Responsiveness of canine bronchial vasculature to excitatory stimuli and to cooling

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Janssen, Luke J., Hwa Lu-Chao, and Stuart Netherton. Responsiveness of canine bronchial vasculature to excitatory stimuli and to cooling. Am J Physiol Lung Cell Mol Physiol 280: L930–L937, 2001.—Changes in bronchial vascular tone, in part due to cooling during ventilation, may contribute to altered control of airflow during airway inflammation, asthma, and exercise-induced bronchoconstriction. We investigated the responses of canine bronchial vasculature to excitatory stimuli and cooling. Electrical stimulation evoked contractions in only some (8 of 88) tissues; these were phenylephrine sensitive and augmented by Nω-nitro-L-arginine. However, sustained contractions were evoked in all tissues by phenylephrine [concentration evoking a half-maximal response (EC50) ~2 μM] or the thromboxane A2 mimetic U-46619 (EC50 ~5 nM) and less so by β,γ-methylene-ATP or histamine. Cooling to room temperature markedly suppressed (~75%) adrenergic responses but had no significant effect against U-46619 responses. Adrenergic responses, but not those to U-46619, were accompanied by an increase in intracellular Ca2+ concentration. Chelerythrine (protein kinase C antagonist) markedly antagonized adrenergic responses (mean maxima reduced 39% in artery and 86% in vein) but had no significant effect against U-46619 responses, whereas genistein (a nonspecific tyrosine kinase inhibitor) essentially abolished responses to both agonists. We conclude that cooling of the airway wall dramatically interferes with adrenergic control of bronchial perfusion but has little effect on thromboxane-mediated vasoconstriction.

The bronchial arteries originate from the aorta and/or the intercostal, internal mammary, or pericardial arteries and wind around the airways to form an adventitial or peribronchial plexus (10). The latter, in turn, sends branches down through the airway smooth muscle layer to form a submucosal plexus beneath the epithelium and then empties into the pulmonary vein (10). It is as yet unclear whether these two vascular beds flow in series, in parallel, or in some combination of the two. Blood flow to the trachea, on the other hand, is supplied by the tracheal arteries and veins.

The bronchial and tracheal vascular bed serves at least three important physiological functions: 1) nourishment of the airway wall, 2) conditioning of inspired air (warming and humidification), and 3) defense and clearance of the airways (entry point for inflammatory cells; removal of autacoids and mediators) (27). Some propose that defects in bronchial vascular function contribute to asthma, particularly exercise- and cold and/or dry air-induced bronchoconstriction (27). Also, restoration of bronchial blood flow is now recognized to be vital to success of lung transplantation (25).

Like most systemic arteries, bronchial arteries receive excitatory sympathetic and inhibitory parasympathetic innervation (5, 9). Excitatory input is mediated by the action of norepinephrine (NE) on α1-adrenoceptors (2, 20, 24, 30) and of neuropeptides coreleased with NE, such as neuropeptide Y (21). Neurotransmitters and inflammatory mediators can also act through the endothelium, causing it to release excitatory (endothelin) and inhibitory (nitric oxide) autacoids (6, 30).

In contrast to most other vascular smooth muscle beds in which temperature is maintained at 37°C, bronchial vascular temperature is expected to vary considerably, particularly during exercise. This is because heat is transferred to the inspirate to warm it to physiological temperatures; heat is also lost from the airway wall during the process of humidification of the inspired air. The magnitude of this heat transfer increases substantially during inspiration of very cold air and/or during exercise (when ventilation can increase from 5 to 200 l/min). Some, but not all, of this heat and moisture are recaptured during expiration; any deficit must ultimately be compensated for by bronchial perfusion. To our knowledge, there have not yet been any direct measurements of the temperature of blood coming out of the bronchial perfusion. However, measurements have been made of the airstream at various points within the lungs: the average temperature in the trachea can be ~32°C during quiet breathing of room air and drop to ~29°C and ~20°C during increased ventilation with room air or frigid air, respectively (23); corresponding temperatures in the subsegmental bronchi can be ~34°C and less than 30°C, respectively (12, 22, 23). The effect that these temper-
ature changes might have on bronchial vascular muscle function has not yet been investigated in detail. We hypothesized that the effects could include altered neurogenic control (which has not been investigated in any detail) and sensitivity to autacoids (e.g., inflammatory mediators). We therefore sought to investigate the responsiveness of canine bronchial and tracheal vasculature to various physiologically relevant agonists and to cooling.

METHODS

Preparation of tissues. Whole lobes of lung and tracheas were obtained from dogs that had been euthanized using pentobarbital sodium (100 mg/kg). After the overlying loose connective tissue was removed, sections of tracheal vein (0.5–2 mm OD) were excised and cut into ring segments (4–5 mm in length); the tracheal artery was usually too small to be used. Lobes of lung were treated in similar fashion; overlying connective tissue and some parenchyma were removed, after which sections of the bronchial vasculature (0.5–2 mm OD) were excised and cut into ring segments (4–5 mm long). Unless indicated otherwise, the bronchial vasculature that we excised was presumed to represent bronchial artery (because bronchial veins are generally sparse, do not travel far down the bronchial tree, and are easily distinguished when they are present from the bronchial artery with which they ramify) (27). Tissues were either used immediately or stored at 4°C for use the next day; we found no functional differences in tissues that were studied immediately compared with those used after 24 h refrigeration.

Muscle bath technique. Ring segments were mounted into 2-ml muscle baths using stainless steel hooks inserted into the lumen; care was taken not to damage the endothelium while inserting the hooks. One hook was fastened to a Grass FT.03 force transducer using silk thread (Ethicon 4–0); the other was attached to a Plexiglas rod, which served as an anchor. Tissues were bathed in Krebs-Ringer buffer (see Solutions and chemicals) containing (in mM) 125 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, 0.25 EDTA, 10 d-glucose, and 10 l-taurine, pH 7.0. Single cells were studied in Ringer buffer containing (in mM) 130 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, 20 HEPES, and 10 d-glucose, pH 7.4. Intact tissues were studied using Krebs-Ringer buffer containing (in mM) 116 NaCl, 4.2 KCl, 2.5 CaCl2, 1.6 NaH2PO4, 1.2 MgSO4, 22 NaHCO3, and 11 d-glucose, bubbled to maintain pH at 7.4. Indomethacin (10 μM) was also added to the latter to prevent generation of cyclooxygenase metabolites of arachidonic acid.

Chemicals were obtained from Sigma Chemical. The 10 mM stock solutions were prepared in aqueous medium [phenylephrine (PE), N-nitro-L-arginine (L-NNA), and phenol, DMSO (chelerythrine and genistein), or 95% EtOH (U-46619); the final bath concentration of DMSO and EtOH did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity.

Data analysis. Responses are reported as means ± SE; n refers to the number of animals. Statistical comparisons were made using Student’s t-test; P < 0.05 was considered statistically significant.

RESULTS

Excitatory regulation. We first sought to establish those autacoids that mediate excitation of these tissues. In general, vascular tissues receive excitatory neural input from the sympathetically innervated, which releases the neurotransmitters NE and/or ATP (2, 20, 24); NE mediates its excitatory effects via α-adrenoceptors (2, 20, 24, 30). We therefore examined the mechanical responses to electrical stimulation in our excised tissues.

Over the course of the 2-h equilibration period, “resting” tone increased spontaneously from the initial preload tension of 0.5 g to a mean value of 1.1 ± 0.1 g; development of this tone was dramatically accelerated on warming the baths to 37°C (e.g., see Fig. 1). Other than this spontaneous tonic activity, the tissues were quiescent, showing no spontaneous phasic activity. Only a minority of tissues contracted when stimulated electrically (5 of 36 bronchial arteries but none of 47 tracheal veins; see Fig. 1). These contractions reached a peak within 10 s and resolved to baseline within 1 min after the onset of electrical stimulation. The magnitudes of these responses ranged considerably (from 0% of the response to 60 mM KCl in most tissues up to nearly 100% of KCl in a few tissues). Thus we did not calculate the mean magnitudes of these responses. In a group of tissues from two animals, we were able to examine the frequency-response relationship of these contractions, finding an optimal frequency of 2–5 pps (Fig. 2). L-NNA (10−4 M), an inhibitor of endogenous
nitric oxide production, enhanced the magnitude of responses to electrical stimulation without altering their time course (note that all tissues had already been exposed to 10^{-5} M indomethacin for several hours); in the bronchial artery, l-NNA augmented EFS contractions in three of the five “responders” and unmasked contractions in two of the 36 “nonresponders” (Fig. 3), whereas in the tracheal vein it unmasked small EFS contractions in one of the 47 tracheal vein segments. These EFS-evoked responses were highly sensitive to the \(\alpha\)-adrenoceptor antagonist phentolamine (10^{-6} M, Fig. 3, \(n = 4\)).

Although only a minority of tissues exhibited an excitatory response to electrical stimulation even in the presence of both indomethacin and l-NNA, almost all tissues exhibited substantial contractile responses on application of the \(\alpha\)-adrenoceptor agonist PE (Fig. 2). These adrenergic responses were sustained and dose dependent (Fig. 4), with a concentration evoking a half-maximal response (EC_{50}) value of 2.0 \(\pm\) 0.5 and 3.0 \(\pm\) 0.5 \(\mu\)M in the tracheal vein and bronchial artery, respectively (\(n = 7\)).

\(\beta,\gamma\)-Methylene-ATP, a stable analog of ATP, generally had no effect on tissues at concentrations below 10^{-6} M but evoked transient contractions (lasting <5 min) at higher concentrations (Fig. 4, B and C). Because we were not able to observe a sustained “plateau” response to \(\beta,\gamma\)-methylene-ATP, we could not calculate an EC_{50} value for this agonist.

Vascular tissues are also regulated by a variety of inflammatory mediators, including thromboxane (Tx)-A2 and histamine. We found that the TxA2 mimetic U-46619 evoked sustained and dose-dependent contractions in both the tracheal vein and bronchial artery (Fig. 4), with an EC_{50} of 6.0 \(\pm\) 0.5 and 3.0 \(\pm\) 0.5 nM in the tracheal vein and bronchial artery, respectively.
Histamine, on the other hand, elicited mixed responses: 7 of 8 tracheal vein segments exhibited contractions (Fig. 4), with an EC\textsubscript{50} of 20 \( \mu \)M, whereas the remaining tissue relaxed in response to histamine.

**Effect of cooling on physiological responses.** In vivo, most vascular beds experience little or no change in temperature; in contrast, the temperature in the tracheal and bronchial vasculature can regularly fall substantially below the normal body temperature of \( \sim 37^\circ \text{C} \) (12, 22, 23). We therefore sought to examine the effects of cooling on the physiological responses previously described.

After the water pump of our muscle bath apparatus was turned off, the bath temperature fell to ambient room temperature (22–25°C) within 5 min, during which time we noticed a drop in basal tension of the tissues from 1.1 \( \pm \) 0.1 to 0.8 \( \pm \) 0.1 g (Fig. 5A); the responsiveness of the tissues was examined 20–30 min after cessation of warming (Fig. 5). At room temperature, the responses to PE were substantially and significantly reduced compared with those observed at 37°C (Fig. 5); this was not due to desensitization of the adrenergic receptors because full responsiveness was restored when the tissues were rewarmed (Fig. 5, *).

Given that U-46619 responses were largely irreversible on washout of the agonist, we could not use this same protocol to compare U-46619 sensitivity both before and after cooling. However, we did find that U-46619 could evoke substantial contractions at room temperature in tissues that had not been stimulated previously; these responses were not significantly different from those obtained in other tissues studied at 37°C (Fig. 5), and these did not demonstrate as dramatic an increase in magnitude on rewarming as did the PE responses (Fig. 5).

**Mechanisms underlying excitatory responses.** In an attempt to understand the differential sensitivity of PE and U-46619 contractions to cooling, we next examined the intracellular signaling mechanisms underlying these responses. Generally, contraction in smooth muscle is a \( \text{Ca}^{2+} \)-dependent event; we therefore examined the effect of these agonists on intracellular \( \text{Ca}^{2+} \) concentration \([\text{Ca}^{2+}]_i\) (Fig. 6). These responses were similar in amplitude and time course to those that have been examined in many other vascular and nonvascular preparations, and we did not examine them in any more detail here. Fluorometric responses could also be elicited by PE (10\(^{-5}\) M, \( n = 9 \), Fig. 6B) but not by U-46619 (10\(^{-7}\) to 10\(^{-5}\) M, \( n = 12 \), Fig. 6A). \( \beta,\gamma \)-Methylene-ATP (10\(^{-4}\) M) had no effect on 13 of 16 cells tested (\( n = 4 \)); 3 of the remaining cells showed a small fluorometric response.

These observations indicate that U-46619, though a far more potent and efficacious spasmogen than PE, does not act by mobilizing \( \text{Ca}^{2+} \). Instead, it may increase the \( \text{Ca}^{2+} \) sensitivity of the contractile apparatus (26); in other preparations, such an event is mediated by one or more protein kinases (26). We therefore examined the effects of inhibitors of protein kinase C (chelerythrine, 10\(^{-6}\) M) and of tyrosine kinases (genistein, 10\(^{-4}\) M) on responses to U-46619 and to PE. Chelerythrine suppressed basal tone (to 0.7 \( \pm \) 0.1 and 0.6 \( \pm \) 0.1 g in bronchial artery and tracheal vein, respectively).
respectively) and reduced PE contractions substantially in the case of the bronchial artery and completely in the case of tracheal vein. In contrast, U-46619 contractions in the vein were only slightly (and not significantly) reduced, whereas those in the artery were unaffected. Interestingly, the degree of inhibition produced by chelerythrine in these four test groups approximated that caused by cooling. Genistein, on the other hand, exerted substantial suppression of basal tone (to $0.6 \pm 0.1$ g in both bronchial artery and tracheal vein) as well as of the contractile responses to U-46619 and PE (Fig. 7).

**DISCUSSION**

The bronchial circulation is important for the supply of oxygen and nutrients to the various cell types of the airways (epithelium, smooth muscle, and innervation), and it has a key role in the warming and humidification of inspired air (10). In dogs, it also is important in cooling the animal because they do not sweat. The bronchial circulation can also influence airflow in several ways. Vasodilation of this vascular bed increases the thickness of the mucosa and the stiffness of the airway wall (5, 7, 16, 17, 29) and removes spasmogenic agents (inflammatory mediators, allergens, neurotransmitters, etc.), thereby accelerating recovery from bronchoconstriction (27). Finally, it contributes to allergic asthma (as a source of inflammatory cells) and exercise-induced asthma [exercise-induced vasodilation leads to thickening of mucosa, narrowing of lumen, and stiffening of airway walls (27)]. For these reasons, it is important to gain a better understanding of the control of bronchial perfusion and its response to cooling.

**Excitatory regulation of bronchial vascular tone.** As is true for many vascular beds, we found that the bronchial vasculature develops spontaneous tonic activity. We did not characterize in detail the mechanisms underlying this tone, but its insensitivity to phentolamine and to indomethacin indicates little or no role for adrenergic mechanisms or cyclooxygenase metabolites, whereas its sensitivity to both chelerythrine and genistein indicates important roles for protein kinase C and tyrosine kinases. The target(s) of these kinases is as yet unclear.
In contrast to many other vascular beds, however, we found the bronchial vasculature to be relatively poorly regulated by excitatory neurogenic input; only a minor fraction of the excised tissues exhibited any excitatory response to electrical stimulation even though almost all of them responded to an exogenously applied adrenergic agonist. Responsiveness to electrical stimulation did not improve dramatically during simultaneous inhibition of nitric oxide synthase and cyclooxygenase activities, suggesting that the endothelial factors nitric oxide and prostacyclin were not inhibiting adrenergic neurotransmission. Furthermore, because we have previously been able to demonstrate substantial excitatory neurogenic responses in airway and pulmonary vascular segments (14, 15) even after 1 or 2 days of storage in a refrigerator, we do not believe that the nerve endings were inadvertently damaged or destroyed in this study. In fact, in this study, we often observed electrically evoked inhibitory responses in these tissues (data not shown). Instead, we interpret these data to mean that the canine bronchial vasculature is poorly regulated by excitatory innervation. The nerve endings that do exist in these tissues appear to be primarily adrenergic because the neurogenic responses were essentially abolished by phentolamine.

Inflammatory cells in the airway mucosa release a wide variety of mediators, including histamine (28) and several prostaglandins (2, 17). Histamine can mediate contractions via an action on H1 receptors (2, 18, 19, 28); TxA2 is also a potent spasmogen in many vascular beds. In the bronchial vasculature, we found the TxA2 mimetic U-46619 to be the most potent and powerful spasmogen, much more so than either histamine or PE. The receptors through which U-46619 acts in this tissue (likely TxA2 receptors, given the very low EC50 value for U-46619) are apparently not coupled to phospholipase C or protein kinase C because the responses were not accompanied by a [Ca2+] transient and were completely insensitive to chelerythrine. On the other hand, the sensitivity of these responses to genistein suggests that the receptors are coupled to tyrosine kinase(s); again, the target(s) of these kinases is as yet unclear.

The endothelium may also release factors that affect vasomotor tone, nitric oxide and PGI2 being the most widely recognized mediators of its inhibitory effects and endothelin the most popular excitatory endothelial autacoid (6, 30). We did not study these regulatory pathways in detail in the present study but did obtain evidence of an ongoing synthesis (and action) of nitric oxide, as indicated by the change in basal tone on addition of l-NNA. It is as yet unclear how nitric oxide or other endothelial factors might have influenced the responses to PE or U-46619, or how cooling might affect the synthesis and/or release of endothelial signaling molecules (although the potential contributions of PGI2 were prevented using indomethacin). Endothelial regulation of this tissue needs to be examined in a subsequent study.

Relevance of the bronchial circulation to asthma. Exercise decreases airflow in >70% of individuals with symptomatic asthma (27). Two overall mechanisms...
have been proposed to account for these changes based on the findings that they can be mimicked in humans and animals by inspiration of cold and/or dry air (3) or exposure to hyperosmolar fluids (11). On the one hand, the cooling and drying associated with increased ventilation are believed to stimulate mast cells in the airway wall, either directly or through increased osmolarity of the mucosal lining, leading to degranulation and release of mediators. On the other hand, some patients still exhibit exercise-induced bronchospasm (EIB) while breathing air which has already been fully warmed and/or humidified (1), suggesting that the trigger for EIB is something other than cooling, drying, or altered osmolarity of the airway wall. A competing theory posits that bronchial arterial dilation during exercise leads to thickening of the mucosa via engorgement of the vessels per se and/or increased edema formation; this would lead to encroachment of the mucosa into the airway lumen as well as to decreased airway compliance, both of which would impair airflow (29). Bronchial arterial dilatation is accompanied by a near doubling of tracheal mucosal thickness (7) and impairment of airflow (8) in sheep; similar changes have been described in dogs (5, 16, 17).

Pertinent to this, we found that cooling of the excised tissues markedly suppressed spontaneous myogenic tone as well as that tone triggered by activation of adrenoceptors (by electrical stimulation or exogenous application of PE). Thus during increased ventilation (e.g., exercise) or inhalation of cold air, the basal level of resistance to blood flow as well as the increased resistance evoked by sympathetic/adrenergic stimulation would be reduced; the resultant vasodilation would then contribute in part to exercise- and cold air-induced asthma. The vasodilatory response to osmotic changes in the airway wall (e.g., caused by hyperosmolar fluids or dry air), on the other hand, likely involves a different mechanism(s) because a previous study has shown that inhalation of warm dry air in dogs causes an increase in tracheobronchial blood flow, which is not attenuated by α-adrenergic blockade (4). Remarkably, U-46619-evoked contractions were much less sensitive to cooling, being only moderately reduced in the tracheal vein and unaffected in the bronchial artery.

In an attempt to understand the basis for the relative differences in the sensitivity of adrenergic and U-46619 contractions to cooling, we examined the signaling mechanisms underlying these two responses and found two important differences: adrenergic responses were accompanied by an elevation of [Ca^{2+}]_{i} and were highly sensitive to an inhibitor of protein kinase C (chelerythrine), while those to U-46619 were not. It is not clear how and/or whether these functional differences account for the different sensitivity to cooling, but we noted empirically that the degree of inhibition exerted by cooling paralleled closely that exerted by chelerythrine. Both interventions reduced adrenergic contractions dramatically, reduced U-46619 contractions in the tracheal vein partially (but not significantly), and had no effect whatsoever on U-46619 contractions in the bronchial artery.

We conclude that excitation in the canine bronchial vasculature is exerted primarily by myogenic mechanisms and thromboxanes, whereas excitatory neurogenic input is relatively unimportant. Furthermore, cooling of the vasculature markedly suppresses excitatory myogenic and adrenergic responses but has little effect on tone exerted by TXA2. These findings are relevant to exercise- and cold air-induced asthma.

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REFERENCES