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Functional Interactions between 5-Hydroxytryptamine Receptors and the Serotonin Transporter in Pulmonary Arteries

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ABSTRACT

Pulmonary arterial 5-hydroxytryptamine (serotonin) (5-HT) transporter (SERT)-, 5-HT receptor expression, and 5-HT-induced vasoconstriction can be increased in pulmonary hypertension. These variables were studied in normoxic and hypoxic Fawn-Hooded (FH) and Sprague-Dawley (SD) rats. Furthermore, we compared the functional effects of SERT inhibitors and 5-HT receptor antagonists against 5-HT-induced vasoconstriction of pulmonary arteries. SERT and 5-HT_{1B} expression was greater in FH rat lungs than in SD rats, as was 5-HT-mediated vasoconstriction. The 5-HT_{2A} receptor antagonist ketanserin and the 5-HT_{1B} receptor antagonist SB224289 (1′-methyl-5-[[2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydro-spiro-[furo] 2, 3-*f*]indole-3,4′-piperidine]) inhibited responses to 5-HT in all vessels. The combined 5-HT_{1B} receptor/SERT antagonist LY393558 (1-[2-[4-(6-fluoro-1*H*-indol-3-yl)-3,6-dihydro-1(2*H*)-pyridinyl]ethyl]-3-isopropyl-6-(methylsulfonyl)-3,4-dihydro-1*H*-2,1,3-benzothiadiazine-2,2-dioxide) was the most potent inhibitor of constriction in all vessels. SERT inhibitors citalopram and fluoxetine inhibited responses to 5-HT in SD vessels. However, these inhibitors potentiated responses to 5-HT in FH vessels. After exposure of rats to 2 weeks of hypoxia, there was increased 5-HT-mediated vasoconstriction and a profound decrease in SERT expression in both the FH and SD rat lung. Accordingly, citalopram had no effect on 5-HT-induced constriction in SD rat vessels and markedly less effect in FH rat vessels. Ketanserin, SB224289, and LY393558 inhibited responses to 5-HT in all hypoxic rat vessels. LY393558 was the most potent antagonist, and there was synergy between the effects of fluoxetine and SB224289 when given simultaneously. The results suggest that, in FH rats, SERT inhibitors may increase pulmonary vasoconstriction, but this can be inhibited by simultaneous 5-HT_{1B} receptor antagonism. There is synergy between the inhibitory effects of 5-HT_{1B} receptor antagonists and SERT inhibitors on 5-HT-induced pulmonary vasoconstriction.

Pulmonary arterial hypertension (PAH) is characterized by sustained elevation in pulmonary artery pressure. Familial PAH can be related to heterozygous germline mutations in the gene encoding the bone morphogenetic protein type II receptor and/or polymorphisms in the gene encoding the 5-hydroxytryptamine (serotonin) (5-HT) transporter (SERT) (Lane et al., 2000; Eddahibi et al., 2001). Idiopathic PAH has no demonstrable cause and PAH can also occur secondary to many cardiorespiratory disorders. Regardless of the type of PAH, the elevated pulmonary vascular resistance is associated with remodeling of muscular pulmonary arteries and arterioles that exhibit smooth muscle proliferation, medial hypertrophy, and fibrosis (Fishman, 1998).

SERT mRNA is elevated in platelets from patients with PAH (Eddahibi et al., 2001). A polymorphism with long and short forms (Lesch et al., 1996) affects SERT function with the long allele inducing an increased rate of SERT gene transcription. The SERT polymorphism can also predict the severity of PAH in patients with chronic obstructive pulmonary disease (Eddahibi et al., 2003). Hence, it has been hypothesized that inhibitors of SERT may be useful in the treatment of PAH. One mechanism of action of SERT inhibitors treating clinical depression, however, is to cause an extracellular accumulation of 5-HT and increased 5-HT receptor activation (Slattery et al., 2004). The possibility that

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ABBREVIATIONS: PAH, pulmonary arterial hypertension; 5-HT, 5-hydroxytryptamine (serotonin); SERT, serotonin transporter; FH, Fawn-Hooded; SD, Sprague-Dawley; RVP, right ventricular pressure; RV, right ventricle/right ventricular; SB224289, 1′-methyl-5-[[2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydro-spiro[furo[2,3-*f*]indole-3,4′-piperidine]; LY393558, 1-[2-[4-(6-fluoro-1*H*-indol-3-yl)-3,6-dihydro-1(2*H*)-pyridinyl]ethyl]-3-isopropyl-6-(methylsulfonyl)-3,4-dihydro-1*H*-2,1,3-benzothiadiazine-2,2-dioxide.

SERT inhibitors may similarly increase 5-HT activation in pulmonary arteries requires investigation. It is the 5-HT_{1B} and 5-HT_{2A} receptors that mediate contraction of human, rat, and mouse pulmonary arteries (MacLean et al., 1996a,b; Morecroft et al., 1999; Keegan et al., 2001). Very recently, Liu et al. (2004) hypothesized that SERT and 5-HT_{1B} receptor activity interact and cooperate to effect pulmonary artery smooth muscle cell growth, suggesting that combined block would be the optimal therapeutic approach in PAH. Here, we also examine whether SERT and 5-HT_{1B} receptor activities interact or cooperate to affect contractile responses.

The Fawn-Hooded (FH) rat is more sensitive to hypoxia-induced PAH than its Sprague-Dawley (SD) controls. The FH rat has been studied as a model of human PAH because it has altered serotonergic function, an inherited storage defect to serotonin, increased circulating levels of 5-HT (Sato et al., 1992; Fujimori et al., 1998), and increased pulmonary vascular responsiveness to 5-HT (Ashmore et al., 1991). These are all factors observed in human PAH (MacLean et al., 2000). The FH rat also demonstrates increased lung endothelin-1 (Stelzner et al., 1992), and elevated circulating levels of endothelin-1 have also been reported in patients with PAH (Stewart et al., 1991). There is an increase in SERT activity in the brains of the FH rat (Hulihan-Giblin et al., 1993), although, surprisingly, expression of the SERT in the lung has not yet been studied.

Because treatment of PAH with 5-HT_{2A} antagonists results in systemic hypotension (Domenighetti et al., 1997), this is unlikely to be a future therapeutic approach, and hence we chose to examine the synergistic effects of the 5-HT_{1B} and SERT in this study. We examined the effects of LY393558, because this is both a SERT inhibitor (K_i of ~1 nM) and a 5-HT_{1B} receptor antagonist with a K_i value at 5-HT_{1B} receptors of ~1 nM (Mitchell et al., 2001; Pullar et al., 2001). We also studied the combination of fluoxetine and SB224289 in vitro. SB224289 is a selective 5-HT_{1B} receptor antagonist with a K_i value of ~10 nM (Price et al., 1997; Roberts et al., 1997). SB224289 has no reported affinity for SERT sites. To investigate the role of the 5-HT_{2A} receptor, we studied ketanserin, which has a 10,000-fold selectivity for the 5-HT_{2A} receptor over the 5-HT_{1B} receptor (K_i value at 5-HT_{2A} receptors of ~1 to 10 nM; Bard et al., 1996). We compared the effects of citalopram and fluoxetine to compare two SERT inhibitors with different pharmacological profiles. Citalopram is an extremely selective SERT inhibitor with a K_i value of ~1.8 nM, with no reported affinity for 5-HT receptors. Fluoxetine has a K_i value at SERT sites of ~0.9 to 2 nM, but it also has a relatively high affinity against the 5-HT_{2A} receptor (K_i of ~140 nM) (Owens et al., 1997).

In this study, we wished to test the following hypotheses. 1) SERT inhibitors may potentiate contractile responses to 5-HT. 2) In the presence of SERT inhibitors, contractile responses will be inhibited by coadministration of a 5-HT_{1B} receptor antagonist, i.e., the actions of SERT and receptor inhibitors synergize to inhibit 5-HT-induced contractile responses. 3) The effects of SERT and 5-HT_{1B} receptor antagonism will be modified i) in the FH rat, which exhibits increased SERT expression; and ii) after hypoxic exposure, which inhibits SERT expression.

Materials and Methods

Isolated Vessel Study

All animal care and procedures were in accordance with institutional and international guidelines. FH rats (and control SD rats) were studied (body weights were 250–350 g). They were killed by sodium pentobarbitone (200 mg kg^{-1}), and the lungs were removed. Small pulmonary arteries of ~250 μm i.d. were dissected and set up using wire myography as described previously (MacLean et al., 1996b; Keegan et al., 2001). Control vessels were set up at tensions equivalent to their mean in vivo right ventricular pressure (RVP; 15–20 mm Hg), whereas hypoxic rat vessels were set up at tensions equivalent to the elevated in vivo mean pressures observed after exposure to hypoxia (30–35 mm Hg). After a 45-min equilibration period, the response to 50 mM KCl was determined, the concentration that produced maximal contraction in these vessels. Cumulative response curves to 5-HT (1 nM–0.1 mM; Sigma Chemical, Poole, Dorset, UK) were constructed. One curve to 5-HT was constructed either in the absence or presence of inhibitor in each vessel. All inhibitors were allowed a 45-min equilibrium period before constructing the curves to 5-HT.

Exposure to Hypoxia

FH and SD rats (30–33 days old) were maintained in hypoxic conditions (equivalent to 10% O_2, 0.3% CO_2 balance N_2) in a hypobaric chamber for 2 week as described previously (MacLean et al., 1996b; Keegan et al., 2001).

Ex Vivo Assessment of PHT

Measurement of Right Ventricular Hypertrophy. Right ventricular hypertrophy is assessed as right ventricular (RV) weight divided by left ventricular plus septal weight. Because the body weight of the age-matched SD and FH rats was significantly different, we corrected this ratio for body weight (grams).

Pulmonary Artery Remodeling. The percentage of remodeled vessels (<50 μm in diameter) was assessed by measuring vessels with a double elastic lamina and expressing this as a percentage of vessels examined.

In Vivo Measurements

Rats were premedicated with an intraperitoneal injection of fentanyl (0.315 mg ml^{-1})/fluanizone (10 mg ml^{-1}) (Hypnorm; Janssen Pharmaceuticals, Antwerp, Belgium), 0.9 to 1.1 ml kg^{-1}, and midazolam (0.5 mg kg^{-1}). The rats were placed on a thermostatically controlled pad and fitted with a rectal thermometer. Anesthesia was maintained via a face mask with a mixture of nitrous oxide, oxygen (1:1 ratio) and 1% halothane. Systemic blood pressure was monitored through a 3-French i.v. cannula (Portex Ltd., Hythe, UK) inserted into the ascending aorta via the right carotid artery. The RV was catheterized through the right external jugular vein and right atria using a 3-French catheter. The catheter position within the RV was confirmed by the morphology of the pressure trace. Basal RV pressure and systemic blood pressures were made after a period of stabilization using an Elcomatic E751A pressure transducer connected to an MP100 data acquisition system (BIOPAC Systems Inc., Santa Barbara, CA). Heart rate was derived from the pressure traces. Results were analyzed using the built-in software package (Acq*Knowledge* 3.5, BIOPAC Systems, Inc., Santa Barbara, CA).

Quantification of SERT Expression by Radioligand Binding and TaqMan Reverse Transcriptase-Polymerase Chain Reaction

We have established, using immunohistochemistry, that SERT expression is almost entirely selective to the pulmonary arteries, with no significant expression being observed on other structures in the lung in FH or SD rats, either before or after hypoxic exposure (see Fig. 5G for illustration). Because it is the small pulmonary

arteries that are of primary interest in PAH, and it is not possible to dissect these out due to their size and fragility, we chose to look at whole lung in the knowledge that this reflected pulmonary artery expression.

Radioligand Binding

Membrane Preparation. Lungs were washed and pulverized under liquid nitrogen and resuspended in assay buffer: 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, and 10 mM $MgCl_2$, containing 500 μg/ml soybean trypsin inhibitor, 10 mM benzamidine, 1 μg/ml leupeptin, bacitracin, pepstatin A, and antipain, and 10% glycerol, pH 7.4. After homogenization, homogenate was filtered and centrifuged at 1200*g* for 5 min at 4°C. The supernatant fraction was centrifuged twice at 56,000*g* for 30 min at 4°C, and resulting membrane pellet resuspended in Tris-HCl buffer and homogenized. Protein estimation was by Pierce protein assay kit (Pierce Chemical, Tattenhall, UK).

Saturation Binding Studies. Saturation studies were performed with membranes (25 μg/ml) incubated in duplicate with [^{3}H]citalopram (83 Ci/mmol) (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) (0.025–20 nM) in Tris-HCl assay buffer. For each assay, membranes were prepared from $n = 8$ to 10 lungs. The reaction mixture was incubated in a final volume of 0.5 ml for 60 min at 22°C for the measurement of total binding. Nonspecific binding was defined in the presence of 10 μM fluoxetine-hydrochloride (Tocris Cookson Inc., Bristol, UK). The reaction was terminated using a Brandel cell harvester, and bound [^{3}H]citalopram was separated from free by vacuum filtration over Whatman GF/C filters

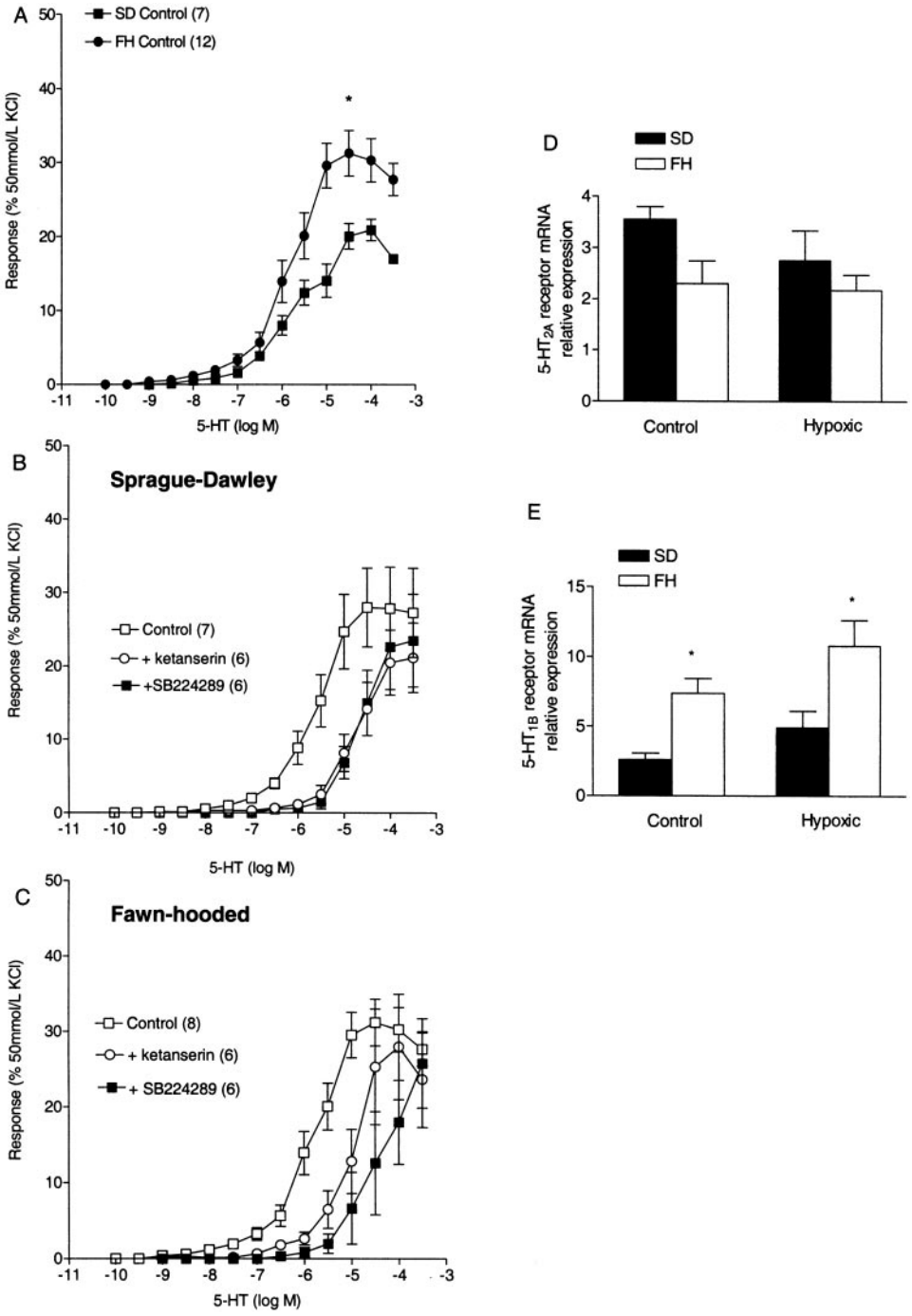

Fig. 1. Contractile responses to 5-HT in pulmonary resistance arteries from FH and SD rats. A, responses in FH and SD vessels. B, effect of the 5-HT_{2A} receptor antagonist ketanserin (10 nM) and the 5-HT_{1B} receptor antagonist SB224289 (200 nM) in SD rat vessels. C, effect of ketanserin and SB224289 in FH rat vessels. All responses (A–C) are expressed as percentage of a response to 50 mM KCl in each vessel, and the number of animals is in parentheses. D, relative expression of lung 5-HT_{2A} receptor mRNA in lungs ($n = 4$) from control and hypoxic SD and FH rats. E, relative expression of lung 5-HT_{1B} receptor mRNA in lungs ($n = 4$) from control and hypoxic SD and FH rats. Statistical analysis by one-way analysis of variance and Newman-Keuls multiple comparisons test is shown for the maximum response in FH versus SD vessels. *, $P < 0.05$. All data are expressed as mean ± S.E. Full statistical analysis is detailed in Table 1.

(Whatman, Maidstone, UK). Binding isotherms were analyzed by a nonlinear least square parametric curve-fitting program GraphPad Prism (GraphPad Software Inc., San Diego, CA), to derive a dissociation constant (K_D) and receptor number (B_{max}).

Quantitative mRNA Expression. After extraction of total RNA from whole lung using TRIzol reagent (Invitrogen, Carlsbad, CA), real-time fluorogenic reverse transcriptase-polymerase chain reaction was performed using Assays on Demand gene expression probes for rat SERT, 5-HT_{2A}, and 5-HT_{1B} receptor (Rn00564737, Rn00568473, and Rn00573666, respectively; Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Relative mRNA abundance was determined using the comparative delta CT method using 18S ribosomal RNA as internal control.

Immunohistochemistry

Immunohistochemistry was performed to identify the location of SERT and to confirm that the inhibition of SERT expression and binding after hypoxia could be observed at the protein level.

Paraffin sections (5 μm in thickness) were mounted on poly-L-lysine slides. Slides were dewaxed in Histoclear, and sections were rehydrated by immersion in ethanol (100, 95, and 70%) and then in distilled water. Antigen retrieval was carried out by microwaving in 10 mM citric acid buffer, pH 6.0. Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 30 min. After two washes in PBS, the sections were preincubated in PBS supplemented with 0.5% bovine serum albumin, 10% normal horse serum for 1 h. Endogenous biotin was blocked using an avadin/biotin blocking kit (Vector Laboratories, Petersborough, UK) and then incubated overnight with goat polyclonal anti-SERT antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:50 in PBS containing 0.5% bovine serum albumin, 15% normal horse serum. The sections were exposed for 1 h to biotin-labeled anti-goat secondary antibodies (Vector Laboratories), diluted 1:100 in PBS, and then transferred to streptavidin/biotin horseradish peroxidase solution. Peroxidase staining was carried out using 3′3′-diaminobenzidine tetrahydrochloride dihydrate and hydrogen peroxide. Finally, the sections were stained with hematoxylin.

Analysis

Unless otherwise stated, statistical comparisons were made by one-way analysis of variance, and differences ($P < 0.05$) were established using the Newman-Keuls multiple comparisons test. Statistical analysis was always carried out between test groups and controls simultaneously as shown in figures and not against pooled data sets as shown in tables. Only when maximal responses were obtained were pEC_{50}, B_{max}, and apparent pK_B values calculated. Apparent pK_B values were calculated according to the equation $pK_B = \log (DR - 1) - \log[B]$, where DR is the ratio of the mean EC_{50} value in the presence of antagonist to the mean EC_{50} value in the absence of antagonist for a particular agonist.

Results

Contractile Responses to 5-HT in Normoxic Animals

5-HT induced a small contractile response in adult SD and FH rats, and the responses were of a greater magnitude in the FH rats (Fig. 1A; Table 1).

Effect of Selective 5-HT Antagonists. In the SD rat and the FH rat vessels, both ketanserin and SB224289 inhibited responses to 5-HT. In the SD rat, the value for the pK_B of SB224289 was 7.65 ± 0.20, consistent with an effect at contractile 5-HT_{1B} receptors (pK_B in FH rats was not calculated because no maximum response was achieved). The pK_B value for ketanserin was 8.86 ± 0.20 and 8.20 ± 0.20 in SD and FH rat vessels, respectively, consistent with an effect at contractile 5-HT_{2A} (Fig. 1, B and C; Table 1).

TABLE 1
pEC_{50} and E_{max} values for 5-HT-induced vasoconstriction in FH and SD rat pulmonary resistance arteries: effect of the 5-HT receptor inhibitors ketanserin (5-HT_{2A}, 10 nM) and SB224289 (5-HT_{1B}, 200 nM)
All data are expressed as mean ± S.E, with n representing number of animals.

	pEC_{50}	E_{max}	n
SD rat			
Control	5.50 ± 0.19	20.9 ± 1.4	7
Control + ketanserin	4.75 ± 0.16**	22.5 ± 2.7	6
Control + SB224289	4.6 ± 0.16**	24.6 ± 3.0	6
Hypoxic	6.71 ± 0.08***	75.7 ± 6.4***	8
Hypoxic + ketanserin	5.64 ± 0.16‡‡‡	57.5 ± 7.8	6
Hypoxic + SB224289	5.43 ± 0.08‡‡‡	71.5 ± 8.5	6
FH rat			
Control	5.98 ± 0.15	31.8 ± 3.0†	15
Control + ketanserin	5.02 ± 0.16***	27.7 ± 7.0	6
Control + SB224289	N.M.	N.M.	6
Hypoxic	6.39 ± 0.10**	132 ± 10***	10
Hypoxic + ketanserin	5.37 ± 0.10‡‡‡	144 ± 12	7
Hypoxic + SB224289	4.91 ± 0.15‡‡‡	115 ± 20	6

N.M., no maximum response achieved.
† $P < 0.05$ versus SD control; ** $P < 0.01$; *** $P < 0.001$ versus own control; ‡‡‡ $P < 0.001$ versus own hypoxic control group.

Receptor mRNA Expression. In both the normoxic and hypoxic rats, there was a higher magnitude of 5-HT_{1B} receptor mRNA expression in lung tissue from the FH rats compared with in SD. No difference in 5-HT_{2A} receptor expression was detected (Fig. 1, D and E). There was no significant increase in the expression of either receptor after exposure to hypoxia. The mRNA relative expression data for the 5-HT_{2A} receptor, shown in Fig. 1D, are as follows ($n = 4$): control SD, 3.55 ± 0.25; control FH, 2.3 ± 0.44; hypoxic SD, 2.75 ± 0.58; and hypoxic FH, 2.17 ± 0.3. The mRNA relative expression data for the 5-HT_{1B} receptor, shown in Fig. 1E, are as follows ($n = 4$): control SD, 2.5 ± 0.49; control FH, 7.38 ± 1.06; hypoxic SD, 4.9 ± 1.21; and hypoxic FH, 10.79 ± 1.83.

Effects of SERT Inhibitors. Figure 2A illustrates the effects of the SERT inhibitors on responses to 5-HT in SD control rats. These data are summarized and statistically analyzed in Table 2. Although 0.1 μM fluoxetine had no effect, 1 μM inhibited responses to 5-HT. Citalopram also inhibited responses to 5-HT. LY393558 had the most potent inhibitory effect at both 10 and 100 nM.

Because LY393558 inhibits both the transporter and 5-HT_{1B} receptor, the profound inhibitory effect of LY393558 suggested a synergy between these two. To verify this, we conducted separate experiments in SD rat vessels and examined the effects of fluoxetine alone and SB224289 alone and then examined the effects of the two antagonists when applied together at the same concentrations. The concentration of SB224289 selective for the 5-HT_{1B} receptor was chosen (200 nM), and we selected a concentration of fluoxetine that had no inhibitory effect on its own (0.1 μM). The results are shown in Fig. 2B. It can be seen that the effects of the two combined are greater than the added effects of the two given separately, suggesting a synergistic interaction. The pEC_{50} values for the 5-HT control, SB224289 alone, fluoxetine alone, and SB224289 plus fluoxetine were 5.56 ± 0.13, 4.74 ± 0.33, 5.72 ± 0.16, and 5.03 ± 0.5, respectively, and the E_{max} values (expressed as percentage of response of contraction to 50 mM KCl) were 30 ± 4, 22 ± 5, 34 ± 9, and 10 ± 4 ($P < 0.05$ versus 5-HT control). The response to KCl was not affected by hypoxia in either FH or SD rat vessels. Figure 2C illustrates the effects of the 5-HT transport inhibitors on responses to

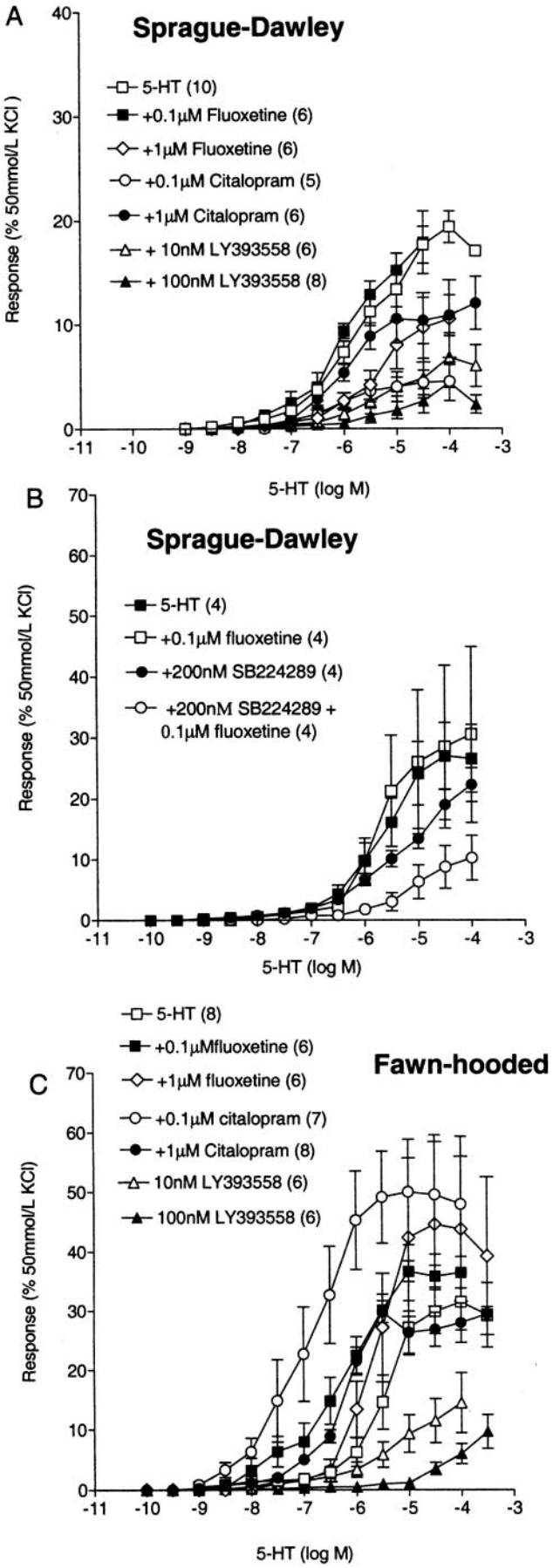

TABLE 2

pEC_{50} and E_{max} values for 5-HT-induced vasoconstriction in FH and SD rat pulmonary resistance arteries: effect of SERT inhibitors

All data are expressed as mean ± S.E, with n representing number of animals.

	pEC_{50}	E_{max}	n
SD rat			
Control	5.67 ± 0.1	21.0 ± 1.0	10
Control + 0.1 μM citalopram	6.15 ± 0.26	4.5 ± 0.6***	5
Control + 1 μM citalopram	6.00 ± 0.17	11.3 ± 0.7***	6
Control + 0.1 μM fluoxetine	5.90 ± 0.19	17.8 ± 3.0	6
Control + 1 μM fluoxetine	5.36 ± 0.27	11.0 ± 1.5***	6
Control + 10 nM LY393558	5.10 ± 0.05	7.2 ± 2.2***	6
Control + 100 nM LY393558	5.00 ± 0.5	3.9 ± 2.2***	8
Hypoxic	6.71 ± 0.08***	75.7 ± 6.4***	8
Hypoxic + 0.1 μM fluoxetine	6.55 ± 0.10	90.0 ± 8.3	6
Hypoxic + 1 μM fluoxetine	5.73 ± 0.06‡‡‡	68.0 ± 4.9	6
Hypoxic + 0.1 μM citalopram	6.69 ± 0.10	90.2 ± 5.2	6
Hypoxic + 1 μM citalopram	6.48 ± 0.07	85.7 ± 7.3	6
Hypoxic + 10 nM LY393558	5.76 ± 0.20‡‡‡	39.3 ± 8.9‡‡‡	7
Hypoxic + 100 nM LY393558	4.73 ± 0.14‡‡‡	21.2 ± 4.9‡‡‡	6
FH rat			
Control	5.47 ± 0.11	31.5 ± 0.11†	8
Control + 0.1 μM citalopram	6.91 ± 0.27**	50.0 ± 9.0	7
Control + 1 μM citalopram	6.30 ± 0.07*	26.6 ± 4.1	8
Control + 0.1 μM fluoxetine	6.24 ± 0.13*	35.8 ± 1.8	6
Control + 1 μM fluoxetine	5.71 ± 0.19	44.6 ± 13.8	6
Control + 10 nM LY393558	N.M.	N.M.	6
Control + 100 nM LY393558	N.M.	N.M.	6
Hypoxic	6.34 ± 0.08	126 ± 8.5	11
Hypoxic + 0.1 μM fluoxetine	6.62 ± 0.15	125 ± 18	5
Hypoxic + 1 μM fluoxetine	6.02 ± 0.10	90 ± 10	6
Hypoxic + 0.1 μM citalopram	6.84 ± 0.14	123 ± 17	5
Hypoxic + 1 μM citalopram	7.00 ± 0.14‡	124 ± 12	5
Hypoxic + 10 nM LY393558	5.88 ± 0.18	87 ± 13‡	6
Hypoxic + 100 nM LY393558	5.31 ± 0.30‡‡‡	64 ± 12‡‡	6

N.M., no maximum response achieved.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus own control; † $P < 0.05$ versus SD control; ‡ $P < 0.05$; ‡‡ $P < 0.01$; ‡‡‡ $P < 0.001$ versus own hypoxic control group.

5-HT in FH rats. These data are summarized and statistically analyzed in Table 2. Unlike the inhibitory effect in the SD rat, citalopram actually increased the response to 5-HT in the FH rat vessels (Fig. 2C). Fluoxetine (0.1 μM) also increased the potency of 5-HT. LY393558 inhibited the responses to 5-HT in a dose-dependent manner.

Hemodynamics and the Effects of Chronic Hypoxia

RV remodeling was elevated in the FH rat compared with the SD rat, although there was no significant difference in the percentage of remodeled vessels or RV pressure (Table 3). When exposed to hypoxia, the FH rats developed a greater degree of RV hypertrophy compared with the SD controls (Table 3). Hypoxia induced a greater degree of remodeling in the FH rats versus the SD rats (Table 3). Mean systemic arterial pressure was the same in all groups of rats as was heart rate. Although RV pressure was elevated by ~2-fold in hypoxic FH and SD rats, the degree of elevation was the same in the SD and FH rat groups (Table 3).

Contractile Responses to 5-HT in Hypoxic Animals

There was a 3-fold increase in the contractile response to 5-HT in the SD rats after exposure to hypoxia. The affinity

Fig. 2. Effect of SERT inhibitors on contractile responses to 5-HT in pulmonary resistance arteries. A, effects in SD rat pulmonary arteries. B, effects of combining fluoxetine and the 5-HT_{1B} receptor antagonist SB224289 in SD vessels. C, effects in Fawn-Hooded rat pulmonary arteries. All responses (A–C) are expressed as percentage of a response to 50 mM KCl in each vessel, and the number of animals is in parentheses. Statistical analysis is detailed in Table 2. All data are expressed as mean ± S.E.

TABLE 3
Body weight, vascular and right ventricular remodeling, and hemodynamics in SD and FH rats: effects of 2-week hypoxia
All data are expressed as mean ± S.E., with *n* representing number of animals.

	SD Normoxic	FH Normoxic	SD Hypoxic	FH Hypoxic	*n*
Body weight (g)	363 ± 11	255 ± 5†††	297 ± 8***	251 ± 4†††	7–9
Percentage of remodeled arteries	2.4 ± 2.4	6.8 ± 0.7	21.8 ± 1.2***	30.4 ± 3.2***††	4–5
[RV/LV + S]/body weight ($\times 10^{-4}$)	7.11 ± 0.54	10.72 ± 0.54†	15.98 ± 1.52***	21.94 ± 1.15***††	7–9
Heart rate (beats/min)	379 ± 18	351 ± 12	426 ± 14	392 ± 12	7–9
Systolic RVP (mm Hg)	26.2 ± 3.0	33.2 ± 2.2	57.8 ± 3.0***	62.4 ± 2.8***	7–9
Mean SAP (mm Hg)	90.4 ± 9.5	79.3 ± 2.3	91.4 ± 5.1	72.4 ± 1.6	7–9

RV/LV + S, right ventricular/left ventricular plus septal weight (grams); SAP, systemic arterial pressure.
*** $P < 0.001$ versus own normoxic control; † $P < 0.05$; †† $P < 0.01$; ††† $P < 0.001$ versus SD normoxic/hypoxic.

for 5-HT was also increased (Fig. 3A; Table 1). Both ketanserin and SB224289 significantly inhibited the response to 5-HT in the hypoxic vessels (Fig. 3A; Table 1). The value for the pK_B of SB224289 was 8.16 ± 0.20, and the pK_B for ketanserin was 9.03 ± 0.26, consistent with an effect at 5-HT$_{1B}$ and 5-HT$_{2A}$ receptors, respectively. In the FH rat, there was an even greater (4-fold) increase in the maximum response to 5-HT after hypoxic exposure as well as an increase in affinity (Fig. 3B; Table 1). Expression of 5-HT$_{1B}$ receptor mRNA was higher in the hypoxic FH lung compared with the hypoxic SD lung (Fig. 1E). Both ketanserin and SB224289 inhibited responses to 5-HT in these vessels. The pK_B of ketanserin was 8.2 ± 0.2. The pK_B of SB224289 was 8.16 ± 0.2.

Effects of SERT Inhibitors. The effects of the 5-HT transport inhibitors on responses to 5-HT in hypoxic SD rat vessels are shown on Fig. 3C and summarized in Table 2. Citalopram had no effect on responses to 5-HT. Fluoxetine inhibited responses at 1 μM. LY393558 inhibited the responses to 5-HT in a dose-dependent manner.

The effects of the 5-HT transport inhibitors on responses to 5-HT in hypoxic FH rat vessels are shown on Fig. 3D and summarized in Table 2. Citalopram potentiated the potency of 5-HT but only at 1 μM. Fluoxetine did not have an effect on

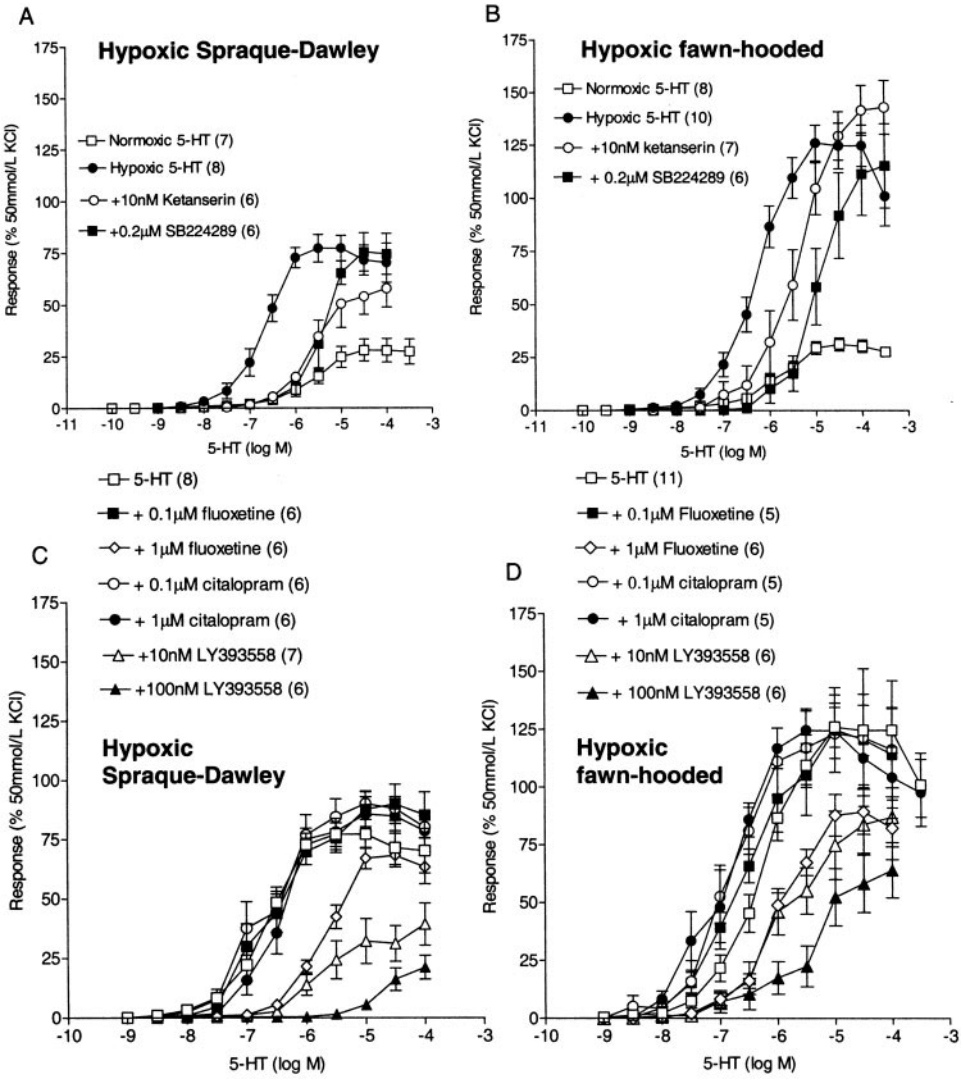

Fig. 3. Effects of hypoxia on responses to 5-HT in rat pulmonary arteries. Effect of 2-week hypoxic exposure in vivo on responses to 5-HT (hypoxic 5-HT) compared with responses from normoxic rats (normoxic 5-HT): effect of the 5-HT$_{2A}$ receptor antagonist ketanserin (10 nM) and the 5-HT$_{1B}$ receptor antagonist SB224289 (200 nM) in hypoxic SD rat vessels (A) and hypoxic Fawn-Hooded rat vessels (B); effect of SERT inhibitors in hypoxic SD rat vessels (C) and hypoxic FH rat vessels (D). All data are expressed as mean ± S.E. Statistical analysis is detailed in Tables 1 and 2.

responsiveness to 5-HT. LY393558 inhibited the maximum responses to 5-HT in a dose-dependent manner.

Quantification of SERT Expression

SERT Binding Sites. Figure 4A illustrates that lung [^{3}H]citalopram binding was higher in the FH rat than in the SD rat. The affinity for [^{3}H]citalopram was not altered; the K_D value for [^{3}H]citalopram binding was 0.77 ± 0.26 (SD) and 0.89 ± 0.17 (FH). After 2 weeks of hypoxia, however, specific binding was not detectable in either the FH or SD rat lungs.

SERT mRNA Expression. Consistent with the immunohistochemistry and the binding studies, there was greater SERT mRNA expression in the FH rats compared with their controls and a marked inhibition after exposure to hypoxia (Fig. 4B).

Localization of SERT and Changes with Hypoxia. SERT immunoreactivity was noted in the SD rat pulmonary arteries, especially at the medial/adventitial border (Fig. 5A). Immunoreactive staining was more widespread in the pulmonary arteries of the FH rats, extending into the medial layer itself (Fig. 5B). Staining was negligible in the vessels from the FH hypoxic rats (Fig. 5C), and there was no visible SERT staining in the SD hypoxic vessels (Fig. 5F). Figure 5G shows that SERT immunoreactivity was exclusive to the pulmonary arteries, with negligible immunoreactivity visible in other lung structures, including the airways. The example shown is from the SD rat but the FH rat exhibited the same distribution of SERT immunoreactivity.

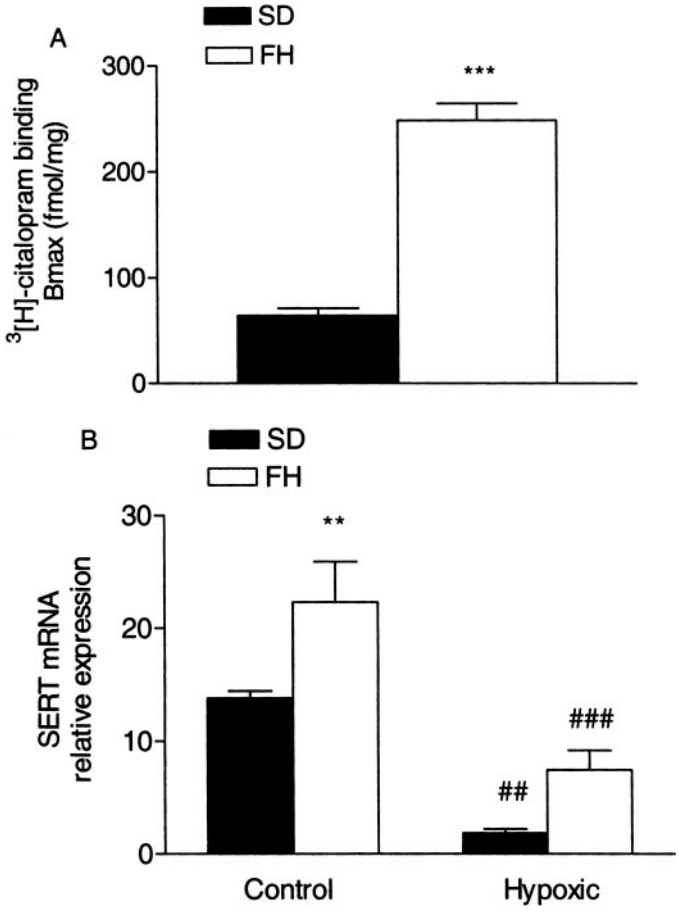

Fig. 4. SERT expression in the FH rat lung compared with SD rat lung and the effects of 2-week exposure to hypoxia. A, [^{3}H]citalopram binding in lungs (n = 4) (binding was absent in lungs from hypoxic SD and FH rats). B, relative expression of lung SERT mRNA in lungs (n = 4). All data are expressed as mean ± S.E. Statistical analysis was made by one-way analysis of variance and Newman-Keuls multiple comparison test. **, $P < 0.01$ and ***, $P < 0.001$ versus SD vessels; ##, $P < 0.01$ and ###, P versus control vessels.

Discussion

Previous studies have indicated that there is higher SERT ligand binding in various regions of the FH rat brain compared with control rat strains (Hulihan-Giblin et al., 1993; Chen and Lawrence, 2000). Here, we have shown that expression of SERT mRNA and [^{3}H]citalopram binding sites was higher in the FH rat lung compared with the SD rat lung controls. When sections of lung were examined, SERT immunoreactivity was seen to be concentrated in the pulmonary arteries, with negligible immunoreactivity observed in other lung structures. In SD rat pulmonary arteries, immunoreactive SERT was localized to the cells at the medial/adventitial border, which is a similar distribution to that we have previously described in mouse (MacLean et al., 2004). However, in FH rat pulmonary arteries, the immunoreactivity extended further into the medial layer. SERT immunoreactivity is also concentrated in the pulmonary arteries in human, and this is increased in patients with both primary and secondary PAH. In these patients, SERT immunoreactivity is distributed throughout the medial layer of pulmonary arteries (Eddahibi et al., 2001, 2003). Hence, with respect to SERT expression, the blood vessels from the FH rat provide a good model in which to study the effects of SERT expression on pulmonary vascular contractile responses.

Compared with the SD rats, there was evidence of RV hypertrophy. After a 2-week exposure to hypoxia, the FH rats developed a greater degree of PAH as indicated by RV hypertrophy and increased pulmonary vascular remodeling. This is consistent with previous studies (Ashmore et al., 1991; Sato et al., 1992; Stelzner et al., 1992; Fujimori et al., 1998). We show here that the development of hypoxia-induced PAH in both the SD and FH rat was associated with a decrease in the expression of lung SERT as determined by TaqMan reverse transcriptase-polymerase chain reaction, ligand binding, and immunohistochemistry. In the hypoxic SD rat lungs, SERT expression was almost completely absent, whereas expression of SERT mRNA and SERT immunoreactivity was evident in FH rat vessels but markedly reduced. We have recently described a mouse that overexpresses the gene for human SERT, and it also is similarly predisposed to hypoxia-induced PAH. There is also a decrease in SERT expression after hypoxic exposure in these mice (MacLean et al., 2004). The current study confirms that this was not a phenomenon only observed in mice and suggests that there is dissociation between SERT activity and PAH caused by hypoxic exposure. Mice overexpressing SERT developed spontaneous increases in RVP (MacLean et al., 2004). Although this was not the case for the FH rat, there was evidence of increased RV weight. Animal and clinical studies all suggest, therefore, that overexpression of the SERT cause a predisposition to PAH be it familial or secondary to hypoxic exposure. Consistent with this, SERT knockout in mice protects against hypoxia-induced PAH (Eddahibi et al., 2000). Mouse and rat hypoxic models suggest, however, that persistence of

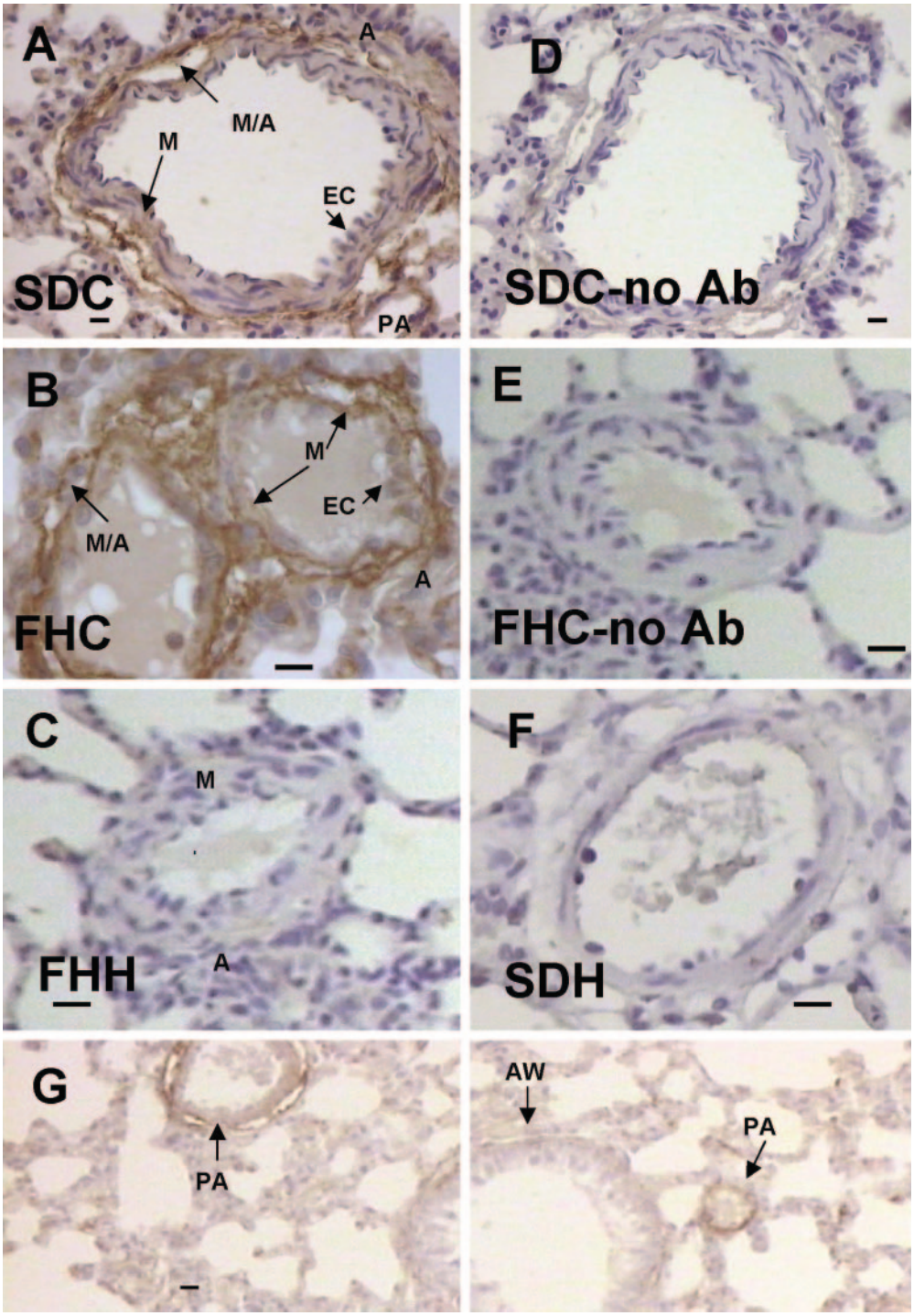

Fig. 5. Immunohistochemical localization of the SERT in small pulmonary arteries from Sprague-Dawley control rat (SDC) (A), Fawn-Hooded control rat (FHC) (B), Fawn-Hooded rat after 2-week hypoxia (FHH) (C), and Sprague-Dawley rat after 2-week hypoxia (SDH) (F). D and E, SDC and FHC control sections incubated with secondary but not primary antibody. G, SERT immunoreactivity in SDC lung shown to be concentrated in pulmonary arteries. Scale bars, 10 μm (A–F), 25 μm (G). A, adventitia; AW, airway; EC, endothelial cell layer; M, medial layer; M/A, medial/adventitial border; PA, pulmonary artery.

SERT overexpression is not required for the progression of hypoxia-induced PAH (MacLean et al., 2004; this study).

5-HT causes pulmonary artery smooth muscle cell proliferation in a SERT-dependent manner (Fanburg and Lee, 2000; Eddahibi et al., 2001). However, one mechanism of action of SERT inhibitors against clinical depression is an increase in extracellular accumulation of 5-HT (Slattery et al., 2004). If this occurred at pulmonary arteries, then such elevations in 5-HT might cause increased pulmonary arterial vasoconstriction. Therefore, we wished to examine the effects of SERT inhibitors on contractile response to 5-HT in isolated pulmonary arteries.

All the SERT inhibitors reduced the contractile response to 5-HT in SD rat vessels. However, we show that in FH rat vessels, where the 5-HT_{1B} receptor is overexpressed, citalopram (0.1 and 1 μM) and fluoxetine (0.1 μM) actually increased the potency of 5-HT. The effects of citalopram were, surprisingly, not concentration-dependent. This suggests either that citalopram is not as specific to the SERT as previously reported or that SERT activity affects vasoconstriction via more than one mechanism. There is more evidence for the latter, given that SERT activity can affect the 5-HT available for receptor activation (Slattery et al., 2004), and the amount of 5-HT passing into the cell may also affect 5-HT activation

through superoxide production (Lee et al., 1999; Liu and Folz, 2004). The higher concentration of fluoxetine (1 μM) inhibited 5-HT contraction in the FH rat vessels. This is likely to be due to fluoxetine inhibiting the 5-HT_{2A} receptor at this concentration (K_i against 5-HT_{2A} receptor, ~0.14 μM; Owens et al., 1997) or inhibiting calcium sensitivity/uptake (Ungvari et al., 1999, 2000). As well as direct effects on 5-HT receptors, chronic treatment with SERT inhibitors may also result in up-or down-regulation of 5-HT receptors (Gray and Roth, 2001). For example, chronic fluoxetine can alter 5-HT_{2A} receptor signaling (Damjanoska et al., 2003), increase 5-HT_{1A} receptors (Hirano et al., 2002), and both up- and down-regulate the 5-HT_{2B} receptor (Kong et al., 2002). If SERT inhibitors are to be used for the treatment of PAH, these effects are worthy of consideration.

It required a combined block of both the SERT and the 5-HT_{1B} receptor with LY393558 to inhibit the contractile response to 5-HT in the FH rat vessels. In addition, we demonstrated a synergistic interaction between fluoxetine and SB224289 in SD rat vessels. Neither drug alone had an effect on the maximum response to 5-HT, but together, they inhibited this by ~60%. The 5-HT_{1B} receptor is expressed in the pulmonary arteries of patients with PAH (Launey et al., 2002; Marcos et al., 2004). In these patients, expression is shown either to increase (Launey et al., 2002) or remain constant (Marcos et al., 2004). Regardless of whether 5-HT_{1B} receptor expression is increased, its cooperativity with the SERT in affecting both proliferation (Liu et al., 2004) and constriction (this study) indicates that maximum clinical effect may be gained by blocking both the 5-HT_{1B} receptor and SERT.

So why did citalopram and fluoxetine potentiate responses to 5-HT in FH rat vessels? Our working hypothesis is that, by inhibiting 5-HT reuptake, more 5-HT is accessible to stimulate 5-HT receptors. This would explain the requirement to block the receptors simultaneously to confer inhibition of 5-HT-induced vasoconstriction as with LY393558 or higher concentrations of fluoxetine. Consistent with this, hypoxia both reduced SERT expression and increased 5-HT-induced vasoconstriction, mimicking the effects of citalopram and fluoxetine in the FH rat vessels. The vasoconstriction was increased most in the FH rats where there was enhanced 5-HT_{1B} receptor expression. The reduced SERT expression would mean that less 5-HT was removed from the 5-HT receptor sites.

We demonstrate that SERT expression is higher in lungs from the FH rat than the SD rat, yet contractile responses to 5-HT were greatest in the FH rat vessels. Hence, the higher SERT activity, which could remove 5-HT away from the receptors, must be overridden by receptor activation. Analysis of 5-HT_{1B} and 5-HT_{2A} expression confirmed that both receptors were present in the lungs of FH and SD rats. Accordingly, both ketanserin and SB224289 inhibited responses to 5-HT in FH and SD vessels. Lung 5-HT_{1B} receptor expression was, however, 3-fold higher in the FH rat. It is possible that the increased 5-HT_{1B} receptor stimulation overcame any effects of higher SERT expression. In addition, we have previously shown that increased levels of ET-1 can increase 5-HT_1 receptor activation in rat pulmonary arteries (MacLean and Morecroft, 2000). There is increased lung ET-1 production in FH rats (Stelzner et al., 1992). Hence, this may also have increased 5-HT_{1B} receptor activation and contributed to the increased response to 5-HT observed in the FH rat vessels.

Consistent with our observation that hypoxia had no significant effect on either 5-HT_{1B} or 5-HT_{2A} receptor expression, both SB224289 and ketanserin inhibited responses to 5-HT in hypoxic SD and FH rats with similar pK_B values to those observed in normoxic controls. In the absence of SERT sites in the hypoxic SD vessels, citalopram had no effect. Fluoxetine blocked the response to 5-HT, but again, only at the higher concentration that would inhibit the 5-HT_{2A} receptor. LY393558 was again the most potent antagonist. Citalopram only slightly potentiated 5-HT potency consistent with a reduction in SERT expression. Again, LY393558 inhibited the maximum response to and potency of 5-HT in a dose-dependent manner.

In summary, we have shown that SERT and 5-HT_{1B} expression are higher in FH rat lung than in SD rat lung. The contractile response of pulmonary arteries to 5-HT is increased in the FH rat, probably due to increased 5-HT_{1B}-mediated constriction. 5-HT-mediated constriction is enhanced in hypoxic SD and FH vessels, and this is associated with a decreased expression of SERT. The results also suggest that 1) SERT inhibitors can potentiate contractile responses to 5-HT when the 5-HT_{1B} receptor is overexpressed, and 2) this potentiation is due to inhibition of 5-HT uptake increasing the 5-HT available to activate the increased number of 5-HT receptors. An important additional finding is that combined 5-HT_{1B} and SERT inhibition synergize in blocking 5-HT-mediated constriction. The addition of 5-HT_{1B} antagonism prevents the increased 5-HT-mediated vasoconstriction induced by SERT inhibitors.

Acknowledgments

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