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σ_1-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. σ_1-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 σ_1-receptor (σ_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 σ_1R 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, σ_1R 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 σ_1R expression in the LV. Fluvoxamine also attenuated hypertrophy-induced impaired LV fractional shortening. The fluvoxamine cardioprotective effect was nullified by treatment with a σ_1R antagonist [NE-100 (1 mg/kg)]. Importantly, another SSRI with very low affinity for σ_1Rs, 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 σ_1R expression and stimulation of σ_1R-mediated Akt-eNOS signaling in mice. This is the first report of a potential role for σ_1R 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 σ_1-receptor (σ_1R) as well as serotonin uptake inhibitors. The order of affinity of SSRIs for σ_1R is as follows: fluvoxamine (K_i: 36 nM) > sertraline (K_i = 57 nM) > fluoxetine (K_i = 120 nM) > citalopram (K_i = 292 nM) > paroxetine (K_i = 1,893 nM) (30). Thus, σ_1R is a potential physiological target for SSRIs not only in the brain but also in the heart.

σ-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 σ-receptor ligands was first reported in rat neonatal cultured cardiomyocytes (11). Later, σ-receptors were found in the membranes of adult rat ventricular cardiomyocytes (32). Approximately 80% of σ-receptors in the rat ventricular myocardium are the σ_1-subtype and 20% are the σ_2-subtype, based on an analysis of membrane binding of specific σ-ligands in isolated rat cardiomyocyte preparations (32). Several studies (12, 33) have indicated the effects of σ-ligands in isolated rat hearts and reported desensitization of σ-receptors in heart muscle by repeated treatment with σ-ligands. However, the physiological function of cardiac σ-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 σ-receptor ligands, no study has described the expression level of σ-receptors, nor have their downstream targets in cardiomyocytes been identified. Most studies of σ-receptors have been carried out using isolated heart preparations (11, 12, 13, 31, 32) and indirect interpretations using nonspecific σ-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 σ_1R 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 σ_1Rs, we recently analyzed σ_1R expression in the heart (2), kidney (3), and thoracic aorta (5). We found that pressure overload-induced hypertrophy significantly decreased the expression of σ_1Rs in the left ventricle (LV) of ovariectomized rats and observed a significant correlation between heart dysfunction and decreased σ_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 σ_1Rs and evaluate the pathophysiological relevance of σ_1Rs in cardiac hypertrophy and heart failure. In the present study, we found that fluvoxamine's potent σ_1R 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 σ_1Rs, 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-σ_1R 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 (Thr^{202}/Tyr^{204}), total p44/42 MAPK (ERK1/2), anti-phospho-Akt antibody (Ser^{437}), and total Akt antibody (Cell Signaling Technology, Beverly, MA); anti-phospho-Akt antibody (Thr^{308}), anti-phospho-PKC-α antibody (Ser^{657}), 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 σ_1R agonist fluvoxamine maleate was supplied by Meiji Seika Kaisha (Tokyo, Japan), and the specific σ_1R antagonist *N,N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine monohydrochloride (NE-100) was generously supplied by Taisho Pharmaceutical (Tokyo, 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 35–40 g were obtained from Nippon SLC (Hamamatsu, Japan). Ten-week-old male mice were acclimated to the local environment for 1 wk, which included housing in polypropylene cages at 23 ± 1°C in a humidity-controlled environment maintained on a 12:12-h light-dark schedule (lights on 8:00 AM to 8:00 PM). Mice were provided food and water ad libitum.

Primary cardiomyocyte cultures from neonatal rats. Neonatal ventricular myocytes were isolated from the hearts of 1- to 3-day-old Wistar rats by collagenase digestion and cultured according to the methods of Lu et al. (25). Briefly, neonatal rats were decapitated, and their hearts immediately removed. Ventricles were separated from the heart and washed in HBSS (137.0 mM NaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.3 mM $Na_2HPO_4 \cdot 12H_2O$, 6.12 mM glucose, and 4.2 mM $NaHCO_3$; pH 7.4), and myocytes were dissociated from the ventricles by serial digestion with 0.1% trypsin and 0.05% DNase I in HBSS. After digestion, dissociated cardiomyocytes were collected and suspended in DMEM with 10% FBS and 0.02% trypsin inhibitor to inhibit further digestion. Cells were collected by centrifugation (4°C, 1,000 *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 CO_2 incubator to remove nonmyocytes, which attached to the plates. Unattached myocytes were collected and plated at 1–2 × 10^6 cells/5-mm dish and incubated with DMEM and 10% FBS in a humidified incubator with DMEM and 5% CO_2 at 37°C for 48 h. Cells were cultured in serum-free DMEM for 24 h before treatment with ANG II (100 nM).

Morphological analysis of cultured cardiomyocytes. Cultured myocytes were plated on collagen-coated coverglasses at a density of 1–2 × 10^6 cells/coverslip of 12-mm diameter. After an incubation in the presence or absence of ANG II for 48 h, cultured cells were washed three times in 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% BSA 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 the cells was quantitated using the Image J program. All cells from randomly selected fields were examined in each condition (at least 100 myocytes/group). The surface area of control cells was expressed as 100% and compared with that of treated cells.

Small interfering RNA transfection in cultured cardiomyocytes. σ_1R small interfering (si)RNA (sense: 5′-ACACGTGGATGGTGGAGTA-3′ and antisense: 5′-TACTCCACCATCCACGTGT-3′) was purchased from Exigen (Tokyo, Japan). Cultured myocytes were plated on uncoated 90-mm culture dishes on collagen-coated coverglasses. Transfections were performed with 100 nM σ_1R siRNAs according to the methods of Wang et al. (49). Briefly, 20 μM σ_1R 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% CO_2 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 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 previously described (35). After acclimatization for 7 days, animals were anesthetized with tribromoethanol (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 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 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 σ_1Rs 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 σ_1R-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*) sham 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% calboxymetylcellulose, 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

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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: FS = [(LVEDD − LVESD)/LVEDD] × 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% lowfat 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 Imagegause 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 σ_1R expression. To define the role of σ_1R expression in cardiac hypertrophy and heart failure, we evaluated the time course of LV function, cardiac hypertrophy, and σ_1R expression after TAC. Echocardiographic data for the time course experiment is provided in the Supplemental Material (Supplemental Table 1)[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

[1] Supplemental Material for this article is available online at the *American Journal of Physiology-Heart and Circulatory Physiology* website.

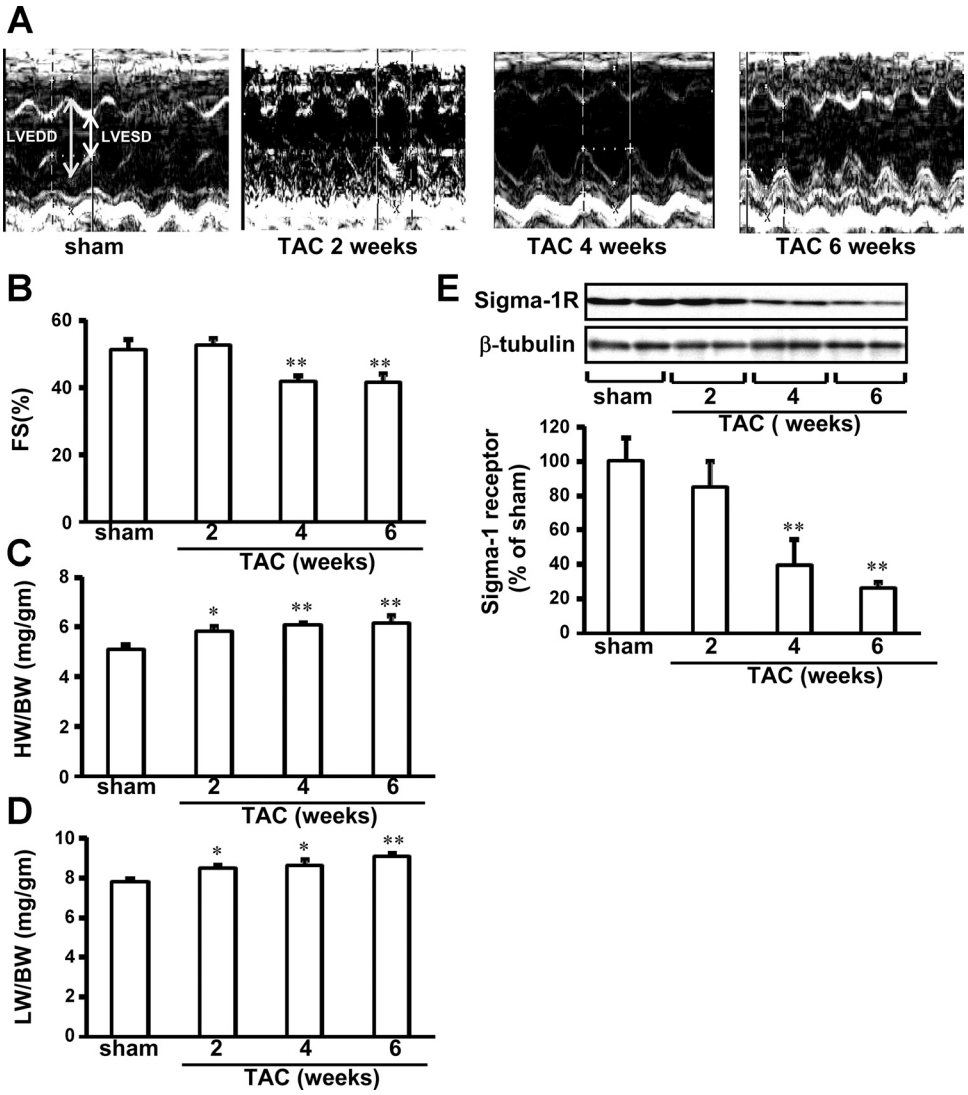

Fig. 1. Temporal changes in cardiac hypertrophy and failure induced by transverse aortic constriction (TAC) in mice. *A*: representative M-mode echocardiograms of mice before and after 2–6 wk of TAC. LVEDD, left ventricular (LV) end-diastolic diameter; LVESD, LV end-systolic diameter. *B*: changes in 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 σ_1-receptor (σ_1R) 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.

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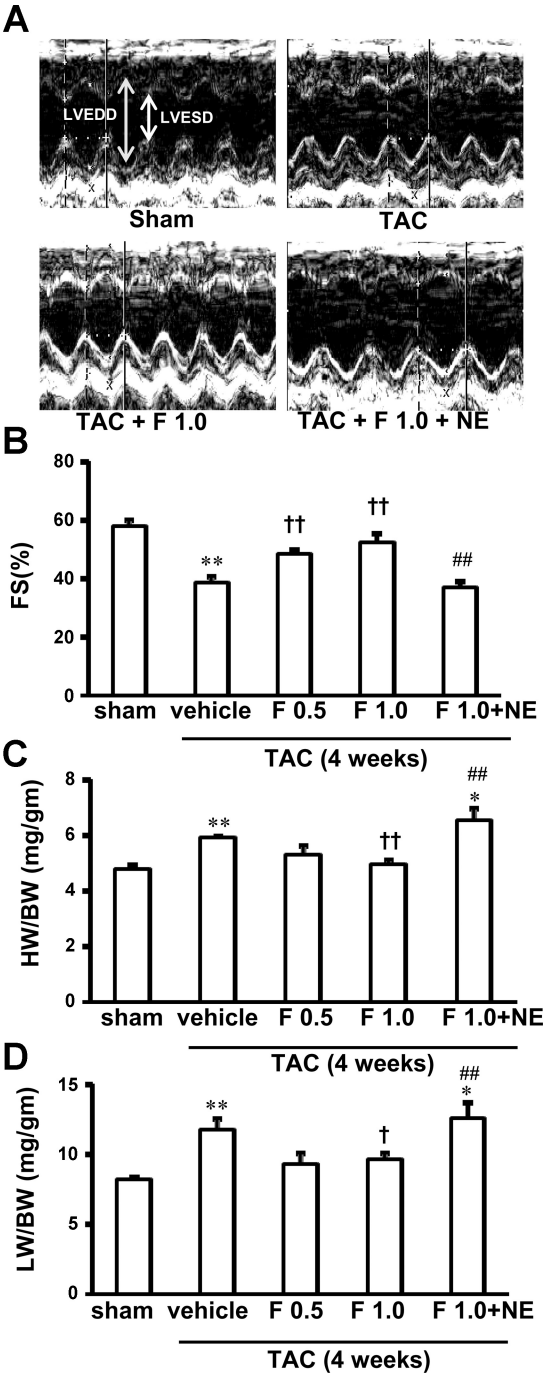

Fig. 2. Effect of fluvoxamine (Flux) and NE-100 (NE) on cardiac hypertrophy and failure induced by TAC in mice. *A*: representative M-mode echocardiograms of mice with and without Flux and/or NE treatment. *B*: changes in the percentage of LV FS. TAC mice were treated with Flux [0.5 mg/kg (F 0.5 group) 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.

($P < 0.01$ vs. the sham group; Fig. 1, *A* and *B*). Time course experimetns to assess HWs and lung weights (LWs) indicated that HW/BW (Fig. 1*C*) increased time dependently from 1 to 6 wk after TAC in mice ($P < 0.01$ vs. the sham group). We also found time-dependent increases in the LW-to-BW ratio (LW/BW; Fig. 1*D*) from 1 to 6 wk after TAC in mice. Interestingly, σ_1R 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. 1*E*). Thus, our study revealed a significant negative linear correlation of heart dysfunction with reduced σ_1R expression in the LV.

Effect of σ_1R stimulation on myocardial hypertrophy and dysfunction. To confirm the cardioprotective role of fluvoxamine and paroxetine, which are typical SSRIs, we treated mice

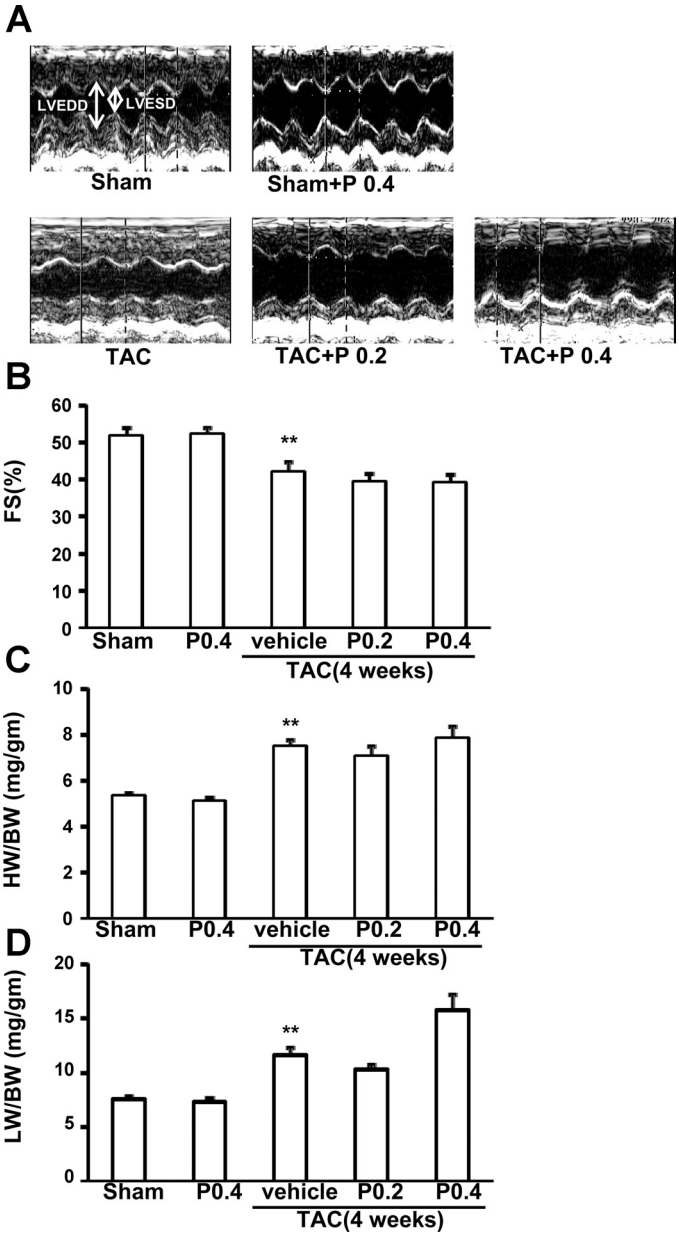

Fig. 3. Effect of paroxetine (Parox) on cardiac hypertrophy and failure induced by TAC in mice. *A*: Representative M-mode echocardiograms of mice with and without drug (Parox) treatments. *B*: changes in the percentage of LV FS. TAC or sham mice were treated with Parox at 0.2 mg/kg (P 0.2 group) or 0.4 mg/gk (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.

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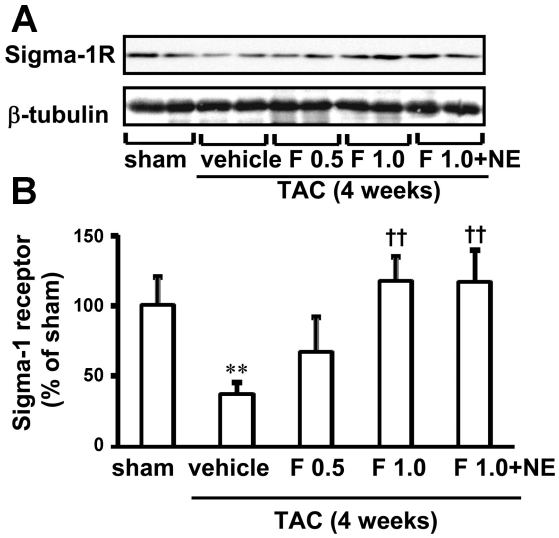

Fig. 4. Effects of Flux and NE-100 on σ_1R expression. *A*: Western blot analysis of σ_1R expression and β-tubulin as a loading control in the LVs of sham and TAC mice with or without drug treatment. Immunoblot analysis with anti-β-tubulin antibody showed equal protein loading in each lane. *B*: densitometric quantification of σ_1R-immunoreactive bands. Data are expressed as percentages of values of sham mice (means ± SE). Each group consisted of 5–10 mice. $^{**}P < 0.01$ vs. the sham group; $^{\dagger\dagger}P < 0.01$ vs. the TAC-vehicle group.

with either drug or with the σ_1R 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. S1*B*). Consistent with temporal changes in heart function, as shown in Fig. 1*B*, 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 ($P < 0.01$ vs. the TAC-vehicle group for both the F 0.5 and F 1.0 groups; Fig. 2*B*). Paroxetine treatment, however, failed to restore FS (Fig. 3*B*). 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. 2*B*).

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. 2*C*) and elevated LW/BW ($P < 0.05$ vs. the TAC-vehicle group for the F 1.0 group; Fig. 2*D*). Paroxetine administration failed to inhibit increases in both HW/BW (Fig. 3*C*) and LW/BW (Fig. 3*D*). 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. 2*C*) and LW/BW ($P < 0.05$ vs. the sham group and $P < 0.01$ vs. the F 1.0 group; Fig. 2*D*). 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. S1*C*) and LW/BW (Supplemental Fig. S1*D*) ratio in the sham group. Taken together, we conclude that the antihypertrophic effect of fluvoxamine on TAC mice is mediated by σ_1R stimulation.

Effect of fluvoxamine treatment on σ_1R expression. Since σ_1R stimulation by fluvoxamine ameliorated TAC-induced LV hypertrophy in mice, we asked whether treatment with fluvoxamine and NE-100 had any effect on σ_1R expression in the LV. TAC-induced hypertrophy significantly decreased σ_1R 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 σ_1R 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 sham mice had no effect on σ_1R expression in the LV (Supplemental Fig. S1*E*). Thus, fluvoxamine-induced restoration of σ_1R levels is correlated with its cardioprotective action in the context of TAC-induced hypertrophy; however, that effect is likely not through stimulation of σ_1Rs.

σ_1R 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

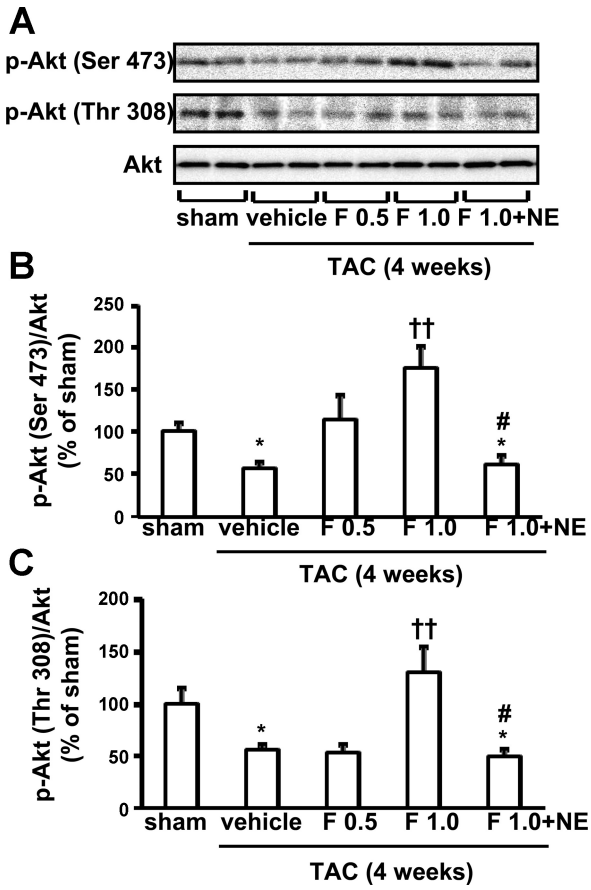

Fig. 5. Effects of Flux and NE on Akt phosphorylation. TAC mice were treated with Flux (0.5 or 1.0 mg/kg) or Flux (1.0 mg/kg) + NE (1.0 mg/kg) as shown. *A–C*: Western blot analysis (*A*) and densitometric quantification of phosphorylated (p-)Akt (Ser^{473}; *B*) and p-Akt (Thr^{308}; *C*) in the LVs 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}; *B*) and p-Akt (Thr^{308}; *C*) was relative to total Akt in the LV. Each group consisted of 5–10 mice. Each bar represents the mean ± SE. $^{*}P < 0.05$ vs. the sham group; $^{\dagger\dagger}P < 0.01$ vs. the TAC-vehicle group; $^{\#}P < 0.05$ vs. the TAC + F 1.0 group.

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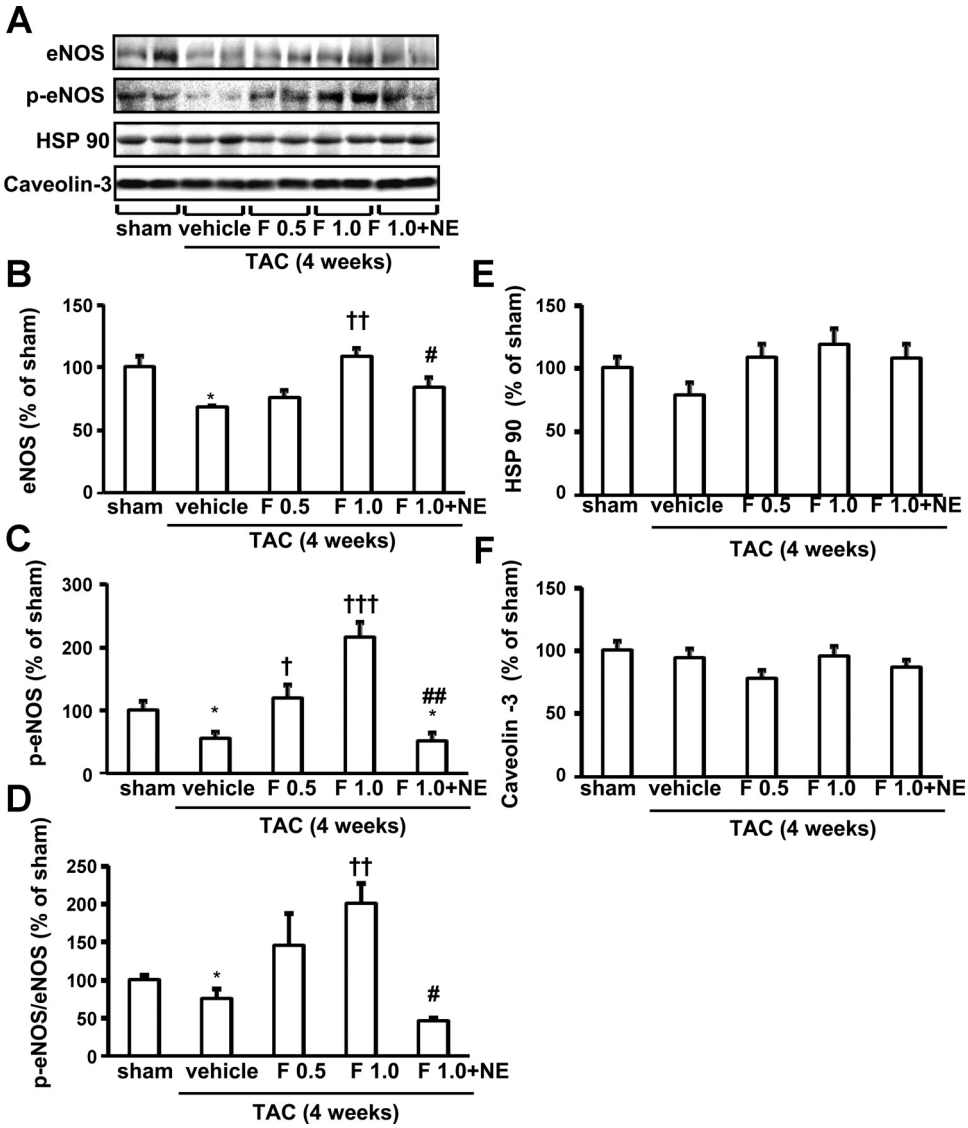

Fig. 6. Effects of Flux and NE on the levels of endothelial nitric oxide synthase (eNOS), eNOS phosphorylation, heat shock protein 90 (HSP90), and caveolin-3. TAC mice were treated with Flux (0.5 or 1.0 mg/kg) or Flux (1.0 mg/kg) plus NE (1.0 mg/kg) as shown. *A–F*: Western blot analysis (*A*) and densitometric quantification of eNOS (*B*), p-eNOS (Ser[1179]; C), ratio of p-eNOS to eNOS (*D*), HSP90 (*E*), and caveolin-3 (*F*) in the LVs of TAC mice with or without drug treatment. Data are expressed as percentages of the value of sham mice. Each group consisted of 5–10 mice. Each bar represents the mean ± SE. $*P < 0.05$ vs. the sham group; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, and $\dagger\dagger\dagger P < 0.001$ vs. the TAC-vehicle group; $\#P < 0.05$ and $\#\#P < 0.01$ 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 Ser[473] ($P < 0.01$ vs. the TAC-vehicle group for the F 1.0 group) and Thr[308] ($P < 0.01$ vs. the TAC-vehicle group for the F 1.0 group; Fig. 5, *A–C*) in LV tissues. Interestingly, coadministration of NE-100 with fluvoxamine nullified the fluvoxamine-induced rescue of Akt phosphorylation at Ser[473] ($P < 0.05$ vs. the sham group and $P < 0.05$ vs. the F 1.0 group) and Thr[308] ($P < 0.05$ vs. the sham group and $P < 0.05$ 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.

σ_1R 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 Ser[1177] ($P < 0.05$ 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 σ_1Rs. Likewise, coadministration of NE-100 with fluvoxamine blocked fluvoxamine-mediated increased eNOS phosphorylation ($P < 0.05$ vs. the sham group and $P < 0.01$ vs. the F 1.0 group; Fig. 6, *A* and *C*). These results suggest that fluvoxamine-mediated increased expression of σ_1Rs promotes increased eNOS phosphorylation by Akt, as indicated by the increased ratio of eNOS phosphorylation to total eNOS expression (Fig. 6*D*). Enhanced Akt activity and concomitant eNOS phosphorylation are likely mediated by σ_1R stimulation.

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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. 6*E*) and caveolin-3 (Fig. 6*F*) after TAC. Moreover, treatment of TAC mice with fluvoxamine and NE-100 had no effect on the expression of HSP90 (Fig. 6*E*) and caveolin-3 (Fig. 6*F*). 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. 7*A*).

PKC-α, 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 σ_1-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. 7*B*).

Role of σ_1R stimulation on ANG II-induced cardiomyocyte hypertrophy in vitro. To confirm the antihypertrophic effect of fluvoxamine through σ_1Rs 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 σ_1R stimulation mediates the fluvoxamine-induced inhibition of cardiomyocyte hypertrophy, we treated cardiomyocytes with σ_1R siRNA to downregulate σ_1Rs. As reported by Wang et al. (49), σ_1Rs were downregulated ~70% by σ_1R siRNA treatment ($P < 0.01$ vs. control; Fig. 9*A*). σ_1R 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 σ_1R siRNA ($P < 0.001$ vs. the F 5.0 group; Fig. 9, *B* and *C*). Taken together, our observations strongly suggest that the antihypertrophic effect of fluvoxamine on ANG II-induced cardiomyocyte hypertrophy is mediated by σ_1R stimulation.

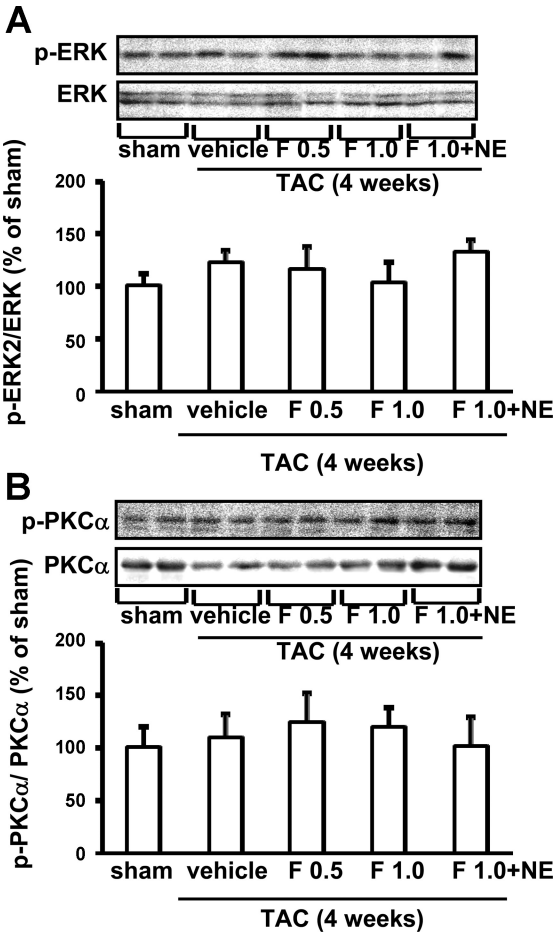

Fig. 7. Effects of Flux and NE on ERK1/2 and PKC-α phosphorylation. *A* and *B*: Western blot analysis (*top*) and densitometric quantification (*bottom*) of ERK1/2 phosphorylation (Thr^{202}/Tyr^{204}; *A*) and PKC-α phosphorylation (Ser^{657}; *B*) relative to respective total proteins in the LVs of TAC mice with or without drug treatment. Data are expressed as percentages of the value of sham mice. Each group consisted of 5–10 mice. Each bar represents the mean ± SE.

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, 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 the potential reduction in MI by SSRIs administered to humans are largely unknown.

We first hypothesized that SSRIs with a high affinity for σ_1Rs have direct cardioprotective effects via σ_1Rs expressed on cardiomyocytes. We confirmed the cardioprotective role of one such high-affinity reagent, fluvoxamine (K_i: 36 nM), for σ_1Rs in pathophysiological conditions mediated by TAC and compared those with the effects of the low- affinity agonist paroxetine (K_i: 1,893 nM). The most significant observations in

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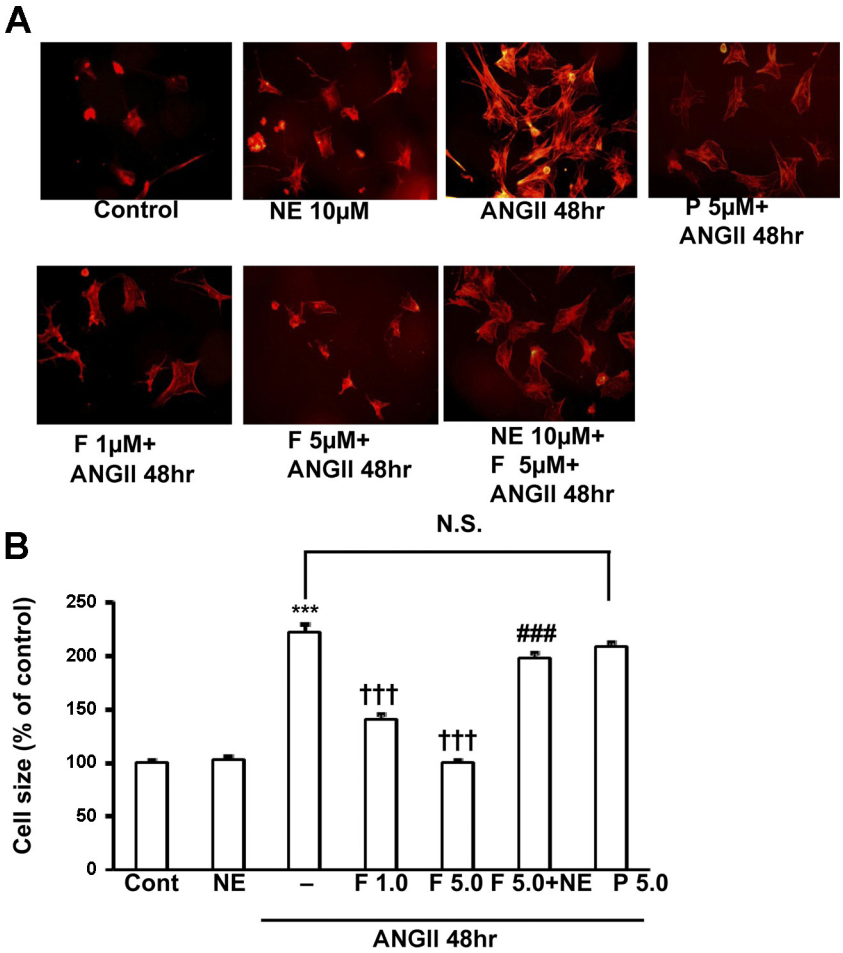

Fig. 8. Effect of Flux and Parox treatments on ANG II-induced hypertrophy in cultured cardiomyocytes. *A*: cells were fixed with 4% paraformaldehyde, stained with rhodamine-conjugated phalloidin, and processed for fluorescence microscopy. One hundred cells from randomly selected fields were evaluated 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 ± 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 μM Flux-treated (F 5.0) group.

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 σ_1Rs in the LV associated with contractile impairment after TAC. We also report, for the first time, that restored Akt activity through σ_1R stimulation by fluvoxamine ameliorates the impairment of eNOS expression and phosphorylation in TAC cardiomyocytes.

Currently, we do not know how chronic fluvoxamine treatment upregulates σ_1R expression. Whether σ_1R 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 σ_1R agonist increased σ_1R mRNA levels in the rat brain. Shirayama et al. (42) reported a decrease in σ_1-binding sites in the rat brain after chronic treatment with imipramine, a weak σ_1R agonist (K_i: 343 nM). Chronic haloperidol (a σ_1R antagonist) treatment reportedly promoted a reduction in σ_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 σ_1R antagonist NE-100 did not alter σ_1R expression in sham mice. Similarly, the combination of NE-100 with fluvoxamine had no effect on fluvoxamine-induced upregulation of σ_1Rs in the LV but nullified fluvoxamine-mediated antihypertrophic effects. These data suggest that antihypertrophic effects and the upregulation of Akt signaling are mediated by σ_1R stimulation and that stabilization or upregulation of σ_1Rs by fluvoxamine is not mediated by σ_1R-stimulated signaling. In another words, the stabilization or upregulation of σ_1Rs is not mediated by Akt signaling. Further studies are required to identify the mechanisms underlying the stabilization or upregulation of σ_1Rs 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 σ_1Rs 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 σ_1R 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, σ_1R knockdown via siRNA reportedly inhibited thrombin-stimulated Akt phosphorylation, increasing cell death in lens cells (49). Taken together, although the σ_1R is not a G protein-coupled transmembrane receptor, σ_1R 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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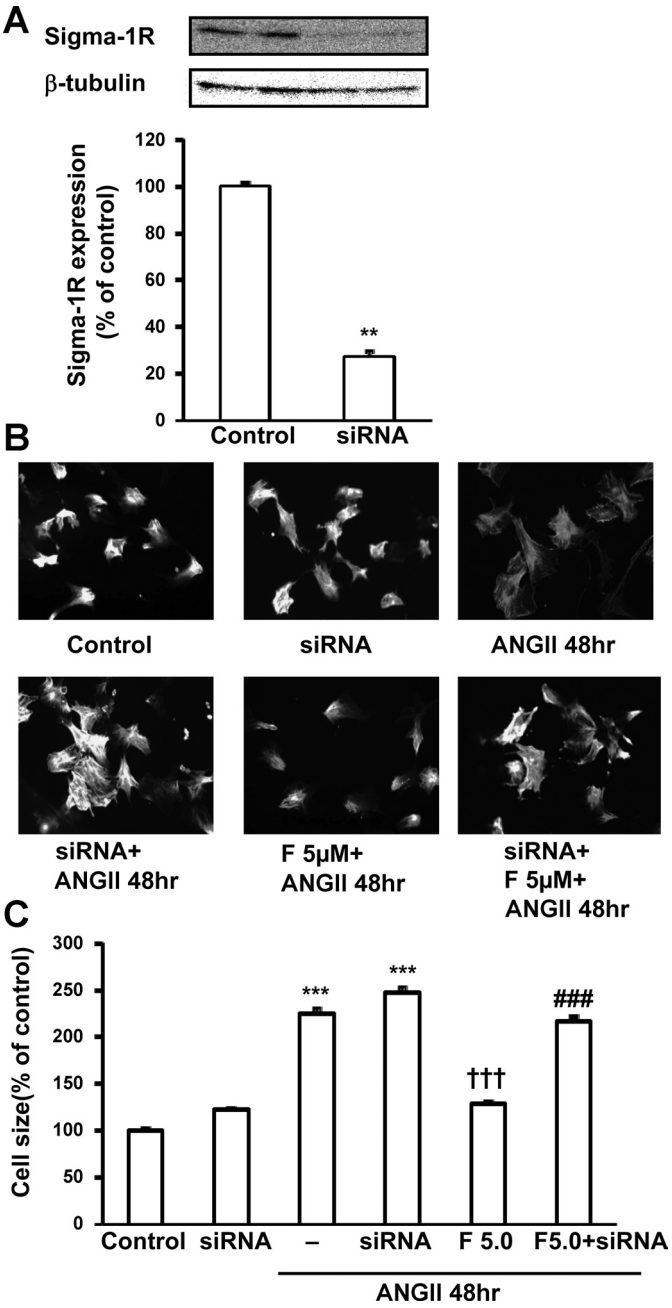

Fig. 9. Effect of σ_1R small interfering (si)RNA on Flux-induced antihypertorphic effects in cultured cardiomyocytes. *A*: Western blot analysis (*top*) and densitometric quantification (*bottom*) of σ_1R expression with or without (control) σ_1R siRNA treatment. Immunoblot analysis with anti-β-tubulin 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 expressed as surface area relative to control cells. Each bar represents the mean ± SE. $^{**}P < 0.01$ and $^{***}P < 0.001$ 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 σ_1R antagonist NE-100 significantly nullified fluvoxamine-mediated eNOS upregulation and Akt-mediated eNOS phosphorylation, confirming th σ_1R-mediated modulation of eNOS activity in the heart. σ_1R ligands, such as dehydroepiandrosterone, rapidly 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 σ-ligands. PKC-α, 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 Ser^{1177} (7). We did not observe significant changes in PKC-α 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 σ_1Rs, and σ_1Rs stimulate 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 σ_1R 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 σ_1Rs but that a paroxetine dose of 20 mg/BW has no effect in the intact human brain (20), suggesting that σ_1Rs 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 σ_1Rs. 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

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strongly suggest that SSRIs that stimulate σ_1Rs 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 σ_1R expression in the heart to attenuate TAC-induced hypertrophy in mice. With regard to the particular function of σ_1Rs in the heart, our data confirmed that fluvoxamine is a σ_1R 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 σ_1Rs and stimulation of σ_1R-mediated Akt-eNOS signaling in mice. We also report, for the first time, that, among SSRIs, fluvoxamine-related σ_1R 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 σ_1R signaling may provide novel therapeutic targets for which a new class of antihypertrophic drugs can be designed.

GRANTS

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DISCLOSURES

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

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