Serotonin and Angiotensin Receptors in Cardiac Fibroblasts Coregulate Adrenergic-Dependent Cardiac Hypertrophy

Fabrice Jaffré, Philippe Bonnin, Jacques Callebert, Haythem Debbabi, Vincent Setola, Stéphane Doly, Laurent Monassier, Bertrand Mettauer, Burns C. Blaxall, Jean-Marie Launay, Luc Maroteaux

Abstract—By mimicking sympathetic stimulation in vivo, we previously reported that mice globally lacking serotonin 5-HT\textsubscript{3}\textsubscript{A} receptors did not develop isoproterenol-induced left ventricular hypertrophy. However, the exact cardiac cell type(s) expressing 5-HT\textsubscript{2B} receptors (cardiomyocytes versus noncardiomyocytes) involved in pathological heart hypertrophy was never addressed in vivo. We report here that mice expressing the 5-HT\textsubscript{2B} receptor solely in cardiomyocytes, like global 5-HT\textsubscript{2B} receptor–null mice, are resistant to isoproterenol-induced cardiac hypertrophy and dysfunction, as well as to isoproterenol-induced increases in cytokine plasma-levels. These data reveal a key role of noncardiomyocytes in isoproterenol-induced hypertrophy in vivo. Interestingly, we show that primary cultures of angiotensinogen null adult cardiac fibroblasts are releasing cytokines on stimulation with either angiotensin II or serotonin, but not in response to isoproterenol stimulation, demonstrating a critical role of angiotensinogen in adrenergic-dependent cytokine production. We then show a functional interdependence between AT\textsubscript{1}R and 5-HT\textsubscript{2B} receptors in fibroblasts by revealing a transinhibition mechanism that may involve heterodimeric receptor complexes. Both serotonin- and angiotensin II–dependent cytokine production occur via a Src/heparin-binding epidermal growth factor–dependent transactivation of epidermal growth factor receptors in cardiac fibroblasts, supporting a common signaling pathway. Finally, we demonstrate that 5-HT\textsubscript{2B} Receptors are overexpressed in hearts from patients with congestive heart failure, this overexpression being positively correlated with cytokine and norepinephrine plasma levels. Collectively, these results reveal for the first time that interactions between AT\textsubscript{1} and 5-HT\textsubscript{2B} receptors coexpressed by noncardiomyocytes are limiting key events in adrenergic agonist-induced, angiotensin-dependent cardiac hypertrophy. Accordingly, antagonists of 5-HT\textsubscript{2B} receptors might represent novel therapeutics for sympathetic overstimulation-dependent heart failure. (Circ Res. 2009;104:113-123.)

Key Words: fibroblast ■ heart failure ■ hypertrophy ■ interleukins ■ sympathetic nervous system

Cardiac hypertrophy is a physiological adaptation of the heart to increased workload. Recent data challenged the widely held belief that cardiac hypertrophy is a necessary compensatory mechanism to maintain normal heart function.\textsuperscript{1,2} When sustained and extensive, cardiac hypertrophy can lead to maladaptation and progressive dysfunction leading to heart failure secondary to cardiomyocyte apoptosis and fibrosis.\textsuperscript{3} In addition to biomechanical stress, several neurohumoral factors acting via G protein–coupled receptors (GPCRs), including \textbeta\textsubscript{-adrenergic (\textbeta-AR)}, endothelin and angiotensin (Ang) II type 1 (AT\textsubscript{1}R) receptors have been identified as potent inducers of cardiac hypertrophy.\textsuperscript{4–7}

The chronic adrenergic stimulation experienced by patients with congestive heart failure (CHF) is a strong predictor of morbidity and mortality. Norepinephrine, through stimulation of \textbeta-ARs, is a well-known trigger of cardiac hypertrophy. The extent of left ventricular dysfunction in human pathology correlates to plasma norepinephrine concentration independently of arterial blood pressure.\textsuperscript{8} Plasma levels of cytokines such as tumor necrosis factor (TNF)-\alpha and interleukin (IL)-6\textsuperscript{9} or transforming growth factor (TGF)-\beta\textsuperscript{10} are also significantly increased in primary idiopathic hypertrophic cardiomyopathy. Noncardiomyocyte (cardiac fibroblast) stimulation by adrenergic agonists or Ang II can release growth factors,\textsuperscript{11} endothelin-1, and cytokines including TNF-\alpha, IL-6, IL-1\beta, and TGF-\beta\textsuperscript{12–14} Interestingly, several authors have demonstrated marked in vitro release of these hypertrophic factors by cardiac fibroblasts and suggested a causal link between this release and cardiomyocyte hypertrophy. The hypertrophic capacity of cytokines was also validated in vivo by

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the observation that mice with cardiac-restricted overexpression of TNF-α, IL-6, or IL-1 exhibited cardiac hypertrophy.\textsuperscript{15–17}

The question of whether β-AR stimulation promotes pathological cardiac hypertrophy by a direct effect on myocytes and/or nonmyocytes remains debatable. In vitro, most of the investigators used neonatal rat cardiomyocytes and found that the β-AR agonist, isoproterenol (ISO), leads to a mild hypertrophy of these cells, the hypertrophy being stronger in presence of cardiac fibroblast-conditioned medium.\textsuperscript{18} However, it was reported that ISO had no hypertrophic effects on adult rat myocytes,\textsuperscript{19,20} suggesting that noncardiomyocytes could participate in β-adrenergic–dependent cardiac hypertrophy in an in vivo adult context.

Using a genetic approach, we previously showed that serotonin 5-HT\textsubscript{2B} receptors (5-HT\textsubscript{2B}Rs) have a trophic action on newborn cardiomyocytes in vitro\textsuperscript{21} and by α-myosin heavy chain (α-MHC)-dependent 5-HT\textsubscript{2B}R overexpression in cardiomyocytes in vivo.\textsuperscript{22} The initial cardiomyopathy of 5-HT\textsubscript{2B}R mutant mice is compensated over time in the absence of hypertrophic stage.\textsuperscript{23} Thus, we studied their response to a pathological hypertrophic stimulus using chronic ISO infusion as a model of sympathetic stimulation in vivo. We reported that either total genetic (5-HT\textsubscript{2B}R mutant mice) or pharmacological (SB206553 or SB215505, 5-HT\textsubscript{2B}R antagonists) blockade of 5-HT\textsubscript{2B}R function completely prevented ISO-induced cardiac hypertrophy.\textsuperscript{13} Recently, 5-HT\textsubscript{2B}Rs were shown to be required for left ventricular hypertrophy in another model of cardiac hypertrophy (Ang II chronic infusion).\textsuperscript{24} Nevertheless, neither the exact cardiac cells requiring 5-HT\textsubscript{2B}Rs (cardiomyocytes versus fibroblasts), the receptor crosstalk nor their transduction pathway has been addressed in in vivo models of pathological cardiac hypertrophy.

The purpose of this study was to determine: (1) whether 5-HT\textsubscript{2B}R expression in cardiomyocytes is required for ISO-induced left ventricular hypertrophy; (2) whether Ang II participates in β-AR-dependent cardiac hypertrophy in vivo; (3) which epistatic relationships exist between β-AR–, AT1R–, and 5-HT\textsubscript{2B}R–dependent hypertrophic factor release; and (4) whether similar mechanisms could be found in human CHF.
Materials and Methods

Generation of 5-HT_{2B}R Transgenic Mice and Genotyping

Generation of α-MHC-5-HT_{2B}^{+/+} (Tg) mice and 5-HT_{2B}^{−/−} mice has been described previously.\textsuperscript{22,25} All animal experiments were performed in accordance with institutional guidelines and European regulations.

Induction of Cardiac Hypertrophy by ISO

In 11-week-old male mice, ISO (30 mg/kg per day), was delivered for 7 days by miniosmotic pumps (1007D, Alzet Corp) implanted subcutaneously under anesthesia (0.75% isoflurane).

Cardiovascular Phenotyping by Echocardiography

Left ventricular dimension and heart rate were assessed before and after ISO infusion under isoflurane anesthesia (0.75%) by echocar-
After echocardiographic analysis, mice were euthanized by CO₂ and weighed.

Analysis of 5-HT₂R and AT₁R Expression by Binding Assays

Membrane proteins prepared from heart ventricles or from adult mouse cardiac fibroblast or myocytes primary cultures were analyzed by binding studies to assess receptor expression.

Patients

Cardiac samples were obtained from explant grafts except for normal controls, the tissue of which was obtained from donors without recipient. ELISAs for plasma concentration for cytokines were performed. The local ethical committee (Comité Consultatif de Protection des Personnes se Prêtant à la Recherche Biomédicale, CCPPRB d’Alsace) approved the study, and all patients gave their informed consent before tissue collection and plasma collection. For frozen human heart sections, all harvest and use of human tissue was performed in accordance with NIH and University of Rochester Medical Center institutional review board guidelines.

Adult Cardiac Fibroblasts Primary Culture

Cultures of ventricular noncardiomyocytes were obtained by differential plating from dissociated heart of male adults mice (10 to 12 weeks) or from neonatal rat hearts (3 to 4 days). Cardiac fibroblasts used during early passages were identified by characteristic morphology and positive staining with antibody to vimentin (>90%) and negative staining for macrophage marker F4/80 (<1%) (Figure II in the online data supplement). One day before the experiments, the cells were serum-starved.

Measurement of Cytokines in Plasma and Culture Supernatants

Concentrations of Ang II, IL-6, IL-1β, TNF-α, and TGF-β, were measured in plasma and culture supernatants by ELISA kits (Bertin, DY 406, DY 401, DY 410 and DY 1679, R&D systems).

Confocal Imaging

Cells or tissues were observed after 4% paraformaldehyde fixation and revealed using either a mouse monoclonal anti–FLAG M2 (Sigma, 1:100), a rabbit anti-GFP antibody (Santa Cruz Biotechnology, 1:100), a monoclonal anti-5-HT₂BR antibody (Pharmingen, 1:100), a rabbit anti-AT₁R (N-10, Santa Cruz Biotechnology, 1:100) or a rabbit anti-Vimentin (Santa Cruz Biotechnology, 1:200).

Immunoprecipitation and Western Blotting

Serum-starved cells were homogenized at 4°C in RIPA buffer, centrifuged at 10 000g, and incubated with either anti-FLAG affinity matrix (40 µL, Sigma) overnight at 4°C or a monoclonal anti–5-HT₂BR antibody (Pharmingen, 2 µg). Western blot analysis of immunoprecipitated samples was performed on SDS-PAGE 10% gels and revealed using either a rabbit anti-GFP antibody (Santa Cruz Biotechnology, 1:1000) or a rabbit anti-Vimentin (Santa Cruz Biotechnology, 1:200).

Data Analysis and Statistics

All results are expressed as means±SEM. Different groups were compared through 1-way ANOVA, followed by Newman–Keuls test. All calculations were performed using the GraphPad Prism 4.0 program.

Results

Expression of 5-HT₂BRs by Noncardiomyocytes Is Required for ISO-Induced Cardiac Hypertrophy

We first verified the expression of 5-HT₂BRs and AT₁Rs either by immunohistochemistry of human adult left ventricles (Figure 3. Expression of 5-HT₂BRs by noncardiomyocytes is required for ISO-induced cardiac hypertrophy in vivo. In the different transgenics (Tg; 5-HT₂B⁺/++; Tg; 5-HT₂B⁺/--; Tg; 5-HT₂B−/--; and global 5-HT₂B−/− mice), ISO infusion led to a significant increase in left ventricular mass-to-body weight ratio (LVM/BW) (a) from 0 (D0) (black bars) to 7 days of ISO (30 µg/g per day) infusion (ISO D7) (white bars), as determined by echocardiography, and a significant decrease in fractional shortening (FS) (b) or in systolic ejection volume (SEV) (c), only in Tg; 5-HT₂B−/− and Tg; 5-HT₂B−/− but not in Tg; 5-HT₂B−/− mice expressing 5-HT₂BRs solely in cardiomyocytes or global 5-HT₂B−/− mice. The cytokine IL-6 (d), IL-1β (e), and TGF-β (f) plasma levels are also increased only in Tg; 5-HT₂B−/− and Tg; 5-HT₂B−/− but not in Tg; 5-HT₂B−/− mice expressing 5-HT₂BRs solely in cardiomyocytes or global 5-HT₂B−/− mice. Values are means±SEM. *P<0.05, D0 vs D7 ISO (30 µg/g per day) (n=6 to 8 mice).)
**Figure 4.** 5-HT_{2B}Rs and AT_{1}Rs are required for ISO-induced cytokine release in cardiac fibroblast culture. A significant increase in IL-6 (a) and IL-1β (d) cytokines could not be observed in adult cardiac fibroblasts isolated from Agt^{-/-} mice unable to generate Ang II after stimulation with ISO (10 μmol/L) (■) but was observed after a 4-hour Ang II (100 nmol/L) (▲) or 5-HT (1 μmol/L) (□) stimulation. Similarly, IL-6 (b) and IL-1β (e) cytokines were significantly increased in adult cardiac fibroblasts isolated from wild-type (WT) (▲) mice after a height-hour stimulation with ISO (10 μmol/L), but not after preincubation with ZD7155 (ZD) (100 nmol/L) (□), for 30 minutes. Finally, stimulation with Ang II (100 nmol/L) increased IL-6 (c) and IL-1β (f) supernatant concentrations in adult cardiac fibroblasts from wild-type mice (5-HT2B^{+/+}) (●/●, ■), but not after 30 minutes of preincubation with the 5-HT_{2B} antagonist SB206553 (SB) (100 nmol/L) (○) or from global mutant for 5-HT_{2B}Rs (5-HT2B^{−/−}) (●/●; ▼). Results are means±SEM expressed in pg/mL of cardiac fibroblasts supernatants (n=3 independent fibroblast cultures each determination in triplicate). *P<0.05 vs antagonist-treated.

1a and 1b) or by binding assays on primary cultures of wild-type and 5-HT_{2B}^{−/−} mice (Figure 1d and 1e). Interestingly, 5-HT_{2B}Rs were found mainly colocalized with AT_{1}Rs and vimentin (a marker of cardiac fibroblasts) in human heart sections. To assess whether 5-HT_{2B}R expression in cardiomyocytes is required for left ventricular hypertrophy, global 5-HT_{2B}^{−/−} mice were crossed with transgenic mice expressing the cardiac fibroblast-specific α-MHC promoter (Tg) (Figure 2a). After genotyping, the 4 resulting strains (5-HT_{2B}^{+/+}; Tg; 5-HT_{2B}^{+/−}; Tg; 5-HT_{2B}^{+/−}; Tg; 5-HT_{2B}^{−/−}; and 5-HT_{2B}^{−/−} [Figure 2b]) were assessed for cardiac 5-HT_{2B}R expression (Figure 2c). As expected after ISO infusion, a similar heart rate increase was observed in all 4 genotypes (+25%) (supplemental Table I). By echocardiography, ISO infusion led to cardiac hypertrophy in Tg; 5-HT_{2B}^{+/+} and in Tg; 5-HT_{2B}^{+/−} mice as shown by increased left ventricular mass-to-body weight ratio (+30%) (Figure 3a and supplemental Table I). However, Tg; 5-HT_{2B}^{+/−} mice expressing 5-HT_{2B}Rs solely in cardiomyocytes were, like global 5-HT_{2B}^{−/−} mice, resistant to ISO-induced cardiac hypertrophy. ISO-induced impairment of left ventricle contractility was observed in the Tg; 5-HT_{2B}^{+/+} and in the Tg; 5-HT_{2B}^{+/−} mice, as demonstrated by a decrease of both fractional shortening and systolic ejection volume in these 2 groups. Conversely, ISO did not modify ventricular functions in Tg; 5-HT_{2B}^{−/−} or in global 5-HT_{2B}^{−/−} mice (Figure 3b and 3c). Importantly, Ang II plasma level was not increased at 7 days of ISO-infusion in any mice (405±32, versus 354±61 fg/mL, P>0.05, n=6 per genotype each in triplicate). However, ISO infusion led to significant increases in plasma concentrations of TNF-α (1.4-fold over basal; supplemental Figure IV), IL-6 (2.5-fold over basal), IL-1β (2.8-fold over basal), and TGF-β (2.5-fold over basal) in Tg; 5-HT_{2B}^{−/−} and in Tg; 5-HT_{2B}^{−/−} mice (Figure 3d and 3f). Furthermore, Tg; 5-HT_{2B}^{−/−} mice expressing 5-HT_{2B}Rs only in cardiomyocytes were, like global 5-HT_{2B}^{−/−} mice, resistant to ISO-induced increase in plasma cytokines.

**Angiotensinogen, AT_{1}Rs, and 5-HT_{2B}Rs Are Required for ISO-Induced Cytokine Release in Noncardiomyocytes**

Interestingly, primary cultures of adult noncardiomyocytes isolated from Angiotensinogen mutant mice (Agt{−/−}), which are unable to generate Ang II, did not exhibit any increase in cytokine release after ISO stimulation, whereas Ang II (100 nmol/L) significantly increased concentrations of IL-6 (4.7-fold over basal), IL-1β (4.1-fold), and TNF-α (1.6-fold) at 4 hours, as did 5-HT (1 μmol/L) stimulation (Figure 4a and 4d and supplemental Figure IV, a). Furthermore, ISO stimulation of wild-type cardiac fibroblasts elicited a significant increase in the release of Ang II (883±24 fg/mL at 4 hours versus 190±7 fg/mL at 0 hour; n=4 independent determinations in triplicate; P<0.05) but not of 5-HT (<1 nmol/L). The potent and selective AT_{1}R antagonist ZD7155 at 100 nmol/L (supplemental Figure I, A and C) significantly reduced ISO-induced cytokine release (IL-6, 4-, IL-1β, 1.8-, and TNF-α; 2.3-fold) at 8 hours (Figure 4b through 4e and
supplemental Figure IV, b) but not basal cytokine levels. Either genetic or pharmacological (using the potent and selective antagonist SB206553; supplemental Figure I, B and D) blockade of 5-HT2BRs inhibited Ang II–induced cytokine release in adult cardiac fibroblasts (Figure 4c and 4f and supplemental Figure IV, c). Moreover, we also verified that 5-HT2BRs were required for cytokine production by newborn cardiac fibroblasts (supplemental Figure III, E and F).

5-HT2BRs and AT1Rs Share a Common Epidermal Growth Factor Receptor Transactivation Pathway–Mediating Cytokine Release

Similar to the effects of 5-HT2BR antagonists on Ang II action, ZD7155 (100 nmol/L) significantly reduced 5-HT-induced cytokine release (Figure 5a through 5c and supplemental Figure IV, d). In noncardiocytes, activation of AT1Rs or β-ARs has been shown to induce shedding of heparin-binding epidermal growth factor (HB-EGF) through activa-
tion of matrix metalloproteinases (MMPs) and subsequent activation of the epidermal growth factor receptor (EGF-R), a phenomenon called transactivation.28,29 In adult cardiac fibroblasts in the presence of AG1478 (an EGF-R blocker, 100 nmol/L), either Ang II– or 5-HT–induced release of IL-6, TNF-α, and IL-1β was totally prevented and that of TGF-β only partially (Figure 5a through 5c and supplemental Figure IV, d). Cytokine release stimulated by either Ang II or 5-HT was totally abrogated in adult cardiac fibroblasts prepared from mice lacking HB-EGF, although EGF stimulation (10 ng/mL) led to normal cytokine release (Figure 5a through 5c and supplemental Figure IV, d). GM6001 (an MMP inhibitor, 100 nmol/L) (supplemental Figure 3, C and D) or PP2 (a Src inhibitor, 200 nmol/L) totally prevented Ang II– and 5-HT–induced cytokine release by cardiac fibroblasts (Figure 5a through 5c and supplemental Figure IV, d). Interestingly, a strong reduction of Ang II– and complete reduction of 5-HT–induced cytokine release were induced by the p38 inhibitor SB203580 (10 μmol/L). Conversely, the extracellular signal-regulated kinase (ERK)1/2 inhibitor PD098059 (10 μmol/L) did not affect Ang II– or 5-HT-induced IL-6, IL-1β, or TGF-β release and only slightly reduced TNF-α cytokine release (Figure 5a through 5c and supplemental Figure IV, d).

**5-HT2BRs and AT1Rs Colocalize and Coimmunoprecipitate**

By confocal microscopy, we first observed membrane colocalization of 5-HT2BRs and AT1Rs in cardiac fibroblasts (Figure 6a). After immunoprecipitation of neonatal rat cardiac fibroblast extracts with an anti-5-HT2BR antibody, we detected bands of 41 to 43 kDa (expected molecular mass for AT1Rs) similar to those observed by direct Western blot analysis (Figure 6a) with an anti-AT1R antibody. We further confirm these putative interactions, using HEK293 cells cotransfected with 5-HT2BR-CFP and AT1R-Flag. Single confocal plane observation reveals the 5-HT2BR-CFP localization (2B-CFP), AT1R-Flag localization (AT1-Flag), and their colocalization by overlay (merge). Scale bar, 2 μm. Bottom, HEK293 cells, nontransfected (NT) or transfected with 5-HT2BR-CFP (2B-CFP), AT1R-Flag (AT1-Flag), or 5-HT2BR-CFP in combination with AT1R-Flag were immunoprecipitated with anti-Flag antibody. Immunoprecipitates (IP) anti-Flag were immunoblotted using anti-CFP antibody. Blots are representative of 3 independent experiments.
single band of 80 kDa (the expected molecular mass of the CFP-5-HT<sub>2B</sub>R) only in cotransfected cells (Figure 6b). These results strongly suggest that AT<sub>1</sub>Rs and 5-HT<sub>2B</sub>Rs colocalize and may directly interact in common signaling complexes in transfected cells.

5-HT<sub>2B</sub>R Overexpression in Human Heart Failure

Because 5-HT<sub>2B</sub>Rs were also expressed in both human cardiac fibroblasts and cardiomyocytes (Figure 1), we assessed a putative contribution of 5-HT<sub>2B</sub>Rs in human CHF. We looked for 5-HT<sub>2B</sub>R expression in left ventricular biopsies of 16 CHF patients, compared to 7 normal control subjects. Expression of 5-HT<sub>2B</sub>Rs, obtained from biopsies of left ventricles, were found to be significantly elevated in samples from failing hearts (Figure 7a). This increase appears to be independent of cardiomyopathy etiology, severity of the disease, or treatments (supplemental Table II). However, significant correlations were found between cardiac 5-HT<sub>2B</sub>R expression levels and plasma concentrations of either norepinephrine, IL-6, TGF-β, or TNF-α in CHF patients (Figure 7b, 7c, and 7e and supplemental Figure IV, e) and reciprocally (Figure 7d and 7f and supplemental Figure IV, f), but not in controls (supplemental Table II). Taken together, these data support the notion that 5-HT<sub>2B</sub>R expression is linked to cardiac cytokine production during the sympathetic overactivity associated with CHF.

Discussion

In light of our previous reports, 5-HT<sub>2B</sub>Rs participate in trophic responses of the myocardium by acting directly on cardiomyocytes<sup>21,22</sup> or indirectly on noncardiomyocytes...
through the release of paracrine factors induced by chronic ISO stimulation. By reexpressing the 5-HT_{2B}R selectively into cardiomyocytes in a 5-HT_{2B}R-null background, we describe here for the first time that its expression by noncardiomyocytes is absolutely required for ISO-induced cardiac hypertrophy. The ISO-mediated increases in TNF-α, TGF-β, IL-6, and IL-1β plasma levels observed in the wild-type mice also require 5-HT_{2B}R expression in noncardiomyocytes.

No increase in plasma Ang II could be detected after ISO infusion in mice but Ang II can be produced in the heart through a local renin-angiotensin system. Interestingly, angiotensinogen mRNA and protein levels are increased by β-AR stimulation in neonatal cardiac fibroblasts, which were shown, as opposed to cardiomyocytes, to serve as the predominant source of IL-6 after ISO stimulation in mouse myocardium. In our study, we uncover the Ang II/AT1R axis critical role for ISO-induced cytokine release in adult cardiac fibroblasts as validated by several observations. (1) A significant increase in Ang II, but not in 5-HT, was detected in supernatants of adult mouse cardiac fibroblast culture after ISO stimulation. (2) This peak of Ang II release occurs at 4 hours of ISO stimulation, similar to that of cytokines after direct Ang II stimulation. (3) The cytokine release peak following ISO stimulation occurred only after 8 hours, suggesting a multistep process. (4) Consistent with these results, Ang–/– cardiac fibroblasts did not release cytokines on ISO stimulation, but cytokine release in these cells was similar to wild-type cells when stimulated with Ang II or 5-HT. (5) Finally, using ZD7155, we show that AT1Rs are also required for ISO-induced cytokine release. Together, these data reveal, for the first time, that ISO-dependent Ang II production by cardiac fibroblasts leading to the autocrine AT1R stimulation is absolutely required for hypertrophic cytokine release in heart.

The present report addresses unknowns regarding the AT1R and 5-HT_{2B}R signaling pathway(s) controlling cytokine release in cardiac fibroblasts. In the present work, we demonstrate that expression of HB-EGF and Src activity are critical for either an Ang II– or a 5-HT–dependent cytokine release process. We show that MMPs are responsible for HB-EGF shedding and subsequent EGF-R transactivation that is induced by GPCR agonists such as Ang II or 5-HT. TNF-α–converting enzyme (TACE) (ADAM-17) was found to control HB-EGF shedding in fibroblasts, and a recent report indicated that 5-HT_{2B}Rs can directly regulate this enzyme activity in neuronal cells. Our work also highlights the importance of p38 but not ERK1/2 pathway for cytokine release. In summary, our data support the following epistatic relationships (Figure 8 and online data and video): ISO ⇒ Ang II ⇒ 5-HT_{2B}R ⇒ AT1Rs ⇒ Src ⇒ MMPs ⇒ HB-EGF ⇒ ErbB-1/4-Rs ⇒ p38 ⇒ IL-6, TNF-α, TGF-β, and IL-1β release. All of these findings support that AT1Rs and 5-HT_{2B}Rs share common EGF-R-dependent signaling pathways in adult cardiac fibroblasts.

Blockade of 1 of the 2 receptors prevents cytokine release induced by the other receptor, supporting interactions between 5-HT_{2B}Rs and AT1Rs. Using coimmunolocalization and a pull-down assay, we show that the 2 receptors interact in a common cell compartment. Recently, reports have suggested that GPCRs exist in heterodimeric complexes that may play a key role in receptor maturation and trafficking to the plasma membrane and/or signaling (for review, see Bulenger et al). The protein network associated with the C terminus of the 5-HT_{2B}Rs includes scaffolding proteins containing 1 or several PDZ domains, signaling proteins and proteins of the cytoskeleton that may be involved in signaling complexes. More work will be necessary to conclude whether in vivo interactions between AT1Rs and 5-HT_{2B}Rs are direct or mediated by adaptor proteins. Only a few reports have described an inhibitory mechanism in trans between 2 GPCRs. To our knowledge, this process was first described between AT1Rs and β2-ARs in COS-7 cells that express equal endogenous levels of AT1Rs and β2-ARs and was also shown to occur in adult cardiomyocytes. Our findings are consistent with the hypothesis that AT1Rs and 5-HT_{2B}Rs exist in common signaling complexes and that they may interact together.

The increase of 5-HT_{2B}R sites in biopsies of left ventricles from CHF patients that we observed appears independent of the type of cardiopathy, its duration, or the treatments (including β blockers or ACE inhibitors). In recent cardiac transcriptome analysis, an increase in 5-HT_{2B}R mRNA was also reported in human and rat failing heart tissue, during the functional recovery of end-stage human heart failure, and in rats after banding of the ascending aorta. The significant correlation between 5-HT_{2B}R expression and cytokines IL-6, TNF-α, or TGF-β plasma levels supports, in humans, our findings in mice. Interestingly, the significant correlations with sympathetic activity found in patients indicate that 5-HT_{2B}Rs are as well required for adrenergic-dependent cytokines production in humans. A cardiac hypertrophy–associated switch of adult to fetal genes has been reported. It is tempting to speculate that similar mechanisms might be operative at 5-HT_{2B}R receptor expression, which is expressed in embryonic heart. Sympathetic overstimulation...
may also participate via cAMP-dependent regulation as an autocrine regulatory loop.35

In summary, our data indicate that a selective 5-HT2B R antagonist blocks both Ang II and adrenergic adverse effects in pathological conditions with no alterations of hemodynamic or blood pressure.

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References
32. Nakashima H, Eguchi K, Eguchi S. ADAM17 mediates epidermal growth factor receptor transactivation and vascular smooth muscle cell hypertro-
phy induced by angiotensin II. *Arterioscler Thromb Vasc Biol*. 2006;26: e133–137.


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**Generation of 5-HT<sub>2B</sub>R transgenic mice and genotyping**- To obtain the transgenic mice, we crossed 5-HT<sub>2B</sub><sup>−/−</sup> mice with α-MHC-5-HT<sub>2B</sub> transgenic mice (Tg). The α-MHC-5-HT<sub>2B</sub><sup>−/−</sup> mice in a CD-1 background were crossed with 129SvPAS pure background mice for more than 6 generation. These mice were then crossed again with 5-HT<sub>2B</sub><sup>−/−</sup> mice on a pure 129SvPAS background. We did all the experiment on littermates including all different genotypes form several independent litters. The presence of 5-HT<sub>2B</sub>R transgene insertion, endogenous 5-HT<sub>2B</sub>R gene and mutant allele was determined by genomic DNA Southern analysis. DNA was extracted from mice tails, digested with BglII enzyme, run on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized to a [³²P]-probe using a 1-Kbp EcoRI-EcoRI restriction fragment of the 3' end of 5-HT<sub>2B</sub>R cDNA. The α-MHC-5-HT<sub>2B</sub> transgene was revealed as a 2.8-Kbp band, the 5-HT<sub>2B</sub><sup>−/−</sup> gene as a 3.2-Kpb band and the endogenous 5-HT<sub>2B</sub>R gene as a 9.3-Kbp band. We obtained 4 different genotypes: wild type mice (⁺⁺), global 5-HT<sub>2B</sub>R mice, mice overexpressing 5-HT<sub>2B</sub>R in cardiomyocytes in a homozygous or heterozygous endogenous 5-HT<sub>2B</sub>R background (Tg; 5-HT<sub>2B</sub><sup>−/+</sup>, Tg; 5-HT<sub>2B</sub><sup>−/−</sup>) and mice overexpressing 5-HT<sub>2B</sub>R solely in cardiomyocytes in a homozygous mutant background (Tg; 5-HT<sub>2B</sub><sup>−/−</sup>).

**Cardiovascular phenotyping by echocardiography**- Left ventricular dimension and heart rate were assessed before and after ISO infusion under isoflurane anesthesia (0.75%) using an echocardiograph (Vivid 7, GE Medical Systems ultrasound<sup>®</sup>, Horten, Norway) equipped with a 12-MHz linear transducer (12L) connected to an image workstation for subsequent analysis (PC EchoPAC, GE Medical Systems ultrasound<sup>®</sup>, Horten, Norway) ¹. The frame rate was adjusted to 50 frames.s<sup>−1</sup> when applying zoom. Two-dimensional echocardiographic loops and parasternal long-axis M-mode (motion-mode) tracings were recorded. The M-mode recordings were used for all calculations of cardiac structure and function. The left ventricular mass (LVM) was calculated as follow: LVM=1.055*[Sd+PWd+EDD<sup>3</sup>-(EDD)<sup>3</sup>]. Spatial flow profile, time-average mean BFV, Vmean, cm.s<sup>−1</sup>) in the pulmonary artery was recorded using pulsed-wave Doppler recordings. Together with the pulmonary artery diameter (Dpa, cm), Vmean were used to calculate cardiac output (CO, mL.min<sup>−1</sup>) as follow: CO = [(Vmean .60).p.(Dpa/2)<sup>2</sup>] ². Systolic ejection volume (SEV) was calculated as follow: SEV= Cardiac output (CO)*Heart rate (HR) (Online Table I).
**Adult cardiac fibroblast and myocyte primary culture**- Cultures of ventricular cells were obtained by differential plating from dissociated heart of male adults (10-12 weeks), 5-HT2B<sup>−/−</sup>, in a pure 129/PAS background, HB-EGF<sup>+/−</sup> in a pure C57/B16 background and Agt<sup>−/−</sup> in a pure FVB background and their corresponding wild-type mice and Sprague Dawley newborn rats. After anesthesia, the heart was quickly removed via thoracotomy and transferred to an ice-cold Tyrode solution, containing (in mM): NaCl, 140; KCl, 5.4; CaCl<sub>2</sub>, 1.8; MgCl<sub>2</sub>, 1.8; HEPES, 10; glucose, 10; pH 7.35. Ventricles were isolated and dissected into small pieces in Ca<sup>2+</sup>-free KREBS-Ringer solution containing (in mM): NaCl, 35; KCl, 7.75; KH<sub>2</sub>PO<sub>4</sub>, 1.18; HEPES, 10; glucose, 10; NaHCO<sub>3</sub>, 25; saccharose, 77; pH 7.4. The ventricular tissue was then incubated for 6 min at 37°C in Ca<sup>2+</sup>-free KREBS-Ringer solution supplemented with 1% bovine serum albumine (BSA), 155 IU/ml Collagenase (type V, Sigma) and 6 IU/ml Protease (type XXIV, Sigma). This was followed by gentle stirring of the tissue in modified Tyrode solution containing (in mM): NaCl, 130; KCl, 4.8; KH<sub>2</sub>PO<sub>4</sub>, 1.2; glucose, 5; HEPES, 25; pH 7.4. Isolated cells were filtered through a nylon filter (pore size 150 µm) and maintained for 45 min in the same solution for sedimentation; cardiomyocytes were mainly concentrated in the pellet, and cardiac fibroblasts primarily in the supernatant. Fibroblasts present in the supernatant were collected by mild centrifugation (40g, 10 min at room temperature), concentrated by centrifugation (150g, 10 min) and washed once in M199 medium. They were seeded on a 10cm-well plate at a density of 120,000 cells/ml. Cardiac fibroblasts used during early passages were identified by characteristic morphology and positive staining with the rabbit polyclonal anti-human Vimentin (H-84, 1/200 dilution, Santa Cruz) (>90%)<sup>4</sup> and negative staining for the macrophage marker F4/80<sup>5</sup> (1/500 dilution, MF 4800, CalTag lab) (<1%) (**Online Fig II**). One day prior to experiments, the cells were serum starved.

**Measurement of cytokines in culture supernatants** - Concentrations of AngII, IL-6, IL-1β, TNF-α and TGF-β<sub>1</sub> were measured in culture supernatants by ELISA kits (Bertin, DY 406, DY 401, DY 410 and DY 1679, R&D systems)<sup>4</sup> after incubation with various inducers and/or inhibitors. Dose response curve were determined for ISO (**Online Fig III A-B**) and for non-standard inhibitors, i.e. MMP inhibitor GM 6001 (**Online Fig III C-D**). Being interested in adult heart hypertrophic responses, we performed most of the experiments using adult cardiac fibroblast primary cultures. In order to exclude particular effects from adult cells, we also verified the responses of newborn cardiac fibroblasts, which indeed responded to ISO in a 5-HT2B receptor dependent manner (**Online Fig III E-F**).
Adult cardiac fibroblast transduction

The current report addresses unknowns regarding the AT-1R and 5-HT_{2B}R signaling pathway(s) controlling cytokine release in cardiac fibroblasts. AG1478 (100 nM), which completely suppresses AngII- and 5-HT-induced cytokine release, is a potent and fairly selective inhibitor of the tyrosine kinase activity of EGF-Rs, ErbB1-R, \((K_i = 3 \text{ nM})\) but also of ErbB4-R tyrosine kinase activity. HB-EGF binds to and activates EGF-Rs containing either homo- or heterodimers of ErbB1-R or ErbB4-R. In the heart, HB-EGF mainly expressed by cardiac fibroblasts has been found to be overexpressed in pathological conditions. In the present work, we found that either AngII- or 5-HT-dependent cytokine release was completely prevented in HB-EGF^{-/-} cardiac fibroblasts, demonstrating that expression of HB-EGF is critical for AngII or 5-HT-dependent cytokine release process. Src activity was also required for either AngII or 5-HT-induced cytokines release, in agreement with previous reports demonstrating its role in the release of soluble HB-EGF by MMP-dependent shedding. GM6001 dose-dependently blocked cytokine release by cardiac fibroblast up to 100 nM (Online Fig III C-D). Because GM6001 is a broad-spectrum MMP inhibitor, we cannot define the exact MMP(s) involved. Indeed, MMP-2/9, MMP-7, MMP-8 or MMP-13 can be critical for HB-EGF shedding and subsequent EGF-R transactivation induced by different GPCR agonists such as AngII or 5-HT. TNF-α converting enzyme (TACE, ADAM-17), was found to control HB-EGF shedding in fibroblasts and a recent report indicated that 5-HT_{2B}Rs can directly regulate this enzyme activity in neuronal cells. Our work also highlights the importance of p38 but not ERK1/2 pathway for cytokine release. Accordingly, genetic inhibition of cardiac ERK1/2 in mice did not prevent ISO- or AngII-induced cardiac hypertrophy in vivo. All these findings support that AT-1Rs and 5-HT_{2B}Rs share common EGFR-dependent signaling pathways in adult cardiac fibroblasts. All these findings support that AT-1Rs and 5-HT_{2B}Rs share common EGFR-dependent signaling pathways in adult cardiac fibroblasts (See Online Video).

Analysis of 5-HT_{2B}R and AT1-R expression by binding assays

Membrane proteins prepared from heart ventricles or from adult mouse cardiac fibroblast or myocyte primary cultures were analyzed by binding studies with specific tritiated antagonists of the 5-HT_{2B} receptor ([^{3}H]-LY266097), of the 5-HT_{2A} receptor ([^{3}H]-MDL 100907) and for 5-HT_{2C} receptor ([^{3}H]-mesulergine) in presence of the 100 nM of the specific 5-HT_{2A}R antagonist MDL 100907 and 100 nM of the specific 5-HT_{2B}R antagonist LY266097. Binding
of $[^{125}\text{I}]$-AngII to membrane of adult cardiac fibroblast or myocyte culture was similarly performed \(^1\). The dissociation constant, \(K_D\) and maximal number of sites, \(B_{\text{max}}\) were obtained from competition curves (GraphPad Prism).

**Inositol phosphate dosage**

After plating in 6 wells plates in DMEM (GIBCO) supplemented in 10% fetal calf serum, cells were serum starved in BME (serum free inositol free) for 20 hours in the presence of 1 mCi/ml of tritiated myo-inositol. After washing of the unincorporated radioactivity, 1ml Krebs buffer was added to the cells, and then supplemented with 10 mM LiCl 10 min before induction. After 1 hour incubation in the presence of various concentrations of ligand, the reaction was stopped with 1 ml Stop solution (1ml formic acid /1 l H2O), then the cells were scraped and extracted by 500 µl Chloroforme. Phosphoinositols were purified from aqueous phase by chromatography on a Dowex columns, washed with water wash solution (Borax/formic acid) and eluted by formic acid, and counted by liquid scintillation. Values were normalized by the total amount of incorporated radioactivity. The effect of various antagonists was tested after agonist stimulation and IC50 was calculated (Online Fig I A-D).

**Patients**

Sixteen patients with advanced congestive heart failure (9 : primary dilated cardiomyopathy, 6 : ischemic cardiopathy and 1 : congenital cardiopathy ; 15 males and 1 female) with chronic heart failure were compared to 7 normal control subjects (7 males, from 35 to 61 years old). Cardiac samples were obtained from explants grafts except for normal controls where tissue was obtained from donors without recipient. ELISA assays for plasma concentration for hypertrophic cytokines were performed. Plasma levels of epinephrine and norepinephrine (Online Table II) were measured by HPLC \(^{19}\). 5-HT\(_{2b}\) Bmax was obtained from biopsies of left ventricles and is the average of sub-epicardial, sub-endocardial and mid-ventricle biopsies (Online Table II). All tissues were immediately frozen in liquid nitrogen and stored at -80°C. Serotonin and catecholamine concentrations in plasma and culture supernatants were determined by HPLC coupled to amperometric detection \(^{19}\).

**Human heart sections immunofluorescence and confocal microscopy**

Frozen sections (8 µm) from non-failing human left ventricle were fixed in 4% paraformaldehyde in PBS pH 7.4 for 10 min in room temperature. The sections were washed three times in ice cold PBS and were permeabilized in 100 µM digitonin in PBS for 10 min
only for the 5-HT$_{2B}$R/Vimentin double staining. Sections were washed three times in ice cold PBS and blocked with 1% BSA in PBS-Tween solution (PBST) for 30 min. Then, the heart sections were incubated with the mixture of two primary antibodies in the blocking solution for 1h at room temperature as followed: the mouse monoclonal anti-5-HT$_{2B}$R (A72, 1/100 dilution Pharmingen) with the rabbit polyclonal anti human AT-1R (N-10, 1/100 dilution, SantaCruz) or with the rabbit polyclonal anti-human Vimentin (H-84, 1/200 dilution, SantaCruz). The primary antibodies solution were decanted and the slides, washed 3 times in PBST and then incubated with the mixture of two secondary antibodies (Texas Red-conjugated against mouse and Alexa Fluor 488 against rabbit, 1/1000 dilution each) in 1% BSA PBST solution in dark. After 30 min, the mixture was decanted and the slices were washed three times with PBST and mounted. Immunofluorescence pictures were taken using a confocal microscope (Olympus).

References

3. Fredj S, Bescond J, Louault C, Delwail A, Lecron JC, Potreau D. Role of interleukin-6 in cardiomyocyte/cardiatic fibroblast interactions during myocyte hypertrophy and fibroblast proliferation. *J Cell Physiol.* 2005;204:428-436.


ONLINE FIG. I. **Pharmacological properties of ZD7155 and of SB206553.**

(A) Determination of the concentration of the AT1-R antagonist ZD7155 inhibiting half of the phosphoinositol (PI) generated upon stimulation (IC50) of transfected cells expressing AT1-R by 100 nM of AngII. (B) Determination of the effect of various concentrations of the 5-HT2B R antagonist SB206553 on the phosphoinositol (PI) generated upon stimulation of transfected cells expressing AT1-R by 100 nM AngII. (C) Determination of the concentration of the 5-HT2B R antagonist SB206553 inhibiting half of the phosphoinositol (PI) generated upon stimulation (IC50) of transfected cells expressing 5-HT2B R by 100 nM 5-HT. (D) Determination of the effect of various concentrations of the AT1-R antagonist ZD7155 on the phosphoinositol (PI) generated upon stimulation (IC50) of transfected cells expressing 5-HT2B R by 100 nM 5-HT. Results are means±S.E.M n=3.
Online Figure II

ONLINE FIG. II. *Characterization of cardiac fibroblast primary cultures.*

Fixed primary cultures of adult mouse cardiac fibroblasts were immunostained using antisera against F4/80 for macrophage or vimentin for fibroblasts and DAPI for nuclear cell counting (blue). More than 90% of the culture cells are vimentin positive and less than 1% is F4/80 positive (n>100 from two independent cultures); Bars 2 µM.
ONLINE FIG. III. **Pharmacological characterization of cytokine release by cardiac fibroblast culture.**

A dose-dependent increase in IL-6 (A), or TNF-α (B) cytokines release could be observed in adult cardiac fibroblasts isolated from wild type mice, after stimulation with ISO (from 10 nM to 10 µM, open bars), or AngII (100 nM, grey bars) stimulation but not with SB206553 (100 nM) or ZD7155 (100 nM) alone. A significant increase in IL-6 (C), or TNF-α (D) cytokines could be observed in adult cardiac fibroblasts isolated from wild type mice, after a four-hour stimulation with AngII (100 nM, grey bars), that was dose-dependently inhibited by GM 6001 (GM) until 100 nM and mimicked by the MMP2/9 inhibitor at 500 nM. Similar results were obtained after 5-HT stimulation and for IL1β or TGFβ. Similarly, a significant increase in IL-
6 (E), or TNF-α (F) cytokines could be observed in cardiac fibroblasts isolated from newborn wild type (WT) mice, after a four-hour stimulation with ISO (10µM, black bars), but not from newborn 5-HT_{2B}R^{-/-} (KO) mice (open bars). Results are means±S.E.M expressed in pg/ml of cardiac fibroblasts supernatants (n=3 independent fibroblast cultures each determination in triplicate). *p<0.05 vs. untreated.
ONLINE FIG. IV. Characterization of TNF-α release in plasma and by cardiac fibroblast culture.
(a) In the different transgenics, Tg; 5-HT$_{2B}^{+/+}$, Tg; 5-HT$_{2B}^{+/−}$, Tg; 5-HT$_{2B}^{−/−}$ and global 5-HT$_{2B}$ mice, an increase in plasma TNF-α occurred from 0 (D0-black bars) to 7 days of ISO (30µg/g/d) infusion (ISO D7-white bars). Values are mean±SEM, * p<0.05, D0 vs. D7 ISO (30µg/g/d), n= 6-8 mice.

(b) A significant increase in TNF-α could not be observed in adult cardiac fibroblasts isolated from Agt$^{−/−}$ mice, unable to generate AngII, after stimulation with ISO (10µM, open square), but was observed after a four-hour AngII (100 nM, black square) or 5-HT (1 µM, black circle) stimulation. (c) TNF-α was significantly increased in adult cardiac fibroblasts isolated from wild-type (WT, black square) mice after a height-hour stimulation with ISO (10 µM), but not after pre-incubation with ZD7155 (100 nM) (black circle, ZD) for 30 minutes.

(d) Finally, stimulation with AngII (100 nM) increased TNF-α supernatant concentrations in adult cardiac fibroblasts from wild-type mice (5-HT2B$^{+/+}$; +/+, black square; ), but not after 30 min pre-incubation with the 5-HT$_{2B}$R antagonist SB206553 (100 nM) (open circle, SB) or from global mutant for 5-HT$_{2B}$Rs (5-HT2B$^{−/−}$;−/−, reverse triangle). Results are means±S.E.M expressed in pg/ml of cardiac fibroblasts supernatants (n=3 independent fibroblast cultures each determination in triplicate). *p<0.05 vs. antagonist-treated.

(e) Wild-type (+/+) adult cardiac fibroblasts were pre-incubated with various inhibitors, SB206553 (100 nM), a selective 5-HT$_{2B}$R antagonist, n=4, ZD7155 (100 nM), a selective AT-1R antagonist, n=3, AG1478 (100 nM), a selective inhibitor of EGF-R, n=3, GM6001 (100 nM), an MMP inhibitor, n=5 (see dose response in Online Fig. IIIC-D), PP2 (200 nM), a Src inhibitor, n=3, PD098059 (10 µM), a specific inhibitor of ERK1/2, n=3, SB203580 (10µM) a specific inhibitor of p38, n=3, for 30 minutes, stimulated 4 hours with AngII (100 nM) (grey bars) or 5-HT (1 µM) (black bars) or not , n=10, (n= number of independent fibroblasts culture, each determination in triplicate). Cultures of HB-EGF mutant fibroblasts (−/−-HB-EGF) were also stimulated with EGF (10 ng/ml), AngII (100 nM) or 5-HT (1 µM) during 4 hours (n=4) and TNF-α supernatant concentrations were measured by ELISA. Results are means±S.E.M expressed in pg/ml of cardiac fibroblasts supernatants. **p<0.001, *p<0.05 vs. control, #p<0.05 vs. AngII or 5-HT.

Correlations between the cardiac 5-HT$_{2B}$R expression levels (in fmol/mg of proteins) and the plasma levels (in pg/ml) of TNF-α (f), are shown. Similarly, correlations between the plasma levels of norepinephrine with TNF-α (g), are given. P value (p) and Spearman rank correlation (r) are presented.
## Online Table I

**Left ventricular echocardiographic parameters of transgenic mice**

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<th>LVPW (mm)</th>
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<td>5-HT$_2$B$^{-/-}$</td>
<td>365 ± 6</td>
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Echocardiographic parameters at day 0 (D0) and day 7 (D7) of ISO infusion. EDD: end-diastolic diameter, ESD: end-systolic diameter, HR: heart rate in beat per min, PWd: posterior wall thickness in diastole, Sd: septum wall thickness in diastole. *: p<0.05 vs. D0.
Online Table II:
Clinical characteristics of the 16 heart failing patients

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<td>10.9</td>
<td>15</td>
<td>184</td>
<td>320</td>
<td>32</td>
<td>1345</td>
<td>147</td>
<td>116</td>
<td>61.2</td>
<td>79.6</td>
</tr>
<tr>
<td>16</td>
<td>63</td>
<td>M</td>
<td>3</td>
<td>14.5</td>
<td>30</td>
<td>47</td>
<td>158</td>
<td>27</td>
<td>649</td>
<td>39</td>
<td>116</td>
<td>8.6</td>
<td>25.3</td>
</tr>
</tbody>
</table>

The characteristics of the 16 heart failing patients {9 : primary dilated cardiomyopathy (patients 1-9), 6 : ischemic cardiopathy (patients 11-16) and 1 : congenital cardiopathy (patient 10); 16 males and 1 female (patient 10)} are the following: VO₂max : maximal capacity for oxygen consumption (ml/min/kg), EF : left-ventricular ejection fraction (%), PCPm : mean pulmonary capillary pressure (mmHg), ANP : plasma atrial natriuretic peptide (pg/ml), BNP : plasma brain natriuretic peptide (pg/ml), NE : plasma norepinephrine (pg/ml), E : plasma epinephrine (pg/ml), Bmax : Bmax of 5-HT₂B receptors in left-ventricular myocardium (fmol/mg prot), plasma IL-6, TNF-α, IL-1β and TGF-β concentrations in pg/ml. NA : not available.