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Chronic Social Stress Induces Cardiomyocyte Contractile Dysfunction and Intracellular Ca²⁺ Derangement in Rats

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Abstract

Chronic psychosocial stress triggers cardiovascular diseases although underlying mechanisms are still elusive. This study examined the effect of social stress on cardiomyocyte contractile function and pathological changes in myocardium using the visible burrow system (VBS) model. Chronic social stress was induced using a mixed-sex VBS housing in adult Sprague-Dawley (SD) rats. Contractile and intracellular Ca²⁺ properties were evaluated in isolated cardiomyocytes including peak shortening (PS), time-to-PS (TPS), time-to-90% relengthening (TR₉₀), maximal velocity of shortening/relengthening ($\pm$ dL/dt), Fura-2 fluorescence intensity, and intracellular Ca²⁺ decay. Myocardial histology was evaluated using Masson trichrome staining. Social stress led to depressed PS, $\pm$ dL/dt, shortened TPS and prolonged TR₉₀ compared with the unstressed controls. Baseline and electrically-stimulated rise in Ca²⁺ were reduced whereas intracellular Ca²⁺ decay was delayed in stressed rats. Histological analyses exhibited overt interstitial fibrosis and cardiomyocyte hypertrophy in stressed rats. The GSH/GSSG ratio (indicative of oxidative stress status) was reduced whereas oxidative protein carbonyl formation was elevated in stressed rats. Western blot analysis showed unchanged expression of superoxide dismutase 1 (SOD1), β_1 -adrenoceptor (β_1 -AR) levels, reduced sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA2a) levels, and elevated phosphorylation of the stress signaling protein kinase JNK but not ERK in myocardium from stressed rats. Short-term *in vitro* treatment of cardiomyocytes with the stress inducer phenylephrine mimicked cell damage and intracellular Ca²⁺ mishandling, the effects of which were mitigated by antioxidant, JNK inhibition, carvedilol and SERCA2a adenovirus. These findings indicate that chronic social stress is detrimental to cardiac structure and function possibly via mechanisms associated with oxidative injury and intracellular Ca²⁺ mishandling.

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Keywords

social stress; cardiomyocytes; contraction; oxidative stress; intracellular Ca^{2+}

1. INTRODUCTION

Chronic social stress has been considered as a common attributing factor for cardiovascular diseases [1]. Ample of evidence has demonstrated that psychosocial stress triggers a wide array of cardiovascular pathologies including cardiac hypertrophy [2], hypertension [3,4], arrhythmia [5], ischemia injury and myocardial infarction [6,7]. In addition, a correlation has been found between social stress and prevalence of obesity [8,9], which further aggravates the undesirable outcome of psychosocial stress on cardiovascular diseases [10]. Up-to-date, the mechanisms of action behind chronic social stress-induced cardiovascular events are not well described although a number of scenarios have been somewhat generally accepted. In particular, psychosocial stress triggers hyper-activation of the hypothalamic-pituitary-adrenal (HPA) axis and dysregulation of autonomic nervous system, leading to an elevated sympathetic tone and high circulating levels of catecholamine [11,12]. This is supported by the fact that β -adrenergic blockers may ameliorate endothelial injury initiated by psychosocial stress [13], favoring the essential role of sympathetic tone in stress-related cardiovascular diseases. On the other hand, oxidative stress is considered to play a pivotal role in social stress-induced oxidative lipid, protein and DNA damages [14,15]. Although there is a wealth of information on the systemic and behavioral effects of social stress, the impact of social stress on cardiac function is not well understood.

Given the apparent limitations in assessing health effects of social stress in human subjects, animal models of social stress have become available to mimic the psychosocial stress condition such as the visual burrow system (VBS). In the VBS system, mixed-sex housing of rats results in formation of dominance hierarchies where subordinate males (stressed) can be identified from dominants according to their agonistic and non-agonistic behaviors, wound patterns, and weight changes [16]. Sustained exposure to such stressful social defeat conditions in the subordinate rats has been shown to closely resemble the loss of social control in humans and therefore prompts development of stress-induced pathological alterations [17]. To better understand the influence of social stress on cardiac structure and contractile function, this study examined myocardial histology, cardiomyocyte contractile and intracellular Ca^{2+} properties in socially stressed rats using the VBS resident-intruder paradigm. To explore the possible mechanism(s) of action, the intracellular Ca^{2+} regulatory protein sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA2a), protein carbonyl formation, oxidative stress status using reduced and oxidized glutathione levels, superoxide dismutase 1 (SOD1) and β_1 -adrenoceptor (β_1 -AR) expression were evaluated in stressed and non-stressed rat hearts. Activation of essential stress signaling cascades including extracellular regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) was also monitored to assess the potential contribution of these signaling molecules to cardiac perturbations in response to chronic social stress, if any. Short-term *in vitro* culture model of high levels of phenylephrine, an α -adrenergic agonist mimicking stress [15,18,19], was also used to examine the possible effect of antioxidant, α -/ β -adrenergic blockade, JNK inhibition and SERCA2a adenovirus transfection in stress-induced intracellular Ca^{2+} dysregulation.

2. MATERIALS AND METHODS

2.1. Induction of social stress

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Wyoming (Laramie, WY). In brief, adult male Sprague-

Dawley (SD) rats (4–6 months of age) were housed in standard plastic cages containing two animals of approximately the same age. The light–dark cycle was reversed (light from 21:00–9:00 h) and water and food were provided *ad libitum*. Castrations were performed in all rats under ketamine (75 mg/kg)-xylazine (5 mg/kg) anesthesia. At the time of castration, rats received implants of two silastic tubes filled to a length of 30 mm with testosterone. All implants were placed into individual subcutaneous pouches on the back at the level of the scapula. Following surgery, rats were returned to the group cages with the same partner they had been cohabiting with since weaning and were left for 3 additional weeks. This protocol has been demonstrated to result in elevated levels of serum testosterone and aggressive behavior comparable to that in intact male rats [20]. Following recovery, rats were housed in a VBS with a total of four testosterone-treated males and four intact female rats. The VBS was consisted of a large open space (62 cm×62 cm×45 cm) interconnected via 9.5 cm high tunnels to two smaller darkened chambers (25 cm×21 cm×18.5 cm and 17.5 cm×14.5 cm×18.5 cm). Food and water were available at all times, albeit only in the open chamber. Studies of gonadally-intact males indicate that inter-male aggression is initially high under these housing conditions, although it subsides as dominance hierarchies are formed [21]. In order to keep the level of inter-male aggression high, the formation of hierarchies was disrupted by changing the composition of males at least three times per week. Rats were classified as dominants or subordinates (stressed) based on aggressive behaviors. The stressed animals were housed in VBS for 4 weeks prior to experimentation. Control animals were handled similar to the animals in the social stress group with the exception of undisturbed dwelling in their home cage.

2.2. Isolation and short-term culture of adult rat cardiomyocytes

Rats were decapitated and hearts were immediately removed. Hearts were perfused (at 37°C) with Krebs–Henseleit bicarbonate buffer (mM: 118 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 10 N-[2-hydro-ethyl]-piperazine-N'-[2-ethanesulfonic acid] (HEPES), 11.1 glucose, pH 7.4). The heart was then perfused for 20 min with Krebs–Henseleit bicarbonate containing 223 U/ml collagenase II (Worthington Biochemical, Freehold, NJ) and 0.5 mg/ml hyaluronidase. After perfusion, the left ventricle was removed and minced. Cells were further digested with 0.02 mg/ml trypsin before being filtered through a nylon mesh (300 µm). Extracellular Ca²⁺ was added incrementally back to 1.25 mM. Isolated cardiomyocytes were maintained in serum-free medium for up to 12 hrs after isolation [22]. The myocyte yield was ~90%, which was comparable between the two groups. Isolated cardiomyocytes were then cultured in a culture medium M199 supplemented with or without the α -adrenergic agonist phenylephrine (20 µM) [23] in the absence or presence of the antioxidant N-acetylcysteine (NAC, 500 µM) [24] the mixed α/β adrenergic antagonist carvedilol (100 nM) [25], the β -adrenergic antagonist propranolol (1 µM) [26], or the specific peptide inhibitor for JNK (JNKI, 2 µM) [27]. A cohort of cardiomyocytes was transfected with the SERCA2a adenovirus or the viral vector encoding the marker gene β -galactosidase (β -GAL) at the viral concentration of 10⁸ pfu/ml for 36 hrs [28,29] where exposure of phenylephrine was initiated after 18 hrs. The SERCA inhibitor thapsigargin (5 µM) [30] was used as a positive control. All cardiomyocytes were maintained for 36 hrs before intracellular Ca²⁺ homeostasis was assessed.

2.3. Cell shortening/relengthening

Mechanical properties of cardiomyocytes were assessed using an IonOptix MyoCam system (IonOptix Corporation, Milton, MA) [22]. In brief, cells were superfused with a contractile buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES at 37°C with a pH of 7.4. The extracellular Ca²⁺ concentration was set at 1.0 mM unless otherwise stated. The cells were field stimulated at 0.5 Hz. The myocyte was displayed on a computer monitor using an IonOptix MyoCam camera, which rapidly scans the image area

every 8.3 ms such that the amplitude and velocity of shortening/relengthening were recorded with good fidelity. Only rod-shaped myocytes with clear edges without spontaneous contraction were selected for study. Cell shortening and relengthening were assessed at 37°C using the following indices: peak shortening (PS), the amplitude myocytes shortened upon electrical stimulation, an indicative of peak ventricular contractility; time-to-PS (TPS), the duration of myocyte shortening, an indicative of systolic duration; time-to-90% relengthening (TR₉₀), the duration to reach 90% relengthening (time between 100% and 10% of the peak height), an indicative of diastolic duration (90% rather 100% relengthening was used to avoid noisy signal at baseline level); and maximal velocities of shortening/relengthening, representing maximal rates of departure/return phase of isotonic contraction.

2.4. Measurement of intracellular Ca²⁺ transients

Cardiomyocytes were loaded with Fura-2/AM (0.5 µM) for 10 min at 30°C and intracellular Ca²⁺ transients were recorded with a dual-excitation fluorescence photomultiplier system (Ionoptix) [22]. Myocytes imaged through an Olympus IX-70 Fluor 40× oil objective were exposed to light emitted by a 75W lamp and passed through either a 360 or a 380 nm filter (bandwidths were ± 15 nm) while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480–520 nm by a photomultiplier tube after first illuminating the cells at 360 nm for 0.5 s then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 nm excitation scan was repeated at the end of the protocol and qualitative changes in intracellular Ca²⁺ fura-2 fluorescence intensity (FFI) were inferred from the ratio of the fluorescence intensity at the two wavelengths. The following indexes were measured using Ionwizard software: resting fura-2 fluorescence intensity (FFI), rise of FFI (ΔFFI) in response to electrical stimuli and intracellular Ca²⁺ transient decay time constant, calculated using a single and bi-exponential equation.

2.5. Histological examination

Hearts were removed and cut into transverse slices prior to snap-freezing in isopentane precooled liquid nitrogen. Transverse sections of 10 µm in thickness were cut using a Leica cryostat and stained with Masson trichrome. An Olympus BX-51 microscope (Olympus America Inc., Melville, NY) coupled to a MagnaFire camera was used to visualize the sections. Masson trichrome-stained sections were used for fibrosis analysis and cardiomyocyte diameters using NIH Image J program. Cardiomyocyte diameter was measured using longitudinally-oriented cardiomyocytes at the nuclear level. The mean myocyte diameter was calculated from 100 cells from each group consisting of 3 rats [31,32]. For fibrosis, twelve random microscopic fields (400×) from each tissue section (three animals per group) were digitally captured under the fixed microscope illumination settings. Stained areas were quantified using the ImageJ software by calculating the total number of pixels of selected stained areas and the number of pixels of the whole image. Results were expressed as the average percentage of collagen staining per total area analyzed.

2.6. Glutathione and glutathione disulfide assay

Glutathione levels were determined and the ratio of glutathione (GSH): oxidized glutathione (GSSG) was used as an indicator of oxidative stress. In brief, hearts were homogenized in 4 volumes (w/v) of 1% picric acid. Acid homogenates were centrifuged at 16,000 g (30 min) and supernatant fractions collected. Supernatant fractions were assayed for total GSH and GSSG by the standard recycling method. The procedure consisted of using one-half of each sample for GSSG determination and the other half for GSH. Samples for GSSG determination were incubated at room temperature with 2 µl of 4-vinyl pyridine per 100 µl sample for 1 h after vigorous vortexing. Incubation with 4-vinyl pyridine conjugates any GSH present in the sample, so that only GSSG is recycled to GSH without interference by

GSH. The GSSG (as GSH $\times 2$) was subtracted from total GSH to determine the actual GSH level and the GSH: GSSG ratio [33].

2.7. Protein carbonyl assay

The carbonyl content of protein was determined as described [34]. Briefly, proteins were extracted and minced to prevent proteolytic degradation. Protein was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA) to protein (0.5 mg) and centrifuged at $11,000 \times g$ for 5 min at 4°C. The TCA solution was removed and the sample was resuspended in 10 mM 4-dinitrophenylhydrazine (2,4-DNPH) solution. Samples were incubated at room temperature for 15–30 min. Following addition of 500 μ l of 20% TCA, samples were centrifuged at $11,000 \times g$ for 3 min at room temperature. The pellet was washed in ethanol: ethyl acetate and allowed to incubate at room temperature for 10 min. Samples were centrifuged again at $11,000 \times g$ for 3 min at room temperature and the ethanol: ethyl acetate steps were repeated twice more. The precipitate was resuspended in 6 M Guanadine HCl solution and incubated at 37°C for 60 min to dissolve pellets before being centrifuged again at $11,000 \times g$ for 3 min at room temperature and insoluble debris removed. The maximum absorbance (360–390 nm) of the supernatant was read against appropriate blanks and the carbonyl content was calculated using the molar absorption coefficient of 22 000 L/mol per cm.

2.8. Western blot analysis

The protein was prepared as described [35]. Samples containing equal amount of proteins were separated on 10% or 15% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad) and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in TBS-T, and were incubated overnight at 4°C with anti-SOD1, anti-SERCA2a, anti- β 1 adrenergic receptor antibody (1:500), anti-c-Jun N-terminal kinase (JNK), anti-pJNK (Thr183/Tyr185), anti-extracellular signal-regulated kinase (ERK) 1/2, anti-pERK1/2 (Tyr204), and anti- α smooth muscle actin (1:1,200 as loading control) antibodies. After immunoblotting, the film was scanned and the intensity of immunoblot bands was detected with a Bio-Rad Calibrated Densitometer.

2.8. Adenoviral transfection of isolated cardiomyocytes

Our previous work showed that the efficiency of adenoviral gene transfer is significant (> 70%) at the viral concentrations of 10^8 pfu/ml or higher [28]. Construction of the replication-incompetent (E1-deleted) adenoviral vectors encoding SERCA2a or the marker gene β -GAL was described in detail previously [29]. Isolated cardiomyocytes were transduced with adenoviral vectors (SERCA2a or β -GAL, 10^8 pfu/ml) in minimal essential medium (MEM) for 36 hrs at 37°C. Non-transduced cells served as control. We tested the relationship between adenoviral incubation duration and SERCA2a gene transfer efficacy and found that 36 hrs of incubation time seemed to provide the optimal gene transfer efficiency without significantly affecting the myocyte mechanics. Our earlier study using the same experimental setting indicated that cardiomyocyte mechanical function remained stable between 12 and 48 hrs of incubation. However, it started to deteriorate rapidly after 48 hrs.

2.9. Statistical analysis

Mean $\pm$ SEM. Statistical significance ($p < 0.05$) for each variable was estimated by t-test or a one-way analysis of variance (ANOVA) where appropriate.

3. RESULTS

3.1. Body and heart weights following social stress

Body weights were not significantly different between stressed (435 ± 19 g, $n = 4$) and non-stressed (412 ± 17 g, $n = 4$) rats. Interestingly, the absolute heart weight was significantly heavier in the stressed rats (2.05 ± 0.15 g, $p < 0.05$ vs. control) than the control unstressed (1.48 ± 0.13 g) group. Similarly, heart size (normalized to body weight) was significantly greater in the stressed rats (4.69 ± 0.16 mg/g, $p < 0.05$ vs. control) than the control unstressed (3.58 ± 0.25 mg/g) group.

3.2. Social stress leads to compromised cardiomyocyte contractile and intracellular Ca^{2+} properties

Resting cell length was similar in both groups (data not shown). Stressed rats displayed significantly depressed PS, $\pm \text{dL}/\text{dt}$, shortened TPS and prolonged TR_{90} compared with those from the control rats (Fig. 1). To explore the possible mechanism of action behind compromised cardiomyocyte contractile function following social stress, intracellular Ca^{2+} handling was evaluated using the Fura-2 fluorescence microcopy. Resting intracellular Ca^{2+} levels were not significantly different between the two rat groups (data not shown). However, the electrically-stimulated rise in intracellular Ca^{2+} (ΔFFI) was significantly reduced in cardiomyocytes from the stressed rats compared with the control unstressed rats. Moreover, the intracellular Ca^{2+} decay (both single and bi-exponential) was overtly prolonged in cardiomyocytes from the stressed rats compared with those from the control unstressed group (Fig. 2).

3.3. Social stress promotes myocardial fibrosis and cardiomyocyte hypertrophy

Chronic psychosocial stress is known to trigger myocardial structural alterations including myocardial hypertrophy and interstitial fibrosis [36]. As shown in Fig. 3A (panel a–b), picrosirius red stained sections manifested an increase in perivascular fibrosis. Our further quantification the fibrotic area using the Masson trichrome stained sections revealed marked increase in interstitial fibrosis in myocardium from stress rats compared with that from control rats (Fig. 3A: panel c–d, Fig 3B). In line with the previous report [36], cardiomyocyte diameter was significantly greater in stressed rats compared with the control unstressed group, indicating the presence of overt cardiomyocyte hypertrophy (Fig. 3C).

3.4. Social stress results in reduced antioxidant defense and protein carbonyl formation

Although individual GSH and GSSG levels were not significantly different between the control and stressed rat myocardium, the GSH/GSSG ratio was significantly lower in stressed rat myocardium compared with the control group (Fig. 4A–C), indicating enhanced oxidative stress following social stress. Protein carbonyl formation, measured as an index for oxidative injury, was significantly elevated (~10-fold increase) in stressed rat hearts compared with the control group (Fig. 4D). Given that the loss of antioxidant capacity such as Cu Zn-superoxide dismutase (SOD1) may be responsible for the stress-associated adrenergic oxidative stress in the heart [37], myocardial expression of SOD1 was evaluated. Our data shown in Fig. 4E failed to note any difference in SOD1 levels between the two rat groups.

3.5. Effect of social stress on expression of β_1 -AR, SERCA2a and stress signaling molecules

As forced immobilization stress may be associated with a decrease in cardiac β_1 -adrenergic receptor (AR) levels [38], levels of myocardial β_1 -AR expression were examined. Our results did not reveal any difference in β_1 -AR expression between the stressed and control

groups. To assess the possible mechanism(s) of action behind the disrupted intracellular Ca^{2+} homeostasis in cardiomyocytes following social stress, expression of the essential intracellular Ca^{2+} handling protein SERCA2a was examined. Consistent with the dampened intracellular Ca^{2+} decay and prolonged TR_{90} , social stress significantly downregulated SERCA2a levels. Given that activation of stress-related mitogen-activated protein kinase (MAPK) signaling cascades has been reported following stress [39,40], total and phosphorylated protein levels of the key members of MAPK family including ERK and JNK were monitored. Our results denoted a significant increase in the phosphorylation of JNK but not ERK in myocardium from stressed rats compared with the non-stressed ones. The pan protein expression of JNK and ERK was comparable between the two groups (Fig. 5).

3.6. Effects of the antioxidant NAC, adrenergic inhibition on in vitro phenylephrine exposure-induced cell damage and intracellular Ca^{2+} derangement

To examine the effect of antioxidant and adrenergic inhibition in stress-induced cardiac mechanical derangement, isolated cardiomyocytes from control unstressed rats were exposed to the stress inducer phenylephrine (20 μM) [23] to mimic a stress environment in the absence or presence of the antioxidant NAC (500 μM) [24] the mixed α -/ β - adrenergic antagonist carvedilol (100 nM) [25], or the β -adrenergic antagonist propranolol (1 μM) [26]. Phenylephrine incubation significantly interrupted intracellular Ca^{2+} homeostasis as evidenced by decreased ΔFFI and peak FFI as well as prolonged intracellular Ca^{2+} decay (single exponential) associated with unchanged baseline FFI levels, reminiscent of the intracellular Ca^{2+} defect observed in socially stressed rat cardiomyocytes. Along the same line, phenylephrine treatment significantly promoted protein damage and oxidative stress as evidenced by enhanced protein carbonyl formation and reduced GSH/GSSG ratio. Interestingly, NAC and carvedilol (but not propranolol) attenuated or ablated phenylephrine-induced carbonyl formation, oxidative stress and intracellular Ca^{2+} derangement without eliciting any effects themselves (Fig. 6). These data favor a likely role for oxidative stress and α -adrenergic rather than β -adrenergic signaling in phenylephrine-induced cell damage and intracellular Ca^{2+} dysregulation.

3.7. Effects of SERCA2a transfection on phenylephrine-induced cell damage and intracellular Ca^{2+} derangement

To examine the role of SERCA2a in stress-induced cardiac anomalies, cardiomyocytes from control unstressed rats were transfected with adenoviral vectors encoding SERCA2a and β -GAL (as marker gene) prior to the exposure of the stress inducer phenylephrine (20 μM) [23] to mimic a stress environment. Cardiomyocytes transfected with SERCA2a (but not β -GAL) adenovirus displayed a three-fold expression of SERCA2a, validating the viral transfection efficacy. Phenylephrine significantly downregulated SERCA2a expression, the effect of which was not affected by the β -GAL viral transfection. Interestingly, SERCA2a viral transfection masked the phenylephrine-induced decrease in SERCA2a level. Our data further depicted that SERCA2a transfection mitigated phenylephrine-interrupted intracellular Ca^{2+} handling including decreased ΔFFI and peak FFI, prolonged intracellular Ca^{2+} decay and unchanged baseline FFI levels. Transfection of viral vector alone (β -GAL) did not affect the phenylephrine-induced intracellular Ca^{2+} anomalies. Interestingly, adenoviral transfection of SERCA2a and β -GAL failed to affect phenylephrine-induced oxidative stress (as evidence by persistent decrease in the GSH/GSSG ratio following viral transfection). Transfection of adenovirus encoding SERCA2a and β -GAL themselves did not elicit any overt effect on GSH/GSSH ratio and intracellular Ca^{2+} properties (Fig. 7). These data favor a likely beneficial role for SERCA2a, possibly downstream of oxidative stress accumulation, in the phenylephrine-induced intracellular Ca^{2+} dysregulation.

3.8. Effects of JNK inhibition on phenylephrine-induced intracellular Ca^{2+} derangement

To further examine if the stress signaling cascade JNK plays a role in intracellular Ca^{2+} mishandling under stress, Fura-2 fluorescence was evaluated in cardiomyocytes from control rats incubated with phenylephrine (20 μM) for 36 hrs with or without the peptide inhibitor of JNK (JNKI, 2 μM). The specific inhibitor for SERCA thapsigargin (5 μM) was used as a positive control. Our data shown in Fig. 8 revealed that JNKI effectively abrogated phenylephrine-induced intracellular Ca^{2+} mishandling including reduced ΔFFI and peak FFI, prolonged intracellular Ca^{2+} clearance associated with unchanged baseline FFI. JNKI itself did not elicit any notable intracellular Ca^{2+} response. To the contrary, JNKI failed to affect thapsigargin-elicited abnormalities in intracellular Ca^{2+} handling (reduced ΔFFI and peak FFI, prolonged intracellular Ca^{2+} clearance associated with unchanged baseline FFI). These data support a role for JNK, possibly upstream of SERCA, in phenylephrine-induced intracellular Ca^{2+} anomalies.

4. DISCUSSION

In this study, a mixed-sex housing VBS model was used to examine the impact of social stress on cardiomyocyte contractile function, intracellular Ca^{2+} homeostasis, oxidative damage and possible underlying mechanism(s) involved. The VBS model is an established protocol to produce social dominance hierarchies, resulting in behavioral and physiological consequences in rodents including changes in HPA-axis and sympathetic tone [16]. Our results revealed that chronic stressed (subordinate) rats displayed in depressed PS, $\pm \text{dL/dt}$, shortened TPS duration and prolonged TR_{90} , which were associated with a decrease in electrically-stimulated rise in Ca^{2+} levels (ΔFFI) as well as prolonged intracellular Ca^{2+} decay. Further investigation revealed that socially stressed rats displayed overt myocardial fibrosis, cardiomyocyte hypertrophy, oxidative stress (reduced GSH/GSSG ratio), protein oxidative damage and downregulated SERCA2a levels associated with unchanged levels of the antioxidant SOD1 and $\beta 1\text{-AR}$. These findings collectively prompt a possible role of oxidative damage and intracellular Ca^{2+} regulatory protein SERCA2a in the pathogenesis of social stress-induced change in cardiac structure and contractile function. These findings received support from our *in vitro* cell culture model where antioxidant, inhibition of stress signaling JNK and SERCA2a transfection reconciled phenylephrine-induced oxidative stress and intracellular Ca^{2+} anomalies.

Stress, a hallmark of the 21st century health problem, is associated with undesirable cardiovascular consequences [41]. Data from our study revealed decreased cardiomyocyte peak shortening, maximal velocity of shortening and relengthening, shortened TPS and prolonged TR_{90} following chronic social stress, depicting the onset of cardiac dysfunction following social stress. A number of scenarios may be speculated for social stress-induced cardiac defects. It has been suggested that compromised cardiac contractile function may be attributed to defective contractile and intracellular Ca^{2+} regulatory proteins [42], reduced intracellular Ca^{2+} availability due to depressed sarcoplasmic reticulum Ca^{2+} load [43], and altered myofilament Ca^{2+} sensitivity [44]. Measurement of intracellular Ca^{2+} revealed decreased intracellular Ca^{2+} rise (ΔFFI) and prolonged intracellular Ca^{2+} decay rate in cardiomyocytes from stressed rats, consistent with decreased PS and prolonged TR_{90} . In addition, the downregulated SERCA2a levels and overt myocardial fibrosis consolidate the presence of diastolic dysfunction following social stress. This is supported by the fact that SERCA2a viral transfection effectively ameliorated phenylephrine-induced cell damage and intracellular Ca^{2+} mishandling. In our study, the shortened systolic duration (or shortened TPS value) in the presence of a reduced contraction velocity ($+ \text{dL/dt}$), may be partially ascribed to the fact that cardiomyocytes from stressed rats exhibit a decreased fraction of shortening. In addition, chronic psychosocial stress may be associated with overt cardiac sympathetic activation [45] while the resultant increase in catecholamine levels may cause

catecholamine desensitization [46]. We thus speculated that β -adrenergic receptor downregulation may be a contributing factor in the cardiomyocyte mechanical dysfunction observed in our study. To our surprise, expression of myocardial β 1-AR was comparable between the stressed and non-stressed rats. The mixed α -/ β -adrenergic blocker carvedilol ablated the cell stressor phenylephrine-induced cell damage and intracellular Ca^{2+} defect. Although it is not surprising that the β -adrenergic blocker propranolol did not elicit any response to α -adrenergic agonist-induced cell injury, it is premature to exclude the possible contribution of β -adrenergic system in social stress-induced cardiac dysfunction at this time. It is possible that reduced β 1 receptor affinity to catecholamine may play a role in the impaired cardiac mechanical and intracellular Ca^{2+} homeostasis following stress [47]. Further studies are warranted to elucidate the possible involvement of β 1 receptor affinity in social stress-induced cardiac anomalies. In light of the fact that pathological change in myocardial structure may be one of the major determinants of cardiac performance [48], we examined pathological changes in myocardium following chronic social stress. Our data revealed increased heart weight and overt cardiomyocyte hypertrophy, in line with an earlier report [2]. Although arterial blood pressure was not monitored, chronic sympathetic dominance in the stressed rats may contribute to the myocardial structural changes through the regulation of cardiac afterload (i.e., blood pressure). Consistent with prior findings [49,36], interstitial fibrosis was markedly increased in the stressed rats compared with the non-stressed controls, favoring the onset of diastolic dysfunction.

Data from our study revealed overtly reduced GSH/GSSG ratio in stressed rats, suggesting presence of oxidative stress. This is consistent with the enhanced protein carbonyl formation in stressed rat hearts as well as the beneficial effect of the antioxidant NAC against phenylephrine-induced cell damage and intracellular Ca^{2+} derangement. Social stress is known to contribute to ROS production and lipid peroxidation leading to tissue injury [50]. Stress exposure (isolation, crowding and confrontation) may increase the levels of corticosterone and oxidized metabolites of bilirubin, favoring ROS production and oxidative stress. Cardiac protein carbonyl formation was found elevated in a rat model of immobilization stress [51]. Supplementation of glutathione, a critical antioxidant, was shown to alleviate oxidative stress induced by immobilization stress [52]. Our finding of unchanged SOD1 levels following social stress does not favor a major role of this antioxidant in the apparent oxidative stress following social stress. Activation of MAPK cascades has been found to accompany NF- κ B activation in monocytes [53] and hearts [54] following psychosocial stress. Our present finding of JNK activation along with the beneficial role of JNK inhibition against phenylephrine-induced intracellular Ca^{2+} mishandling indicates a likely role of JNK signaling in social stress-induced cardiac anomalies. It is not clear at this time with regards to the discrepancy in the phosphorylation of ERK and JNK following social stress. Epinephrine, a main catecholamine released in response to social stress, was shown to increase the phosphorylation of JNK but not ERK in skeletal muscles [55]. It is possible that the disparate responses in MAPK signaling may be underscored by stress-related hormones or neurotransmitters (such as epinephrine or phenylephrine). Although the precise mechanism of action behind social stress-induced cardiac anomalies is still elusive, it is plausible to speculate that nuclear as well as mitochondrial DNA damage triggered by oxidative stress may lead to altered protein expression and function of essential cardiac contractile and Ca^{2+} regulatory proteins, reduced availability of ATP, and ultimately resulting in apoptotic cell death. Apoptotic cell death is known to prompt cardiomyocyte hypertrophy to compensate cell loss, and changes in cardiomyocyte contractile properties during both shortening and relengthening [56].

Experimental limitations

There are a number of pitfalls for our current study. First, the use of phenylephrine as an *in vitro* stress model [15,23,18,19] cannot represent the changes of hormones and neurotransmitters following social stress. Thus, caution has to be taken when interpreting these *in vitro* findings to the setting of *in vivo* social stress. Nonetheless, this *in vitro* approach does provide some advantages such as viral transfer, drug specificity issue and toxicity which may not be overcome in the *in vivo* behavior study. Second, all rats were castrated and then supplemented with testosterone, which may affect protein expression and oxidative stress status. Aggressiveness has been found to be positively correlated with the testosterone levels although increased aggression is not necessarily correlated with dominance. Castration was performed to decrease aggressive behaviors, an effect that can be reversed by testosterone replacement [57,21].

In conclusion, our current study demonstrated, for the first time, onset of cardiomyocyte contractile and intracellular Ca^{2+} anomalies in a rodent model of social stress. Cardiomyocytes from the stressed rats exhibited compromised contractile and intracellular Ca^{2+} properties, associated with oxidative stress, reduced SERCA2a levels, cardiac hypertrophy and interstitial fibrosis. Observations from our *in vitro* model of cell stress using phenylephrine mimicking the elevated catecholamine following social stress [19,15,18] revealed beneficial effects of antioxidant, JNK inhibition and SERCA2a transfection against phenylephrine-induced oxidative stress and/or intracellular Ca^{2+} anomalies, support a possible role of JNK, oxidative stress and SERCA in social stress-induced cardiac dysfunction. However, the precise mechanism(s) responsible for social stress-induced oxidative stress and subsequently contractile protein damage and structural change are still elusive. Future work should focus on the elucidation of the cause-effect relationship between oxidative stress and changes of cardiac structural/functional proteins as well as the signaling mechanisms involved in social stress.

Research Highlights

- Social stress is a risk factor cardiovascular disease.
- Social stress led to cardiomyocyte contractile dysfunction, intracellular Ca^{2+} dysregulation and myocardial pathologies.
- These abnormalities are associated with increased oxidative stress and intracellular Ca^{2+} mishandling.

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Fig. 1.

Cardiomyocyte contractile properties in stressed and non-stressed rats. A: Representative cell shortening traces from control and stressed rats; B: Peak shortening (normalized to cell length); C: Maximal velocity of shortening ($+dL/dt$); D: Maximal velocity of relengthening ($-dL/dt$); E: Time-to-peak shortening (TPS); and F: Time-to-90% relengthening (TR_{90}). Mean $\pm$ SEM, $n = 90$ cells from 3 rats per group; * $p < 0.05$ vs. control group.

Fig. 2. Intracellular Ca^{2+} transient properties in cardiomyocytes from control (no stress) and stressed rats. A: Representative fura-2 traces in cardiomyocytes from control and stressed rats; B: Electrically-stimulated rise in FFI (ΔFFI); C: Single exponential intracellular Ca^{2+} decay rate; and D: Bi-exponential intracellular Ca^{2+} decay rate. Mean $\pm$ SEM, $n = 54-60$ cells from 3 rats per group, * $p < 0.05$ vs. control group.

Fig. 3. Histological characteristics in myocardium from control (no stress) and stressed rats. **A:** Representative micrographs stained with picosirius red (panel a–b) and Masson trichrome (panel c–d) depicting fibrosis and cardiomyocyte hypertrophy (c–d); **B:** Quantification of interstitial fibrotic area; and **C:** Mean cardiomyocyte diameter. Original magnification: 200×, scale bar = 100 μm; Mean ± SEM, n = 100 cells from 3 rats per group, * p < 0.05 vs. control group.

Fig. 4. Levels of GSH (A), GSSG (B), the GSH-to-GSSG ratio (C), protein carbonyl formation (D) and SOD1 protein expression (E) in myocardium from control (no stress) and stressed rats. Inset: Representative gel blots depicting expression of SOD1 and α -actin (used as loading control) using specific antibodies. Mean $\pm$ SEM, $n = 4-5$ rats/group, * $p < 0.05$ vs. control group.

Fig. 5. Western blot analysis of β_1 -AR, SERCA2a and MAPK signaling molecules (ERK and JNK) in myocardium from control (no stress) and stressed rats. A: Representative gel blots depicting expression of β_1 -AR, SERCA2a, pan and phosphorylated JNK and ERK as well as α -actin (used as loading control) using specific antibodies; B: β_1 -AR; C: SERCA2a; D: pERK/ERK ratio; and E: pJNK/JNK ratio. Mean $\pm$ SEM, n = 4–5 rats per group, * p < 0.05 vs. control group.

Fig. 6. Protein carbonyl content, GSH/GSSG ratio and intracellular Ca²⁺ handling in isolated cardiomyocytes from control rats maintained for 36 hrs in a culture medium M199 supplemented with the α -adrenergic agonist phenylephrine (20 μ M) in the absence or presence of the antioxidant NAC (500 μ M), the mixed α -/ β - adrenergic antagonist carvedilol (100 nM), or the β -adrenergic antagonist propanolol (1 μ M). A: Protein carbonyl content; B: GSH/GSSG ratio; C: Resting FFI; D: Peak FFI; E: Electrically-stimulated rise in FFI (Δ FFI); and F: Intracellular Ca²⁺ decay rate; Mean $\pm$ SEM, n = 4 isolations (panel A–B) or 30–36 cells from 3 rats (Panel C–F) per group, * p < 0.05 vs. control group, # p < 0.05 vs. phenylephrine group.

Fig. 7. SERCA2a protein expression, GSH/GSSG ratio and intracellular Ca^{2+} handling in isolated cardiomyocytes from control rats transfected with adenovirus vectors (10^8 pfu/ml) encoding SERCA2a or β -GAL (as marker gene) for 36 hrs in the absence or presence of the α -adrenergic agonist phenylephrine (20 μ M). A: SERCA2a expression; inset: Representative gel blots of SERCA2a and α -actin (loading control) using specific antibodies; B: GSH / GSSG ratio; C: Resting FFI; D: Peak FFI; E: Electrically-stimulated rise in FFI (Δ FFI); and F: Intracellular Ca^{2+} decay rate; Mean $\pm$ SEM, n = 5 isolations (panel A–B) or 30–36 cells from 3 rats (Panel C–F) per group, * p < 0.05 vs. control group, # p < 0.05 vs. phenylephrine group.

Fig. 8.

Effect of JNK inhibition on phenylephrine- and thapsigargin-induced intracellular Ca^{2+} responses in isolated cardiomyocytes from control rats. Cardiomyocytes were maintained in a culture medium (M199) for 36 hrs with the α -adrenergic agonist phenylephrine (20 μM) or the SERCA inhibitor thapsigargin (5 μM) in the absence or presence of the specific peptide inhibitor for JNK (JNKI, 2 μM). A: Resting FFI; B: Peak FFI; C: Electrically-stimulated rise in FFI (ΔFFI); and D): Intracellular Ca^{2+} decay rate (single exponential); Mean $\pm$ SEM, $n = 35\text{--}36$ cells from 3 rats per group, * $p < 0.05$ vs. control group, # $p < 0.05$ vs. phenylephrine group.