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Serotonin Transporter Inhibition Prevents and Reverses Monocrotaline-Induced Pulmonary Hypertension in Rats

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Background—Progression of pulmonary hypertension (PH) is associated with increased lung expression of the serotonin transporter (5-HTT), which leads to hyperplasia of the pulmonary artery smooth muscle cells (PA-SMCs). Given the postulated causal relation between 5-HTT overexpression and PH, we herein investigated whether the highly selective 5-HTT inhibitor fluoxetine prevented and/or reversed PH induced by monocrotaline (MCT) in rats. Selective 5-$HT_{1B/1D}$, 5-HT_{2A}, and 5-HT_{2B} receptor antagonists were used for comparative testing.

Methods and Results—MCT injection (60 mg/kg SC) was followed by an early peak in lung 5-HTT expression on day 1, which preceded the onset of PH. Established PH on day 15 was associated with a sustained 5-HTT increase. Continued fluoxetine treatment completely prevented PA-SMC proliferation and PH development and also suppressed the late 5-HTT increase, without affecting the early peak. The 5-HT receptor antagonists did not affect PH. Fluoxetine (10 mg · kg^{-1} · d^{-1} PO) started 3 weeks after MCT injection completely reversed established PH, normalizing PA pressure and structure. MCT-induced PH was also associated with increased expression of various cytokines, but only interleukin-1β and monocyte chemotactic protein-1 increased at the early phase and stimulated 5-HTT expression by cultured PA-SMCs.

Conclusion—Upregulation of lung 5-HTT induced by MCT appears necessary to initiate the development of pulmonary vascular remodeling, whereas a sustained increase in 5-HTT expression may underlie both the progression and the maintenance of MCT-induced PH. Complete reversal of established PH by fluoxetine provides a rationale for new therapeutic strategies in human PH. **(*Circulation*. 2005;111:2812-2819.)**

Key Words: pulmonary heart disease ■ remodeling ■ muscle, smooth

Pulmonary hypertension (PH) occurs either as a complication of various disease states or as a primary disease for which no underlying cause can be found. Smooth muscle hyperplasia is a hallmark pathological feature of all forms of PH and leads to structural remodeling and occlusion of the pulmonary vessels.[1] We recently reported that serotonin (5-hydroxytryptamine, 5-HT) and its transporter (5-HTT) play a central role in the pathogenesis of pulmonary artery smooth muscle cell (PA-SMC) proliferation, not only in experimental hypoxic PH but also in human PH, whether primary or secondary to pulmonary or systemic diseases.[2,3] The increased 5-HTT expression that governs PA-SMC proliferation in patients with PH is at least partly related to polymorphism of the 5-HTT promoter gene.[3,4] This implies that the 5-HT pathway involving 5-HTT contributes to the pathogenesis of various forms of human PH and/or is an important modifier of the PH phenotype.[5] Recent studies showing that mice overexpressing 5-HTT develop PH in the absence of other stimuli also support the concept that 5-HTT upregulation precedes the development of pulmonary vascular remodeling.[6]

Whether 5-HTT may be a molecular target for the treatment of PH remains to be established. We previously found that hypoxia-induced PH was strikingly attenuated in mice deficient in 5-HTT and in wild-type mice treated with 5-HTT inhibitors.[7] However, some pulmonary vascular remodeling was still observed after genetic or pharmacological inactivation of 5-HTT, and hypoxic pulmonary vasoconstriction was potentiated, possibly because of a stimulatory effect of 5-HT on at least some 5-HT receptors. In some studies, but not all, treatment with drugs antagonizing 5-HT_{1B} and 5-HT_{2B} receptors seemed to also attenuate the development of hypoxic PH.[8,9]

The potential effectiveness of drugs interfering with 5-HT, however, has not been tested in severe experimental PH such as that induced by monocrotaline (MCT) in rats. MCT is a pyrrolizidine alkaloid toxin that produces endothelial injury followed by progressive development of severe and irrevers-

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ible PH. We chose this experimental inflammatory model of PH to assess the role of the 5-HTT versus 5-HT receptors because MCT-induced PH results primarily from marked alterations in PA structure, including medial hypertrophy and intimal fibromuscular hyperplasia, similar to those observed in some forms of human PH.[10] We therefore investigated whether the selective 5-HTT inhibitor fluoxetine or specific antagonists of 5-$HT_{1B/1D}$, 5-HT_{2A}, and 5-HT_{2B} receptors prevented or reversed established MCT-induced PH in rats. Because inflammation contributes to MCT-induced PH and to various forms of human PH, we also measured the expression of inflammatory cytokines in parallel with 5-HTT during the course of MCT-induced PH. Finally, whether cytokines affected 5-HTT expression was also investigated in isolated PA-SMCs.

Methods

Animal Models and Experimental Design

All experiments were performed in adult male Wistar rats (200 to 250 g) according to institutional guidelines that comply with national and international regulations.

In the first part of the study, PH development and pulmonary expression of cytokines and 5-HTT were examined in rats at various times after a single injection of MCT (60 mg/kg SC, Sigma). Then, to assess the potential preventive effect of 5-HTT inhibition or 5-HT receptor blockade on MCT-induced PH, we assigned rats at random to 1 of 6 groups (8 to 10 animals in each group): 2 groups received fluoxetine at 2 or 10 $mg \cdot kg^{-1} \cdot d^{-1}$; 1 group each received the selective 5-$HT_{1B/1D}$ receptor antagonist GR127935 (2 $mg \cdot kg^{-1} \cdot d^{-1}$), the 5-$HT_{2A}$ receptor antagonist ketanserin (2 $mg \cdot kg^{-1} \cdot d^{-1}$), or the 5-$HT_{2B}$ receptor antagonist RS127445 (2 $mg \cdot kg^{-1} \cdot d^{-1}$); and 1 group received vehicle only. All treatments were given once a day by gavage for 3 weeks after a single MCT injection (60 mg/kg SC). Finally, to assess the potential curative effects of 5-HTT inhibition, rats given MCT (60 mg/kg SC) were left untreated for 21 days and then randomly divided into 2 groups, 1 treated with fluoxetine (10 $mg \cdot kg^{-1} \cdot d^{-1}$) and the other with vehicle, from day 21 to day 42.

Assessment of PH

Three or 6 weeks after MCT administration, rats were anesthetized with ketamine (60 mg/kg IM) and xylazine (3 mg/kg IM). A polyvinyl catheter was introduced into the right jugular vein and pushed through the right ventricle into the PA. Another polyethylene catheter was inserted into the right carotid artery. After measurement of PA (PAP) and systemic arterial (SAP) pressures, the thorax was opened and the left lung immediately removed and frozen in LN_2 for measurement of cytokines and 5-HTT expression. The heart was dissected and weighed for calculation of the right ventricular hypertrophy index (ratio of right ventricular free wall weight divided by the sum of the septum plus left ventricular free wall weight [RV/{LV+S}]). The right lung was fixed in the distended state with formalin buffer. After paraffin embedding, 5-μm-thick lung sections were stained with hematoxylin-phloxine-safranin. In each rat, 40 to 60 intra-acinar arteries were analyzed and categorized as muscularized (fully or partially) or nonmuscularized to assess the degree of muscularization. In addition, medial wall thickness of fully muscularized intra-acinar arteries was calculated and expressed as follows: index (%)=(external diameter−internal diameter)/external diameter×100. Cell proliferation and apoptosis were also assessed in the walls of distal pulmonary vessels from rats treated with vehicle or fluoxetine on day 21 or 42 after MCT administration.

Real-Time Quantitative RT-PCR for Measurement of Lung 5-HTT, Interleukin-1β, Monocyte Chemotactic Protein-1, and Interleukin-6 mRNA

RNA extraction was performed with Trizol reagent (Gibco BRL). The concentration and quality of RNA were determined by electrophoresis on agarose gel and spectrophotometry. Then, reverse transcription was performed with random hexamer primers and reverse transcriptase (RT, Biotech Ltd). Primers for polymerase chain reaction (PCR) were designed with Primer Express Software (Applied Biosystems). To avoid inappropriate amplification of residual genomic DNA, intron-spanning primers were selected and internal control 18S rRNA primers provided. For each sample, the amplification reaction was performed in duplicate with SyberGreen mix and specific primers. Signal detection and analysis of results were performed with ABI-Prism 7000 sequence detection software (Applied Biosystems). The relative expression level of the genes of interest was computed relative to the mRNA expression level of the internal standard, r18S, by the following formula: relative $mRNA = ½(Ct_{\text{gene of interest}} - Ct_{r18S})$.

Lung 5-HTT Immunoblotting and Immunolocalization

Detection and quantification of 5-HTT protein in lung tissue were achieved by Western blotting. In brief, lung samples were homogenized in ice-cold phosphate-buffered saline (PBS). Total protein (150 μg) from each sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. After incubation in a blocking solution for 1 hour, the membranes were incubated with a 5-HTT antibody as previously described.[7] Densitometric quantification was normalized for β-actin level in each sample. Localization of 5-HTT was performed by immunostaining of lung tissue sections as previously described.[7]

Evaluation of In Situ PA-SMC Death and Proliferation

To assess PA-SMC proliferation in rats treated with MCT alone or with fluoxetine, the proliferating cell nuclear antigen (PCNA) was evaluated. Tissue sections were deparaffinized in xylene and then treated with a graded series of alcohol washes, rehydrated in PBS (pH 7.5), and incubated with target retrieval solution (Dako) in a water bath at 90°C for 20 minutes. Endogenous peroxidase activity was blocked with H_2O_2 in PBS (3%, vol/vol) for 5 minutes. Slides were then washed in PBS, incubated for 30 minutes in a protein-blocking solution, and incubated for 30 minutes with anti-PCNA mouse monoclonal antibody (PC-10, 1:200, Dako). Antibodies were washed off, and the slides were processed with the alkaline phosphatase LSAB+ system horseradish peroxidase detection kit (Dako). Brown color was generated with a diaminobenzidine substrate, and nuclei were counterstained with hematoxylin.

Detection of cells undergoing apoptosis was achieved with the ApopTag Red in situ apoptosis detection kit (Qbiogene), as specified by the manufacturer. At the end of the procedure, the samples were observed by fluorescence microscopy after Hoechst staining (Sigma).

Effects of Cytokines on 5-HTT Expression by Isolated PA-SMCs

SMCs from control rat PAs were cultured and characterized as previously described.[2] To examine the effects of cytokines on 5-HTT expression, the cells were grown to confluence and the medium was removed. The cells were then exposed to interleukin (IL)-1β, IL-6, or monocyte chemotactic protein (MCP)-1 (10 ng/mL) in serum-free medium for 4 or 24 hours. Then the samples were used for real-time quantitative RT-PCR or Western blotting.

Statistical Analyses

The data are expressed as mean±SEM. A nonparametric Mann-Whitney test was used for comparisons between 2 groups. Comparisons of data at various times after MCT injection or of various treatment groups were performed with a nonparametric Kruskal-Wallis test followed by Dunn test when significant. The mortality rates between vehicle- and fluoxetine-treated animals were compared by χ^2 tests. The effect of fluoxetine on 5-HTT and cytokine expression in the lung at various times after MCT injection was evaluated by 2-way ANOVA, testing for treatment and time effects. When a time-by-treatment interaction was found, comparisons be-

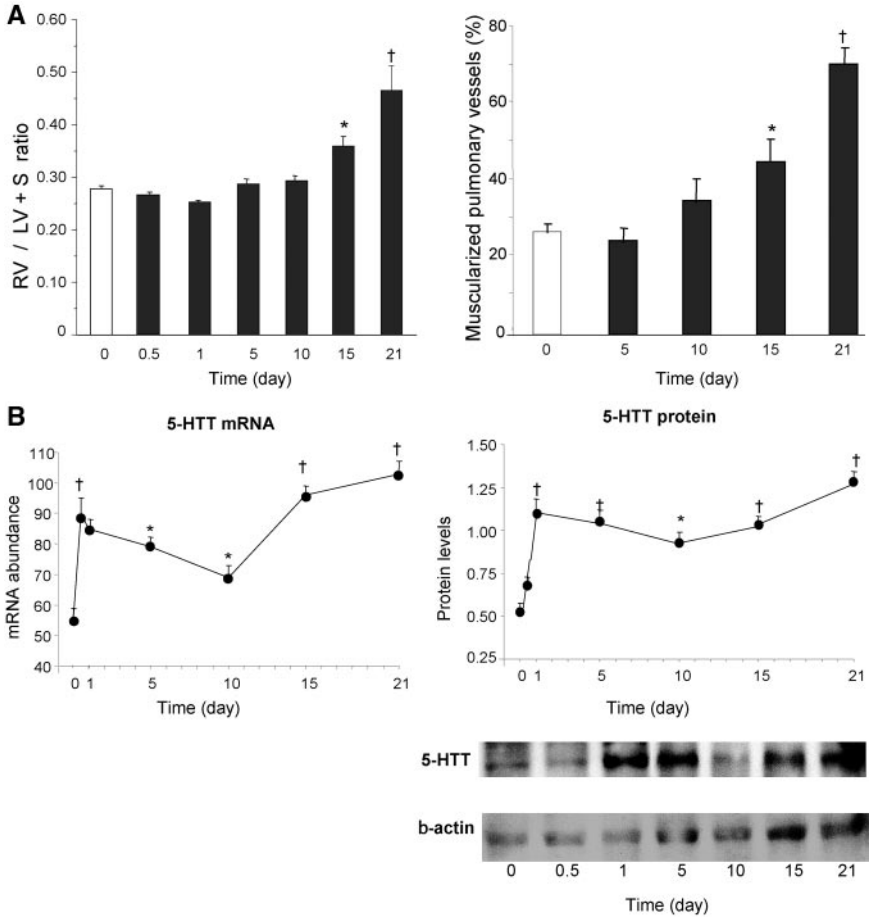

Figure 1. A, Development of right ventricular hypertrophy as assessed by weight ratio RV/(LV+S) (left) and muscularization of distal pulmonary vessels estimated by percentage of muscularized intra-acinar vessels (right). B, 5-HTT mRNA expression in lung tissue (left) performed by real-time quantitative RT-PCR, and protein levels (right) assessed by densitometric quantification of 5-HTT immunoblotting normalized against β-actin level. Each point is mean±SE of at least 5 determinations at various times after MCT administration (mean± SE, n=5 at each time). *$P<0.05$, †$P<0.01$ compared with values obtained in control, untreated rats (day 0).

tween vehicle and active treatment were performed with a nonparametric Mann-Whitney test.

Results

Development of PH and Time-Dependent Increase in Lung 5-HTT Expression After MCT Administration

Administration of MCT was followed by delayed increases in the ratio of RV/(LV+S), reflecting right ventricular hypertrophy (Figure 1A, left) and in the percentage of partially or fully muscularized distal vessels (reflecting pulmonary vessel remodeling, Figure 1A, right). In both cases, the increase became significant on day 15 compared with control rats and continued from day 15 to day 21. Lung levels of 5-HTT mRNA and 5-HTT protein increased early after MCT injection (Figure 1B), reaching a peak within the first 24 hours, decreasing slightly for the following 10 days, and then increasing again until the last day of the study (day 21). As shown in Figure 1B, 5-HTT expression remained significantly enhanced from day 1 to day 21 after MCT administration.

Effects of Treatment With Fluoxetine or 5-HT Receptor Antagonists on Development of MCT-Induced PH

In rats treated with vehicle after MCT administration and studied on day 21, severe PH developed, with marked increases in PAP, RV/(LV+S) (Table), and PA muscularization (Figure 2A and 2B) compared with control rats injected with saline instead of MCT. Long-term treatment with the selective 5-$HT_{1B/1D}$ receptor antagonist GR127935 (2 mg kg^{-1} d^{-1}), the 5-HT_{2A} receptor antagonist ketanserin (2 mg kg^{-1} d^{-1}), or the 5-HT_{2B} receptor antagonist RS127445 (2 mg kg^{-1} d^{-1}) for 21 days after MCT injection did not affect these parameters. In contrast, with fluoxetine, the development of PH was markedly attenuated with the lowest dose (2 mg/kg) and completely abolished with the highest dose (10 mg/kg). PAP, RV/(LV+S), and muscularization of distal PAs were significantly lower in MCT-treated rats given fluoxetine than in those given vehicle; in MCT-treated rats given the high fluoxetine dose (10 mg/kg), these values were similar to those determined in control rats treated with vehicle instead of MCT. 5-HTT immunostaining of lung sections demonstrated a marked increase in 5-HTT immunoreactivity, located mainly in the media of PAs and correlated with media thickening (Figure 3).

During the 21-day follow-up, no deaths occurred in the MCT group. To examine the effect of fluoxetine treatment on survival, we followed up 2 groups of MCT-treated rats for 42 days; 1 group had received vehicle and the other, fluoxetine (10 mg kg^{-1} d^{-1}), from day 1 to day 21. Among vehicle-treated rats, 40% died between days 30 and 35 after MCT, whereas none of the fluoxetine-treated rats died during the 42-day follow-up ($P<0.01$).

Reversal of MCT-Induced PH With Fluoxetine

In rats treated with fluoxetine from day 21 to day 42 (10 mg kg^{-1} d^{-1}) after MCT injection, PAP, RV/(LV+S), PA

Body Weight (BW), Heart Weight, and Hemodynamic Data in Rats at Day 21 After MCT Administration

		MCT					
	Controls (n=6)	Vehicle (n=8)	GR127 (n=6)	RS144 (n=8)	Ket (n=5)	Fluox, 2 mg · kg^{-1} · d^{-1} (n=5)	Fluox, 10 mg · kg^{-1} · d^{-1} (n=5)
BW, g	361±9	304±7*	306±6*	321±5*	311±8*	315±9*	325±5*
PAP, mm Hg	19.1±1.2	30.7±3.6*	28.6±1.8*	27.9±2.3*	29.2±1.9*	20.3±1.4†	18.5±0.9†
SAP, mm Hg	108±6	95±5	104±7	94±5	99±10	101±12	102±5
Heart rate, bpm	308±21	344±15	353±26	333±27	324±14	296±30	300±23
Hematocrit, %	47±2	44±4	48±3	46±4	43±5	46±3	45±4
RV, mg	155±6	246±8*	237±6*	254±7*	256±9*	181±4*†	159±3‡
LV+S, mg	551±14	539±9	524±11	546±8	529±10	522±12	536±13
RV/(LV+S)	0.28±0.01	0.47±0.03*	0.48±0.04*	0.46±0.03*	0.50±0.02*	0.35±0.02*†	0.30±0.02‡

Ket indicates ketanserin; fluox, fluoxetine. All values are mean±SEM.
*P<0.01 compared with corresponding values in control rats (controls) given saline instead of MCT.
†P<0.01, ‡P<0.001 compared with MCT-treated rats given vehicle.

muscularization, SAP, and heart rate on day 42 did not differ from those values in control rats given vehicle instead of MCT (Figure 4). In contrast, in vehicle-treated rats, PH worsened between days 21 and 42 (values reported in the Table and Figure 4), and PA wall thickness increased markedly, as illustrated in Figure 5.

Effect of Fluoxetine Treatment on PA-SMC Proliferation and Apoptosis

In MCT rats treated with vehicle, PCNA labeling showed proliferation of SMCs in distal PA walls, which was more marked at 42 than at 21 days. The number of PCNA-positive cells was markedly lower in PA walls from rats treated with

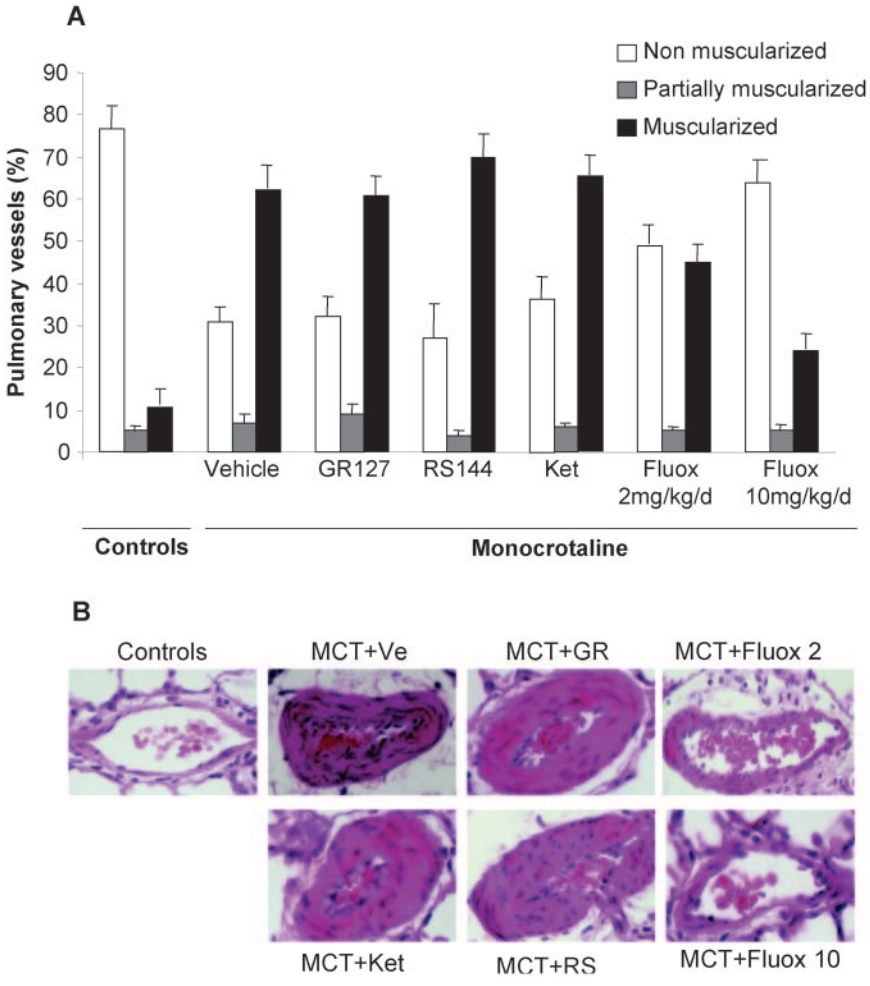

Figure 2. A, Percentage of nonmuscularized (NM), partially muscularized (PM), or fully muscularized (M) distal vessels. Total of 40 to 60 intra-acinar vessels was analyzed in each lung from rats injected with MCT and treated daily with GR127935 (GR; 2 mg kg^{-1} d^{-1}, n=10), RS127445 (RS; 2 mg kg^{-1} d^{-1}, n=10), ketanserin (Ket; 2 mg kg^{-1} d^{-1}, n=8), fluoxetine (Fluox; 2 or 10 mg kg^{-1} d^{-1}, n=10 for each dose), or vehicle (Ve, n=10) for next 21 days. Compared with control rats, MCT caused increase in muscularization (P<0.001). Degree of muscularization in MCT rats was lower in fluoxetine-treated (2 and 10 mg kg^{-1} d^{-1}) groups than in vehicle-treated groups (P<0.05 and P<0.01, respectively). There was no difference between GR127935, RS127445, ketanserin, and vehicle-treated rats. B, Pulmonary vascular remodeling illustrated by representative photomicrographs of pulmonary vessels from each group of rats.

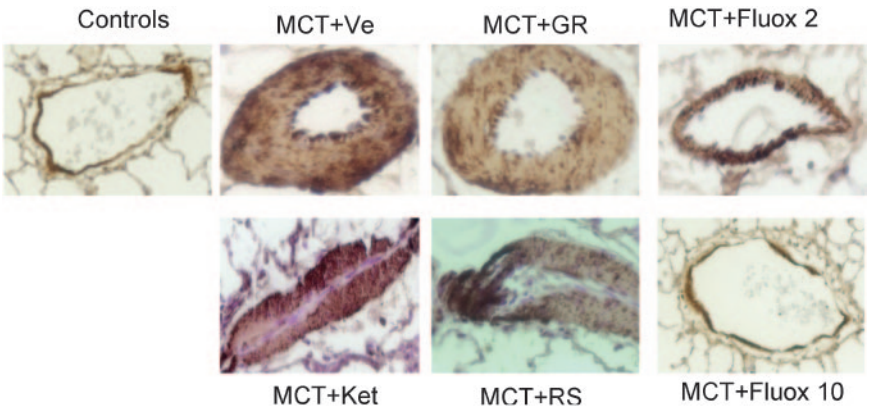

Figure 3. 5-HTT immunostaining in PAs from control rats (controls) and from rats injected with MCT and treated daily with GR127935 (MCT+GR), RS127445 (MCT+RS), ketanserin (MCT+Ket), fluoxetine at 2 or 10 mg kg^{-1} d^{-1} (MCT+Fluox2 and MCT+Fluox 10), or vehicle (MCT+Ve). 5-HTT–like immunoreactivity was mainly confined to SMCs of PAs in all groups. Scale bar=100 μm.

fluoxetine on both days 21 and 42. In contrast to the large number of PCNA-positive cells, the number of apoptotic cells detected by terminal dUTP nick end-labeling (TUNEL) was very low in PA walls from MCT animals treated with vehicle or fluoxetine on both days 21 and 42 (Figure 6).

Lung Expression of Cytokines and 5-HTT After MCT Treatment in Rats Given Vehicle or Fluoxetine

Pulmonary expression of the cytokines IL-1β, IL-6, and MCP-1, previously shown to be involved in the inflammatory response to MCT,[11–13] was measured at various times after MCT injection. The results showed early peaks in IL-1 β and MCP-1 mRNA levels within the first 24 hours, followed by a transient decrease on day 5, and then by a gradual increase from day 5 to day 21 (Figure 7). In contrast, lung IL-6 mRNA remained undetectable until day 5 but then increased sharply to reach a plateau from day 10 to day 21 (Figure 7). Long-term treatment with fluoxetine (10 mg/kg) abolished the late increases in IL-6, MCP-1, and IL-1 β mRNA but did not affect the early peaks in IL-1β and MCP-1 mRNA levels (Figure 7). Similarly, treatment with fluoxetine suppressed the late increase in 5-HTT protein on day 21, from 1.26±0.05 to 0.50±0.03 U (5-HTT/β-actin density; $P<0.01$) but had no influence on the early peak on day 1.

Effect of Inflammatory Cytokines on 5-HTT Expression in Isolated Rat PA-SMCs

The potential effects of various inflammatory cytokines on 5-HTT expression were examined in cultured PA-SMCs from control rats. Incubation of PA-SMCs with IL-1β or MCP-1 for 4 or 24 hours induced an increase in 5-HTT mRNA or protein levels, respectively, compared with cells incubated with vehicle ($P<0.05$) (Figure 8). However, incubation of PA-SMCs with IL-6 did not change the levels of 5-HTT mRNA or protein.

Discussion

The main finding from the present study is that treatment with 5-HTT inhibitors not only prevents but also completely reverses PH induced by MCT in rats. In this model of severe PH, the highest dose of the 5-HTT inhibitor fluoxetine completely prevented death and PH development, as shown by the hemodynamic values similar to those in control animals and by the absence of both right ventricular hypertrophy and pulmonary vessel remodeling. Moreover, fluoxetine treatment resulted in complete reversal of established PH. Whereas PAP, right ventricular hypertrophy, distal PA-SMC proliferation, and wall thickness continued to increase from day 21 to day 42 in rats given vehicle, these values and vessel muscularization returned to normal in rats given fluoxetine.

MCT causes early endothelial injury of PAs and an inflammatory response, which precede the onset of PA-SMC proliferation and the development of PH.[10] In the present study, we showed an early increase in 5-HTT protein expression in lung tissue at a time when PA-SMC proliferation was not yet detectable. Levels of 5-HTT mRNA and protein peaked 12 to 24 hours after MCT injection and then decreased slightly but remained elevated thereafter. In rats that

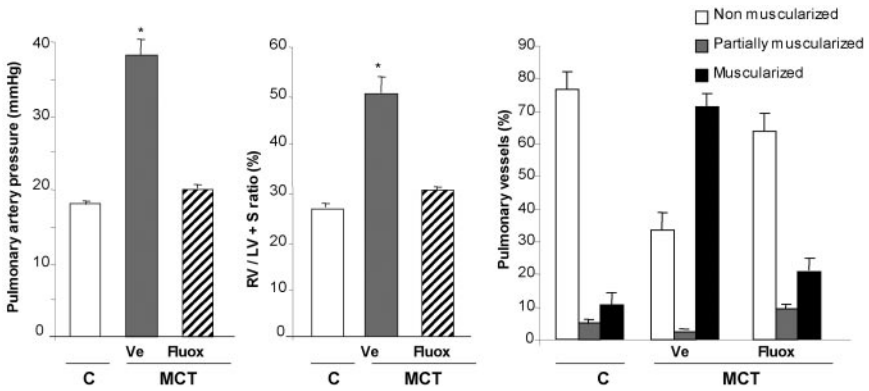

Figure 4. PAP, right ventricular hypertrophy as assessed by weight ratio RV/(LV+S), and percentage of nonmuscularized (NM), partially muscularized (PM), or fully muscularized (M) vessels in rats on day 42 after saline (controls) or MCT administration combined with fluoxetine (Fluox) or vehicle (Ve) treatment from day 21 to day 42 (n=10 in each group). *$P<0.01$ compared with values in control rats.

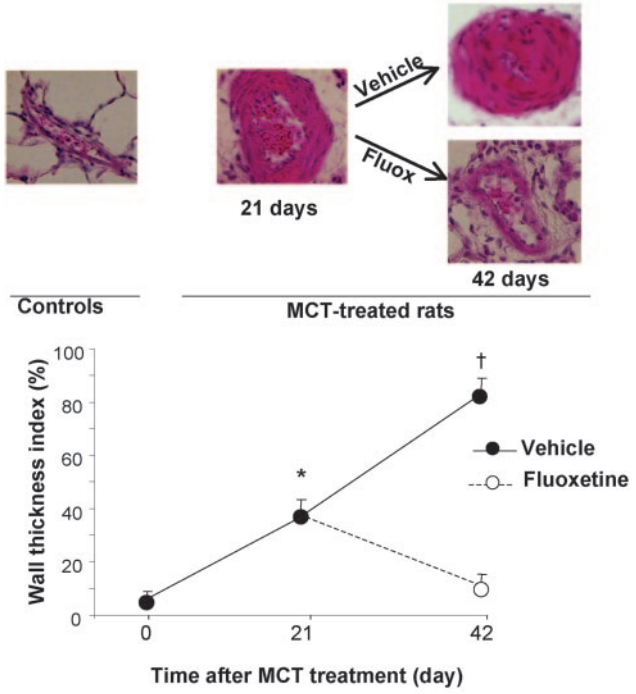

Figure 5. Pulmonary vascular remodeling as assessed by PA wall thickness index 21 and 42 days after MCT administration. Treatment with fluoxetine from day 21 to day 42 completely reversed medial wall hypertrophy of PAs. Control rats were studied 42 days after saline. Each value is mean±SE of 10 independent determinations. *$P<0.01$ compared with values in control, saline-treated rats. †$P<0.01$ compared with values in MCT-treated rats given vehicle.

were given fluoxetine and did not develop PH, the early increase in lung 5-HTT expression was unaffected, whereas the late increase in 5-HTT measured on day 21 was abolished. The most plausible interpretation of these data is that reduction of pulmonary vascular remodeling, and thereby of the number of SMCs, explained the large reduction in 5-HTT expression observed after fluoxetine treatment. These data constitute good evidence that early upregulation of lung 5-HTT in response to MCT is necessary to initiate PA-SMC proliferation and the subsequent development of PH. All fluoxetine-treated rats were still alive 42 days after MCT administration, whereas 40% of the vehicle-treated animals died between days 30 and 35. The fact that in vehicle-treated rats death occurred after establishment of PH highly suggests that the beneficial effect of fluoxetine on survival was related to prevention of PH. Moreover, the observation that fluoxetine not only prevented but also reversed established PH is evidence that a sustained increase in 5-HTT expression is necessary for both the progression and maintenance of MCT-induced PH. In vehicle-treated rats, medial thickness of distal PAs increased markedly between days 21 and 42, and vascular cell proliferation as assessed by PCNA labeling was observed on day 21 but was even more marked on day 42. In contrast, treatment with fluoxetine from day 21 to 42 was associated with a reduction in PCNA labeling on day 42, together with complete reversal of PH and distal vessel remodeling. Because the number of TUNEL-positive SMCs was very small in both conditions, it is likely that fluoxetine reversed PH by inhibiting the sustained marked proliferation of cells that was needed for the maintenance and late aggravation of PH as a consequence of 5-HTT overexpression.

Interestingly, marked PH prevention was observed not only with the highest daily dose, 10 mg/kg PO of fluoxetine, but also with 2 mg/kg PO, a dose within the therapeutic range (20 to 120 mg daily) used in humans with depression and other psychopathological conditions.[14] Direct assessment of 5-HTT blockade and its biochemical/behavioral consequences in vivo in rodents showed that maximal effects were achieved with the systemic administration of 10 mg/kg fluoxetine, although significant 5-HT uptake inhibition was already obtained with 1 to 3 mg/kg.[15,16] Furthermore, it has been demonstrated that the effects of low-dose fluoxetine (on brain neurotransmission) are significantly larger under subchronic versus acute treatment conditions,[17,18] probably because of the long half-lives (at least 2 days) of the drug and its active metabolite norfluoxetine.[14] All of these features are in accordance with our observations that fluoxetine at only 2 mg/kg PO daily for 3 weeks effectively prevented MCT-induced PH as a result of its established ability to inhibit 5-HTT.

In contrast to fluoxetine, the 5-HT_{2A} receptor antagonist ketanserin, the 5-HT_{2B} receptor antagonist RS127445, and the 5-$HT_{1B/1D}$ receptor antagonist GR127935 failed to prevent MCT-induced PH. These results are in accordance with previous data showing that the mitogenic effect of 5-HT on SMCs is not altered by these receptor antagonists but is abolished by the 5-HTT inhibitor fluoxetine.[2,4,19,20] The fact that MCT-induced PH is due chiefly to pulmonary vascular

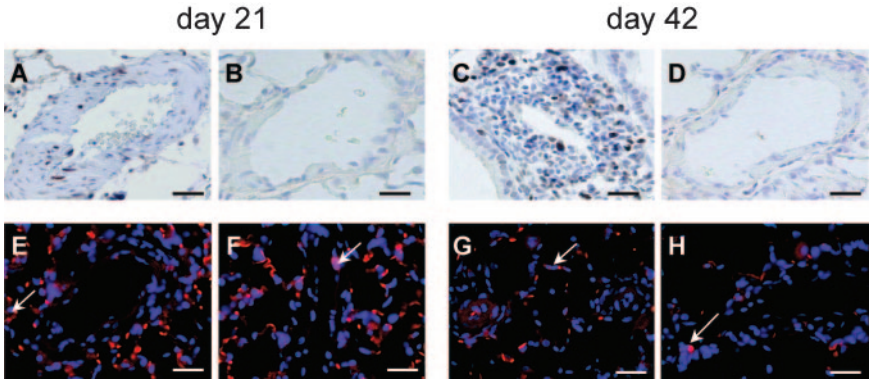

Figure 6. Rat PA-SMC proliferation (PCNA, A–D) and apoptosis (E–H) on days 21 and 42 after MCT injection. Medial hypertrophy was associated with increased number of proliferating vascular cells, shown by immunohistochemistry for PCNA; dark nuclei are PCNA-positive cells, which were more numerous after 42 than after 21 days (A and C). Prevention and regression of medial hypertrophy induced by fluoxetine (10 mg kg^{-1} d^{-1}) was attributed to inhibition of PA-SMC proliferation (B and D). In contrast, number of apoptotic cells (in situ TUNEL assays) was very lower in PA walls from MCT animals treated with vehicle (E and C) or fluoxetine (F and H).

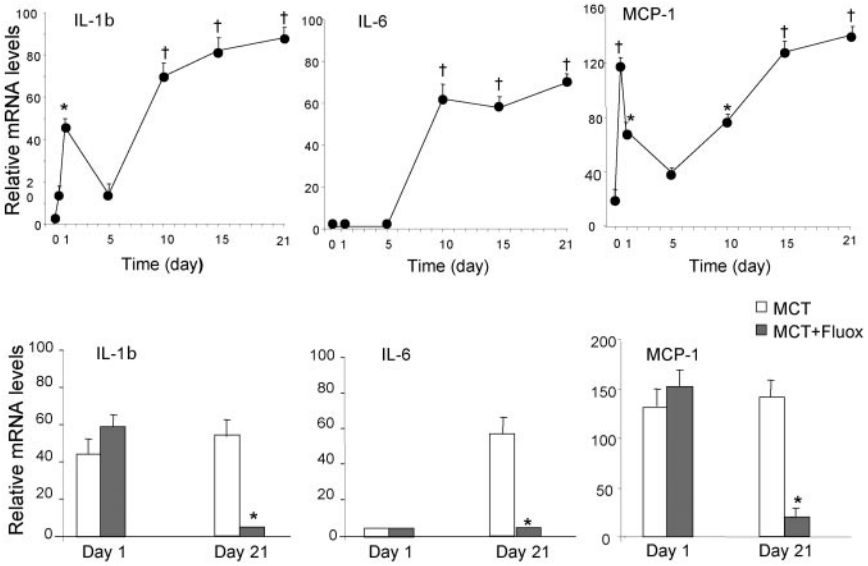

Figure 7. A, Time course of IL-1β, IL-6, and MCP-1 mRNA levels in lungs from rats after MCT administration (n=5 in each group). *$P<0.05$ and †$P<0.01$ compared with values in control, untreated rats (day 0). B, IL-1β, IL-6, and MCP-1 mRNAs in lung tissue from rats treated with fluoxetine (Fluox, 10 mg kg^{-1} d^{-1}) or vehicle (Ve) on days 1 and 21 after MCT administration. *$P<0.01$ compared with corresponding values in MCT-treated rats given vehicle and studied under similar conditions.

remodeling probably explains the efficacy of fluoxetine in this model. Previous findings that rats treated with selective 5-$HT_{1D/1B}$ or 5-HT_{2A} receptor antagonists[8,21] and mice lacking the 5-HT_{1B} receptor gene[8] develop less severe chronic experimental PH and a milder degree of vascular remodeling than relevant paired controls probably reflect the effects of these drugs on 5-HT–mediated pulmonary vasoconstriction or on 5-HT–independent mechanisms. Recent studies have also emphasized the contribution of 5-HT_{2B} receptors to hypoxia-induced PH.[9] In the present work, the antagonists used in those previous studies had no influence on MCT-induced PH. These results are somewhat discordant with previous findings showing attenuation of MCT-induced PH with 5-HT_{2A} antagonists.[21] However, in those studies, none of the antagonists was capable of reversing advanced disease or of completely inhibiting MCT-induced PH. Taken together with our previous findings that 5-HTT inhibitors, but not 5-$HT_{1D/1B}$ or 5-HT_{2A} receptor antagonists, affect hypoxia-induced PH, these results provide evidence for a predominant role of 5-HTT over 5-HT receptors in both hypoxia- and MCT-induced PH.

The hypothesis that inflammatory cytokines are involved in the process of pulmonary vascular remodeling has long been suggested on the basis of experimental and human studies.[11] The present results showing early increases in IL-1β and MCP-1 expression after MCT administration are consistent with the concept that inflammation plays a key role in initiating PH in this model.[10] Indeed, a similar early increase in lung IL-1β mRNA has been reported, as well as a reduction in MCT-induced PH, in rats treated with an anti–MCP-1 antibody or an IL-1 receptor antagonist.[12,13] In our studies, high values of IL-6 mRNA were detected only from day 10 onward, concomitant with second elevations in IL-1β and MCP-1 associated with the development of PH. Because treatment with fluoxetine completely abolished the late increases in IL-1β, MCP-1, and IL-6 mRNA on day 21, it is likely that these late changes occurred as a consequence rather than as a cause of PH. Treatment with fluoxetine, however, did not affect the early increase in IL-1β that coincided with the rise in 5-HTT. Treatment of isolated PA-SMCs with IL-1β or MCP-1 induced a nearly 2-fold increase in 5-HTT mRNA levels, whereas IL-6 had no effect, suggesting that the early rises in IL-1β and MCP-1 in response to MCT may have contributed to the increase in 5-HTT protein and to the subsequent PA-SMC proliferation. Such an increase in 5-HTT mRNA in response to IL-1β has previously been found in human choriocarcinoma cell lines.[22,23] Therefore, these in vitro and in vivo data provide good evidence for a relation between inflammatory mediators and the 5-HT pathway in the pathogenesis of MCT-induced PH.

Whether inflammation contributes to the pathogenesis of human PH via stimulation of 5-HTT expression and whether treatment with 5-HTT inhibitors holds therapeutic potential in

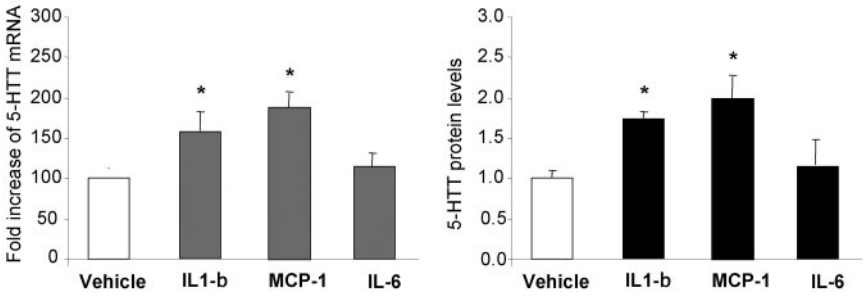

Figure 8. mRNA and protein levels of 5-HTT in cultured PA-SMCs after stimulation by IL-1β, IL-6, and MCP-1 (10 ng/mL) or vehicle. 5-HTT mRNA expression was performed by real-time quantitative RT-PCR, and protein levels were assessed by densitometric quantification of 5-HTT immunoblotting normalized for β-actin level. Results are expressed as percentage of levels in PA-SMCs incubated with vehicle (controls). Values are mean±SEM of 5 independent experiments. *$P<0.05$ compared with values obtained in control PA-SMCs.

the various forms of human PH are extremely important questions. Many patients with PH exhibit clear-cut signs of inflammation, including elevated circulating and lung levels of proinflammatory cytokines such as IL-1β, IL-6, platelet-derived growth factor, macrophage inflammatory protein-1α, RANTES, and fractalkine.[11,24,25] Areas of focal necrosis with inflammatory reactions have long been described in pulmonary vessel walls of patients with primary PH. More recently, perivascular inflammatory cell infiltrates have been found in plexiform lesions. Given that some cytokines promote thrombosis and are considered potential mitogens, our results demonstrating a link between IL-1β and 5-HTT also provide new insight into the mechanisms by which inflammation may contribute to vessel wall remodeling and in situ microthrombosis in PH via stimulation of 5-HTT expression.

Stimulation of 5-HTT expression by inflammatory cytokines may also account for the 5-HTT upregulation observed in patients with PH, whether primary or associated with various pulmonary or systemic diseases. Indeed, we previously reported that overexpression of 5-HTT in humans with PH was only partly related to 5-HTT gene polymorphism.[3,4] That inflammatory cytokines such as IL-1β and MCP-1 stimulate 5-HTT expression is an important finding, as it may help us understand how environmental factors such as inflammation contribute to 5-HTT overexpression and therefore to the pathogenesis of pulmonary vascular remodeling. Reciprocally, the fact that 5-HTT blockade reverses PH and simultaneously normalizes the late expression of these cytokines supports the concept that altered 5-HTT expression is a primary abnormality in the pathogenesis of PH and that selective 5-HTT inhibition may represent a novel treatment strategy directed against the pulmonary vascular remodeling process that underlies all forms of human PH.

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