Serotonin 5-HT$_{2B}$ receptor loss of function mutation in a patient with fenfluramine-associated primary pulmonary hypertension

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Abstract

Objective: Appetite-suppressant drug fenfluramine is implicated in primary pulmonary hypertension (PPH) but the molecular pathways that mediate this effect are unknown. A mouse model incriminates the serotonin 5-HT$_{2B}$ receptor but contrasts with other models where this receptor has been shown to mediate pulmonary arterial relaxation via nitric oxide production. Methods: We analyzed the human 5-HT$_{2B}$ gene in 10 patients with appetite-suppressant drug-associated PPH. Results: A mutation causing premature truncation of the protein product was found in one patient. The mutation was not found in 80 control subjects and no 5-HT$_{2B}$ mutation was found in 18 PPH patients not associated with appetite-suppressants. Functional analysis of the transfected receptor expressed either transiently in COS cells or stably in CHO cells demonstrated that the mutated receptor fails to activate the second messenger inositol-phosphates cascade and subsequent intracellular calcium release, in spite of normal expression at the cell membrane. The mutated receptor had no constitutive activity, and produced no dominant negative effect on the wild-type receptor. Conclusion: Loss of serotonin 5-HT$_{2B}$ receptor function may predispose to fenfluramine-associated PPH in man.

Keywords: G-proteins; Pulmonary circulation; Receptors; Serotonin (5-HT); Human

This article is referred to in the Editorial by O. Eickelberg et al. (pages 466–468) in this issue.

1. Introduction

Primary pulmonary hypertension (PPH; primary PH) is a syndrome of dyspnea, fatigue, chest pain, and syncope resulting from a pre-capillary increase in pulmonary vascular resistance leading to sustained elevation of pulmonary arteriolar pressure, right ventricular failure, and death. From a clinical standpoint, a diagnosis of PPH is made after exclusion of known causes of secondary PH [1]. The overall incidence of PPH is 1–2 cases per million people per year, and increases up to 1 in 10,000 after intake of appetite-suppressant drugs, mainly fenfluramines and aminorex [2,3]. Heterozygous loss-of-function mutations in the gene encoding the Bone Morphogenetic Protein (BMP) Receptor type II (BMPR2 gene) [4,5] result in PPH transmitted as an autosomal dominant trait with low penetrance, around 10–20%. Such hereditary mutations are found in 25–30% of apparently sporadic cases [6]. The molecular causes for the majority of PPH cases, and the causes for reduced penetrance in BMPR2 mutation carriers, are unknown, but some other genetic predisposition is likely.

Fenfluramine and its stereoisomer dexfenfluramine (collectively referred to as fenfluramines) are indirect serotonin...
(5-HT) agonists which act by releasing 5-HT from storage vesicles. Fenfluramines are furthermore rapidly de-ethyalted in vivo to form norfenfluramines, which bind 5-HT2B and 5-HT2C receptors with high affinity and display direct agonist activity [7]. 5-HT2B is expressed in endothelial and smooth muscle cells of porcine and human pulmonary arteries [8,9], and is strongly overexpressed in lungs of PPH patients [10].

The 5-HT2B receptor is a 7-transmembrane domain, G-protein-coupled receptor, which contains a PDZ binding motif that can directly activate the constitutive endothelial nitric oxide synthase (eNOS) [11]. 5-HT2B coupling to Gq furthermore activates the inositol-phosphates (IP) second messenger cascade and calcium release [8].

In the pig pulmonary artery, 5-HT2B agonists produce a reversible, endothelium-dependent relaxation [9]. In contrast with this vasodilative effect, however, 5-HT2B has recently been implicated in vascular proliferation and PH in mice exposed to chronic hypoxia and norfenfluramine, a model that shares features with chronic hypoxia and norfenfluramine, a model that shares features with PPH [13]. To sort out the net effect of 5-HT2B function in man, we investigated the human 5-HT2B receptor gene in 10 patients with fenfluramine-related PPH, and found a loss-of-function mutation in one.

2. Methods

2.1. Study patients

We studied 10 patients who developed pulmonary arterial hypertension and reported intake of fenfluramine or dexfenfluramine, alone or in combination with diethylpropion (amfepramone) or phentermine. A diagnosis of PPH was reached after careful exclusion of known causes of PAH according to current guidelines [1]. The 10 patients, nine females and one male of Western European descent, took appetite suppressants at recommended anorexic dose. Family history regarding PPH was unremarkable over three generations. The investigation conforms with the principles outlined in the Declaration of Helsinki.

2.2. Mutagenesis and 5-HT2B constructs

The different constructs of the human wild-type 5-HT2B and the natural carboxy-terminal domain mutant R393X were performed by PCR as described [12] using the following primers: forward primer containing BamH1 restriction site (underlined) used for untagged and Green Fluorescent Protein (GFP) fusion receptors: 5′TCTAGATCGAGATCCACCA- TGGCTCTTTCATTCAAGGTTC-3′ for wild-type 5-HT2B and 5′TCTAGATCGAGATCCACCA- TGGCTCTTTCATTCAAGGTTC-3′ for mutated 5-HT2B. EGFP was amplified with a forward primer described above and a reverse primer replacing the TGA stop codon by a Xhol restriction site (5′CTCGAGTTACTTTCTTTACAGCTCCTC-3′ for wild-type 5-HT2B and 5′CTCGAGTTACTTTCTTTACAGCTCCTC-3′ for mutated 5-HT2B). EGFP was amplified by a forward primer containing a Xhol restriction site, followed by a flexible triglycine linker replacing the ATG start codon (5′CTCGAGTTACTTTCTTTACAGCTCCTC-3′) and a reverse primer containing a Xba1 restriction site after the stop codon (5′CTCGAGTTACTTTCTTTACAGCCTTC-3′) and a reverse primer containing a Xba1 restriction site after the stop codon (5′CTCGAGTTACTTTCTTTACAGCCTTC-3′). EGFP was then cloned in frame with the 5-HT2B C-terminus using the Xhol restriction site, and the linker sequence LEGGG between the two coding regions. After checking the constructs by sequencing, the fusion proteins coding sequences were transferred into the bicistronic expression vector pEFIN3, using the BamH1 and Xba1 restriction sites.

2.3. Expression of mutant receptors in CHO-K1 cell lines

CHO-K1 cells were cultured using Ham’s F12 medium supplemented with 10% fetal calf serum (Life Technologies, Merelbeke, Belgium), 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies). Cells were serum-starved 24 h before all functional assays. A plasmid encoding apoaequorin under control of the SRα promoter [14] was transfected into CHO-K1 cells, using Fugene 6 (Roche). Puromycin (100 μg/ml, Calbiochem) selection of transfec-tants was initiated 2 days after transfection. Individual clones were isolated 3 weeks later, and the clone with strongest functional response (luminescence signal) to ionomycin A (100 nM) and ATP (10 μM) was selected. Constructs encoding wild-type or mutant 5-HT2B in the pEFIN3 bicis-tronic vector were further transfected using Fugene 6 in this apoaequorin expressing cell line. Selection of transfected cells was made for 14 days with 400 μg/ml G418 (Life Technologies), and the population of mixed cell clones expressing wild-type or mutant receptors was used for binding and functional studies.

2.4. IP assay

COS cells were seeded 2 days before the experiment (6 × 10⁴/well in a 24-well plate). After overnight growing in the recommended culture medium, cells were transfected with various concentrations of plasmids using lipofectamine.
Four hours after transfection, the medium was changed to DMEM (Biowhittaker, cat no. 12604F) containing 3 μCi/ml [³H] myo-inositol (Amersham). On the next day, medium containing [³H] myo-inositol was removed and cells were washed twice with CSS buffer (Tris 25 mM, pH 7.4; NaCl 120 mM; KCl 5.4 mM; CaCl₂ 1.8 mM; MgCl₂ 0.8 mM; D-glucose 16.4 mM). Cells were analyzed for receptor expression by FACS analysis or were incubated for 30 min at 37 °C with various concentrations of agonist in the CSS buffer containing 10 mM of LiCl. Reactions were stopped by addition of HClO₄ 1 N to each well and incubated for 30 min at 4 °C. KOH phosphate buffer was then added to each well and incubated at 4 °C for 1 h to form KClO₄ precipitate. A sample buffer containing 30 mM Dinitetraborate and 3 mM EDTA was then added on each well and the plate was centrifuged at 1500 rpm at 4 °C for 5 min.

At the same time, mini anion exchange columns were prepared as follows. Dowex resin AG1X8 formate form (Biorad, cat no. 140–1454) was weighed and mixed with distilled water (25% w/v). About 1.6 ml of this resin was added to each Poly Prep column (Biorad, cat no. 731–1550). The columns were then washed one time with 5 ml of distilled water. The supernatant of each well was applied to the columns containing anion exchange resin and the columns were rinsed with 5 ml of distilled water. The GPI was eluted with 10 ml of 5 mM Dinitetraaborate/60 mM Ammonium formate. The IPs mixture were then eluted with 0.1 M formic acid/1 M ammonium formate and collected into scintillation vials. Radioactivity was determined by adding 3.5 ml of scintillation cocktail and counted in Tricarb. Results were expressed as the ratio between the radioactivity collected in the IP fraction over the radioactivity recovered from the solubilised cellular membranes. The use of this ratio allows for greater homogeneity in the data, as it reduces variation from differences in cell numbers from individual wells. The normalized IP formation was determined as a percentage of IP formation ratio compared with that obtained with buffer alone and referred to as 100% [15].

2.5. FACS analysis

The cellular expression of wild-type 5-HT₂B-GFP and R393X-5-HT₂B-GFP was measured by flow cytometry using 10 μg/ml of FITC-conjugated anti-FLAG M2 antibody (Sigma). FACS analysis was performed on a FACScan flow cytometer using the CellQuest software (Becton Dickinson). The percentage of positive cells or Mean Channel Fluorescence was determined.
cence (MCF) was used to compare the levels of receptor expression. We defined the non-specific fluorescence as the fluorescence of cells transfected with the vector only (0% of positive cells).

### 2.6. Confocal microscopy

For confocal studies in living cells, stable CHO-K1 cell lines expressing wild-type or mutant 5-HT$_{2B}$-GFP were

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**Fig. 2.** Cellular expression of wild-type 5-HT$_{2B}$ and R393X mutant receptor. (A) Saturation binding curves were performed on membranes from CHO-K1 cells expressing wt5HT2B or R393X, using [N-methyl-$^3$H]-lysergic acid diethylamide as tracer. Results were analysed by the Graphpad Prism software, using a single site model, and the nonspecific binding was measured by addition of a saturating concentration of serotonin (1 μM). All points were run in triplicate (error bars: S.E.M.). Data are representative of two independent experiments. (B) FACS analysis showing the level of expression of wt5HT2B-GFP or R393X-GFP in stably transfected CHO cells. A typical experiment out of the two performed independently is represented. (C) Subcellular distribution of wt5HT2B-GFP and R393X-GFP expressed in CHO cells, as analysed by confocal microscopy. The figure presented is representative of two experiments with similar results. Scale bar: 20 μm.
seeded the day before the analysis on 22 mm round glass coverslips, and grown for 18 h in a CO2 incubator. Cells were rinsed in DMEM/F12 (Life Technologies) and the coverslips were placed in a 1-ml chamber with 200 μl of culture medium. Experiments were performed at 37°C on a temperature-controlled stage. Cells were observed on a MRC 1024 confocal microscope (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, United Kingdom) fitted on an Axiovert 100 inverted microscope (Zeiss, Oberkochen, Germany) equipped with a Plan-Neofluar™ 40 ×/1.3 oil immersion objective (Zeiss). The 488-nm excitation beam of an Argon-Krypton laser and a 522/32 nm band-pass emission filter were used for selective viewing of the green fluorochrome. Fields of interest (512 × 512 pixels) were selected visually. The gray scale datasets generated were transferred to an Indy™ workstation (Silicon Graphics, Mountain View, CA) running the ImageSpace™ software (Molecular Dynamics, Sunnydale, CA). No labeling was observed on untransfected CHO-K1 (data not shown). Figures were prepared on a Power Mac™ (Apple, Cupertino, CA) running Freehand™ (Macromedia, San Francisco, CA) and Illustrator™ (Adobe, San Francisco, CA) softwares.

2.7. Aequorin-based functional assay

The functional response to 5-HT (Sigma) and BW723C86 (Tocris), a selective 5-HT2B agonist, was analyzed by measuring the luminescence of aequorin as described [16]. Cells were collected from plates with Ca2+ and Mg2+ -free PBS supplemented with 5 mM EDTA, pelleted for 2 min at 1000 × g, resuspended in DMEM at a density of 5 × 10^6 cells/ml and incubated for 2 h in the dark in the presence of 5 μM coelenterazine H (Molecular Probes, Eugene, OR). Cells were diluted 5-fold before use. Agonists in a volume of 50 μl DMEM were added to 50 μl of the cell suspension (50,000 cells) and luminescence was measured for 30 s in an EG and G Berthold (Perkin-Elmer, Oosterhout, Netherlands). Functional parameters were determined with the PRISM software (Graphpad Software) running nonlinear regression applied to a sigmoidal dose–response model.

2.8. Preparation of membrane extracts

CHO-K1 cells stably expressing wild type 5-HT2B and R393X mutant were cultured to 90% cell confluence. The medium was removed and the cells were scraped from the plates in Ca2+ and Mg2+-free Phosphate-buffered saline (PBS). The cells were then centrifuged for 3 min at 1500 g and the pellets were resuspended in buffer A (15 mM Tris-HCl pH 7.5; 2 mM MgCl2; 0.3 mM EDTA; 1 mM EGTA) and homogenized in a glass homogenizer. The crude membrane fraction was collected by two consecutive centrifugation steps at 40,000 × g for 25 min separated by a washing step in buffer A. The final pellet was resuspended in buffer B (7.5 mM Tris-HCl pH 7.5; 12.5 mM MgCl2; 0.3 mM EDTA; 1 mM EGTA; 250 mM sucrose) and flash frozen in liquid nitrogen. Protein concentration was determined by the Folin method.

2.9. Binding assays

Saturation binding assays were performed in triplicate in polyethylene MiniSorp tubes (Nunc) containing binding buffer (50 mM Tris-HCl pH 7.4, 0.1% Ascorbic acid and 4 mM CaCl2), wild-type and mutant 5-HT2B membrane extracts (40 μg protein/tube), 0.2 to 10 nM [N-methyl-3H]-lysergic acid diethylamide ([3H]-LSD; Amersham Pharmacia). Total binding was measured in the absence of competitor and nonspecific binding was measured with at least 200-fold excess of 5-HT. The samples were incubated in a final volume of 0.25 ml for 30 min at 37°C and then filtered on GF/C filters presoaked in 0.5% PEI (Polyethylenimine) using a multiple membrane filter set-up (Linca Lamon Instrumentation, Tel Aviv, Israel). Filters were washed three times with 4 ml of ice-cold binding buffer, dried, and bound [N-methyl-3H]-lysergic acid diethylamide was measured by liquid scintillation counting. Maximal binding capacity (Bmax) and dissociation constant (Kd) were determined by non-linear regression using a single-site model (PRISM, Graph Pad Software, San Diego).

3. Results

3.1. Mutation analysis

We investigated whether mutations in the human 5-HT2B gene could be implicated in the development of PPH after intake of anorexigens by sequencing the whole coding region of the 5-HT2B gene in our cohort of 10 patients. We found a heterozygous mutation in one patient, a white Belgian female diagnosed with PPH at the age of 50 years, 5 years after her last intake of anorexigens. She had used fenfluramine and diethylpropion at recommended anorexic doses for a cumulative time of nine months. The mutation consisted of a C to T transition resulting in the substitution of a stop codon in place of an arginine codon at position 393 of the 5-HT2B gene. The mutant allele, R393X, was found in two of our patients.

Table 1

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Kd (nM)a</th>
<th>Bmax (pmol/mg proteins)b</th>
<th>EC50 (nM)1</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt5-HT2B</td>
<td>1.99 ± 0.62</td>
<td>1.10 ± 0.02</td>
<td>4.34 ± 1.12</td>
</tr>
<tr>
<td>R393X-5-HT2B</td>
<td>2.24 ± 0.30</td>
<td>1.43 ± 0.09</td>
<td>&gt;1000</td>
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a Derived from saturation binding assays using [N-methyl-3H]-lysergic acid diethylamide as tracer. Data are mean ± S.E.M. from two separate experiments.
b Derived from saturation binding assays using [N-methyl-3H]-lysergic acid diethylamide as tracer. Data are mean ± S.E.M. from two separate experiments.
1 Derived from dose–response curves in the aequorin assay. Data are mean ± S.E.M. from two separate experiments.
Fig. 3. Intracellular calcium release mediated by wt5-HT2B and R393X receptor mutant. (A) The functional response of the cell lines co-expressing apoaequorin and wild-type 5-HT2B was tested following addition of serotonin (5-HT) and a 5-HT2B agonist (BW723C86). The luminescent signal resulting from the activation of the apoaequorin-coelenterazine complex was recorded for 30 s in a luminometer. Results were analysed by nonlinear regression using the Graphpad Prism software. All points were run in duplicate (error bars: S.E.M.). The displayed curves represent a typical experiment out of two performed independently. (B) The functional response to 5-HT of the two types of fusion proteins, wt5HT2B-GFP and wt5HT2B-FLAG, were tested on CHO-K1 cells stably expressing the fusion receptors, using aequorin assay as described above. All points were run in duplicate (error bars: S.E.M.). The displayed curves represent a typical experiment out of two performed independently. (C) Increase of intracellular calcium following the addition of a saturating concentration of 5-HT or BW723C86 in cells expressing the different wt5HT2B and R393X mutant constructs, as measured by the aequorin assay. The basal level was defined as the luminescence measured in the absence of agonist. pEFIN3, plasmid vector. All points were run in duplicates (error bars: S.E.M.). The displayed histogram represents a typical experiment out of two performed independently.
Fig. 4. Inositol-phosphates production by wt5-HT$_{2B}$ and R393X mutant. (A) Inositol-phosphates (IP) production was analyzed on COS cells 48 h after transfection with different concentrations of the various wild-type and mutant 5-HT$_{2B}$ receptor constructs. Levels of IP production were determined in the basal state and following addition of 5-HT in saturating concentration. Results were expressed as a normalized ratio between the radioactivity collected in the IP fraction over the radioactivity recovered from the solubilised cellular membranes. The normalized IP production was determined as an increase in the IP formation ratio compared with that obtained with buffer alone (defined as an IP production ratio of 1, see methods). All points were run in triplicates (error bars: S.E.M.). The displayed histogram represents a typical experiment out of two performed independently. (B) Cellular expression of wt5HT2B-GFP and R393X-GFP in COS cells transiently transfected with various concentrations of plasmids. FACS analysis was performed on the same day as the IP assay. Results were presented as the percentage of fluorescence-positive cells. All points were run in triplicate (error bars: S.E.M.). The displayed histogram represents a typical experiment out of two performed independently. (C) Plasma membrane expression of wt5HT2B-FLAG and R393X-FLAG in COS cells transiently transfected with various concentrations of plasmids, using FITC-coupled anti-FLAG monoclonal antibody and FACS analysis as described above. The displayed histogram represents a typical experiment out of two performed independently.
the predicted protein sequence (R393X). This mutation resulted in the truncation of most of the carboxy-terminal tail of the receptor, removing putative palmitoylation and phosphorylation sites as well as the PDZ binding motif [11] (Fig. 1). This mutation was not found in 160 control alleles. 5-HT2B gene sequencing in 18 PPH or PAH patients with no reported history of appetite-suppressant intake revealed no further mutation.

Sequencing of the whole coding sequence and intron–exon junctions of BMPR2 [17] in the 10 patients detected no mutation.

3.2. R393X mutant is normally expressed at the plasma membrane and binds LSD with normal affinity

In order to investigate the function of this new mutation (R393X), plasmids encoding wt5-HT2B, R393X mutant, and wt5-HT2B or R393X fused to a Green Fluorescent protein (GFP) or FLAG epitope were transfected in CHO-K1 cells stably expressing the reporter protein apoaequorin. Mixed populations of transfected cells were used for testing surface expression, binding, and functional properties of the receptors.

The binding parameters ($B_{\text{max}}$ and $K_d$) of mixed cell populations expressing wt5-HT2B or R393X mutant receptor were determined by a saturation binding assay, using LSD as tracer. No specific LSD binding was observed on control CHO cells transfected with the empty vector. As shown in Fig. 2a and Table 1, wt5-HT2B and R393X bound LSD with similar affinity (1.99 ± 0.62 nM and 2.24 ± 0.30 nM, respectively) and displayed similar expression levels ($B_{\text{max}}$ of 1.10 ± 0.02 and 1.43 ± 0.09 pmol/mg proteins, respectively). Pools of transfected CHO-K1 cells expressing wt5-HT2B-GFP or R393X-GFP were also assayed for total receptor expression by FACS analysis. As shown in Fig. 2b, both constructs were expressed at similar level. The subcellular distribution of wt5-HT2B-GFP and R393X-GFP was also similar, as analyzed by confocal microscopy (Fig. 2c). Both fusion proteins were detected at the plasma membrane, although a fraction of the receptor was seen intracellularly.

3.3. R393X mutant can not activate intracellular signaling

We next tested the ability of these non-clonal cell populations expressing wt5-HT2B and R393X to trigger intracellular calcium release using the aequorin assay. As shown in Fig. 3a and Table 1, 5-HT and BW723C86, a selective agonist for 5-HT2B, elicited a robust increase of intracellular calcium in cells expressing wt5-HT2B, characterized by EC_{50} of 4.34 and 3.09 nM, respectively. A similar functional response to 5-HT was found in cells expressing wt5-HT2B-GFP and wt5-HT2B-FLAG (Fig. 3b), whereas no response was observed in cells expressing R393X or transfected with vector only, up to 1 μM of either agonist (Fig. 3c).

To investigate whether the absence of calcium release in R393X-expressing cells was the consequence of a higher constitutive activity of the R393X mutant and of subsequent receptor desensitization, we analyzed the IP production of COS cells transiently transfected with various constructs of wt5-HT2B and R393X. A FLAG epitope fused to the aminooxidase sequence of the R393X mutation on wt5-HT2B function. (A) The effect of R393X on the cell surface expression of wt5HT2B-FLAG was determined by FACS analysis as described in Fig. 4b, using FITC-coupled anti-FLAG monoclonal antibody, 48 h after plasmid co-transfection in COS cells. The displayed histogram represents a typical experiment out of two performed independently. (B) The effect of R393X on the IP production mediated by wt5HT2B-FLAG in response to 5-HT stimulation was analyzed in COS cells 48 hours after being co-transfected with different concentration ratios of wt5HT2B-FLAG and R393X mutant. Results were expressed and normalized as presented in Fig. 4a. The displayed histogram represents a typical experiment out of two performed independently.
terminal domain of receptors was used to compare the results of functional assays with cell surface expression.

Different concentrations of the various plasmid constructs were transfected in COS cells and assayed after 48 h for IP production and FACS analysis. As shown in Fig. 4a, basal IP production was not different between the various concentrations of wt5-HT2B, R393X, or control plasmids, demonstrating that neither wt5-HT2B nor R393X mutant presented a constitutive activity in COS cells. By contrast, a significant increase of IP production could be seen for the R393X plasmid together with FLAG-aequorin assays, only cells expressing wt5-HT2B but not R393X showed an increase of IP production in response to 1 μM 5-HT (Fig. 4a) despite similar percentages of GFP-positive cells (Fig. 4b) and cell surface receptor expression as detected by a monoclonal antibody against the FLAG epitope (Fig. 4c).

3.4. R393X mutant produces no dominant negative effect

To investigate whether R393X has a dominant negative effect on wt5-HT2B function, we co-transfected various concentrations of R393X plasmid together with FLAG-wt5-HT2B and assayed wt5HT2B cell surface expression by FACS analysis as well as IP production. Receptor cell surface expression (Fig. 5a) or receptor function (Fig. 5b) ruled out a dominant negative effect even following co-transfection of a 3:1 ratio of R393X-wt5-HT2B.

4. Discussion

We investigated the 5-HT2B gene in 10 patients who developed PPH after intake of fenfluramines and found a mutation in one (patient 2, Table 1). It truncates most of the carboxy-terminal tail of the receptor, including the C-terminal PDZ motif (Fig. 1). This mutation is rare as it was not found in a sample of 160 control chromosomes from the same population. In spite of normal expression at the plasma membrane and of normal agonist binding (Table 1; Fig. 2), the mutation causes loss of 5-HT2B function as measured by IP production (Fig. 4a; Table 1) and by calcium-dependent aequorin luminescence (Fig. 3c). Although endogenous receptor expression precluded these experiments to be performed in cultures of pulmonary vascular cells, our results from COS and CHO cells clearly show that an intact C-terminal tail is necessary for IP cascade activation, and for calcium release, by 5-HT2B. The mutated receptor displays no constitutive activity as compared with the wild-type receptor, and does not produce a dominant negative effect on the latter in co-transfection experiments (Fig. 5).

Considering the rarity of the mutation and of the phenotype, their association is unlikely to result from chance and indicates that heterozygous, i.e. partial, loss of 5-HT2B function played a causal role in PPH disease progression at least in this patient.

Fenfluramines are transported by the 5-HT transporter (5-HTT) into the cytoplasm of 5-HTT-expressing cells, where they release 5-HT from storage vesicles [7]. 5-HT produces a globally vasoconstrictive effect on pulmonary arteries in most species studied [18–20]. The wild-type 5-HT2B receptor subtype, however, elicits a reversible endothelium-dependent relaxation of pulmonary arteries in pigs, associated with an increase in cyclic GMP [9], through coupling to the nitric oxide (NO) signalling pathway [11]. The PDZ motif at the C-terminus of 5-HT2B is required for activation of eNOS [11]. The 5-HT2B receptor is furthermore coupled to G-protein Gq and intracellular calcium release [8], and the calcium/calmodulin complex is thought to activate eNOS in at least some vascular beds [21]. As the R393X mutation abolishes calcium release and removes the PDZ motif, we conclude that the mutated receptor has lost its ability to stimulate eNOS. Expression of the eNOS enzyme is decreased in PPH both at mRNA and protein level to an extent that is inversely proportional to vascular resistance [22]. Therefore, maximal stimulation of eNOS by 5-HT2B might be a limiting factor, consistent with a model where imbalances in the endothelial production of vasodilators and vasoconstrictors play a significant role in PPH disease progression [23]. Of note, the 5-HT2B receptor is strongly overexpressed in pulmonary arteries of PPH patients [10]. We speculate that this strong overexpression is a secondary event that partially counterbalances the vasoconstrictive effect of 5-HT, and that susceptibility to fenfluramines was increased in our patient because of the heterozygous loss of function. Homozygous 5-HT2B loss-of-function mutations are not expected in PPH patients, whose phenotype is normal at birth and in infancy, in view of the severe congenital cardiomyopathy in homozygous 5-HT2B knockout mice [24].

It remains uncertain which receptor subtype mediates pulmonary vasoconstriction by 5-HT. Both the 5-HT1B and 5-HT2A receptors are expressed in human small muscular pulmonary arteries, and both have been implicated in vasoconstriction [20,25]. In mice chronically submitted to hypoxia, a model which shares features with PPH, 5-HT2B was shown to be required for smooth muscle cell proliferation and structural remodeling of pulmonary arteries [10], consistent with the ability of 5-HT2B to initiate cell cycle progression in mouse fibroblasts [26]. In that model 5-HT2B was furthermore required for the potentiation of PH by dexfenfluramine [10]. If this myoproliferative effect of 5-HT2B is relevant to human smooth muscle cells from PPH patients, we hypothesize that it can be produced by one allele only, i.e. that it is not haploinsufficient, although no data are reported in heterozygous mice [10]. It is hence not excluded that the 5-HT2B receptor, besides endothelium-dependent vasodilation, also participated in pulmonary arterial smooth muscle cells proliferation or remodeling in our patient. This hypothesis could not be tested because our patient’s lungs were explanted years before the present study, and were hence not available for analysis.
A germline **BMPR2** mutation can be detected in up to 30% of patients with apparently sporadic PPH [6]. No mutation of this gene was found in our five patients exposed to fenfluramines. Although **BMPR2** and 5-HT$_{2B}$ mutations might theoretically be associated with distinct subgroups of PPH patients, these genes are more likely part of a complex network of signalling pathways where several, non-mutually exclusive mutations may predispose to PPH. Indeed, **BMPR2** mutations have been found in some patients with fenfluramine-triggered PPH [27]. Of note, these patients had a mean duration of exposure to fenfluramine of less than 3 months, while the patient in the present study reported a 9-month exposure (Table 1). More importantly, BMPR-II [28] and BMPR-IA [29] protein expression at the plasma membrane of pulmonary endothelial cells is decreased not only in hereditary PPH with germline **BMPR2** mutations but also in non-hereditary PPH and in secondary PH cases, highlighting the essential role of the BMP/TGF-$\beta$/SMAD pathway [30] in possibly all forms of PH. Experimentally, BMPs suppress proliferation of pulmonary artery smooth muscle cells from normal controls but not from PPH patients [31]. We assume that these changes were present in our patient with the 5-HT$_{2B}$ mutation, but her explanted lungs were not available for study.

A frequent polymorphism of the 5-HTT gene increases the expression of 5-HTT in pulmonary artery smooth muscle cells, and is associated with PPH, albeit not specifically with appetite-suppressant-associated PPH [32]. A search for polymorphisms in candidate serotonergic genes, followed by genetic association studies, should address whether some multigenic variability in 5-HT signalling predisposes to PPH.

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