Abstract—Estrogen regulation of myocardial chymase and chymase effects on cardiac remodeling are unknown. To test the hypothesis that estrogen prevents pressure overload–induced adverse cardiac remodeling by inhibiting mast cell (MC) chymase release, transverse aortic constriction or sham surgery was performed in 7-week-old intact and ovariectomized (OVX) rats. Three days before creating the constriction, additional groups of OVX rats began receiving 17β-estradiol, a chymase inhibitor, or a MC stabilizer. Left ventricular function, cardiomyocyte size, collagen volume fraction, MC density and degranulation, and myocardial and plasma chymase levels were assessed 18 days postsurgery. Aortic constriction resulted in ventricular hypertrophy in intact and OVX groups, whereas collagen volume fraction was increased only in OVX rats. Chymase protein content was increased by aortic constriction in the intact and OVX groups, with the magnitude of the increase being greater in OVX rats. MC density and degranulation, plasma chymase levels, and myocardial active transforming growth factor-β1 levels were increased by aortic constriction only in OVX rats. Estrogen replacement markedly attenuated the constriction-increased myocardial chymase, MC density and degranulation, plasma chymase, and myocardial active transforming growth factor-β1, as well as prevented ventricular hypertrophy and increased collagen volume fraction. Chymostatin attenuated the aortic constriction–induced ventricular hypertrophy and collagen volume fraction in the OVX rats similar to that achieved by estrogen replacement. Nedocromil yielded similar effects, except for the reduction of chymase content. We conclude that the estrogen-inhibited release of MC chymase is responsible for the cardioprotection against transverse aortic constriction-induced adverse cardiac remodeling. (Hypertension. 2015;65:00-00. DOI: 10.1161/HYPERTENSIONAHA.114.04238) ● Online Data Supplement

Key Words: cardiac remodeling ■ chymase ■ estrogen ■ mast cell

The loss of cardioprotection in postmenopausal women represents a significant health issue that is in need of further research to delineate the responsible mechanisms. To this end, we have reported gender differences in the pattern of global myocardial remodeling in response to a sustained cardiac volume overload and demonstrated the protection of estrogen (E2) against volume overload–induced adverse cardiac remodeling.1,4 Others have reported similar sex remodeling differences in mice with sustained pressure overload secondary to transverse aortic constriction (TAC).5 We also found the cardiac mast cell (MC) to be pivotal in initiating the adverse myocardial remodeling that leads to ventricular decompensation in male and ovariectomized (OVX) rats but not in intact (Int) premenopausal female rats.5-9 Furthermore, we demonstrated that supplemental E2 administered to male and OVX rats was able to attenuate volume overload–induced adverse cardiac remodeling by its ability to prevent the increase in cardiac MC density and degranulation.10,11 Chymase, which is stored in a macromolecular complex in MC cytoplasmic secretory granules and is released after MC activation, plays a crucial role in cardiovascular diseases12-18 via its ability to form angiotensin II, activate transforming growth factor (TGF)-β1, IL-1β, and endothelin-1, and degrade the extracellular matrix, as well as its involvement in lipid metabolism.16-21 Recently, it has been reported that myocardial fibrosis was decreased in hypertensive rats after a reduction in chymase activity.22 However, with the exception of a recent study that suggested an association of E2 with chymase in renin-overexpressing mRen2. Lewis female rats,23 little is known regarding E2’s ability to regulate cardiac MC chymase and its association with cardiac remodeling. Accordingly, the purpose of this study was to test the hypothesis that E2 prevents the adverse cardiac remodeling induced by pressure overload by inhibiting cardiac MC chymase release.

Methods

Animals

The protocol was approved by the Institution’s Animal Care and Use Committee, and all studies were conducted in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

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of Laboratory Animals. A detailed Methods section is available in the online-only Data Supplement, which includes ovariectomy and TAC procedures, estrogen replacement and drug treatment, transthoracic echocardiography, histology analysis, Western blot, and ELISA.

**Results**

**Ovarian Hormones Prevented TAC-Induced Adverse LV Remodeling**

There were no significant differences in body weight between the Int and OVX rats before TAC surgery. Also, as can be seen in Figure 1A and 1B, ovariectomy did not result in a significant change in the left ventricle (LV)/tibia length (TL) ratio and myocyte cross-sectional area (CSA), respectively. However, it did cause a significant increase in LV interstitial collagen volume fraction (CVF; Figure 1C) compared with the Int-sham (Int-Sh) rats (2.36±0.12% versus 1.08±0.12%; Figure 1D). LV hypertrophy occurred in both TAC groups as evidenced by significant increases in LV/TL, myocyte CSA (Figure 1A and 1B), and LV posterior wall thickness (Figure S1C in the online-only Data Supplement), with the increases in the OVX-TAC group being greater than those in the Int-TAC group. LV CVF also significantly increased after 18 days of TAC in the OVX-TAC group (35% increase versus OVX-Sh). In contrast, there was no increase in CVF in the Int-TAC rats relative to the Int-Sh rats (Figure 1C and 1D). LV internal diameter, ejection fraction, and fractional shortening, as determined by echocardiography (Figure S1C), and lung weight/TL (Figure S2A) were similar in the 4 groups, indicating that LV function remained compensated in both TAC groups.

**Ovarian Hormones Inhibited TAC-Induced Myocardial MC Chymase Synthesis and Release**

LV tissue chymase protein content for the 4 groups, as determined by Western blot (Figure 2A), is provided in Figure 2B. Also, the plasma chymase levels for the study groups, which are assumed to include the chymase released from myocardial cells, are shown in Figure 2C. As can be seen, ovariectomy had no effect on myocardial chymase protein content and plasma chymase levels compared with the Int-Sh group. However, myocardial chymase protein content was significantly increased in both the Int-TAC and OVX-TAC groups, with the magnitude of the increase being greater in the OVX-TAC group (Figure 2B), whereas the plasma chymase level was significantly increased only in the OVX-TAC group (Figure 2C).

Chymase is known to be primarily synthesized and stored in MCs. This is demonstrated in Figure 2D, where LV tissue immunostained with a chymase antibody revealed it to be localized in MCs and secreted MC granules. Accordingly, the TAC-induced elevation in tissue chymase protein levels would be indicative of a greater MC chymase synthesis rate, a greater MC density or both, whereas the increase in the plasma chymase level in the OVX-TAC group would reflect enhanced MC degranulation. As depicted in Figure 2E, MC density was increased only in the OVX-TAC group. Thus, although chymase production was elevated in response to TAC in both the

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**Figure 1.** Transverse aortic constriction (TAC) or sham (Sh) surgery was performed in 7-week-old rats with either ovaries intact (Int) or bilateral ovariectomy (OVX). Changes in the ratio of left ventricular weight (LV) to tibia length (TL), myocyte cross-sectional area (CSA), and LV interstitial collagen volume fraction (CVF) after 18 days post TAC or Sh surgery are shown in A, B, and D, respectively. Representative images of myocardium stained with collagen-specific picrosirius red are depicted for the 4 study groups in C (×100 magnifications). All values are mean±SEM, N=6 to 7; *P<0.05 vs Int-Sh, †P<0.05 vs OVX-Sh, and #P<0.05 vs Int-TAC.
Int and OVX groups, the additional increment in the OVX-TAC group (Figure 2B) was likely caused by the greater MC density. An estimate of MC degranulation indicated that it was significantly greater in the OVX-TAC group (74.5±1.4%) compared with the other groups (Int-Sh, 62.8±2.6%; OVX-Sh, 63.2±1.5%; and Int-TAC, 61.0±2.4%), which in all likelihood contributed to the plasma chymase levels seen in all 4 groups (Figure 2C).

Estrogen Inhibited TAC-Induced Myocardial MC Chymase Synthesis and Release and Prevented Adverse LV Remodeling

The above results clearly reflect the cardioprotective role of ovarian hormones to the adverse myocardial remodeling associated with TAC. Accordingly, estrogen replacement experiments in OVX rats with TAC were performed. Estrogen replacement significantly prevented the TAC-induced increases in LV/TL (Figure 3A), myocyte CSA (Figure 3B), systolic and diastolic LV posterior wall thickness (Figure S3C), LV interstitial CVF (Figure 3C and 3D), plasma chymase level (Figure 4C), MC density (Figure 4D), and MC degranulation (Figure 4E). Chymostatin also was able to prevent the TAC-induced increases in LV/TL, myocyte CSA, LV interstitial CVF (Figure 3), and systolic and diastolic LV posterior wall thickness (Figure S3C). However, it did not prevent the TAC-induced increases in myocardial chymase protein content (Figure 4B), plasma chymase level (Figure 4C), and MC density (Figure 4D) and degranulation (Figure 4E). Lung weight/TL (Figure S2B), systolic and diastolic LV internal diameter, ejection fraction, and fractional shortening (Figure S3C) were not different between the 4 OVX-TAC groups, indicating that LV function remained compensated in OVX-TAC groups with or without treatment. These results clearly delineate the estrogen-MC-chymase axis as being contributory to the modulation of TAC-induced myocardial remodeling.

Chymase Activated TGF-β1

Activated TGF-β1 is known to stimulate collagen synthesis in fibroblast and myofibroblasts. Accordingly, we determined its myocardial levels in the 4 study groups and also the effect of supplementary estrogen, nedocromil, and chymostatin on these levels in OVX-TAC rats. Depicted in Figure 5A and 5B are the
levels of myocardial active TGF-β1 in the 4 groups as determined by Western blotting (Figures 5A). As can be seen, the myocardial active TGF-β1 level was only significantly increased in the OVX-TAC rats, which was prevented by supplementary estrogen, nedocromil, and chymostatin (Figure 5C and 5D).

Discussion
Accumulating evidence has demonstrated the ability of estrogen to markedly attenuate or prevent maladaptive remodeling in response to sustained abnormal elevations in myocardial stress.2–5,24 Cardiac MCs play a pivotal role in...
adverse remodeling, which is countered by estrogen’s ability to induce protective, phenotypic MC changes. Given that myocardial chymase is primarily produced and localized in cardiac MCs and that decreased myocardial fibrosis was reported after a reduction in chymase activity in hypertensive rats, we hypothesized that estrogen prevents adverse myocardial remodeling secondary to a sustained cardiac pressure overload via its ability to diminish the MC synthesis of chymase and attenuate MC degranulation. To this end, intact female and OVX rats were subjected to sham or TAC surgery, and the myocardial remodeling, chymase, and active TGF-β1 levels in OVX rats treated with estrogen (E2), nedocromil (Ne), or chymostatin (Ch) started 3 days before 18 days of TAC. All values are mean±SEM, N=6 to 7; *P<0.05 vs Int-Sh, OVX-Sh, and Int-TAC; +P<0.05 vs OVX-TAC.

Because chymase has been known to exert its actions after it is released into the interstitium, it is necessary to measure extracellular chymase activity to assess its influence on cardiac remodeling. In view of the fact that chymase is activated and stored in MC secretory granules, assessing chymase activity in tissue homogenates will not reflect extracellular chymase activity in the heart. Therefore, recent reports have indicated that chymase is also present within cardiomyocytes, where it can promote angiotensin II formation. Therefore, to determine the ability of estrogen to regulate myocardial chymase, chymase synthesis and release were assessed by measuring myocardial chymase protein content, plasma chymase levels, and cardiac MC degranulation. The fact that OVX did not have an effect on myocardial chymase synthesis, plasma chymase levels, and MC density and degranulation indicated ovarian hormones did not influence synthesis and release of chymase under basal condition. Myocardial chymase synthesis was increased by TAC in both intact and OVX rats with the magnitude of the increase being greatest in the OVX rats. In contrast, chymase release, as reflected by increased plasma chymase levels and MC degranulation, was elevated by TAC only in the OVX rats. Interestingly, the TAC-induced increase in the synthesis of myocardial chymase paralleled that of LV hypertrophy, and the TAC-induced increase in the release of chymase occurred concomitantly with the enhanced collagen deposition. Estrogen replacement markedly attenuated the TAC-induced increase in myocardial chymase synthesis and release and prevented LV hypertrophy and elevations in collagen deposition in the OVX rats. These results demonstrated that estrogen inhibited TAC-induced myocardial chymase synthesis and release and prevented TAC-induced adverse cardiac remodeling.

Previous studies reported chymase activity to be increased in the human atherosclerotic aorta and infarcted hearts and that chymase inhibition suppressed cardiac fibrosis in animal models of myocardial infarction, cardiomyopathy, and tachycardia-induced heart failure. In the present study, the increases in myocardial chymase synthesis and release were found to correlate with TAC-induced LV remodeling. To establish a causal relationship between myocardial chymase and TAC-induced adverse cardiac remodeling, chymostatin was administrated to the OVX-TAC rats. Chymostatin markedly attenuated the TAC-induced LV hypertrophy and collagen deposition, indicating myocardial chymase as being responsible for the TAC-induced adverse cardiac remodeling. Moreover, myocardial active TGF-β1 levels were also reduced by chymostatin, suggesting that activation of TGF-β1 is one of the pathways by which chymase promotes cardiomyocyte hypertrophy and collagen deposition. This was further supported by the observations that the TAC-induced increases in active TGF-β1 levels were also attenuated secondary to the estrogen- and nedocromil-induced reductions in chymase release. In addition to chymase activating TGF-β1, other actions of chymase include the conversion of angiotensin I to angiotensin II. Therefore, to the TAC-induced adverse cardiac remodeling either indirectly by stimulating the production of latent TGF-β1 or directly by stimulating fibroblast collagen synthesis and myocyte hypertrophy. The measurement of active TGF-β1 should reflect, if any, the contribution of angiotensin II to the latent TGF-β1 pool. However, the direct contribution of angiotensin II to TAC-induced fibrosis and hypertrophy in this study, if any, was not assessed.

The fact that chymase is primarily synthesized and released from MCs was further evidenced by the immunohistochemistry staining showing chymase to be localized in MCs and the externalized MC granules. As a result of elevated myocardial stress secondary to sustained pressure or volume overload or injury, a variety of factors, such as reactive oxygen species and...
We conclude that the estrogen-inhibited release of myocardial MC chymase is responsible for the cardioprotection against TAC-induced adverse cardiac remodeling in intact females.

Potential Limitations

Although the results clearly indicate that estrogen attenuates TAC-induced fibrosis and myocyte hypertrophy, it remains unknown if the continued use of chymostatin, MC stabilizing drug, or supplementary estrogen would prevent or retard LV dysfunction from occurring. However, previous studies have demonstrated that inhibition of chymase by Qiliqingxin,22 a specific traditional Chinese drug, and the chymase inhibitors CI-B27 and SUNC825732 reduced myocardial fibrosis and thus attenuated LV dysfunction in spontaneously hypertensive rats, myocardial infarction, and tachycardia-induced heart failure, respectively. Therefore, one could infer from these reports that our results indicating that chymostatin, estrogen replacement, and MC membrane stabilization markedly attenuated TAC-induced myocardial fibrosis indirectly demonstrate that the inhibition of chymase would effectively retard or prevent LV dysfunction. Also, the study did not examine the effects of chymase on other aspects of TAC-induced cardiac remodeling, such as inflammation and myocyte death. Finally, as mentioned earlier, the direct contribution of chymase-induced increases in myocardial angiotensin II, if any, on TAC-induced fibrosis and hypertrophy was not assessed.

Perspective

As summarized in Figure 6, the results herein demonstrate that estrogen inhibits cardiac MC chymase release to prevent TAC-induced adverse cardiac remodeling, indicating for the first time that estrogen regulation of the cardiac MC chymase release plays an important role in the cardiac remodeling under pressure overload conditions in female rats. Since accumulating evidence has demonstrated the crucial role of chymase in cardiac remodeling, the present findings increase our understanding as to why the postmenopausal incidence of heart failure is significantly increased. Also, these findings suggest that MC stabilization or chymase inhibition could potentially serve as an alternative to or in combination with estrogen replacement therapy to attenuate the fibrosis and myocyte hypertrophy associated with hypertensive heart disease.

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Disclosures

None.

References

1. Janicki JS, Spinale FG, Levick SP. Gender differences in non-ischemic myocardial remodeling: are they due to estrogen modulation of cardiac mast cells and/or membrane type 1 matrix metalloproteinase. Pflugers Arch. 2013;465:687–697.
What Is New?

- This is the first report to document the estrogen-inhibited release of myocardial mast cell chymase as being responsible for the cardioprotection against TAC-induced adverse cardiac remodeling.

What Is Relevant?

- The findings of this study suggest that myocardial mast cell stabilization or chymase inhibition could potentially serve as an alternative to or in combination with estrogen replacement therapy to confer cardioprotection.

Novelty and Significance

Estrogen inhibits cardiac mast cell chymase release to prevent transverse aortic constriction–induced adverse cardiac remodeling, indicating for the first time that estrogen regulation of the cardiac mast cell chymase release plays an important role in the adverse cardiac remodeling secondary to chronic pressure overload conditions in female rats.
Estrogen Inhibits Mast Cell Chymase Release to Prevent Pressure Overload-Induced Adverse Cardiac Remodeling
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Estrogen Inhibits Mast Cell Chymase Release to Prevent Pressure Overload-Induced Adverse Cardiac Remodeling

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Methods

Animals. Five wk old female Sprague-Dawley rats, purchased from Harlan Laboratories, were housed under standard environmental conditions and maintained on a rodent diet that did not contain alfalfa or soybean meal (Phytoestrogen-free diet no. 2014, Harlan Teklad) and tap water ad libitum. Anesthesia for all surgical procedures was achieved by an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and postoperative analgesia was maintained by the intramuscular injection of buprenorphine hydrochloride (0.025 mg/kg). At the endpoint of the experiments, blood, hearts and lungs were collected under deep anesthesia and the wet weights of the heart, left ventricle (LV) and lungs (LW) were obtained. The basal portion of the LV was fixed in 10% formalin for histological analysis and the apical portion was snap frozen in liquid nitrogen and stored at −80 °C for subsequent analysis. The tibia length (TL) was used to normalize tissue weights.

Ovariectomy and transverse aortic constriction. At five wks of age, the rats were randomly assigned to undergo bilateral ovariectomy (OVX) or sham (ovaries intact, Int) surgery¹. After a two-wk recovery period, a transverse aortic constriction (TAC) or sham (Sh) surgical procedure was performed in a random fashion as described elsewhere². Briefly, a medial skin incision from the neck to the upper chest was made and the manubrium of the sternum was opened. This approach allowed for the direct visualization of the transverse aorta without having to enter the pleural space. The transverse aorta between the right innominate and left carotid artery was constricted to the outside diameter of a 22-gauge needle using 6-0 silk suture. Sham surgery was performed without banding the aorta. The animals were grouped as follows: intact rats
with transverse aortic constriction (Int-TAC, n=7); intact sham operated (Int-Sh, n=6); OVX rats with transverse aortic constriction (OVX-TAC, n=7); and OVX sham operated (OVX-Sh, n=6). Eighteen days after TAC or sham surgery, the rats were sacrificed and tissue and blood samples were collected as described above.

**Estrogen replacement and drug treatment.** Three days prior to the creation of TAC, additional groups of OVX rats began receiving either 17β-Estradiol (E2) (OVX-TAC-E2, n=7), nedocromil (Ne), a mast cell stabilizing drug (OVX-TAC-Ne, n=7) or chymostatin (Ch), a peptidic chymotrypsin inhibitor (OVX-TAC-Ch, n=7). E2 was delivered at a rate of 0.02 mg·kg\(^{-1}\)·day\(^{-1}\) via time release pellets (Innovative Research of America, Sarasota, FL) implanted subcutaneously. This dose yields blood levels of ∼35 pg/ml which are comparable to the average estrogen blood level in intact female rats\(^{3,4}\). Ne was administrated at a rate of 30 mg·kg\(^{-1}\)·day\(^{-1}\) via time release pellets (Innovative Research of America, Sarasota, FL) that were implanted subcutaneously\(^5\). Ch was administrated by intraperitoneal injection (1 mg/kg, twice daily)\(^6\). These treatments were continued for three wks prior to sacrifice and the collection of samples as described above.

**Transthoracic echocardiography.** Rats were sedated with isoflurane (~ 1%) and LV function was evaluated echocardiographically using a Vevo 770 High-Resolution Imaging System with a 37.5-MHz high-frequency linear transducer (VisualSonics Inc. Toronto, ON, Canada). LV internal diameter (LVID) and posterior wall thickness (LVPW) at end-systole (s) and –diastole (d) were measured from short-axis M-mode images.
recorded at the papillary muscle level. Fractional shortening (FS) and ejection fraction (EF) were calculated using VisualSonics Measurement Software formulae.

**Histology analysis.** Formalin-fixed LV tissue was embedded in paraffin and 5 μm thick cross sections were obtained. The sections were stained with hematoxylin–eosin for assessment of myocyte size. The average cross sectional area (CSA) of myocytes was calculated from the area of 50 myocytes measured by Image J (NIH). LV collagen matrix was stained with picrosirius red\(^7\,8\). Twenty microscopic fields (100 ×) per section devoid of perivascular collagen were imaged. Interstitial fibrillar collagen volume fraction (CVF) was determined from these images using ImageProPlus 6.0. (Media Cybernetics, Inc., Bethesda, MD) and expressed as percent of the collagen stained area within the imaged tissue area. LV MCs were stained with toluidine blue\(^9\). MC density in the section was calculated by dividing the number of MCs per section by the section area measured with a calibrated imaging densitometer (Bio-Rad). If one or more extruded granules were visible adjacent to the mast cell, the cell was considered to be degranulating\(^10\). The degranulating MCs were counted and divided by the total MC population in the section to yield the percent degranulation. Formalin-fixed LV tissue sections were immunostained with chymase antibody (Proteintech Group, Chicago IL) and Vectastain ABC kit (Vector Laboratories, Burlingame CA) to determine the distribution of myocardial chymase.

**Western blot and ELISA.** Total protein from LV tissue was extracted using RIPA buffer and a protease inhibitor cocktail (Pierce, Rockford, IL). Protein samples were fractionated by SDS-PAGE and then transferred to nitrocellulose membranes. The
membranes were incubated with primary antibodies against chymase (Proteintech Group, Chicago IL), TGF-β1 and GAPDH (SCBT Inc, Dallas TX), subsequently incubated with HRP-conjugated secondary antibodies and detected with the ECL Detection Kit (Thermo Scientific, Rockford, IL). The protein expression was quantified with Image J (NIH) and adjusted to GAPDH. Plasma chymase protein levels were measured using a rat chymase ELISA kit (Uscn Life Science Inc, Wuhan, PRC) according to the manufacturer’s instructions.

**Statistical analysis.** Results are presented as mean ± SEM. For comparison between groups, one-way analysis of variance (ANOVA) was performed, followed by the Tukey post-hoc test. Statistical significance was set at a $P$ value < 0.05.

**References**


Figure S1. Transverse aortic constriction (TAC) or sham (Sh) surgery was performed in 7-wk-old rats with either ovaries intact (Int) or bilateral ovariectomy (OVX). Shown in Panel A and B are the representative photomicrographs of left ventricle (LV) coronal section and LV echocardiograms taken at the papillary muscle level, respectively, after 18 days post TAC or Sh surgery. The changes in LV internal diameters (LVID) and posterior wall thickness (LVPW) at the end-diastole (LVIDd, LVPWd) and -systole (LVIDs, LVPWs) and LV ejection fraction (EF) and fractional shortening (FS), determined by echocardiography, are shown in Panel C. All values are mean ± SEM, N=6-7, *p<0.05 vs. Int-Sh and OVX-Sh, +p<0.05 vs. Int-TAC.
Figure S2. Panel A and B – The ratios of wet weight of lungs (LW) to tibia length (TL) in rats 18 days following transverse aortic constriction (TAC) or sham (Sh) surgery with ovaries intact (Int) or removed (OVX) and in OVX rats treated with estrogen (E2), nedocromil (Ne) or chymostatin (Ch) started 3 days prior to 18 days of TAC, respectively. All values are mean ±SEM, N=6-7.
Figure S3. Treatment with estrogen (E2), nedocromil (Ne) and chymostatin (Ch) started 3 days prior to transverse aortic constriction (TAC) in ovariectomized (OVX) rats and continued for 3 wks. Shown in Panel A and B are the representative photomicrographs of left ventricle (LV) coronal section and LV echocardiography taken at the papillary muscle level with or without treatment, respectively. The changes in LV internal diameters (LVID) and posterior wall thickness (LVPW) at the end-diastole (LVIDd, LVPWd) and -systole (LVIDs, LVPWs) and LV ejection fraction (EF) and fractional shortening (FS), determined by echocardiography, are shown in Panel C. All values are mean ± SEM, N=6-7, *p<0.05 vs. OVX-TAC.