Fenfluramine-induced pulmonary vasoconstriction: role of serotonin receptors and potassium channels

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Bělohlávková, Simona, Jan Šimák, Alena Košková, Olga Hnilíčková, and Václav Hampl. Fenfluramine-induced pulmonary vasoconstriction: role of serotonin receptors and potassium channels. J Appl Physiol 91: 755–761, 2001.—The anorectic agent fenfluramine considerably increases the risk of primary pulmonary hypertension. The mechanism of this effect is unknown. The appetite-reducing action of fenfluramine is mediated by its interaction with the metabolism of serotonin [5-hydroxytryptamine (5-HT)] in the brain. We tested the hypothesis that the pulmonary vasoconstrictive action of fenfluramine is at least in part mediated by 5-HT receptor activation. In addition, we sought to determine whether pharmacological reduction of voltage-gated potassium (Kv) channel activity would potentiate the pulmonary vascular reactivity to fenfluramine. Using isolated rat lungs perfused with Krebs-albumin solution, we compared the inhibitory effect of ritanserin, an antagonist of 5-HT2 receptors, on fenfluramine- and 5-HT-induced vasoconstriction. Both 5-HT (10⁻⁷ mol/l) and fenfluramine (5 × 10⁻⁴ mol/l) caused significant increases in perfusion pressure. Ritanserin at a dose (10⁻⁷ mol/l) sufficient to inhibit >80% of the response to 5-HT reduced the response to fenfluramine by ~50%. A higher ritanserin dose (10⁻⁵ mol/l) completely abolished the responses to 5-HT but had no more inhibitory effect on the responses to fenfluramine. A pharmacological blockade of Kv channels by 4-aminopyridine (3 × 10⁻³ mol/l) markedly potentiated the pulmonary vasoconstrictor response to fenfluramine but was without effect on the reactivity to 5-HT. These data indicate that the pulmonary vasoconstrictor response to fenfluramine is partly mediated by 5-HT receptors. Furthermore, the pulmonary vasoconstrictor potency of fenfluramine is elevated when the Kv-channel activity is low. This finding suggests that preexisting Kv-channel insufficiency may predispose some patients to the development of pulmonary hypertension during fenfluramine treatment.

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¹The 5-HT₂C receptors were originally described as 5-HT₁C receptors and were reclassified as 5-HT₂C later, based on structural and operational properties (18). Receptors originally described as 5-HT₂ are now called 5-HT₂A. This accounts for some degree of confusion in the literature. We use the term 5-HT₂ receptors as denomination for the whole family consisting of 5-HT₂A, 5-HT₂B, and 5-HT₂C receptors (2).

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d-fenfluramine is able to directly activate 5-HT receptors (13, 41). Inhibition of 5-HT uptake by fenfluramine has been demonstrated not only in neuronal cells but also in vascular endothelial cells and platelets.

Interestingly, anomaly of 5-HT handling has been implicated in the etiology of pulmonary hypertension (9, 17). Circulating plasma levels of 5-HT are markedly increased in patients with primary pulmonary hypertension (17). The fawn-hooded strain of rats, known for its genetic platelet storage pool defect, has an elevated plasma 5-HT concentration and spontaneously develops pulmonary hypertension when exposed to a very mild hypoxia (36). Continuous 5-HT infusion potentiates the development of pulmonary hypertension induced in normal rats by a chronic exposure to hypoxia (9). In pulmonary arteries isolated from dogs or rats, 5-HT causes a dose-dependent vasoconstriction (21, 36). Continuous 5-HT infusion potentiates the development of pulmonary hypertension induced in normal rats by a chronic exposure to hypoxia (9). In pulmonary arteries isolated from dogs or rats, 5-HT causes a dose-dependent vasoconstriction (21, 24). The 5-HT action on vascular smooth muscle is mediated mainly through peripheral 5-HT1A, 5-HT2A, and 5-HT7 receptors (18, 23, 39). In light of these data, it is relevant to hypothesize that the effect of fenfluramine on the pulmonary circulation may be mediated by its action on the 5-HT receptors in the pulmonary vascular smooth muscle. In the present study, we used ritanserin, an antagonist of 5-HT2 receptors (4, 18), to test this hypothesis.

One of the most puzzling aspects of the fenfluramine-associated pulmonary hypertension is the fact that only a minority of patients taking fenfluramines actually develops pulmonary hypertension. What makes certain subjects particularly vulnerable is completely unknown, yet of utmost interest. One hypothesis attempting to explain this phenomenon is based on the observation that fenfluramine reduces the 4-aminopyridine (4-AP)-sensitive potassium (K) currents in the pulmonary arterial smooth muscle cells (43). 4-AP is a relatively selective inhibitor of the voltage-gated family of potassium channels (Kv channels) (29). It has been postulated that individuals with preexisting Kv-channel dysfunctions may be more vulnerable to the Kv-channel-inhibiting action of fenfluramine and, consequently, the development of fenfluramine-induced pulmonary hypertension (44). The second goal of the present study, therefore, was to find whether reduced Kv-channel activity potentiates the pulmonary vasoconstrictor effect of fenfluramine.

METHODS

Isolated perfused rat lung. Isolated perfused rat lungs were prepared as previously described (15, 16, 43). Adult female Wistar rats (210–300 g body wt) were anesthetized with ketamine (100 mg/kg) and xylazine (16 mg/kg) intramuscularly. A tracheostomy was performed, and the lungs were ventilated with a continuous-flow, pressure-limited, time-cycled ventilator at 40 breaths/min (peak inspiratory pressure = 12.5 cmH2O; inspiration-to-expiration time ratio = 1:2) with a warmed, humidified mixture of 95% air and 5% CO2. A median sternotomy was performed, heparin (200 IU) was injected into the right ventricle, and cannulas were placed into the pulmonary artery and left ventricle. The heart, lungs, and mediastinal structures were removed en bloc and suspended in a humid chamber at 37°C.

The preparation was perfused through a pulmonary artery cannula using a peristaltic pump at a constant flow rate of 0.04 ml·min−1·g body wt−1 with a warm (37°C) Krebs solution containing bovine serum albumin (4%). To minimize a possible confounding influence of the endothelial cells’ alteration by pharmacological agents used in the experiments, meclofenamate sodium (1.7 × 10−5 mol/l), a cyclooxygenase blocker, and Nω-nitro-L-arginine methyl ester (L-NAME, 5 × 10−5 mol/l), a nitric oxide synthase inhibitor, were added to the perfusate before the beginning of perfusion. The first portion of the perfusate (50 ml) was used to wash out remnants of blood from the preparation and was discarded. Additional perfusate (50 ml) was then used for recirculation. Pulmonary artery perfusion pressure was measured from a side port of the pulmonary artery line, and maximum changes were recorded. Because the flow rate was held constant, the changes in perfusion pressure directly reflect changes in vascular resistance.

Ritanserin (10−7 or 10−5 mol/l) or its vehicle, tartaric acid (10−3 mol/l), was added to the reservoir at the beginning of recirculation. After 15 min of equilibration, a bolus injection of 5-HT (10−5 mol/l final perfusate concentration), fenfluramine (5 × 10−4 mol/l), or angiotensin II (2 × 10−9 mol/l) was given into the pulmonary artery cannula. Each lung received only one of these agonists. A higher dose of the same agonist used in each lung in the first injection was administered 15 min later (Fig. 1). Control groups received saline only, ritanserin (10−5 mol/l) only, or tartaric acid only.

In a separate set of experiments, 4-AP at a final concentration of 3 × 10−3 mol/l was added to the reservoir at minute 15 of perfusion. This dose is at the upper limit for the selectivity for Kv-channel inhibition (28, 29). After the vasoconstrictor response to 4-AP reached a plateau (8–15 min after 4-AP administration), fenfluramine (5 × 10−3 mol/l) or 5-HT (10−4 mol/l) was injected into the pulmonary artery cannula (Fig. 2).

In all experiments, perfusion was finished after 45 min and pulmonary edema was evaluated as a wet-to-dry weight ratio of the right lung tissue.

Chemicals. All drugs were purchased from Sigma Chemical, Prague, Czech Republic. Angiotensin II, 5-HT, meclofenamate sodium, L-NAME, and fenfluramine hydrochloride were dissolved in Krebs solution. Ritanserin was dissolved in 10−2 mol/l tartaric acid, tartaric acid was dissolved in distilled water, and 4-AP was dissolved in normal saline.

Statistical analysis. Data are expressed as means ± SE. The groups were compared using factorial ANOVA followed by Fisher’s least significant difference post hoc test. There were five lungs in each experimental group, which was shown to be a sufficient number to guard reasonably against type II statistical error by power analysis (Power ≥ 0.74). Differences were considered significant at P < 0.05.

RESULTS

Fenfluramine increased perfusion pressure in the isolated rat lungs perfused at a constant flow rate. A bolus injection resulting in a final perfusate concentration of 5 × 10−4 mol/l caused a steady elevation of perfusion pressure from 6.5 ± 0.5 to 9.8 ± 0.7 mmHg (Fig. 1A). Another bolus, resulting in a perfusate concentration of 10−3 mol/l, caused a further, transient increase of perfusion pressure to 12.1 ± 0.9 mmHg followed within minutes by a steady, elevated plateau of 11.2 ± 1.3 mmHg (Fig. 1A). Lower fenfluramine concentrations tested (10−7 and 10−6 mol/l) did not
cause any significant changes in perfusion pressure (data not shown).

5-HT caused a transient increase in perfusion pressure from the baseline of 6.2 ± 0.2 mmHg to a peak of 9.6 ± 0.1 mmHg at a perfusate concentration of 10⁻⁵ mol/l and a sustained increase to 8.0 ± 0.4 mmHg at 10⁻⁴ mol/l (Fig. 1B).

The vasoconstrictor response to fenfluramine (5 × 10⁻⁴ mol/l) was ~50% inhibited by the 5-HT₂ receptor antagonist, ritanserin, given at a dose of 10⁻⁷ mol/l (Figs. 1A and 3). To confirm the specificity of ritanserin at this dose as a 5-HT receptor blocker in our preparation, additional experiments were performed with 5-HT and angiotensin II. Ritanserin at 10⁻⁷ mol/l prevented most of the vasoconstrictor response to 10⁻⁸ M 5-HT (Figs. 1B and 3) but had no significant effect on the reactivity to 5 × 10⁻¹⁰ M angiotensin II (Figs. 1C and 3). The next higher ritanserin dose studied (10⁻⁵ mol/l), on the other hand, reduced the response to angiotensin II by ~70% (Figs. 1C and 3), indicating that at this higher dose ritanserin was not selective for the 5-HT receptors. However, this high, nonselective ritanserin dose did not have any more inhibiting effect on the fenfluramine-induced pulmonary vasoconstriction than the lower, specific dose of 10⁻⁷ mol/l (Figs. 1A and 3).

Adding 4-AP (3 × 10⁻³ mol/l), a KV-channel inhibitor, to the perfusate of isolated lungs significantly increased perfusion pressure by 1.8 ± 0.6 mmHg. The presence of 4-AP in the perfusate did not alter the vasoconstrictor response to 5-HT (10⁻⁴ mol/l; Fig. 4). In contrast, the vasoconstrictor reactivity to both 5 × 10⁻⁴ and 10⁻³ mol/l fenfluramine was significantly potentiated by 4-AP (Fig. 4).

In control lungs treated with tartaric acid alone (vehicle for ritanserin) or with ritanserin alone, there was a slight initial decrease in perfusion pressure during stabilization (observed in most lungs). Thereaf-
ter, the perfusion pressure was stable (Fig. 1D). The wet-to-dry weight ratio of the lung tissue was 6.4 ± 0.3 in lungs treated with 10⁻⁷ M ritanserin plus 5 × 10⁻⁴ mol/l fenfluramine and was not significantly different from lungs of any other group including lungs treated with ritanserin vehicle (tartaric acid) or saline alone (data not shown).

**DISCUSSION**

It is well known that the appetite suppressant fenfluramine has multiple effects on the serotonergic signaling and that 5-HT is capable of contributing to the development of pulmonary hypertension. However, the possibility that the effects of fenfluramine on the pulmonary vessels are mediated by 5-HT receptors has not been directly tested. Our present finding that 10⁻⁷ mol/l ritanserin reduced fenfluramine-induced vasoconstriction in isolated rat lungs by 50% (Fig. 3) shows that 5-HT receptors participate in the pulmonary vasoconstrictor response to fenfluramine. The selectivity of this low ritanserin dose for 5-HT receptor blockade in our preparation was confirmed by our experiments showing that it abolished most of the response to 5-HT without having any significant effect on the reactivity to angiotensin II (Fig. 3). Interestingly, the inhibitory effect of ritanserin on the fenfluramine-induced pulmonary vasoconstriction could not be further augmented by rising the ritanserin dose to a level that completely erased reactivity to 5-HT and started to show some degree of nonspecificity, evidenced by the diminished responses to angiotensin II. Taken together, these data strongly indicate that the effect of the lower ritanserin dose (10⁻⁷ mol/l) is indeed attributable to the inhibition of the 5-HT receptors.

Although the response of the systemic vessels to 5-HT is believed to be mediated mainly by the 5-HT_2A receptors (18), there is also evidence for the presence of the 5-HT_2B receptors in vascular tissues (4, 18). Therefore, we sought to inhibit all these receptors. We chose ritanserin, which has an approximately even affinity to all members of the 5-HT receptor family (4).

In addition to the role of 5-HT_2 receptors in vasoconstrictor response to 5-HT [and 5-HT_7 receptors, for-
merly known as 5-HT₁-like receptors (2), in intracranial vessels (18, 39), there are data implying the existence of receptors of the 5-HT₁ group in the pulmonary vessels (8, 20, 21, 27), although in the rat these receptors only appear active during chronic hypoxic exposure (21). Nevertheless, the possibility should be considered that the vasoconstrictor response to fenfluramine cannot be fully inhibited by ritanserin because it involves 5-HT₁ receptors. If 5-HT₁ receptors were present and functional in our preparation, they might be expected to be activated by exogenous 5-HT and thus contribute to the total response to 5-HT. In such a case, a portion of the 5-HT response should be resistant to 5-HT₂ receptor blocker such as ritanserin. In fact, in the presence of 10⁻⁷ mol/l ritanserin, a rudimentary response to 5-HT was still detectable. The fact that the 5-HT response was completely abolished by the higher ritanserin dose (10⁻⁵ mol/l) could be interpreted as evidence against the involvement of other receptors than 5-HT₂ if it were not for the fact that this ritanserin dose also reduced the angiotensin II response. Because angiotensin II reactivity is unrelated to 5-HT receptors, it is possible that the additional reduction in 5-HT reactivity caused by the higher compared with the lower ritanserin dose is due to some nonselective effects rather than to a more complete blockade of the 5-HT₂ receptors. A small contribution of 5-HT reactivity caused by the higher compared with the lower ritanserin dose also reduced the 5-HT and fenfluramine responses in our preparation thus cannot be conclusively excluded. However, if such a contribution does indeed exist, it can account for only a minimal portion of the fenfluramine response, as judged from the difference between the degree of inhibition by ritanserin of the 5-HT and fenfluramine responses (Fig. 3).

What accounts for the part of the fenfluramine response persisting in the presence of ritanserin is not clear from our data. One possibility is that the inhibition of Kv channels and consequent membrane depolarization described in the pulmonary arterial smooth muscle cells by Weir et al. (43) is unrelated to 5-HT₂ receptor activation. This alternative is supported by our data showing that 4-AP, a Kv-channel blocker, augments the response to fenfluramine but not to 5-HT. Nevertheless, the possibility that Kv-channel inhibition is a consequence of 5-HT₂ receptor activation also should be considered. These receptors activate the inositol 1,4,5-trisphosphate second messenger system (18), which in turn releases calcium from the endoplasmic reticulum. Post et al. (30) provided evidence that an increased intracellular calcium concentration may suppress Kv-channel activity in the pulmonary arterial smooth muscle cells. Thus Kv-channel inhibition might be a consequence of 5-HT₂ receptor activation by fenfluramine. On the other hand, Reeve et al. (32) found that the δ-fenfluramine-induced increase in intracellular calcium concentration can be inhibited by caffeine. This finding suggests that fenfluramines activate the ryanodine-sensitive calcium channel of the endoplasmic reticulum, even though the specificity of caffeine as a probe to study this channel is poor (31). Because the ryanodine-sensitive channel is independent of inositol 1,4,5-trisphosphate and thus of 5-HT₂ receptors, this mechanism may explain the ritanserin-insensitive part of fenfluramine-induced pulmonary vasoconstriction.

The second principal finding of this study is that the pulmonary vasoconstrictor reactivity to fenfluramine is augmented by pretreatment with the Kv-channel blocker 4-AP (Fig. 4). In general, the baseline vascular tone in the normal lung is very low, especially with blood-free perfusion. Increasing the baseline tone tends to improve vasoconstrictor reactivity (25). However, in the present study, only the reactivity to fenfluramine, but not to 5-HT, was potentiated by 4-AP. This implies that the potentiation of the fenfluramine response by 4-AP was not a nonselective effect of increased vascular tone. Fenfluramine is known to reduce Kv-channel activity in pulmonary arterial smooth muscle cells (43). Kv-channel activity is a major determinant of the pulmonary arterial smooth muscle cells’ membrane potential (3, 45). That is a likely reason why fenfluramine causes pulmonary arterial smooth muscle cell depolarization (43). In theory, it might be expected that the impact of a given dose of fenfluramine on vascular smooth muscle membrane potential (and consequently vascular tone) would be augmented under conditions of diminished starting membrane potassium conductance. Our data provide experimental support for this notion by showing that, when the Kv-channel activity is reduced (by 4-AP administration) before the exposure to fenfluramine, the resulting pulmonary vasoconstrictor response is higher than the sum of the response to 5-HT-channel inhibition and to fenfluramine. This is consistent with the idea that some individuals may have an abnormally low activity of Kv channels, which is not functionally (and clini-

The main limitation of this work is the one shared with most other experimental studies of the anorectic-induced pulmonary hypertension: the doses of fenfluramine needed to elicit an effect in experiments are higher than doses causing pulmonary hypertension in patients. The sustained plasma concentration of fenfluramine in humans who use it as a weight-reducing agent is about 10⁻⁶ mol/l, whereas we used 5 × 10⁻³ mol/l. The reason for this discrepancy is unknown. It could be partly related to the binding of fenfluramine in plasma (~40%) (38). Also, the metabolism of fenfluramine is much faster in rats than in humans (22). There are data supporting species differences in the density of 5-HT receptors (19, 35). It is important to consider that, in our study, each of a relatively small series of lungs responded with vasoconstriction to the relatively high dose of fenfluramine. On the other hand, in the human population, only one in several hundred of those ingesting fenfluramines develops pulmonary hypertension (13, 37). It is possible that, if
fenfluramine could be administered to a number of rat lungs resembling the number of human users, some of them might respond even to a clinically relevant low dose. In addition, the isolated lungs perfused with a blood-free solution without pharmacological preconstriction appear to have a considerably lower vasoconstrictor reactivity than lungs in vivo, at least judging from the physiologically important responses to acute hypoxia (25). Nevertheless, our finding that the inhibitory effect of ritanserin on a higher fenfluramine dose ($10^{-3}$ mol/l; data not shown) was similar to the effect on the lower dose suggests that our conclusion about the involvement of 5-HT receptors might be extrapolated also to fenfluramine doses lower than tested in this study.

A related issue is the fact that we studied acute effects of fenfluramine on vascular wall tension, whereas pulmonary hypertension, in which remodeling of vascular structure is an important feature, develops only after a prolonged anorectic intake (1). Studying acute effects is a common strategy because attempts to reproduce anorectic-induced pulmonary hypertension by chronic exposure of experimental animals to anorectics have been mostly unsuccessful. Thus, although the current data show a role for 5-HT receptors in a vasoconstrictor response to fenfluramine, the mechanism of chronic fenfluramine-induced pulmonary hypertension is most likely to be more complex. Nevertheless, in the pulmonary circulation, stimuli that cause acute vasoconstriction (such as hypoxia or 5-HT) lead to or augment chronic pulmonary hypertension (10, 11).

The pulmonary vascular reactivity to fenfluramines is known to be modulated by the endothelium. Specifically, inhibition of nitric oxide synthase (presumably in the endothelium) has been shown to augment the vasoconstrictor response to $\alpha$-fenfluramine (43). Thus it is likely that the effects observed in this study are less pronounced in vivo. The influence of endothelium-produced prostaglandins on the fenfluramine reactivity has not been studied. We excluded their potentially confounding effect by inclusion of cyclooxygenase blocker in the perfusate because endogenous prostaglandins are known to modulate pulmonary vasoactivity to a number of other stimuli (42). It is still possible that the responses observed in this study were affected by other endothelial products, such as the insufficiently characterized endothelium-derived hyperpolarizing factor (which does not currently have any specific blocker) or endothelin.

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