11				
Neutrophils	4.55±0.11	4.61±0.16	5.29±0.18	5.87±0.15	0.007	0.0001	0.84	0.01
Epithelial	5.30±0.63	5.24±0.13	5.37±0.13	5.23±0.13	0.72	0.97	0.78	0.40
Macrophages	6.98±0.03	7.07±0.05	6.91±0.11	7.13±0.08	0.60	0.68	0.58	0.07

Data and statistical analysis as for table 1.

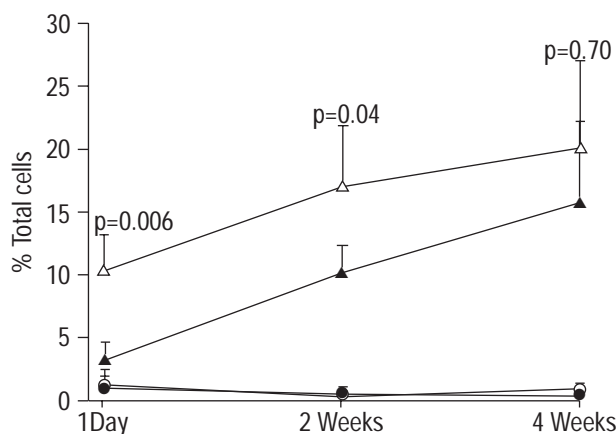


Fig. 2. – Percentage of neutrophils out of total number of cells collected in the airway lavage fluid after exposure to SO₂ or air for 1 day, 2 weeks or 4 weeks. The p-values represent differences between the vehicle-treated SO₂-exposed rats and the capsaicin-treated SO₂-exposed rats. ●: vehicle-air; ○: capsaicin-air; ▲: vehicle-SO₂; △: capsaicin-SO₂.

day and 2 weeks of SO₂ exposure. The number of neutrophils in the BAL fluid of capsaicin rats was significantly increased earlier than in the vehicle rats, *i.e.* at 2 weeks of SO₂ exposure ($p=0.015$) (*versus* 4 weeks in the vehicle rats), and remained elevated at 4 weeks ($p=0.0001$) of SO₂ exposure. No changes in BAL epithelial cell or macrophage counts were seen.

Effect of capsaicin treatment

Air exposure. The data from rats exposed to air were analysed to determine if there were any baseline differences between the capsaicin and vehicle rats ("capsaicin effect-air" in tables 1–3). The only significant difference between the two groups was in the number of neutrophils recovered from the BAL fluid after one day of air exposure (table 3). Even though statistically significant, this difference was probably not biologically significant, as these neutrophils represented <1% of the total number of BAL cells in either group of animals after air exposure. Therefore, it was concluded that there were no important baseline differences between capsaicin and vehicle and that it was not necessary to adjust for baseline differences in the subsequent analyses of SO₂-exposed rats.

SO₂ exposure. The data from rats exposed to SO₂ were analysed to determine if capsaicin and vehicle rats responded differently to this inhaled irritant ("capsaicin effect-SO₂" in tables 1–3). After exposure to SO₂, the NL from the capsaicin rats consistently contained more neutrophils than did the NL from vehicle rats (table 1), although this difference was statistically significant only in the rats exposed to SO₂ for 2 weeks ($p=0.0009$). No differences were seen in the numbers of epithelial cells or macrophages.

Capsaicin rats had significantly more neutrophils in AL than vehicle rats ($p=0.012$) after one day of SO₂ exposure (table 2). However, this difference was no longer apparent after 2 or 4 weeks of SO₂. In order to control for differences in the total number of cells recovered in the AL which may have occurred by trapping of cells in

mucus, neutrophils were also measured and compared as a fraction of total cells (fig. 2). The percentage of neutrophils (analysed as the base 10 log of the per cent cells) in the AL fluid of capsaicin rats exposed to SO₂ for 1 day and 2 weeks was significantly higher than that in vehicle rats ($p=0.006$ and $p=0.04$, respectively), but the difference was no longer significant at 4 weeks of exposure. Although both groups of animals had an increased number of epithelial cells in their AL after SO₂ exposure, there was no significant difference in these cell numbers between the capsaicin and vehicle rats at any time point. No significant differences in macrophage numbers were seen.

There was no significant difference in BAL neutrophils between capsaicin and vehicle rats exposed to SO₂ for 1 day. However, after 2 or 4 weeks of SO₂ exposure, neutrophils were significantly higher in the BAL fluid from capsaicin compared to vehicle rats ($p=0.004$ at 2 weeks, and $p=0.01$ at 4 weeks) (table 3). When expressed as a percentage of total BAL cells, BAL neutrophils from capsaicin and vehicle were significantly different from each other at 2 weeks ($p=0.007$), but not at 4 weeks (fig. 3). No differences were seen in the number of epithelial cells or macrophages.

Body weight changes

The body weight of the rats before exposure to air or SO₂, and the change in weight after the 4-week exposure period are shown in table 4. Prior to exposure, there was no significant difference in the body weight of the capsaicin and vehicle rats. Both groups of rats lost weight over the course of the SO₂ exposure period, but this weight change was not significantly different from that seen in the air-exposed animals.

Ventilatory responses to SO₂

Table 5 lists baseline ventilatory parameters of capsaicin and vehicle rats measured in the 20-min period

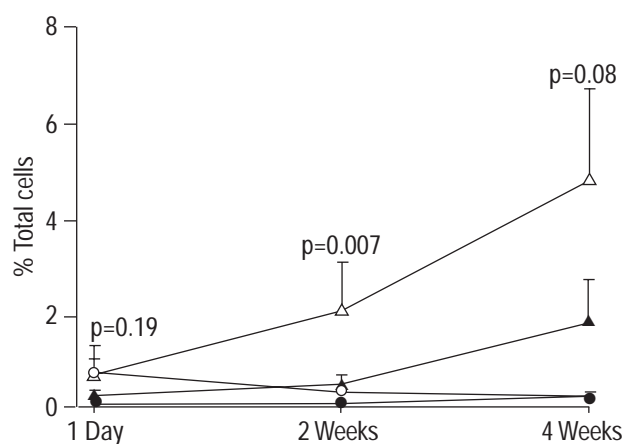


Fig. 3. – Percentage of neutrophils out of total number of cells collected in the bronchoalveolar lavage fluid after exposure to SO₂ or air for 1 day, 2 weeks or 4 weeks. The p-values represent differences between the vehicle-treated SO₂-exposed rats and the capsaicin-treated SO₂-exposed rats. ●: vehicle-air; ○: capsaicin-air; ▲: vehicle-SO₂; △: capsaicin-SO₂.

Table 4. – Change in body weight after 4 weeks of exposure to air or SO₂

Pretreatment	n	Pre-exposure weight g	Δ Body weight (4 Weeks) g
Air			
Vehicle	6	289±25	-1±12
Capsaicin	7	265±28	+7±10
SO ₂			
Vehicle	14	274±11	-9±5
Capsaicin	11	254±11	-13±7

Values given are mean±SEM.

prior to SO₂. Capsaicin pretreatment had no significant effect on baseline ventilatory parameters at any time point examined. Baseline f_R was significantly reduced at week 3 ($p<0.02$) and week 5 ($p<0.01$) compared to day 1 in both groups. The decrease in frequency was primarily the result of a significant increase in t_E at week 3 ($p<0.001$) and week 5 ($p<0.01$) compared to day 1. There was no significant effect of week of exposure on the baseline values of any other ventilatory parameter.

Figure 4 shows the effect of the first SO₂ (250 ppm) or filtered air exposure on ventilatory parameters. SO₂ exposure caused a transient increase in V_T ($p<0.02$) and a sustained decrease in f_R ($p<0.0001$), but no significant change in V_E compared to air-exposed control rats. There was no significant effect of capsaicin treatment on any of these parameters. The decrease in frequency induced by SO₂ exposure was the result of significant increases in both t_I ($p<0.004$) and t_E ($p<0.003$). The increase in t_I predominated in vehicle rats, whereas the increase in t_E predominated in capsaicin rats.

Figure 5 shows the effect of chronic SO₂ exposure on V_E , V_T , and f_R . Because no significant effect of drug (vehicle or capsaicin) treatment on these parameters was observed, data from both groups of rats were combined in order to demonstrate the effect of chronic SO₂ exposure. There was a significant effect of exposure time on SO₂ induced changes in f_R ($p<0.0005$) and V_T ($p<0.01$). Chronic exposure to SO₂ for 3 or 5 weeks significantly attenuated both the sustained decrease in f_R and the transient increase in V_T that was observed during SO₂ exposure on the first exposure day. V_E was not significantly affected by SO₂ exposure on any exposure day.

Discussion

These results demonstrate that exposure to SO₂ gas causes neutrophil influx and epithelial sloughing into the lavage fluids of rats. The inflammatory response to SO₂ progressed deeper into the respiratory tract with increased length of exposure. Despite the fact that C-fibres and their tachykinin neuropeptides have the capacity to cause inflammation, and are purported to be mediators of neurogenic inflammation, no evidence was found in this study that SO₂ stimulation of C-fibres is responsible for the inflammatory changes associated with SO₂-induced chronic bronchitis. In contrast, it was observed that rats which had been depleted of tachykinins by capsaicin pretreatment actually showed a greater degree of respiratory tract inflammation than did the vehicle rats.

The data collected in this study allowed the tracing of the progression of airway responses to SO₂, both anatomically and over time. Elevated neutrophil counts appeared in the NL fluid after one day of exposure, in the AL fluid after 2 weeks, and finally the BAL fluid at 4 weeks. The finding that SO₂ had its greatest effects in the nose and large airways is consistent with what would be expected from a highly soluble gas such as SO₂ [25], and with studies that have shown that SO₂ is removed from inhaled air primarily in the upper respiratory tract [26]. Hence the lower airways and alveoli are likely to have received a much smaller dose of SO₂. The progression of the inflammation down the respiratory tract with time may represent continuous airway injury and repair caused by the cumulative effect of these smaller doses.

Exposure to SO₂ for 1 day, 2 weeks or 4 weeks caused sloughing of epithelial cells into the AL (table 2), indicating a toxic effect of the SO₂ on the epithelium, as previously described [27]. The present study found that the epithelial cells recovered in the lavage fluids of rats exposed to SO₂ for 2 or 4 weeks often lacked cilia, suggesting that the epithelial cells themselves underwent morphological changes over the course of the exposure period. These findings are consistent with work by LAMB and REID [4], who found severe damage to airway epithelium after acute exposure to 400 ppm SO₂, followed by some degree of regeneration, characterized by a thicker than normal epithelium, with sparse, stunted cilia after 6 weeks. The investigators proposed that these changes in the epithelial lining made it more resistant to injury. They also speculated that the thickening of the

Table 5. – Baseline ventilatory patterns of capsaicin- and vehicle-treated rats measured immediately prior to SO₂ exposure

	V_E mL·min ⁻¹	V_T mL	f_R breaks·min ⁻¹	t_I s	t_E s
Vehicle					
Day 1	142±15	1.20±0.21	129±15	0.256±0.049	0.276±0.041
Week 3	128±6	1.35±0.15	102±11	0.247±0.026	0.400±0.034
Week 5	135±10	1.39±0.15	100±10	0.301±0.046	0.340±0.019
Capsaicin					
Day 1	138±22	1.22±0.16	116±9	0.293±0.043	0.287±0.025
Week 3	122±8	1.37±0.08	93±8	0.338±0.051	0.391±0.033
Week 5	142±8	1.63±0.09	89±6	0.327±0.031	0.394±0.033

Results are mean±SEM of data from six vehicle and six capsaicin pretreated rats in the 20 min period immediately prior to SO₂ exposure. Rats were studied on the first day of SO₂ exposure and in the 3rd and 5th week of exposures. Exposures were to 250 parts per billion SO₂, 5 h·day⁻¹, 5 day·wk⁻¹. V_E : minute ventilation; V_T : tidal volume; f_R : respiratory frequency; t_I , t_E : inspiratory and expiratory times, respectively.

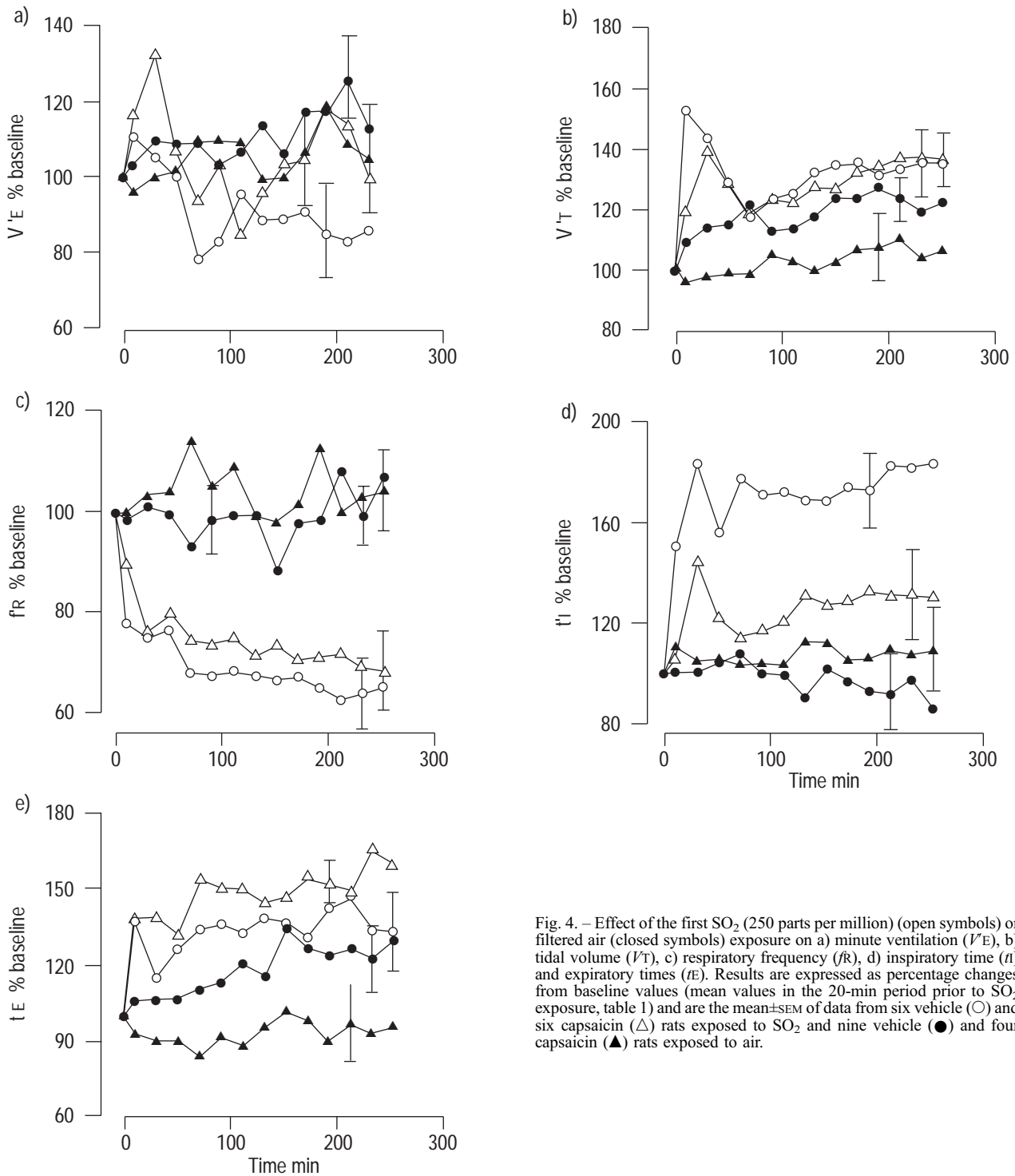


Fig. 4. – Effect of the first SO₂ (250 parts per million) (open symbols) or filtered air (closed symbols) exposure on a) minute ventilation ($V'E$), b) tidal volume ($V'T$), c) respiratory frequency (fR), d) inspiratory time ($t'I$) and expiratory times ($t'E$). Results are expressed as percentage changes from baseline values (mean values in the 20-min period prior to SO₂ exposure, table 1) and are the mean \pm SEM of data from six vehicle (\circ) and six capsaicin (Δ) rats exposed to SO₂ and nine vehicle (\bullet) and four capsaicin (\blacktriangle) rats exposed to air.

mucus blanket over the course of the exposure period might protect the regenerating epithelial cells. No differences were observed in the number of epithelial cells recovered from lavage fluid of capsaicin or vehicle rats. These observations suggest that the acute toxic effect of SO₂ on epithelial cells is not influenced by neurotransmitters released from C-fibres.

To the authors' knowledge, the present study is the first systematic analysis of lavage fluid collected during the

course of the development of chronic bronchitis by SO₂ exposure in rats. The finding of an increased number of neutrophils, with little change in the number of macrophages or lymphocytes in the lavage fluids matches observations made by SHORE *et al.* [7], who used the same SO₂ model in dogs. An increase in neutrophils is also the most pronounced change observed in the lung and airway lavage fluid of patients with chronic bronchitis [22, 23]. In contrast, tissue sections of airways from patients with

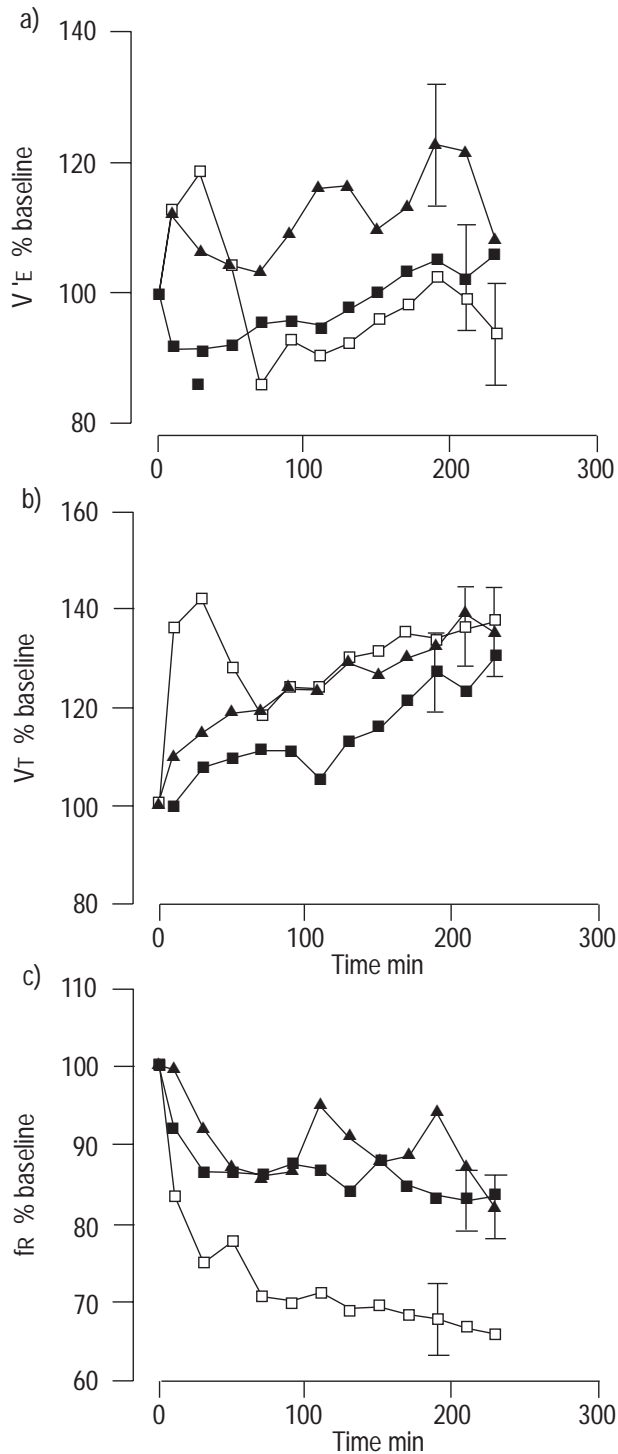


Fig. 5. – Time course of the effect of SO_2 exposure on a) minute ventilation (V_E), b) tidal volume (V_T), and c) respiratory frequency (f_R). Six vehicle and six capsaicin rats were chronically exposed to SO_2 gas (250 parts per million, 5 h-day⁻¹, 5 day-week⁻¹ for 5 weeks). Values shown are mean \pm SEM. On the first day of SO_2 exposure and once during the 3rd and 5th week of exposure, the animals were placed in nose only exposure tubes that served as body plethysmographs during the SO_2 exposure. Results are expressed as percentage changes from baseline values (mean values for the 20-min period immediately prior to SO_2 exposure; table 5). Because no significant effect of drug (vehicle or capsaicin) treatment on these parameters was observed, data from both groups of rats were combined in order to demonstrate the effect of chronic SO_2 exposure.

chronic bronchitis demonstrate infiltration by lymphocytes and macrophages [24]. The airway tissue of these rats was not examined, but in dogs exposed to SO_2 , subepithelial mononuclear cell infiltration is observed [6]. One explanation for the differences in the nature of the inflammatory cells in the airway lumen *versus* the airway tissue is that there are differences in the nature of the chemotactic factors and adhesion molecules expressed by epithelial *versus* subepithelial cells.

It was found that inflammation was greater, and progressed more rapidly down the respiratory tract of capsaicin than of vehicle rats. These findings are consistent with an earlier report from the authors' laboratory using the same model of chronic bronchitis [19]. This study showed that while SO_2 exposure increases the pulmonary responsiveness of both capsaicin and vehicle rats to inhaled aerosolized methacholine, the change in responsiveness was greater in the capsaicin rats. In addition, capsaicin rats had increased smooth muscle in their airway walls, and increased tracheal contractility, which may contribute to the increase in airway responsiveness. The enhancement of these changes in the structure and function of the airways could result, in part, from the increased inflammatory response that occurs in the capsaicin rats (tables 1–3).

Studies using other models of inflammation have shown similar alterations in the inflammatory response to irritants or infectious agents in capsaicin-treated animals. It was observed that the inflammatory response to the intratracheal instillation of endotoxin was greater in capsaicin than vehicle rats [28]. Other investigators have reported a greater degree of pulmonary or airway inflammation associated with infection with *Mycoplasma pulmonis* or with inhalation of hydrogen sulphide gas or ozone in capsaicin *versus* vehicle rats (for a review see [29]).

There are several possible explanations for the higher degree of pulmonary inflammation seen in capsaicin rats after SO_2 exposure. Firstly, SO_2 stimulates C-fibres [16] which in the lungs evokes a variety of physiological responses that may protect the lung from damage by noxious agents. For example, stimulation of C-fibres increases mucous secretion, ciliary beat frequency, and cough [10, 30], which would help to dilute and clear the irritant from the lung. C-fibre stimulation also has central effects such as the induction of a rapid shallow breathing pattern [16], which might tend to limit penetration of the gas into the lungs. However, the present results indicate that ventilatory responses to SO_2 stimulation of C-fibres is not an important determinant of the more pronounced inflammatory response and the greater increase in airway responsiveness [6] observed in capsaicin rats. In the vehicle rats, the initial response to SO_2 exposure was not rapid shallow breathing, but rather a breathing pattern characterized by slower and deeper breaths (fig. 4), and this response was not different in rats treated neonatally with capsaicin and lacking C-fibres.

A second factor which could contribute to the greater degree of inflammation seen in capsaicin rats is that animals that lack C-fibres may be less able to resolve tissue injury, resulting in elevated or prolonged release of inflammatory mediators, including those that are chemotactic for neutrophils. The concept that capsaicin rats are less able to resolve the airway injury induced by SO_2 exposure is supported by studies suggesting that SP is

involved in wound healing [31, 32]. SP, NKA, CGRP and nitric oxide, all of which are released by C-fibres, have a number of effects that could contribute to the resolution of tissue injury including synthesis of connective tissue, proliferation of epithelial cells, and increases in blood flow to the airways [12–32, 33].

Even though SO₂ has been shown to stimulate C-fibres and stimulation of C-fibres has been shown to result in rapid shallow breathing [16], this rapid shallow breathing was not observed in naive rats exposed for the first time to SO₂ (fig. 4). High concentrations of SO₂ have also been shown to inhibit slowly adapting stretch receptors (SARs) [34]. It is likely that the latter effect was dominant in altering the pattern of breathing, because the increase in \dot{V}_T and prolongation of t_I , which were the main consequences of acute SO₂ exposure in vehicle rats, are consistent with changes that occur following inhibition of the Hering–Breuer inflation reflex by which SARs normally terminate a breath [34]. Laryngeal receptors with responses similar to SARs are also inhibited by SO₂ [35] and may have contributed to the observed effects of SO₂ on \dot{V}_T and the timing of breathing. The observation that in capsaicin animals, increases in t_E predominated over increases in t_I despite similar changes in f_R , suggests that SO₂ stimulation of C-fibres does modify the breathing pattern, but such changes are unlikely to have had important effects on the ultimate delivery of SO₂ to the respiratory tract.

After 3 or 5 weeks of SO₂ exposure, the ventilatory response to SO₂ exposure was markedly diminished compared to the first day of exposure (fig. 5). It is possible that the increased presence of mucus in the airways [8] of animals exposed chronically to SO₂ diluted the gas to such an extent as to prevent it reaching and inhibiting the activity of SARs. It is also possible that chronic airway injury induced by SO₂ exposure caused a more permanent damage to these receptors.

In summary, these data show that exposure to high concentrations of SO₂ causes epithelial sloughing and inflammation of the respiratory tract. The inflammatory response progressed along the respiratory tract over the course of the exposure period, and more inflammation appeared earlier in the capsaicin rats. These data support the hypothesis that the presence of C-fibres limits or delays the inflammation that occurs during the development of chronic bronchitis induced by SO₂ exposure. The mechanism of this response is not known, but does not appear to involve stimulation of ventilatory reflexes.

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