0026-895X/00/010075-07$3.00/0

MOLECULAR PHARMACOLOGY, **57:**75–81 (2000).

ACCELERATED COMMUNICATION

Possible Role of Valvular Serotonin 5-HT_{2B} Receptors in the Cardiopathy Associated with Fenfluramine

LAWRENCE W. FITZGERALD, TIMOTHY C. BURN, BARRY S. BROWN, JOHN P. PATTERSON, MARTHA H. CORJAY, PATRICIA A. VALENTINE, JUNG-HUI SUN, JOHN R. LINK, ILGAR ABBASZADE, JEANNINE M. HOLLIS, BRIAN L. LARGENT, PAUL R. HARTIG, GREGORY F. HOLLIS, PAUL C. MEUNIER, ALBERT J. ROBICHAUD, and DAVID W. ROBERTSON

CNS Diseases Research (L.W.F., B.S.B., J.P.P., P.A.V., B.L.L., P.R.H.), Chemical and Physical Sciences (J.-H.S., A.J.R., D.W.R.), Applied Biotechnology (T.C.B., J.R.L., I.A., J.M.H., G.F.H.), and Department of Safety Assessment (P.C.M.), Cardiovascular Diseases Research (M.H.C.), The DuPont Pharmaceuticals Research Laboratories, Experimental Station, Wilmington, Delaware

Received August 20, 1999; accepted September 30, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

Dexfenfluramine was approved in the United States for long-term use as an appetite suppressant until it was reported to be associated with valvular heart disease. The valvular changes (myofibroblast proliferation) are histopathologically indistinguishable from those observed in carcinoid disease or after long-term exposure to 5-hydroxytryptamine (5-$HT)_2$-preferring ergot drugs (ergotamine, methysergide). 5-HT_2 receptor stimulation is known to cause fibroblast mitogenesis, which could contribute to this lesion. To elucidate the mechanism of "fen-phen"-associated valvular lesions, we examined the interaction of fenfluramine and its metabolite norfenfluramine with 5-HT_2 receptor subtypes and examined the expression of these receptors in human and porcine heart valves. Fenfluramine binds weakly to 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. In contrast, norfenfluramine exhibited high affinity for 5-HT_{2B} and 5-HT_{2C} receptors and more moderate affinity for 5-HT_{2A} receptors. In cells expressing recombinant 5-HT_{2B} receptors, norfenfluramine potently stimulated the hydrolysis of inositol phosphates, increased intracellular Ca^{2+}, and activated the mitogen-activated protein kinase cascade, the latter of which has been linked to mitogenic actions of the 5-HT_{2B} receptor. The level of 5-HT_{2B} and 5-HT_{2A} receptor transcripts in heart valves was at least 300-fold higher than the levels of 5-HT_{2C} receptor transcript, which were barely detectable. We propose that preferential stimulation of valvular 5-HT_{2B} receptors by norfenfluramine, ergot drugs, or 5-HT released from carcinoid tumors (with or without accompanying 5-HT_{2A} receptor activation) may contribute to valvular fibroplasia in humans.

Appetite suppressant medications have been used worldwide for decades for the treatment of obesity. Interest in pharmacological approaches to obesity has been largely driven by an increased cultural pressure for dieting, an ever-increasing fraction of individuals defined as obese, the identification of a causal relationship between obesity and cardiovascular disease and diabetes, and the recognition that nonpharmacological treatments alone have limited efficacy. One of the most widely prescribed anorectic agents was fenfluramine either alone or in combination with the noradrenergic drug phentermine ("fen-phen"). These agents were combined clinically with the presumption that the resulting reduction in the daily dosing of either drug alone would mitigate untoward side effects while maintaining clinical efficacy. In 1996, the United States approved the use of the *d*-isomer of fenfluramine, dexfenfluramine, for chronic use in the long-term management of obesity. However, this decision was largely rescinded in 1997 when the Food and Drug Administration issued a public health advisory indicating that 33 women who had taken fenfluramine and phentermine in combination had unusual heart valve morphology and regurgitation (Connolly et al., 1997). These observations were histopathologically identical with the fibroplasia seen in carcinoid (with accompanying 5-HT-secreting tumors) or ergotamine-induced valve disease (Connolly et al., 1997; Kulke and Mayer, 1999). Later studies showed that although

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; HEK 293E cells, human embryonic kidney 293 Epstein-Barr nuclear antigen cells; DMEM, Dulbecco's modified Eagle's medium; IA, intrinsic activity, PCR, polymerase chain reaction; PI, phoshoinositide, 5-CT, 5-carboxymidotryptamine, MAP, mitogen-activating protein; FLIPR, fluorescence image plate reader.

the incidence of valve defects may not be as great as initially thought, it appears to be a threat with extended use (>3 mon) of fenfluramine alone or in combination with phentermine (Wee et al., 1998).

In contrast to its well documented 5-hydroxytryptamine (5-HT)-releasing properties, other pharmacological properties of fenfluramine and its metabolite norfenfluramine, particularly regarding their interactions with postsynaptic human 5-HT receptors, are still poorly defined (Curzon et al. 1997). Furthermore, except for a presumptive involvement of 5-HT, no specific mechanisms have been proposed for fenfluramine- and ergot-related cardiopathy. Because the 5-HT_2 receptor subfamily plays a prominent role in the feeding, cardiovascular, and mitogenic effects of 5-HT, we examined the interaction of fenfluramine, norfenfluramine, and ergot drugs with 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. We also measured the mRNA expression levels of these receptor subtypes in porcine and human heart valves.

Experimental Procedures

Materials. [^{125}I](1-(4-iodo-2,5-Dimethoxyphenyl)-2-aminopropane (2200 Ci/mmol) was purchased from New England Nuclear (Boston, MA). *myo*-[2-^{3}H]Inositol (15–20 Ci/mmol), [^{3}H]5-HT (122 Ci/mmol), and [^{3}H]carboxymidotryptamine (5-CT; 57 Ci/mmol) were purchased from Pharmacia Amersham (Arlington Heights, IL). Electrophoresis reagents were purchased from Novex (San Diego, CA). Chirally pure isomers, *d* and *l*, of norfenfluramine were synthesized by the Department of Chemical and Physical Sciences at the DuPont Pharmaceuticals Co. Most cell culture supplies were purchased from Life Technologies (Grand Island, NY). All other reagents were purchased from Research Biochemicals Inc. (Natick, MA) or Sigma Chemical Co. (St. Louis, Mo) unless otherwise noted. Stable cell lines were generated by transfecting human embryonic kidney 293 Epstein-Barr nuclear antigen cells (HEK 293E) cells with episomal plasmids containing human 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, or 5-HT_{2C} (VNV edited isoform) cDNA using calcium phosphate as described previously (Fitzgerald et al., 1999).

Radioligand Binding Studies. Radioligand binding assays were conducted in disposable polypropylene 96-well plates (Costar Corp., Cambridge, MA) as described previously (Fitzgerald et al., 1999). The 5-HT_{2A}, 5-HT_{2C}, 5-HT_{1E}, and 5-HT_{1F} assays were initiated by the addition of membrane homogenate in tissue buffer to assay buffer (50 mM Tris · HCl, 0.5 mM EDTA, 10 mM pargyline, 10 mM $MgSO_4$, 0.05% ascorbic acid, pH 7.5) containing [^{125}I](1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane (0.3–0.5 nM final, for the 5-HT_{2A} and 5-HT_{2C} assays) or [^{3}H]5-HT (2–10 nM final, for the 5-HT_{1E} and 5-HT_{1F} assays) with or without competing ligand. The 5-HT_{2B} and 5-HT_{1D} assays were conducted similarly except for the substitution of 4 mM $CaCl_2$ for the $MgSO_4$ and the use of [^{3}H]*d*-lysergic acid (2–2.5 nM) or [^{3}H]5-CT (1–2 nM) as the radioligands, respectively. Apparent dissociation constants (K_i values) from the competition experiments were calculated using an iterative nonlinear regression curve-fitting program (Prism; GraphPAD Software, San Diego, CA).

Phosphoinositide (PI) Hydrolysis studies. The ability of ligands to stimulate PI hydrolysis was monitored in whole cells expressing recombinant 5-HT receptors as described previously (Fitzgerald et al., 1999). Cells were treated with 0.5 μCi/well *myo*-[^{3}H]inositol for 16 to 18 h, washed with serum/inositol-free Dulbecco's modified Eagle's medium (DMEM) containing 10 mM LiCl and 10 μM pargyline, and incubated for 30 min with the same medium but now containing test compound. Reactions were terminated, and [^{3}H]phosphoinositides were extracted and then separated by anion exchange chromatography as described previously (Fitzgerald et al., 1999). EC_{50} values were determined by nonlinear regression analysis with Prism (GraphPAD). E_{max} (maximal response) was derived from the fitted curve maxima for each compound. Intrinsic activity (IA) was determined by expressing the E_{max} of a compound as a percentage of the E_{max} of 5-HT (IA = 1.0).

Measurement of Ca^{2+}-Evoked Fluorescence by Fluorescence Imaging Plate Reader (FLIPR). The ability of norfenfluramine to increase intracellular Ca^{2+} was assessed by FLIPR. Cells expressing recombinant 5-HT receptors were seeded onto poly(D-lysine)-coated, 96-well plates (Costar Corp., Cambridge, MA) and incubated overnight or until near confluence was established. Growth medium was then replaced with dye loading buffer (Hanks' solution) containing 20 mM HEPES, 4 μM Fluo-3 (Molecular Probes, Eugene, OR), and 0.04% (w/v) pluronic acid (Molecular Probes) for 60 min at 37°C. Dye buffer was removed and replaced four times with a dye- and pluronic acid-free Hanks solution maintained at 37°C. Cell and drug plates were then placed in a 96-well FLIPR (Molecular Devices, Sunnyvale, CA) and equilibrated for 10 to 15 min at 37°C. Fluorescence readings were taken for 10 s before and 4 min after the agonist addition. Each drug plate contained 6 wells of 3 μM 5-HT that served as an internal standard for the determination of IA.

Measurement of Agonist-Activated Mitogen-Activating Protein (MAP) Kinase Signaling. The ability of ligands to acutely activate (phosphorylate) MAP (Erk1/Erk2) kinase was examined by Western blotting in cells expressing human recombinant 5-HT receptors. Cells were grown to 75% confluency in DMEM containing 10% dialyzed FCS in 100-mm dishes. Cells were serum-starved overnight, and the medium was aspirated and then incubated for 5 min with serum-free DMEM containing test compound. Cells were washed with PBS containing 1 mM orthovanidate, lysed (with 125 mM Tris, 2% SDS, 5% glycerol, 1 mM orthovanidate, and 10 μg/ml aprotinin, leupeptin, and pepstatin), and then sonicated. Lysates (2 μg protein) were boiled, loaded onto 4 to 12% acrylamide gels, electrophoresed, and transferred onto nitrocellulose membranes according to the manufacturer's instructions (Novex, San Diego, CA). Blots were blocked in PBS-Tween (0.05% v/v) containing 2% nonfat milk for 45 min and then incubated overnight (4°C) with antidiphosphorylated MAP kinase antibody (diluted 1:10,000; Sigma Chemical Co.). After extensive washing (2 h), blots were incubated with secondary antibody (Vector Laboratories, Burlingame, CA; 90 min), washed again in PBS-Tween (2 h), and detected by enhanced chemiluminescence (New England Nuclear).

Expression of 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} mRNA in Porcine and Human Heart Valves. Real-time polymerase chain reaction (PCR) was performed essentially as described (Gibson et al., 1996). Sequences for pig 5-HT_{2A} (Ullmer et al., 1995), 5-HT_{2B} (Ullmer et al., 1995), and GAPDH (accession number AF017079) were obtained from GenBank. Sequences for the 5-HT_{2C} receptor were obtained by reverse transcription-PCR using rat primers, with the resulting products being cloned and sequenced. Primers and probes were designed for 5-HT_{2A} (primers: TGCCCCTTCTTCATCACCA and CCGATGACATCCTCGTTGC, probe: TCATGGCCGTCATCTGCAAAGAGTCC), 5-HT_{2B} (primers: GCCATTTCAGTGGATCGTTACATA and AATGCTGTAGCTCGTGAGTTATATTGA, probe: CCATCAAAAGCCAATCCAGGCCA), 5-HT_{2C} (primers: GCACTTCAGGAAATCCAGGC and ATGCTCCTGCGCGGC, probe: TCCCGGCGGTTCCTCGGTG), and GAPDH (primers: GCAATGCCTCCTGTACCACC and TGCCGAAGTTGTCATGGATG, probe: ACTGCTTGGCACCCCTGGCC). All probes were labeled at the 5′ end with the reporter dye 6-FAM and on the 3′ end with the quencher dye TAMRA (Perkin-Elmer/Applied Biosystems, Foster City, CA).

Total RNA was isolated from pig or human aortic valves and mitral valves as described (Davis et al., 1986). Then, 1 μg of total RNA was treated with RNase-free DNase I (Life Technologies, Gaithersburg, MD) and reverse transcribed essentially as described (Huang et al., 1996) with primers and Moloney murine leukemia virus (Clontech, Palo Alto, CA) being added after the DNase I treat-

ment. Each PCR used the cDNA from 100 ng of starting RNA. Valves were obtained from six pigs. The individual leaflets of the aortic valves were combined and pooled for the first three pigs, with data from the entire valve being analyzed. For the three remaining pigs, the individual leaflets of the aortic valves were combined from the three pigs, with the data from the left, right, and middle valve pools being compared. Copy numbers were calculated by comparing the threshold values for each reaction with a standard curve produced using linearized cDNA for the respective genes essentially as described previously (Gibson et al., 1996). The Molecular Probes PicoGreen assay was used to measure the concentration of the linearized DNAs before use in the standard curves as described by the manufacturer. Copy numbers obtained from "no reverse transcriptase" controls were subtracted from the copy numbers obtained for the experimental samples. Expression levels were normalized with GAPDH. All expression levels are relative, because the initial efficiency of the reverse transcription reaction was not accounted for in the copy number calculations.

Results

Radioligand binding experiments were conducted to evaluate the affinities of fenfluramine and norfenfluramine for human 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors expressed in HEK 293E cells. The *d*- and *l*-isomers of fenfluramine demonstrated weak affinities ($K_i > 0.7$–1.5 μM) for all three 5-HT receptor subtypes (see Table 1). In contrast, the isomers of norfenfluramine were most potent at the 5-HT$_{2B}$ (27 and 65 nM, *d*- and *l*-, respectively) and 5-HT$_{2C}$ (56 and 99 nM) receptors and less potent at 5-HT$_{2A}$ (187 and 267 nM) receptors. Phentermine was found to be inactive at these receptors at concentrations up to 10 μM.

In vitro functional assays were then used to assess ligand efficacy and potency. We examined the ability of norfenfluramine to hydrolyze inositol phosphates (IP) and mobilize intracellular Ca^{2+} in whole cells. Both isomers of norfenfluramine were potent high efficacy partial-to-full agonists with respect to their ability to stimulate IP production and increase intracellular Ca^{2+}-evoked fluorescence (Table 2 and Fig. 1). As observed in the binding studies, norfenfluramine was most potent at the 5-HT$_{2B}$ receptor, moderately potent at the 5-HT$_{2C}$ receptor, and least potent at the 5-HT$_{2A}$ receptor. Furthermore, although the *d*-isomer of norfenfluramine was consistently more potent than the *l*-isomer, their kinetics of receptor activation (see Fig. 1B) were identical. The isomers of the parent compound, fenfluramine, also behaved as agonists on these measures, but their weak activity precluded an accurate assessment of potency and efficacy (data not shown).

Because the lesion reported in human valves is fibroplasia, we assessed whether norfenfluramine would activate the MAP kinase (Erk1/2) pathway, a proliferative/mitogenic pathway linked to tyrosine kinase and G protein-coupled receptor activation, including the 5-HT$_{2B}$ receptor (Launay et al., 1996; Lopez-Ilasaca, 1998). Cells were stimulated with 1 μM 5-HT or *d*-norfenfluramine, lysed, and tested for phospho-MAP kinase by Western blotting. This stimulation time (10 min) was shown in a preliminary experiment to elicit maximal levels of activated enzyme (data not shown). Like 5-HT, *d*-norfenfluramine increased the levels of immunoreactive phospho-MAP kinase via all three 5-HT$_2$ receptor subtypes (Fig. 2).

Last, because the long-term use of ergotamine and methysergide in humans has been associated with a risk for valvular effects similar to those of fenfluramine, we examined their binding affinities and functional properties at human 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors. Ergotamine (10.9, 28.4, and 19 nM), methysergide (6.8, 0.7, and 1.2 nM), and the principal desmethyl metabolite of methysergide, methylergonovine (1.0, 1.2, and 9.0 nM), possess significant affinity (K_i) for all three 5-HT$_2$ subtypes (mean K_i values at 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors, respectively; $n = 2$). IP hydrolysis assays revealed that methysergide is a silent antagonist and that ergotamine is a partial agonist (IA = 70–90% of 5-HT) at 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors. Methylergonovine was found to be a partial agonist at the 5-HT$_{2B}$ and 5-HT$_{2A}$ receptors (40 and 20% of 5-HT, respectively) but an antagonist at 5-HT$_{2C}$ receptors. Because ergots are also known to exert potent vascular effects via 5-HT$_{1D\text{-like}}$ receptors, we examined the interactions of methysergide, ergotamine, and methylergonovine at human 5-HT$_{1D}$, 5-HT$_{1E}$, and 5-HT$_{1F}$ receptors. Although we confirmed that ergotamine, methylergonovine, and methysergide can show high-to-moderate affinity for these receptors, *d*-norfenfluramine is comparatively weak (K_i = 5–10 μM).

We next used real-time PCR to quantify transcript levels of the 5-HT$_2$ subfamily of receptors in porcine and human aortic and mitral valves. The relative expression levels of the genes were compared within tissues by determining the number of copies present in the starting cDNA relative to the standard curve. We observed 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor mRNA levels that were significantly higher than 5-HT$_{2C}$ receptor levels in oligo(dT) primed cDNA from both aortic and mitral valves (Fig. 3). After normalizing between experiments with GAPDH levels, we observed between 13 and 20 copies of 5-HT$_{2C}$ receptor transcript in 100 ng of total RNA from aortic and mitral valves relative to plasmid-based standard curves. In aortic valves, 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor transcripts

TABLE 1

Norfenfluramine, but not the parent molecule fenfluramine, potently binds to the human 5-HT$_{2B}$ receptor and other members of the 5-HT$_2$ receptor subfamily

Competition experiments were conducted as described in *Materials and Methods*. Depicted are mean ± S.E. K_i values of enantiomers of fenfluramine and norfenfluramine versus [^{125}I]DOI for the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor or [^{3}H]LSD binding for the 5-HT$_{2B}$ receptor (from three to six independent experiments).

Receptor Subtype	K_i			
	d-Fenfluramine	*l*-Fenfluramine	*d*-Norfenfluramine	*l*-Norfenfluramine
	nM			
5-HT$_{2A}$	2470 ± 240	1430 ± 330	187 ± 10	267 ± 16
5-HT$_{2C}$	2080 ± 480	1620 ± 340	56 ± 19	99 ± 12
5-HT$_{2B}$	3920 ± 830	680 ± 16	27 ± 7	65 ± 23

were expressed at 757- and 375-fold higher levels than 5-HT_{2C} levels, respectively. Similarly in mitral valves, 5-HT_{2A} and 5-HT_{2B} were expressed at 440- and 360-fold higher levels than 5-HT_{2C} levels. We also compared data for the left, right, and middle leaflets of the aortic valves and saw no differences in 5-HT receptor expression among the different leaflets. We repeated the real-time PCRs using random-primed and gene-specific primed cDNA to assure ourselves that oligo(dT) priming did not bias our results. The relative expression patterns were consistent between datasets. When compared with 5-HT_{2C} receptor expression, 5-HT_{2A} and 5-HT_{2B} receptors were shown to be expressed at approximately 750 and 250 times higher levels in random primed aortic valve cDNAs. In mitral valve cDNA, 5-HT_{2A} and 5-HT_{2B} were expressed at levels that were 250- and 150-fold higher than 5-HT_{2C} levels. In a limited number of experiments using gene-specific primers for the reverse transcription, we obtained similar results. However, the limiting amount of RNA that we had available made the use of independent reverse transcription reactions for each amplification impractical. The 5-HT_{2C} primer/probe reagents efficiently detected more than 10^5 copies of 5-HT_{2C} in choroid plexus cDNA, with the assay being linear between 10 copies and 10^7 copies per PCR. Therefore, inefficiencies in the assay were not responsible for the low 5-HT_{2C} levels observed in the valve RNAs.

We repeated the real-time PCR experiments with RNA isolated from human valves that had been obtained from patients undergoing valve replacement. The human valves had high levels of calcification and yielded low amounts of poor quality RNA. However, the data obtained from human valves were consistent with that of the pig. We observed 200 to 700 copies of 5-HT_{2A} and 5-HT_{2B} transcripts in human cDNA samples, respectively, whereas 5-HT_{2C} levels were below the level of detection for the assay (data not shown).

TABLE 2

The enantiomers of norfenfluramine are potent agonists at the recombinant human 5-HT_{2B} receptor

Potencies (EC_{50} values) and efficacies [intrinsic activity (IA)] of fenfluramine and norfenfluramine at eliciting inositol phosphate (IP) hydrolysis and intracellular Ca^{2+} mobilization. Intrinsic activities were calculated by comparing net maximal stimulation of [^{3}H]IP hydrolysis or Ca^{2+}-mediated fluorescence by norfenfluramine with the net maximal effects elicited by the full agonist 5-HT at the human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. Mean potencies for 5-HT in stimulating IP production via human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors were 178.3, 39.4, and 21.7 nM, respectively. Mean potencies for 5-HT in stimulating Ca^{2+} mobilization via human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors were 30.9, 2.8, and 21.2 nM, respectively. Mean potencies and IAs (±S.E.) were derived from three to five independent experiments.

Functional Assay	EC_{50} (IA)	
	d-Norfenfluramine	*l*-Norfenfluramine
	nM	
5-HT_{2A} receptor		
IP hydrolysis	3,100 ± 330 (0.7 ± 0.02)	26,600 ± 4,290 (0.5 ± 0.01)
Ca^{2+} mobilization	720 ± 77 (0.8 ± 0.04)	1,990 ± 320 (0.4 ± 0.08)
5-HT_{2C} receptor		
IP hydrolysis	190 ± 16 (0.9 ± 0.03)	727 ± 45 (0.8 ± 0.05)
Ca^{2+} mobilization	300 ± 29 (0.9 ± 0.09)	995 ± 56 (0.8 ± 0.05)
5-HT_{2B} receptor		
IP hydrolysis	24 ± 3.7 (0.9 ± 0.01)	292 ± 50 (0.8 ± 0.02)
Ca^{2+} mobilization	23 ± 4 (1.0 ± 0.1)	239 ± 39 (0.7 ± 0.04)

Discussion

The goal of the present study was to examine the pharmacology of fenfluramine and its metabolite, norfenfluramine, with 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, a subfamily of

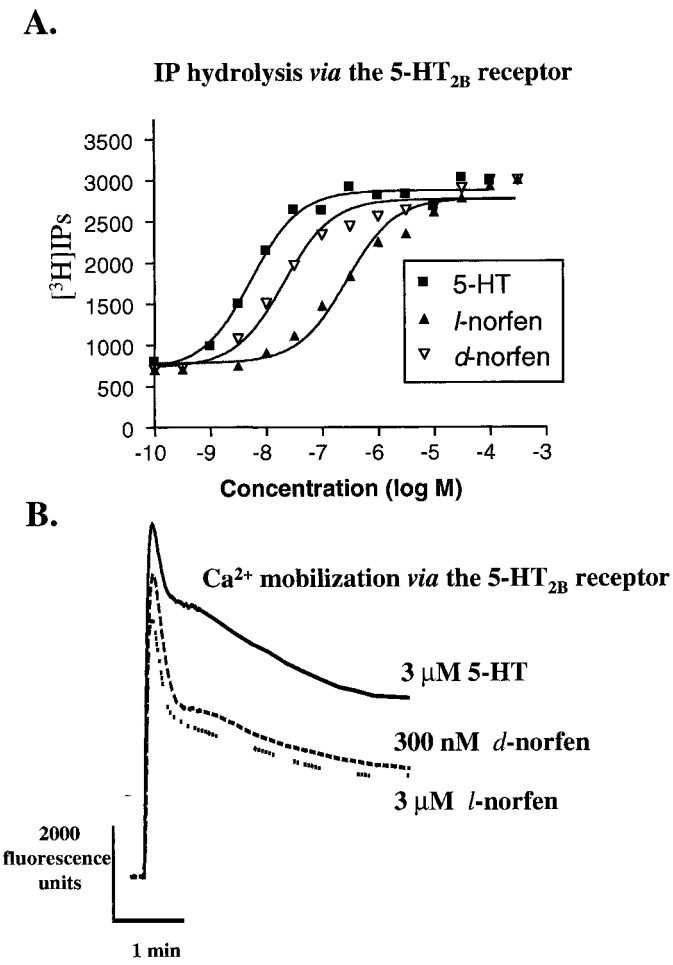

Fig. 1. A, norfenfluramine-mediated stimulation of PI hydrolysis in adherent HEK 293E cells expressing the human 5-HT_{2B} receptor. Cells were incubated with different concentrations of ligand for 30 min. The reactions were terminated, and inositol phosphates were isolated using anion exchange chromatography. B, comparison of the time course for calcium-induced fluorescence evoked by 5-HT, *d*-norfenfluramine, and *l*-norfenfluramine in HEK 293E cells expressing the 5-HT_{2B} receptor. Refer to Table 2 for a detailed presentation of the functional data. Each depicted curve or trace is representative of three experiments with similar results.

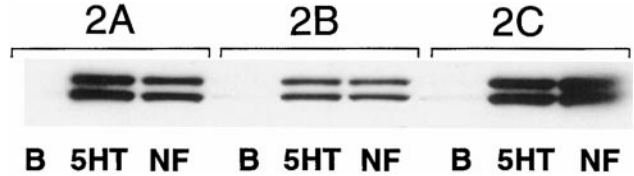

Fig. 2. Activation of the MAP kinase by 1 MM 5HT or norfenfluramine in cells expressing 5-HT_{2A}, 5-HT_{2B}, or 5-HT_{2C} receptors as assessed by Western blotting. Cells were treated with 5-HT or *d*-norfenfluramine (NF) for 10 min, lysed, and loaded onto SDS-polyacrylamide gels. Nitrocellulose filters were blotted for immunoreactive MAP kinase using a monoclonal antibody that recognizes the diphosphorylated (activated) form of MAP kinase.

5-HT receptors implicated in the feeding, cardiovascular, and mitogenic effects of 5-HT.

Radioligand binding experiments were first conducted to determine the affinities of fenfluramine and norfenfluramine for human recombinant 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors. The *d*- and *l*-stereoisomers of fenfluramine demonstrated weak affinities ($K_i > 0.7$–1.5 μM) for all three 5-HT receptor subtypes. In contrast, the isomers of norfenfluramine exhibited high-to-moderate affinity for 5-HT$_{2B}$ (27 and 65 nM, *d*- and *l*-isomer, respectively) and 5-HT$_{2C}$ receptors (56 and 99 nM) but lesser affinity for 5-HT$_{2A}$ receptors (187 and 267 nM). Based on reported steady-state blood concentrations of norfenfluramine after average clinical doses in humans (Caccia et al., 1985), norfenfluramine could sufficiently occupy 5-HT$_{2C}$ and 5-HT$_{2B}$ receptors and contribute to the pharmacological and toxicological actions of fenfluramine. Phentermine, the other component of the "fen-phen" combination, was found to be inactive at these receptors.

In vitro functional assays were used to assess ligand efficacy and potency. We examined the ability of norfenfluramine to stimulate hydrolysis of IP and the subsequent increase in intracellular Ca^{2+} in whole cells. Stereoisomers of norfenfluramine were potent agonists with respect to their ability to stimulate IP production and increase intracellular Ca^{2+}. Norfenfluramine was most potent at the 5-HT$_{2B}$ receptor, was moderately potent at the 5-HT$_{2C}$ receptor, but was very weak in activating the 5-HT$_{2A}$ receptor. We intentionally used lines expressing modest levels of receptor and exhibiting low receptor reserve. The intrinsic activities of other known 5-HT agonists (e.g., partial agonists, *m*-chlorophenylpiperazine and *d*-lysergic acid) at these receptors were shown previously to compare favorable with efficacies observed in native tissues (Fitzgerald et al., 1999).

Because the lesion underlying the cardiac valvular regurgitation in humans is fibroplasia (myofibroblast proliferation), we assessed whether norfenfluramine would activate the MAP kinase (ERK1/2) pathway, a mitogenic pathway linked to tyrosine kinase and G protein-coupled receptor activation (Lopez-Ilasaca, 1998). Like 5-HT, *d*-norfenfluramine increased the levels of immunoreactive phospho-MAP kinase via all three 5-HT$_2$ receptor subtypes. The proliferative potential of the 5-HT$_2$ subfamily of 5-HT receptors was demonstrated when it was shown that the 5-HT$_{2C}$ receptor could transform NIH3T3 fibroblasts (Julius et al., 1989). More recently, activation of the *ras*-MAP kinase pathway has been implicated in the mitogenic and transforming properties of the 5-HT$_{2B}$ receptor expressed at moderate densities in fibroblasts and endogenously in carcinoid tumors (Launay et al., 1996). The 5-HT$_{2B}$ receptor also mediates important trophic functions in cardiovascular morphogenesis. Embryonic expression of 5-HT$_{2B}$ predates that of the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors and is localized to the heart primordia and neural fold before neural tube closure (Choi et al., 1998).

Because fenfluramine-induced valvular changes are histopathologically identical with those seen after chronic exposure to 5-HT$_2$ receptor-preferring ergots (ergotamine, methysergide), we examined their affinities, along with that of the active metabolite of methysergide, methylergonovine, at the 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors. Methylergonovine concentrations in plasma have been reported to be substantially higher than the parent drug after the oral administration of methysergide; it is postulated to be the main contributor of methysergide efficacy and side effects (Muller-Schweinitzer and Tapparelli, 1986). All three ergot compounds exhibited high affinity for all three 5-HT$_2$ receptor subtypes (see also Kursar et al., 1992; Newton et al., 1996; Schmuck et al., 1996). IP hydrolysis assays revealed that methysergide is a silent antagonist and ergotamine is a partial agonist at 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors. Methylergonovine was found to be a partial agonist at the 5-HT$_{2B}$ and 5-HT$_{2A}$ receptors but an antagonist at 5-HT$_{2C}$ receptors.

Although these data collectively are consistent with the hypothesis that the valvular lesions are due to stimulation of myofibroblast mitogenesis via activation of 5-HT$_{2B}$ receptors, the interaction of norfenfluramine and ergots with other receptor subtypes may also contribute to these changes. Because ergots are known to exert potent vascular effects via 5-HT$_{1D}$-like receptors, we examined the interactions of methysergide, ergotamine, methylergonovine, and norfenfluramine at human 5-HT$_{1D}$, 5-HT$_{1E}$, and 5-HT$_{1F}$ receptors. We confirmed that although ergotamine, methylergonovine, and methysergide can exhibit high-to-moderate affinities for

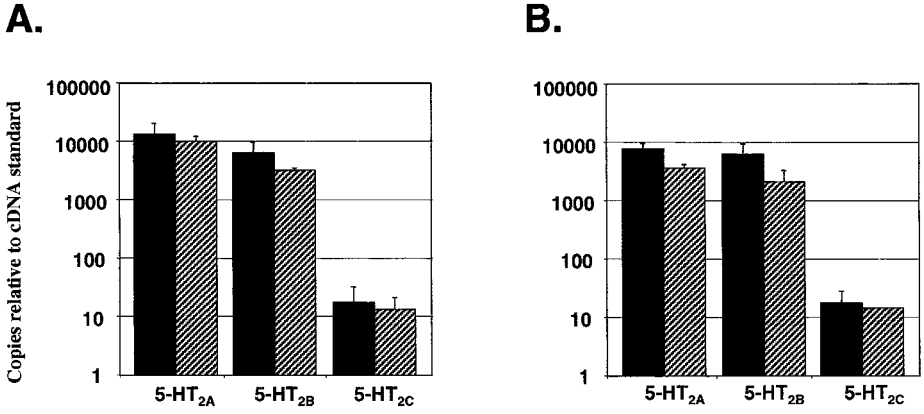

Fig. 3. Real-time PCR was performed as described in the test using oligo(dT) or random primed cDNA. Data are shown as mean relative copies (±S.E.) per 100 ng of starting RNA for aortic (A) and mitral (B) valves. Solid columns represent data from random primed cDNA experiments, and hatched columns denote oligo(dT) data. Expressions for 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors are shown as relative copy numbers because they were calculated directly from standard curves generated with cDNA clones.

these receptors, *d*-norfenfluramine is comparatively weak (K_i = 5–10 μM). This inactivity of norfenfluramine along with the observation that tryptamines used clinically for migraine (e.g., sumatriptan) interact with 5-HT_{1D}-like receptors (Johnson et al., 1998) but do not cause valve defects suggests that fenfluramine- and ergot-related valve defects are not mediated by a common 5-HT_{1D} mechanism. Furthermore, sumatriptan is also inactive (>10 μM) at 5-HT_{2B} receptors (Schmuck et al., 1996). Some ergot drugs (e.g., bromocriptine) can activate dopamine receptors, and are used chronically to treat Parkinson's disease and hyperprolactinemia. However, bromocriptine and dopamine receptor agonists in general have not been associated with valvular heart disease.

We next used real-time PCR to quantify mRNA levels of the 5-HT_2 subfamily of receptors in porcine aortic and mitral valves. 5-HT_{2A} and 5-HT_{2B} receptor mRNA levels were more than two orders of magnitude higher than 5-HT_{2C} receptor transcript levels in oligo(dT)-primed cDNA from both aortic and mitral valves. We repeated the real-time PCR amplifications using random-primed and gene-specific primed cDNA to ensure that oligo(dT) priming did not bias our results. The overall patterns of expression were consistent among the various methods: 5-HT_{2B} and 5-HT_{2A} transcripts were abundant relative to 5-HT_{2C} transcript levels, which were extremely low to undetectable. Determination of absolute copy numbers for each gene is difficult because we have no measure of the reverse transcription efficiency. However, even if we assume that it is as low as 1%, the expression levels of the 5-HT_{2C} receptor would be predicted to be less than one transcript per cell in both mitral and aortic valves. Real-time PCR experiments with RNA isolated from human valves that had been obtained from patients undergoing valve replacement yielded similar results. It is unclear what cell types reflect the 5-HT_2 receptor transcript expression pattern in the heart valves because RNA from whole tissues was used; future work using in situ hybridization or immunohistochemical techniques may help resolve this question.

The combined data suggest that the agonist interaction of norfenfluramine with 5-HT_{2B} receptors may contribute to the proliferative valvular heart valve disease seen with fenfluramine. For both fenfluramine- and methysergide-induced cardiomyopathy, our analysis suggests that the primary metabolite of these drugs may be responsible for the myofibroblast proliferation characteristic of this toxicity. Although all three 5-HT_2 receptor subtypes could trigger the cell proliferation characteristic of ergot and norfenfluramine cardiopathy, the 5-HT_{2B} receptor presents with the most compelling dataset for this role. The relative absence of 5-HT_{2C} receptor transcripts in heart valves and the complete lack of agonist activity by methysergide or its metabolite methylergonovine at the 5-HT_{2C} receptor argue against a role for this receptor subtype in the common valvular pathology caused by ergots and fenfluramine. In contrast, the 5-HT_{2A} receptor transcript is abundantly expressed in heart valves, and both ergots and norfenfluramine show moderate-to-potent affinity for this receptor subtype. Steady-state blood levels of free (plasma protein unbound) norfenfluramine (≈48 nM, assuming free concentration equates with levels at receptor sites) in humans (Caccia et al., 1985; Spinelli et al., 1988) suggest a far lesser role for 5-HT_{2A} receptors, because it exceeds the midpoint for activation of the 5-HT_{2B} receptor (i.e., ≈24 nM) although remaining well below levels (i.e., >700 nM) capable of significantly activating the 5-HT_{2A} receptor. However, this argument rests solely on in vitro potency differences at recombinant receptors. We cannot exclude a role for the 5-HT_{2A} receptor without a comprehensive pharmacological analysis (e.g., protein expression and reserve) of native receptors in heart valves. Characterization of valvular 5-HT_{2B} and 5-HT_{2A} receptors was judged impractical for the present study because of the number of pigs needed to obtain sufficient numbers of acutely isolated myofibroblasts in culture. Last, we cannot exclude the possibility that interactions with other molecular targets (known or orphan) are contributing factors. Nevertheless, the pharmacological arguments presented herein suggest that direct agonist actions of norfenfluramine at the 5-HT_{2B} receptor may contribute to this valvular lesion.

In summary, agonistic interactions of norfenfluramine with 5-HT_{2B} receptors, perhaps with modest 5-HT_{2A} receptor activation, may directly induce mitogenic activities responsible for the cell proliferation and the cardiotoxic effects of fenfluramine in humans. 5-HT_{2B} receptor activation could also contribute to explain the cardiopathy seen in carcinoid disease as well as after treatment with ergots. Corroborating studies are necessary to validate the mechanistic involvement of this receptor and perhaps identify other genetic and environmental factors that predispose certain individuals for this lesion. This understanding will ultimately lead to the design of safer medicines for future therapeutic use.

Acknowledgments

We thank Deborah Conklin, Carol Krause, Anne Marshall, Scott Knauer, and Margo Altaweel for excellent technical assistance. We also thank Drs. Ken Rohrbach, Stephen Arneric, Robert Stein, and Keith Miller for their helpful suggestions and insights. Last, we thank Dr. Gerard Lemole (Department of Cardiac Surgery, Christiana Care Health System, Wilmington, DE) for the procurement of human heart valves. The pig 5-HT_{2C} receptor sequences have been deposited in GenBank (accession no. AF188614).

References

Caccia S, Conforti I, Duchier J and Garattini G (1985) Pharmacokinetics of fenfluramine and norfenfluramine in volunteers given d- and d,l-fenfluramine for 15 days. *Eur J Clin Pharmacol* **29:**221–224.

Choi DS, Kellermann O, Richard S, Colas JF, Bolanos-Jimenez F, Tournois C, Launay JM, Maroteaux L (1998) Mouse 5-HT2B receptor-mediated serotonin trophic functions. *Ann NY Acad Sci* **861:**67–73.

Connolly HM, Crary JL, McGoon MD, Hensrud DD, Edwards BS, Edwards WD and Schaff HV (1997) Valvular heart disease associated with fenfluramine-phentermine. (1997) *N Engl J Med* **337:**581–588.

Curzon G, Gibson EL and Oluyomi AO (1997) Appetite suppression by commonly used drugs depends on 5-HT receptors but not on 5-HT availability. *Trends Pharmacol Sci* **18:**21–25.

Davis LG, Dibner MD and Battey JF (1986) *Basic Methods in Molecular Biology*, Elsevier, New York.

Fitzgerald LW, Conklin DS, Krause CM, Marshall AP, Patterson JP, Tran DP, Iyer G, Kostich, WA, Largent BL and Hartig PR (1999) High-affinity agonist binding correlates with efficacy (intrinsic activity) at the human serotonin 5-HT_{2A} and 5-HT_{2C} receptors: Evidence favoring the ternary complex and two-state models of agonist action. *J Neurochem* **72:**2127–2134.

Gibson UE, Heid CA and Williams PM (1996) A novel method for real time quantitative RT-PCR. *Genome Res* **6:**995–1001.

Huang Z, Fasco MJ and Kaminsky LS (1996) Optimization of DNase I removal of contaminating DNA from RNA for use in quantitative RNA-PCR. *Biotechniques* **20:**1012–1020.

Kulke MH and Mayer RJ (1999) Carcinoid tumors. *N Engl J Med* **340:**858–867.

Kursar JD, Nelson DL, Wainscott DB, Cohen ML and Baez M (1992) Molecular cloning, functional expression, and pharmacological characterization of a novel serotonin receptor (5-hydroxytryptamine$_{2B}$) from rat fundus. *Mol Pharmacol* **42:**549–557.

Johnson KW, Phebus LA and Cohen ML (1998) Serotonin in migraine: Theories, animal models and emerging therapies. *Prog Drug Res* **51:**219–244.

Julius D, Livelli TJ, Jessell TM and Axel R (1989) Ectopic expression of the serotonin 1c receptor and the triggering of malignant transformation. *Science (Wash DC)* **244:**1057–1062.

Launay J-M, Birraux G, Bondoux D, Callebert J, Choi D-S, Loric S and Maroteaux L (1996) Ras involvement in signal transduction by the serotonin 5-HT2B receptor. *J Biol Chem* **271:**3141–3147.

Lopez-Ilasaca M (1998) Signaling from G-protein-coupled receptors to mitogen-activated protein (MAP)-kinase cascades. *Biochem Pharmacol* **56:** 269–277.

Muller-Schweinitzer E and Tapparelli C (1986) Methyergonovine, an active metabolite of methysergide. *Cephalalgia* **6:**35–41.

Newton RA, Phipps SL, Flanigan TP, Newberry NR, Carey JE, Kumar C, McDonald B, Chen C and Elliott JM (1996) Characterisation of human 5-hydroxytryptamine2A and 5-hydroxytryptamine2C receptors expressed in the human neuroblastoma cell line SH-SY5Y: Comparative stimulation by hallucino-genic drugs. *J Neurochem* **67:**2521–2531.

Schmuck K, Ullmer C, Kalkman HO, Probst A and Lubbert H (1996) Activation of meningeal 5-HT2B receptors: An early step in the generation of migraine headache? *Eur J Neurosci* **8:**959–967.

Spinelli R, Fracasso C, Guiso G, Garattini S and Caccia S (1988) Disposition of (−)-fenfluramine and its active metabolite (−)-norfenfluramine in rat: A single dose-proportionality study. *Xenobiotica* **18:**573–584.

Ullmer C, Schmuck K, Kalkman HO and Lubbert H (1995) Expression of serotonin receptor mRNAs in blood vessels. *FEBS Lett* **370:**215–221.

Wee CC, Phillips RS, Aurigemma G, Erban S, Kriegel G, Riley M and Douglas PS (1998) Risk for valvular heart disease among users of fenfluramine and dexfenfluramine who underwent echocardiography before use of medication. *Ann Intern Med* **129:**870–874.

Send reprint requests to: Lawrence W. Fitzgerald, Ph.D., The DuPont Pharmaceuticals Co., Experimental Station, E400/4442, P.O. Box 80400, Wilmington DE 19880. E-mail: lawrence.w.fitzgerald@dupontpharma.com