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Functional Activity of Bronchi from an Organ Donor with Fatal Asthma: Studies on Cryopreserved Bronchi

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ABSTRACT

Human bronchi were taken from the lungs of a single asthmatic and 5 nonasthmatic organ donors. The tissues were slowly frozen to –70°C and stored for 1–28 months in liquid nitrogen (–196°C) while suspended in Krebs-Henseleit solution containing 1.8 M dimethyl sulfoxide and 0.1 M sucrose as cryoprotectants. After thawing, bronchial rings were suspended in 10 ml organ baths for isometric tension recording. Spontaneously developed tone (1.13 ± 0.12, n = 22, vs. 0.56 ± 0.07 g, n = 33, $p < 0.001$) and maximal contractile responses to histamine (1.93 ± 0.12, n = 34, vs. 1.02 ± 0.14 g, n = 30, $p < 0.001$) were significantly stronger in asthmatic than in nonasthmatic bronchi. The potency of histamine was 4 times less in asthmatic than in nonasthmatic

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bronchi ($p < 0.001$). Comparison of the maximal responses to histamine after storage at –196°C for up to 28 months revealed no significant reduction of the contractile function by time of cryostorage. Salbutamol and the potassium channel opener SDZ PCO 400 were 3–4 times less potent in asthmatic than in nonasthmatic bronchi. For antagonism of histamine by ketotifen in asthmatic bronchi ($pD'_2 = 8.04 \pm 0.13$, $n = 5$) 4 times higher concentrations were necessary than in nonasthmatic bronchi ($pD'_2 = 8.63 \pm 0.06$, $n = 15$, $p < 0.001$). These data support the contention that in spite of a diminished sensitivity to histamine after fatal asthma, isolated bronchi show enhanced spontaneous and agonist-induced contractile responses whereas relaxant responses appear to be impaired.

INTRODUCTION

Only few comparative in vitro studies on the responsiveness of airway tissue from asthmatic and nonasthmatic patients have been published up to now. Furthermore, the limited availability of airway tissue from asthmatic patients in addition to differences in airway size and severity of asthma have provided conflicting results on the in vitro responsiveness of asthmatic bronchial smooth muscle (1–11). Pharmacological investigations on asthmatic bronchi require the availability of human airway tissues as no appropriate animal model exists. However, the main problems with use of human tissues are the irregularity of its supply and the quantity of material that can be utilized at one time, as the tissues deteriorate rapidly after removal from the body. Cryopreservation has become an important tool for the storage of animal and human vascular tissues in pharmacological research (12), and recently, evidence has been presented that airway smooth muscle preparations may also be stored by the same technique with a wide variety of functional activities being well preserved (13–16). Using this technique, we now present pharmacological data comparing postthaw functional responses of human bronchi from the lungs of a single organ donor who died during an asthmatic attack with responses of bronchi obtained from 5 nonasthmatic multiple-organ donors.

MATERIALS AND METHODS

Tissue Preparation and Storage Method

Human lungs were taken from a patient who died during an asthmatic attack (female, 42 years) and from 5 nonasthmatic organ donors (3 male, 2 female, 39–66 years old, mean age 57 ± 5 years) after permission was obtained from the local ethical committee. Information about therapeutic drug administration was not available for these subjects. Immediately after retrieval, the explanted lungs were transported to the laboratory, where small bronchi (outer diameter ≈ 2–5 mm) were excised within 6 hr after explantation. The bronchi were cleaned of surrounding tissue and placed in 2 ml liquid nitrogen storage ampules (Life Technologies AG, Basel, Switzerland) filled with Krebs-Henseleit (KH) solution (composition mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, EDTA 0.03) containing 1.8 M dimethyl sulfoxide (DMSO) and 0.1 M sucrose as cryoprotecting agents. After an equilibration time of 10–30 min at room temperature the ampules were placed in a polystyrol box (11 × 11 × 22 cm) and slowly frozen at a mean cooling rate of about 1.3°C/min in a freezer maintained at –70°C. About 3–15 hr later the ampules were transferred into liquid nitrogen (–196°C) where they were stored for up to 28 months until used for in vitro experiments. Before use the tissues were thawed within 4 min by placing the ampules in a 40°C water bath.

Organ Bath Studies

The bronchial segments were cut into rings (about 2–3 mm in length), mounted between two hooks of stainless steel wire, and suspended in 10 ml organ baths containing KH solution at 37°C, bubbled with 5% CO_2 in oxygen. Changes in tone of the preparations were recorded isometrically with electromechanical transducers (Statham model UC 3) and a potentiometric recorder. At the beginning of the experiments the rings were stretched to an initial tension of 2 g and allowed to equilibrate and develop spontaneous tone for about 2–3 hr in the bathing solution. During this time the preparations were tested once with carbachol or histamine (1–10 μM) and allowed to develop spontaneous tone, the baseline tension of the rings being readjusted to 1 g if required. Concentration-response curves for agonists were determined by cumulative additions, each concentration being added when the maximum effect had been produced by the previous concentration. When antagonism of histamine was investigated, the maximal contractile response of each bronchial ring to 100 μM histamine was first determined and this effect was taken as point of comparison for subsequent responses. After repeated washouts cumulative concentration-response curves for histamine were determined without and in the presence of the antagonist added 20 min before the first administration of histamine. In each series of experiments with nonasthmatic bronchi, preparations from at least 2 donors were used. Each bronchial ring was exposed only once to an antagonist. Responses to relaxant agonists were investigated during spontaneous tone and papaverine (300 μM) was added at the end of the concentration-response curves to induce complete relaxation of the bronchial rings. This effect was defined as a 100% relaxation. At the end of some organ bath experiments the bronchial rings were blotted between two paper tissues and the wet weight was recorded.

Drugs

The following pharmacological agents were used: histamine dihydrochloride, isoprenaline sulfate, salbutamol, propranolol hydrochloride, nordihydroguaiaretic acid (NDGA), dexamethasone 21-phosphate disodium, aminophylline hydrate, carbamylcholine chloride (carbachol), papaverine hydrochloride (Sigma, Munich, Germany). The potassium channel activator SDZ PCO 400 ((–)-(3**S**,4**R**)-3,4-dihydro-3-hydroxy-2,2-dimethyl-4-(3-oxocyclopent-1-enyloxy)-2H-1-benzopyran-6-carbonitrile) (17) and ketotifen fumarate were synthesized at Sandoz Pharma Ltd, Basel, Switzerland. SDZ PCO 400 was dissolved in DMSO (Merck-Schuchardt, Hohenbrunn, Germany) and diluted to give a 1 mM solution containing 5% DMSO.

Data Analysis

Concentration-response curves were analyzed with a computer program in RS/1 (BBN Software Products Corporation, Cambridge MA) and E_{max} (maximal effects) and pD_2 values (negative logarithm of the molar concentration of the agonist producing 50% of E_{max}) were derived from this analysis. For the insurmountable antagonism of histamine by ketotifen pD'_2 values (negative logarithm of the molar concentration of the antagonist that reduces the maximal response of the agonist by 50%) were calculated according to the equation $pD'_2 = pD'_x + \log(x - 1)$, where pD'_x is the negative logarithm of the molar concentration of the antagonist used and x is the ratio of E_{max} of the agonist in the absence and presence of the antagonist. Apparent pA_2 values (negative logarithm of the molar concentration of the antagonist that reduces the effect of a double concentration of the agonist to that of a single concentration) were calculated according to the equation $pA_2 = pA_x + \log(x - 1)$, where x is the ratio of the EC_{50} values in the presence and absence of the antagonist and pA_x is the negative logarithm of the molar concentration of the antagonist used. Where appropriate, one-way analysis of variance (ANOVA) was performed, followed by the Bonferroni-corrected t-test to assign differences to individual between-group comparisons when overall significance ($p < 0.05$) was attained. Data are presented as mean

values ± SEM; n values refer to the number of bronchial rings employed.

RESULTS

Following suspension and preloading of human bronchial rings in the organ bath, the tissues developed spontaneously *active tone*. This tone was significantly stronger in rings from the asthmatic than in rings from nonasthmatic organ donors. As estimated by the relaxation following addition of papaverine (300 μM) at the end of the experiments, this effect amounted to 1.13 ± 0.12 g (n = 22) in bronchi from the asthmatics whereas in bronchi from nonasthmatics only 0.56 ± 0.07 g (n = 33) of spontaneous tone was unmasked in that way. In contrast, comparison of the wet weight of bronchial rings after termination of the experiments (53 ± 5 mg, n = 46 in asthmatic vs. 50 ± 4 mg, n = 42 in nonasthmatic bronchi) revealed no significant differences between both groups of bronchi (Table 1), indicating that the bronchial rings in both groups were of similar size.

Bronchial rings from the asthmatic organ donor were tested after 1–28 months following explantation and cryopreservation. Comparison of the maximal contractile responses to histamine revealed no correlation between time of cryostorage at –196°C and postthaw functional recovery of these bronchi. Moreover, maximal responses to histamine of bronchi that had been stored for 28 months in liquid nitrogen were statistically not different from those determined after 1 month of cryo-storage (Fig. 1).

As observed with the spontaneous tone, maximal *contractile responses* to histamine were also significantly higher in asthmatic bronchi, although the amine proved to be 4 times less potent than in nonasthmatic bronchi (Table 1, Fig. 2a). An additional series of experiments was performed to investigate contractile responses to histamine in the presence of various drugs. Neither the phosphodiesterase inhibitor aminophylline (10 μM, n = 2, not illustrated) nor the corticosteroid dexamethasone (1 μM) modified responses to histamine of bronchi in either group, whereas 1 μM of the lipoxygenase inhibitor NDGA (18) caused a slight, but significant reduction of the maximal histamine effect in the asthmatic airway tissues (E_{max} = 90 ± 2%, n = 4). When ketotifen was used as the antagonist, in both asthmatic and nonasthmatic bronchi the drug reduced the maximal contractile responses to histamine in a concentration-dependent manner (Fig. 3). However, as indicated by the calculated pD'_2 values, ketotifen was about 4 times more

Table 1. Parameters of Contractile and Relaxant Responses of Human Bronchi from Asthmatic and Nonasthmatic Organ Donors

	ASTHMATIC BRONCHI	NONASTHMATIC BRONCHI
Potency (pD_2 values)		
Histamine	4.73 ± 0.04 (20)	5.34 ± 0.05 (18)**
Isoprenaline	7.19 ± 0.08 (5)	7.47 ± 0.14 (14)
Salbutamol	6.54 ± 0.08 (6)	6.96 ± 0.09 (12)*
SDZ PCO 400	6.25 ± 0.10 (6)	6.86 ± 0.12 (6)*
Efficacy (E_{max})		
Histamine (g)	1.97 ± 0.13 (20)	1.02 ± 0.17 (18)**
Papaverine (g)	–1.13 ± 0.12 (22)	–0.56 ± 0.07 (33)**
Isoprenaline (%)	–86 ± 3 (5)	–96 ± 1 (9)*
Salbutamol (%)	–38 ± 3 (6)	–83 ± 4 (12)**
SDZ PCO 400 (%)	–73 ± 2 (6)	–82 ± 1 (6)*
Wet weight (mg)	53 ± 5 (46)	50 ± 4 (42)

Data are presented as mean values ± SEM, with the number of determinations in parentheses. Difference against values determined on asthmatic bronchi significant at *$p < 0.05$; **$p < 0.001$.

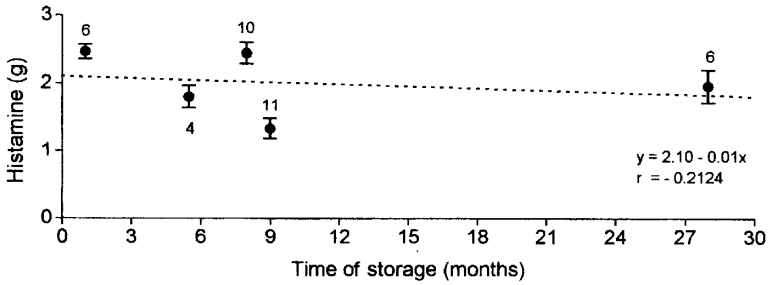

Figure 1. Maximal contractile responses to histamine, expressed in g, of human asthmatic bronchi after different times of cryostorage. Points represent mean values ± SEM; the numbers indicate number of determinations.

potent in nonasthmatic tissues (pD'_2 = 8.63 ± 0.06, n = 15) than in asthmatic bronchi (pD'_2 = 8.04 ± 0.13, n = 5).

When *relaxant responses* were investigated, the potassium channel opener SDZ PCO 400 relaxed bronchi from both groups with nearly similar efficacy. However, as indicated by the calculated pD_2 values for SDZ PCO 400, relaxation of bronchi from the asthmatic patient required 4 times higher concentrations of the potassium channel opener than relaxation of nonasthmatic bronchi (Table 1, Fig. 2b). While the nonselective beta-adrenoceptor agonist isoprenaline relaxed both groups of bronchi nearly completely (Fig. 2c), the $beta_2$-adrenoceptor selective agonist salbutamol proved to be markedly

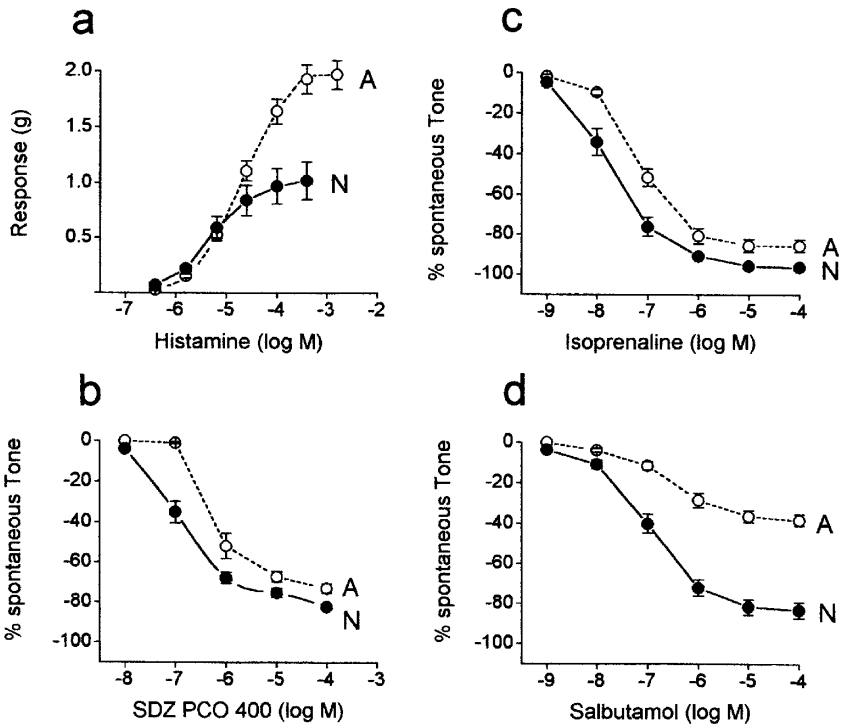

Figure 2. Contractile response curves for (a) histamine, expressed in g, and relaxant response curves for (b) SDZ PCO 400, (c) isoprenaline, and (d) salbutamol, expressed as percentages of maximum papaverine effect on rings from human bronchi from an asthmatic (A, open circles) and nonasthamtic donors (N, filled circles). Number of determinations for each curve as specified in Table 1; the bars represent mean values ± SEM.

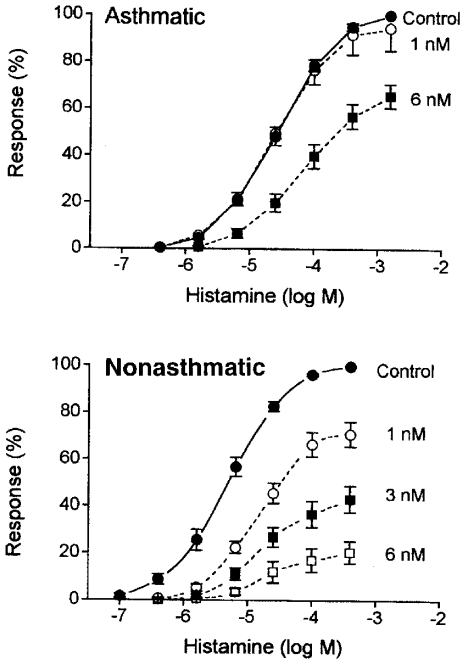

Figure 3. Concentration-response curve for histamine on rings from human bronchi from an asthmatic (top, for each curve $n = 4$) and nonasthmatic donors (bottom) without (solid line, $n = 9$) and in the presence (broken line, each curve $n = 5$) of ketotifen (1 nM, 3 nM, and 6 nM). The effects are expressed as percentages of the controls. The bars represent mean values ± SEM.

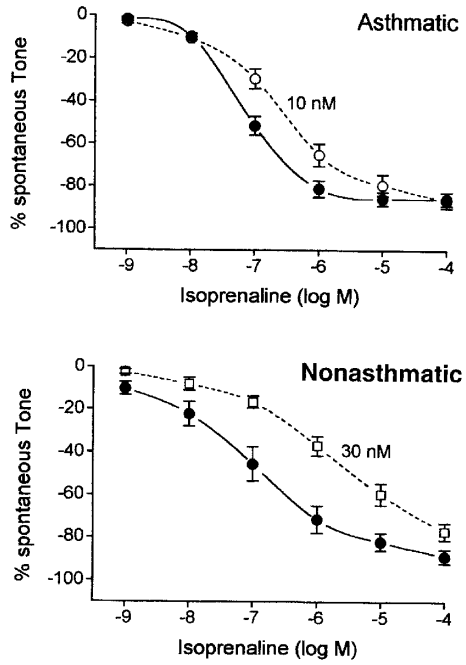

Figure 4. Concentration-response curve for isoprenaline on rings from human bronchi from an asthmatic (top) and nonasthmatic donors (bottom) without (●) and in the presence of propranolol (○, 10 nM and □, 30 nM). The effects are presented as percentages of the maximum papaverine effect. For each curve $n = 5$; the bars represent mean values ± SEM.

less effective in the asthmatic bronchi, eliciting only about 45% relaxation of that produced in nonasthmatic tissues (Fig. 2d). Additional experiments were performed to compare the blocking potency of the nonselective beta-adrenoceptor blocker propranolol in bronchi from both groups. In each group only one concentration of propranolol was used against isoprenaline as the agonist. In bronchi from both groups propranolol caused a parallel shift to the right of the isoprenaline curve indicating competitive antagonism. The difference between the calculated pA_2 values of 8.42 ± 0.12 ($n = 5$) in asthmatic and 8.62 ± 0.26 ($n = 5$) in nonasthmatic bronchi was not significant (Fig. 4).

DISCUSSION

Cryopreserved human bronchi were used to compare responses to various pharmacological agents of airway smooth muscle from an organ donor who died during an asthma attack to those obtained from nonasthmatic organ donors. The technique of cryopreservation has been shown to allow storage of airway smooth muscle preparations for virtually indefinite time with both morphological characteristics (15) and a wide variety of functional activities being well preserved (13–16). Further evidence for this was provided in the present study showing undiminished maximal contractile responses to histamine even after more than 2 years of cryostorage. Therefore, no comparative experiments on unfrozen bronchi from these organ donors have been performed in the present study. After frozen/thawed human bronchi were mounted in the organ baths, tension developed spontaneously during the first 30–60 min, confirming published observations on unfrozen human bronchi (19–21). Various mechanisms such as intrinsic

production of leukotrienes and cyclooxygenase products of arachidonic acid metabolism, spontaneous release of histamine and acetylcholine, and altered Ca^{2+} influx have been postulated to contribute to generation of this active basal tone in human bronchial tissue (19–21). In the present study the spontaneous tone was significantly stronger in bronchi from the asthmatic lungs than in bronchi from the nonasthmatic lungs. While in nonasthmatic bronchi both spontaneous and histamine-induced tone were resistant to blockade of lipoxygenase by 1 μM NDGA, a slight but significant reduction of the maximal histamine effect in asthmatic bronchi by NDGA suggested that endogenous leukotrienes, the major mediators of allergen-induced bronchoconstriction (1), contributed at least in part to the histamine response of these tissues.

In the present study contractile responses of cryopreserved asthmatic bronchi to histamine were significantly stronger than those of nonasthmatic tissues. This is in agreement with the hyperresponsiveness to spasmogens of unfrozen asthmatic bronchi reported by other authors (2,3,8,10,22). On the other hand, for stimulation of asthmatic bronchi 4 times higher histamine concentrations were required than in nonasthmatic bronchi. This reduced potency of histamine in asthmatic tissue is similar to observations reported by Goldie et al. (23) and Whicker et al. (9) albeit in other studies histamine stimulated asthmatic and nonasthmatic bronchi with similar potency (8,10,22). Furthermore, for antagonism of histamine effects in asthmatic bronchi about 4 times higher concentrations of the antiasthmatic drug with H_1-receptor-blocking-activity ketotifen (24) were required than in nonasthmatic bronchi. The reason for this difference is not clear, but might be due to therapeutic drug administration before death of the asthmatic organ donor and/or to receptor desensitization by histamine released from mast cells during the freezing/thawing process. Recently, it has been demonstrated that in spite of a good postthaw preservation of smooth muscle function, the responses to antigen of bronchi from atopic patients are not preserved (16). Since the contraction to antigen of bronchi from atopic patients is suggested to result from the release of mediators from mast cells within the bronchi (25), this observation supports the suggestion of injury occurring in human mast cells during the freezing/thawing process. Indeed, it has been observed that the anti-human IgE antibody-induced release of histamine, which is suggested to derive from bronchial mast cells, is abolished after cryopreservation of human lung tissue under the conditions used in the present study (H.-J. Pfannkuche, personal communication). This is further in line with the finding that human granulocytes have little recovery after cryopreservation (26).

While asthmatic bronchi are hyperreactive to contractile agonists, effects of relaxant agonists are usually impaired. Previous studies on cryopreserved human pulmonary arteries have shown that the potassium channel activator SDZ PCO 400 relaxes pulmonary vascular smooth muscle by only 40–50% (27). In the present study relaxant responses to SDZ PCO 400 of human airway smooth muscle occurred within the same concentration range as observed in human pulmonary arteries (27); however, the potassium channel activator relaxed human bronchi considerably more effectively than pulmonary arteries, eliminating about 80% of the spontaneous tone in both asthmatic and nonasthmatic tissues. In contrast, the $beta_2$-adrenoceptor agonist salbutamol proved to be markedly less effective in relaxing asthmatic bronchi compared to nonasthmatic tissues. This reduced relaxant efficacy of salbutamol in asthmatic bronchi is in agreement with the observation that responses to beta-adrenoceptor stimulation of isolated airways from patients with severe and fatal asthma are diminished (4–6,10,11,23). Despite this attenuation of beta-adrenoceptor-mediated effects, propranolol proved to be equipotent when tested against isoprenaline in bronchi of both groups suggesting that the site of defect in the beta-adrenoceptor-mediated relaxation system in asthmatic airway smooth muscle is unlikely to be caused by a decrease in receptor number, which are, in fact, rather increased in these patients (7,11,23).

In summary, the present data support the contention that in spite of a diminished sensitivity to histamine, isolated bronchi from asthmatic patients show enhanced spontaneous and agonist-induced contractile responses while relaxant responses appear to be

impaired and that these changes in airway smooth muscle reactivity may contribute to the hyperreactivity of airways in asthmatic patients.

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