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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_{2B} receptors did not develop isoproterenol-induced left ventricular hypertrophy. However, the exact cardiac cell type(s) expressing 5-HT_{2B} receptors (cardiomyocytes versus noncardiomyocytes) involved in pathological heart hypertrophy was never addressed in vivo. We report here that mice expressing the 5-HT_{2B} receptor solely in cardiomyocytes, like global 5-HT_{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_1Rs and 5-HT_{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_{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_1 and 5-HT_{2B} receptors coexpressed by noncardiomyocytes are limiting key events in adrenergic agonist-induced, angiotensin-dependent cardiac hypertrophy. Accordingly, antagonists of 5-HT_{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.[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.[3] In addition to biomechanical stress, several neurohumoral factors acting via G protein–coupled receptors (GPCRs), including β-adrenergic (β-AR), endothelin and angiotensin (Ang) II type 1 (AT_1R) receptors have been identified as potent inducers of cardiac hypertrophy.[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 β-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.[8] Plasma levels of cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6[9] or transforming growth factor (TGF)-β_1[10] are also significantly increased in primary idiopathic hypertrophic cardiomyopathy. Noncardiomyocyte (cardiac fibroblast) stimulation by adrenergic agonists or Ang II can release growth factors,[11] endothelin-1, and cytokines including TNF-α, IL-6, IL-1β, and TGF-β1.[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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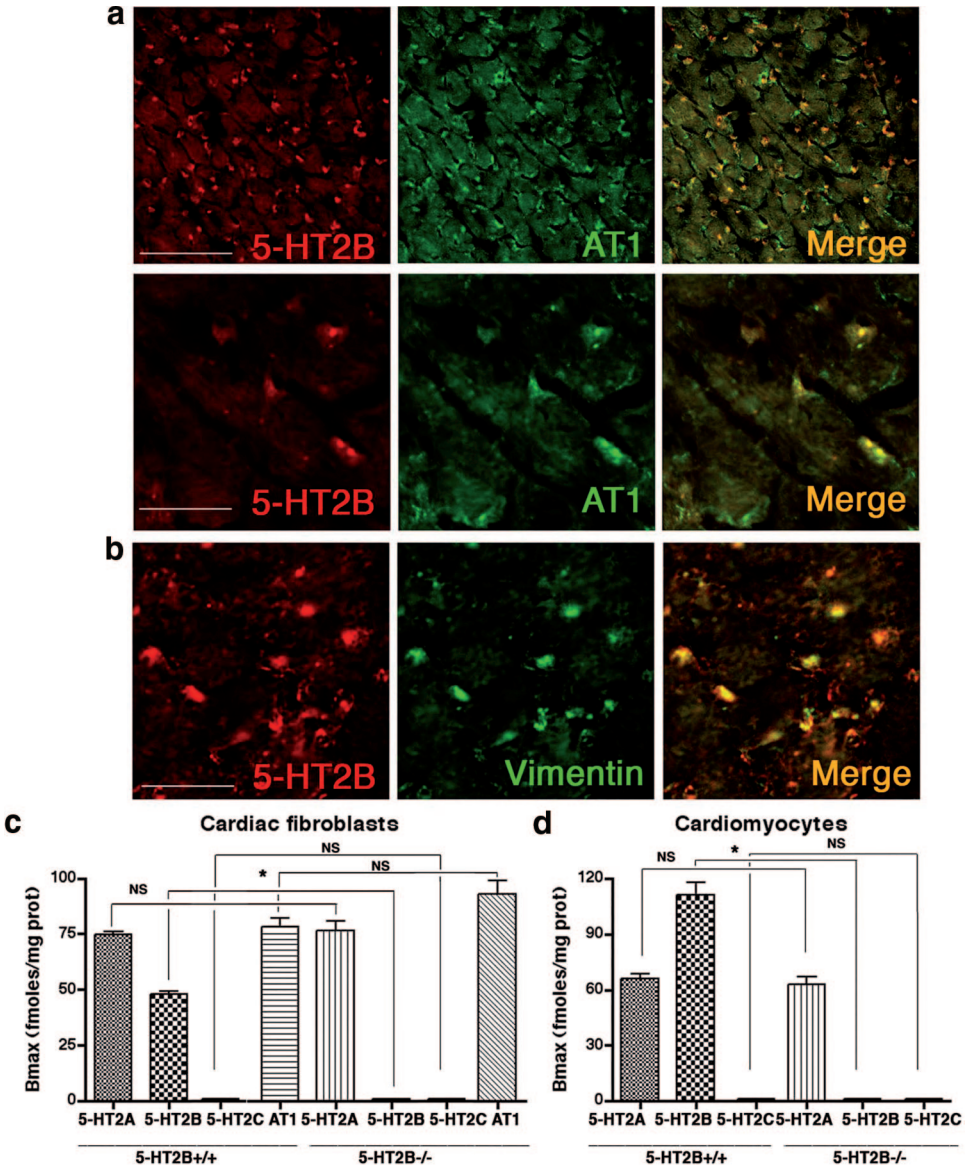

Figure 1. 5-HT_{2B}Rs and AT_1Rs colocalization in fibroblasts. a, Fixed frozen human left ventricle sections were immunostained using antisera against 5-HT_{2B}R or AT_1R. Single confocal plane observation reveals that 5-HT_{2B}R localization (5-HT2B) is mainly colocalized (merge) with AT_1R-positive cells (AT1); top scale bar, 10 μm; bottom images, higher magnification (scale bar, 5 μm). b, Fixed human left ventricle sections were immunostained using antisera against 5-HT_{2B}R or vimentin. Single confocal plane observation reveals that 5-HT_{2B}R is mainly colocalized (merge) with vimentin-positive cells; scale bar, 5 μm. Radioligand binding revealed the expression level (Bmax) of 5-HT_{2A}Rs, 5-HT_{2B}Rs, 5-HT_{2C}Rs, or AT_1Rs in primary culture of adult mouse cardiac fibroblasts (c) or cardiomyocytes (d) of wild-type and mutant mice for 5-HT_{2B}Rs (n=3 independent cultures; each determination in triplicate). Results are means±SEM in maximal density of receptor sites Bmax expressed as fmol/mg of membranes proteins *$P<0.05$; NS, $P>0.05$ vs wild-type mice.

the observation that mice with cardiac-restricted overexpression of TNF-α, IL-6, or IL-1 exhibited cardiac hypertrophy.[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.[18] However, it was reported that ISO had no hypertrophic effects on adult rat myocytes,[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_{2B} receptors (5-HT_{2B}Rs) have a trophic action on newborn cardiomyocytes in vitro[21] and by α-myosin heavy chain (α-MHC)-dependent 5-HT_{2B}R overexpression in cardiomyocytes in vivo.[22] The initial cardiomyopathy of 5-HT_{2B}R mutant mice is compensated over time in the absence of hypertrophic stage.[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_{2B}R mutant mice) or pharmacological (SB206553 or SB215505, 5-HT_{2B}R antagonists) blockade of 5-HT_{2B}R function completely prevented ISO-induced cardiac hypertrophy.[13] Recently, 5-HT_{2B}Rs were shown to be required for left ventricular hypertrophy in another model of cardiac hypertrophy (Ang II chronic infusion).[24] Nevertheless, neither the exact cardiac cells requiring 5-HT_{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_{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–, AT_1R–, and 5-HT_{2B}R–dependent hypertrophic factor release; and (4) whether similar mechanisms could be found in human CHF.

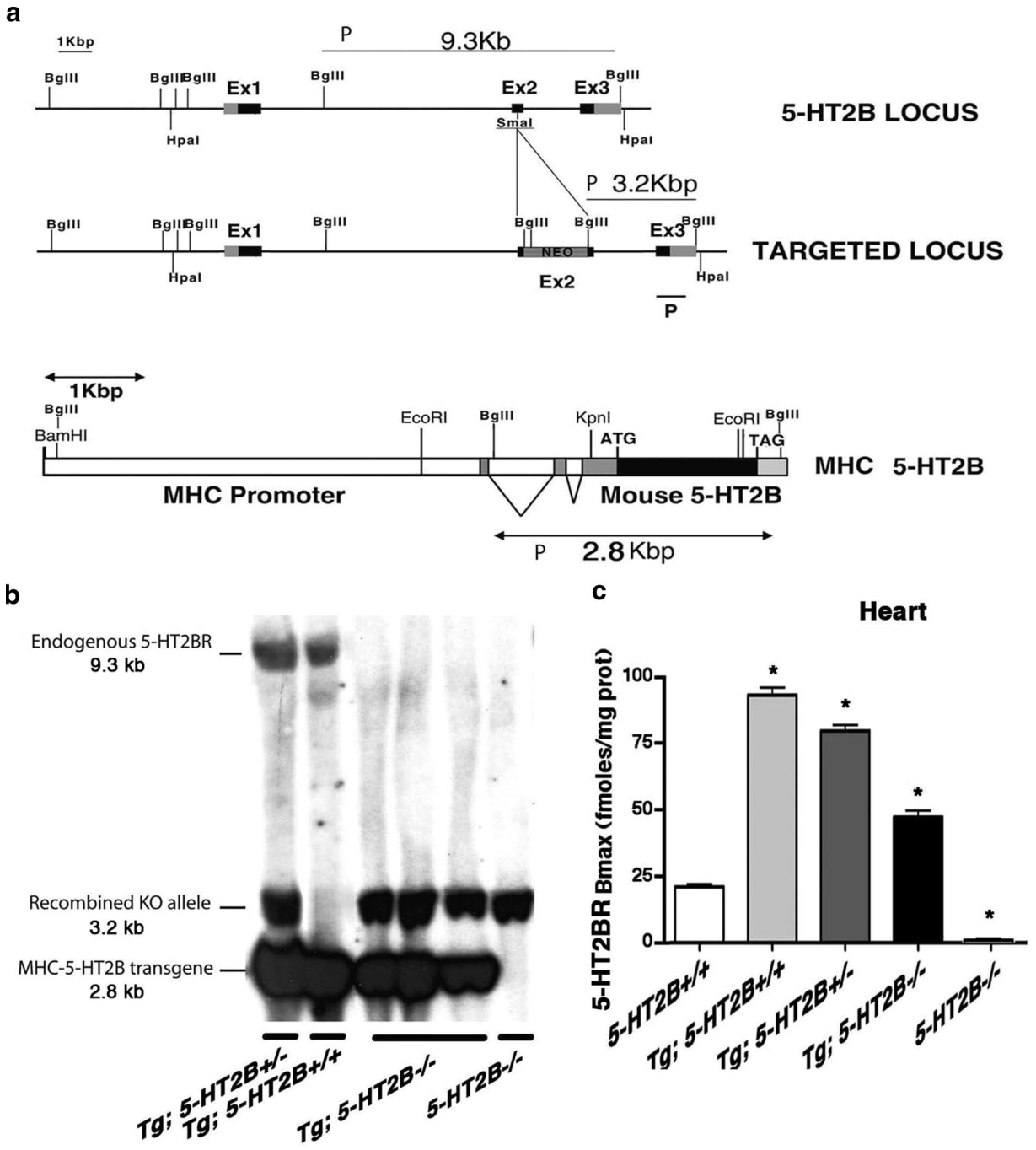

Figure 2. Rescued expression of 5-HT$_{2B}$Rs in cardiomyocytes in vivo. a, Top, Restriction map of the 5-HT$_{2B}$ receptor genomic locus of interest. Middle, Mutated locus after homologous recombination in $-/-$ mice. Bottom, Mouse α-MHC-5-HT$_{2B}$ transgene. b, The different genotypes were detected by Southern blot analysis. The probe (P) detects a 9.3-kbp *Bgl*II fragment from wild-type DNA, whereas it is reduced to 3.2 kbp in the homologous recombined allele and to 2.8 kbp for α-MHC2B transgene. Radioligand binding revealed the expression level of 5-HT$_{2B}$Rs as maximal density of receptor sites (Bmax) in heart of different transgenics (c) (n=6 to 8 mice, each determination in triplicate). Results are means±SEM in maximal density of receptor site Bmax expressed as fmol/mg of membranes proteins. *$P<0.05$ vs wild-type control mice (5-HT$_{2B}$R$^{+/+}$): transgenic α-MHC-5-HT$_{2B}$R over wild-type (Tg; 5-HT$_{2B}$$^{+/+}$); transgenic α-MHC-5-HT$_{2B}$R over heterozygous (Tg; 5-HT$_{2B}$$^{+/-}$); transgenic α-MHC-5-HT$_{2B}$R over homozygous (Tg; 5-HT$_{2B}$$^{-/-}$); global homozygous 5-HT$_{2B}$$^{-/-}$ mice.

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.[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-

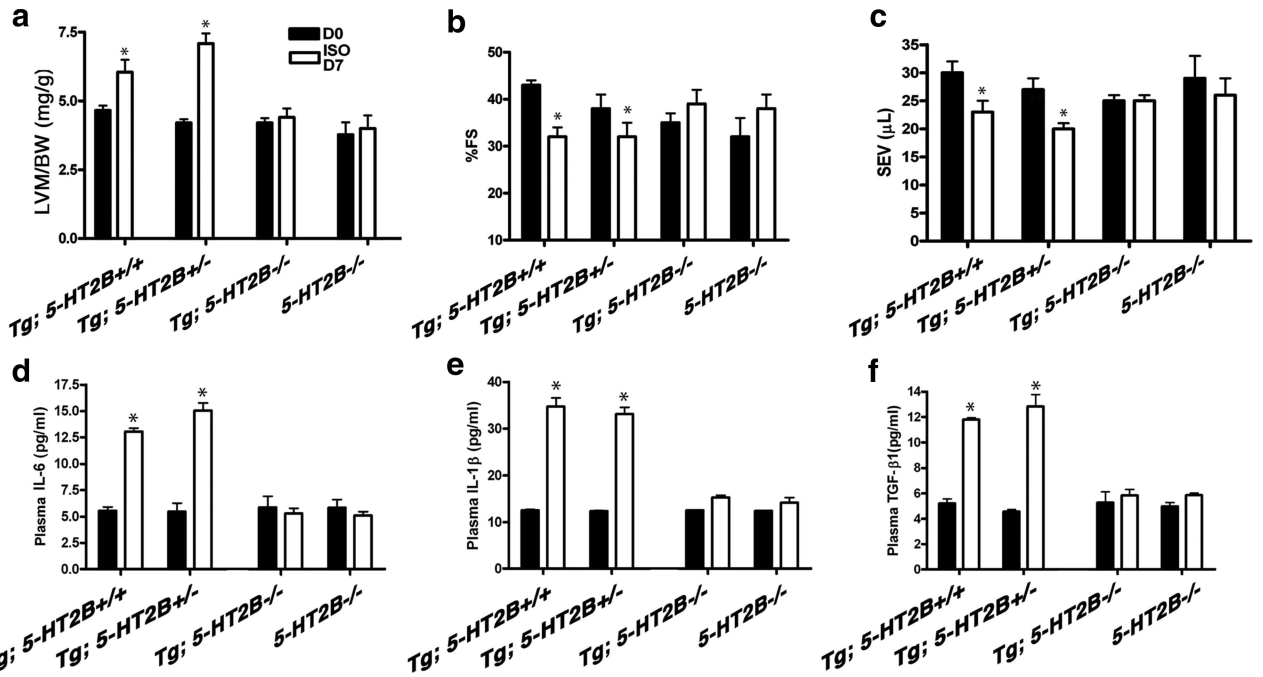

Figure 3. Expression of 5-HT_{2B}Rs by noncardiomyocytes is required for ISO-induced cardiac hypertrophy in vivo. In the different transgenics (Tg; 5-$HT_{2B}^{+/+}$; Tg; 5-$HT_{2B}^{+/-}$; Tg; 5-$HT_{2B}^{-/-}$; and global 5-$HT_{2B}^{-/-}$ mice), ISO infusion led to a significant increase in left ventricular 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-$HT_{2B}^{+/+}$ and Tg; 5-$HT_{2B}^{+/-}$ but not in Tg; 5-$HT_{2B}^{-/-}$ mice expressing 5-HT_{2B}Rs solely in cardiomyocytes or global 5-$HT_{2B}^{-/-}$ mice. The cytokine IL-6 (d), IL-1β (e), and TGF-β (f) plasma levels are also increased only in Tg; 5-$HT_{2B}^{+/+}$ and Tg; 5-$HT_{2B}^{+/-}$ but not in Tg; 5-$HT_{2B}^{-/-}$ mice expressing 5-HT_{2B}Rs solely in cardiomyocytes or global 5-$HT_{2B}^{-/-}$ mice. Values are means±SEM. *P<0.05, D0 vs D7 ISO (30 μg/g per day) (n=6 to 8 mice).

diography.[13] After echocardiographic analysis, mice were euthanized by CO_2 and weighed.

Analysis of 5-HT_2R and AT_1R 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.[23]

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 Prétant 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%)[13] and negative staining for macrophage marker F4/80[26] (<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_1 were measured in plasma and culture supernatants by ELISA kits (Bertin, DY 406, DY 401, DY 410 and DY 1679, R&D systems).[13]

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-HT_{2B}R antibody (Pharmingen,1:100), a rabbit anti-AT_1R (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-HT_{2B}R 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-AT_1R (N-10, Santa Cruz Biotechnology, 1:1000).

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-HT_{2B}Rs by Noncardiomyocytes Is Required for ISO-Induced Cardiac Hypertrophy

We first verified the expression of 5-HT_{2B}Rs and AT_1Rs either by immunohistochemistry of human adult left ventricles (Figure

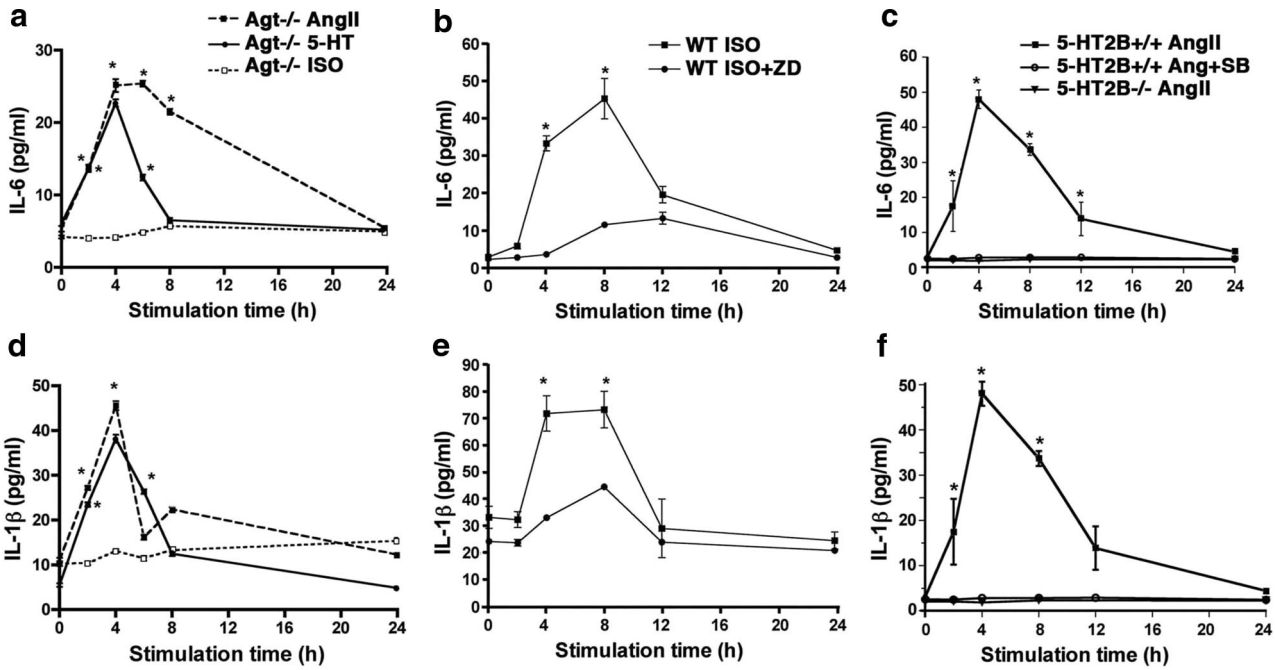

Figure 4. 5-HT$_{2B}$Rs and AT$_1$Rs are required for ISO-induced cytokine release in cardiac fibroblast culture. A significant increase in IL-6 (a) and IL-1β (d) cytokines could not be observed in adult cardiac fibroblasts isolated from Agt$^{-/-}$ mice unable to generate Ang II after stimulation with 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$_{2B}$R 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$_{2B}$R$^{-/-}$ 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$_{2B}$R$^{-/-}$ mice were crossed with transgenic mice expressing 5-HT$_{2B}$Rs selectively in cardiomyocytes via the cardiomyocyte-specific α-MHC promoter (Tg) (Figure 2a).[22] 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,[27] 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

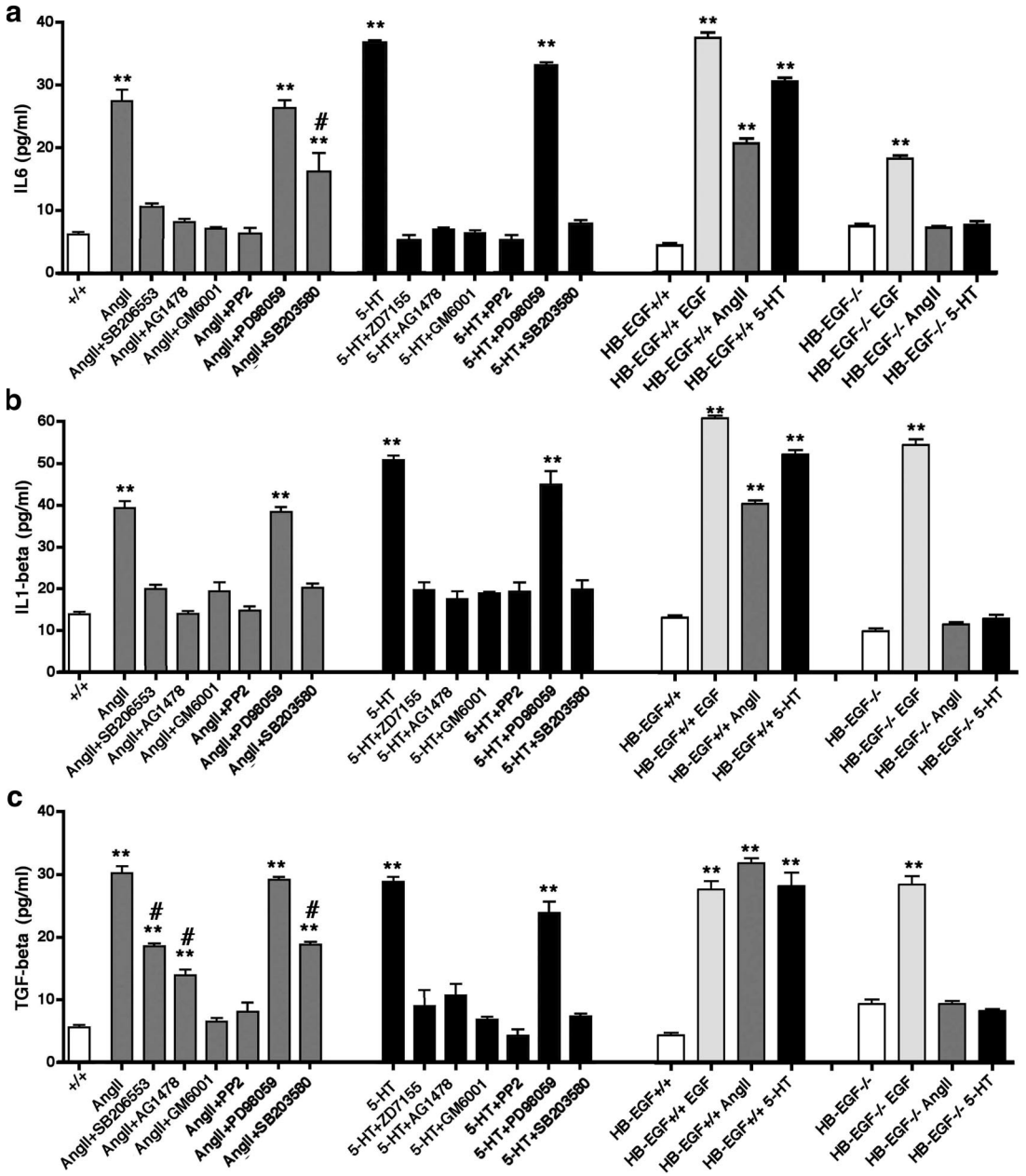

Figure 5. Ang II– or 5-HT–induced cytokine release is mediated by EGF-R transactivation. Wild-type (+/+) adult cardiac fibroblasts were preincubated with various inhibitors (SB206553 [100 nmol/L], a selective 5-HT_{2B}R antagonist [n=4]; ZD7155 [100 nmol/L], a selective AT_1R antagonist [n=3]; AG1478 [100 nmol/L], a selective inhibitor of EGF-R [n=3]; GM6001 [100 nmol/L], an MMP inhibitor [n=5] [see dose response in supplemental Figure III, C and D]; PP2 [200 nmol/L], a Src inhibitor [n=3]; PD098059 [10 μmol/L], a specific inhibitor of ERK1/2 [n=3]; SB203580 [10 μmol/L], a specific inhibitor of p38 [n=3]) for 30 minutes, stimulated for 4 hours with Ang II (100 nmol/L) (gray bars) or 5-HT (1 μmol/L) (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), Ang II (100 nmol/L) or 5-HT (1 μmol/L) during 4 hours (n=4). IL-6 (a), IL-1β (b), and TGF-β (c) supernatant concentrations were measured by ELISA. Results are means±SEM expressed in pg/mL of cardiac fibroblasts supernatants. $^{**}P<0.001$, $^{*}P<0.05$ vs control; #$P<0.05$ vs Ang II or 5-HT.

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-HT_{2B}Rs 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-HT_{2B}Rs were required for cytokine production by newborn cardiac fibroblasts (supplemental Figure III, E and F).

5-HT_{2B}Rs and AT_1Rs Share a Common Epidermal Growth Factor Receptor Transactivation Pathway–Mediating Cytokine Release

Similar to the effects of 5-HT_{2B}R 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 noncardiac cells, activation of AT_1Rs or β-ARs has been shown to induce shedding of heparin-binding epidermal growth factor (HB-EGF) through activa-

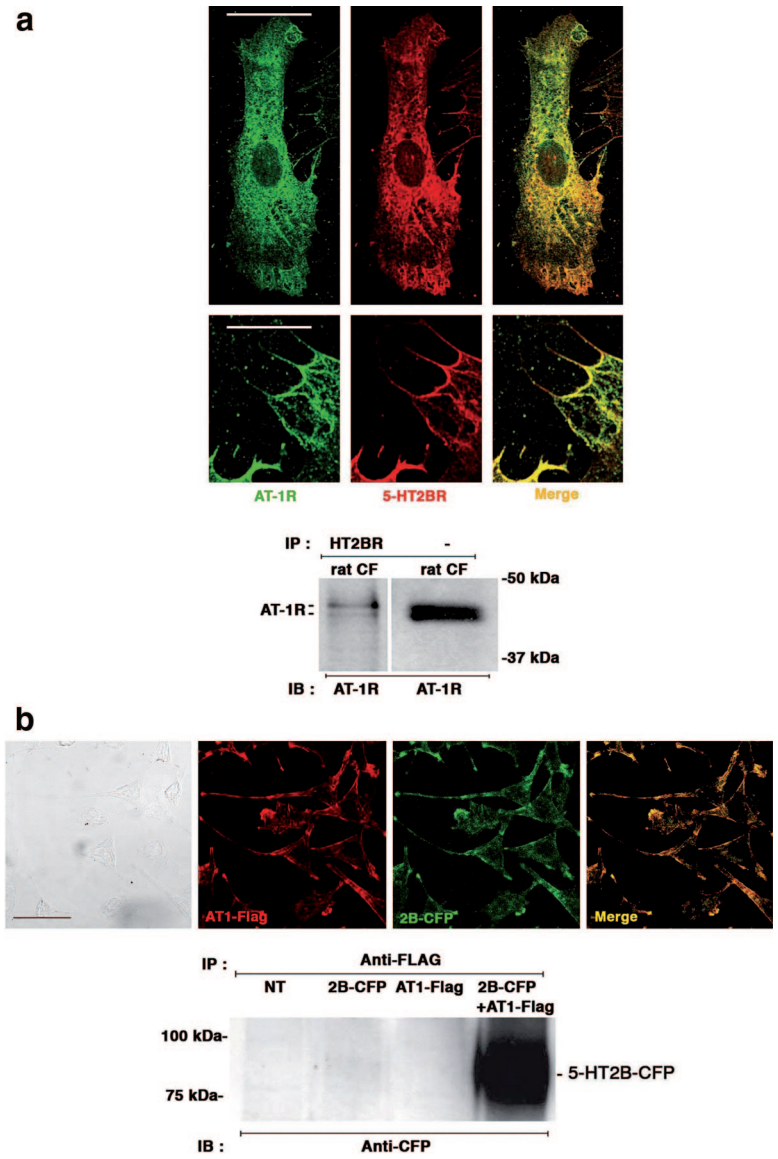

Figure 6. 5-HT$_{2B}$Rs and AT$_1$Rs colocalize and coimmunoprecipitate in transfected cells. a, Fixed newborn rat cardiac fibroblast primary culture were immunostained using antisera against 5-HT$_{2B}$R or AT$_1$R. Single confocal plane observation reveals that 5-HT$_{2B}$R localization (5-HT2BR) is mainly colocalized (merge) with AT$_1$R-positive cells (AT$_1$R); top scale bar, 2 μm; bottom, higher magnification (scale bar, 1 μm). Newborn cardiac fibroblast (CF) were immunoprecipitated with anti–5-HT$_{2B}$R antibody (IP) (left lane), and then crude extract (right lane) or immunoprecipitate were immunoblotted (IB) using anti-AT$_1$R antibody. b, Top, HEK293 cells were cotransfected with 5-HT$_{2B}$R-CFP and AT$_1$R-Flag. Single confocal plane observation reveals the 5-HT$_{2B}$R-CFP localization (2B-CFP), AT$_1$R-Flag localization (AT-1-Flag), and their colocalization by overlay (merge). Scale bar, 2 μm. Bottom, HEK293 cells, nontransfected (NT) or transfected with 5-HT$_{2B}$R-CFP (2B-CFP), AT$_1$R-Flag (AT1-Flag), or 5-HT$_{2B}$R-CFP in combination with AT$_1$R-Flag were immunoprecipitated with anti-Flag antibody. Immunoprecipitates (IP) anti-Flag were immunoblotted using anti-CFP antibody. Blots are representative of 3 independent experiments.

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-HT$_{2B}$Rs and AT$_1$Rs Colocalize and Coimmunoprecipitate

By confocal microscopy, we first observed membrane colocalization of 5-HT$_{2B}$Rs and AT$_1$Rs in rat cardiac fibroblasts (Figure 6a). After immunoprecipitation of neonatal rat cardiac fibroblast extracts with an anti-5-HT$_{2B}$R antibody, we detected bands of 41 to 43 kDa (expected molecular mass for AT$_1$Rs) similar to those observed by direct Western blot analysis (Figure 6a) with an anti-AT$_1$R antibody. We further confirm these putative interactions, using HEK293 transfected cells with FLAG-tagged human AT$_1$Rs and CFP-tagged human 5-HT$_{2B}$Rs, that showed membrane colocalization (Figure 6b). After immunoprecipitation with an anti-FLAG antibody, we probed Western blots of immunoprecipitations with an anti-GFP antibody. We detected a

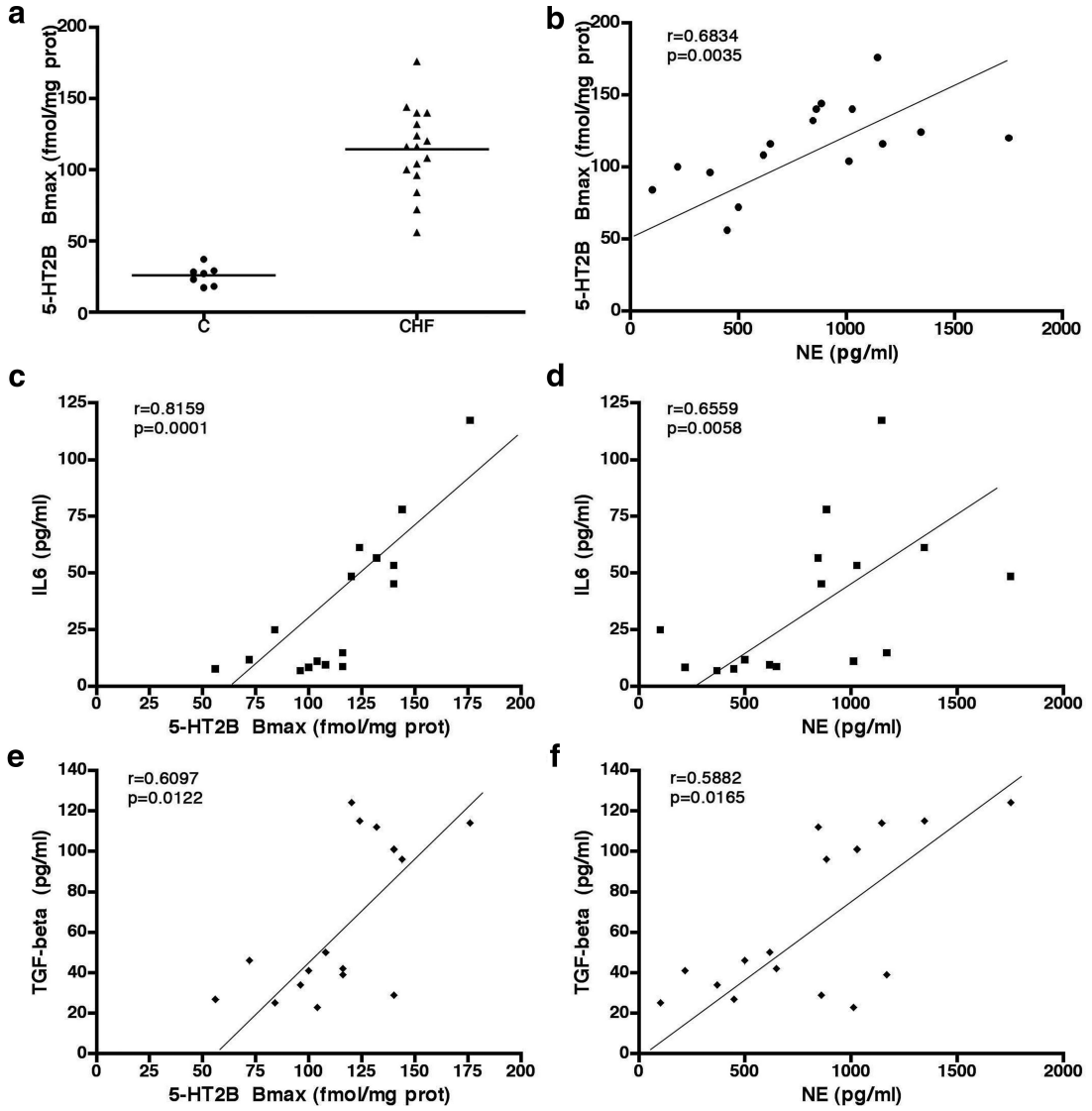

Figure 7. The overexpression of 5-HT_{2B}Rs observed in human cardiac hypertrophy is correlated with plasma norepinephrine and cytokine levels. a, Expression of 5-HT_{2B}Rs (Bmax), obtained from biopsies of left ventricles, was found to be significantly elevated and is expressed the average of subepicardial, subendocardial, and midventricle biopsies. Correlations between the cardiac 5-HT_{2B}R expression levels (in fmol/mg of proteins) and the plasma levels (in pg/mL) of either norepinephrine (b), IL-6 (c), TGF-β (e), or TNF-α (Supplemental Figure 4f) are shown. Similarly, correlations between the plasma levels of norepinephrine with either IL-6 (d), TGF-β (f), or TNF-α (Supplemental Figure 4g), are given. Probability value (p) and Spearman rank correlation (r) are presented.

single band of 80 kDa (the expected molecular mass of the CFP-5-HT_{2B}R) only in cotransfected cells (Figure 6b). These results strongly suggest that AT_1Rs and 5-HT_{2B}Rs colocalize and may directly interact in common signaling complexes in transfected cells.

5-HT_{2B}R Overexpression in Human Heart Failure

Because 5-HT_{2B}Rs were also expressed in both human cardiac fibroblasts and cardiomyocytes (Figure 1), we assessed a putative contribution of 5-HT_{2B}Rs in human CHF. We looked for 5-HT_{2B}R expression in left ventricular biopsies of 16 CHF patients, compared to 7 normal control subjects. Expression of 5-HT_{2B}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_{2B}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_{2B}R expression is linked to cardiac cytokine production during the sympathetic overactivity associated with CHF.

Discussion

In light of our previous reports, 5-HT_{2B}Rs participate in trophic responses of the myocardium by acting directly on cardiomyocytes[21,22] or indirectly on noncardiomyocytes

through the release of paracrine factors induced by chronic ISO stimulation.[13] 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.[30] Interestingly, angiotensinogen mRNA and protein levels are increased by β-AR stimulation in neonatal cardiac fibroblasts,[31] 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/AT$_1$R 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, Agt$^{-/-}$ 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 AT$_1$Rs 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 AT$_1$R stimulation is absolutely required for hypertrophic cytokine release in heart.

The present report addresses unknowns regarding the AT$_1$R 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.[32,33] TNF-α–converting enzyme (TACE) (ADAM-17) was found to control HB-EGF shedding in fibroblasts,[34] and a recent report indicated that 5-HT$_{2B}$Rs can directly regulate this enzyme activity in neuronal cells.[35] 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$\Rightarrow$Ang II$\Rightarrow$5-HT$_{2B}$+AT$_1$Rs$\Rightarrow$Src$\Rightarrow$MMPs$\Rightarrow$HB-EGF$\Rightarrow$ErbB-1/4-Rs$\Rightarrow$p38$\Rightarrow$IL-6, TNF-α, TGF-β, and IL-1β release. All of these findings support that AT$_1$Rs and 5-HT$_{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 AT$_1$Rs. 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[36]). The protein network associated with the C terminus of the 5-HT$_2$Rs includes scaffolding proteins containing 1 or several PDZ domains, signaling proteins and proteins of the cytoskeleton that may be involved in signaling complexes.[37] More work will be necessary to conclude whether in vivo interactions between AT$_1$Rs 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 AT$_1$Rs and β_2-ARs in COS-7 cells that express equal endogenous levels of AT$_1$Rs and β_2-ARs and was also shown to occur in adult cardiomyocytes.[38] Together, our findings are consistent with the hypothesis that AT$_1$Rs and 5-HT$_{2B}$Rs exist in common signaling complexes and that they may interact together.

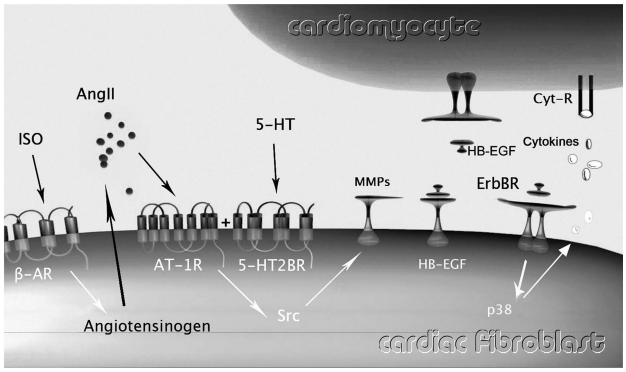

Figure 8. Deduced signaling pathway for cytokine production after 5-HT or Ang II stimulation in adult cardiac fibroblasts. Stimulation of β-ARs on cardiac fibroblasts by ISO leads to the release of Agt-dependent Ang II that stimulates the AT$_1$R-5-HT$_{2B}$R complexes activating MMPs, which induces the pro-HB-EGF cleavage. The mechanism that leads to MMPs activation by AT$_1$Rs or 5-HT$_{2B}$Rs involves Src. Soluble HB-EGF activates ErbB-Rs to induce cytokine release via p38 mitogen-activated protein kinase activation. Released cytokines and HB-EGF bind their receptors in cardiomyocytes to activate hypertrophy in a paracrine way (see also the online data supplement and Movie).

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[39] or rat[40] failing heart tissue, during the functional recovery of end-stage human heart failure,[41] and in rats after banding of the ascending aorta.[42,43] 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$_{2B}$ 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-HT_{2B}R antagonist blocks both Ang II and adrenergic adverse effects in pathological conditions with no alterations of hemodynamics or blood pressure.

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Disclosures

None.

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