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σ₁-Receptor stimulation with fluvoxamine ameliorates transverse aortic constriction-induced myocardial hypertrophy and dysfunction in mice

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Tagashira H, Bhuiyan S, Shioda N, Hasegawa H, Kanai H, Fukunaga K. σ₁-Receptor stimulation with fluvoxamine ameliorates transverse aortic constriction-induced myocardial hypertrophy and dysfunction in mice. Am J Physiol Heart Circ Physiol 299: H1535–H1545, 2010. First published August 27, 2010; doi:10.1152/ajpheart.00198.2010.—Selective serotonin reuptake inhibitors (SSRIs) are known to reduce post-myocardial infarction-induced morbidity and mortality. However, the molecular mechanism underlying SSRI-induced cardioprotection remains unclear. Here, we investigated the role of σ₁-receptor (σ₁R) stimulation with fluvoxamine on myocardial hypertrophy and cardiac functional recovery. Male ICR mice were subjected to transverse aortic constriction-induced myocardial hypertrophy and dysfunction in mice. To confirm the cardioprotective role of fluvoxamine by σ₁R stimulation, we treated mice with fluvoxamine (0.5 or 1 mg/kg) orally once per day for 4 wk after the onset of aortic banding. Interestingly, in untreated mice, σ₁R expression in the left ventricle (LV) decreased significantly over the 4 wk as TAC-induced hypertrophy increased. In contrast, fluvoxamine administration significantly attenuated TAC-induced myocardial hypertrophy concomitant with recovery of σ₁R expression in the LV. Fluvoxamine also attenuated hypertrophy-induced impaired LV fractional shortening. The fluvoxamine cardioprotective effect was nullified by treatment with a σ₁R antagonist [NE-100 (1 mg/kg)]. Importantly, another SSRI with very low affinity for σ₁Rs, paroxetine, did not elicit antihypertrophic effects in TAC mice and cultured cardiomyocytes. Fluvoxamine treatment significantly restored TAC-induced impaired Akt and endothelial nitric oxide synthase (eNOS) phosphorylation in the LV. Our findings suggest that fluvoxamine protects against TAC-induced cardiac dysfunction via upregulated σ₁R expression and stimulation of σ₁R-mediated Akt-eNOS signaling in mice. This is the first report of a potential role for σ₁R stimulation by fluvoxamine in attenuating cardiac hypertrophy and restoring contractility in TAC mice.

protein kinase B; Akt; endothelial nitric oxide synthase

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 σ₁-receptor (σ₁R) as well as serotonin uptake inhibitors. The order of affinity of SSRIs for σ₁Rs is as follows: fluvoxamine (Ki: 36 nM) > sertraline (Ki = 57 nM) > fluoxetine (Ki = 120 nM) > citalopram (Ki = 292 nM) > paroxetine (Ki = 1,893 nM) (30). Thus, σ₁R is a potential physiological target for SSRIs not only in the brain but also in the heart. σ₁-Receptors, which are unique in having two transmembrane segments that show no homology with any mammalian G protein-coupled receptor, are widely expressed in heart muscle. In cardiac tissue, modulation of contractility by σ₁-receptor ligands was first reported in rat neonatal cultured cardiomyocytes (11). Later, σ₁-receptors were found in the membranes of adult rat ventricular cardiomyocytes (32). Approximately 80% of σ₁-receptors in the rat ventricular myocardium are the σ₁₁-subtype and 20% are the σ₁₂-subtype, based on an analysis of membrane binding of specific σ₁-ligands in isolated rat cardiomyocyte preparations (32). Several studies (12, 33) have indicated the effects of σ₁-ligands in isolated rat hearts and reported desensitization of σ₁-receptors in heart muscle by repeated treatment with σ₁-ligands. However, the physiological function of cardiac σ₁-receptor agonists in the normal heart and their therapeutic potential in cardiac disease remain unclear.

Despite information available relevant to the modulation of cardiomyocyte function by σ₁-receptor ligands, no study has described the expression level of σ₁-receptors, nor have their downstream targets in cardiomyocytes been identified. Most studies of σ₁-receptors have been carried out using isolated heart preparations (11, 12, 13, 31, 32) and indirect interpretations using nonspecific σ₁-ligands (such as haloperidol, 1,3-di-o-tolylguanidine, and (+)-3,3-(3-hydroxyphenyl)-N-(1-propyl)piperidine [(+)-3-PPP]) (22, 27). Moreover, the expression of σ₁R in the 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 σ₁Rs, we recently analyzed σ₁R expression in the heart (2), kidney (3), and thoracic aorta (5). We found that pressure overload-induced hypertrophy significantly decreased the expression of σ₁Rs in the left ventricle (LV) of ovariectomized rats and observed a significant correlation between heart dysfunction and decreased σ₁R 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 σ₁Rs and evaluate the pathophysiological relevance of σ₁Rs in cardiac hypertrophy and heart failure. In the present study, we found that fluvoxamine’s potent σ₁R stimulatory effect largely blunts the development of pathological LV hypertrophic remodeling and promotes cardiac functional recovery. In contrast, paroxetine, which has a low affinity for σ₁Rs, had no antihypertrophic effect. We also

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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-σ1R antibody (Abcam, Cambridge, UK); anti-endothelial nitric oxide synthase (eNOS) antibody and anti-β-tubulin antibody (Sigma, St. Louis, MO); anti-phospho-eNOS antibody, anti-phospho-p44/42 MAPK (ERK1/2) antibody (Thr202/Tyr204), total p44/42 MAPK (ERK1/2), anti-phospho-Akt antibody (Ser473), and total Akt antibody (Cell Signaling Technology, Beverly, MA); anti-phospho-Akt antibody (Thr308), anti-phospho-PKC-α antibody (Ser657), total PKC-α antibody, and anti-heat shock protein 90 (HSP90) antibody (Upstate Biotechnology, Lake Placid, NY), anti-caveolin-3 antibody (BD Biosciences, San Jose, CA); and anti-rabbit antibody (Amersham Biosciences, Piscataway, NJ). The σ1R agonist fluvoxamine maleate was supplied by Meiji Seika Kaisha (Tokyo, Japan). Cultured myocytes were plated on uncoated 90-mm culture dishes on collagen-coated coverslips. Transfections were performed with 100 nM σ1R siRNAs according to the methods of Wang et al. (49). Briefly, 20 μM σ1R siRNA in 5 μl was added to 180 μl opti-MEM (Invitrogen), and 5 μl Lipofectamine 2000 (Invitrogen) was added to 10 μl opti-MEM. Both solutions were incubated separately at room temperature for 5 min, mixed, and then incubated at room temperature for 15–20 min. Meanwhile, medium containing serum was removed from the cells and replaced with 1 ml opti-MEM. This solution was then removed and replaced with 800 μl fresh opti-MEM to which 200 μl of the siRNA solution was added. Cells were then incubated at 35°C in a 5% CO2 atmosphere for 4 h to initiate transfection. DMEM (500 μl) supplemented with 5% FBS was then added to each well, and the transfection was continued for up to 72 h. Cultured cells were then washed in the cell culture medium (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 previously described (35). After acclimatization for 7 days, animals were anesthetized with trichloroethylene (0.25–0.3 g/kg ip). The animal was placed supine, and an endotracheal intubation was rapidly performed. The cannula was connected to a volume-cycled rodent ventilator with a tidal volume of 0.5 ml of 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 the aortic arch was isolated, the transverse aorta was isolated between the carotid arteries and constricted with a 7-0 silk suture 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 their body temperature was kept at 37°C. Sham-operated animals, which underwent a similar surgical procedure without aortic constriction, served as controls.

Experimental design. Initially, we observed a downregulated expression of σ1R in TAC-induced hypertrophy. ICR mice were randomly separated into four groups: 1) sham-operated animals (sham; n = 9), 2) TAC for 2 wk (n = 6), 3) TAC for 4 wk (n = 6), and 4) TAC for 6 wk (n = 7). We then observed the cardioprotective role of σ1R-mediated signaling and randomly separated ICR mice into 10 treatment groups: 1) sham (n = 15), 2) sham plus fluvoxamine (1 mg/kg, n = 5), 3) sham plus NE-100 (1 mg/kg, n = 5), 4) TAC plus vehicle (TAC-vehicle; n = 12), 5) TAC plus 0.5 mg/kg fluvoxamine (F 0.5; n = 7), 6) TAC plus 1.0 mg/kg fluvoxamine (F 1.0; n = 7), 7) TAC plus 1.0 mg/kg fluvoxamine plus NE-100 (F 1.0-NE; n = 7), 8) TAC plus 0.4 mg/kg paroxetine (n = 5), 9) TAC plus 0.2 mg/kg paroxetine (n = 4), and 10) TAC plus 0.4 mg/kg paroxetine (n = 4).

Drug administration. Fluvoxamine and paroxetine maleate were dissolved in 0.5% 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 wk (once daily) in a volume of 1 ml/100 g mouse body wt (BW), starting from the onset of aortic banding.

Measurement of cardiac hypertrophy. After 4 wk of drug or vehicle administration, animals were subjected to terminal surgery. Mice were weighed and anesthetized with a mixture of ketamine (100 mg/kg ip) and xylazine (5 mg/kg ip) (34). The thoracic cavity was opened, and hearts were immediately harvested and weighed. Cardiac indexes, expressed as the heart weight (HW)-to-BW ratio (HW/BW; in mg/g), 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 American Society of Echocardiography guidelines (26). The percentage of LV fraction shortening (FS) was calculated as follows: 

$$\text{FS} = \left( \frac{\text{LVEDD} - \text{LVESD}}{\text{LVEDD}} \right) \times 100.$$

Western blot analysis. Four weeks after aortic banding, mice were anesthetized, and hearts were excised and quickly perfused with PBS 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 have previously described (4, 6). An equal amount of protein for each sample (25 µg total protein) was separated on 7.5–15% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). After being blocked 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 an incubation with donkey anti-rabbit IgG coupled to horseradish peroxidase, and blots were developed using an 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 Imagegausage version 3.41 (Fuji Film). Relative amounts of proteins were expressed as percent increases over sham values.

Statistical analysis. Values are represented as means ± SE. Morphometric changes and SDS-PAGE results were evaluated for differences by one-way ANOVA combined with Dunnett’s post hoc test. P values of <0.05 were considered statistically significant.

RESULTS

Time course analysis of cardiac hypertrophy and $\sigma_1$R expression. To define the role of $\sigma_1$R expression in cardiac hypertrophy and heart failure, we evaluated the time course of LV function, cardiac hypertrophy, and $\sigma_1$R expression after TAC. Echocardiographic data for the time course experiment is provided in the Supplemental Material (Supplemental Table 1) and Fig. 1. We found a time-dependent impairment of LV function, as indicated by decreased FS 4 and 6 wk after TAC in mice.

Fig. 1. Temporal changes in cardiac hypertrophy and failure induced by transverse aortic constriction (TAC) in mice. A: representative M-mode echocardiograms of mice before and after 2–6 wk of TAC. LVEDD, left ventricular (LV) end-diastolic diameter; LVESD, LV end-systolic diameter. B: changes in the percentage of LV fraction shortening (FS). C and D: TAC-induced myocardial hypertrophy as indicated by the heart weight-to-body weight ratio (HW/BW; C) and lung weight-to-body weight ratio (LW/BW; D). E: Western blot analysis (top) and densitometry quantification (bottom) of $\sigma_1$-receptor ($\sigma_1$R) expression in the LV. Each group consisted of 5–10 mice. Immunoblot analysis with anti-β-tubulin antibody showed equal protein loading in each lane. Data are expressed as percentages of values in sham-operated animals (sham group; means ± SE). *$P < 0.05$ and **$P < 0.01$ vs. the sham group.
found time-dependent increases in the LW-to-BW ratio (LW/BW; Fig. 1D) from 1 to 6 wk after TAC in mice. Interestingly, $\sigma_1$R expression in the LV markedly decreased time dependently with significantly decreased expression observed 4 and 6 wk after TAC in mice ($P < 0.01$ vs. the sham group; Fig. 1E). Thus, our study revealed a significant negative linear correlation of heart dysfunction with reduced $\sigma_1$R expression in the LV.

Effect of $\sigma_1$R stimulation on myocardial hypertrophy and dysfunction. To confirm the cardioprotective role of fluvoxamine and paroxetine, which are typical SSRIs, we treated mice

Fig. 2. Effect of fluvoxamine (Flux) and NE-100 (NE) on cardiac hypertrophy and failure induced by TAC in mice. A: representative M-mode echocardiograms of mice with and without Flux and/or NE treatment. B: changes in the percentage of LV FS. TAC mice were treated with Flux [0.5 mg/kg (F 0.5 group) or 1.0 mg/kg (F 1.0 group)] or Flux (1.0 mg/kg) plus NE (1.0 mg/kg; F 1.0 + NE group) as shown. C and D: TAC-induced myocardial hypertrophy as indicated by HW/BW (C) and LW/BW (D). Each group consisted of 5–10 mice. Each bar represents the mean ± SE. *$P < 0.05$ and **$P < 0.01$ vs. the sham group; †$P < 0.05$ and ††$P < 0.01$ vs. the TAC-vehicle group; ##$P < 0.01$ vs. the TAC + F 1.0 group.

Fig. 3. Effect of paroxetine (Parox) 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 the percentage of LV FS. TAC or sham mice were treated with Parox at 0.2 mg/kg (P 0.2 group) or 0.4 mg/kg (P 0.4 group) as shown. C and D: TAC-induced myocardial hypertrophy as indicated by HW/BW (C) and LW/BW (D). Each group consisted of 4–5 mice. Each bar represents the mean ± SE. **$P < 0.01$ vs. the sham group.
In Supplemental Fig. S1E, F when compared with the sham group, NE-100 treatment nullified fluvoxamine-mediated cardioprotective effects (Fig. 4). Interestingly, although NE-100 treatment nullified fluvoxamine-mediated cardioprotective effects (Fig. 2), coadministration of NE-100 with fluvoxamine did not nullify fluvoxamine-dependent increases in \( \sigma_1 \)R expression (Fig. 4). Moreover, oral administration of fluvoxamine (1 mg/kg) and NE-100 (1 mg/kg) for 4 wk in mice had no effect on \( \sigma_1 \)R expression in the LV (Supplemental Fig. S1E). Thus, fluvoxamine-induced restoration of \( \sigma_1 \)R levels is correlated with its cardioprotective action in the context of TAC-induced hypertrophy; however, that effect is likely not through stimulation of \( \sigma_1 \)Rs.

**\( \sigma_1 \)R activation induces Akt phosphorylation.** Our previous studies (4, 6) showed that LV Akt phosphorylation levels markedly decrease after pressure overload-induced hypertrophy in ovariectomized rats. We speculated that fluvoxamine with either drug or with the \( \sigma_1 \)R antagonist NE-100 for 4 wk, starting at the onset of TAC. Echocardiographic data are shown in Supplemental 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 wk had no effect on echocardiographic parameters (Supplemental Table S2) and LV FS (Supplemental Fig. S1B). Consistent with temporal changes in heart function, as shown in Fig. 1B, TAC treatment significantly decreased LV FS (\( P < 0.01 \)) compared with the sham group (Figs. 2, A and B, and 3, A and B). Fluvoxamine treatment restored decreased LV FS dose dependently (\( P < 0.01 \)) versus the TAC-vehicle group for both the F 0.5 and F 1.0 groups; Fig. 2B). Paroxetine treatment, however, failed to restore FS (Fig. 3B). Coadministration of NE-100 with fluvoxamine nullified the fluvoxamine-mediated amelioration of heart dysfunction, as indicated by the decreased LV FS (\( P < 0.01 \)) vs. the F 1.0 group; Fig. 2B).

Similarly, fluvoxamine treatment significantly restored both the elevated HW/BW (\( P < 0.01 \)) vs. the TAC-vehicle group for the F 1.0 group; Fig. 2C) and elevated LW/BW (\( P < 0.05 \)) vs. the TAC-vehicle group for the F 1.0 group; Fig. 2D). Paroxetine administration failed to inhibit increases in both HW/BW (Fig. 3C) and LW/BW (Fig. 3D). Coadministration of NE-100 with fluvoxamine nullified the latter’s inhibition of TAC-induced hypertrophy, as indicated by HW/BW (\( P < 0.05 \) vs. the sham group and \( P < 0.01 \)) vs. the F 1.0 group; Fig. 2C) and LW/BW (\( P < 0.05 \)) vs. the sham group and \( P < 0.01 \) vs. the F 1.0 group; Fig. 2D). Moreover, oral administration of fluvoxamine (1 mg/kg) and NE-100 (1 mg/kg) alone to mice that had not undergone TAC for 4 wk had no effect on HW/BW (Supplemental Fig. S1C) and LW/BW (Supplemental Fig. S1D) ratio in the sham group. Taken together, we conclude that the antihypertrophic effect of fluvoxamine on TAC mice is mediated by \( \sigma_1 \)R stimulation.

**Effect of fluvoxamine treatment on \( \sigma_1 \)R expression.** Since \( \sigma_1 \)R stimulation by fluvoxamine ameliorated TAC-induced LV hypertrophy in mice, we asked whether treatment with fluvoxamine and NE-100 had any effect on \( \sigma_1 \)R expression in the LV. TAC-induced hypertrophy significantly decreased \( \sigma_1 \)R expression in the LV (\( P < 0.01 \) vs. the sham group), whereas fluvoxamine administration significantly and dose dependently increased it (\( P < 0.01 \) vs. the TAC-vehicle group for the F 1.0 group; Fig. 4). Interestingly, although NE-100 treatment nullified fluvoxamine-mediated cardioprotective effects (Fig. 2), coadministration of NE-100 with fluvoxamine did not nullify fluvoxamine-dependent increases in \( \sigma_1 \)R expression (\( P < 0.01 \)) vs. the TAC-vehicle group in the LV (Fig. 4). Moreover, oral administration of fluvoxamine (1 mg/kg) and NE-100 (1 mg/kg) for 4 wk in mice had no effect on \( \sigma_1 \)R expression in the LV (Supplemental Fig. S1E). Thus, fluvoxamine-induced restoration of \( \sigma_1 \)R levels is correlated with its cardioprotective action in the context of TAC-induced hypertrophy; however, that effect is likely not through stimulation of \( \sigma_1 \)Rs.

**\( \sigma_1 \)R activation induces Akt phosphorylation.** Our previous studies (4, 6) showed that LV Akt phosphorylation levels markedly decrease after pressure overload-induced hypertrophy in ovariectomized rats. We speculated that fluvoxamine

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**Fig. 4. Effects of Flux and NE-100 on \( \sigma_1 \)R expression.** A: Western blot analysis of \( \sigma_1 \)R expression and \( \beta \)-tubulin as a loading control in the LVs of sham and TAC mice with or without drug treatment. Immunoblot analysis with anti-\( \beta \)-tubulin antibody showed equal protein loading in each lane. B: densitometric quantification of \( \sigma_1 \)R-immunoreactive bands. Data are expressed as percentages of values of sham mice (means \( \pm \) SE). Each group consisted of 5–10 mice. **\( P < 0.01 \) vs. the sham group; ††\( P < 0.01 \) vs. the TAC-vehicle group.

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**Fig. 5. Effects of Flux and NE on Akt phosphorylation.** TAC mice were treated with Flux (0.5 or 1.0 mg/kg) or Flux (1.0 mg/kg) + NE (1.0 mg/kg) as shown. A–C: Western blot analysis (A) and densitometric quantification of phosphorylated (p-)Akt (Ser\( ^{473} \)) and p-Akt (Thr\( ^{308} \)); B) and p-Akt (Thr\( ^{308} \)-expressedAkt C) in the LVs of TAC mice with or without drug treatment. Data are expressed as percentages of the value of sham mice. Densitometric quantification of p-Akt (Ser\( ^{473} \)-expressedAkt C) was relative to total Akt in the LV. Each group consisted of 5–10 mice. Each bar represents the mean \( \pm \) SE. *\( P < 0.05 \) vs. the sham group; ††\( P < 0.01 \) vs. the TAC-vehicle group; #\( P < 0.05 \) vs. the TAC + F 1.0 group.
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 Ser473 ($P < 0.01$ vs. the TAC-vehicle group for the F 1.0 group) and Thr308 ($P < 0.01$ vs. the sham group and $P < 0.01$ vs. the TAC-vehicle group; Fig. 5, A–C). Notably, coadministration of NE-100 with fluvoxamine nullified the fluvoxamine-induced rescue of Akt phosphorylation at Ser473 ($P < 0.05$ vs. the sham group and $P < 0.05$ vs. the F 1.0 group) and Thr308 ($P < 0.05$ vs. the sham group and $P < 0.01$ vs. the F 1.0 group; Fig. 5, A–C). These findings suggest that fluvoxamine-mediated cardioprotection is closely associated with the maintenance of Akt phosphorylation/activity in mice.

**α1R 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. the sham group; Fig. 6, A and B) as well as markedly reduced Akt-mediated eNOS phosphorylation at Ser1177 ($P < 0.05$ vs. the sham group; Fig. 6, A and C). Notably, fluvoxamine administration increased both eNOS levels ($P < 0.01$ vs. the TAC-vehicle group for the F 1.0 group) and Akt-mediated eNOS phosphorylation ($P < 0.05$ vs. the TAC-vehicle group for the F 0.5 group and $P < 0.01$ vs. the TAC-vehicle group for the F 1.0 group; Fig. 6, A–C). NE-100 treatment antagonized fluvoxamine-mediated eNOS upregulation ($P < 0.05$ vs. the F 1.0 group), confirming that fluvoxamine-mediated increased eNOS expression in the LV is mediated by $\alpha_1$R stimulation. Likewise, coadministration of NE-100 with fluvoxamine blocked fluvoxamine-mediated increased eNOS phosphorylation in the LV by $\alpha_1$Rs. These results suggest that fluvoxamine-mediated increased eNOS expression 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 $\alpha_1$R stimulation.
In cardiomyocytes, the localization and activity of eNOS are regulated by a complex formation between it and scaffold proteins, such as HSP90 and caveolin-3, especially in caveolae (16). Since eNOS protein levels are reduced by TAC, we determined whether fluvoxamine administration modulates HSP90 and caveolin-3 levels after TAC in mice. We observed no changes in the expression of HSP90 (Fig. 6E) and caveolin-3 (Fig. 6F) after TAC. Moreover, treatment of TAC mice with fluvoxamine and NE-100 had no effect on the expression of HSP90 (Fig. 6E) and caveolin-3 (Fig. 6F). Taken together, fluvoxamine treatment increased the expression and Akt-mediated phosphorylation of eNOS without affecting eNOS scaffold proteins, including HSP90 and caveolin-3, in cardiomyocyte caveolae.

**Effect of fluvoxamine treatment on ERK1/2 and PKC-α phosphorylation.** To determine the involvement of other protein kinase signaling in fluvoxamine effects, we examined the role of ERK1/2 phosphorylation in the LV after TAC-induced cardiac hypertrophy and after fluvoxamine treatment. We did not observe significant changes in ERK1/2 phosphorylation in the LV when expressed as the ratio of phosphorylated to total ERK1/2 after aortic banding and after treatment of mice with fluvoxamine and/or NE-100 (Fig. 7A).

PKC-α, a major PKC isoform expressed in endothelial cells, regulates eNOS phosphorylation at Ser1177 in endothelial cells in vitro and in vivo (7). Moreover, in rat pheochromocytoma PC12 cell lines, a σ1-agonist induces PKC-α activation and underlies cell survival in serum deprivation-induced apoptotic conditions (8). Thus, we evaluated PKC-α phosphorylation associated with activation after TAC-induced hypertrophy and fluvoxamine treatment. We did not observe significant changes in PKC-α phosphorylation in the LV when expressed as the ratio of phosphorylated to total PKC-α after TAC and after treatment with fluvoxamine and/or NE-100 in mice (Fig. 7B).

**Role of σ1R stimulation on ANG II-induced cardiomyocyte hypertrophy in vitro.** To confirm the antihypertrophic effect of fluvoxamine through σ1Rs 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 with untreated (control) cells 48 h after treatment, as previously described (25) (P < 0.001 vs. control; Figs. 8, A and B, and 9, A and B). Fluvoxamine treatment dose dependently inhibited this effect (P < 0.001 vs. ANG II for both the F 1.0 and F 5.0 groups; Fig. 8, A and B). Combined NE-100 and fluvoxamine treatment reversed the fluvoxamine-mediated inhibition of cardiomyocyte hypertrophy (P < 0.001 vs. the F 5.0 group; Fig. 8, A and B). Consistent with our in vivo findings, paroxetine treatment did not inhibit hypertrophy (Fig. 8, A and B).

To obtain direct evidence that σ1R stimulation mediates the fluvoxamine-induced inhibition of cardiomyocyte hypertrophy, we treated cardiomyocytes with σ1R siRNA to downregulate σ1Rs. As reported by Wang et al. (49), σ1Rs were downregulated ~70% by σ1R siRNA treatment (P < 0.01 vs. control; Fig. 9A). σ1R siRNA treatment alone resulted in a 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 σ1R siRNA (P < 0.001 vs. the F 5.0 group; Fig. 9, B and C). Taken together, our observations strongly suggest that the anti hypertrophic effect of fluvoxamine on ANG II-induced cardiomyocyte hypertrophy is mediated by σ1R stimulation.

**DISCUSSION**

σ-Receptors have been suggested to regulate the cardiovascular system, as evidenced by the fact that several σ-receptor ligands influence cardiovascular function and that cardiomyocytes exhibit binding sites for σ-receptor ligands (10, 11). σ-Receptor ligands such as (+)-3-PPP, (+)-pentazocine, and haloperidol alter contractility, Ca2+ 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 the potential reduction in MI by SSRIs administered to humans are largely unknown.

We first hypothesized that SSRIs with a high affinity for σ1Rs have direct cardioprotective effects via σ1Rs expressed on cardiomyocytes. We confirmed the cardioprotective role of one such high-affinity reagent, fluvoxamine (Kᵀ: 36 nM), for σ1Rs in pathophysiological conditions mediated by TAC and compared those with the effects of the low-affinity agonist paroxetine (Kᵀ: 1,893 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 the downregulation of \( \alpha_1 \)Rs in the LV associated with contractile impairment after TAC. We also report, for the first time, that restored Akt activity through \( \alpha_1 \)R stimulation by fluvoxamine ameliorates the impairment of eNOS expression and phosphorylation in TAC cardiomyocytes.

Currently, we do not know how chronic fluvoxamine treatment upregulates \( \alpha_1 \)R expression. Whether \( \alpha_1 \)R expression is regulated by agonists and antagonists is not clear from the literature. Zamanillo et al. (51) reported that chronic treatment using E-5842 as a \( \alpha_1 \)R agonist increased \( \alpha_1 \)R mRNA levels in the rat brain. Shirayama et al. (42) reported a decrease in \( \alpha_1 \)-binding sites in the rat brain after chronic treatment with imipramine, a weak \( \alpha_1 \)R agonist \((K_i: 343 \text{ nM})\). Chronic haloperidol (a \( \alpha_1 \)R antagonist) treatment reportedly promoted a reduction in \( \alpha_1 \)-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 \( \alpha_1 \)R antagonist NE-100 did not alter \( \alpha_1 \)R expression in sham mice. Similarly, the combination of NE-100 with fluvoxamine had no effect on fluvoxamine-induced upregulation of \( \alpha_1 \)Rs in the LV but nullified fluvoxamine-mediated antihypertrophic effects. These data suggest that antihypertrophic effects and the upregulation of Akt signaling are mediated by \( \alpha_1 \)R stimulation and that stabilization or upregulation of \( \alpha_1 \)Rs by fluvoxamine is not mediated by \( \alpha_1 \)R-stimulated signaling. In another words, the stabilization or upregulation of \( \alpha_1 \)Rs is not mediated by Akt signaling. Further studies are required to identify the mechanisms underlying the stabilization or upregulation of \( \alpha_1 \)Rs by fluvoxamine administration.

Our novel findings indicate that fluvoxamine prevents the 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 \( \alpha_1 \)Rs 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 \( \alpha_1 \)R antagonists such as rimcazole (BW 234U) promote calcium-independent inhibition of phosphatidylinositol 3'-kinase (PI3K) signaling pathways, inhibiting Akt phosphorylation in tumor cell lines (45). Moreover, \( \alpha_1 \)R knockdown via siRNA reportedly inhibited thrombin-stimulated Akt phosphorylation, increasing cell death in lens cells (49). Taken together, although the \( \alpha_1 \)R is not a G protein-coupled transmembrane receptor, \( \alpha_1 \)R stimulation activates PI3K/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 wk not only increased Akt-mediated eNOS phosphorylation on Ser\(^{1177} \) but also enhanced eNOS protein

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**Fig. 8. Effect of Flux and Parox treatments on ANG II-induced hypertrophy in cultured cardiomyocytes.**

A: cells were fixed with 4% paraformaldehyde, stained with rhodamine-conjugated phallolidin, and processed for fluorescence microscopy. One hundred cells from randomly selected fields were evaluated for cell size in each condition. B: cell size expressed as a percentage of the relative surface area in each condition compared with control cells. Each bar represents the mean \( \pm \) SE. **P < 0.001 vs. the control group; †††P < 0.001 vs. the ANG II-treated group; ###P < 0.001 vs. the ANG II + 5 \( \mu \)M Flux-treated (F 5.0) group.
activate eNOS through a nontranscriptional mechanism requiring ERK1/2 activity but independent of the PI3K/Akt-mediated pathway (44). However, we did not observe changes in ERK1/2 phosphorylation in the LV after TAC as well as after continuous treatment with \( \sigma_1 \)-ligands. PKC-\( \alpha \), a major PKC isoform expressed in endothelial cells, also regulates eNOS activity in endothelial cells in vitro and in vivo by increasing the phosphorylation of eNOS at Ser1177 (7). We did not observe significant changes in PKC-\( \alpha \) phosphorylation in the LV after aortic banding or after treatment with fluvoxamine. Therefore, the fluvoxamine-mediated cardioprotective effect is partly mediated by the increased expression of \( \sigma_1 \)-Rs, and \( \sigma_1 \)-Rs stimulate both increased eNOS expression and increased Akt-mediated eNOS phosphorylation at Ser1177 in the heart.

Here, we used therapeutic doses of fluvoxamine and paroxetine, which have \( \sigma_1 \)-R activities with a \( K_i \) of 36 nM (potent) and 1,893 nM (weak), respectively (20). A positron emission tomography study in humans demonstrated that fluvoxamine (50–200 mg/BW) binds to \( \sigma_1 \)-Rs but that a paroxetine dose of 20 mg/BW has no effect in the intact human brain (20), suggesting that \( \sigma_1 \)-Rs function in the pharmacological actions of fluvoxamine (20). Notably, 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 decreased platelet activation in patients treated for depression and in healthy volunteers (1, 34, 40, 41). Sauer et al. (37) reported a significant reduction of the risk of MI in SSRI-treated smokers, whereas in the Sertraline Antidepressant Heart Attack Randomized Trial (17), the incidence of severe cardiac events was only 14.5% in the sertraline (\( K_i = 57 \) nM)-treated group compared with 22.4% in the placebo group. Likewise, when 457 fatal and nonfatal cardiovascular events were followed up for 29 mo, the risk of death or recurrent MI was significantly lower in patients taking SSRIs (47). However, the mechanisms underlying reversal of cardiac dysfunction by SSRIs are largely unknown. Our study is the first to define the SSRI-induced cardioprotection through \( \sigma_1 \)-Rs. Current American College of Cardiology/American Heart Association guidelines for coronary artery bypass graft surgery, acute MI, and chronic angina all recommend an evaluation of the symptoms of depression and consideration of treatment of depression (36). Future large-scale followup studies should define SSRI cardioprotective mechanisms and reveal the 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 the expression of inflammatory markers (15, 17, 36, 39, 48). Our observations

Fig. 9. Effect of \( \sigma_1 \)-R small interfering (si)RNA on Flux-induced antihypertrophic effects in cultured cardiomyocytes. A: Western blot analysis (top) and densitometric quantification (bottom) of \( \sigma_1 \)-R expression with or without (control) \( \sigma_1 \)-R siRNA treatment. Immunoblot analysis with anti-\( \beta \)-tubulin antibody showed equal protein loading in each lane. Data are expressed as percentages of the control value. **P < 0.01 and ****P < 0.0001 vs. the control group; ††††P < 0.001 vs. the ANG II-treated group; †††P < 0.001 vs. the ANG II + F 5.0 group.

expression in the LV. Treatment with the \( \sigma_1 \)-R antagonist NE-100 significantly nullified fluvoxamine-mediated eNOS upregulation and Akt-mediated eNOS phosphorylation, confirming the \( \sigma_1 \)-R-mediated modulation of eNOS activity in the heart. \( \sigma_1 \)-R ligands, such as dehydroepiandrosterone, rapidly
strongly suggest that SSRIs that stimulate $\sigma_1$Rs can reduce the risk of MI 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 $\sigma_1$R expression in the heart to attenuate TAC-induced hypertrophy in mice. With regard to the particular function of $\sigma_1$Rs in the heart, our data confirmed that fluvoxamine is a $\sigma_1$R agonist in the heart, because its effect was 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 an upregulation of $\sigma_1$Rs and stimulation of $\sigma_1$R-mediated Akt-eNOS signaling in mice. We also report, for the first time, that, among SSRI s, fluvoxamine-related $\sigma_1$R stimulatory effects could be beneficial to patients with cardiovascular disease. Our observations bring a new therapeutic perspective to an intervention into the hypertrophic process. At the same time, modulation of $\sigma_1$R signaling may provide novel therapeutic targets for which a new class of antihypertrophic drugs can be designed.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


α₁-Receptor Stimulation Ameliorates Myocardial Hypertrophy


