

# Vasostatin-1 Stops Structural Remodeling and Improves Calcium Handling via the eNOS-NO-PKG Pathway in Rat Hearts Subjected to Chronic $\beta$ -Adrenergic Receptor Activation

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## Abstract

**Purpose** Chronically elevated catecholamine levels activate cardiac  $\beta$ -adrenergic receptors, which play a vital role in the pathogenesis of heart failure. Evidence suggests that vasostatin-1 (VS-1) exerts anti-adrenergic effects on isolated and perfused hearts in vitro. Whether VS-1 ameliorates hypertrophy/remodeling by inducing the chronic activation of  $\beta$ -adrenergic receptors is unknown. The present study aims to test the efficacy of using VS-1 to treat the advanced hypertrophy/remodeling that result from chronic  $\beta$ -adrenergic receptor activation and to determine the cellular and molecular mechanisms that underlie this response.

**Methods and Result** Rats were subjected to infusion with either isoprenaline (ISO, 5 mg/kg/d), ISO plus VS-1 (30 mg/kg/d) or placebo for 2 weeks. VS-1 suppressed chamber dilation, myocyte hypertrophy and fibrosis and improved in vivo heart function in the rats subjected to ISO infusion. VS-1 increased phosphorylated nitric oxide synthase levels and induced the activation of protein kinase G. VS-1 also deactivated multiple hypertrophy signaling pathways that were triggered by the chronic activation of  $\beta$ -adrenergic receptors, such as the

phosphoinositide-3 kinase (PI3K)/Akt and  $\text{Ca}^{2+}$ /calmodulin-dependent kinase (CaMK-II) pathways. Myocytes isolated from ISO + VS-1 hearts displayed higher  $\text{Ca}^{2+}$  transients, shorter  $\text{Ca}^{2+}$  decays, higher sarcoplasmic reticulum  $\text{Ca}^{2+}$  levels and higher L-type  $\text{Ca}^{2+}$  current densities than the ISO rat hearts. VS-1 treatment restored the protein expression of sarcoplasmic reticulum  $\text{Ca}^{2+}$  uptake ATPase, phospholamban and Cav1.2, indicating improved calcium handling.

**Conclusions** Chronic VS-1 treatment inhibited the progression of hypertrophy, fibrosis, and chamber remodeling, and improved cardiac function in a rat model of ISO infusion. In addition,  $\text{Ca}^{2+}$  handling and its molecular modulation were also improved by VS-1. The beneficial effects of VS-1 on cardiac remodeling may be mediated by the enhanced activation of the eNOS-cGMP-PKG pathway.

**Keywords** Vasostatin-1 · Isoprenaline · Cardiac remodeling · Left ventricular function ·  $\text{Ca}^{2+}$  handling

## Introduction

Chronically elevated catecholamine (CA) levels have long been known harm the heart [1–3]. The heart initially develops cardiac hypertrophy when exposed to prolonged and excessive adrenergic stress [4, 5]. If left uncontrolled, this hypertrophy will develop into heart failure. To ensure sufficient cardiac output, the sympathetic nervous system (SNS) and renin-angiotensin-aldosterone system (RAAS) are activated and sustained [6]. A growing amount of evidence indicates that the sustained activation of the SNS and the associated excess activation of adrenergic signaling pathways by CAs, have adverse prognostic effects and accelerate progress toward heart failure [7]. These findings are of substantial clinical significance,

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and they provide theoretical support that anti-adrenergic therapies, including the use of  $\beta$ -adrenergic-blockers, remain the most effective drug treatments for heart failure.

Chromogranin A (CgA) is a major soluble protein that is co-stored in and co-released with CAs from secretory vesicles located in adrenal medulla chromaffin cells [8]. CgA gives rise to several bioactive peptides via a post-translational proteolytic processing mechanism. One of these peptides, vasostatin, is a novel cardiac modulator and a stabilizer of adrenergic tone, which contributes to cardio-circulatory homeostasis. Evidence has indicated that the N-terminal regions of human recombinant CGA1–78 (hr-CGA1–78, vasostatin-1, VS-1) and hrCGA1–113 (vasostatin-2, VS-2) exert cardiodepressive and anti-adrenergic effects on isolated and perfused hearts in eel, frog and rat [9–11]. VS-1 and VS-2 inhibit myocardial contractility (negative inotropism) and relaxation (negative lusitropism) in a concentration-dependent manner under both basal and adrenergically stimulated conditions. Studies have demonstrated that the anti-adrenergic effect that is induced by VS-1 is caused by the  $\text{Ca}^{2+}$ -independent, PI3K-dependent endothelial release of NO and not on a direct interaction with cardiac cells. In addition, VS-1 also exerts a protective effect in ischemic hearts via an adenosine/nitric oxide signaling mechanism [12]. Four N-terminal CGA-derived peptides (CGA(4–113), CGA(1–124), CGA(1–135), and CGA(1–199)) that contain the vasostatin sequence have been characterized in rat hearts [13]. This strongly suggests that these fragments play roles in the autocrine/paracrine regulation of cardiac function. However, most studies have focused on the transient, anti-adrenergic effects of vasostatin on perfused hearts. Whether vasostatin protects the heart against chronic  $\beta$ -ARs overstimulation *in vivo* remains unknown.

In the present study, we tested the effects of VS-1 treatment in hearts subjected to chronic  $\beta$ -ARs activation. We show that VS-1 suppressed chamber, cellular, and molecular remodeling and improved cardiac function in the treated hearts. In addition,  $\text{Ca}^{2+}$  cycling and its molecular regulation were improved. A molecular signaling pathway analysis indicated that the beneficial effects of VS-1 on cardiac remodeling may be mediated via the enhanced activation of the eNOS-cGMP-PKG pathway.

## Materials and Methods

### Animal Models

All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals that was published by the US National Institutes of Health (NIH

Publication #85–23, revised 1996), and the protocol was approved by the Animal Care and use Committee of Renmin Hospital of Wuhan University, China.

Male Sprague-Dawley rats (Experimental Animal Center of the Medical College of Wuhan University) weighing 250–300 g each were randomly divided into three groups: a control group in which the rats received a volume of 0.9 % saline; an ISO group in which the rats received ISO (Sigma) alone (5 mg/kg/d, intraperitoneally [ip]); and an ISO + VS-1 (synthesized by Wuhan Moon Biosciences Co. Ltd., China) group in which the rats received ISO (5 mg/kg/d, ip) and VS-1 (30 mg/kg/d, ip). Drugs and saline were administered for 2 weeks (1/3 total doses every 8 h). VS-1 treatment was initiated 30 min prior to ISO administration. The human recombinant Ser-Thr-Ala CGA 1–78 (VS-1) peptide was produced and characterized as previously described [14].

### Physiological Studies

We performed two-dimensional, guided M-mode echocardiography in anesthetized rats. Measurements were acquired using a sequoia C256 (Siemens) with a 15-MHz linear-array transducer. Intact heart hemodynamic analyses were performed as previously described [15]. A catheter was inserted into the right carotid artery and then advanced into the left ventricle (LV) to record LV systolic pressure and LV end-diastolic pressure (Biopac systems, Inc. MP150).

### Histology

Hearts were fixed in 10 % formalin overnight, embedded in paraffin, sectioned to a thickness of 5  $\mu\text{m}$  and stained with hematoxylin/eosin or Masson's trichrome. Myocyte diameters and interstitial collagen fractions were determined using a digital image analysis (Adobe Photoshop 7.0, NIH image J), with the observer blinded to the tissue source. In four different hearts from each group, five separate fields were analyzed (a total of 50–70 cells per heart).

### Quantitative PCR

mRNA expression was analyzed using real-time quantitative PCR as previously described [16]. Total RNA was extracted from cells, and 0.2  $\mu\text{g}$  of RNA was analyzed. The following PCR primers were used in this study: rat atrial natriuretic peptide (ANP), 5'-GGGCTTCTTCTCTTCCTG-3' and 3'-CTGAGACGGGTTGACTTCC-5'; rat brain natriuretic peptide (BNP), 5'-CTCA AAGGACCAAGGCCCTACAA-3' and 3'-AAAC AACCTCAGCCCCTCACAG-5'; and rat  $\beta$ -myosin heavy chain (MHC), 5'-CAGTATGAGGAGGAGACAGAG-3' and 3'-AGGAACACTTGGCATTGACAGC-5'. To adjust

for equal loading, the results were normalized to the levels of glyceraldehyde-3phosphate dehydrogenase (GAPDH), which was amplified using the following primers: 5'-ACAGCAACAGGGTGGTGGAC-3' and 3'-TTGAGGGTGCAGCGAACTT-5'.

### Western Blot Analysis

Protein was prepared from snap-frozen heart tissue as previously described [17]. Protein extracts were run on 4–12 % Bis-Tris NuPage gels, blotted onto nitrocellulose membranes, and probed using the following primary antibodies: eNOS, Ser1179-phospho-eNOS, PI3K, P-PI3K, Akt, Ser-473-phospho-Akt (Cell Signaling Technology), PKG, CaMK-II, Thr-286-phospho-CaMK-II, SERCA2a, PLB, p-PLB (phospho S16 + T17) and Ca<sub>v</sub>1.2 (Abcam). The primary antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies and enhanced using chemiluminescence (Pierce). Total protein levels (eNOS, PI3K, Akt, and CaMK-II) were assessed after stripping the same membranes and using them to detect phospho-proteins. The blots were quantified using NIH Image J software, and the ratios of phosphorylated/total protein levels were calculated and normalized to the control group results.

### Myocyte Isolation

Rat ventricular myocytes were isolated as previously described [18]. Briefly, hearts were quickly excised and perfused at 37 °C on a Langendorff's apparatus. After 5 min of perfusion with Ca<sup>2+</sup>-free Tyrode's solution (NaCl, 130 mM; KCl, 5.4 mM; CaCl<sub>2</sub>, 1.8 mM; MgCl<sub>2</sub>, 2 mM; Na<sub>2</sub>HPO<sub>4</sub>, 0.3 mM; Hepes, 10 mM; and glucose, 10 mM; pH, 7.40 ± 0.05), the perfusate was switched to Tyrode's solution containing type II collagen enzyme (Sigma) to digest connective tissues. After 15 min of digestion, single ventricular myocytes were isolated from dissected and triturated ventricles and stabilized in BSA containing Tyrode's solution.

### Systolic Ca<sup>2+</sup> Transients and SR Ca<sup>2+</sup> Content

Cardiomyocytes were incubated with Fluo-4 AM (10 μmol/L, Molecular Probes) for 25 min at room temperature. After the cells were stained, the cardiomyocytes were superfused for 25 min to allow the fluorescent dye to wash out and then de-esterified. The Fluo-4 was excited with the 460–480 nm line of an argon laser, and emission was collected at 500–550 nm (Leica Microsystems, Germany). Systolic Ca<sup>2+</sup> transients were recorded under steady-state conditions and constant field stimulation (at 0.5, 1, or 2 Hz; 20 V). To assess SR Ca<sup>2+</sup> content, we measured the amplitude of caffeine-induced Ca<sup>2+</sup> transients. Two seconds after the stimulation was stopped

under steady-state conditions, caffeine (10 μmol/L) was directly applied onto the cell, which led to immediate and complete SR Ca<sup>2+</sup> release. The recorded Ca<sup>2+</sup> transients were analyzed using Leica Microsystems software.

### Measurement of I<sub>Ca,L</sub>

Membrane currents were obtained and analyzed using an EPC-9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Single cardiac ventricular myocytes were placed in the experimental chamber and perfused with external solution (Tyrode's solution) for 5 min at 2–3 ml/min. The measurements were acquired at RT. Glass microelectrodes were made using two-stage pulling with a resistance of 3.0–5.0 MΩ and filled with internal solution (120 mM CsCl, 1.0 mM CaCl<sub>2</sub>, 5.0 mM MgCl<sub>2</sub>, 5.0 mM Na<sub>2</sub>ATP, 11 mM EGTA, 10 mM HEPES and 11 mM glucose; adjusted to pH 7.2 with CsOH). The mean capacitance of the cells was 102.64 ± 32.68 pF, and the series resistances were <25 MΩ. I<sub>Ca,L</sub> was recorded using a whole-cell patch clamp configuration.

### Statistical Analysis

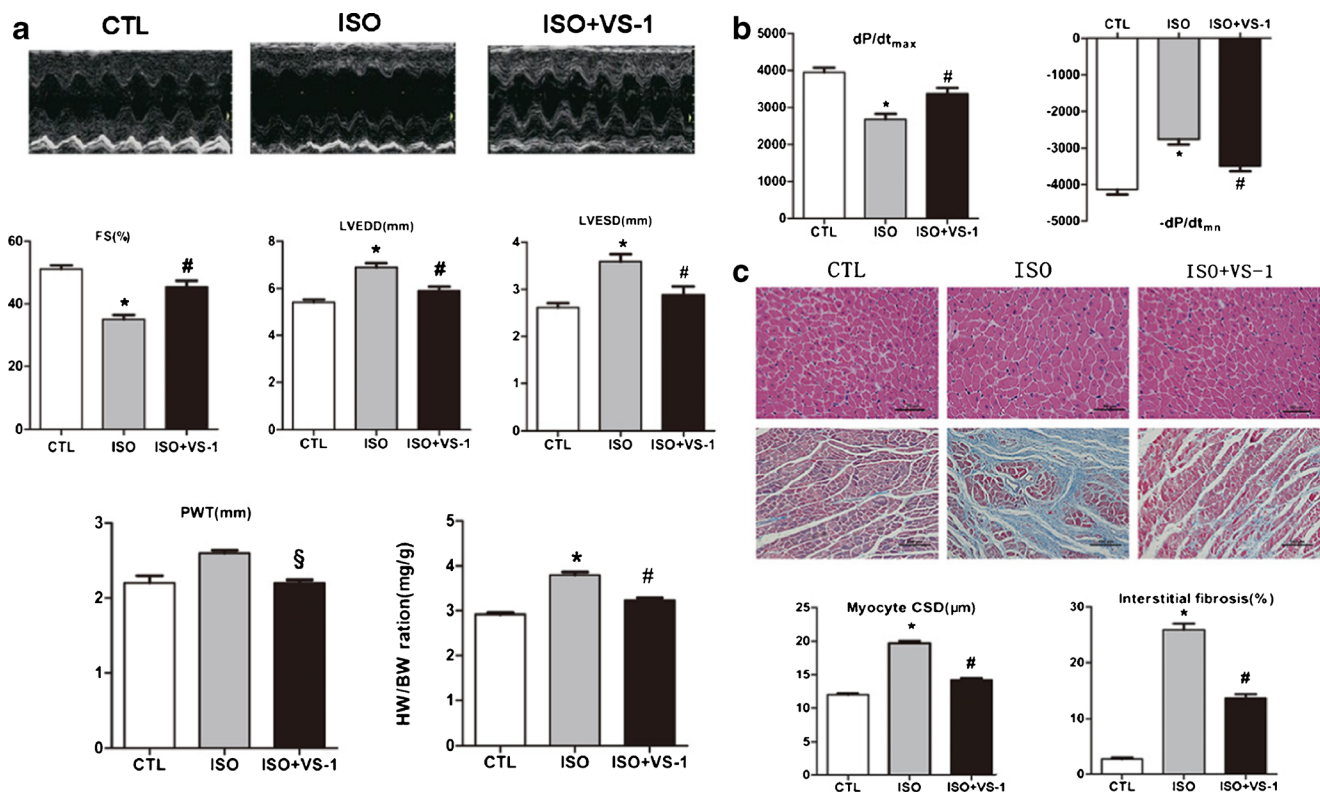
SPSS 19.0 for windows (SPSS Inc., Chicago, Illinois) was used for the statistical analyses. The data are presented as the mean ± S.E.M. Differences between groups were assessed using one-way analysis of variance followed by Tukey's multiple comparisons test. When within-group variance differed substantially, a non-parametric Kruskal-Wallis test and Bonferroni correction was used.

## Results

### VS-1 Inhibits Hypertrophy, Remodeling and Fibrosis and Improves Cardiac Function

Chronic activation of β-ARs induced heart chamber dilation (LVESD: +26.17 %; LVEDD: +28.3 %; Fig. 1a) with reduced fractional shortening (−31.5 %; Fig. 1a). Both the chamber remodeling and the reduced FS were inhibited by treatment with VS-1 (Fig. 1a). VS-1 blocked 8.5 % of the increase in the HW/BW ratio that was observed in the ISO-infused rats (Fig. 1a). Chronic β-ARs activation also induced myocardial fibrosis and myocyte hypertrophy, and both were suppressed by VS-1 (by 46 % and 28 %, respectively; Fig. 1c).

Figure 1b shows the results of our in vivo tests of cardiac function. Rats with sustained β-AR activation showed reduced contractility (dP/dt<sub>max</sub>) and relaxation (dP/dt<sub>min</sub>). Treatment with VS-1 increased contractility by 26 % and relaxation by 27 % (Fig. 1b).



**Fig. 1** Chronic VS-1 treatment suppresses progressive cardiac dilation, dysfunction, fibrosis and hypertrophy and improves cardiac systolic and diastolic function in hearts subjected to sustained  $\beta$ -ARs activation. **a** Representative echocardiographic data in CTL and ISO rats treated with or without VS-1; FS (%), LVEDD, LVESD, and PWT were analyzed using echocardiography, and HW/BW were measured in three groups (mean  $\pm$  S.E.M.;  $n = 7$ ). \*,  $P < 0.001$  versus the CTL group; #,  $P < 0.001$  versus the ISO group; and §,  $P < 0.01$  versus the ISO group. VS-1, vasostatin-1; AR, adrenergic receptor; CTL, control; ISO, isoprenaline; FS, fractional shortening; LVEDD, left ventricular end-diastole

diameter; LVESD, left ventricular end-systole diameter; PWT, posterior-wall thickness; and HW/BW, heart weight/body weight. **b** In vivo cardiac systole function (dP/dt<sub>max</sub>) and diastole function (dP/dt<sub>min</sub>). \*,  $P < 0.001$  versus the CTL group; and #,  $P < 0.001$  versus the ISO group. dP/dt<sub>max</sub>, peak rate of pressure rise; and dP/dt<sub>min</sub>, peak rate of pressure decline. **c** HE- and Masson's-stained myocardium showing reduced interstitial fibrosis and myocyte hypertrophy following treatment with VS-1 ( $n = 5$  for each). \*,  $P < 0.001$  versus the CTL group; #,  $P < 0.001$  versus the ISO group. HE, hematoxylin and eosin

### Treatment with VS-1 Enhances eNOS-cGMP-PKG Pathway Activity and Blocks Fetal Gene Expression and PI3K/Akt Pathway Activity

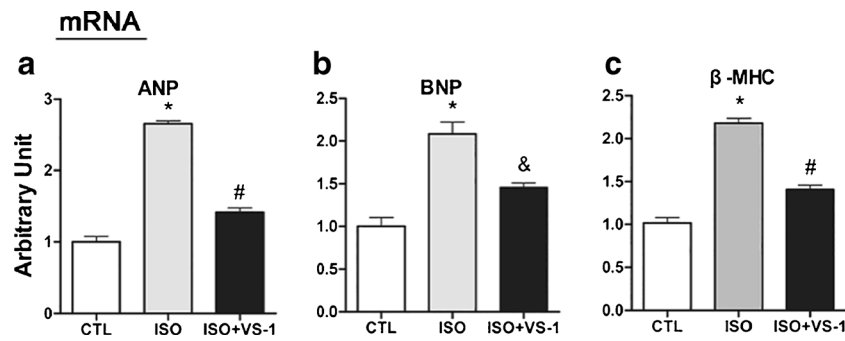
The expression and activity of eNOS were suppressed in rat hearts that were subjected to chronic  $\beta$ -ARs activation (Fig. 3a). The activity of PKG-1 $\alpha$ , a downstream effector of eNOS, was also inhibited by  $\beta$ -ARs activation (Fig. 3a). In hearts subjected to ISO infusion, treatment with VS-1 had no impact on the total expression level of eNOS ( $P = 0.717$ , Fig. 3a). However, VS-1 significantly increased the level of phosphorylated eNOS. In addition, chronic treatment with VS-1 restored a lower level of PKG-1 $\alpha$  protein expression (Fig. 3a). These data suggest that treatment with VS-1 enhances eNOS-PKG pathway activity and suppresses the ISO infusion-induced re-expression of fetal genes, including ANP, BNP and  $\beta$ -MHC (Fig. 2).

PI3K/Akt is a prominent signaling cascade that is stimulated by chronic  $\beta$ -AR activation. The hyperactivation of this pathway is associated with cardiac hypertrophy and

remodeling. ISO infusion increased PI3K/Akt activity, as shown by the increased ratio of phosphorylated to total PI3K/AKT protein levels (Fig. 3b). VS-1 suppressed this response to nearly baseline levels (Fig. 3b).

### VS-1 Improves $\text{Ca}^{2+}$ Release and SR $\text{Ca}^{2+}$ Content

To investigate the mechanism underlying  $\text{Ca}^{2+}$  handling, we isolated cardiomyocytes from animals in each of the three groups and measured epifluorescence (Fig. 4a). The cardiomyocytes were stimulated at 0.5, 1, 2 and 3 HZ, and systolic  $\text{Ca}^{2+}$  transients were recorded (Fig. 4b). When cells were stimulated at 0.5 HZ, the amplitudes of the systolic  $\text{Ca}^{2+}$  transients were 42 % lower in the ISO group than in the CTL group (47 % lower at 1 HZ, 49 % lower at 2 HZ and 49 % lower at 3 HZ; Fig. 3b). In addition, in response to 0.5 HZ stimulation,  $\text{Ca}^{2+}$  transient half-time decay was 32 % higher in the ISO group (Fig. 4c). Treatment with VS-1 alleviated the depressions in the amplitudes of systolic  $\text{Ca}^{2+}$  transients that were caused by



**Fig. 2** Treatment with VS-1 prevents fetal gene re-expression. The expression levels of fetal ANP (a), BNP (b), and β-MHC (c) mRNAs were normalized to the level of GAPDH mRNA expression in whole heart isolates (n = 5 for each group). \*, P < 0.001 versus the control group;

#, P < 0.001 versus the ISO group; and &, P < 0.01 versus the ISO group. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; MHC, myosin heavy chain

stimulation with β-ARs (+30 % at 0.5 HZ, +33 % at 1 HZ, +40 % at 2 HZ, and +45 % at 3 HZ; Fig. 4c) and decreased the prolonged half-time decays of Ca<sup>2+</sup> transients by 15 % when cells were stimulated at 0.5 HZ (Fig. 4c).

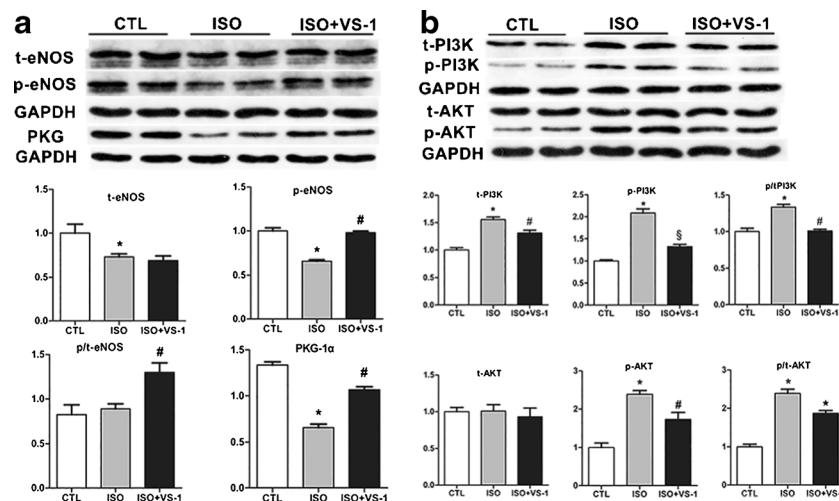
Caffeine was applied to quantify SR Ca<sup>2+</sup> content (Fig. 4d). We detected lower SR Ca<sup>2+</sup> content in the ISO group, and 57 % lower caffeine-induced Ca<sup>2+</sup> transients were detected in the ISO group than in the CTL group (Fig. 4e). In addition, the SR Ca<sup>2+</sup> release fraction was also reduced by 24 % in the ISO group (Fig. 4e). Treatment with VS-1 resulted in 50 % higher SR Ca<sup>2+</sup> content and increased the release fraction by 23 % (Fig. 4e).

**VS-1 Reduces CaMK-II Pathway Activity and Restores Ca<sup>2+</sup>-Handling Proteins**

As [Ca<sup>2+</sup>]<sub>i</sub> was elevated, CaMK-II, a factor that plays a vital role in cardiac remodeling, was chronically

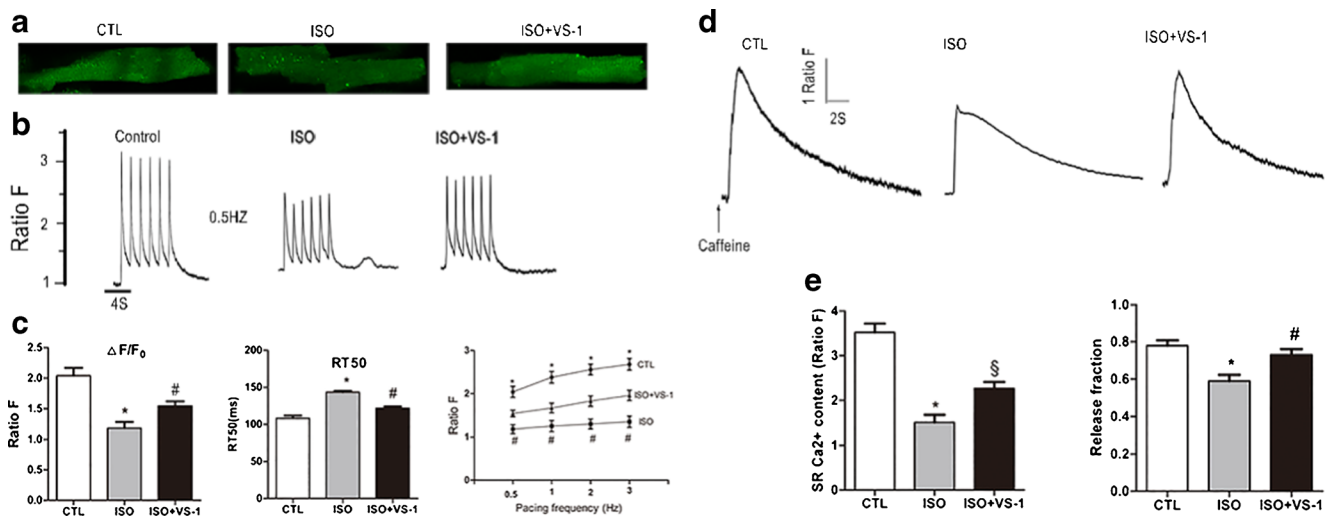
activated. We detected the expression levels and activity of CaMK-II in the present study. We found that both were increased in rat hearts that were subjected to β-ARs activation (Fig. 5a). In the ISO group, this enhanced level of activity in the CaMK-II pathway was suppressed by treatment with VS-1 (Fig. 5a).

Because Ca<sup>2+</sup> cycling was improved in VS-1 treated hearts, we examined the effects on sarcoplasmic reticulum handling proteins that could have been modified by the therapy. SERCA2a expression was 45 % lower after 3 weeks of ISO infusion (Fig. 5b). Levels of PLB, which combines with SERCA2a to induce an inhibitory effect on SERCA2a, were 107 % higher in the ISO group than in the CTL group (Fig. 5b). In addition, treatment with VS-1 decreased t-PLB and increased p-PLB expression, resulting in a higher p/t:PLB ratio (which reflects Ca<sup>2+</sup> uptake) than was observed in the placebo-treated ISO group (Fig. 5b).



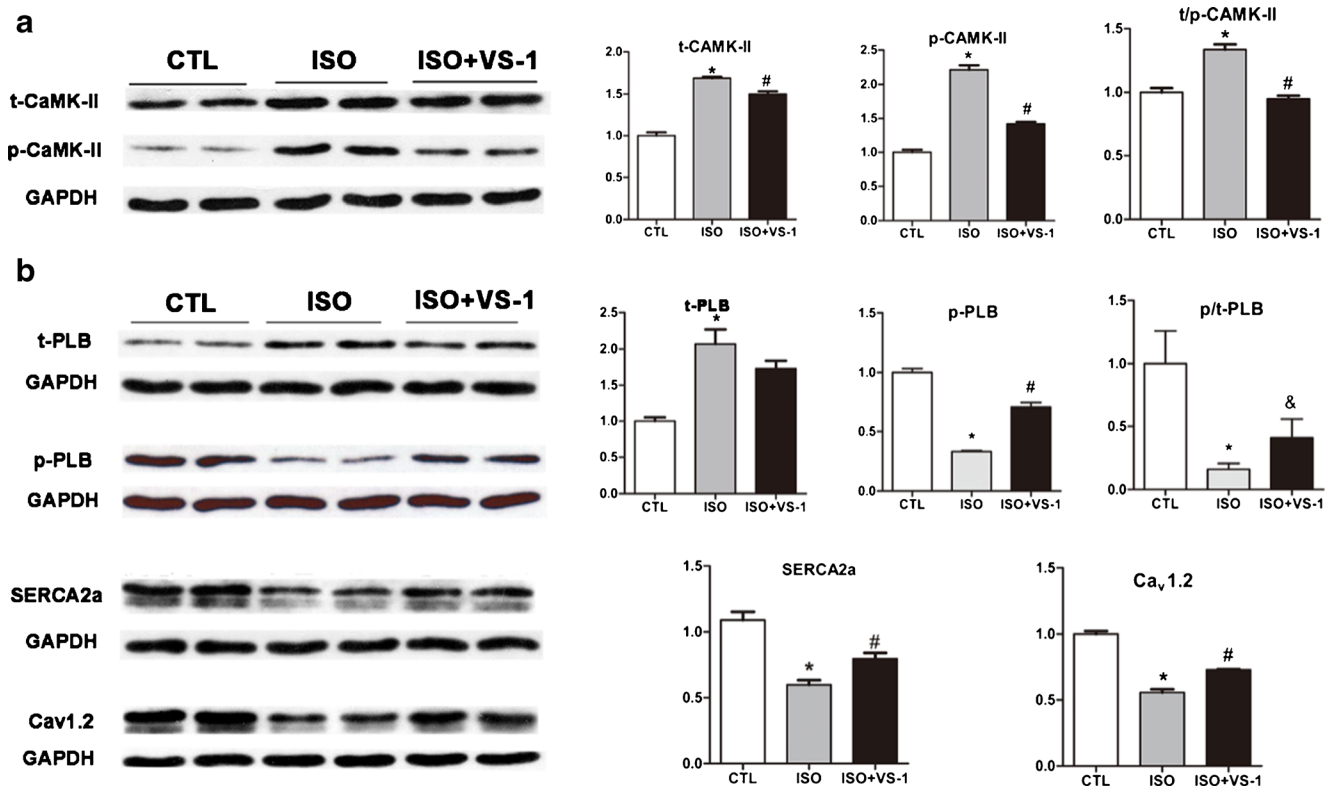
**Fig. 3** Chronic VS-1 treatment enhances the activation of the eNOS-PKG pathway and prevents the expression of the hypertrophy signaling pathway. **a** Western blot analysis of eNOS and PKG-1α protein expression. The expression levels were normalized to GAPDH expression in whole heart isolates (n = 5 for each). \*, P < 0.05 versus the control group; #, P < 0.05 versus the ISO group; eNOS, endothelial nitric oxide synthase;

and PKG, protein kinase G. **b** Western blot analysis of PI3K/Akt protein expression (n = 5 for each). The PI3K/Akt summary results are shown as the ratio of phosphorylated (p) to total (t) protein. \*, P < 0.01 versus the control group; §, P < 0.001 versus the ISO group; #, P < 0.01 versus the ISO group; and ★, P < 0.05 versus the ISO group. PI3K, phosphatidylinositol 3-kinase



stimulation. Statistics of  $\text{Ca}^{2+}$  transient amplitude stimulation at 0.5, 1, 2 and 3 HZ ( $n \geq 23$  for each). \*,  $P < 0.001$  versus the control group; and #,  $P < 0.001$  versus the ISO group. **d** Example traces of  $\text{Ca}^{2+}$  transients induced in response to the application of 10 mM caffeine. **e** Summary data for SR  $\text{Ca}^{2+}$  contents and release fractions ( $n \geq 23$  for each group). \*,  $P < 0.001$  versus the control; §,  $P < 0.001$  versus the ISO group; and #  $P < 0.01$  versus the ISO group. SR, sarcoplasmic-endoplasmic reticulum

stimulation. Statistics of  $\text{Ca}^{2+}$  transient amplitude stimulation at 0.5, 1, 2 and 3 HZ ( $n \geq 23$  for each). \*,  $P < 0.001$  versus the control group; and #,  $P < 0.001$  versus the ISO group. **d** Example traces of  $\text{Ca}^{2+}$  transients induced in response to the application of 10 mM caffeine. **e** Summary data for SR  $\text{Ca}^{2+}$  contents and release fractions ( $n \geq 23$  for each group). \*,  $P < 0.001$  versus the control; §,  $P < 0.001$  versus the ISO group; and #  $P < 0.01$  versus the ISO group. SR, sarcoplasmic-endoplasmic reticulum



$\text{Ca}_v1.2$  protein expression (western blot, left and summary data, right;  $n = 5$  for each). \*,  $P < 0.001$  versus the control group; #,  $P < 0.001$  versus the ISO group; §,  $P < 0.01$  versus the ISO group; SERCA2a, sarcoplasmic-endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase; and PLB, phospholamban

$\text{Ca}_v1.2$  protein expression (western blot, left and summary data, right;  $n = 5$  for each). \*,  $P < 0.001$  versus the control group; #,  $P < 0.001$  versus the ISO group; §,  $P < 0.01$  versus the ISO group; SERCA2a, sarcoplasmic-endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase; and PLB, phospholamban

## VS-1 Increases $I_{Ca,L}$ and the Expression of $Ca_v1.2$

Figure 6a shows voltage-dependent  $I_{Ca,L}$  traces that were recorded in the CTL and ISO rats with or without VS-1 treatment. Peak  $I_{Ca,L}$  amplitudes were normalized by cell membrane capacitance and expressed as the peak  $I_{Ca,L}$  densities. Chronic  $\beta$ -ARs activation resulted in 41 % lower peak  $I_{Ca,L}$  densities (Fig. 6b) and 44 % lower Cav1.2 expression (Fig. 5b) than were observed in the CTL group. Treatment with VS-1 increased peak  $I_{Ca,L}$  densities by 1.3-fold (Fig. 6b) and Cav1.2 expression by 1.3-fold (Fig. 5b) over the results observed in the placebo-treated group.

## Discussion

Chronically elevated CA levels result in the activation of cardiac  $\beta$ -ARs, which may play a major role in the pathogenesis of heart failure. Impaired myocardial structure and contractility have been observed in animals in which  $\beta$ -ARs are overactivated [19, 20]. Previous studies have suggested that VS-1 exerts an anti-adrenergic effect in isolated and perfused hearts. In the present study, we first showed that VS-1 played a protective role in rat hearts that were subjected to chronic  $\beta$ -ARs activation. Briefly, VS-1 suppressed chamber dilation, myocyte hypertrophy and fibrosis and improved in vivo heart functions in rats that were subjected to ISO infusion. Treatment with VS-1 increased phosphorylated nitric oxide synthase levels and activated protein kinase G. VS-1 also deactivated multiple hypertrophy signaling pathways that are normally triggered by chronic  $\beta$ -ARs activation, such as the PI3K/Akt [21] and CaMK-II pathways [22].  $Ca^{2+}$  handling and its molecular modulation were also improved by treatment with VS-1.

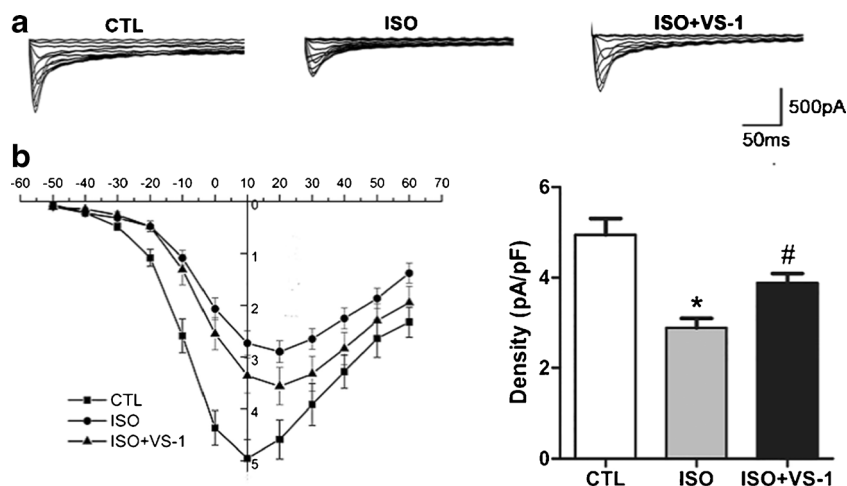
The heart is viewed as an endocrine organ because a growing number of studies have demonstrated that cardiac cells possess the ability to synthesize and release many hormones,

such as CAs [23] and natriuretic peptides (NPs) [24], which can function to regulate cardiovascular homeostasis under both normal and disease conditions [25]. The identification of Chromogranin-A (CgA) [26] and its derivative peptides [13] in the heart contribute to the notion that the heart is an endocrine organ. Among these peptides, VS-1 is one of the most well-studied, and it has been shown to be a novel cardiac modulator and stabilizer of adrenergic tone in cardio-circulatory homeostasis. It is worth noting that acute administration of VS-1 has been shown to have cardio-inhibitory effects under  $\beta$ -ARs stimulation conditions in previous studies. However, in the present study, chronic administration of VS-1 improved functions in hearts that were subjected to chronic  $\beta$ -ARs stimulation. The apparent paradox between the negative effects of acute exposure and positive effects of chronic exposure on contractility responses has some similarities with the effects of  $\beta$ -blockers. Acute administration of  $\beta$ -blockers is negatively inotropic, whereas chronic blockade in a stressed heart enhances contraction, likely by suppressing sustained catecholamine cytotoxicity [27].

## Anti-Hypertrophy Is Effected via the eNOS-NO- PKG Pathway

Previous studies have shown that VS-1 exerts cardio-inhibitory effects via the eNOS-NO-PKG pathway under both basal and  $\beta$ -ARs stimulation conditions [9–11]. Similar to the results of previous studies, we found that VS-1 increased the levels of phosphorylated nitric oxide synthase and induced the activation of protein kinase G. Evidence suggests that eNOS-derived NO acts by elevating PKG and thereby exerts beneficial effects on cardiac remodeling. For example, mice that globally lack eNOS (eNOS<sup>-/-</sup>) develop greater cardiac hypertrophy and fibrosis and show more deterioration in cardiac functions following thoracic aorta coarctation (TAC) [28, 29]. Conversely, mice with cardiac-specific over-expression of eNOS showed improved left ventricular performance and

**Fig. 6** Chronic VS-1 treatment prevents the  $\beta$ -ARs activation-induced suppression of  $I_{Ca,L}$  densities. **a** Examples of  $I_{Ca,L}$  recorded in the control, ISO and ISO + VS-1 groups. **b** Current-voltage (I-V) correlations for  $I_{Ca,L}$  and quantitative analysis of peak  $I_{Ca,L}$  densities in the three groups ( $n = 8$  each). \*,  $P < 0.001$  versus the control group; and #,  $P < 0.05$  versus the ISO group



reduced compensatory hypertrophy after myocardial infarction [30]. Thus, we hypothesized that the beneficial effects of VS-1 on cardiac remodeling are mediated by the enhancement of the eNOS-NO-PKG pathway.

Because no classical surface receptors of VS-1 have been documented, the cellular processes that lie upstream of the activation of eNOS by this peptide are not fully understood. Previous studies have shown that in endothelial cells, VS-1 acts as a cell-penetrating peptide by utilizing its cationic and amphipathic properties, binds to heparin sulfate proteoglycans (HSPGs) [31], and then activates eNOS phosphorylation (Ser1179) through a PI3K/Akt-dependent, endocytosis-coupled mechanism [10, 32].

In the present study, VS-1 suppressed the expression and activity of PI3K/Akt. These results appear to contradict the results of previous studies [8, 29], which have suggested that VS-1 does not act directly on cardiomyocytes but rather on endothelial cells. The increased expression and activity of PI3K/Akt reflected the acute effect of VS-1 on endothelial cells [10, 31, 32] that has been observed in previous studies. However, in our study, the samples contained various cell types but were mainly composed of myocardial cells. Consequently, the depressed expression and activity of PI3K/Akt that were observed in the present study reflected a chronic and indirect effect of VS-1 on myocardium and not its effect on endothelial cells. In addition, a growing amount of evidence suggests that sustained activation of the PI3K/Akt pathway is associated with the development of pathological cardiac hypertrophy [33–35]. Furthermore, chronic activation of the PI3K/Akt pathway is associated with TAC-inducing pressure-overload and was prevented by the phosphodiesterase-5 A (PDE5) inhibitor sildenafil, which increased cGMP synthesis [17, 36]. Consequently, we hypothesized that VS-1 suppressed the activation of PI3K/Akt that was stimulated by chronic ISO infusion, possibly by enhancing the eNOS-cGMP-PKG pathway and thereby exerting protective effects on the myocardium.

### VS-1 Improves $\text{Ca}^{2+}$ Handling and its Molecular Regulation

Calcium cycling in cardiomyocytes is crucial to both cardiac contraction and relaxation. Defective intracellular  $\text{Ca}^{2+}$  homeostasis is a central cause of contractile dysfunction and arrhythmias in failing myocardium and can result from pathological alterations in the expression and activity of structural proteins, ion channels, and enzymes. A growing amount of evidence suggests that the reduced expression and activity of SERCA2a and an increase in the inhibitory activity of PLB impairs uptake by the sarcoplasmic-endoplasmic reticulum (SR) in both animal models and human heart failure [37–39]. Defects in cytosolic  $\text{Ca}^{2+}$  removal result in increased diastolic  $[\text{Ca}]^{2+}_i$  levels, and reduced SR  $\text{Ca}^{2+}$  content and systolic  $[\text{Ca}]^{2+}_i$  transients. In addition, elevated diastolic  $[\text{Ca}]^{2+}_i$

causes a sustained activation of CaMK-II, which in turn causes additional damages to calcium cycling. In the present study, we found that chronic administration of VS-1 improved  $\text{Ca}^{2+}$  pump activity after ISO infusion by repairing SERCA2a expression and alleviating the increased inhibitory activity of PLB. Furthermore, the depressed systolic  $[\text{Ca}]^{2+}_i$  transients and reduced SR  $\text{Ca}^{2+}$  content that were caused by ISO infusion were also significantly improved by treatment with VS-1. Finally, we found that VS-1 prevented the reduction of  $I_{\text{Ca,L}}$  that was caused by ISO administration, which might improve  $\text{Ca}^{2+}$  transients in cardiomyocytes.

### cGMP-PKG-Dependent Modulation of the CaMK-II Pathway

Consistent with the results of previous studies [40], in the present study, we found that both the activity and expression of CaMK-II were increased by chronic stimulation with  $\beta$ -ARs. A growing amount of evidence suggests that CaMK-II regulates not only immediate cellular functions but also chronic processes, such as ventricular remodeling, that can lead to heart failure [41–43]. As shown in our study, the activity and expression of CaMK-II that were induced by chronic  $\beta$ -ARs stimulation were substantially suppressed by treatment with VS-1.

The inhibitory role of VS-1 on CaMK-II might be mediated by the enhancement of the eNOS-cGMP-PKG pathway. NO/cGMP have a substantial impact on intracellular calcium homeostasis. On the one hand, NO/cGMP strongly inhibit L-type  $\text{Ca}^{2+}$  channels [44, 45], which alleviates the intracellular calcium overload that is caused by the chronic infusion of ISO. On the other hand, evidence suggests that the cGMP-dependent pathway has an inhibitory effect on  $\text{Na}^+/\text{H}^+$  exchange (NHE) [46–48]. NHE activity can, when  $[\text{Na}^+]_i$  is elevated, operate in reverse to extrude  $\text{Na}^+$  in exchange for the inflow of  $\text{Ca}^{2+}$ , thereby leading to elevated  $[\text{Ca}^{2+}]_i$ . VS-1 increased the activity of the cGMP pathway and deactivated the CaMK-II pathway by decreasing the level of  $[\text{Ca}^{2+}]_i$ . Previous studies have come to similar conclusions. For example, sildenafil, which inhibits the cGMP-specific phosphodiesterase 5 A to increase  $[\text{cGMP}]_i$ , inhibited the pressure overload-induced activity and expression of CaMK-II [49]. Further evidence supporting the notion that cGMP signals attenuate CaMK-II activation comes from  $\text{pGC}^{-/-}$  mice, in which the observed hypertrophic phenotype was associated with the upregulation of CaMK-II expression, the increased autophosphorylation of CaMK-II and the phosphorylation of downstream targets of CaMK-II [50, 51].

### Clinical Implications

Heart failure is a global problem with an estimated prevalence of 38 million patients. This number is increasing with the aging of the population. Despite the fact



that some progress has been made in this field, the prognosis for heart failure is worse than that for most cancers. A growing amount of evidence suggests that chronic neurohumoral activation plays a vital role in the progression of heart failure. Strategies aimed at blocking neurohumoral activation exert cardio-protective effects to improve cardiac function. Previous studies have shown that VS-1 exerts anti-adrenergic effects in isolated and perfused hearts. In the present study, we showed that chronic administration of VS-1 stopped chamber, cellular, and molecular remodeling and improved calcium handling in rat hearts that were subjected to chronic  $\beta$ -ARs activation. These studies collectively suggest that VS-1 has the potential to become a new therapeutic drug for heart failure.

## Conclusion

Chronic VS-1 treatment suppressed chamber dilation, myocyte hypertrophy and fibrosis and improved in vivo heart function in rats that were subjected to ISO infusion.  $\text{Ca}^{2+}$  cycling and its molecular modulation were also improved by VS-1. The beneficial effects of VS-1 on cardiac remodeling are dependent on the eNOS-cGMP-PKG pathway.

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## Compliance with Ethical Standards

**Disclosures** None

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