Overexpression of the Serotonin 5-HT\textsubscript{2B} Receptor in Heart Leads to Abnormal Mitochondrial Function and Cardiac Hypertrophy

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Background—Identification of factors regulating myocardial structure and function is important to understand the pathogenesis of heart disease. We previously reported that 5-HT\textsubscript{2B} receptor ablation in mice leads to dilated cardiomyopathy. In this study, we investigated the pathological consequence of overexpressing 5-HT\textsubscript{2B} receptors in heart in vivo.

Methods and Results—We have generated transgenic mice overexpressing the Gq-coupled 5-HT\textsubscript{2B} receptor specifically in heart. We found that overexpression of 5-HT\textsubscript{2B} receptor in heart leads to ventricular hypertrophy as the result of increased cell number and size. Increased atrial natriuretic peptide and myosin heavy chain expression demonstrated activation of the molecular program for cardiac hypertrophy. Echocardiographic analysis indicated the presence of thickened ventricular free wall without alteration of the systolic function, showing that transgenic mice have compensated hypertrophy. Electron microscopic analysis revealed structural abnormalities including mitochondrial proliferation, as also manifested by histological staining. Transgenic mouse heart displayed a specific reduction in the expression levels of the adenine nucleotide translocator associated to increase in the succinate dehydrogenase and cytochrome C oxidase mitochondrial activities.

Conclusions—Our results constitute the first genetic evidence that overexpression of the 5-HT\textsubscript{2B} receptor in the heart leads to compensated hypertrophic cardiomyopathy associated with proliferation of the mitochondria. This observation suggests a role for mitochondria in the hypertrophic signaling that is regulated by serotonin. These transgenic mice provide a new genetic model for hypertrophic heart disease. (Circulation. 2003;107:3223-3229.)

Key Words: cardiomyopathy ■ genetics ■ hypertrophy ■ receptors ■ signal transduction
vascular endothelial cells and can be co-released.\textsuperscript{8} The Gq-coupled 5-HT\textsubscript{2B} receptor (5-HT\textsubscript{2B}R) belongs to one of the four classes of 5-HT receptors (5-HT\textsubscript{1A}, 5-HT\textsubscript{1B}, 5-HT\textsubscript{1D}, and 5-HT\textsubscript{2A} - 6).\textsuperscript{9} Genetic ablation of 5-HT\textsubscript{3A}R in mice leads to partial embryonic and neonatal death as the result of the following heart defects: (1) 5-HT\textsubscript{3A}R mutant embryos exhibit a lack of trabeculae in the heart, leading to mid-gestation lethality.\textsuperscript{10} (2) In newborn mice, contractility and structural deficits at cellular junctions in 5-HT\textsubscript{3A}R mutant cardiomyocytes lead to cardiac dilation. (3) In the adult 5-HT\textsubscript{2B}R mutant mice, echocardiography and electrocardiography both confirm the presence of left ventricular dilation and decreased systolic function typical of dilated cardiomyopathy.\textsuperscript{11} These results constituted the first genetic evidence that 5-HT through 5-HT\textsubscript{2B}R can regulate differentiation and proliferation of developing heart and structure and function of adult heart.

Although 5-HT\textsubscript{3A}R ablation (loss of function) in mice leads to dilated cardiomyopathy, the consequence of overexpression of Gq-coupled 5-HT\textsubscript{2B}R (gain of function) in heart remains undefined. In this study, we used an in vivo approach by developing TG mice overexpressing 5-HT\textsubscript{2B}R in heart to further investigate the pathophysiological role of 5-HT through 5-HT\textsubscript{2B}R.

Methods

Generation of TG Mice

A 5.5-kilobasepair (Kbp) BamHI-KpnI restriction fragment of the mouse α-myosin heavy chain (MHC) was fused to a 2.0-kbp XbaI-XbaI restriction fragment of mouse 5-HT\textsubscript{3A}R cDNA. The recombinant plasmid p-MHC-5-HT\textsubscript{3A} was linearized, microinjected into pronuclei of fertilized CD1 mouse eggs, and implanted into pseudopregnant CD1 foster mothers. Mouse tail DNA hybridized into pronuclei of fertilized CD1 mouse eggs, and implanted into cardiac dilation. (3) In the adult 5-HT\textsubscript{2B}R mutant mice, developing heart and structure and function of adult heart. Accordance with institutional guidelines and the French Animal Care ascertain gene transfer. All animal experimentation was performed in accordance with the guidelines of the American Society of Echocardiography.

Echocardiography Methods

Animals were weighed and analyzed for cardiac anatomy and function on a Sonos 5500 (Philips Electronics, Koniklijke, the Netherlands) with a 15-MHz linear transducer (15L6), as previously described.\textsuperscript{11} All the examinations were performed in mice anesthetized with sodium pentobarbital (30 mg/kg IP). All the measurements were performed according to the guidelines of the American Society of Echocardiography.

Blood Pressure Measurements

Systolic arterial pressure and heart rate were recorded by the tail-cuff technique in awake mice, as previously described.\textsuperscript{11}

Morphological Analysis of Mouse Heart

Transmission electron microscopy (TEM) and histological techniques were performed as previously described.\textsuperscript{10} Hearts were dissected, fixed, paraffin-embedded, and sectioned (7 μm/diameter) on a microtome, using standard techniques. Ragged-red fibers (RRF) were revealed by staining the sections using the trichrome of Gomori’s technique.\textsuperscript{12}

Western Blot Analysis

For Western blots, ~20 μg of heart protein was separated on 10% SDS/PAGE and blotted to nitrocellulose membranes. To verify loading homogeneity, the same blot was stripped and reprobed with an antibody against β-actin. Blots were stripped with 6.25 mmol/L Tris pH 7.5, 2% SDS, and 100 mmol/L 2-mercaptoethanol for 30 minutes at 45°C and washed for 1 hour. Antibody-antigen complexes were detected with an ECL kit according to manufacturer instructions. We used the following antibodies: 5-HT\textsubscript{2A}R (Pharmingen, San Diego, Calif), MHC MF-20 (Hybridoma bank, Iowa City, Iowa), atrial natriuretic factor (ANF) (Amersham Biosciences, Orsay, France), adenine nucleotide translocator (ANT) (Santa Cruz Biotech, Santa Cruz, Calif), β-actin (Sigma-Aldrich, Lyon, France), and voltage-dependent anion channel (VDAC) (Santa Cruz Biotech). The ECL signals were quantified through the use of an image analyzer (GS-700, Bio-Rad, Hercules, Calif) and calculated as arbitrary units.

Analysis of Hypertrophic Cardiac Genes by RT-PCR

Semi-quantitative RT-PCR was performed on 1 μg of total RNA extracted from age-matched control and knockout mice, using the ribosomal elongation factor 1A as an internal control as previously described.\textsuperscript{10} The following primers were used: for ANF 5'-caggccatattggagcaaa and 5'-gaagctgtagccgactcgtg; for α-MHC 5'-ctgctggagaggttattcctcg and 5'-ggaaagtaggagcggccgatcaagg; for β-MHC 5'-tgagaaggctgacggtgg and 5'-gccaaccaccgccgcaagtag.

Enzyme Activity and Histochemistry

The histochemical enzyme analysis of succinate dehydrogenase (SDH) and cytochrome C oxidase (COX) activities were performed on cryosections of unfixed heart as described\textsuperscript{13} and on heart whole-cell extract as described.\textsuperscript{14}

Cardiomyocyte Morphology Determination and Microscopic Analysis

Immunohistochemistry was performed on heart cryosections with the antiserumomic HMC MF-30 (Hybridoma Bank), ANF, or ANT antibody as described.\textsuperscript{10} Confocal microscope images were used to evaluate the total number of HMC-positive cells. The percentage of cardiomyocytes in a given field was calculated by determining the number of cardiomyocytes that showed green cytoplasmic MHC staining and dividing by the total number of cells in the field, which were visualized by red nuclear propidium iodide (PI) staining. Isolated cardiomyocyte size was determined as described.\textsuperscript{10,15}

Data Analysis and Statistics

All values represent the average values of independent experiments (±SEM, n = number of experiments as indicated in the text). Comparisons between groups were performed by using the Student’s unpaired t test or ANOVA and a Fisher’s test. Significance was set at a value of P<0.05.

Results

Generation of TG Mice

We used a cardiomyocyte-specific promoter to generate TG mice to determine the consequence of abnormal expression of 5-HT\textsubscript{2B}R in heart. The transgene consisted of the cardiomyocyte-specific α-MHC promoter\textsuperscript{16} linked to the entire coding sequence for the mouse 5-HT\textsubscript{2B}R (Figure 1A). A TG founder mouse was obtained that successfully transmitted the transgene to its progeny (Figure 1B). TG mice were recovered at mendelian frequency and survived to adulthood. Immunoblot analysis performed with a 5-HT\textsubscript{2B}R antibody showed that the
5-HT$_{2B}$ R protein level was increased in heart lysate of TG compared with nontransgenic (NTG) mice (Figure 1C). Binding assay with a 5-HT$_{2B}$ R–specific labeled compound revealed 6.8-fold overexpression of 5-HT$_{2B}$ R but no significant changes in 5-HT$_{2A}$ R protein in the heart (Figure 1C). These data show a specific 5-HT$_{2B}$ R overexpression in the TG heart.

Heart Morphology and Cardiac Functions
Cardiac functions and anatomy were assessed in 12-week-old male mice. Histological analysis revealed an increase in the thickness of the left ventricular free wall from TG mice (Figure 2). Echocardiographic analysis confirmed that the posterior wall thickness (28±5%) and the total left ventricular mass (22.8±8.8%) were significantly increased in adult TG mice. TG mice also showed increased left ventricle weight-to-body weight ratio by 178%. Left ventricular end-systolic or end-diastolic diameters, fractional shortening, or systolic blood pressure was not altered, whereas heart rate was slightly increased in the TG mice. No loss of systolic performance occurred in the TG mice, indicating a compensated hypertrophy (Table).

Cardiomyocyte Number and Size
We next investigated the mechanism leading to the increased ventricular mass found in 5-HT$_{2B}$ R TG heart. Total cell and cardiomyocyte numbers were determined from frozen sections stained with PI and cardiomyocyte-specific MHC antibody, respectively. As shown in Figure 3, heart from TG had 11.0±0.4% more cardiomyocytes than from NTG mice. Isolated TG cardiomyocytes had a significant increase in size by 23±5%. These data indicate that the increase in ventricular mass observed in TG mice results from increases in both cell density and size of cardiomyocytes.

Hypertrophic Gene Expression in Heart
To determine whether the hypertrophic growth is associated with altered expression of hypertrophic markers, expression was evaluated in adult TG heart. Semiquantitative RT-PCR analysis of TG heart mRNA demonstrated an increase in ANF expression and $\alpha$-MHC and no change in $\beta$-MHC or desmin levels. Western blot analysis confirmed the increase

Figure 1. Generation of TG mice. A, Mouse $\alpha$-MHC promoter was used as cardiac-specific promoter to control expression of mouse 5-HT$_{2B}$ R cDNA. B, 5-HT$_{2B}$ R transgene insertion was determined by genomic DNA Southern analysis. Using as a probe a 0.35-Kbp PstI–XbaI restriction fragment of the 5' region of 5-HT$_{2B}$ R cDNA, the 5-HT$_{2B}$ R transgene was revealed after EcoRI digest as a 3.1-Kbp band and the endogenous gene as a 6.5-Kbp band. C, Number of 5-HT$_{2B}$ R–specific sites was measured by binding studies (expressed as fmol/mg of protein ±SEM). *$P<0.05$, TG vs NTG, n=4, each determination in triplicate (left). 5-HT$_{2B}$ R overexpression was verified on Western blot with anti-5-HT$_{2B}$ R antiserum (right).

Figure 2. 5-HT$_{2B}$ R TG mice with increased ventricular mass. Representative transverse sections from adult heart at low and high magnifications of Masson’s trichrome–stained sections are shown for NTG and TG mice (n=3, 12-week-old male). Arrow indicates thickness of left ventricular posterior wall. lv indicates left ventricle; rv, right ventricle. Bars=1000 μm, top; 250 μm, bottom.
in ANF expression by 40% and MHC by 70% (Figure 4A) as well as immunohistochemical staining on the cryosectioned heart (Figure 4B). These changes in expression demonstrate the activation of a molecular program for cardiac hypertrophy in TG heart.

Heart Ultrastructural Analysis
Ultrastructural study by TEM revealed structural abnormalities along with mitochondrial proliferation in TG mice ventricular myocardium compared with NTG. Moreover, TG heart exhibited lipid deposition and T-tubules enlargement (Figure 5, A and B). The sarcomere structure of TG appeared normal, but mitochondria were rounded, irregular, and higher in number. Notably, no evidence for myocardial apoptosis, fibrosis, or significant inflammatory cell infiltrates was found.

Heart Mitochondrial Function
To verify mitochondrial proliferation, we stained cryosections of the heart with the modified Gomori’s trichrome stain that reveals red staining material associated with proliferation of mitochondria (RRF).12 TG mice heart demonstrated typical RRF staining that was not observed in the NTG mouse heart (Figure 6A). To investigate the mitochondrial function, enzymatic activities for COX and SDH were measured. Activity assay on whole-heart extract showed a significantly increased activity of both SDH and COX compared with NTG. Enzymatic histochemical staining for SDH and COX activity confirmed the increased activity of both SDH and COX by 54.0 ± 0.5% and 28.8 ± 4.0%, respectively, in the TG heart (Figure 6A).

It has previously been reported that the expression of ANT was increased in association with mitochondrial defects.18 Western blot analysis on total heart lysate showed a decreased ANT protein expression by 32% in the TG mice without alterations in the VDAC levels, which was confirmed by immunostaining of heart cryosections (Figure 6B). Together, these data indicate increased respiratory chain and oxidative phosphorylation in the mitochondria of TG heart that are associated with 5-HT2BR overexpression.

Discussion
Our previous study has shown that loss of function for the Gq-coupled 5-HT2BR leads to dilated cardiomyopathy.11 In this study, we used a TG animal model overexpressing the 5-HT2BR in heart to provide novel insights in the determinants for cardiomyocyte growth in response to 5-HT. Overexpression of 5-HT2BR in heart leads to cardiac hypertrophy accompanied by abnormal mitochondrial proliferation and enzyme activity, indicating an essential role for 5-HT in the hypertrophic signaling.

TG mice have heart hypertrophy as the result of an increased number of cardiomyocytes and increased growth. The overexpression of 5-HT2BR raises the expression of hypertrophic genes such as ANF and β-MHC as an evidence of hypertrophic response in the TG heart. This response should be a direct effect of 5-HT2BR overexpression because hypertrophy was observed without evidence of hemodynamic

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**Cardiovascular Parameters for 5-HT2BR TG Mice: Basal Cardiovascular Phenotype in 12-Week-Old Male Mice**

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<th>LVEDD, mm</th>
<th>LVEDS, mm</th>
<th>FS, %</th>
<th>PW, mm</th>
<th>Septum, mm</th>
<th>SBP, mm Hg</th>
<th>HR, bpm</th>
<th>LVM, mg</th>
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<tr>
<td>NTG, n=7</td>
<td>3.45±0.10</td>
<td>2.16±0.17</td>
<td>38±3</td>
<td>0.71±0.03</td>
<td>0.68±0.04</td>
<td>96±4</td>
<td>521±17</td>
<td>76.7±3.9</td>
</tr>
<tr>
<td>TG, n=8</td>
<td>3.39±0.04</td>
<td>2.16±0.12</td>
<td>37±3</td>
<td>0.91±0.04*</td>
<td>0.72±0.05</td>
<td>111±5</td>
<td>560±6</td>
<td>94.2±6.8*</td>
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Echocardiographic analysis was used to determine left ventricular end-systolic (LVEDS) and end-diastolic (LVEDD) diameters, fractional shortening (FS), and posterior wall (PW) or septum thickness and to deduce left ventricular mass (LVM). In the conscious state, systolic blood pressure (SBP) and heart rate (HR) were assessed by tail cuff on awake animals. Values are expressed as mean±SEM.

*P<0.05: difference between TG and NTG mice (Student’s t test).
overload. Cardiac fibrosis was not observed in TG mouse heart, indicating that myocardial overexpression of 5-HT$_{2B}$R did not alter remodeling in noncardiomyocyte cells through paracrine mechanisms.

The involvement of the Gq-coupled receptor in regulating cardiomyocyte hypertrophy is not yet fully understood despite the use of both loss and gain of function to study the same receptor in vivo. For example, cardiac hypertrophy develops in TG mice overexpressing angiotensin AT1 receptors in the myocardium, but pressure overload and stretch-induced hypertrophy still occur in AT1 knockout mice. Our previous work showed that ablation of Gq-coupled 5-HT$_{2B}$Rs in mice leads to dilated cardiomyopathy without hypertrophic response, and the current study shows that overexpression of this receptor leads to a compensated cardiac hypertrophy.

Several factors might be responsible for the development of compensatory hypertrophy after 5-HT$_{2B}$R overexpression. A first possibility is a change in signaling pathways and Gq-protein repertoire coupling. In TG mice, cardiac-specific overexpression of constitutively active ε-PKC and ε-PKC isoforms caused identical nonpathological cardiac hypertrophy. TG mice with cardiac-restricted expression of an activated MEK1 had concentric hypertrophy without signs of cardiomyopathy or lethality, indicating a hypertrophic response associated with augmented cardiac function and partial resistance to apoptosis. Overexpression may modify the coupling of 5-HT$_{2B}$Rs that can activate p60$^{src}$ and MAPK pathways, phospholipase C and A$_2$, and nitric oxide synthesis.

A second possible cause of hypertrophy after overexpression of 5-HT$_{2B}$R is an altered proliferation/survival of cardiomyocytes. Recently, we have shown that activation of 5-HT$_{2B}$R inhibits apoptosis induced by serum withdrawal in isolated cardiomyocytes. Moreover, 5-HT$_{2B}$R knockout newborn mice have ventricular hypoplasia as the result of impaired proliferation of cardiomyocytes. It is possible that overexpression of 5-HT$_{2B}$R in heart activates the mitogenic pathway before birth and then inhibits apoptosis, because cardiomyocytes became terminally differentiated and nonproliferative shortly after the birth. Overexpression of this receptor in the 5-HT$_{2B}$R TG mouse heart leads to hypertrophy associated with mitochondrial proliferation and increased mitochondrial enzyme activity such as COX and SDH.

The role of mitochondria in transition from compensatory hypertrophy to maladaptation has not been clearly elucidated. It is likely that mitochondrial functional changes are associated with compensatory hypertrophy (adaptive response). However, failing mitochondrial functions occur with heart failure (decompensation) and induction of apoptotic signaling. To modify or abort decompensation, intrinsic determinants of mitochondrial apoptosis have recently been elucidated. Nix/Bnip3, a critical component of the apoptotic program for Gq-mediated apoptosis of cardiomyocytes, is...
observed with ANT downregulation in the heart of 5-HT_{2b}R-overexpressing TG mice. In cardiomyocytes, ANT may thus regulate functions of mitochondria, which are important cellular components that act at hypertrophy or decompensation. In conclusion, our findings show that overexpression of 5-HT_{2b}R leads to hypertrophic cardiomyopathy and is associated with altered mitochondrial function.

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