Dexfenfluramine-Associated Changes in 5-Hydroxytryptamine Transporter Expression and Development of Hypoxic Pulmonary Hypertension in Rats

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ABSTRACT

The appetite suppressant dexfenfluramine, which inhibits neuronal 5-HT uptake and elevates plasma 5-HT levels, has been associated with an increase in the relative risk of developing primary pulmonary hypertension. 5-HT is a mitogen for pulmonary artery smooth muscle cells (PA-SMCs), an effect that depends upon activity of the 5-HT transporter (5-HTT). To investigate the relationship between dexfenfluramine and pulmonary hypertension, we examined 1) the effect of dexfenfluramine on 5-HT uptake by PA-SMCs and the mitogenic response of these cells to 5-HT, and 2) 5-HTT mRNA in lung tissue from normoxic and chronically hypoxic rats during and at discontinuation of a 4-week dexfenfluramine treatment (2 mg/kg/day). In cultured PA-SMCs, dexfenfluramine (10^-6 M) markedly reduced [3H]5-HT uptake and [3H]thymidine incorporation in response to 5-HT (10^-6 M). In lungs from rats exposed to 4-week hypoxia (10% O_2), 5-HTT mRNA levels were higher than in normoxic rats (233.5 ± 22.5 versus 121.8 ± 4.8 amol/mg of RNA, P < 0.05), but were not affected by concomitant treatment with dexfenfluramine. One week after discontinuation of dexfenfluramine, 5-HTT mRNA levels increased substantially, this effect being additive with that of hypoxia (364.0 ± 13.1 in hypoxic versus 164.2 ± 10 amol/mg of RNA in normoxic rats). When exposure to 2 weeks of hypoxia followed discontinuation of a 4-week treatment, right ventricular hypertrophy was more severe and muscularization of distal pulmonary arteries more marked (P < 0.01) than in rats pretreated with the vehicle. These data show that, in rats, the increased 5-HTT expression that follows dexfenfluramine discontinuation promotes the development of hypoxic pulmonary hypertension.

Dexfenfluramine is the active enantiomer of a substituted phenethylamine, fenfluramine, which has been extensively used as a weight-reducing agent in obese patients (Blundell and Lawton, 1995). Support for the existence of a link between the use of appetite suppressants and the development of PPH was provided by an epidemiological study in which obese patients having used these drugs for more than 3 months had an at least 23.1-fold increase in the absolute risk of PPH as compared with nonusers (Abenhaim et al., 1996). Withdrawal of these drugs from the market was also prompted by reports of heart valve incompetence in patients who had taken fenfluramine and phentermine (Connolly et al., 1997).

The appetite-suppressant effect of fenfluramine derivatives is thought to be dependent upon inhibition of neuronal 5-HT reuptake, increased indoleamine release, and subsequent 5-HT receptor stimulation (Caccia et al., 1993; Rothman et al., 1999). Inhibition of 5-HT uptake by fenfluramine is not confined to nerve cells but also occurs in platelets, and possibly in pulmonary endothelial and smooth muscle cells since these various cell types share the same 5-HT transporter encoded by a single gene (Lesch et al., 1994). The increase in 5-HT plasma levels observed during treatment with fenfluramine derivatives may therefore be a consequence of impaired 5-HT uptake by platelets and pulmonary vascular cells (Celada et al., 1994). Several conditions leading to the development of pulmonary hypertension have been shown to be associated with an increase in 5-HT plasma levels. Fawn-hooded rats, which are characterized by genetically deficient 5-HT storage in plate-
lets, develop pulmonary hypertension when exposed to mild hypoxia (Sato et al., 1992). PPH has also been observed in humans with a similar platelet 5-HT storage deficit (Hervé et al., 1990). The possibility that 5-HT may promote the development of pulmonary hypertension is further supported by data from our group showing that continuous intravenous 5-HT infusion during a 2-week exposure to hypoxia aggravated pulmonary hypertension in rats (Eddahibi et al., 1997). However, the causal relationship between dexfenfluramine treatment and pulmonary hypertension in humans remains unclear. A direct vasoconstrictor effect of the drug mediated by potassium channel blockade or an increase in intracellular Ca²⁺ (Reeve et al., 1999) in smooth muscle cells has been suggested but not proven. Chronic dexfenfluramine treatment did not affect the development of pulmonary hypertension in rats chronically exposed to mild or severe hypoxia (Eddahibi et al., 1998). In rats with hypoxic pulmonary hypertension, concomitant dexfenfluramine treatment did not potentiate but on the contrary prevented the aggravating effect of 5-HT infusion (Eddahibi et al., 1998). This protective effect would be consistent with dexfenfluramine-induced inhibition of 5-HT transport into the smooth muscle cells of pulmonary vessels. In a previous study, we found that the mitogenic and comitogenic effects of 5-HT on cultured pulmonary smooth muscle cells in culture were abolished by fluoxetine or paroxetine, two specific inhibitors of the 5-HT transporter (5-HTT) (Eddahibi et al., 1999). We have also shown that both in vitro and in vivo exposure to hypoxia induced, via a transcriptional mechanism, increased 5-HTT expression in pulmonary artery smooth muscle cells, an effect that potentiates the stimulatory action of 5-HT on smooth muscle proliferation (Eddahibi et al., 1999). Moreover, our recent finding of attenuated pulmonary hypertension and vascular remodeling in chronically hypoxic mice lacking the 5-HTT supports a key role for 5-HTT in the pulmonary hypertensive process of chronic hypoxia (Eddahibi et al., 2000).

To further clarify the relationship between dexfenfluramine treatment and pulmonary hypertension development, we first studied the effect of dexfenfluramine on 5-HT uptake by smooth muscle cells from rat pulmonary arteries (PA-SMCs) and the proliferation of these cells in response to 5-HT under normoxic and hypoxic conditions. We then investigated whether prolonged in vivo dexfenfluramine treatment affected 5-HTT expression in the adult rat lung. Since we previously found that exposure to hypoxia was associated with increased 5-HTT expression (Eddahibi et al., 1999), we examined the effect of dexfenfluramine under both normoxic and hypoxic conditions. Finally, we determined whether the increased lung expression of 5-HTT that followed cessation of long-term dexfenfluramine treatment affected the development of hypoxic pulmonary hypertension.

Materials and Methods

Isolation and Culture of Rat PA-SMCs

The method used for PA-SMC isolation and culture has been described previously (Rothman et al., 1992). In brief, male Wistar rats weighing 250 to 300 g were killed by an overdose of pentobarbital. The lungs were immediately removed, and the proximal pulmonary arteries isolated under aseptic conditions. After removal of surrounding fat, adventitia, and connective tissue, the pulmonary arteries were cut into small pieces, which were then incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with elastase type III (0.125 mg/ml), collagenase type I (1 mg/ml), and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). After a 90-min incubation at 37°C, the tissue suspension was centrifuged (1200g, 10 min at room temperature), and the pellet was resuspended in DMEM supplemented with 15% (v/v) fetal calf serum (FCS), 2 mM l-glutamine, and the same antibiotics as mentioned above. Cells in the pellet suspension were cultured in 100-mm Petri dishes at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, until they were confluent. The medium was changed every day, and the cells were harvested with trypsin (0.2 g/l)-EDTA (0.5 g/l). Cells after four to five passages were used for the experiments. All cells exhibited specific immunostaining by anti-α-smooth muscle actin antibodies, as expected of PA-SMCs (Rothman et al., 1992).

Effect of Dexfenfluramine on [³H]5-HT Uptake by PA-SMCs

Smooth muscle cells in medium containing 15% FCS were seeded in 24-well plates at a density of 5 × 10⁴ cells/well and allowed to grow for 72 h. At the end of this period, the medium was removed, and cell growth was arrested in medium containing 0.2% FCS. After 8 to 24 h of incubation under normoxic (5% CO₂, 20% O₂, 75% N₂) or hypoxic (5% CO₂, 95% N₂) conditions, the cells were washed twice with phosphate-buffered saline and exposed to 10 nM 5-hydroxy[G-³H]tryptamine creatinine sulfate ([³H]5-HT, 15–16 Ci/mmol; Amersham, Buckinghamshire, UK) in a medium containing 120 mM NaCl, 5 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 5.6 mM glucose, 4 mM Tris-HCl, 6.25 mM HEPES, and 0.5 mM ascorbic acid, pH 7.4 (uptake buffer). [³H]5-HT uptake by PA-SMCs was linear for at least 15 min. Therefore, incubation was performed for 10 min at 37°C with or without either the specific 5-HTT inhibitor flutixetine (10⁻⁶ M) or dexfenfluramine (10⁻⁶ M). At the end of the incubation period, the medium was removed and the cells were washed three times with phosphate-buffered saline. The cells were then lysed by addition of 0.5 ml of 0.1 N NaOH, and lysate radioactivity was counted by liquid scintillation spectrometry. Uptake is reported as femtomoles of [³H]5-HT taken up per milligram of protein measured by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Effect of Dexfenfluramine on 5-HT-Induced Increase in [³H]Thymidine Incorporation by PA-SMCs

Smooth muscle cells in medium supplemented with 15% FCS were seeded in 24-well plates at a density of 5 × 10⁴ cells/well and allowed to adhere. Then, cell growth was arrested for 48 h in medium containing 0.2% FCS. At the end of this period, the cells were incubated with 5-HT (10⁻⁸–10⁻⁶ M) alone or with fluoxetine (10⁻⁶ M) or dexfenfluramine (10⁻⁶ M), which were added 20 min before the 5-HT, in DMEM supplemented with 0.2% FCS, antibiotics (as mentioned above), 0.6 mM ascorbic acid, 0.1 mM iproniazid (a monoamine oxidase inhibitor; Sigma, St. Louis, MO), and 0.6 μCi/ml [³H]thymidine (50 Ci/mmol; Amersham). After a 24-h incubation under normoxic (5% CO₂, 20% O₂, 75% N₂) or hypoxic (5% CO₂, 95% N₂) conditions, the cells were washed twice with phosphate-buffered saline, followed by ice-cold 10% trichloroacetic acid. The cells were then dissolved in 0.1 N NaOH (0.5 ml/well), and the incorporated radioactivity was counted.

In Vivo Dexfenfluramine Treatments

Male Wistar rats weighing 250 to 300 g at the start of the experiments were given either dexfenfluramine (2 mg/kg of body weight/day) or its vehicle by gastric gavage once a day and concomitantly exposed to normoxia or hypoxia for 4 weeks. The dexfenfluramine dose was chosen based on previous studies showing that it effectively reduced food intake in various animal models (Rowland and Carlton, 1988).

The rats were randomly divided into four groups, of which one was
exposed to 10% O_2 and dexfenfluramine (n = 5), one to 10% O_2 and the vehicle (n = 5), one to normoxia (21%) and dexfenfluramine (n = 5), and one to normoxia and the vehicle (n = 5). The animals were sacrificed at the end of the 4-week treatment period, and 5-HTT mRNA in their lungs was quantitated.

In a second series of experiments, the rats were treated with dexfenfluramine or the vehicle during 30 days of exposure to normoxia or hypoxia, and then withdrawn from the study drug but kept under the same O_2 condition as previously during an additional week. Quantitation of 5-HTT mRNA in lung tissue was performed at the end of this drug-free week.

In the last series of experiments, rats maintained under normoxic conditions were treated with dexfenfluramine or its vehicle during 30 days, and then withdrawn from the study drug and exposed to normoxia or hypoxia for 15 days. Assessment of pulmonary hypertension and quantitation of 5-HTT mRNA in lung tissue were performed at the end of this drug-free period.

**Exposure of Rats to Chronic Hypoxia**

Rats were exposed to chronic hypoxia (10% O_2) in a ventilated chamber (500-liter capacity; Flufrance, Cachan, France), as described previously (Adnot et al., 1991). To create the hypoxic environment, the chamber was flushed with a mixture of room air and nitrogen, and the gas was recirculated. The chamber environment was monitored using an oxygen analyzer (Servomex OA150, Crowborough, UK). Carbon dioxide was removed using soda lime granules and was monitored using an oxygen analyzer (Servomex OA150, Crowborough, UK).

To create the hypoxic environment, the chamber was flushed with a mixture of room air and nitrogen, and the gas was recirculated. The chamber environment was monitored using an oxygen analyzer (Servomex OA150, Crowborough, UK). Carbon dioxide was removed using soda lime granules, and excess humidity was prevented by cooling the recirculation circuit. The chamber temperature was kept at 22–24°C. The chamber was opened every other day for 1 h to clean the cages and to replenish food and water stores. Normoxic rats were kept in the same room, with the same light/dark cycle. Rat chow and tap water were provided ad libitum.

**Quantitation of 5-HTT mRNA in Lung Tissue**

After an intraperitoneal injection of sodium pentobarbital (20 mg/kg), the thorax was opened, the heart and lungs were quickly removed and dissected, the heart was weighed, and the lungs were stored at −80°C.

**RNA Extraction.** Lung tissue was homogenized with guanidinium isothiocyanate (Interchim, Montluçon, France). Total RNA was extracted according to the method of Chomczynski and Sacchi (1987) and electrophoresed in 1% agarose gel stained with ethidium bromide. Quantitation was performed with reference to a scale of total RNAs prepared on a cesium chloride gradient and was estimated by optical density measurement at 260 nm.

**Quantitative Determination of 5-HTT mRNA.** The method was based on a competitive polymerase chain reaction (PCR) with reverse transcription of RNAs and amplification of synthesized cDNAs in the presence of an internal standard consisting of the same target mRNA synthesized with deletion of about 100 bases, as described in detail elsewhere (Gérard et al., 1996).

Total RNA (0.8 μg/sample) and internal standard RNA (0.01–1 pg) were reverse-transcribed (45 min at 48°C) and amplified using the Access RT-PCR kit (Promega, Lyon, France) with the primers 5’-TTACACAGCATTCATGCG (nucleotides 2008–1991) and 5’-TTATGTGCTGACCCACACTG (nucleotides 1541–1558), in the presence of 2.5 mM MgCl_2. Cycle amplifications were performed at 94, 66, and 72°C (1 min each, 28 cycles). PCR products from the 5-HTT mRNA and the corresponding synthetic deleted RNA contained 484 bases, and 400 base pairs, respectively. They were electrophoresed in 2% agarose gel stained with ethidium bromide, and then quantitated using a gel analyzer (GDS 5000; UVP, Cambridge, UK).

**Assessment of Pulmonary Hypertension in Response to Chronic Hypoxia**

At the end of the 2-week hypoxia exposure, the rats were anesthetized with ketamine (60 mg/kg i.m.) and xylazine (3 mg/kg i.m.). A polyvinyl catheter was inserted into the right jugular vein and pushed through the right ventricle into the pulmonary artery. A polyethylene catheter was also inserted into the right carotid artery. Pulmonary (Pap) and systemic (Sap) arterial pressures were measured, and blood was sampled for hematocrit determination. The rats were anesthetized with sodium pentobarbital (20 mg/kg i.p.); the thorax was opened; and the heart was quickly removed, dissected, and weighed. The ratio of right ventricular free wall weight over the sum of left ventricular free wall plus septum weight (fresh tissue) was used as an index of right ventricular hypertrophy. Then, the lungs were fixed in the distended state by infusion of 4% aqueous-buffered Formalin into the trachea at a pressure of 25 cm of H_2O, and subsequently immersed in the same fixative for 1 week. A midsagittal slice of the right lung, including the apical, azygous, and diaphragmatic lobes was processed for paraffin embedding. Sections (5 μm in thickness) were cut for light microscopy and stained with hematoxylin phloxin saffron and orcein-picroindigo-carmine. In each rat, a total of 35 to 65 intra-acinar vessels accompanying either alveolar ducts or alveoli was examined. Their type was identified as muscular, partially muscular, or nonmuscular. Muscular arteries had a complete layer of smooth muscle cells bound by two orcein-stained elastic lamina. Smooth muscle cells were identified as elongated cells that stained red with phloxin and had square-ended nuclei. They were seen in only part of the arterial circumference of partially muscular arteries and were absent from nonmuscular arteries.

Lungs from rats exposed to 2 weeks of hypoxia after discontinuation of a 4-week dexfenfluramine or vehicle treatment were removed under the same conditions as described above and frozen at −80°C for quantitative determination of 5-HTT mRNA.

**Statistical Analysis**

The statistical significance of the effects of treatment or pretreatment with dexfenfluramine under normoxic or hypoxic conditions on [3H]5-HT uptake and [3H]thymidine incorporation into PA-SMCs and on 5-HTT mRNA levels in lung tissue was assessed using 2-way ANOVA, testing for drug and O_2 environment effects. Comparisons of hemodynamic values and ratios of the right ventricle weight over the sum of left ventricle plus septum weight between the groups pretreated with dexfenfluramine or vehicle before exposure to hypoxia were performed using the nonparametric Mann-Whitney test. To compare the degree of pulmonary vessel muscularization between these two groups of animals, pulmonary vessels were ordinarily classified as nonmuscular, partially muscular, or muscular. Comparisons of muscularization were performed separately at the alveolar duct and alveolar wall levels using the non-parametric Mann-Whitney test.

**Results**

**Effect of Dexfenfluramine on [3H]5-HT Uptake by PA-SMCs.** Compared with PA-SMCs under normoxic conditions, [3H]5-HT uptake was markedly increased in PA-SMCs exposed to hypoxia (Fig. 1). This increase developed gradually, up to a maximum reached after 16 h of hypoxia. At that time, [3H]5-HT uptake was three times higher than under normoxic conditions (P < 0.001). Fluoxetine (10⁻⁶ M) caused profound inhibition of [3H]5-HT uptake, the residual uptake being similar under normoxic or hypoxic conditions (Fig. 1).

Dexfenfluramine (10⁻⁶ M) also markedly attenuated [3H]5-HT uptake, but the residual uptake in the presence of this drug remained higher under hypoxic than under normoxic conditions (P < 0.05, Fig. 1).

**Effect of Dexfenfluramine on 5-HT-Induced [3H]Thymidine Incorporation by PA-SMCs.** In quiescent PA-SMCs maintained in a normoxic environment and incubated...
with serum-free medium (0.2% FCS), 5-HT produced a concentration-dependent increase in [3H]thymidine incorporation of up to 5-fold with 10⁻⁶ M indoleamine (Fig. 2). As illustrated in Fig. 2, fluoxetine (10⁻⁶ M) prevented this effect of 5-HT. In addition, dexfenfluramine (10⁻⁶ M) also inhibited the stimulating effect of 5-HT on [3H]thymidine incorporation, albeit less effectively than fluoxetine.

The stimulating effect of 5-HT on [3H]thymidine incorporation by PA-SMCs persisted under hypoxic conditions. Furthermore, as previously reported (Eddahibi et al., 1999), [3H]thymidine incorporation in response to low (10⁻⁸ M) and intermediate (10⁻⁷ M) concentrations of 5-HT was significantly greater under hypoxic than normoxic conditions. As already noted for normoxia, the stimulating effect of 5-HT on [3H]thymidine incorporation under hypoxia was completely abolished by fluoxetine and markedly attenuated by dexfenfluramine (Fig. 2).

**Effect of Dexfenfluramine on 5-HTT mRNA Levels in Lung Tissue.** In lungs from chronically hypoxic rats, 5-HTT mRNA levels measured by competitive RT-PCR were significantly higher (+40%, *p < 0.05) than in lungs from normoxic rats. Neither this effect nor the absolute 5-HTT mRNA levels was altered by dexfenfluramine treatment during 4 weeks (Fig. 3). However, 5-HTT mRNA levels increased markedly during the week following dexfenfluramine discontinuation, in both the normoxic and the hypoxic rats (Fig. 3). This effect was of limited duration: 2 weeks after dexfenfluramine withdrawal, 5-HTT mRNA levels had returned to the values observed during the 4-week period of treatment with the drug or its vehicle (125 ± 7 versus 115 ± 6 amol/mg of total RNA in normoxic rats, and 231 ± 8 versus 214 ± 9 amol/mg of total RNA in hypoxic rats, 2 weeks after discontinuation of vehicle or dexfenfluramine administration, respectively, N.S.).

**Fig. 1.** [3H]5-HT uptake in smooth muscle cells previously exposed to normoxia or hypoxia for 16 h with or without fluoxetine or dexfenfluramine. Vehicle, □; 10⁻⁶ M fluoxetine, △; 10⁻⁶ M dexfenfluramine, ■. Values are the means ± S.E.M. of four independent experiments with n = 6 wells for each treatment and condition. *P < 0.05 and **P < 0.01 compared with the vehicle under similar O₂ conditions. †P < 0.05 and ††P < 0.001 compared with [3H]5-HT uptake in cells cultured under normoxic or hypoxic conditions.

**Fig. 2.** Effects of 5-HT with or without dexfenfluramine or fluoxetine on [3H]thymidine incorporation by smooth muscle cells cultured under normoxic (□) or hypoxic (■) conditions. [3H]Thymidine incorporation is expressed as the fold increase over that measured without 5-HT. Fluoxetine (10⁻⁶ M) or dexfenfluramine (10⁻⁶ M) was added 20 min before 5-HT (10⁻⁶ M). Each column is the mean ± S.E.M. of data obtained in four separate experiments (six wells per experiment). *P < 0.01 and **P < 0.001 compared with values obtained with the same concentration of 5-HT and the same oxygenation conditions. †P < 0.01 compared with corresponding values under normoxic conditions.

**Fig. 3.** 5-HTT mRNA levels in lung tissue from rats after 4 weeks of continuous treatment with dexfenfluramine (■) (2 mg/kg/day) or its vehicle (□) and 1 week after discontinuation of this treatment. Rats were exposed to normoxia (top) or hypoxia (bottom) throughout the experiments. Each column is the mean ± S.E.M. of data obtained in five rats per group.
Effect of Pretreatment with Dexfenfluramine on Development of Pulmonary Hypertension. Although all the rats had similar body weights at treatment initiation, after the 30-day treatment period body weight was significantly lower in the group given dexfenfluramine than in the group given the vehicle alone (374 ± 5.8 versus 437 ± 11.5 g, respectively P < 0.001). However, under hypoxia, this difference was no longer apparent 2 weeks after treatment discontinuation.

After a 2-week exposure to hypoxia, no differences were found in hematocrit, Sap, or Pap between the rats pretreated with dexfenfluramine and those pretreated with the vehicle during 4 weeks (Table 1). However, the ratio of the weight of the right ventricle over that of the left ventricle plus the septum was significantly higher in the rats pretreated with dexfenfluramine than in those pretreated with the vehicle (P < 0.05, Fig. 4). In addition, the degree of pulmonary artery muscularization at both the alveolar duct and the alveolar wall level was also significantly higher in the rats pretreated with dexfenfluramine than in those pretreated with the vehicle (P < 0.01, Fig. 5).

Discussion

Our results show that discontinuation in rats of a 30-day dexfenfluramine treatment (2 mg/kg/day) was followed by a transient increase in 5-HTT mRNA levels in lung tissues, whereas no alterations in the levels of this transcript were noted during the treatment. In agreement with previous studies (Eddahibi et al., 1999), we also observed an increase in lung 5-HTT mRNA levels in the rats exposed to chronic hypoxia. The two effects were additive, leading to a marked, transient increase in lung 5-HTT mRNA levels after dexfenfluramine withdrawal in the rats maintained under hypoxia. When exposure to 2 weeks of hypoxia followed discontinuation of a 4-week dexfenfluramine treatment, right ventricular hypertrophy was more severe and muscularization of distal pulmonary arteries more marked than in rats pretreated with the vehicle. These results suggest that a causal relationship may exist between increased 5-HTT expression and PA-SMC proliferation in response to discontinuation of dexfenfluramine treatment combined with hypoxia.

Investigations on the effects of 5-HT and 5-HTT on the pulmonary circulation are of special interest because of the reported increased risk of PPH development in patients who used appetite suppressants responsible for 5-HT transport inhibition (Abenhaim et al., 1996). In addition to its vasoactive effects, 5-HT has been shown to exert mitogenic and comitogenic effects on PA-SMCs (Lee et al., 1991; Eddahibi et al., 1999). The mitogenic and comitogenic effects require internalization of indoleamine by a high-affinity 5-HT transporter (5-HTT), which can be competitively inhibited by specific drugs such as fluoxetine and paroxetine. In a recent study, we showed that hypoxia increased the rate of 5-HTT gene transcription in PA-SMCs and potentiated the growth-promoting effect of 5-HT (Eddahibi et al., 1999). An increase in 5-HTT mRNA levels was also observed in the smooth muscle of remodeled pulmonary arteries from rats exposed to chronic hypoxia (Eddahibi et al., 1999). Moreover, that 5-HTT plays a key role in the vascular remodeling induced by chronic hypoxia is supported by our previous findings in mice deficient in 5-HT transporter gene (Eddahibi et al., 2000). Despite a potentiation of their pressor response to acute hypoxia, these animals develop less severe pulmonary hypertension and have attenuated muscularization of distal pulmonary vessels when exposed to chronic hypoxia.

However, to date, the mechanisms by which fenfluramine derivatives may lead to pulmonary hypertension remain unelucidated. Efforts to induce pulmonary hypertension in animals by chronic administration of these drugs have consistently failed. Furthermore, we have previously reported that chronic dexfenfluramine treatment does not aggravate the development of pulmonary hypertension in rats exposed to either mild or severe hypoxia (Eddahibi et al., 1998). Thus, rats treated with dexfenfluramine in a daily dose of 2 mg/kg/day for 2 weeks exhibited the same degree of pulmonary hypertension, right ventricular hypertrophy, and structural remodeling of distal pulmonary arteries as vehicle-treated rats exposed to the same level of hypoxia. Moreover, there is some evidence that the aggravating effect of a continuous 5-HT infusion on pulmonary vascular remodeling in response

![Fig. 4. Ratio of the weight of the right ventricle over that of the left ventricle plus septum in rats pretreated with dexfenfluramine (■) (2 mg/kg/day) or vehicle (□) for 4 weeks under normoxic conditions, and then exposed to hypoxia for 2 weeks. n = 7 in each group.]

### TABLE 1

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<th>Final BW</th>
<th>Pap</th>
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<th>Heart Rate</th>
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<td>Dexfenfluramine (n = 7)</td>
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<td>105.0 ± 19.9</td>
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BW, body weight.
to chronic exposure to 10% \( O_2 \) may be prevented by concomitant dexfenfluramine treatment. Indeed, pulmonary artery muscularization at both the alveolar duct and the alveolar wall level was less marked after a 2-week exposure to 10% \( O_2 \) in rats given both 5-HT and dexfenfluramine than in those given 5-HT only. The protection afforded by dexfenfluramine treatment against 5-HT-potentiation of pulmonary vascular remodeling is likely related to inhibition by dexfenfluramine of 5-HT transport into PA-SMCs. Supporting this hypothesis, chronic dexfenfluramine treatment has been shown to increase 5-HT levels in plasma (Eddahibi et al., 1998) and to decrease 5-HT levels in lung tissue and blood platelets (Celada et al., 1994), suggesting that dexfenfluramine may block 5-HT uptake not only in platelets but also in pulmonary vessel cells. Additional evidence of this is provided by our finding that dexfenfluramine markedly reduced \( ^{3}H \)5-HT uptake in smooth muscle cells derived from rat pulmonary arteries. Concomitantly with its effect on 5-HT uptake, and similarly to the specific 5-HT transport inhibitor fluoxetine, dexfenfluramine also abolished the mitogenic effect of 5-HT on PA-SMCs. This observation, together with our previous data (Eddahibi et al., 1999), is conclusive evidence that internalization of 5-HT through 5-HTT is essential to the mitogenic effect of indoleamine.

Previous studies have shown that 5-HTT levels and activity in serotonergic neurons can be modulated by hormones and pharmacological agents (Blakely et al., 1996). Dexfenfluramine given in high doses has been shown to produce long-lasting decreases in both concentration and uptake of 5-HT in forebrain regions, as well as in 5-HTT mRNA levels within the dorsal raphe nucleus (Semple-Rowland et al., 1996). However, the effect of chronic dexfenfluramine treatment on 5-HTT expression in lung tissue has not been investigated previously. We found that the levels of 5-HTT transcript in lung tissue from rats given chronic dexfenfluramine treatment for 4 weeks remained unchanged compared with those in animals treated with the vehicle alone and maintained under similar normoxic conditions. In contrast, in the rats exposed to 10% \( O_2 \), lung levels of 5-HTT mRNA showed a marked increase, which was of similar magnitude in the groups concomitantly treated by dexfenfluramine versus its vehicle. The effect of hypoxia on 5-HTT mRNA levels is in accordance with our previous results showing that exposure of PA-SMCs to hypoxia resulted in a rapid and transient increase in 5-HTT gene transcription followed by a prolonged increase in 5-HT uptake by the cells (Eddahibi et al., 1999). Moreover, the present data are also consistent with our previous in situ hybridization results demonstrating an increase in 5-HTT mRNA concentration in remodeled pulmonary arteries of rats previously exposed to chronic hypoxia (Eddahibi et al., 1999).

Contrasting with the unchanged 5-HTT gene expression during prolonged dexfenfluramine treatment, the 5-HTT mRNA levels were significantly increased 1 week after dexfenfluramine discontinuation compared with the values in rats treated with the vehicle under similar \( O_2 \) exposure conditions. This increase upon dexfenfluramine withdrawal suggests that regulation of 5-HTT expression in the lung may differ markedly from that in the brain: decreased 5-HTT mRNA levels have been reported in the dorsal raphe nucleus 5 days after cessation of repeated dexfenfluramine administration in rats (Rattray et al., 1994). It may reflect a compensatory response to the long-term 5-HTT blockade by the drug, the effect being to reduce the increased 5-HT plasma levels to normal, thus diminishing the vasoactive effects of 5-HT. However, it is unlikely that this effect is mediated by binding of 5-HT to cell surface receptors because no up-regulation of 5-HTT expression was observed during chronic dexfenfluramine treatment, despite the marked increase in 5-HT plasma levels due to 5-HT uptake blockade by the drug.

The present in vivo data demonstrate that increased 5-HTT expression also affected the development of pulmonary hypertension. When rats were exposed to chronic hypoxia at the time of increased 5-HTT gene expression associated with dexfenfluramine withdrawal, both right ventricular hypertrophy and pulmonary vessel remodeling were more severe compared with the alterations observed in untreated rats under the same hypoxic conditions. The fact that pulmonary artery pressure showed no significant difference between rats pretreated with dexfenfluramine versus its vehicle may be related to the limited duration of 5-HTT overexpression following dexfenfluramine discontinuation: 5-HTT mRNA levels were increased 1 week after discontinuation but returned to baseline during the following week.

Although the clinical picture and histological findings of human primary pulmonary hypertension differ significantly
from this animal model of hypoxic pulmonary hypertension, the present study provides experimental evidence supporting a link between treatment with fenfluramine derivatives and the development of pulmonary hypertension. Whereas in a previous study we found that deficiency in 5-HT transport is associated with attenuated pulmonary hypertension in response to chronic hypoxia (Eddahibi et al., 2000), our present results demonstrate that a factor associated with an increase in 5-HT transport into PA-SMCs promotes the hypertensive process. Differences may exist across species. Susceptibility factors may also play a role in the association of primary pulmonary hypertension with use of fenfluramine derivatives. Although the risk of pulmonary hypertension seems to increase steadily with the cumulative appetite suppressant dose, in the epidemiological study by Abenhaim et al. (1996) 6.2% of controls without primary pulmonary hypertension had used appetite suppressants. It has recently been suggested that low transport capacity and site density of 5-HTT in subjects with depression may be related to intronic tandem repeat polymorphism of the 5-HTT gene (Lesch et al., 1996). Low transporters and site density of 5-HTT may also play a role in the association of primary pulmonary hypertension with use of fenfluramine derivatives. Although the risk of pulmonary hypertension seems to increase steadily with the cumulative appetite suppressant dose, in the epidemiological study by Abenhaim et al. (1996) 6.2% of controls without primary pulmonary hypertension had used appetite suppressants. It has recently been suggested that low transport capacity and site density of 5-HTT in subjects with depression may be related to intronic tandem repeat polymorphism of the 5-HTT gene (Lesch et al., 1996). Further studies are needed to investigate whether high levels of 5-HTT expression and activity may increase susceptibility to primary pulmonary hypertension, and whether this disease is associated with 5-HTT gene polymorphism.

References

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