Microvascular dilation evoked by chemical stimulation of C-fibers in rats

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Roberts AM, Yu J, Joshua IG. Microvascular dilation evoked by chemical stimulation of C-fibers in rats. J Appl Physiol 118: 55–-60, 2015. First published October 30, 2014; doi:10.1152/japplphysiol.00077.2014.—Activation of pulmonary C-fibers can reflexively decrease heart rate, blood pressure, and peripheral vascular resistance. However, the effects of these afferents on microvascular tone remain incompletely understood. In this study, we examined the effects of these afferents on microvascular tone in a striated muscle vascular bed. The right cremaster muscle in pentobarbital-anesthetized rats with intact circulation and innervation was suspended in a tissue bath, and diameters of small arterioles were measured by intravital video microscopy. Stimulation of pulmonary C-fibers by injecting capsaicin (5 μg/kg) or phenylbiguanide (20 μg/kg) into the right atrium dilated small arterioles and decreased blood pressure and heart rate. The effects persisted when the cervical vagus nerves were cooled to 5 to 7°C (blocking myelinated fibers), but were prevented by cooling to 0°C (blocking C-fibers and myelinated fibers), by cutting the genital femoral nerve (GFN) supplying the cremaster to block the nerve supply to the muscle, or by adding 6-hydroxydopamine to the bathing medium to selectively block sympathetic effects by depleting norepinephrine from adrenergic nerve terminals. Our results show that stimulation of pulmonary C-fibers reflexively dilates small arterioles in striated muscle by a mechanism that could involve withdrawal of sympathetic adrenergic tone. In conclusion, pulmonary C-fibers can exert an inhibitory influence on neural tone of the microcirculation at an important site where microvascular resistance and tissue blood flow are regulated.

pulmonary chemoreflex; vagal afferents; pulmonary C-fibers; microcirculation; arterioles

Stimulation of pulmonary C-fibers by injecting certain chemicals into the pulmonary circulation reflexively causes hypotension and bradycardia, which are well-known components of the pulmonary chemoreflex (11, 25). Hypotension is not entirely abolished when bradycardia is prevented, suggesting that pulmonary C-fibers evoke peripheral vasodilation (9). By measuring vascular responses of whole organs or perfused tissue beds, chemical stimulation of pulmonary C-fibers has been found to reflexively dilate hindlimb resistance and splanchnic capacitance vessels (3), and to dilate vessels in skeletal muscle (5), the heart (7, 27), and the diaphragm (8). The afferent pathway of the reflex is in the vagus nerves, and depending on the vascular bed, changes in resistance have been attributed to withdrawal of sympathetic tone, or to a reflex cholinergic mechanism.

Although stimulation of pulmonary C-fibers has been found to cause regional changes in blood flow, little is known about the reflex effects of pulmonary C-fibers on individual vessels or at specific sites of the circulation, such as small arterioles (25). The present study was designed to test the hypothesis that stimulation of pulmonary C-fibers can reflexively dilate small arterioles in striated muscle. In addition to determining a major site of reflex control of peripheral resistance by directly observing the microcirculation, our objective was to investigate a mechanism by which pulmonary afferents can alter microvascular resistance.

METHODS

This protocol was approved by the University of Louisville Institutional Animal Care and Use Committee in compliance with United States Public Health Service standards and National Institutes of Health guidelines. It is in compliance with federal laws and regulations and the Guiding Principles in the Care and Use of Vertebrate Animals, published by the American Physiological Society. Male Sprague-Dawley rats (159–277 g body wt) were anesthetized intraperitoneally with pentobarbital sodium (50 mg/kg). Supplemental doses of anesthesia (~10 mg/kg ip) were given as needed to maintain an adequate level of anesthesia as defined by absence of an active corneal reflex, whisker twitching, and a withdrawal reflex to a toe pinch. The trachea was cannulated and the rats breathed room air spontaneously. The left carotid or femoral artery was cannulated to record arterial blood pressure with a Statham P23ID pressure transducer and a Grass model 7 polygraph recording system (Quincy, MA). Heart rate was recorded from the blood pressure pulse. The cervical vagus nerves were separated for cooling (described below), or the right genital femoral nerve (GFN) was separated for cutting for different protocols as needed. After conclusion of the protocols described below, the rats were euthanized by an overdose of anesthetic.

Cremaster Preparation

The right cremaster muscle was prepared for microvascular observations according to a standard technique that we have used previously (15, 34). Briefly, the skin of the right scrotum was cut medially to expose the cremaster muscle, which surrounds the testicle. Then the muscle was gently dissected free from the surrounding tissue, taking care to maintain the vascular and neural connections, and kept moist with physiological salt solution. After the muscle was incised by heat cautery, the testicle was pushed into the abdominal cavity. The cremaster muscle was suspended with silk ligatures in a flat position over an optical port in a tissue bath. The bath was filled with physiological salt solution having the following composition (in mM): 25.5 NaHCO3, 112.9 NaCl, 4.7 KCl, 1.19 KH2PO4, 1.19 MgSO4·7H2O, 2.55 CaCl2·2H2O, and 11.6 dextrose. Bath O2 tension (30–40 mmHg) and pH (7.35–7.45) were controlled by varying the amounts of N2 and CO2 bubbled through the bath.

The microvascular preparation, suspended over the optical port in the bottom of the bath, was positioned on the stage of a trinocular microscope (Leitz). Light was transmitted through the port and muscle and into the microscope. The image of the microvessels was transferred via a color video camera mounted on the microscope to a calibrated video monitor (magnification ~2,300) so that the internal
diameter of the arteriole could be measured. Microvascular responses were recorded with a videotape recorder with a stop-action feature. Arteriole selection was based on the vascular branching pattern. The largest arteriole that supplied blood to the cremaster muscle was termed the first-order arteriole. Its branches were termed second-order arterioles, and their branches were termed third-order arterioles (3As).

In this study, a single 3A from each cremaster preparation was selected for observation. Observations began at least 1 h after the cremaster had been suspended in the tissue bath.

Stimulation of Pulmonary C-Fibers

To stimulate pulmonary C-fibers, we injected capsaicin (5 µg/kg) or phenylbiguanide (20 µg/kg) (Sigma-Aldrich, St. Louis, MO) into the pulmonary circulation through a catheter placed in or near the right atrium via the right jugular vein. Capsaicin was dissolved in ethanol and Tween 80 (Fisher) as described previously (9) and then mixed with 0.9% NaCl to form a 1 mg/ml stock solution. Phenylbiguanide was dissolved in 0.9% NaCl (1 mg/ml). Capsaicin and phenylbiguanide were subsequently diluted with 0.9% NaCl and injected in volumes of 0.1 ml. Control injections of vehicles did not affect microvascular diameters. At the conclusion of experiments, an aliquot of sodium nitroprusside (10⁻³ M) was added to the bath to determine the maximal active arteriolar dilation, which also verified the vessel as having a preexisting tone.

Blockade of Reflex Pathways

Nerve conduction in vagal afferents was blocked by cooling. Conduction in myelinated fibers was blocked by cooling the vagi to 5 to 7°C (leaving input from most unmyelinated C-fibers intact), and nerve conduction was completely blocked by cooling to 0°C (16). A section of each cervical vagus nerve was gently freed from the carotid sheath and placed on the platform of a chrome-plated brass tube (3 mm OD), through which polyethylene glycol of different temperatures was circulated by a refrigerated circulating bath (Endocal RTE-4; Neslab, Newington, NH). The nerve and adjacent surface of the tube were kept moist with mineral oil. The temperature of each platform was measured with a thermistor (YSI-421; Yellow Springs Instruments, Yellow Springs, OH).

To block the major efferent nerve supply to the cremaster muscle, the right GPN was identified through an abdominal incision and then cut close to the cremaster muscle. In other experiments, to block adrenergic nerve transmission to the cremaster muscle by depleting norepinephrine from nerve terminals, 6-hydroxydopamine (Sigma) cut close to the cremaster muscle. In other experiments, to block the right GFN was identified through an abdominal incision and then

Measurements and Statistical Analysis

The arteriolar diameters were measured every 0.6 to 2 s. To obtain the baseline (or control) diameter, the vessel diameters were averaged over a 30-s period immediately prior to injecting capsaicin or phenylbiguanide. The maximum change in diameter in response to capsaicin or phenylbiguanide was taken as the largest initial response occurring within 10 s after injection. Subsequent changes within 30 s of injection are referred to as secondary or late responses. The vessel data were normalized by expressing the maximum response as a percentage of the average value during the control period. The diameter of the 3As was measured as an index of microvascular resistance. Maximum responses of blood pressure and heart rate responses during stimulation were calculated by averaging values over a 2- to 5-s period at the peak of a response and comparing them with the average for the control period. Data for groups are reported as means ± SE. A Student’s paired t-test was used to compare differences between two responses within an animal, and a r-test was used to compare data between two groups of animals. A one-way ANOVA with a Bonferroni post hoc test was used for both pairwise comparisons and comparisons vs. a control group when more than two factors were compared. Differences were considered statistically significant at P < 0.05.

RESULTS

Injection of capsaicin (Fig. 1) or phenylbiguanide (Fig. 2) into the pulmonary circulation caused arteriolar dilation in conjunction with hypotension and bradycardia (pulmonary depressor chemoreflex). This response typically began within 2 s, and the initial components of the response were similar with injection of either chemical. The effects of capsaicin and phenylbiguanide on blood pressure and heart rate in the various experiments are summarized in Table 1.

Because the pulmonary chemoreflex changes the breathing pattern, we examined the arteriolar response to phenylbiguanide during spontaneous breathing and during mechanical ventilation (Fig. 2). The increases in 3A diameters were not significantly different (58.3 ± 22.1% and 43.3 ± 21.8% of...
baseline, respectively, \( n = 3 \)). Decreases in arterial blood pressure and heart rate in response to phenylbiguanide were also not significantly different (Table 1).

Twelve 3As were examined before and during differential vagal cooling to determine the effect of selective vagal blockade on responses to capsaicin (Fig. 3) or phenylbiguanide (Fig. 4). In control experiments, when capsaicin was injected, the vessels dilated by 28.0 ± 4.7% above baseline (Fig. 3). In all of the 3As, dilation was average diameter increased from 19.5 ± 1.8 \( \mu \text{m} \) to 24.8 ± 1.8 \( \mu \text{m} \) at 3.0 ± 0.3 s, \( n = 4 \)). In all of the 3As, dilation was followed by constriction (to 10.4 ± 0.8 \( \mu \text{m} \) at 13.5 ± 1.9 s), which averaged 45.9 ± 5.3% of baseline (\( P < 0.05 \)). The late dilation (to 24.5 ± 3.2 \( \mu \text{m} \)) reached its peak 26.7 ± 1.0 s after injection and averaged 24.7 ± 7.5% above baseline (\( P < 0.05 \)).

![Table 1. Effects of capsaicin and phenylbiguanide on blood pressure and heart rate](http://jap.physiology.org/)

<table>
<thead>
<tr>
<th>Mechanical ventilation</th>
<th>Blood Pressure, mmHg</th>
<th>Heart Rate, beats/min</th>
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<tbody>
<tr>
<td><strong>Rats, n</strong></td>
<td><strong>Control</strong></td>
<td><strong>Change</strong></td>
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<tr>
<td><strong>Mechanical ventilation</strong></td>
<td></td>
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<tr>
<td>PBG Before</td>
<td>3</td>
<td>113 ± 6</td>
</tr>
<tr>
<td>PBG After</td>
<td>3</td>
<td>104 ± 6</td>
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<tr>
<td><strong>Vagal nerve temperature</strong></td>
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<tr>
<td>CAPS 37°C</td>
<td>5</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>CAPS 37°C</td>
<td>4</td>
<td>98 ± 9</td>
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<tr>
<td>CAPS 0°C</td>
<td>4</td>
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<td>7</td>
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<tr>
<td>PBG 0°C</td>
<td>4</td>
<td>85 ± 5*</td>
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<tr>
<td><strong>Genital femoral nerve</strong></td>
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<tr>
<td>CAPS Intact</td>
<td>6</td>
<td>108 ± 9</td>
</tr>
<tr>
<td>CAPS Cut</td>
<td>6</td>
<td>98 ± 11</td>
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<td>6-Hydroxydopamine</td>
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<tr>
<td>PBG Before</td>
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<td>115 ± 13</td>
</tr>
<tr>
<td>PBG After</td>
<td>4</td>
<td>107 ± 7</td>
</tr>
</tbody>
</table>

Values are means ± SE. CAPS, capsaicin; PBG, phenylbiguanide. *\( P < 0.05 \) vs. response at 37°C. †\( P < 0.05 \) vs. response at 5 to 6°C.
When the vagi were cooled to 0°C (Fig. 3), baseline diameter averaged 20.9 ± 2.3 μm (n = 4) and the initial vasodilation induced by capsaicin injection was prevented (P < 0.05). Constriction (to 10.7 ± 1.2 μm), which averaged 48.3 ± 5.1% of baseline (P < 0.05) at 12.9 ± 1.6 s, was unchanged by vagal cooling. The late dilation (21.0% above baseline at 20.0 s) still occurred in one of the four 3As. At the end of each experiment, after addition of sodium nitroprusside to the bath (10^{-5} M), the average of the diameters increased to 27.8 ± 2.0 μm.

In the capsaicin experiments, blood pressure and heart rate both significantly decreased (Table 1) unless the vagi were cooled to 0°C. Cooling attenuated the decreases and unmasked a pressor response that followed the initial hypotension observed before cooling in about half of the experiments.

The response of seven 3As to the injection of phenylbiguanide was examined (Fig. 4). Phenylbiguanide caused a 40.6 ± 7.1% dilation (P < 0.05) that peaked 4.7 ± 0.7 s after injection and resulted in an increase in arteriolar diameter from 18.2 ± 2.3 μm to 25.1 ± 2.7 μm.

When the vagi were cooled to 5 to 7°C (n = 6), phenylbiguanide still significantly dilated the 3As by 16.6 ± 3.9% (P < 0.05) at 6.0 ± 1.3 s. The arteriolar diameter increased from 24.0 ± 4.0 μm to 28.1 ± 4.7 μm. However, the dilation in response to phenylbiguanide was prevented (P < 0.05) by cooling the vagi to 0°C (n = 4). At 0°C, baseline diameter averaged 21.3 ± 3.1 μm. At the end of each experiment, addition of sodium nitroprusside to the bath increased the diameters to 29.2 ± 3.2 μm.

In six 3As, we examined the arteriolar response to capsaicin before and after cutting the right GFN. All of the tested 3As dilated immediately after cutting the nerve and then gradually regained tone at a new steady state (Fig. 5). Similar to cooling the vagi to 0°C, cutting the GFN also prevented the initial dilation (a 39.1 ± 13.3% increase in diameter, P < 0.05) caused by injection of capsaicin. The constriction was unaltered by cutting the GFN. On average, there was a 70.0 ± 17.5% decrease (P < 0.05) in diameter (at 12.1 ± 1.2 s) in four of six 3As with GFN intact. In all six 3As after the GFN were cut, a 69.3 ± 14.2% decrease (P < 0.05) occurred 13.4 ± 1.2 s after injection. The late dilation (74.5 ± 22.2% at 17.4 ± 1.9 s after injection, P < 0.05) appeared in all 3As with GFN intact and in three of six 3As (13.2 to 69.8% at 10 to 25 s after injection, P < 0.05) after GFNs were cut.

In four 3As (average diameter 10.4 ± 0.1 μm), we examined the arteriolar response to phenylbiguanide before and after adding 6-hydroxydopamine to the bath (Fig. 6). Treatment of
the tissue with 6-hydroxydopamine abolished the dilation (48.7 ± 7.8 μm, \( P < 0.05 \)) in response to phenylbiguanide.

DISCUSSION

Capsaicin and phenylbiguanide have been widely used in afferent and reflex studies to stimulate vagal C-fiber endings (10). Both substances stimulate pulmonary C-fibers and initiate a pulmonary chemoreflex in rats within 2 s when injected into the pulmonary circulation via the right atrium (29). In the present study, we found that both of these chemicals caused hypotension and bradycardia, as reported previously in rats and other species (10, 11, 29).

The results of this study show that stimulation of vagal C-fibers can evoke dilation of small arterioles in striated muscle and that microvascular dilation is a component of the pulmonary depressor chemoreflex. Dilation coincides with the onset of the hypotension and bradycardia and is elicited by two different chemicals that have the common property of stimulating pulmonary C-fibers. The results also demonstrate that both the initial dilation to capsaicin and the dilation to phenylbiguanide are reflexively mediated via a vagal afferent pathway. Like the other components of the pulmonary chemoreflex, these initial dilations persisted when the vagus nerves were cooled to 5 to 7°C, but not to 0°C. Because myelinated axons are blocked at 5 to 7°C (16), it appears that unmyelinated C-fibers mediated the arteriolar dilation.

Right atrial injection of capsaicin in dogs causes a vagally mediated reflex dilation of hindlimb resistance vessels and splenic capacity elements that coincides with the onset of hypotension and bradycardia (3). Stimulation of pulmonary C-fibers by injecting capsaicin into the pulmonary circulation also causes vasodilation in the coronary circulation (7, 27) and diaphragm (8). Our results extend these observations of perfused vascular beds and provide direct evidence that pulmonary C-fibers can reflexively alter microvascular tone at the level of 3As, an important site for regulating microvascular resistance in striated muscle.

Changes in breathing have been well-described as a component of the pulmonary chemoreflex (10, 11). In rats, Sapru et al. (29) reported an initial apnea followed by rapid, shallow breathing. Thus microvascular tone could be altered by changes in breathing through reflex pathways (34) or through changes in blood gases (26, 33). Our results suggest that the microvascular dilation evoked by stimulating pulmonary C-fibers was not caused by altered breathing. The vasodilation was essentially the same in magnitude and onset when the animals breathed spontaneously or when the lungs were ventilated at constant rate and tidal volume.

Our finding that cutting the GFN, which innervates the cremaster (18), prevented the initial dilation to capsaicin, further demonstrates that the response was a reflex and that the efferent pathway was mediated by sympathetic nerves. This conclusion is also supported by blockade of dilation to phenylbiguanide by treatment of the tissue with 6-hydroxydopamine, which selectively destroys noradrenergic neurons, reducing their norepinephrine content and binding sites (24). The concentration of 6-hydroxydopamine and method of administration that we used in our experiments is similar to what has been used in other microvascular studies to selectively destroy catecholaminergic neurons in the peripheral circulation (1, 12, 30). Arterioles still retained their ability to dilate to topical application of sodium nitroprusside, indicating that 6-hydroxydopamine did not have a nonspecific depressive effect on vascular smooth muscle. Therefore, the ability of 6-hydroxydopamine to prevent peripheral sympathetic postganglionic input to the cremaster vascular bed from modulating norepinephrine release suggests that the efferent arm of the reflex involves withdrawal of sympathetic vasoconstrictor tone. This is more specific than cutting the GFN, which would remove neural input altogether. Other evidence for sympathetic withdrawal during the reflex dilation is the gross reduction in sympathetic output to the cardiovascular system as indicated by decreases in systemic blood pressure and heart rate. These decreases still occurred after the GFN was cut or 6-hydroxydopamine was added to the bath. Because the GFN has motor efferent fibers in addition to sympathetic efferent fibers, a decrease in sympathetic nerve activity in itself would not verify the issue of sympathetic withdrawal. Our focus was to define the reflex effects of the pulmonary chemoreflex on a site of microvascular resistance in a striated muscle vascular bed. Although our data indicate that the efferent arm of the reflex involves sympathetic withdrawal of adrenergic tone, further studies beyond the scope of the present investigation are needed to define the mechanism of this reflex component.

Capsaicin has complex actions on the cardiovascular system that vary with the route of administration, dose, and species (6, 13, 31). In rats, a triphasic effect caused by intravenous injection has been described that, in addition to hypotension, includes a transient, and then a sustained, pressor response (6). Although it is established that the initial hypotension and bradycardia are mediated by vagal afferent pathways, the pressor responses are not as well understood.

In our experiments, capsaicin caused delayed arteriolar constriction and dilation that were present after vagal block or cutting the GFN. We did not try to identify the mechanism for these nonvagal microcirculatory effects, which need to be clarified by other studies. Capsaicin can act at other sites when it reaches the systemic circulation (21), or cause cardiovascular changes by acting on the central nervous system (28). There is evidence that vasoconstriction could result from a direct effect of capsaicin on peripheral vessels by an action involving calcium entry into smooth muscle (31), or from a sympathetic reflex or adrenergic mechanism (6). Capsaicin has also been found to cause local dilation of arterioles in the rat cremaster muscle via release of calcitonin gene-related peptide from primary afferent nerve fibers (22, 32), and nonvagal vasodilator effects of capsaicin may occur via release of other neuropeptides or effects on vascular smooth muscle (13, 14, 19). In addition, capsaicin may be involved in both constriction and dilation responses by releasing substance P, which can have excitatory effects at peripheral nerves (17) and sympathetic ganglia (20), or inhibitory effects directly on small arterioles (4).

In conclusion, the present results show that stimulation of pulmonary C-fibers can reflexively cause dilation of small arterioles in striated muscle. Thus it provides direct evidence that microvascular dilation is a component of the pulmonary depressor chemoreflex. These findings indicate that pulmonary C-fibers can exert an inhibitory influence on the neural tone of the microcirculation at an important site where microvascular resistance and tissue blood flow are regulated. In view of the
variety of endogenous substances and conditions that can activate pulmonary C-fibers (10, 25), it is likely that this afferent input has a role in modulating microvascular blood flow in a number of peripheral vascular beds during physiological and pathophysiological conditions. This modulation may occur in response to changes in the pulmonary environment and alter the distribution of blood flow in various vascular beds. Further investigation of this inhibitory mechanism will improve our understanding and perspective of the role of pulmonary vagal afferents in the neural regulation of the microcirculation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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