Induction of Serotonin Transporter by Hypoxia in Pulmonary Vascular Smooth Muscle Cells
Relationship With the Mitogenic Action of Serotonin

S. Eddahibi, V. Fabre, C. Boni, M.P. Martres, B. Raffestin, M. Hamon, S. Adnot

Abstract—The increased delivery of serotonin (5-hydroxytryptamine, 5-HT) to the lung aggravates the development of hypoxia-induced pulmonary hypertension in rats, possibly through stimulation of the proliferation of pulmonary artery smooth muscle cells (PA-SMCs). In cultured rat PA-SMCs, 5-HT (10^{-8} to 10^{-6} mol/L) induced DNA synthesis and potentiated the mitogenic effect of platelet-derived growth factor-BB (10 ng/mL). This effect was dependent on the 5-HT transporter (5-HTT), since it was prevented by the 5-HTT inhibitors fluoxetine (10^{-6} mol/L) and paroxetine (10^{-7} mol/L), but it was unaltered by ketanserin (10^{-6} mol/L), a 5-HT_{2A} receptor antagonist. In PA-SMCs exposed to hypoxia, the levels of 5-HTT mRNA (measured by competitive reverse transcriptase–polymerase chain reaction) increased by 240\% within 2 hours, followed by a 3-fold increase in the uptake of [\textsuperscript{3}H]5-HT at 24 hours. Cotransfection of the cells with a construct of human 5-HTT promoter-luciferase gene reporter and of pCMV-\beta-galactosidase gene allowed the demonstration that exposure of cells to hypoxia produced a 5.5-fold increase in luciferase activity, with no change in \beta-galactosidase activity. The increased expression of 5-HTT in hypoxic cells was associated with a greater mitogenic response to 5-HT (10^{-8} to 10^{-6} mol/L) in the absence as well as in the presence of platelet-derived growth factor-BB. 5-HTT expression assessed by quantitative reverse transcriptase–polymerase chain reaction and in situ hybridization in the lungs was found to predominate in the media of pulmonary artery, in which a marked increase was noted in rats that had been exposed to hypoxia for 15 days. These data show that in vitro and in vivo exposure to hypoxia induces, via a transcriptional mechanism, 5-HTT expression in PA-SMC proliferation. In vivo exposure of 5-HTT by PA-SMC may play a key role in serotonin-mediated pulmonary vascular remodeling. (\textit{Circ Res}. 1999;84:329-336.)

Key Words: 5-hydroxytryptamine transporter \hspace{1em} hypoxia \hspace{1em} pulmonary arterial smooth muscle cell \hspace{1em} pulmonary hypertension

Hypoxia is well known to affect markedly both the tone and the structure of blood vessels. In the lung, acute hypoxia causes arteriolar vasoconstriction with a subsequent rise in pulmonary artery pressure. Moreover, chronic hypoxia results in smooth muscle cell proliferation and sustained elevation in pulmonary artery pressure. One possible mechanism that may account for the latter effect is a direct action of hypoxia on the expression of specific genes involved in smooth muscle cell proliferation.\(^1\)

That serotonin (5-hydroxytryptamine, 5-HT) plays an important role in the remodeling of the pulmonary circulation, notably during exposure to hypoxia, is suggested by several studies. Fawn-Hooded rats, which have a genetic deficit in 5-HT platelet storage, are characterized by high plasma levels of the indolamine. Interestingly, these animals, but not Sprague-Dawley rats (which have markedly lower levels of plasma 5-HT), develop pulmonary hypertension when they are exposed to mild hypoxia.\(^2\) Similarly, primary pulmonary hypertension has been reported in patients with increased plasma 5-HT levels associated with platelet storage deficit.\(^3\) The possibility that 5-HT may favor the development of pulmonary hypertension is further supported by recent data showing that continuous intravenous infusion of the indolamine during a 2-week exposure to hypoxia aggravates pulmonary hypertension in rats.\(^4\) In contrast, no effects of 5-HT infusion were observed in animals maintained under normoxia. Moreover, the aggravating effect of 5-HT infusion on hypoxic pulmonary hypertension is prevented when rats are treated with dexfenfluramine, an inhibitor of 5-HT transport.\(^5\) These results suggest that the effects of hypoxia on pulmonary vascular remodeling are facilitated by 5-HT through an intracellular mechanism that requires internalization of the indolamine by the dexfenfluramine-sensitive high-affinity 5-HT transporter (5-HTT).
Although pulmonary endothelial cells play an important role in the removal of 5-HT from the circulation, pulmonary smooth muscle cells have also been shown to participate in this process. Indeed, using cells derived from bovine pulmonary arteries, Lee et al. even found that 5-HT uptake by smooth muscle cells was 7- to 8-fold higher than uptake by endothelial cells. Additionally, uptake of 5-HT by both types of cells has also been reported to be potentiated by hypoxia. To date, the physiological role of 5-HTT in pulmonary smooth muscle cells has not yet been completely elucidated. In addition to contributing to the uptake and subsequent inactivation of 5-HT passing through the lung, 5-HTT might also mediate the proliferation of smooth muscle cells through its capacity to internalize the indolamine. As 5-HTT is also a target for drugs that have recently been shown to induce pulmonary hypertension in humans, the potential clinical relevance of this transporter provides further interest for studying both its genetic regulation and its role in the proliferation of pulmonary vascular smooth muscle cells.

The aim of the present study was to investigate 5-HTT expression in cultured pulmonary vascular smooth muscle cells exposed to hypoxia to assess whether 5-HTT could play a role in the control of their proliferation. Moreover, we also examined whether in vivo exposure to hypoxia could affect the expression of 5-HTT in remodeled pulmonary vessels.

Materials and Methods

Isolation and Culture of Rat Pulmonary Artery Smooth Muscle Cells (PA-SMCs)

All animal care and procedures were in accordance with institutional and international guidelines. The method used for PA-SMC isolation and culture has been previously described. In brief, male Wistar rats weighing 250 to 300 g were killed by an overdose of pentobarbital. Lungs were immediately removed, and proximal pulmonary arteries were isolated under aseptic conditions. After removal of fat, adventitia, and connective tissue surrounding the arteries, the pulmonary arteries were cut into small pieces that were then incubated in DMEM supplemented with elastase type III (0.125 mg/mL), and culture has been previously described. In brief, male Wistar rats weighing 250 to 300 g were killed by an overdose of pentobarbital. Lungs were immediately removed, and proximal pulmonary arteries were isolated under aseptic conditions. After removal of fat, adventitia, and connective tissue surrounding the arteries, the pulmonary arteries were cut into small pieces that were then incubated in 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). At a 90-minute incubation at 37°C, the tissue suspension was centrifuged, and the pellet was resuspended in DMEM supplemented with 15% (vol/vol) FCS, 2 mmol/L L-glutamine and the same antibiotics as 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, up to confluency. The medium was changed every day, and the cells were harvested with trypsin (0.2 g/L)-EDTA (0.5 g/L). Cells after 4 to 5 passages were used for the experiments. All cells exhibited specific immunostaining by anti-α-smooth muscle actin antibodies, as expected of PA-SMCs.

Microculture Tetrazolium (MTT) Assay

The MTT assay was performed for evaluation of the effect of hypoxia on PA-SMC viability and density. Cells seeded in 96-well plates were cultured up to confluency, and the medium was replaced by fresh serum-free medium. The cells were then exposed to hypoxia (5% CO₂ and 95% N₂) or normoxia (5% CO₂, 20% O₂, and 75% N₂) for 24 hours. At the end of this period, MTT (0.2 mg/mL) was added to each well, and incubation proceeded for 4 hours at 37°C. Thereafter, the culture medium was removed and the cells were solubilized in 100 μL of DMSO. The extent of reduction of MTT to formazan within cells was quantified spectrophotometrically at 520 nm and taken as an indicator of cell viability.

Effect of 5-HT on [³H]Thymidine Incorporation by SMCs Subjected to Normoxia or Hypoxia

SMCs 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, cells were subjected to 48 hours of growth arrest in medium containing 0.2% FCS. At the end of this period, cells were incubated with 5-HT (10⁻⁸ to 10⁻⁶ mol/L) alone or in the presence of platelet-derived growth factor (PDGF)-BB (10 ng/mL, Sigma) in medium containing 0.2% FCS, antibiotics (as above), 0.6 mmol/L ascorbic acid, 0.1 mmol/L iproniazid (a monoamine oxidase inhibitor, Sigma), and 0.6 μCi/mL of [³H]thymidine (50 Ci/mmol, Amersham). The effect of 5-HT was also examined in the presence of 1 of 2 specific 5-HT inhibitors, fluoxetine (10⁻⁶ mol/L) and paroxetine (10⁻⁷ mol/L), or in the presence of ketanserin (10⁻⁶ mol/L), a 5-HT₂ receptor antagonist. These were added 20 minutes before 5-HT. After 24 hours of incubation under normoxic (5% CO₂, 20% O₂, and 75% N₂) or hypoxic (5% CO₂ and 95% N₂) conditions, cells were washed twice with PBS, followed by ice-cold 10% trichloroacetic acid. Cells were then dissolved in 0.1N NaOH (0.5 mL/well), and the incorporated radioactivity was counted.

Measurement of [³H]5-HT Uptake

SMCs in medium containing 15% FCS were seeded in 24-well plates at a density of 5×10⁴ cells/well and allowed to proliferate for 72 hours. At the end of this period, the medium was removed, and cells were subjected to growth arrest in medium containing 0.2% FCS. After 8 to 24 hours of incubation under normoxic (5% CO₂, 20% O₂, and 75% N₂) or hypoxic (5% CO₂ and 95% N₂) conditions, cells were washed twice with PBS and exposed to 10 nmol/L 5-hydroxy-G-³H[tryptamine creatinine sulfate ([³H]5-HT, 15 to 16 Ci/mmol, Amersham) in a medium containing (in mmol/L) NaCl 120, KCl 5, CaCl₂ 1.2, MgSO₄ 1.2, glucose 5.6, Tris-HCl 4, HEPES 6.25, and ascorbic acid 0.5, pH 7.4 (uptake buffer). Under these conditions, [³H]5-HT uptake by PA-SMCs was linear for at least 15 minutes. Therefore, assays were performed for 10 minutes at 37°C in the absence or the presence of fluoxetine (10⁻⁴ to 10⁻³ mol/L). At the end of the incubation period, the medium was removed and cells were washed 3 times with the uptake buffer. Cells were lysed by adding 0.5 mL of 0.1N NaOH, and radioactivity of lysates was counted by liquid scintillation spectrometry. Uptake is expressed as fmol [³H]5-HT taken up per mg protein (measured by the method of Lowry et al.) with BSA as standard.

Extraction of RNA From Cultured SMCs

To examine the effect of hypoxia on 5-HTT mRNA expression, the medium of cells grown to confluency was removed and replaced by serum-free medium. The cells were then exposed to hypoxia (5% CO₂ and 95% N₂) or normoxia (5% CO₂, 20% O₂, and 75% N₂). After a 2- to 24-hour exposure, cells were washed with PBS and lysed with guanidine isothiocyanate (Interchim). Total RNA was extracted according to the method of Chomczynski and Sacchi and electrophoresed in 1% agarose gel stained with ethidium bromide. Quantification was performed with reference to a scale of total RNAs prepared on a cesium chloride gradient and estimated by optical density measurement at 260 nm.

Quantitative Determination of 5-HTT mRNA

The method was based on competitive polymerase chain reaction (PCR), which RNAs were reverse transcribed and the synthesized cDNAs were amplified in the presence of an internal standard consisting of the same target mRNA, synthesized with a deletion of ~100 bases as described in detail elsewhere.

Total RNAs (0.8 μg per sample) and internal standard RNA (0.01 to 1 pg) were reverse transcribed (45 minutes at 48°C) and amplified using an Access reverse transcriptase (RT)-PCR kit (Promega) with the primers S'-TTAACAACAGCTTACATCGG (nucleotides 2008 to 1991) and S'-GGATCCCTGCTCACACTG (nucleotides 1541 to 1558) at 2.5 mmol/L MgCl₂. Cycle amplifications were performed at 94°C, 56°C, and 72°C (1 minute each, 28 cycles). PCR products from the 5-HTT mRNA and the corresponding synthetic deleted
RNA were of 484 and 400 bp, respectively. They were electrophoresed in 2% agarose gel stained with ethidium bromide and quantified with a gel analyzer (Gel Doc 5000, UVP). RNA levels in smooth muscle cells exposed to hypoxia are expressed as a percentage of those found under normoxic control conditions.

Transfection of SMCs

SMCs were transfected with a plasmid pGL3 in which the promoter of the human-5-HTT was fused to the reporter luciferase gene.20,21 The human 5-HTT promoter genomic fragment (bp −4288 to +217 with respect to the transcription initiation site) is defined by a TATA-like motif and several potential binding sites for transcription factors including activator protein (AP)-1, AP-2, SP1, and a cAMP TATA-like motif.26 To correct for variations in luciferase activity, luciferase activity was measured after incubation of 50 μL of lysate samples to 100 μL luciferin reagent. Chemiluminescence was counted 15 s after reagent mixing in a liquid scintillation spectrometer. β-Galactosidase activity was measured after incubation of 50 μL of lysate samples with chloroform red-β-o-galactopyranoside at a final concentration of 5 mM/L. Absorbance at 574 nm was measured after a 60-minute incubation of the assay mixture at room temperature.

Exposure of Rats to Chronic Hypoxia

Male Wistar rats, weighing 250 to 300 g at the beginning of the experiments, were exposed to chronic hypoxia (10% O2; 2 weeks) in a ventilated chamber (volume = 500 L; Flufrance) as previously described.23 To establish the hypoxic environment, the chamber was flushed with a mixture of room air and nitrogen, which was recirculated. The chamber environment was monitored with an oxygen analyzer (Servomex OA150). Carbon dioxide was removed by soda lime granules, and excess humidity was prevented by cooling of the recirculation circuit. The temperature of the chamber was maintained at 22°C to 24°C. The chamber was opened every day for 1 hour so that the cages could be cleaned and food and water provided. Normoxic rats were kept in the same room, with the same light-dark cycle. Rat chow and tap water were provided ad libitum.

TABLE 1. Effect of 5-HT on [3H]Thymidine Incorporation in Smooth Muscle Cells Exposed to Normoxia or Hypoxia in the Absence or Presence of PDGF-BB

<table>
<thead>
<tr>
<th>5-HT Concentration, mol/L</th>
<th>0.2% FCS</th>
<th>0.2% FCS + PDGF-BB (10 ng/mL)</th>
<th>0.2% FCS</th>
<th>0.2% FCS + PDGF-BB (10 ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>505±61</td>
<td>320±55*</td>
<td>6758±286</td>
<td>4701±196*</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>720±78</td>
<td>955±96*</td>
<td>14 494±542</td>
<td>20 757±1048†</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>1220±59†</td>
<td>2326±146†</td>
<td>21 215±728</td>
<td>24 342±1361†</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>2425±179†</td>
<td>2636±568†</td>
<td>24 566±219</td>
<td>24 257±477†</td>
</tr>
</tbody>
</table>

The incorporation of [3H]thymidine was determined during a 24-hour incubation under normoxic (5% CO2, 20% O2, and 75% N2) or hypoxic (5% CO2 and 95% N2) conditions. Values (cpm/well) are mean±SEM of 1 typical experiment with n = 6 wells for each concentration of 5-HT tested. *P < 0.05 compared with respective values under normoxia. †P < 0.05 compared with respective basal values (without 5-HT). ‡P < 0.01 compared with respective values under normoxia.

In Situ Hybridization Histochemistry

Lungs isolated from normoxic or hypoxic rats were digested by infusion of Tissue-Tek (Miles) diluted in PBS (1:1) into the trachea, rapidly frozen in isopentane at −30°C, and stored at −80°C. Lung sections (15 μm) were cut at −20°C in a cryostat and thaw mounted onto ready-to-use Super Frost plus slides (Consortium de Matériel pour Laboratoire, Nemours, France). Sections were then fixed for 30 minutes in ice-cold 4% paraformaldehyde in PBS, dehydrated in a graded series of ethanol solutions (60%, 80%, 95%, and 100%), and stored at −80°C until use.

For the hybridization step, each lung section was covered with hybridization buffer containing 0.6 mol/L NaCl, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, 100 μg/mL denatured salmon sperm DNA, 50 μg/mL yeast total RNA, 2.5× Denhardt’s solution, 10% dextran sulfate, 10 mmol/L DTT, 50% formamide, and 4×10⁶ cpm of [35S]labeled antisense riboprobe. Incubation then proceeded overnight in a humid chamber at 57°C. Nonhybridized probe was subsequently eliminated by RNase A (50 μg/mL in 100 mmol/L Tris-HCl and 0.5 mol/L NaCl, pH 8.0) for 60 minutes at 37°C, followed by extensive washes.24 Then, sections were dipped in Kodak autoradiographic emulsion (Amersham) and stored in the dark at 4°C before development 1 to 2 weeks later. Sections were finally counterstained with hematoxylin phloxin saffron and examined using light- and dark-field microscopy.

Statistical Analysis

[3H]5-HT uptake after various times of exposure to normoxia were compared using 1-way ANOVA. Since there were no significant differences between these values, they were pooled. Values obtained after various times of exposure to hypoxia were compared, with the pooled normoxic values using 1-way ANOVA followed by Dun-
nett’s t test. [3H]Thymidine incorporation was compared at various concentrations of 5-HT under normoxic or hypoxic conditions using a 2-way ANOVA, followed, in case of significant interaction, by the Mann-Whitney nonparametric test for comparison between normoxic and hypoxic conditions at each concentration of 5-HT.

Results

Effect of 5-HT on [3H]Thymidine Incorporation by PA-SMCs During Normoxia or Hypoxia

In quiescent cells 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 (Table 1). In the presence of PDGF-BB (10 ng/mL), the stimulatory effect of 5-HT was still observed, despite a 13-fold increase in [3H]thymidine incorporation induced by PDGF-BB alone. Addition of 10⁻⁶ mol/L 5-HT to the culture medium increased DNA synthesis by 5-fold and 3.5-fold in the presence of 0.2% FCS and PDGF-BB, respectively. Pretreatment of the cells with a 5-HT transport inhibitor, either fluoxetine (10⁻⁶ mol/L) or paroxetine (10⁻⁷ mol/L), completely inhibited the increase in [3H]thymidine incorporation induced by 5-HT in the presence of either 0.2% FCS or PDGF-BB (Figure 1A and 1B). In contrast, the response to 5-HT was unaltered in the presence of the 5-HT₂A receptor antagonist ketanserin at 10⁻⁶ mol/L. Neither fluoxetine/paroxetine nor ketanserin affected the response to PDGF-BB alone (data not shown).

A decrease (–30% and –37%, P<0.05) in [3H]thymidine incorporation was observed in PA-SMCs exposed to hypoxia in the absence and in the presence of PDGF-BB. However, the stimulatory effect of 5-HT on [3H]thymidine incorporation persisted under hypoxic conditions. Indeed, in cells incubated with 0.2% FCS, [3H]thymidine incorporation in response to low (10⁻⁸ mol/L) and intermediate (10⁻⁷ mol/L) concentrations of 5-HT was greater under hypoxic than normoxic conditions (Table 1 and Figure 1). Hypoxia also potentiated the stimulatory effect of 10⁻⁸ mol/L 5-HT in cells incubated with PDGF-BB. During hypoxia, the stimulatory effect of 5-HT on DNA synthesis was completely abolished by fluoxetine or paroxetine but unaltered by ketanserin. Thus, in cells incubated with 0.2% FCS or PDGF-BB, [3H]thymidine incorporation remained unchanged when 5-HT (10⁻⁶ mol/L) was associated with pretreatment with fluoxetine or paroxetine under both normoxic and hypoxic conditions (Figure 1).

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

[3H]5-HT uptake remained stable in SMCs that had been exposed to normoxia for various times (8, 16, or 24 hours). In contrast, a progressive increase in [3H]5-HT uptake was observed in cells exposed to hypoxia (Figure 2). Thus, at its maximal value that was reached after hypoxia for 16 hours, [3H]5-HT uptake was 3-fold higher than that found under normoxic conditions (P<0.001, Figure 2). Fluoxetine caused a concentration-dependent inhibition of [3H]5-HT uptake, with similar IC₅₀ values under normoxic and hypoxic conditions (2.2×10⁻⁸ and 2.8×10⁻⁸ mol/L in normoxia and after 24 hours of exposure to hypoxia, respectively). At the highest concentration of fluoxetine tested (10⁻⁵ mol/L), residual [3H]5-HT uptake was similar under hypoxic versus normoxic conditions.

Effect of Hypoxia on 5-HTT mRNA Levels in PA-SMCs

Cells exposed to hypoxia showed no evidence of cellular damage. In particular, the number of cells incubated in serum-free DMEM remained similar after 24 hours of exposure to normoxia or hypoxia (MTT measurements). Levels of 5-HTT mRNA did not change significantly with time in cells exposed to normoxic conditions. In contrast, exposure to hypoxia for 2 hours resulted in a 2.4-fold increase of 5-HTT mRNA levels (Figure 3). A significant increase (+50%) was still observed after 4 hours of hypoxia; then 5-HTT mRNA...
levels returned down to the values measured in PA-SMCs exposed to normoxic conditions (Figure 3).

**Induction by Hypoxia of the 5-HTT Promoter Gene Construct Transfected to SMCs**

In cells cotransfected with human 5-HTT promoter-luciferase and pCMV-LacZ genes, exposure to hypoxia led to a marked increase in luciferase activity compared with normoxic controls, whereas β-galactosidase activity remained unchanged (Figure 4). As illustrated in Figure 4, the increase in luciferase activity developed progressively during hypoxia, up to a maximum that was reached at 16 to 24 hours. At this time, luciferase activity in cells exposed to hypoxia was 5- to 6-fold higher than that measured in cells maintained under normoxic conditions (Figure 4). However, exposure to hypoxia of cells cotransfected with pCMV-LacZ and pGL3-luciferase reporter vectors did not change the activities of expressed enzymes compared with the values obtained under normoxic environment (not shown).

**Effect of Chronic Hypoxia on 5-HTT mRNA Levels in Pulmonary Arteries and Lungs**

mRNA encoding 5-HTT was present in proximal pulmonary arteries from chronically hypoxic rats but was not detected in arterial tissue from normoxic animals (Table 2). In lungs from chronically hypoxic rats, the levels of 5-HTT mRNA, measured by competitive RT-PCR, were found to be significantly higher (+38%, P<0.05) than those determined in the same tissues of normoxic rats (Table 2).

As illustrated in Figure 5, in situ hybridization histochemistry performed on rat lung sections showed that 5-HTT mRNA was predominant in pulmonary vascular walls. In rats that had been maintained under hypoxic conditions, the hybridization signal was observed in both endothelial and SMCs and was more marked than in arteries from normoxic rats.

**Discussion**

The present results show that, in rat PA-SMCs, the mitogenic action of 5-HT depends on the 5-HTT activity. Exposure of PA-SMCs to hypoxia increases the transcriptional rate of the 5-HTT gene and potentiates the growth-promoting effect of 5-HT. Increased 5-HTT gene expression by PA-SMCs is also observed in remodeled pulmonary arteries of rats subjected to chronic hypoxia and developing pulmonary hypertension. These in vitro and in vivo data support a model whereby low oxygen tension induces the expression of 5-HTT, which, in turn, may sensitize PA-SMCs to the mitogenic action of 5-HT.

The effects of 5-HT on pulmonary vessels is currently the matter of renewed interest, notably because anorectic drugs acting through inhibition of 5-HT transport have been reported to cause an increased risk of developing pulmonary hypertension in humans.8,9 Studies have long been concerned with the
vasoactive properties of 5-HT, mediated mainly through its binding to specific receptors on pulmonary vascular endothelial and SMCs. More recently, 5-HT was reported to promote growth of pulmonary vascular SMCs as well as of other cell types. 

Serotonin is a known mitogen for SMCs isolated from bovine, porcine, and rat aorta as well as rat and bovine pulmonary arteries. The mechanism by which 5-HT causes smooth muscle cell proliferation may vary with cell types and species. Several studies have concluded that the mitogenic action of 5-HT is initiated through its binding to a cell surface receptor, notably the 5-HT2A type, whereas evidence has also been provided that it results from an energy-dependent transport of 5-HT into the cell. In the present study, we found that 5-HT was a potent inducer of rat PA-SMC proliferation and that this effect was dose-dependently inhibited by the highly selective inhibitors of 5-HT transport, paroxetine, and fluoxetine, but not by the 5-HT2A receptor antagonist ketanserin. Indeed, fluoxetine and paroxetine inhibited [3H]5-HT uptake and 5-HT-induced cell proliferation at similar concentrations, suggesting that both phenomena were tightly related. These results are consistent with those of Lee et al., showing that the proliferative response of bovine pulmonary vascular SMCs to 5-HT was inhibited by agents that block the transport of 5-HT but not by 5-HT receptor antagonists. In accordance with these previous studies, we found that the mitogenic response to 5-HT also occurred when rat SMCs were incubated in the presence of PDGF-BB. Both fluoxetine and paroxetine inhibited the proliferative action of serotonin in the presence of PDGF-BB, whereas none of these inhibitors affected PDGF-BB-induced cell proliferation. Taken together, these results clearly indicate that low concentrations of 5-HT stimulate proliferation of PA-SMCs and that this effect is dependent on the active transport of 5-HT within cells. At this time, the mechanisms by which 5-HT exerts its mitogenic effect after being transported inside SMCs remain speculative. Lee et al have observed that 5-HT–induced DNA synthesis is associated with tyrosine phosphorylation of GTPase-activating protein and that both effects are blocked by 5-HT transport or tyrosine kinase inhibitors. Therefore, although 5-HT–induced mitogenesis in SMCs requires cellular internalization through 5-HTT rather than binding to a membrane receptor, tyrosine phosphorylation of GTPase-activating protein appears as a downstream intermediate in the signaling pathway. Recently, involvement of superoxide anion formation in association with 5-HT transport has also been suggested to play a role in the mitogenic effects of 5-HT.

Hypoxia is a well-recognized stimulus for pulmonary blood vessel remodeling. One possible mechanism that may account for this effect is a direct action of hypoxia on the expression of specific genes involved in smooth muscle cell proliferation. We found that exposure of PA-SMCs to hypoxia resulted in a rapid and transient increase in the level of 5-HTT mRNA, followed by a prolonged 2.5- to 3.0-fold increase in 5-HT transport activity. While the increase in 5-HTT mRNA levels peaked at 2 hours, the maximal increase in 5-HT transport was measured at 16 to 24 hours of hypoxia, a finding consistent with the time required for protein synthesis and integration of the transporter into the plasma membrane. Interestingly, the IC50 of fluoxetine remained unchanged in cells that had been subjected to hypoxia, indicating that the pharmacological properties of the transporter were not affected by hypoxia. Previous studies already documented an increased rate of 5-HT uptake in cultured endothelial and SMCs derived from bovine pulmonary arteries during exposure to hypoxia. Whether this was due to

**Figure 4.** Effects of hypoxia on luciferase and β-galactosidase activities in SMCs. PA-SMCs were cotransfected with human 5-HT promoter-luciferase gene reporter and pCMV-β-galactosidase gene. Transfected cells were then exposed to normoxia (N) or hypoxia (H) at the durations indicated. Each column is the mean ± SEM of 3 independent experiments (each performed in duplicate).

**TABLE 2. Levels of 5-HTT mRNA in Proximal Pulmonary Arteries and Lungs From Normoxic and Chronically Hypoxic Rats**

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Chronic Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal pulmonary arteries</td>
<td>Not detected (n=4)</td>
<td>0.103±0.025 (n=4)</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.156±0.012 (n=5)</td>
<td>0.216±0.018* (n=5)</td>
</tr>
</tbody>
</table>

5-HTT mRNA levels (attomol/μg of total RNA) are the mean±SEM of n independent determinations.

*P<0.05 compared with normoxia.
an increased activity or expression of the transporter protein was not specified. In the present study, we provided evidence that the increased 5-HT uptake was directly related to an increased transcriptional rate of the 5-HTT gene. Thus, in cells transfected with a luciferase-reporter gene construct containing the human 5-HTT promoter, exposure to hypoxia was associated with a marked increase in luciferase activity. This effect on the 5-HTT promoter was specific, because when the cells were transfected with the same luciferase reporter gene under the dependence of the SV40 promoter, hypoxia did not change luciferase activity. These results can be taken as evidence that hypoxia-mediated activation of 5-HTT transcription is not restricted to the rat but may also apply to humans. Several mechanisms could account for this effect. Previous studies have indicated that expression of the AP-1 proteins c-Jun and c-Fos is upregulated by hypoxia.32

Since there are 2 potential AP-1–binding sites in the promoter region of 5-HTT,33 it can be speculated that induction of 5-HTT expression under hypoxic conditions is mediated by AP-1. A more specific mechanism of hypoxia-induced gene expression might involve the transcription factor hypoxia-inducible factor HIF-1, which binds to identified hypoxia-sensitive elements in the promoter of several hypoxia-inducible genes.34,35 The consensus sequence of these elements is 5′-TACGTGCT-3′. Interestingly, our computer-aided search in the 5-HTT promoter revealed in 2 locations, the presence of core sequence 5′-CGTG-3′, which has previously been shown to bind HIF-1.36 Accordingly, it can be inferred that hypoxia increases the transcriptional rate of the 5-HTT gene probably through one of these potential mechanisms.

Previous studies have shown that hypoxia reduces the proliferating effect of growth factors on cultured SMCs.37,38 In line with these data, we found that PA-SMC proliferation in response to PDGF-BB was attenuated in hypoxic cells compared with their normoxic controls. In contrast, 5-HT added to the cultured cell medium stimulated PA-SMC proliferation to a greater extent under hypoxia than under normoxic conditions. This increased growth-stimulatory effect of 5-HT was directly related to the increased expression of the transporter, since it was completely suppressed in the presence of fluoxetine or paroxetine. That 5-HT may act as a mitogenic factor with increased efficiency during hypoxia should have physiological relevance regarding in vivo stimulation of smooth muscle cell proliferation and vascular remodeling. In particular, the cell proliferation due to 5-HT might contribute to neomuscularization of nonmuscular pulmonary vessels, which occurs as a consequence of prolonged hypoxic exposure.4,5

An important finding of the present study was that the 5-HTT gene was expressed in pulmonary vessels of rats exposed to chronic hypoxia. Our in situ hybridization studies confirmed that the 5-HTT transcript is predominantly located in the media of newly remodeled distal pulmonary arteries in pulmonary hypertensive rats. Interestingly, RT-PCR allowed no detection of basal 5-HTT expression in proximal pulmonary arteries of control normoxic rats, whereas 5-HTT transcripts could be easily detected in the same tissues of hypoxic rats. The absence of 5-HTT mRNA in SMCs from proximal pulmonary arteries of normoxic rats is at variance with results obtained in cultured cells from the same tissue showing expression of 5-HTT mRNA under basal conditions. These data suggest that in vivo PA-SMCs do not phenotypically express 5-HTT under normoxic conditions and that induction occurs in response to hypoxia. In that case, one would infer that 5-HT may behave as a mitogenic factor for PA-SMCs only during conditions of increased 5-HTT mRNA expression. Previous in vivo studies performed in our laboratories are consistent with this hypothesis.4 Indeed, we recently observed that 5-HT promoted the development of pulmonary hypertension when infused continuously in rats during a 2-week exposure to 10% O2.4 Thus, rats treated with 5-HT demonstrated a greater degree of pulmonary hypertension, right ventricular hypertrophy, and structural distal pulmonary artery remodeling than saline-treated rats exposed to a similar level of hypoxia. In contrast, no effect was seen in normoxic rats subjected to the same infusion of 5-HT. Moreover, Fawn-Hooded rats, which have a deficit in platelet 5-HT storage and increased plasma 5-HT levels, developed pulmonary hypertension when they were raised at the altitude of Denver, Colo (an

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Figure 5. In situ hybridization histochemistry of 5-HTT mRNA performed with a specific antisense 35S-labeled riboprobe on lung sections from a normoxic (left) and a hypoxic rat (right). In the rat that had been maintained under hypoxic conditions, the hybridization signal was observed in both endothelial and smooth muscle cell and was more marked than in the normoxic rat. The autoradiograms illustrate the data obtained in 4 independent experiments.
environment with a mild reduction in oxygen tension), whereas Sprague-Dawley rats exhibited normal pulmonary artery pressure under the same conditions. Interestingly, exposure of Fawn-Hooded rats to a moderately O2-enriched environment prevented the development of pulmonary hypertension at the altitude of Denver. It is also noteworthy that treatment of hypoxic rats with dexfenfluramine, an inhibitor of 5-HTT transport, prevented the exacerbation of pulmonary hypertension normally expected from the drug-induced increase in plasma 5-HT levels. These findings strongly suggest that 5-HT may act as a potent mitogenic factor in vivo, leading to pulmonary vascular remodeling and pulmonary hypertension, provided that 5-HTT is active in PA-SMCs.

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