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Involvement of the Serotonin 5-HT_{2B} Receptor in Cardiac Hypertrophy Linked to Sympathetic Stimulation

Control of Interleukin-6, Interleukin-1 β , and Tumor Necrosis Factor- α Cytokine Production by Ventricular Fibroblasts

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Background—The serotonergic 5-HT_{2B} receptor regulates cardiomyocyte development and growth. A putative contribution of this receptor to fibroblast-dependent cardiac function has not been identified.

Methods and Results—By mimicking sympathetic stimulation with chronic isoproterenol perfusion in vivo, we found that mice developed a cardiac hypertrophy, which was prevented by exposure to the 5-HT_{2B} receptor antagonists SB206553 or SB215505 or in 5-HT_{2B} receptor–knockout mice. The isoproterenol-induced hypertrophy was associated with an increase in the plasma levels of interleukin-1 β and tumor necrosis factor- α but not interleukin-6. In contrast, the plasma isoproterenol-induced cytokine increase was not observed in either 5-HT_{2B} receptor–mutant or wild-type mice perfused with isoproterenol+SB206553. We demonstrated that stimulation of wild-type cardiac fibroblasts by isoproterenol markedly increased the production of the interleukin-6, interleukin-1 β , and tumor necrosis factor- α cytokines. Strikingly, we found that this isoproterenol-induced cytokine production was abolished by SB206553 or in 5-HT_{2B} receptor–knockout fibroblasts. Serotonin also stimulated production of the 3 cytokines in wild-type fibroblasts, which was effectively reduced in 5-HT_{2B} receptor–knockout fibroblasts.

Conclusions—Our results demonstrate for the first time that 5-HT_{2B} receptors are essential for isoproterenol-induced cardiac hypertrophy, which involves the regulation of interleukin-6, interleukin-1 β , and tumor necrosis factor- α cytokine production by cardiac fibroblasts. (*Circulation*. 2004;110:969-974.)

Key Words: fibroblasts ■ hypertrophy ■ interleukins ■ nervous system, sympathetic ■ remodeling

Myocardial hypertrophy has long been considered an adaptive process to increased wall stress in the heart.¹ Sustained cardiac hypertrophy is often maladaptive and leads to significant ventricular dysfunction and failure.² This phenomenon involves not only an increase in size and mass of the cardiomyocytes but equally, the activation of cardiac fibroblasts.³ Recent studies have emphasized the role of G protein–coupled receptors in the initiation of processes that play crucial roles in myocyte hypertrophy⁴ and fibroblast stimulation.⁵ Moreover, it was demonstrated that G_q/G₁₁ proteins activated after stimulation of either AT₁ angiotensin II (AGII), α_1 -adrenergic (AR), or ET_A endothelin 1 receptors are key regulators of these hypertrophic responses.⁶ Although the action of these mediators on cardiomyocytes is direct, it also implicates autocrine and paracrine factors. For example, AR stimulation has been demonstrated to induce the release of the hypertrophic cytokines interleukin-6 (IL-6),

interleukin-1 β (IL-1 β), and tumor necrosis factor- α (TNF- α) from cardiac fibroblasts,⁷ which are the predominant source of cytokines in myocardium.⁸ In vivo, the 3 cytokines are produced after either myocardial infarction or a cardiac remodeling process⁹ triggered by sympathetic overstimulation. An increase in serotonin (5-HT) levels has been identified in normal and failing heart,¹⁰ and its release could be associated with sympathetic overstimulation,¹¹ contributing to myocardial remodeling in left ventricular dysfunction.

The effects of 5-HT are mediated by actions on numerous cognate receptors belonging to the G protein–coupled receptors and ionotropic receptors. Activation of the 5-HT₂ G_q/G₁₁-coupled subtypes participates in cell proliferation.¹² We have previously shown that 5-HT, via the 5-HT_{2B} receptor (5-HT_{2B}R), regulates cardiac embryonic development and adult functions; gene targeting of the 5-HT_{2B}R gene by homologous recombination leads to a dilated cardiomyopathy without

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hypertrophy.^{12,13} Alternatively, the selective overexpression of the 5-HT_{2B}R in cardiomyocytes induces a myocardial hypertrophy,¹⁴ and a direct survival effect of 5-HT in cardiomyocytes was placed in evidence.¹⁵ Therefore, the G_q-coupled 5-HT_{2B}R could be implicated in trophic responses of the myocardium by acting directly on cardiomyocytes or indirectly on noncardiomyocytes through the release of paracrine factors.

The aims of the present work were to study whether, on β -adrenergic stimulation, the 5-HT_{2B}R could (1) modify the cardiac hypertrophic responses *in vivo* and (2) contribute to IL-6, IL-1 β , and TNF- α cytokine production by ventricular fibroblasts.

Methods

5-HT_{2B}R-Knockout Mice

Targeted mutagenesis has been described previously.¹² Animal experimentation was performed in accordance with institutional guidelines. Protocols were approved by the French Animal Care Committee in accordance with European regulations. 129/PAS (genetic background of KO mice) served as wild-type (WT) controls.

Induction of Cardiac Hypertrophy by Isoproterenol

In 11-week-old mice, vehicle (saline), isoproterenol (ISO) (30 $\mu\text{g} \cdot \text{g}^{-1} \cdot \text{d}^{-1}$) alone, or ISO associated with the 5-HT_{2B}R antagonist SB206553 or SB215505 (1 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) (all chemicals from Sigma) was delivered by osmotic minipumps (1007D, Alzet Corporation) implanted subcutaneously under anesthesia (sodium pentobarbital, 40 mg/kg IP). After 5 days, the heart was excised and weighed, and the apex was quickly frozen. The remaining left ventricle was fixed in 4% paraformaldehyde PBS solution. For planimetric quantification of cardiomyocyte area, at least 40 cardiomyocytes were measured on 1 midventricular section. Heart rate was recorded by the tail-cuff method (Leticia Model 5002).¹³ For telemetric measurements, mice anesthetized with ketamine (100 mg/kg) and xylazine (0.4 mg/kg) were fitted with the TA11PA-C20 sensor model (Data Sciences) placed in the right common carotid artery.¹⁶ Seven days later, basal parameters were recorded every 5 minutes over a period of 24 hours before implantation of an osmotic minipump. The mean values of systolic and diastolic blood pressures and heart rate were calculated for the whole of this period. Similarly, the animals were recorded over a period of 24 hours after 7 days of treatment. Transthoracic echocardiograms were performed with a 15-MHz linear transducer on a Sonos 5500 (Philips) in anesthetized mice (sodium pentobarbital, 30 mg/kg IP).¹³ This anesthetic was selected to exemplify, and reveal, any cardiodepressive property of SB206553 better than echocardiography in the conscious state.

5-HT_{2B}R Expression in Fibroblasts

Semiquantitative reverse transcription-polymerase chain reaction was performed on 2 μg of total RNA.¹³ The following primers were used: 5'-AAGCCTCGAAGTGGACAATTGATG-3' (5-HT_{2A}R forward), 5'-AATGATTTTCAGGAAGGCTTTGGTT-3' (5-HT_{2A}R reverse), 5'-ACAATTCTGAGCACATTTTAAG-3' (5-HT_{2B}R forward), 5'-AATTAACCATACCCTGTAATC-3' (5-HT_{2B}R reverse), 5'-CCCTTATTGACCTCAACTACATGGT-3' (GAPDH forward), and 5'-GAGGGGCCATCCACAGTCTTCTG-3' (GAPDH reverse).

Adult Cardiac Fibroblast Primary Culture

Cultures of fibroblasts were obtained from the ventricles of adult (10- to 12-week-old) mice using a modification of a previously described protocol.¹⁷ After anesthesia, the heart was excised and the ventricles, free from the atria and atrioventricular valves, were minced and incubated with 0.1 mg/mL type IV collagenase and 1 mg/mL pancreatin (Sigma) at 37°C. Cells were plated in Dulbecco/

Ham F12 medium with 10% calf serum and gentamicin. After a 2-hour incubation period at 37°C in 5% CO₂/95% air, the unattached cardiomyocytes were removed, and the attached cells (mostly fibroblasts) were grown. All experiments were performed using cells of the first passage. Cardiac fibroblasts were identified by characteristic morphology and positive staining with antibody to vimentin (Sigma).¹⁷ One day before the experiments, cells were transferred to serum-free medium.

Dosage of Cytokines

Cultured fibroblasts corresponding to each time point were plated in 6-well plates. Supernatants and cells were collected at 0, 2, 4, 8, 12, and 24 hours after onset of stimulation. This was performed after stimulation with ISO (10 $\mu\text{mol/L}$) in the absence or presence of the 5-HT_{2B}R antagonist SB 206553 (100 nmol/L). Cells were stimulated with 5-HT (1 $\mu\text{mol/L}$) and BW723C86 (BW) (100 nmol/L) (Sigma). The plasma cytokine quantification was performed after centrifugation (2000g, 10 minutes) of 1 mL total blood. Concentrations of IL-6, IL-1 β , and TNF- α were measured in plasma and cell culture supernatants by ELISA kits (DY 406, DY 401, and DY 410, R&D).

Binding Assays

Radioligand binding studies of β_1 - and β_2 -ARs in WT and KO mice hearts were performed according to Molenaar et al.¹⁸ β -ARs were labeled with (-)-[¹²⁵I]-cyanopindolol (Amersham). The proportions of β_1 - and β_2 -ARs were determined by use of CGP 20712A (β_1 -selective antagonist) and ICI 118,551 (β_2 -selective antagonist) (Sigma). K_d and B_{max} were obtained from competition curves.

Data Analysis and Statistics

All results are expressed as mean \pm SEM. Different groups were compared by 1-way ANOVA followed by a Newman-Keuls test. With 2 groups, the comparison was performed using a Student's *t* test. All calculations were made with the GraphPad Prism program (San Diego).

Results

Role of 5-HT_{2B}R in ISO-Mediated Cardiac Hypertrophy *In Vivo*

We used the classic chronic β -AR agonist ISO perfusion (30 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) as a model of cardiac hypertrophy.¹⁹ In WT mice, 5 days of perfusion produced a marked increase in the ratio of cardiac mass to tibia length (CMTL) (59 \pm 17%, ISO versus WT) (Figure 1A). The ISO perfusion induced an important tachycardia (62 \pm 4% in WT-ISO versus WT) (Figure 1B). The increase in cardiac mass was primarily because of the hypertrophy of cardiomyocytes (Figure 2, A and B). The ISO-induced increase in CMTL was completely prevented by the 5-HT_{2B/2C}R antagonist SB206553 (1 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) (Figures 1A and 2B) but not the ISO-induced tachycardia (Figure 1B). Similarly, the selective 5-HT_{2B}R antagonist SB215505 (1 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) prevented the ISO-induced increase in CMTL but not the tachycardia. In 5-HT_{2B}RKO mice, the ISO perfusion produced a slight increase (19%) in CMTL, which was significantly lower than WT ISO-treated mice (Figures 1A and 2B). However, in 5-HT_{2B}RKO mice, ISO induced a tachycardia similar to that observed in WT mice (Figure 1B). The β_1 - and β_2 -AR expression levels (B_{max}) were not significantly different in 5-HT_{2B}RKO mice compared with control animals (Figure 2C); the respective affinity constants (K_d) were also not modified (data not shown). The putative cardiovascular effects of SB206553 (1 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) were investigated in WT mice by telemetry and echocardiography. Chronic expo-

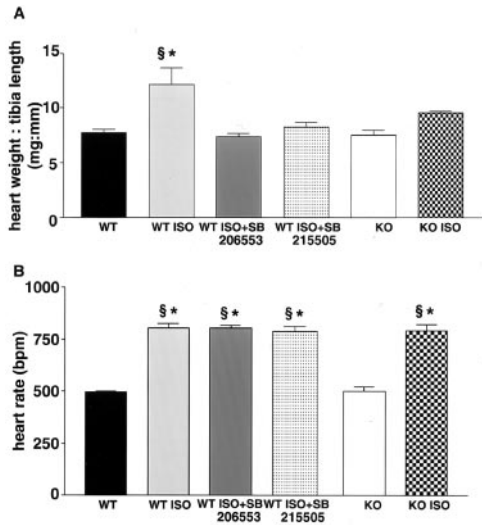


Figure 1. In vivo cardiac effects of chronic ISO perfusion. ISO-induced effects were evaluated by measuring heart weight:tibia length (mg/mm) (A) and assessing heart rate in beats per minute (bpm) by tail-cuff method (B) in WT and in 5-HT_{2B}RKO (KO) mice in absence or presence of 5-HT_{2B}R antagonists SB206553 (n=6) or SB215505 (n=3). **P*<0.05 vs KO, §*P*<0.05 vs WT.

sure to this drug modified neither the cardiac function nor the blood pressure in WT mice (Table). The ISO-induced hypertrophy observed in the WT mice is thus dependent on 5-HT_{2B}Rs.

Role of 5-HT_{2B}Rs in ISO-Mediated Increase in Cytokine Plasma Levels

Five days of ISO perfusion in WT mice increased the plasma levels of TNF- α significantly (2-fold over basal) and IL-1 β (2-fold) (Figure 3, B and C). These increases were prevented by SB206553. In 5-HT_{2B}RKO, the basal values of these 2 cytokines were not different from those of WT mice, and ISO was unable to increase their plasma concentrations (Figure 3, B and C). In WT mice, the perfusion of ISO, either alone or in combination with SB206553, did not significantly modify IL-6 plasma concentrations, nor did it do so in 5-HT_{2B}RKO. However, basal IL-6 plasma concentration was approximately twice as high in 5-HT_{2B}RKO mice as in WT mice (Figure 3A). Therefore, this ISO increase of TNF- α and IL-1 β plasma levels observed in the WT mice was prevented entirely by pharmacological or genetic ablation of 5-HT_{2B}Rs.

Role of 5-HT_{2B}Rs on IL-6, IL-1 β , and TNF- α Cytokine Production by Cardiac Fibroblasts Stimulated by ISO

We focused on cardiac fibroblasts, the main source of hypertrophic cytokines that are involved in the cardiac remodeling processes.¹⁷ Supernatants of primary cultures displayed no difference in basal levels of IL-6, TNF- α , and IL-1 β between WT and 5-HT_{2B}RKO. In WT fibroblasts, ISO (10 μ mol/L) induced a peak of cytokine production (IL-6, 12-fold over basal; TNF- α , 4-fold; and IL-1 β , 2-fold) between 4 and 8 hours of stimulation (Figure 4, A through C). In WT cells treated with SB206553 (100 nmol/L) or in 5-HT_{2B}RKO fibroblasts, a prevention of the ISO-induced

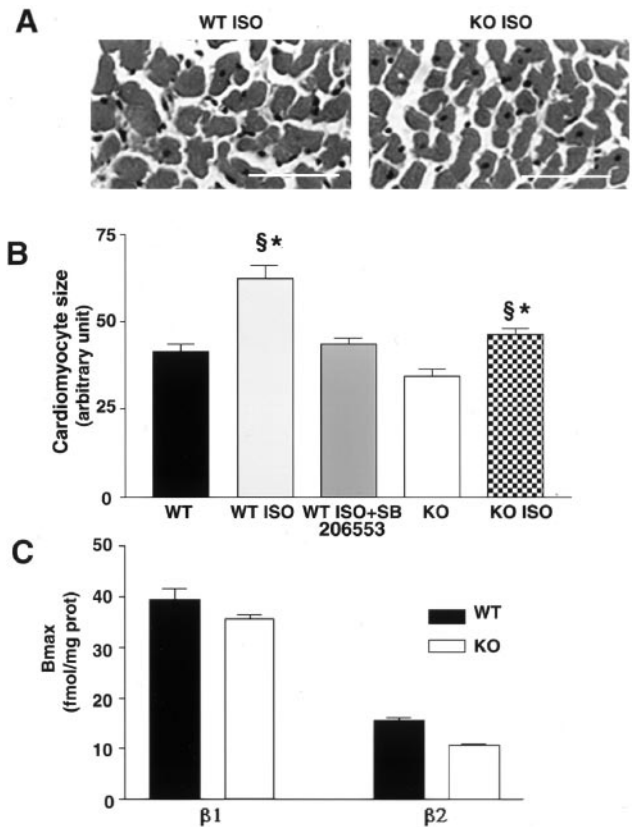


Figure 2. 5-HT_{2B}R blockade reduces cardiomyocyte hypertrophy. Representative sections from adult heart left ventricles of WT ISO-treated and KO ISO-treated mice (A) were measured by planimetric quantification of cardiomyocyte transversal surface (B) (n=6 each). **P*<0.05 vs KO, §*P*<0.05 vs WT. Bar=50 μ m. Observed hypertrophy is independent of modifications in β ₁- and β ₂-AR levels of expression assessed by binding studies (C); values are mean \pm SEM of 3 independent experiments performed in triplicate.

production of IL-6, TNF- α , and IL-1 β was observed. Therefore, 5-HT_{2B}Rs appear necessary for the induction by ISO of the 3 cytokines in cardiac fibroblasts.

Role of 5-HT_{2B}Rs on Cytokine Production by Cardiac Fibroblasts Stimulated by 5-HT

Adult WT cardiac ventricular fibroblasts constitutively express 5-HT_{2A} and 5-HT_{2B}Rs (Figure 5A). 5-HT (1 μ mol/L) markedly increased IL-6 (6-fold over basal), TNF- α (12-fold) and IL-1 β (4-fold) levels in WT fibroblasts at 4 hours after agonist exposure (Figure 5, B through D). In WT fibroblasts, the 5-HT-induced IL-1 β production was mimicked by stimulation with the 5-HT_{2B}R-selective agonist BW (100 nmol/L). BW reproduced 66% of the IL-6 response observed with 5-HT in WT. The response to 5-HT of KO fibroblasts reached only 30% of the WT IL-6 response. For TNF- α , BW induced a peak significantly smaller than the response to 5-HT in WT (\approx 50%), although similar to that in 5-HT_{2B}RKO fibroblasts. These findings indicate a role for 5-HT_{2B}Rs in the production of these 3 cytokines by cardiac fibroblasts in response to 5-HT.

Discussion

This work demonstrates for the first time that 5-HT_{2B}R blockade in vivo can reduce cardiac hypertrophy caused by

Cardiac Function and Blood Pressure

	Basal	SB206553 (7 d)
MBP, mm Hg	108±1	104±0.6
HR, bpm	556±8	551±17
LVM, mg	63±6	63±4
FS, %	37±1	34±2
CO, mL/min	36±11	30±6
E/A	1.5±0.2	1.8±0.1
IVRT, ms	17.3±2.0	17.3±0.6

Mean blood pressure [MBP=diastolic BP+(1/3)(systolic BP – diastolic BP)] and heart rate (HR) were obtained by telemetry. Echocardiographic parameters: left ventricular mass [LVM=1.055×[(S_d+PW_d+LVEDD)³–(LVEDD)³], where S_d indicates septal thickness in diastole; PW_d, posterior wall thickness in diastole; and LVEDD, left ventricular end-diastolic diameter], left ventricular fractional shortening [FS=(LVEDD–LVESD/LVEDD)×100; LVESD, left ventricular end-systolic diameter], cardiac output [CO=(AoD/2)²×π×VTI×HR, where AoD is the aortic diameter and VTI the velocity time integral of the aortic flow], ratio of the early rapid peak mitral filling wave velocity (E) to the late filling velocity caused by atrial contraction (A) (E/A), and the isovolumetric relaxation time (IVRT).

β-AR stimulation. Moreover, 5-HT_{2B}Rs are required for the in vivo regulation of TNF-α and IL-1β cytokine production. Our in vitro results indicate that the 5-HT_{2B}R is a key regulator for IL-6, TNF-α, and IL-1β production by myocardial fibroblasts in response to 5-HT and equally to β-AR activation, which explains, at least in part, our in vivo findings.

A role for 5-HT₂Rs in cardiac hypertrophy linked to hypertension and left ventricular dysfunction was suggested

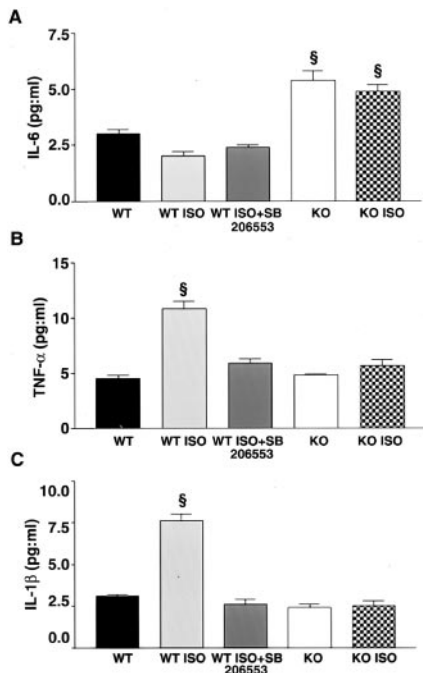


Figure 3. In vivo effects of chronic ISO perfusion on plasma cytokines. Plasma levels of IL-6 (A), TNF-α (B), and IL-1β (C) were evaluated by ELISA in WT and KO mice after 5 days of ISO treatment in absence or presence of SB206553. WT, n=5; KO, n=4; KO ISO, n=6; WT ISO, n=7; WT ISO+SB206553, n=7; §P<0.05 vs WT.

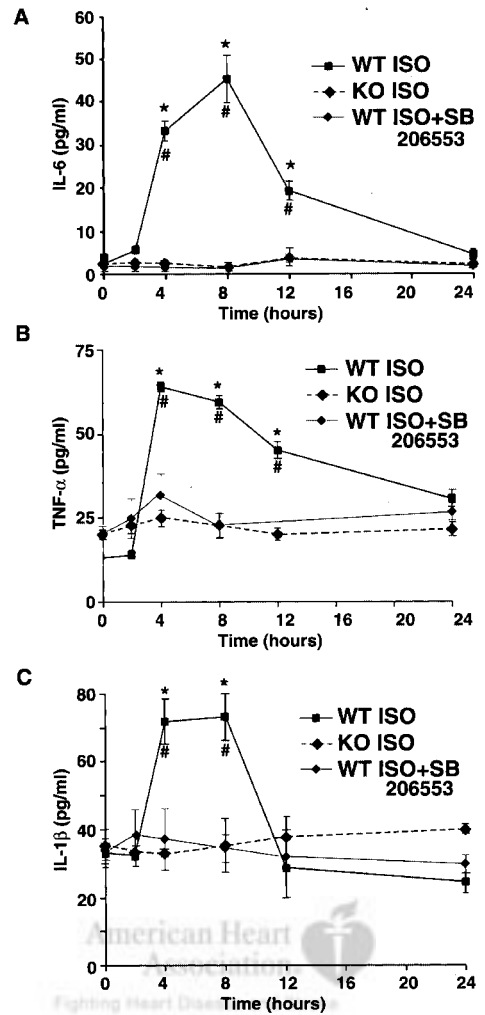


Figure 4. Cytokine production by cardiac fibroblasts in response to ISO. Production of IL-6 (A), TNF-α (B), and IL-1β (C) was measured in supernatant of cardiac fibroblast primary cultures after various times of exposure to ISO, n>4. *P<0.05, WT vs KO ISO; #P<0.05 vs WT ISO+SB206553.

by earlier reports on the antihypertrophic effects of the 5-HT₂ antagonist ketanserin.²⁰ More recently, sarpogrelate, a non-selective 5-HT₂ receptor antagonist, was reported to reduce the hypertrophic responses in cultured cardiomyocytes, alone or in combination with fibroblasts.²¹ β-ARs contribute to the cardiac hypertrophy linked to catecholamine overstimulation²² and to the activation of cardiac fibroblasts in chronic pressure overload.²³ Moreover, in rats, the chronic β-AR stimulation by ISO induces myocardial generation of IL-6, TNF-α, and IL-1β.⁷ In WT, we demonstrate that the cardiac hypertrophy induced by continuous ISO perfusion is completely prevented by the 5-HT_{2B}R antagonists SB206553 or SB215505 at doses that were previously shown to prevent the BW-induced hyperphagia in freely feeding rats.²⁴ The markedly reduced ISO-induced cardiac hypertrophy in 5-HT_{2B}RKO mice demonstrates the selectivity of the SB compounds. The small residual cardiac hypertrophy to ISO in KO mice could result from unknown compensatory mechanisms to the dilated cardiomyopathy of KO. Such a difference was previously described in

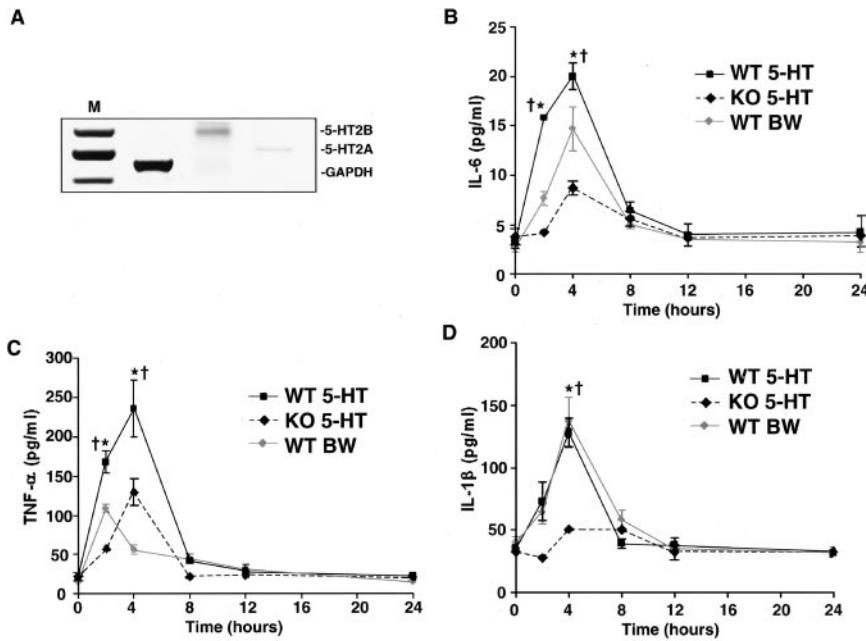


Figure 5. Cytokine production by cardiac fibroblasts in response to 5-HT. Reverse transcription–polymerase chain reaction reveals expression of 5-HT_{2A}R and 5-HT_{2B}R subtypes in cardiac ventricular fibroblasts with GAPDH as internal control (A); M, molecular weight marker. Production of IL-6 (B), TNF-α (C), and IL-1β (D) was measured in supernatant of cardiac fibroblast primary cultures after various times of exposure to 5-HT or BW, n>4. *P<0.05, WT vs KO 5-HT; †P<0.05 vs WT BW.

angiotensin II 1a receptor–knockout mice (AT1KO) in which the cardiac hypertrophy was not completely prevented after transverse aortic constriction²⁵ or myocardial infarction,²⁶ whereas the AT₁ antagonist losartan suppressed entirely the hypertrophic response of WT in the same models.^{27,28} Furthermore, strong alterations of the AR signaling pathway in 5-HT_{2B}RKO mice are unlikely to explain the reduction of ISO-induced hypertrophic effect, as suggested by the similarities in the number of β-adrenergic cardiac binding sites and ISO-induced tachycardia in both WT and KO mice.

The ISO-induced increase in TNF-α and IL-1β plasma levels, which is observed in WT, is completely prevented by pharmacological (SB) or genetic ablation of the 5-HT_{2B}R, supporting the *in vivo* role of this receptor in cardiac IL-6, IL-1β, and TNF-α cytokine production. Similar to rats,⁷ the increase in plasma IL-6 was not found in response to ISO in either WT or KO mice. The high resting level of plasma IL-6 in KO mice could be of noncardiac origin, because we were unable to detect its mRNA expression in their myocardium (data not shown). Moreover, no inflammatory cells were identified in the myocardium of these animals.¹³

In ISO-induced cardiac hypertrophy, fibroblasts constitute a main source of hypertrophic cytokines. As expected, we found that ISO increased the production of IL-6, TNF-α, and IL-1β in WT fibroblasts. According to our *in vivo* results, this increase is prevented by the 5-HT_{2B/2C} antagonist SB206553 at 100 nmol/L,²⁹ which, here, can be considered as a pure 5-HT_{2B}R antagonist, 5-HT_{2C}Rs not being expressed either in heart³⁰ or in vasculature.³¹ Although changes in IL-6 plasma levels could not be detected *in vivo*, our *in vitro* results confirm that at least part of the ISO-induced plasma cytokines originate from cardiac fibroblasts.

Our work reveals for the first time that 5-HT markedly increased the production of IL-6, TNF-α, and IL-1β in WT cardiac fibroblasts, which was mimicked by the 5-HT_{2B}R preferential agonist BW. BW exhibits a significantly higher

affinity at 5-HT_{2B}R than 5-HT_{2A}R.²⁴ Our work demonstrates that in adult mice cardiac fibroblasts, the 5-HT–induced production of IL-1β involves only 5-HT_{2B}Rs, because the IL-1β production is eliminated in 5-HT_{2B}RKO fibroblasts, whereas BW provoked a response superimposable to 5-HT in WT cells. The 5-HT_{2B}R is also a main contributor of 5-HT–induced IL-6 and TNF-α cytokine production. BW only partially reproduced the maximum 5-HT–induced TNF-α or IL-6 responses in WT fibroblasts, with different kinetics for TNF-α. In KO cells, these maximum responses are reduced but not eliminated. These findings indicate that stimulation of 5-HT_{2B}Rs constitutes an essential trigger for the complete production of TNF-α and IL-6 in combination with other 5-HTRs. This result is in agreement with observations indicating that 5-HT stimulates IL-6 production in vascular smooth muscle cells.³² Taken together, these data suggest that 5-HT participates in *in vivo* cardiac hypertrophy.

The mechanisms by which 5-HT_{2B}Rs control ISO-induced IL-6, IL-1β, and TNF-α cytokine production remain to be elucidated. Crosstalk between G_q-coupled 5-HT_{2B}R and G_s-coupled β-AR signaling pathways could be suggested, because a recent report demonstrated a dual transinhibition of AT₁ and β-AR by an antagonist targeting a single receptor³³; the effects of valsartan are obtained in the absence of angiotensin II. These findings support our result showing that the 5-HT_{2B}R antagonist SB206553 blocks the ISO-induced IL-6, IL-1β, and TNF-α cytokine production by cardiac fibroblasts in the absence of 5-HT. The possible existence of complexes formed between β-ARs and 5-HT_{2B}Rs that would explain these interactions is under investigation. Whether hypertrophic responses to agents such as angiotensin II or endothelin 1 could also be affected by 5-HT_{2B} receptor blockade will be examined in subsequent studies.

In conclusion, the lack of a cardiodepressive property of the 5-HT_{2B}R antagonist, together with the essential role of 5-HT_{2B}Rs in cardiac hypertrophy triggered by β-AR stimu-

lation, suggests that 5-HT_{2B}R antagonists could constitute new opportunities to prevent or reduce myocardial remodeling associated with left ventricular dysfunction and sympathetic overactivity.

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