Carbon dioxide enhances substance P-induced epithelium-dependent bronchial smooth muscle relaxation in Sprague–Dawley rats

Tamer Y. El Mays, Mahmoud Saifeddine, Parichita Choudhury, Morley D. Hollenberg, and Francis H.Y. Green

Abstract: Hypocapnia and hypercapnia constrict and relax airway smooth muscle, respectively, through pH- and calcium (Ca^{2+})-mediated mechanisms. In this study we explore a potential role for the airway epithelium in these responses to carbon dioxide (CO_2). Contractile and relaxant responses of isolated rat bronchial rings were measured under hypocapnic, eucapnic, and hypercapnic conditions. Substance P was added to methacholine precontracted bronchial rings with and without epithelium. The role of Ca^{2+} was assessed using Ca^{2+}-free solutions and a Ca^{2+} channel blocker, nifedipine. The effects of pH were assessed in solutions with HEPES buffer. Hypocapnic challenge increased the organ bath's pH and increased bronchial smooth muscle resting tension. This effect was abolished with HEPES buffer and partially inhibited by nifedipine. Hypocapnic conditions suppressed substance P-induced epithelium-dependent relaxation, whereas hypercapnia augmented the response. The epithelial hypocapnic effect was pH dependent, whereas the hypercapnic effect was pH independent. CO_2 had no effect on the epithelial independent smooth muscle agonists methacholine and isoproterenol. In conclusion our data indicate that, in addition to the effects of pH and Ca^{2+}, CO_2 affects airway smooth muscle by a pH-independent, epithelium-mediated mechanism. These findings could potentially lead to new treatments for asthma involving CO_2-sensing receptors in the airways.

Key words: asthma, hypercapnia, hypocapnia, airway smooth muscle, carbon dioxide, pH, calcium channel, epithelium, substance P.

Résumé : L'hypocapnie et l'hypercapnie provoquent la contraction et la relaxation du muscle lisse bronchique respectivement, par des mécanismes véhiculés par le pH et le calcium (Ca^{2+}). Dans la présente étude, nous examinons si l'épithélium bronchique joue un rôle dans la contraction et la relaxation induite par le dioxyde de carbone (CO_2). Nous avons mesuré les réponses d'anneaux bronchiques isolés de rats dans des conditions d'hypocapnie, de normocapnie et d'hypercapnie. Nous avons ajouté de la substance P aux anneaux bronchiques précontractés par la métacholine, en absence et en présence d'épithélium. Nous avons évalué le rôle du Ca^{2+} en utilisant des solutions sans Ca^{2+} et un bloqueur de canaux calciques, nifédipine. Nous avons évalué les effets du pH dans des solutions tampon HEPES. L'épreuve hypocapnique a augmenté le pH du bain d'organes ainsi que la tension de repos du muscle lisse bronchique. Le tampon HEPES a supprimé cet effet, et la nifédipine l'a partiellement inhibé. L'hypocapnie a supprimé la relaxation dépendante de l'épithélium induite par la substance P, alors que l'hypercapnie l'a augmentée. L'effet hypocapnique épithélial a été dépendant du pH, alors que l'effet hypercapnique a été indépendant de celui-ci. Le CO_2 n'a pas eu d'effet sur les agonistes du muscle lisse indépendant de l'épithélium, métacholine et isoprotérénol. Nos résultats indiquent que le CO_2 influe sur le muscle lisse bronchique par un mécanisme, véhiculé par l'épithélium, indépendant du pH. Ces résultats pourraient mener à l'élaboration de nouveaux traitements de l'asthme impliquant les récepteurs sensibles au CO_2 dans les bronches.

Mots-clés : asthme, hypercapnie, hypocapnie, muscle lisse bronchique, dioxyde de carbone, canal calcique, épithélium, substance P.

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Introduction

Dysfunctional breathing in asthma is characterized by hyperventilation and hypocapnia (Thomas et al. 2001). There are several reports that hyperventilation may precipitate (Demeter and Cordasco 1986; Han et al. 1999; Thomas et al. 2001) or exacerbate (Carr 1998) asthmatic symptoms and that hypocapnia can result in bronchoconstriction in asthmatic humans (van den Elshout et al. 1991). Furthermore, 2 studies have shown that low alveolar partial pressure of car-

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T.Y. El Mays, P. Choudhury, and F.H.Y. Green.* Respiratory Research Group, University of Calgary, Calgary, AB, Canada.
M. Saifeddine and M.D. Hollenberg. Smooth Muscle Research Group, University of Calgary, Calgary, AB, Canada.

Corresponding author: Francis H.Y. Green (e-mail: fgreen@ucalgary.ca).

*Present address: Department of Pathologyand Laboratory Medicine, 3330 Hospital Drive N.W., Calgary, AB T2N 4N1, Canada.

bon dioxide (P_ACO_2) may be associated with increased airway tone in asthmatic patients (Fisher et al. 1970; McFadden et al. 1977). A study by van den Elshout and colleagues (van den Elshout et al. 1991) demonstrated that hypercapnia reduced airway resistance in both healthy and asthmatic subjects. Furthermore, Fisher and Hansen showed that inhalation of 6% CO_2 can relieve exercise-induced airflow obstruction in young asthmatics at rest and during exercise (Fisher and Hansen 1976). However, the underlying physiological mechanisms for these findings have not been demonstrated. In addition, inhaled CO_2 (5%, 10%, and 15%) reduced total lung resistance induced by pulmonary artery occlusion in cats and dogs (Astin et al. 1973). Hypocapnia has been shown to increase collateral and small airway resistance in dog lung lobes, while hypercapnia (10% CO_2) had the opposite effect (Kikuchi et al. 1995). Ingram and colleagues (Ingram 1975) studied the effect of varying airway and arterial CO_2 in dogs and showed that low airway P_ACO_2 combined with high arterial P_ACO_2 resulted in a large increase in airway resistance (Ingram 1975). These findings indicate that alveolar CO_2 may have a different effect on airway resistance compared with CO_2 in arterial blood. We hypothesized that these differences might be mediated by the airway epithelium, a tissue known to play an important role in airway smooth muscle homeostasis and asthma (Folkerts and Nijkamp 1998; Holgate 2008).

Studies in vitro have shown that hypocapnia can induce contraction of subpleural strips of canine lung parenchyma and that the contractile response can be reversed by normocapnia (Duane et al. 1979). In other studies, hypocapnia was shown to increase resting tension in precontracted porcine tracheal strips, in step with an increase in intracellular pH (Croxton et al. 1995), whereas hypercapnia was found to reduce tension in rat tracheal rings precontracted by acetylcholine (Twort and Cameron 1986).

Our aim was to explore the role of hypocapnia on rat bronchial smooth muscle contractility in tissues with or without an intact epithelium while controlling for changes in ambient pH and extracellular calcium (Ca^{2+}). We also explored the effects of hypocapnia, normocapnia, and hypercapnia on substance P-induced epithelium-dependent relaxation of methacholine-precontracted bronchial smooth muscle.

Materials and methods

Materials

Animals

Pathogen-free male Sprague–Dawley rats weighing 250–350 g were purchased from Charles River Laboratories (Saint-Constant, Que., Canada). All rats were housed in plastic cages at the University of Calgary Health Sciences Animal Resource Centre and had access to water and rodent laboratory chow (Prolab RMH 2500 5P14) under a 12 h light : 12 h dark cycle.

Drug source

Substance P (SP), the nonselective muscarinic acetylcholine receptor agonist, acetyl--methylcholine (MCh), and the -adrenoceptor agonist, isoproterenol hydrochloride, were purchased from Sigma–Aldrich Canada (Toronto, Ont., Canada). Gas cylinders (95% O_2+ 5% CO_2; 95% O_2 + 5% N_2; 100% O_2; and 92% O_2 + 8% CO_2) were from Praxair Canada (Scarborough, Ont., Canada). Other chemicals and reagents were from standard commercial sources.

Instruments

Changes in bronchial smooth muscle isometric tension were measured using Grass force displacement transducers (Grass Technologies, West Warwick, R.I., USA) and recorded on a Gould chart recorder as previously described (Koetzler et al. 2006).

Methods

All procedures involving animals conformed to Canadian ethical guidelines for animal experimentation and were approved by the University of Calgary Animal Care Committee.

Preparation of rat bronchial rings

Sprague–Dawley rats were sacrificed by decapitation. The lungs were removed, and bronchial rings (2 mm × 3 mm) were dissected and debrided from surrounding tissue. Left and right first-generation bronchial tissues were used, as previously described in this laboratory (Koetzler et al. 2006). The epithelium was removed by gently rubbing the bronchial ring lumen by rotating it against the tip of a small dissection forceps. Bronchial rings were mounted in plastic organ baths containing 4 mL of a bicarbonate- and phosphate-buffered Krebs–Henseleit bioassay solution of the following composition: 115 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 25 mmol/L $NaHCO_3$, 1.2 mmol/L KH_2PO_4, and 10 mmol/L glucose. With this buffer, the pH was maintained at 7.4 by aeration with 95% O_2 and 5% CO_2 at a constant temperature of 37 °C. Tissues mounted under an initial tension of 10 mN were allowed to equilibrate for 60 min before being exposed to agonists. Upon equilibration, the tissue tension had reduced to approximately 5 mN.

Effect of hypocapnic challenge on rat bronchial smooth muscle resting tension

Bronchial rings equilibrated in Krebs solution aerated with carbogen (95% O_2 and 5% CO_2) and exhibiting about 5 mN resting tension at 37 °C were switched to 100% O_2, and changes in isometric tension were measured using a Grass force displacement transducer. Experiments were conducted on tissues with and without respiratory epithelium. To show that changes in muscle tension were not due to hyperoxia, the same experiment was repeated with 95% O_2 + 5% N_2. The tissues were challenged by hypocapnia either in the absence or presence of 24 mmol/L HEPES in the Krebs–Henseleit solution to maintain the constancy of the pH.

MCh (1 μmol/L) (EC_{60}) was utilized to induce muscle contraction. After reaching steady-state tension (about 5 min), SP (5 μmol/L) was added to induce epithelium-dependent relaxation of bronchial smooth muscle and to verify the presence of the epithelium. Alternatively, preparations contracted with MCh were exposed to isoproterenol (1 μmol/L) to induce an epithelium-independent relaxation, verifying the ability of the preparations to relax. After agonist exposure, tissues were washed and equilibrated in fresh buffer for 20 min. To test the effects of hypocapnia, the aeration gas

was switched to 100% O_2 (0% CO_2), and the bronchial smooth muscle resting tension was monitored for 30 min. The gas was then switched back to carbogen (95% O_2 + 5% CO_2), and bronchial smooth muscle resting tension was continually monitored. To assess changes in bronchial ring tension due to changes in the concentration of CO_2, without permitting changes in pH, the organ bath Krebs solution was fortified with HEPES buffer (24 mmol/L). The pH of the organ bath solution was periodically monitored during the course of all experiments. pH measurements were done using an Orion Micro Combination 16 GA Needle Tip pH electrode (Thermo Scientific, Beverly, Mass., USA) and an Accumet digital pH meter 915 (Fisher Scientific, Ottawa, Ont., Canada). In separate experiments, continuous pH measurement was performed for 30 min in organ baths without tissue to evaluate the effect of CO_2 concentration on pH.

To assess the role of extracellular Ca^{2+}, comparable experiments were performed either in the presence of Ca^{2+}-free Krebs–Henseleit buffer or in the presence of the voltage-dependent Ca^{2+} channel blocker nifedipine (10 μmol/L). To control for the solvent used for nifedipine (ethanol), the impact of an identical concentration of ethanol (16.8 mmol/L) on the preparation was evaluated. Furthermore, similar experiments were performed after incubating the tissue for 20 min with a nonselective cyclooxygenase inhibitor, indomethacin (5 μmol/L). This procedure was followed by a MCh-induced contraction and addition of SP (5 μmol/L) as a positive control.

The role of CO_2 in the epithelium-dependent relaxation of rat bronchial smooth muscle

Bronchial rings that contained either intact epithelium or were denuded of epithelium were equilibrated in the organ bath as described above and precontracted with MCh (1 μmol/L). When the contraction had stabilized (after 5 min), SP (5 μmol/L) was added and the presence or absence of a relaxant response monitored. The absence of an intact epithelium was ascertained by an absence of relaxation in response to SP, and the functionality of the preparation (relaxation) was verified by the addition of isoproterenol (1 μmol/L), which causes an epithelium-independent relaxation via the smooth muscle -adrenoceptors. Five minutes thereafter, the tissue was washed and reequilibrated in the organ bath for 20 min before continuing the experiment with the addition of other agonists. To assess the impact of hypocapnia or hypercapnia on the SP-induced relaxation of bronchial smooth muscle, the aeration of the epithelium-intact preparations was switched to gas with increasing concentrations of CO_2 ranging from 0% CO_2 to 8% CO_2 (100% O_2 (0% CO_2); 95% O_2+ 5% CO_2; or 92% O_2 + 8% CO_2). The same protocol was repeated with HEPES (24 mmol/L) buffer added to the organ bath solution to assess the role of pH.

The effect of CO_2 on methylcholine-induced contraction and isoproterenol-induced relaxation of bronchial smooth muscle

Bronchial rings with intact epithelium were equilibrated in the organ bath as described above and were precontracted with MCh (1 μmol/L). When the contraction had stabilized (after 5 min), isoproterenol (1 μmol/L) was added and the relaxant response was monitored. This protocol was repeated with tissue being aerated with varying CO_2 concentrations of gases (100% O_2 (0% CO_2); 95% O_2+ 5% CO_2; or 92% O_2 + 8% CO_2).

The effect of resting tension on methylcholine-induced contraction and substance P-induced relaxation of bronchial smooth muscle

Bronchial rings with intact epithelium were equilibrated in the organ bath as described above and were precontracted with MCh (1 μmol/L). When the contraction had stabilized (after 5 min), Sp (5 μmol/L) was added and the relaxant response was monitored. This protocol was repeated with different tissue resting tensions (5, 10, 15, and 20 mN).

Statistical analysis

The paired Student's t test was used for a comparison of "before and after" experiments. If the normality assumption failed, a signed rank test was performed. The effects of the concentration of CO_2 on the airway smooth muscle tone were compared using the 2-way ANOVA. The Student–Newman–Keuls method was used for post hoc comparisons. Results are given as means ± SE. Results with a p value <0.05 were considered significant. All statistical analyses were conducted using SPSS 16.0 (SPSS, Inc., Chicago, Ill., USA).

Results

Hypocapnic challenge increased bronchial smooth muscle resting tension

Hypocapnia induced a slow increase in resting tension, which reached a maximum at 30 min and rapidly returned back to baseline when the tissue was reaerated with 5% CO_2. The increase in tension (Fig. 1) was accompanied by a rise in pH (Fig. 2). The rise in tissue tension caused by hypocapnia was significantly diminished either in the presence of the Ca^{2+} channel blocker nifedipine (Fig. 3) or when tissues were incubated in a Ca^{2+}-free Krebs–Henseleit solution (Fig. 3). Supplementing the Krebs–Henseleit solution with sufficient HEPES to stabilize the pH at about 7.4 during the hypocapnic challenge prevented the rise in bronchial smooth muscle resting tension (Fig. 3). This result indicated that the effect of hypocapnia on smooth muscle tension was due primarily to the elevation of pH, as would be predicted from the bicarbonate–phosphate composition of the Krebs–Henseleit solution. There was no significant ($p = 0.28$) difference in the response to hypocapnia between tissues denuded of their epithelium compared with those with intact epithelium (Fig. 3). Furthermore, indomethacin, used to block the prostaglandin E2 pathway, had no significant ($p = 0.34$) effect on the observed hypocapnic response (Fig. 3).

CO_2-enhanced epithelium-dependent relaxation of bronchial smooth muscle

The relaxation of bronchial smooth muscle induced by SP was significantly ($p < 0.001$) decreased in preparations that had been denuded of epithelium (Fig. 4). Furthermore, in epithelium-intact preparations, the SP-induced epithelium-dependent relaxation of bronchial smooth muscle maintained in Krebs–Henseleit solution was decreased under hypocapnic conditions (Fig. 5). The SP-induced relaxation was (mean ± SE) 9.8% ± 1.3%, 16.9% ± 2.05%, and 21.3% ± 2.97% when the tissue was

Fig. 1. The top trace (solid line) indicates an increase in bronchial smooth muscle resting tension with hypocapnic challenge. Hypocapnia induced a slow increase in resting tension, which reached a maximum at 30 min and rapidly returned back to baseline when the tissue was regassed with 5% CO_2 . The rise in tissue tension caused by hypocapnia was significantly diminished in the presence of the Ca^{2+} channel blocker nifedipine (broken line). This figure shows a representative experiment.

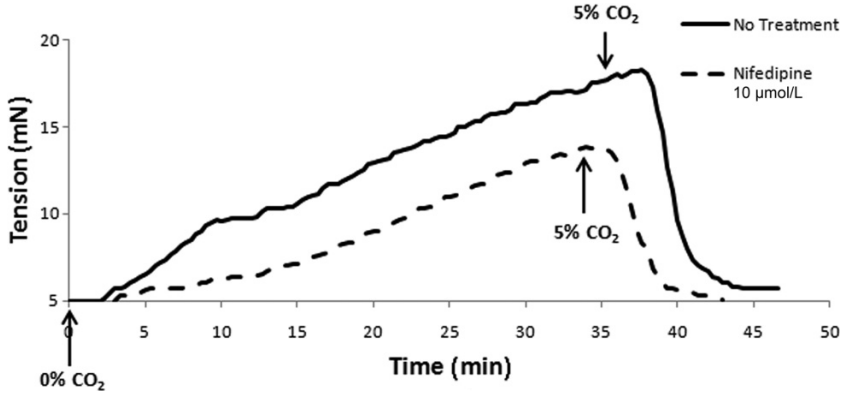
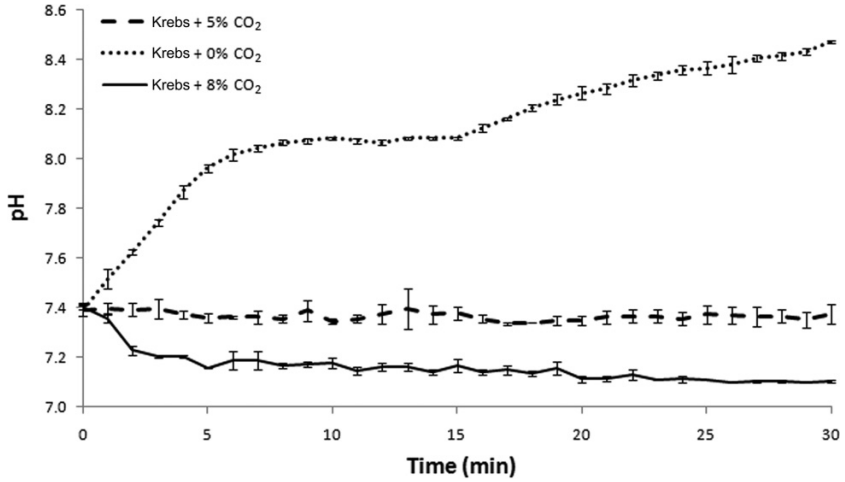


Fig. 2. Changes in pH over time with different CO_2 concentrations. This figure indicates the pH of Krebs solution over a 30-min period under hypocapnic (dotted line), normocapnic (broken line), and hypercapnic conditions (solid line). Data show means ± standard deviation (SD) (n = 3). In these experiments tissue was excluded from the solutions.



aerated with 100% O_2, 95% O_2+ 5% CO_2, and 92% O_2 + 8% CO_2, respectively (Fig. 6). With stable pH at about 7.4, a decrease in CO_2 concentration from 5% to 0% resulted in no significant change in SP-induced epithelium-dependent relaxation of bronchial smooth muscle (Fig. 6). In contrast, with a stable pH at about 7.4, an increase in CO_2 concentration from 5% to 8% resulted in a significant increase in the epithelium-dependent relaxation of the bronchial smooth muscle (Fig. 6).

In hypocapnic conditions, stabilizing the pH with HEPES at about 7.4 resulted in a significantly greater epithelium-dependent relaxation of the bronchial smooth muscle compared with relaxation with no HEPES (Fig. 6). Similarly, in hypercapnic conditions, stabilizing the pH with HEPES at about 7.4 resulted in a significantly greater epithelium-dependent relaxation of the bronchial smooth muscle compared with relaxation with no HEPES (Fig. 6). In contrast, under normocapnic conditions, stabilizing the pH with HEPES at about 7.4 had no effect on the epithelium-dependent relaxation of the bronchial smooth muscle compared with relaxation with no HEPES (Fig. 6).

The effect of CO_2 on methylcholine-induced contraction and isoproterenol-induced relaxation of bronchial smooth muscle

MCh (1 µmol/L) caused contraction of the bronchial preparation (Fig. 5). Changes in CO_2 concentration had no significant (p = 0.86) effect on the MCh-induced contraction of bronchial smooth muscle. MCh-induced contraction was (mean ± SE) 11.10 ± 0.75 mN, 11.95 ± 1.37 mN, and 11.28 ± 1.37 mN when the tissue was aerated with 100% O_2 (0% CO_2), 95% O_2+ 5% CO_2, or 92% O_2 + 8% CO_2, respectively. Furthermore, changes in CO_2 concentration had no significant (p = 0.72) effect on isoproterenol-induced relaxation of bronchial smooth muscle. Isoproterenol-induced relaxation was (mean ± SE) 36.67% ± 4.00%, 40.06% ± 2.76%, and 36.87% ± 3.44% when the tissue was aerated with 100% O_2 (0% CO_2), 95% O_2+ 5% CO_2, or 92% O_2 + 8% CO_2, respectively. Under hypocapnic and hypercapnic conditions, the bronchial smooth muscles retained their ability to contract or relax in response to MCh or isoproterenol, respectively. In control experiments we confirmed that responses to MCh or isoproterenol were epithelium independent.

Fig. 3. Figure depicts maximal changes in bronchial smooth muscle (BSM) resting tension with hypocapnic challenge under various conditions. There was no significant difference between the increase in smooth muscle resting tone with 0% CO_2 (n = 11), 5% N_2 + 95% O_2 (n = 9), 0% CO_2 + ethanol (n = 3), 0% CO_2 + indomethacin (Indo) (n = 5), and 100% O_2 + tissue denuded of epithelium (Epi(–)) (n = 5). When HEPES buffer was added to the solution and constant pH was maintained, the BSM resting tension stayed at the baseline (preset at 0 mN) (n = 12). Addition of nifedipine (10 μmol/L) to the solution significantly (p = 0.04) lowered the increase in tension due to hypocapnia (n = 6). Similarly, in the presence of a calcium-free solution, the increase in smooth muscle resting tone due to hypocapnia was blunted significantly compared with that under 0% CO_2 and 0% CO_2 + nifedipine (Nifed) (p = 0.001 and p = 0.03, respectively). All values are shown as means ± SE. *, significantly different from 0% CO_2.

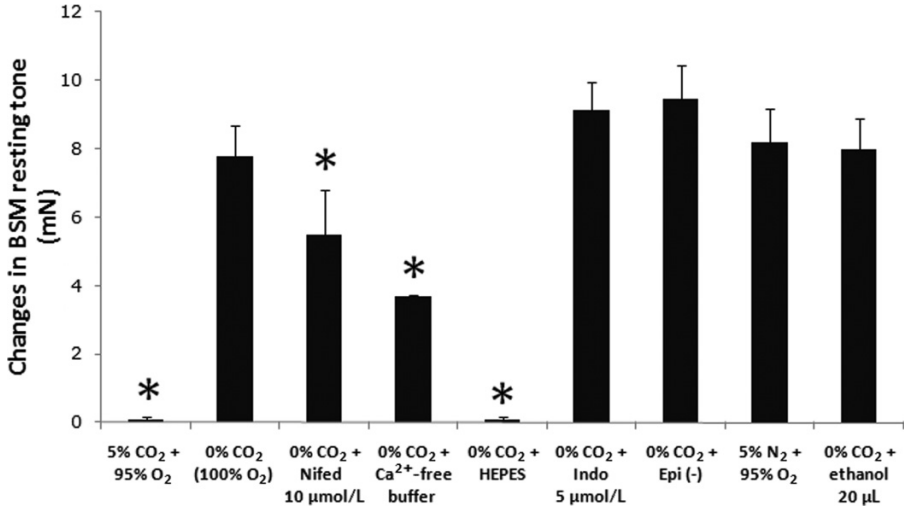


Fig. 4. Substance P-induced epithelium-dependent relaxation of bronchial smooth muscle precontracted with methacholine was significantly lower (p < 0.001) in tissues with denuded epithelium (Epithelium (–)) (5.17% ± 0.59%) compared with tissue having intact epithelium (16.85% ± 2.05%). The values shown are means ± SE for a set of 7 tissues. *, significantly different from Epithelium (–).

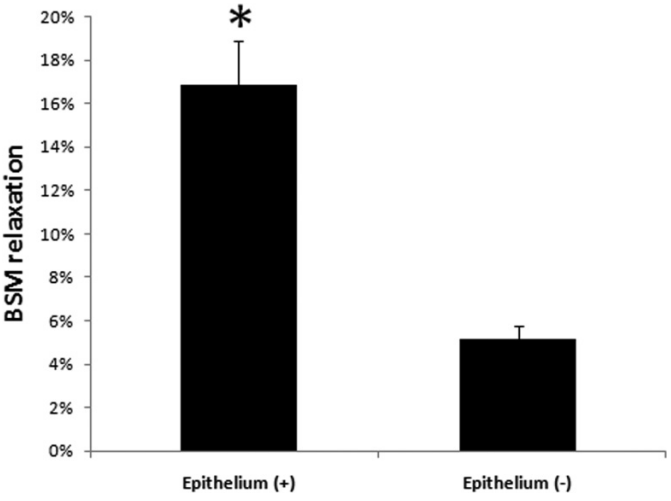
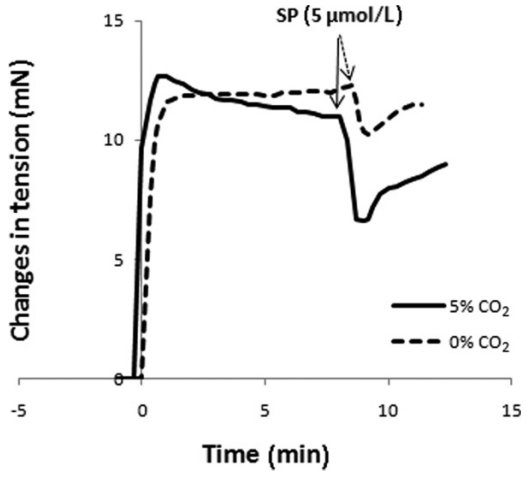


Fig. 5. The figure shows one set of representative experiments of the effect of substance P on bronchial smooth muscle tension under normocapnic (solid line) and hypocapnic (broken line) conditions. In the absence of CO_2, the substance P-induced epithelium-dependent relaxation of bronchial smooth muscle was blunted. The baseline tension for the normocapnic (5% CO_2) tracing was set at approximately 5 mN. The baseline tension for the hypocapnic (0% CO_2) tracing was 12.78 ± 3.02 mN (mean ± SD).



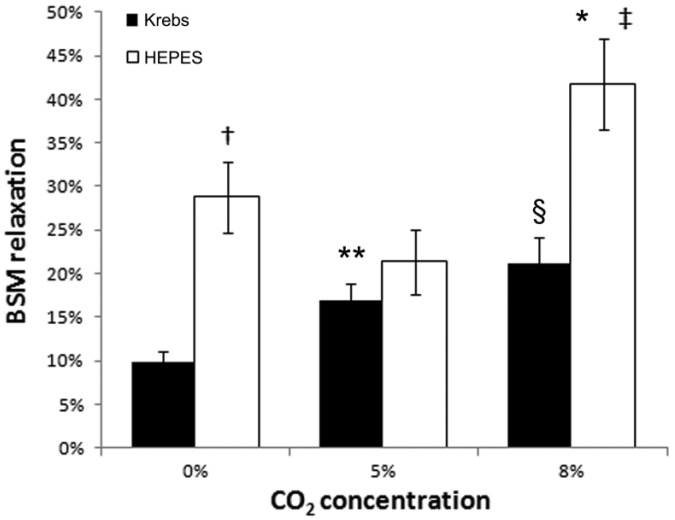
The effect of resting tension on methylcholine-induced contraction and substance P-induced relaxation of bronchial smooth muscle

Changes in resting tension had no significant effect on either MCh-induced contraction (p = 0.94) or SP-induced relaxation (p = 0.86). MCh-induced contraction was (mean ± SE) 16.52 ± 0.26 mN, 17.45 ± 0.28 mN, 16.35 ± 0.25 mN, and 15.30 ± 0.18 mN when the tissue tension was set at 5, 10, 15, and 20 mN, respectively. SP-induced relaxation was (mean ± SE) 21.48% ± 4.16%, 16.94% ± 3.60%, 19.05% ± 4.79%, and 22.59% ± 6.94% when the tissue tension was set at 5, 10, 15, and 20 mN, respectively.

Discussion

In this study we showed that epithelial-dependent SP-induced bronchial smooth muscle relaxation in Sprague–Dawley rats is blunted under hypocapnic conditions and enhanced by hypercapnic conditions. Furthermore, we showed that the hypocapnic effect is pH dependent, whereas the hypercapnic

Fig. 6. Comparison of the effects of CO_2 on substance P-induced epithelium-dependent relaxation of precontracted bronchial smooth muscle (BSM) ($n = 7$) without HEPES (Krebs) and with HEPES buffer. The epithelium-dependant relaxation was significantly enhanced with increasing CO_2 concentrations. Stabilization of the pH with HEPES buffer prevented the attenuation of the epithelium-dependent relaxation of BSM during hypocapnic challenge (0% CO_2) and enhanced it during hypercapnic challenge (8% CO_2). All values are represented as means ± SE. *, $p = 0.007$: different from 0% CO_2 and 5% CO_2 in HEPES; †, $p = 0.007$: different from 0% CO_2 in Krebs; ‡, $p = 0.003$: different from 8% CO_2 in Krebs; §, $p = 0.001$: different from 0% CO_2 in Krebs; **, $p = 0.026$: different from 0% CO_2 in Krebs.



effect is independent of extracellular pH. These effects of CO_2 on airway smooth muscle tension required an intact epithelium. By contrast, we showed that hypocapnic or hypercapnic conditions had no effect on the epithelial-independent mechanisms of MCh-induced contraction or on isoproterenol-induced bronchial smooth muscle relaxation. This finding of an epithelial-dependent, pH-independent mechanism of CO_2-induced bronchial smooth muscle relaxation has not been previously reported.

Our study also confirmed the well-described pH-dependent and Ca^{2+}-mediated effects of hypocapnia on bronchial smooth muscle resting tension (Crouch and Kirpatrick 1975; Yamakage et al. 1995; Freshney 2005). We showed that the increase in bronchial smooth muscle resting tension induced by hypocapnia is entirely extracellular pH dependent and is independent of the prostaglandin E2 pathway inhibited by indomethacin. Other investigators have shown this pH-dependent effect of hypocapnia both in vitro (Duckles et al. 1974) and in vivo (Sterling et al. 1972). There is some evidence that pH shifts play a role in modulating airway tone in patients with asthma (Brijker et al. 2009).

We also report that nifedipine decreased hypocapnia-induced bronchial smooth muscle contraction, a finding similar to that reported by Lindeman and colleagues (Lindeman et al. 1998). Nifedipine blocks L-type Ca^{2+} channels, thus inhibiting the release of intracellular Ca^{2+} from the sarcolemma, a process mediated by the ryanodine receptor (RyR2) (Karaki 2004; Moura et al. 2005), required for smooth muscle contraction. We thus confirm a partial role of L-type Ca^{2+} channels in hypocapnia-induced changes in bronchial smooth muscle tension in this model. Furthermore, the hypocapnia-induced contraction in Ca^{2+}-free solution was significantly smaller than that observed in the presence of nifedipine. This result suggests that mechanisms or Ca^{2+} channels other than the L-type Ca^{2+} channel are involved in this process and that further work is warranted to explore this possibility.

The mechanism(s) that underlie the epithelial–CO_2 effects on airway smooth muscle appear complex and are not fully addressed in this study. As described earlier, changing the concentration of CO_2 was accompanied by changes in extracellular pH. As shown in Fig. 6, stabilization of the pH with HEPES buffer prevented the attenuation of the SP-induced epithelium-dependent relaxation of bronchial smooth muscle during hypocapnic challenge, resulting in a relaxation response that was not significantly different from that seen under normocapnic conditions. These results suggest that the change in H^+ concentration induced by hypocapnia played a primary role in attenuating the epithelial-dependent relaxation of airway smooth muscle by SP.

In contrast to the hypocapnic response described above, tissues aerated under hypercapnic conditions (92% O_2 + 8% CO_2) in Krebs and without HEPES showed significantly greater epithelium-dependent bronchial smooth muscle relaxation compared with bronchial rings incubated under normocapnic or hypocapnic conditions (Fig. 6). Furthermore the response to the higher concentration of CO_2 was enhanced by stabilization of the pH with HEPES buffer (Fig. 6). This finding indicates an additional mechanism whereby hypercapnia relaxes bronchial smooth muscle independently of changes in pH. This mechanism requires an intact respiratory epithelium.

Airway epithelium is believed to play an important role in the development, progression, and exacerbation of asthma (Holgate et al. 2009). Derangement of the barrier function of the epithelium by sloughing (Busse et al. 1999) or by defective tight junction formation (Holgate 2008) may facilitate environmental irritant and allergen penetration and stimulation of airway smooth muscle and intraepithelial nerves (Folkerts and Nijkamp 1998). In addition to its barrier function, airway epithelium secretes epithelium-derived relaxing factors such as prostaglandin E2 and nitric oxide, which in turn act on smooth muscle, causing its relaxation (Folkerts and Nijkamp 1998).

SP modulates airway tone through a complex set of effects on airway smooth muscle depending upon species (Watanabe et al. 2006), strain (Devillier et al. 1992), and route of administration (Frossard and Muller 1986; Joos et al. 1988). In humans, SP is usually proinflammatory and is considered to be a mediator of allergic asthma (Joos et al. 2000). However, a transient relaxant effect of SP has been demonstrated in human airways in vitro (Chitano et al. 1994). In Sprague–Dawley rats, SP is known to induce epithelium-dependent relaxation (Szarek et al. 1995) through the release of prostaglandin E2 from the epithelium (Devillier et al. 1992). Prostaglandin E2 plays a similar role in the human airway, where it has been shown to inhibit histamine-induced contraction of human bronchial smooth muscle (Knight et al. 1995).

As the SP-induced bronchial relaxation response requires an intact epithelium, it is interesting to speculate the presence

of an epithelial receptor, capable of sensing CO_2, mediating the effects we observed. A variety of cells in the body are able to detect changes in CO_2 concentration, including the glomus cells of the carotid body (Dasso et al. 2000) and cells in the central brainstem (Nattie 1999). It has been suggested that increased molecular CO_2, independent of its effect on pH, activates L-type Ca^{2+} channels in the glomus cells of the carotid body, which in turn are responsible for detection of hypercarbia (Obeso et al. 1992; Dasso et al. 2000; Summers et al. 2002). Pulmonary neuroepithelial bodies (NEBs) are a group of highly specialized and densely innervated clusters of neuroendocrine cells that represent an extensive population of intraepithelial airway receptors in humans and other mammals (Adriaensen and Scheuermann 1993). NEBs are intimately connected to pulmonary afferent C-fibres and contain tachykinin neuropeptides (Larson et al. 2003). A study by Lauweryns and colleagues (Lauweryns et al. 1977) demonstrated that NEBs in the rabbit neonatal lung undergo ultrastructural changes upon exposure to hypercapnia and hypoxia. Since then, oxygen-sensing abilities of neuroendocrine cells have been demonstrated in several studies (Cutz and Jackson 1999; Kemp et al. 2003). Exposure of NEBs to low levels of oxygen can lead to nifedipine-sensitive Ca^{2+} influx (Fu et al. 2002). Although the CO_2 sensitivity of neuroepithelial bodies has not been clearly demonstrated yet, it can be postulated that NEBs evoke appropriate local responses to conditions of hypocapnia, hypercapnia, and hypoxia through regional neural networks.

In conclusion, in this in vitro model, we confirmed the important role of CO_2 on bronchial resting smooth muscle tone and the important roles for pH and intracellular and extracellular Ca^{2+} in this process. We provide evidence that hypercapnia enhances the epithelium-dependent relaxation of Sprague–Dawley rat bronchial smooth muscle independently of pH. In addition we show that hypocapnia abolishes the epithelial-dependent smooth muscle relaxation induced by SP. Assuming that similar pathways exist in humans, our findings provide useful insights into the relationships between hyperventilation, hypocapnia, and exercise-induced asthma.

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