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Serotonin 5-HT$_{2B}$ Receptor Blockade Prevents Reactive Oxygen Species–Induced Cardiac Hypertrophy in Mice

Laurent Monassier, Marc-André Laplante, Fabrice Jaffré, Pascal Bousquet, Luc Maroteaux, Jacques de Champlain

Abstract—We established previously that 5-HT$_{2B}$ receptors are involved in cardiac hypertrophy through the regulation of hypertrophic cytokines in cardiac fibroblasts. Moreover, the generation of reactive oxygen species and tumor necrosis factor–α through the activation of reduced nicotinamide-adenine dinucleotide phosphate [NAD(P)H] oxidase has been implicated in cardiac hypertrophy. In this study, we investigated whether 5-HT$_{2B}$ receptors could be involved in the development of cardiac hypertrophy associated with superoxide anion production. Therefore, we measured the effects of serotonergic 5-HT$_{2B}$ receptor blockade on left-ventricular superoxide anion generation in 2 established pathological models of cardiac hypertrophy, ie, angiotensin II and isoproterenol infusions in mice. Angiotensin II infusion for 14 days increased superoxide anion concentration (+32%), NAD(P)H oxidase maximal activity (+84%), and p47$^{phox}$ NAD(P)H oxidase subunit expression in the left ventricle together with hypertension (+37 mm Hg) and cardiac hypertrophy (+17% for heart weight:body weight). The 5-HT$_{2B}$ receptor blockade by a selective antagonist (SB215505) prevented the increase in cardiac superoxide generation and hypertrophy. Similarly, infusion for 5 days of isoproterenol increased left-ventricular NAD(P)H oxidase activity (+48%) and cardiac hypertrophy (+31%) that were prevented by the 5-HT$_{2B}$ receptor blockade. Finally, in the primary culture of left-ventricular cardiac fibroblasts, angiotensin II and isoproterenol stimulated NAD(P)H oxidase activity. This activation was prevented by SB215505. These findings suggest that the 5-HT$_{2B}$ receptor may represent a new target to reduce cardiac hypertrophy and oxidative stress. Its blockade affects both angiotensin II and β-adrenergic trophic responses without significant hemodynamic alteration. (Hypertension. 2008;52:301-307.)

Key Words: 5-HT$_{2B}$ ■ NAD(P)H oxidase ■ superoxide anion ■ angiotensin ■ adrenergic ■ cardiac ■ hypertrophy

Pathological left ventricular hypertrophy has been associated with increased production of reactive oxygen species (ROS). Recent works have shown that antioxidants inhibit in vitro cardiomyocyte hypertrophy and in vivo aortic banding-induced left ventricular hypertrophy. The cardiac ROS production can be triggered by factors such as angiotensin II (Ang II), catecholamines, endothelin-1, tumor necrosis factor-α (TNF-α), and serotonin but also by mechanical stretch. Among these factors, those that signal through the G$_q$/PLC pathway seem to play a crucial role in the initiation and maintenance of cardiac hypertrophy and are known to stimulate the cardiac ROS generation through the phagocyte-type reduced nicotinamide-adenine dinucleotide phosphate [NAD(P)H] oxidase. The gp91$^{phox}$-containing NAD(P)H oxidase plays a pivotal role in the response to Ang II via a pathway involving protein kinase C, c-Src, and phosphatidyl-inositol 3-kinase. NAD(P)H oxidase reduces oxygen O$_2^-$, leading to the formation of superoxide anion (O$_2^{−}$), which can be either dismutated spontaneously to hydrogen peroxide or in a reaction involving superoxide dismutase (SOD).

The 5-HT$_{2B}$ receptor (5-HT$_{2BR}$) is a G$_q$/G$_{11}$ protein-coupled receptor that has been shown to be functionally coupled to ROS synthesis through NAD(P)H oxidase stimulation in a neuroectodermal cell line (1C11). Interestingly, 5-HT$_{2BR}$s appear to control the TNF-α shedding in the extracellular space via NAD(P)H oxidase–dependent TNF-α–converting enzyme activation. This 5-HT$_{2BR}$–dependent NAD(P)H oxidase activation could contribute to the previously described effect of 5-HT$_{2BR}$ blockade on TNF-α release by ventricular fibroblasts after isoproterenol (ISO) stimulation. We established that 5-HT$_{2BR}$s are essential for ISO-induced cardiac hypertrophy and are involved in the regulation of hypertrophic cytokines, interleukin-6, interleukin-1β, and TNF-α production by cardiac fibroblasts. We hypothesized that

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5-HT<sub>2A</sub>R blockade could affect NAD(P)H-oxidase function and, therefore, participate in the modulation of hypertrophic pathways implying this key enzyme.

The aim of the present study was to determine whether 5-HT<sub>2A</sub>Rs could participate in O<sub>2</sub><sup>-</sup> generation during the course of pharmacological-induced cardiac hypertrophy by ISO and Ang II. Its roles in the regulation of the oxidative balance between NAD(P)H oxidase and SOD activities and in the O<sub>2</sub><sup>-</sup> production by left-ventricular fibroblasts have been investigated.

**Methods**

**Animals**

Studies were performed in 129S1/SvImJ mice in accordance with the Canadian Council for Animal Care Guidelines and monitored by the ethical committee for experimental research from the Clinical Research Institute of Montreal Canada (No. 2004-11).

**Induction of Cardiac Hypertrophy by ISO and Ang II**

Mice were infused by either vehicle, ISO (30 mg·kg<sup>-1</sup>·d<sup>-1</sup>), or Ang II (0.2 mg·kg<sup>-1</sup>·d<sup>-1</sup>; please see the detailed procedures available in the online data supplement at http://hyper.ahajournals.org). The following drugs were tested on cardiovascular responses to ISO and/or Ang II: the 5-HT<sub>2A</sub>R antagonist SB215505 (1 mg·kg<sup>-1</sup>·d<sup>-1</sup>), the antagonist of β-adrenergic receptors propranolol (5 mg·kg<sup>-1</sup>·d<sup>-1</sup>), or the NAD(P)H oxidase inhibitor apocynin (1.5 mmol/L, drinking water).

Heart rate and systolic arterial pressure were recorded by the tail-cuff method (Visitech), and transthoracic echocardiograms were performed in 2% isoflurane anesthetized mice, as described previously<sup>3</sup> and transferred to serum-free medium before a direct measurement of the heart weight:body weight ratio (LVMBW) increase as assessed by echocardiographic measurement of the left ventricle mass:body weight ratio (LVM:BW) increase (A) without effect on the systolic arterial pressure (SAP) during Ang II infusion (B) and ISO-induced tachycardia (HR; difference: 9%; P=0.07; C). #P<0.05 vs controls; ∗P<0.05 vs Ang II (n=8) or ISO alone (n=9; all controls were pooled in A). Statistical analysis was performed on each corresponding control group (n=8 each); P>0.05 between the 2 control groups.

5-HT<sub>2A</sub>R antagonist, SB215505 (10<sup>-5</sup> mol/L) alone or simultaneously with the NAD(P)H oxidase inhibitor apocynin (7 mol/L). After treatments, cells were washed in oxygenated Krebs-Hepes buffer. The O<sub>2</sub><sup>-</sup> production was measured with the lucigenin method and adjusted by the protein concentration (counts per minute per microgram) of the samples.

**Data Analysis and Statistics**

Data are expressed as means±SEMs. Statistical comparisons were made by ANOVA followed by the Bonferroni’s method with the GraphPad Prism program (GraphPad Software). A value of P<0.05 was considered significant.

**Effect of the 5-HT<sub>2A</sub>R Antagonist, SB215505, on Chronic Ang II–Induced Cardiovascular Alterations**

The Ang II infusion induced cardiac hypertrophy as assessed by echocardiographic measurement of the left ventricle mass: body weight ratio (+52% over controls; Figure 1A) and direct measurement of the heart weight:body weight ratio (+17% versus controls; Table). A simultaneous 37-mm Hg increase in blood pressure (Figure 1B) and no significant heart rate change (595±14 bpm in Ang II versus 605±22 bpm in controls; P>0.05) were observed. In response to the afterload increase, the fractional shortening was also increased (Table). SB215505 reduced the Ang II–induced left ventricular hypertrophy (Table and Figure 1A) without affecting the increased blood pressure that remained elevated.

**Figure 1.** In mice, SB215505 (SB) prevents Ang II- and ISO-induced left-ventricular mass:body weight ratio (LVM:BW) increase (A) without effect on the systolic arterial pressure (SAP) during Ang II infusion (B) and ISO-induced tachycardia (HR; difference: 9%; P=0.07; C). #P<0.05 vs controls; ∗P<0.05 vs Ang II (n=8) or ISO alone (n=9; all controls were pooled in A). Statistical analysis was performed on each corresponding control group (n=8 each); P>0.05 between the 2 control groups.

**SOD Activity Measurements**

SOD enzymatic activity was measured according to the hematoxylin method of Chattopadhyay et al.<sup>7</sup> The enzymatic reaction was assessed with 10 µg of proteins and 50 µmol/L of hematoxylin with a UV-visible recording spectrophotometer (Shimadzu Corp).

**gp47-phox Ventricular Expressions**

Ventricular tissue was crushed in liquid nitrogen. Lysis and Western blots were performed as described previously<sup>6</sup> with 35<sup>S</sup>-labeled rabbit [H-195] sc-14015). Animals

**Conclusion**

Heart rate and systolic arterial pressure were recorded by the tail-cuff method (Visitech), and transthoracic echocardiograms were performed in 2% isoflurane anesthetized mice, as described previously<sup>3</sup> and transferred to serum-free medium before a direct measurement of the heart weight:body weight ratio (LVMBW) increase as assessed by echocardiographic measurement of the left ventricle mass:body weight ratio (LVM:BW) increase (A) without effect on the systolic arterial pressure (SAP) during Ang II infusion (B) and ISO-induced tachycardia (HR; difference: 9%; P=0.07; C). #P<0.05 vs controls; ∗P<0.05 vs Ang II (n=8) or ISO alone (n=9; all controls were pooled in A). Statistical analysis was performed on each corresponding control group (n=8 each); P>0.05 between the 2 control groups.

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Data are expressed as means±SEMs. Statistical comparisons were made by ANOVA followed by the Bonferroni’s method with the GraphPad Prism program (GraphPad Software). A value of P<0.05 was considered significant.

**Results**

Effect of the 5-HT<sub>2A</sub>R Antagonist, SB215505, on Chronic Ang II–Induced Cardiovascular Alterations

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by 40 mm Hg over controls (Figure 1B). The heart rate was unaffected by the SB215505 treatment (584±17 bpm; *P*>0.05 versus controls), and the fractional shortening was still increased compared with controls (Table).

**Effect of the 5-HT$_{2B}$R Antagonist, SB215505, on Chronic ISO-Induced Cardiovascular Alterations**

ISO induced a cardiac hypertrophy measured by echocardiography (+117% over controls for left ventricle mass:body weight ratio; Figure 1A) and direct measurement of the heart weight:body weight ratio (+31%; Table). This hypertrophy was associated with left ventricular dilatation, as shown by the increase in end-diastolic diameter (+16%; Table and Figure 2) and tachycardia (+17%; *P*<0.05; Figures 1C and 2). No significant changes in blood pressure (115±7 mm Hg in controls versus 114±6 mm Hg in ISO; *P*>0.05) and cardiac contractility (fractional shortening) were detected. The cardiac output was preserved (Table). To confirm that these effects were mediated by β-adrenergic receptors, mice were simultaneously treated by ISO and the nonselective β-adrenergic antagonist propranolol. This compound reduced cardiac hypertrophy (Table) and tachycardia induced by ISO (744±38 bpm in ISO versus 619±24 bpm in ISO+propranolol; *P*<0.05) but also slightly reduced the cardiac output. SB215505 prevented the left ventricular hypertrophy caused by ISO (Figure 1A and Table) and the cardiac dilatation, the end-diastolic diameter (end-diastolic diameter) being similar by ISO (Figure 1A and Table) and the cardiac dilatation, the end-diastolic diameter (end-diastolic diameter) being similar.

**Involvement of the 5-HT$_{2B}$R and NAD(P)H Oxidases on Cardiac O$_2$\textsuperscript{−} Production in Ang II/ISO-Induced Cardiovascular Alterations**

Ang II increased the basal O$_2$\textsuperscript{−} generation, as well as the NADPH-stimulated NAD(P)H oxidase activity in the left ventricle (Figure 3A and 3B). SB215505 completely normalized O$_2$\textsuperscript{−} concentrations in the left ventricle. Ang II induced an increase of the p47\textsuperscript{phox} subunit expression, which was not affected by the simultaneous SB215505 treatment (Figure 4). In the aorta, Ang II also increased basal (623±54 cpm·mg$^{-1}$ in controls to 1145±88 cpm·mg$^{-1}$ in Ang II; *P*<0.05) and NAD(P)H oxidase-mediated (11 8076±23 709 cpm·mg$^{-1}$ in controls to 146 749±10 559 cpm·mg$^{-1}$; *P*<0.05) O$_2$\textsuperscript{−} concentration, but SB215505 was without effect on this Ang II response (820±107 cpm·mg$^{-1}$ in basal and 181 449±19 596 cpm·mg$^{-1}$ after NAD(P)H; *P*>0.05 versus Ang II alone). To verify that the SB215505 effects were not related to a nonspecific antioxidant property, we showed in an in vitro based assay that, in contrast to glutathione and ascorbic acid, it was unable to reduce the level of spontaneous hematoxylin oxidation (please see the data supplement). Moreover, mice subcutaneously infused with 1 mg·kg$^{-1}$ SB215505 alone (5 or 14 days), did not demonstrate any decrease in basal or
NAD(P)H-stimulated O$_2^-$ ventricular concentration (please see the data supplement). In conclusion, the pharmacological blockade of 5-HT$_{2B}$Rs abolished the NAD(P)H oxidase over-activation and, therefore, the O$_2^-$ generation induced by Ang II in the left ventricle.

In contrast to Ang II, the chronic ISO infusion did not modify the basal concentration of O$_2^-$ in the left ventricle (Figure 3A). However, when the maximal activity of NAD(P)H oxidases was tested by the addition of a saturating NAD(P)H concentration (100 μmol/L), we observed a 48% increase in the left ventricular O$_2^-$ production as compared with control animals (Figure 3B). SB215505 prevented the ISO-induced NAD(P)H oxidase activation without significant change in the basal O$_2^-$ concentration after ISO (Figure 3).

To analyze whether the increase in left-ventricular NAD(P)H oxidase activity was because of a change in NAD(P)H complex expression, the p47$^{phox}$ subunit expression was assessed by Western blots. ISO induced a 20% ($P<0.05$) increase in left ventricular p47$^{phox}$ subunit expression (Figure 4). Similar to Ang II+SB215505-treated mice, this overexpression was not affected by SB215505 (Figure 4). Taken together, these data show that Ang II and ISO induced an increase in left ventricular O$_2^-$ generation in association with an increase in the p47$^{phox}$ NAD(P)H oxidase subunit expression.

**Effect of 5-HT$_{2B}$R Blockade on O$_2^-$ Production and NAD(P)H Oxidase Activity in Primary Culture of Left Ventricular Fibroblasts**

We analyzed the role of the 5-HT$_{2B}$R on NAD(P)H oxidase activity after a 24-hour stimulation with either Ang II or ISO in left-ventricular fibroblasts. Ang II and ISO did not affect the basal O$_2^-$ production (data not shown) but induced, respectively, a 34% and a 42% increase in NAD(P)H oxidase–mediated O$_2^-$ concentration compared with controls (Figure 6). These increases were completely prevented by the simultaneous SB215505 treatment, indicating that 5-HT$_{2B}$Rs...
The notion that Ang II type 1 and 5-HT2BRs are not interacting could be tested whether the blockade of 5-HT2BRs could reduce Ang II–induced hypertrophy and cardiac ROS generation in mice. Ang II induced a left ventricular hypertrophy associated with an increase in O2− production but no increase of SOD-mediated generation in basal and stimulated conditions. The reduction of the NAD(P)H oxidase activity by 5-HT2BR blockade does not take place at the expression level, because p47phox subunit was still overexpressed. This drug was selected because of its higher selectivity (∼10) for 5-HT2BRs compared with 5-HT2C.10 Moreover, this drug is a potent antagonist, and we have shown previously that only 5-HT2A and 5-HT2B receptors are expressed in the heart. Interestingly, this antihypertrophic action was achieved without reduction of arterial blood pressure, attesting to the load-independent cardiac antihypertrophic effect of SB215505. In the aorta, Ang II also increased basal and NAD(P)H oxidase–mediated O2− concentration, which was insensitive to SB215505, supporting the notion that Ang II type 1 and 5-HT2BRs are not interacting in aorta. Also, SB215505 neither affected the basal O2− concentration nor the NAD(P)H oxidase maximal activity in control animals (please see the data supplement), and experiments using hematoxylin autooxidation did not reveal any direct chemical antioxidative properties of the compound. Therefore, SB215505 is not a nonspecific antioxidant and does not directly inhibit the NAD(P)H oxidase complex. These data demonstrate that the 5-HT2BR is a key regulator of Ang II–mediated cardiac hypertrophy and NAD(P)H oxidase activity.

Discussion

5-HT2BR Blockade Prevents O2−-Mediated Ang II–Induced Cardiac Hypertrophy

Ang II is recognized as a cardiac hypertrophic factor in vitro, as well as in vivo,8 and gp91phox/Nox2-mediated ROS production in cardiomyocytes contributes to this response.9 We tested whether the blockade of 5-HT2Rs could reduce Ang II-induced hypertrophy and cardiac ROS generation in mice. Ang II induced a left ventricular hypertrophy associated with an increase in O2− production but no increase of SOD-mediated generation in basal and stimulated conditions. The blockade of 5-HT2Rs by SB215505 completely prevented the increased left ventricular O2− generation in basal and stimulated conditions. The reduction of the NAD(P)H oxidase activity by 5-HT2BR blockade does not take place at the expression level, because p47phox subunit was still overexpressed. This drug was selected because of its higher selectivity (∼10) for 5-HT2Rs compared with 5-HT2C.10 Moreover, this drug is a potent antagonist, and we have shown previously that only 5-HT2A and 5-HT2B receptors are expressed in the heart. Interestingly, this antihypertrophic action was achieved without reduction of arterial blood pressure, attesting to the load-independent cardiac antihypertrophic effect of SB215505. In the aorta, Ang II also increased basal and NAD(P)H oxidase–mediated O2− concentration, which was insensitive to SB215505, supporting the notion that Ang II type 1 and 5-HT2Rs are not interacting in aorta. Also, SB215505 neither affected the basal O2− concentration nor the NAD(P)H oxidase maximal activity in control animals (please see the data supplement), and experiments using hematoxylin autooxidation did not reveal any direct chemical antioxidative properties of the compound. Therefore, SB215505 is not a nonspecific antioxidant and does not directly inhibit the NAD(P)H oxidase complex. These data demonstrate that the 5-HT2BR is a key regulator of Ang II–mediated cardiac hypertrophy and NAD(P)H oxidase activity.
neous increase in SOD activity in rats. We reproduced the same result in mice. The physiological relevance of this activation is not yet understood but, 2 aspects could be considered: a deviation of $\text{O}_2^-$ to hydrogen peroxide, the last being involved in hypertrophy, or a compensatory mechanism to $\text{O}_2^-$ increase that would not be sufficient to prevent cardiac hypertrophy. These questions were addressed recently in the study by Cabassi et al\textsuperscript{15} performed on prehypertensive spontaneously hypertensive rats. These animals exhibit an overactivity of the sympathetic nervous system together with increased oxidative stress status and cardiomyocyte hypertrophy. In this model, the SOD mimetic, hydroxyltetramethyl piperidinoxyl, was unable to prevent cardiac hypertrophy, indicating that “pushing” $\text{O}_2^-$ to hydrogen peroxide neither prevented nor amplified the hypertrophic phenotype. Therefore, in our experimental conditions, the SOD overactivation is probably not sufficient to suppress enough $\text{O}_2^-$ production and prevent ventricular hypertrophy. After treatment with a 5-HT\textsubscript{3A} antagonist, the SOD activity returned to control values, showing that the reduction of $\text{O}_2^-$ concentrations was not because of an increased rate of degradation but rather because of a reduction in the production of $\text{O}_2^-$. 

**The 5-HT\textsubscript{3A}R Regulates NAD(P)H Oxidase Activation in Cardiac Fibroblasts**

In mice knockout for the Nox2 subtype of the gp91\textsuperscript{phox} catalytic subunit of the NAD(P)H oxidase complex (Nox2\textsuperscript{−/−}), ISO induced cardiac hypertrophic responses that were prevented by a treatment with the NAD(P)H oxidase inhibitor apocynin (please see http://hyper.ahajournals.org). In cardiac myocytes, the gp91\textsuperscript{phox}/Nox2 is the prominent isoform, whereas Nox1 and Nox4 are expressed at lower levels.\textsuperscript{12} In Nox2\textsuperscript{−/−} mice, stimulation with ISO induces Nox1 overexpression (please see the data supplement). Other Nox isoforms are also known to be expressed in human cardiac fibroblasts, whereas Nox2 is barely detectable.\textsuperscript{16} Therefore, considering the following: (1) apocynin prevents cardiac hypertrophy induced by ISO infusion in Nox2\textsuperscript{−/−} mice; (2) non-Nox2/gp91\textsuperscript{phox} homologues are expressed by fibroblasts; (3) the suppression of Nox2, mainly expressed in cardiomyocytes, cannot suppress ISO-mediated cardiac hypertrophy; and (4) the stimulation of $\beta$-adrenergic receptors located on cardiomyocytes induces a ROS-independent hypertrophy,\textsuperscript{17} it is likely that noncardiomyocytes play an important role in the cardiac hypertrophic responses to $\beta$-adrenergic activation.

Our previous study\textsuperscript{5} indicated that fibroblasts constitute a target of hypertrophic responses to $\beta$-adrenergic activation, because 5-HT\textsubscript{3A}R blockers diminished the release of hypertrophic cytokines. We tested the effect of 5-HT\textsubscript{3A}R blockade in $\text{O}_2^-$ production by cardiac fibroblasts during 24-hour stimulations with Ang II or ISO. Ang II and ISO increased the maximal NAD(P)H oxidase–mediated $\text{O}_2^-$ production, and this effect was prevented by SB215505. This result indicates a role of this receptor in ISO and Ang II–induced NAD(P)H oxidase activation in these cells. This effect was obtained without stimulation of the receptor by its natural agonist, because in our experimental conditions the serotonin concentration in cell culture medium was found <1 nM at the end of the experiments. Therefore, SB215505, through its interaction with the 5-HT\textsubscript{3A}R, seems to interfere with a crosstalk among $\beta$-adrenergic, 5-HT\textsubscript{3A}, and Ang II receptors.

**Perspectives**

The involvement of 5-HT\textsubscript{3A}Rs in the development of cardiac hypertrophy is a new finding with potential value for the treatment or prevention of the disease. In the present study, the blockade of this receptor can prevent the increase of NAD(P)H oxidase activity and the development of cardiac hypertrophy induced by Ang II type 1 or $\beta$-adrenergic receptors, but several questions remain to be answered. Little is known about the basal activity of the 5-HT\textsubscript{3A}R and the possible agonists involved in its stimulation (serotonin) or regulation in the context of the development of cardiac hypertrophy. Also, the signaling factors responsible for the interactions among Ang II type 1, $\beta$-adrenergic, and 5-HT\textsubscript{3A}Rs must still be identified. A major impact of this effect was to demonstrate for the first time that the Gq-coupled 5-HT\textsubscript{3A}R blockade can modify both Ang II type 1 and $\beta$-adrenergic oxidant and hypertrophic responses. The mechanism that triggers this cross-regulation will have to be investigated.

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**Disclosures**

None.

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