Sigma-1 receptor stimulation with fluvoxamine ameliorates transverse aortic constriction-induced myocardial hypertrophy and dysfunction in mice.

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Selective serotonin reuptake inhibitors (SSRIs) are known to reduce post-myocardial infarction (MI)-induced morbidity and mortality. However, the molecular mechanism underlying SSRI-induced cardioprotection remains unclear. Here, we investigated the role of sigma-1 receptor (Sig-1R) stimulation with fluvoxamine on myocardial hypertrophy and cardiac functional recovery. Male ICR mice were subjected to transverse aortic constriction (TAC) in the cardiac aortic arch. To confirm the cardioprotective role of fluvoxamine by Sig-1R stimulation, we treated mice with fluvoxamine (0.5 or 1 mg/kg) orally once a day for 4 weeks after the onset of aortic banding. Interestingly, in untreated mice, Sig-1R expression in the left ventricle (LV) decreased significantly over the 4 weeks as TAC-induced hypertrophy increased. By contrast, fluvoxamine administration significantly attenuated TAC-induced myocardial hypertrophy concomitant with recovery of Sig-1R expression in LV. Fluvoxamine also attenuated hypertrophy-induced impaired LV fractional shortening. The fluvoxamine cardioprotective effect was nullified by treatment with a Sig-1R antagonist (NE-100, 1mg/kg). Importantly, another SSRI with very low affinity for Sig-1R, paroxetine, did not elicit antihypertrophic effects in TAC mice and cultured cardiomyocytes. Fluvoxamine treatment significantly restored TAC-induced impaired Akt and eNOS phosphorylation in the LV. Our findings suggest that fluvoxamine protects against TAC-induced cardiac dysfunction via upregulated Sig-1R expression and stimulation of Sig-1R-mediated Akt-eNOS signaling in mice. This is the first report of a potential role for Sig-1R stimulation by fluvoxamine in attenuating cardiac hypertrophy and restoring contractility in TAC mice.
Keywords: sigma-1 receptor (Sig-1R), fluvoxamine, myocardial hypertrophy, protein kinase B (Akt), endothelial nitric oxide synthase (eNOS)
Introduction

Accumulating clinical evidence suggests that depression after myocardial infarction (MI) is associated with higher morbidity and mortality and that selective serotonin reuptake inhibitors (SSRIs) are safe for use in patients with cardiovascular diseases and may even reduce post-MI morbidity and mortality (47). However, the benefit of SSRIs to patients with cardiovascular disease has not been established, because the molecular mechanism underlying SSRI-induced cardioprotection is largely unknown. Notably, SSRIs such as sertraline (38) and fluvoxamine (24) are potent agonists of the sigma-1 receptor (Sig-1R) as well as serotonin uptake inhibitors. The order of affinity of SSRIs for Sig-1R is as follows: fluvoxamine (Ki=36 nM) > sertraline (Ki=57 nM) > fluoxetine (Ki=120 nM) > citalopram (Ki=292 nM) > paroxetine (Ki=1893 nM) (30). Thus, Sig-1R is a potential physiological target for SSRIs not only in the brain but also in heart.

Sigma receptors, which are unique in having two transmembrane segments that show no homology any mammalian G-protein-coupled receptor, are widely expressed in heart muscle. In cardiac tissue, modulation of contractility by sigma receptor ligands was first reported in rat neonatal cultured cardiomyocytes (11). Later sigma receptors were found in the membranes of adult rat ventricular cardiomyocytes (32). Approximately 80% of sigma receptors in the rat ventricular myocardium are sigma-1 subtype and 20% are sigma-2, based on analysis of membrane binding of specific sigma ligands in isolated rat cardiomyocyte preparations (32). Several studies indicate the effects of sigma ligands in isolated rat hearts and report desensitization of sigma receptors in heart muscle by repeated treatment with sigma ligands (12, 33). However, the physiological function of cardiac sigma receptor agonists in normal heart and their therapeutic potential in cardiac disease remain unclear.
Despite information available relevant to modulation of cardiomyocyte function by sigma receptor ligands, no study has described the expression level of sigma receptors, nor have their downstream targets in cardiomyocytes been identified. Most studies of sigma receptors have been carried out using isolated heart preparations (11, 12, 13, 31, 32) and indirect interpretations using nonspecific sigma ligands (such as haloperidol, 1,3-di-o-tolylguanidine [DTG] and (+)-3-3(3-hydroxyphenyl)-N-(1-propyl)piperidine [(+)3-PPP]) (22,27). Moreover, expression of Sig-1R in heart is upregulated by strong stress stimuli such as immobilization and hypoxia and is not affected by aging (31). To assess both the normal biological function and pathophysiological role of sigma-1 receptors, we recently analyzed Sig-1R expression in the heart (2), kidney (3) and thoracic aorta (5). We found that pressure overload-induced hypertrophy significantly decreased expression of Sig-1R in the LV of ovariectomized rats and observed a significant correlation between heart dysfunction and decreased Sig-1R expression in the LV (2).

Since SSRIs including fluvoxamine are used to treat depressive patients with or without cardiac disease, our goal was to define the molecular mechanism underlying fluvoxamine-induced cardioprotection through Sig-1R and evaluate the pathophysiological relevance of Sig-1R in cardiac hypertrophy and heart failure. In the present study, we found that fluvoxamine’s potent Sig-1R stimulatory effect largely blunts development of pathological LV hypertrophic remodeling and promotes cardiac functional recovery. By contrast, paroxetine, which has low affinity for Sig-1R, had no antihypertrophic effect. We also defined a fluvoxamine-induced cardioprotective mechanism through Akt signaling in LV hypertrophic remodeling.
Materials and Methods

Materials

Reagents and antibodies were obtained from the following sources: anti-Sigma-1 receptor antibody (Abcam, Cambridge, UK); anti-eNOS antibody (Sigma, St. Louis, MO); anti-phospho-eNOS antibody (Cell Signaling Technology, Beverly, MA); anti-phospho-Akt antibody (Ser-437) and total-Akt antibody (Cell Signaling Technology, Beverly, MA); anti-phospho-Akt antibody (Thr-308) (Upstate Biotechnology, Lake Placid, NY); anti-phospho-p44/42 MAPK (ERK 1/2) antibody (Thr-202/Tyr-204) and total-p44/42 MAPK (ERK 1/2) antibody (Cell Signaling Technology, Beverly, MA); anti-phospho-PKCα antibody (Ser-657), total-PKCα antibody and anti-heat shock protein-90 antibody (Upstate Biotechnology, Lake Placid, NY); anti-Caveolin-3 antibody (BD Biosciences, San Jose, CA); anti-β-tubulin antibody (Sigma, St. Louis, MO); anti-rabbit antibody (Amersham Biosciences Inc., Piscataway, NJ). The Sig-1R agonist fluvoxamine maleate was supplied by Meiji Seika Kaisha, Ltd (Tokyo, Japan), and the specific Sig-1R antagonist NE-100 (N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride) was generously supplied by Taisho Pharmaceutical Co. Ltd (Ohmiya, Japan). Other reagents were of the highest quality available (Wako Pure Chemicals, Osaka, Japan).

Animals

All procedures for handling animals complied with the Guide for Care and Use of Laboratory Animals and were approved by the Animal Experimentation Committee of Tohoku University Graduate School of Pharmaceutical Sciences. Adult male ICR mice weighing 35 to 40 g were obtained from Nippon SLC (Hamamatsu, Japan). Ten-week-old males were acclimated to the local environment for 1 week, which included housing in
polypropylene cages at 23 ± 1°C in a humidity-controlled environment maintained on a 12-h light/dark schedule (lights on 8:00 AM–8:00 PM). Mice were provided food and water ad libitum.

Primary cardiomyocyte cultures from neonatal rats

Neonatal ventricular myocytes were isolated from hearts of 1- to 3-day-old Wistar rats by collagenase digestion and cultured according to the method of Lu et al (25). Briefly, neonatal rats were decapitated and their hearts removed immediately. Ventricles were separated from the heart and washed in Hank’s balanced salt solution (137.0mM NaCl, 5.4mM KCl, 0.4mM KH₂PO₄, 0.3mM Na₂HPO₄・12H₂O, 6.12mM glucose, 4.2mM NaHCO₃, pH 7.4), and myocytes were dissociated from the ventricles by serial digestion with 0.1% trypsin and 0.05% DNase I in Hank’s balanced salt solution. After digestion, dissociated cardiomyocytes were collected and suspended in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 0.02% trypsin inhibitor to inhibit further digestion. Cells were collected by centrifugation (4°C, 1000 x g for 10 min). After the supernatant was discarded, DMEM containing 10% FBS was added. Cells were gently agitated and then plated on uncoated 90-mm culture dishes. Plates were allowed to stand for 90 min at 37°C in a CO₂ incubator to remove nonmyocytes, which attached to the plates. Unattached myocytes were collected and plated at 1-2 x 10⁶ cells per 5-mm dish and incubated with DMEM and 10% FBS in a humidified incubator with DMEM and 5% CO₂ at 37°C for 48h. Cells were cultured in serum-free DMEM for 24 h before treatment with angiotensin II (Ang II) (100nM).

Morphological analysis of cultured cardiomyocytes
Cultured myocytes were plated on collagen-coated cover glasses at a density of 1-2 x10^6 cells per coverslip of 12 mm diameter. After incubation in the presence or absence of angiotensin II for 48 h, cultured cells were washed 3 times in phosphate-buffered saline (PBS; pH 7.4) and fixed in PBS plus 4% formaldehyde. After permeabilization with 0.1% Triton X-100 in PBS, fixed cells were incubated with 1% bovine serum albumin in PBS for 30 min. For cell size measurement, cells were incubated 3 h at room temperature with Rhodamine-conjugated Phalloidin (1:300; Molecular Probes, Eugene, OR) in PBS containing 1% BSA. After cell images were acquired using an Olympus fluorescence microscope, the surface area of cells was quantitated using Image J program. All cells from randomly selected fields were examined in each condition (at least 100 myocytes per group). The surface area of control cells was expressed as 100% and compared with that of treated cells.

siRNA transfection in cultured cardiomyocytes

Sig-1R siRNA (sense, 5’-ACACGTGGATGGTGGAGTA-3’ and anti-sense, 5’-TACTCCACCATCCACGTGT-3’) was purchased from Exigen (Exgen Ltd., Tokyo, Japan). Cultured myocytes were plated on uncoated 90-mm culture dishes on collagen-coated cover glass. Transfections were performed with 100nM Sig-1R siRNAs according to the method of Wang et al (49). Briefly, 20μM Sig-1R siRNA in 5μl was added to 180μl opti-MEM (Invitrogen), and 5μl Lipofectamine 2000 (Invitrogen) was added to 10μl opti-MEM. Both solutions were incubated separately at room temperature for 5 min and then mixed and incubated at room temperature for 15 to 20 min. Meanwhile, medium containing serum was removed from the cells and replaced with 1ml of opti-MEM. This solution was then removed and replaced with 800μl of fresh opti-MEM to which 200μl of the siRNA solution was added. Cells were then incubated at 35°C in a 5% CO2 atmosphere
for 4 h to initiate transfection. 500µl DMEM supplemented with 5% FBS was then added to each well and the transfection was continued for up to 72 h. Cultured cells were washed with cold PBS and stored at -80°C until western blot analysis was performed.

Surgical procedures

Transverse aortic constriction (TAC) was performed on male ICR mice as described previously (35). After acclimatization for 7 days, animals were anesthetized with tribromoethanol (0.25–0.3 g/kg i.p.). The animal was placed supine and endotracheal intubation was performed rapidly. The cannula was connected to a volume-cycled rodent ventilator with a tidal volume of 0.5ml room air and respiratory rate of 110 breaths/min. The chest cavity was opened using scissors to make a small incision at the level of the second intercostal space at the left upper sternal border. After isolating the aortic arch, the transverse aorta was isolated between the carotid arteries and constricted by a 7-0 silk suture ligature tied firmly against a 27-gauge needle. The needle was promptly removed to produce an aortic constriction of 0.4 mm in diameter. The chest cavity was then closed with a 6-0 nylon suture, and mice were allowed to recover from anesthesia while keeping the body temperature at 37°C. Sham-operated animals, which underwent a similar surgical procedure without aortic constriction, served as controls.

Experimental design

Initially, we observed downregulated expression of Sig-1R in TAC-induced hypertrophy. ICR mice were randomly separated into four groups: 1) sham (n=9), 2) TAC for 2 weeks (n=6), 3) TAC for 4 weeks (n=6) and, 4) TAC for 6 weeks (n=7). We then observed the cardioprotective role of Sig-1R-mediated signaling and randomly separated ICR mice
into ten treatment groups: 1) sham (n=15), 2) sham plus fluvoxamine (1mg/kg) (n=5), 3) sham plus NE-100 (1mg/kg) (n=5), 4) TAC plus vehicle treatment group (TAC-vehicle) (n=12), 5) TAC plus fluvoxamine (0.5 mg/kg) treatment group (F 0.5) (n=7), 6) TAC plus fluvoxamine (1.0 mg/kg) treatment group (F 1.0)(n=7), 7) TAC plus fluvoxamine (1.0 mg/kg) plus NE-100 treatment group (F 1.0-NE)(n=7), 8) sham plus paroxetine (0.4mg/kg) treatment group (P 0.2) (n=5), 9) TAC plus paroxetine (0.2mg/kg) treatment group (P 0.2) (n=4) and 10) TAC plus paroxetine (0.4mg/kg) treatment group (P 0.4) (n=4).

**Drug administration**

Fluvoxamine and paroxetine maleate were dissolved in 0.5% CMC (calboxymethylcellulose) and NE-100 was dissolved in 0.9% saline water. Vehicle, fluvoxamine (0.5 and 1.0 mg/kg), paroxetine (0.2 and 0.4 mg/kg) and NE-100 (1.0 mg/kg) were administered orally for 4 weeks (once daily) in a volume of 1ml/100g of mouse body weight, starting from the onset of aortic banding.

**Measurement of cardiac hypertrophy**

After four weeks of drug or vehicle administration, animals were subjected to terminal surgery. Mice were weighed and anesthetized with a mixture of ketamine (100 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) (34). The thoracic cavity was opened, and hearts were immediately harvested and weighed. Cardiac indices, expressed as the ratio of heart (in milligrams) to body (in grams) weight (HW/BW), were used to estimate the degree of cardiac hypertrophy.

**Echocardiography**
Noninvasive echocardiographic measurements were performed in mice anesthetized with 2.5% avertin (8 µl/g) (50) using an echocardiograph (SSD-6500; Aloka, Tokyo, Japan) equipped with a 10-Hz linear transducer (UST-5545; Aloka). The heart was imaged in the two-dimensional parasternal short-axis view, and an M-mode echocardiogram of the midventricle was recorded at the level of the papillary muscles. Diastolic and systolic LV wall thickness, LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD) were measured. All measurements were done from leading edge to leading edge according to the American Society of Echocardiography guidelines (26). The percentage of LV fraction shortening (LV%FS) was calculated as (\([\text{LVEDD} - \text{LVESD}] / \text{LVEDD}\) x 100.

**Western blot analysis**

Four weeks after aortic banding, mice were anesthetized, and hearts were excised and quickly perfused with phosphate-buffered saline to wash out blood from coronary vessels. Heart tissue was sliced at 2-mm thickness using a slicer (RBS-2; Zivic-Miller Laboratories, Zelienople, PA). LV tissue samples were then rapidly frozen in liquid nitrogen and stored at -80°C before use. For assays, each frozen sample was homogenized by methods we previously described (4, 6). An equal amount of protein for each sample (25 µg of total protein) was separated on 7.5-15% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA). After blocking with 5% low-fat milk in Tris-buffered saline plus Tween-20, membranes were incubated with specific primary antibodies overnight at 4°C. This procedure was followed by incubation with donkey anti-rabbit IgG coupled to horseradish peroxidase, and the blots were developed using the ECL immunoblotting detection system (Amersham Biosciences) and visualized on X-ray film (Fuji Film). Autoradiographic films were scanned by densitometry (Lasergraphics, Irvine, CA) and quantitated using Imagegause V3.41 (Fuji Film). The relative amounts of
proteins were expressed as percent increase over sham values.

**Statistical analysis**

Values are represented as means ± standard error of the mean (S.E.M.).

Morphometric changes and SDS-PAGE electrophoresis results were evaluated for differences by one way analysis of variance (ANOVA) combined with Dunnett’s post hoc test. A value of P<0.05 was considered statistically significant.
Results

Time course analysis of cardiac hypertrophy and sigma-1 receptor expression

To define the role of Sig-1R receptor expression in cardiac hypertrophy and heart failure, we evaluated the time course of LV function, cardiac hypertrophy, and Sig-1R expression after TAC. Echocardiographic data for the time course study is provided in Supplementary Table 1 and in Fig. 1. We found time-dependent impairment of LV function, as indicated by decreased fractional shortening (FS%) 4- and 6-weeks after TAC in mice ($P<0.01$ vs. sham) (Fig. 1A and 1B). Time course studies to assess heart and lung weights indicated that the heart weight (HW)-to-body weight (BW) ratio (Fig. 1C) increased time-dependently from 1 to 6 weeks following TAC in mice ($P<0.01$ vs. sham). We also found time-dependent increases in the lung weight (LW)-to-body weight (BW) ratio (Fig. 1D) from 1 to 6 weeks following TAC in mice. Interestingly, Sig-1R expression in the LV markedly decreased time-dependently with significantly decreased expression observed 4- and 6-weeks after TAC in mice ($P<0.01$ vs. sham) (Fig. 1E). Thus, our study reveals a significant negative linear correlation of heart dysfunction with reduced Sig-1R expression in the LV.

Effect of sigma-1 receptor stimulation on myocardial hypertrophy and dysfunction

To confirm the cardioprotective role of fluvoxamine and paroxetine, which are typical SSRIs, we treated with either drug or with the Sig-1R antagonist NE-100 for 4 weeks, starting at the onset of TAC in mice. Echocardiographic data are provided in Supplementary Table S3. Oral administration of fluvoxamine (1 mg/kg) or NE-100 (1 mg/kg) alone to mice that had not undergone TAC for 4 weeks had no effect on echocardiographic parameters (Supplementary Table S2) and LV fractional shortening (Supplementary Fig. S1B).
Consistent with temporal changes in heart function, as shown in Fig. 1B, TAC treatment significantly decreased LV fractional shortening (%) ($P<0.01$ vs. sham) compared with sham-operated mice (Fig. 2A, 2B, 3A and 3B). Fluvoxamine treatment restored decreased LV fractional shortening dose-dependently ($P<0.01$ vs. TAC-vehicle for both F 0.5 and F 1.0) (Fig. 2B). Paroxetine treatment, however, failed to restore fractional shortening (Fig. 3B). Co-administration of NE-100 with fluvoxamine nullified fluvoxamine-mediated amelioration of heart dysfunction, as indicated by decreased LV percent FS ($P<0.01$ vs. F 1.0) (Fig. 2B).

Similarly, fluvoxamine treatment significantly restored both the elevated HW-to-BW ratio ($P<0.01$ vs. TAC-vehicle for F 1.0) (Fig. 2C) and the elevated LW-to-BW ratio ($P<0.05$ vs. TAC-vehicle for F 1.0) (Fig. 2D). Paroxetine administration failed to inhibit increases both in HW-to-BW (Fig. 3C) and LW-to-BW (Fig. 3D) ratio.

Co-administration of NE-100 with fluvoxamine nullified the latter’s inhibition of TAC-induced hypertrophy, as indicated by HW-to-BW ratio ($P<0.05$ vs. sham; $P<0.01$ vs. F 1.0) (Fig. 2C) and LW-to-BW ratio ($P<0.05$ vs. sham; $P<0.01$ vs. F 1.0) (Fig. 2D). Moreover, oral administration of fluvoxamine (1 mg/kg) and NE-100 (1 mg/kg) alone to mice that had not undergone for 4 weeks had no effect on the HW/BW (Supplementary Fig. S1C) and the LW/BW (Supplementary Fig. S1D) ratio in sham mice. Taken together, we conclude that the anti-hypertrophic effect of fluvoxamine on TAC mice is mediated by Sig-1R stimulation.

Effect of fluvoxamine treatment on sigma-1 receptor expression

Since Sig-1R stimulation by fluvoxamine ameliorated TAC-induced LV hypertrophy in mice, we asked whether treatment with fluvoxamine and NE-100 had any effect on Sig-1R expression in LV. TAC-induced hypertrophy significantly decreased Sig-1R expression in the LV ($P<0.01$ vs. sham), while fluvoxamine administration
significantly and dose-dependently increased it ($P<0.01$ vs. TAC-vehicle for F 1.0) (Fig. 4).

Interestingly, although NE-100 treatment nullified fluvoxamine-mediated cardioprotective effects (Fig. 2), co-administration of NE-100 with fluvoxamine did not nullify fluvoxamine-dependent increases in Sig-1R expression ($P<0.01$ vs. TAC-vehicle) in the LV (Fig. 4). Moreover, oral administration of fluvoxamine (1 mg/kg) and NE-100 (1 mg/kg) for 4 weeks in sham-operated mice had no effect on Sig-1R expression in the LV (Supplementary Fig. S1E). Thus, fluvoxamine-induced restoration of Sig-1R levels is correlated with its cardioprotective action in the context of TAC-induced hypertrophy; however, that effect is likely not through stimulation of Sig-1R.

**Sigma-1 receptor activation induces Akt phosphorylation**

Our previous study showed that LV Akt phosphorylation levels markedly decrease following pressure overload-induced hypertrophy in ovariectomized rats (4, 6). We speculate that fluvoxamine administration may act to maintain Akt in a phosphorylated state, which is required for its activation. Consistent with our hypothesis, fluvoxamine administration (1.0 mg/kg) significantly increased Akt phosphorylation at Ser 473 ($P<0.01$ vs. TAC-vehicle for F 1.0) and at Thr 308 ($P<0.01$ vs. TAC-vehicle for F 1.0) (Fig. 5A-5C) in LV tissues. Interestingly, co-administration of NE-100 with fluvoxamine nullified fluvoxamine-induced rescue of Akt phosphorylation at Ser 473 ($P<0.05$ vs. sham; $P<0.05$ vs. F 1.0) and Thr 308 ($P<0.05$ vs. sham; $P<0.05$ vs. F 1.0) (Fig. 5A-5C). These findings suggest that fluvoxamine-mediated cardioprotection is closely associated with maintenance of Akt phosphorylation/activity in mice.

**Sigma-1 receptor activation induces eNOS expression and phosphorylation**
Since eNOS is a physiological substrate for Akt in human vascular endothelial cells (21, 52), we asked whether fluvoxamine-induced Akt activation results in increased eNOS phosphorylation in hypertrophied cardiomyocytes of ovariectomized rats (4, 6). Consistent with our previous observation in this model (4, 6), we found that TAC-induced myocardial hypertrophy was associated with significantly decreased eNOS expression ($P<0.05$ vs. sham) (Figure 6A-6B) as well as marked reduction of Akt-mediated eNOS phosphorylation at Ser 1177 ($P<0.05$ vs. sham) (Fig. 6A and 6C). Notably, fluvoxamine administration increased both eNOS levels ($P<0.01$ vs. TAC-vehicle for F 1.0) and Akt-mediated eNOS phosphorylation ($P<0.05$ vs. TAC-vehicle for F 0.5 and $P<0.01$ vs. TAC-vehicle for F 1.0) (Figure 6A-6C). NE-100 treatment antagonized fluvoxamine-mediated eNOS upregulation ($P<0.05$ vs. F 1.0), confirming that fluvoxamine-mediated increased eNOS expression in the LV is mediated by Sig-1R. Likewise, co-administration of NE-100 with fluvoxamine blocked fluvoxamine-mediated increased eNOS phosphorylation ($P<0.05$ vs. sham; $P<0.01$ vs. F 1.0) (Fig. 6A and 6C). These results suggest that fluvoxamine-mediated increased expression of Sig-1R promotes increased eNOS phosphorylation by Akt, as indicated by the increased ratio of eNOS phosphorylation to total eNOS expression (Fig. 6D). Enhanced Akt activity and concomitant eNOS phosphorylation are likely mediated by Sig-1R stimulation.

In cardiomyocytes, localization and activity of eNOS are regulated by complex formation between it and scaffold proteins, such as heat shock protein 90 (HSP 90) and caveolin-3, especially in caveolae (16). Since eNOS protein levels are reduced by TAC, we determined whether fluvoxamine administration modulates HSP 90 and caveolin-3 levels following TAC in mice. We observed no changes in expression of HSP 90 (Fig. 6E) and caveolin-3 (Fig. 6F) following TAC. Moreover, treatment of TAC mice with fluvoxamine and NE-100 had no effect on expression of HSP 90 (Fig. 6E) and caveolin-3 (Fig. 6F).

Taken together, fluvoxamine treatment increased expression and Akt-mediated
phosphorylation of eNOS without affecting eNOS scaffold proteins, including HSP-90 and caveolin-3, in cardiomyocyte caveolae.

**Effect of fluvoxamine treatment on ERK 1/2 and PKCα phosphorylation**

In order to determine involvement of other protein kinase signaling in fluvoxamine effects, we examined the role of ERK 1/2 phosphorylation in the LV after TAC-induced cardiac hypertrophy and after fluvoxamine treatment. We did not observe significant changes in ERK 1/2 phosphorylation in the LV when expressed as a ratio of phosphorylated to total ERK 1/2 following aortic banding and after treatment of mice with fluvoxamine and/or NE-100 (Fig. 7A).

PKCα, a major PKC isoform expressed in endothelial cells, regulates eNOS phosphorylation at Ser 1177 in endothelial cells *in vitro* and *in vivo* (7). Moreover, in rat pheochromocytoma PC12 cell lines, a sigma-1 agonist induces PKCα activation and underlies cell survival in serum deprivation-induced apoptotic conditions (8). Thus, we evaluated PKCα phosphorylation associated with activation following TAC-induced hypertrophy and fluvoxamine treatment. We did not observe significant changes in PKCα phosphorylation in the LV when expressed as a ratio of phosphorylated to total PKCα following TAC and after treatment with fluvoxamine and/or NE-100 in mice (Fig. 7B).

**The role of Sig-1R stimulation on Ang II-induced cardiomyocyte hypertrophy in vitro**

To confirm the antihypertrophic effect of fluvoxamine through Sig-1R on Ang II-induced hypertrophy, we treated neonatal rat cultured cardiomyocytes with Ang II in the presence or absence of various combinations of fluvoxamine, paroxetine and NE-100. The size of Ang II-treated cells was significantly increased compared to untreated (control) cells
48 hr after treatment, as described previously (25) ($P<0.001$ vs. control) (Fig. 8A, B and 9A, B). Fluvoxamine treatment dose-dependently inhibited this effect ($P<0.001$ vs. Ang II for both F 1.0 and F 5.0) (Fig. 8A, B). Combined NE-100 and fluvoxamine treatment reversed fluvoxamine-mediated inhibition of cardiomyocyte hypertrophy ($P<0.001$ vs. F 5.0) (Fig. 8A, B). Consistent with our in vivo findings, paroxetine treatment did not inhibit hypertrophy (Fig. 8A, B).

To obtain direct evidence that Sig-1R stimulation mediates fluvoxamine-induced inhibition of cardiomyocyte hypertrophy, we treated cardiomyocytes with Sig-1R siRNA to downregulate Sig-1R. As reported by Wang et al (49), Sig-1R was downregulated approximately 70% by Sig-1R siRNA treatment ($P<0.01$ vs. control) (Fig. 9A). Sig-1R siRNA treatment alone resulted in slightly enlarged cell size of cardiomyocytes in the absence or presence of Ang II, but these changes were not statistically significant.

Fluvoxamine-mediated inhibition of cardiomyocyte hypertrophy was largely abolished by pretreatment with Sig-1R siRNA ($P<0.001$ vs. F 5.0) (Fig. 9B, C). Taken together, our observations strongly suggest that the anti-hypertrophic effect of fluvoxamine on Ang II-induced cardiomyocyte hypertrophy is mediated by Sig-1R stimulation.
Discussion

Sigma receptors are suggested to regulate the cardiovascular system, as evidenced by the fact that several sigma receptor ligands influence cardiovascular function and that cardiomyocytes exhibit binding sites for sigma receptor ligands (10, 11). Sigma receptor ligands such as (+)-3-PPP, (+)-pentazocine and haloperidol alter contractility, Ca\(^{2+}\) influx, and rhythmic activity in cultured cardiac myocytes, but these actions are complex and some are controversial (11,12,28,32). In addition, mechanisms underlying potential reduction in myocardial infarction by SSRIs administered to humans are largely unknown.

We first hypothesized that SSRIs with high affinity for Sig-1R have direct cardioprotective effects via Sig-1R expressed on cardiomyocytes. We confirmed the cardioprotective role of one such high affinity reagent, fluvoxamine (Ki=36 nM), for Sig-1R in pathophysiological conditions mediated by TAC and compared those with the effects of the low affinity agonist paroxetine (Ki=1893 nM). The most significant observations in the present study are that chronic fluvoxamine but not paroxetine treatment totally inhibited LV hypertrophy and allowed functional recovery and that fluvoxamine administration may rescue downregulation of Sig-1R in LV associated with contractile impairment following TAC. We also report for the first time that restored Akt activity through Sig-1R stimulation by fluvoxamine ameliorates impairment of eNOS expression and phosphorylation in TAC cardiomyocytes.

Currently, we do not know how chronic fluvoxamine treatment upregulates Sig-1R expression. Whether Sig-1R expression is regulated by agonists and antagonists is not clear from the literature. Zamanillo et al. (51) report that chronic treatment using E-5842 as a Sig-1R agonist increased Sig-1R mRNA levels in rat brain. Shirayama et al. (42) report a decrease in sigma-1 binding sites in rat brain after chronic treatment with imipramine, a
weak Sig-1R agonist (Ki=343 nM). Chronic haloperidol (a Sig-1R antagonist) treatment reportedly promotes reduction in Sig-1R binding sites (23). These apparent discrepancies may be due to different methodologies or cell types used, including *in vivo* versus *in vitro* analysis and binding assays versus immunodetection. Our study indicates that treatment with the Sig-1R antagonist NE-100 did not alter Sig-1R expression in sham-operated mice. Similarly, combining NE-100 with fluvoxamine had no effect on fluvoxamine-induced upregulation of Sig-1R in the LV but nullified fluvoxamine-mediated antihypertrophic effects. These data suggest that antihypertrophic effects and upregulation of Akt signaling are mediated by Sig-1R stimulation and that stabilization or upregulation of Sig-1R by fluvoxamine is not mediated by Sig-1R stimulated signaling. In another words, stabilization or upregulation of Sig-1R is not mediated by Akt signaling. Further studies are required to identify mechanisms underlying stabilization or upregulation of Sig-1R by fluvoxamine administration.

Our novel findings indicate that fluvoxamine prevents development of not only TAC-induced LV hypertrophy *in vivo* but also Ang II-induced cardiomyocyte hypertrophy *in vitro* and that Akt and eNOS signaling via Sig-1R likely mediates antihypertrophic effects. Indeed, fluvoxamine treatment stimulated Akt phosphorylation *in vitro* in PC12 cells (29). Our hypothesis regarding fluvoxamine-mediated Akt activation is supported by the fact that Sig-1R antagonists such as rimcazole (BW 234U) promote calcium-independent inhibition of phosphatidylinositol 3'-kinase signaling pathways, inhibiting Akt phosphorylation in tumor cell lines (45). Moreover, Sig-1R knockdown via siRNA reportedly inhibits thrombin-stimulated Akt phosphorylation, increasing cell death in lens cells (49). Taken together, although Sig-1R is not a G-protein-coupled transmembrane receptor, Sig-1R stimulation activates PI3 kinase/Akt signaling in diverse cell lines.
Akt directly phosphorylates recombinant human eNOS or eNOS in situ at Ser-1177 (bovine residue 1179) (16), enhancing eNOS activity. Interestingly, continuous administration of fluvoxamine for 4 weeks not only increased Akt-mediated eNOS phosphorylation on Ser-1177 but also enhanced eNOS protein expression in the LV. Treatment with the Sig-1R antagonist NE-100 significantly nullified fluvoxamine-mediated eNOS upregulation and Akt-mediated eNOS phosphorylation, confirming Sig-1R-mediated modulation of eNOS activity in heart. Sig-1R ligands such as dehydroepiandrosterone rapidly activate eNOS through a nontranscriptional mechanism requiring ERK 1/2 activity but independent of the PI3K/Akt-mediated pathway (44). However, we did not observe changes in ERK 1/2 phosphorylation in the LV following TAC, as well as following continuous treatment with sigma ligands. PKCα, a major PKC isoform expressed in endothelial cells, also regulates eNOS activity in endothelial cells in vitro and in vivo by increasing phosphorylation of eNOS Ser-1177 (7). We did not observe significant changes in PKCα phosphorylation in the LV following aortic banding or after treatment with fluvoxamine. Therefore, the fluvoxamine-mediated cardioprotective effect is partly mediated by increased expression of Sig-1R, and Sig-1R stimulates both increased eNOS expression and increased Akt-mediated eNOS phosphorylation at Ser 1177 in the heart.

Here, we used therapeutic doses of fluvoxamine and paroxetine, which have Sig-1R activities with a Ki of 36 nM (potent) and 1893 nM (weak), respectively (20). A positron emission tomography study in humans demonstrated that fluvoxamine (50 to 200 mg/body) binds to sigma-1 receptors, but a paroxetine dose of 20mg/body has no effect in the intact human brain (20), suggesting that sigma-1 receptors function in pharmacological actions of fluvoxamine (20). Notably, selective serotonin reuptake inhibitors (SSRIs) such as fluvoxamine appear to be generally safe and effective in treating depression in patients with cardiovascular disease. SSRIs generally do not slow cardiac conduction, cause orthostatic
hypotension, decrease heart rate variability, or alter QT variability measures (9, 14). In regard to treatment, SSRIs appear to be safe for use in cardiac patients and can improve both depressive symptoms and quality of life (17, 36, 46).

SSRIs have several physiological effects, as evidenced by studies indicating that in non-depressive individuals, SSRIs can decrease sympathetic nervous system activity at rest (as indicated by reduced plasma norepinephrine concentrations) and during mental stress tasks (as measured by lowered heart rate, blood pressure, and plasma catecholamine concentrations) (18,19,43). Moreover, SSRIs reportedly decrease platelet activation in patients treated for depression and in healthy volunteers (1, 34, 40, 41). Sauer et al. (37) report a significant reduction of risk of myocardial infarction in SSRI-treated smokers, while in the Sertraline Antidepressant Heart Attack Randomized Trial (SADHART) the incidence of severe cardiac events was only 14.5% in the sertraline (Ki=57 nM) group compared to 22.4% in the placebo group (17). Likewise, when 457 fatal and nonfatal cardiovascular events were followed up for 29 months, the risk of death or recurrent MI was significantly lower in patients taking SSRIs (47). However, mechanisms underlying reversal of cardiac dysfunction by SSRIs are largely unknown. Our study is the first to define the SSRI-induced cardioprotection through Sig-1R. Current American College of Cardiology/American Heart Association guidelines for coronary artery bypass graft (CABG) surgery, acute myocardial infarction, and chronic angina all recommend evaluation of symptoms of depression and consideration of treatment of depression (36). Future large-scale follow-up studies should define SSRI cardioprotective mechanisms and reveal links between depression and cardiovascular disease. SSRIs increase brain monoaminergic levels and reverse many physiological derangements associated with depression. In addition SSRI therapy normalizes urinary cortisol excretion and improves heart rate variability, reduces platelet activation, and antagonizes expression of inflammatory markers (15, 17, 36, 39, 48). Our observations
strongly suggest that SSRIs that stimulate Sig-1R can reduce myocardial infarction risk not only in depressive patients but in a wide range of cardiovascular disease patients.

In conclusion, we provide, for the first time, evidence for the potential role of Sig-1R expression in the heart to attenuate TAC-induced hypertrophy in mice. With regard to the particular function of Sig-1R in heart, our data confirmed that fluvoxamine is a Sig-1R agonist in heart, because its effect is not only abrogated by NE-100 treatment but also by siRNA knockdown of the receptor protein. In addition, fluvoxamine treatment protects the heart from TAC-induced hypertrophy and tissue injury via upregulation of Sig-1R and stimulation of Sig-1R-mediated Akt-eNOS signaling in mice. We also report for the first time that, among SSRIs, fluvoxamine-related Sig-1R stimulatory effects could be beneficial to patients with cardiovascular disease. Our observations bring new therapeutic perspective to intervention into the hypertrophic process. At the same time, modulation of Sig-1R signaling may provide novel therapeutic targets for which a new class of antihypertrophic drugs can be designed.

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Disclosures

None

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reuptake inhibitor sertraline after acute coronary events: the Sertraline AntiDepressant Heart Attack Randomized Trial (SADHART) Platelet Substudy. *Circulation* 108:


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Figure legends

Fig. 1: Temporal changes in cardiac hypertrophy and failure induced by TAC in mice. A, Representative M-mode echocardiograms of mice before and after 2-6 weeks of TAC. LVEDD: left ventricular end-diastolic diameter; LVESD: left ventricular end-systolic diameter. B, Changes in percentage of LV fraction shortening (FS%). TAC-induced myocardial hypertrophy indicated by heart weight to body weight ratio (HW/BW) (C) and by lung weight to body weight ratio (LW/BW) (D). E, Western blot analysis (upper) and densitometry quantification (lower) of Sig-1R expression in the LV. Each group consists of 5-10 mice. Immunoblotting with anti-β-tubulin antibody showed equal protein loading in each lane. Data are expressed as percentages of values of sham-operated animals (mean ± S.E.M.) *, P < 0.05 and **, P < 0.01 versus the sham group.

Fig. 2: Effect of fluvoxamine and NE-100 on cardiac hypertrophy and failure induced by TAC in mice. A, Representative M-mode echocardiograms of mice with and without fluvoxamine (Flux) and/or NE-100 treatments. B, Changes in percentage of LV fractional shortening (FS%). TAC mice were treated with fluvoxamine 0.5 or 1.0mg/kg, or fluvoxamine (1.0mg/kg) plus NE-100 (1.0mg/kg) as indicated. TAC-induced myocardial hypertrophy indicated by HW/BW (C) and LW/BW (D) ratio. Each group consists of 5-10 mice. Each bar represents the mean ± S.E.M. **, P < 0.01 versus sham group; †, P < 0.05 and ‡, P < 0.01 versus the TAC-vehicle treated group; ″, P < 0.01 versus the TAC plus fluvoxamine 1mg/kg (F1.0) treated group.

Fig. 3: Effect of paroxetine on cardiac hypertrophy and failure induced by TAC in mice. A, Representative M-mode echocardiograms of mice with and without drug (Parox) treatments. B, Changes in percentage of LV fractional shortening (FS%). TAC or sham mice were treated with paroxetine at 0.2 (P 0.2) or 0.4 (P 0.4) mg/kg, as indicated. TAC-induced
myocardial hypertrophy is indicated by HW/BW (C) and LW/BW (D) ratios. Each group consists of 4-5 mice. Each bar represents the mean ± S.E.M. **, $P < 0.01$ versus sham group.

**Fig. 4:** Effects of fluvoxamine and NE-100 on Sig-1R expression. A. Western blot analysis of Sig-1R expression and β-tubulin as a loading control in the LV of sham and TAC-mice with or without drug treatment. Immunoblotting with anti-β-tubulin antibody showed equal protein loading in each lane. B. Densitometric quantification of Sig-1R immunoreactive bands. Data are expressed as percentages of values of sham-operated mice (mean ± S.E.M). Each group consists of 5-10 mice. **, $P < 0.01$ versus sham group; ††, $P < 0.01$ versus the TAC-vehicle treated group.

**Fig. 5:** Effects of fluvoxamine and NE-100 on Akt phosphorylation. TAC mice were treated with fluvoxamine 0.5 or 1.0mg/kg, or fluvoxamine (1.0mg/kg) plus NE-100 (1.0mg/kg) as indicated. Western blot analysis (A) and densitometric quantification of phospho-Akt (Ser-473) (B) and phospho-Akt (Thr-308) (C) in the LV of TAC-mice with or without drug treatment. Data are expressed as percentages of the value of sham mice. Densitometric quantification of phospho-Akt (Ser-473) (B) and phospho-Akt (Thr-308) (C) was relative to total Akt in the LV. Each group consists of 5-10 mice. Each column represents the mean ± S.E.M. *, $P < 0.05$ versus the sham group; ††, $P < 0.01$ versus the TAC-vehicle treated group; ‡‡, $P < 0.05$ versus the TAC plus fluvoxamine 1 mg/kg (F 1.0) treated group.

**Fig. 6:** Effects of fluvoxamine and NE-100 on levels of eNOS, eNOS phosphorylation, HSP 90 and caveolin-3. TAC mice were treated with fluvoxamine 0.5 or 1.0mg/kg, or fluvoxamine (1.0mg/kg) plus NE-100 (1.0mg/kg) as indicated. Western blot analysis (A) and densitometric quantification of eNOS (B), phospho-eNOS (Ser-1179) (C), ratio of phospho-eNOS to eNOS (D), HSP 90 (E) and caveolin-3 (F) in the LV of TAC mice with or without drug treatment. Data are expressed as percentages of the value of sham mice. Each
group consists of 5-10 mice. Each column represents the mean ± S.E.M. *, $P < 0.05$ versus
the sham group; †, $P < 0.05$, ‡, $P < 0.01$ and ‡‡, $P < 0.001$ versus the TAC-vehicle treated
group; ††, $P < 0.05$ and ‡‡‡, $P < 0.01$ versus the TAC plus fluvoxamine 1 mg/kg (F 1.0) treated
group.

Fig. 7: Effects of fluvoxamine and NE-100 on ERK 1/2 and PKC$\alpha$ phosphorylation. Western
blot analysis and densitometric quantification of ERK 1/2 phosphorylation
(Thr-202/Tyr-204) (A) and PKC$\alpha$ phosphorylation (Ser-657) (B) relative to respective total
proteins in the LV of TAC-mice with or without drug treatment. Data are expressed as
percentages of the value of sham mice. Each group consists of 5-10 mice. Each column
represents the mean ± S.E.M.

Fig. 8: Effect of fluvoxamine and paroxetine treatments on Ang II-induced hypertrophy in
cultured cardiomyocytes. (A) Cells were fixed with 4% paraformaldehyde, stained with
Rhodamine-conjugated Phalloidin, and processed for fluorescence microscopy. One hundred
cells from randomly selected fields were evaluated for cell size in each condition. (B) Cell
size is expressed as a percentage of the relative surface area in each condition compared to
control cells. Each column represents the mean ± S.E.M. ***, $P < 0.001$ versus the control
group; ††, $P < 0.001$ versus the Ang II-treated group; ‡‡‡, $P < 0.001$ versus the Ang II plus
fluvoxamine 5 µM (F 5.0)-treated group.

Fig. 9: Effect of Sig-1R siRNA on fluvoxamine-induced antihypertrophic effects in cultured
cardiomyocytes. (A) Western blot analysis and densitometric quantification of Sig-1R
expression with (siRNA) or without (Control) siRNA Sig-1R treatment. Immunoblotting
with anti-β-tubulin antibody showed equal protein loading in each lane. Data are expressed
as percentages of the control value. (B) Cells were fixed with 4% paraformaldehyde, stained
with Rhodamine-conjugated Phalloidin, and processed for fluorescence microscopy. One
hundred cells from randomly selected fields were examined in each condition. (C) Cell size
results are expressed as surface area relative to control cells. Each column represents the
mean ± S.E.M. **, $P < 0.01$ and ***, $P < 0.001$ versus the control group; ††, $P < 0.001$
versus the Ang II-treated group; ###, $P < 0.001$ versus the Ang II plus fluvoxamine 5 µM (F
5.0)-treated group.
Figure 1:

A

B

C

D

E

Sigma-1R

β-tubulin

Sigma-1 receptor (% of sham)

<table>
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<th>sham</th>
<th>2</th>
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<tr>
<td>Sigma-1 rec</td>
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* P < 0.05
** P < 0.01
*** P < 0.001
Figure 2:

A

Sham

TAC

TAC + F 1.0

TAC + F 1.0 + NE

B

FS(%)**††

sham vehicle F 0.5 F 1.0 F 1.0+NE

C

HW/BW (mg/gm)**

sham vehicle F 0.5 F 1.0 F 1.0+NE

D

LW/BW (mg/gm)**

sham vehicle F 0.5 F 1.0 F 1.0+NE

TAC (4 weeks)
Figure 3:

A

B

C

D
Figure 4:

A

Sigma-1R
β-tubulin

sham vehicle F 0.5 F 1.0 F 1.0+NE

TAC (4 weeks)

B

Sigma-1 receptor (% of sham)

sham vehicle F 0.5 F 1.0 F 1.0+NE

TAC (4 weeks)
Figure 5:

A

p-Akt (Ser 473)
p-Akt (Thr 308)
Akt

sham vehicle F 0.5 F 1.0 F 1.0+NE

B

TAC (4 weeks)

p-Akt (Ser 473)/Akt (% of sham)

sham vehicle F 0.5 F 1.0 F 1.0+NE

C

TAC (4 weeks)

p-Akt (Thr 308)/Akt (% of sham)

sham vehicle F 0.5 F 1.0 F 1.0+NE
Figure 6:

A. Western blot analysis of eNOS, p-eNOS, HSP 90, and Caveolin-3 in sham vehicle, F 0.5, F 1.0, F 1.0+NE groups after TAC (4 weeks).

B. Bar graph showing eNOS (% of sham) in sham vehicle, F 0.5, F 1.0, F 1.0+NE groups after TAC (4 weeks).

C. Bar graph showing p-eNOS (% of sham) in sham vehicle, F 0.5, F 1.0, F 1.0+NE groups after TAC (4 weeks).

D. Bar graph showing p-eNOS/eNOS (% of sham) in sham vehicle, F 0.5, F 1.0, F 1.0+NE groups after TAC (4 weeks).

E. Bar graph showing HSP 90 (% of sham) in sham vehicle, F 0.5, F 1.0, F 1.0+NE groups after TAC (4 weeks).

F. Bar graph showing Caveolin-3 (% of sham) in sham vehicle, F 0.5, F 1.0, F 1.0+NE groups after TAC (4 weeks).
Figure 7:

A. p-ERK/ERK (% of sham)

B. p-PKCα/PKCα (% of sham)
Figure 8:

A

Control  NE 10\mu M  AngII 48hr  P 5\mu M+

F 5\mu M+  AngII 48hr  NE 10\mu M+  F 5\mu M+

B

N.S.

Cell size

Cont  NE  –  F 1.0  F 5.0  F 5.0+NE  P 5.0

AngII 48hr
Figure 9:

A

Sigma-1R

β-tubulin

Sigma-1R expression (% of control)

Control  siRNA

B

Control  siRNA  AngII 48hr

siRNA+ AngII 48hr  F 5µM+ AngII 48hr  siRNA+ F 5µM+ AngII 48hr

C

Cell size (% of control)

Control  siRNA  − siRNA  F 5.0  F 5.0+siRNA

AngII 48hr