Molecular Mechanisms of Stress-Induced Myocardial Injury in a Rat Model Simulating Posttraumatic Stress Disorder

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ABSTRACT

Objective: Posttraumatic stress disorder (PTSD) is an independent risk factor for cardiovascular diseases. This study investigated the molecular mechanisms underlying myocardial injury induced by simulated PTSD.

Methods: Sprague-Dawley rats were randomly divided into two groups: control group (n=18) and PTSD group (n=30). The PTSD model was replicated using the single prolonged stress (SPS) method. On the 14th day poststress, the apoptotic cells in myocardium were assessed using both TUNEL method and transmission electron microscopy; the protein levels of the endoplasmic reticulum stress (ERS) molecules were measured by using Western blotting analysis.

Results: Exposure to SPS resulted in characteristic morphologic changes of apoptosis in cardiomyocytes assessed by transmission electron microscopy. Moreover, TUNEL staining was also indicative of the elevated apoptosis rate of cardiomyocytes from the SPS rats (30.69% versus 7.26%, p < .001). Simulated PTSD also induced ERS in myocardium, demonstrated by up-regulation of protein levels of glucose-regulated protein 78 (0.64 versus 0.26, p = .017), calreticulin (p = .040), and CCAAT/enhancer-binding protein-homologous protein (0.95 versus 0.43, p = .047), phosphorylation of protein kinase RNA–like ER kinase (p = .003), and caspase 12 activation (0.30 versus 0.06, p < .001) in myocardium from the SPS rats. The ratio of Bcl-2 to Bax decreased significantly in myocardium from the SPS rats (p = .005).

Conclusions: The ERS-related apoptosis mediated by the protein kinase RNA–like ER kinase/CCAAT/enhancer-binding protein-homologous protein and caspase 12 pathways may be associated with myocardial injury in a rat model simulating PTSD. This study may advance our understanding of how PTSD contributes to myocardial injury on a molecular level.

Key words: posttraumatic stress disorder, myocardial injury, endoplasmic reticulum stress, apoptosis.

INTRODUCTION

Posttraumatic stress disorder (PTSD) is a common condition that develops after a traumatic event (such as combat, natural disasters, or sexual assault) and is characterized by such symptoms as reexperiencing the traumatic event (e.g., intrusive thoughts or nightmares), avoidance of reminders of the event, negative alterations in cognition and mood, and alterations in arousal and reactivity, according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (1). In addition to the above behavioral and psychological symptoms, cardiovascular symptoms may also become prominent among patients with PTSD. For instance, young veterans suffering from combat-related PTSD exhibited higher blood pressures and heart rates compared with veterans without PTSD (2). They also observed that trauma-exposed patients without PTSD exhibited significantly higher blood pressure than did nonexposed patients. Blechert and colleagues (3)
observed that patients with PTSD exhibited lower respiratory sinus arrhythmia (a measure of cardiac vagal control), higher electrodermal activity (an assessment of sympathetic activity), elevated resting heart rates, and increased cardiovascular sympathetic activation. These results indicated that elevated sympathetic activity and profound cardiac vagal withdrawal are associated with PTSD. Another study indicated that attenuated parasympathetic control and elevated sympathetic control were both linked to an increased risk of sudden cardiac death (4). In addition, numerous studies found that there was a positive correlation between PTSD and coronary heart disease and mortality in veterans and other individuals with PTSD (5–9). PTSD has also been increasingly recognized as an independent risk factor for cardiovascular diseases (CVDs) (9–12). A recent study by Cho et al. (13) demonstrated that traumatic experiences resulted in acute myocardium injury in a mouse model simulating PTSD. Whether this stress exerts any long-term effects on the heart has not been determined. Based on this background, the present study examined the long-term effects of PTSD on heart tissue using a previously validated animal model.

One of the biological mechanisms linking PTSD with CVD involves dysregulation of basic cell biology. The endoplasmic reticulum (ER) is a multifunctional signaling organelle that controls a wide range of cellular processes such as the synthesis of proteins and steroids, the entry and release of Ca²⁺, and apoptosis. Under physiological conditions, glucose-regulated protein 78 (GRP78), the ER resident chaperone, is bound to the three transmembrane ER proteins, inositol-requiring protein-1α (IRE1α), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6), preventing their activation. A wide variety of cellular stressors may interrupt protein folding process in the ER, resulting in the accumulation of unfolded or misfolded proteins in the ER lumen. Unfolded proteins in the ER cause GRP78 to release IRE1α, pro- 

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protein-kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6), preventing their activation. By binding with the protein TRAF, IRE1 activates a JNK signaling pathway and the downstream caspases (ASK1)/JNK cascade and subsequently induces cell apoptosis; activated IRE1 also may activate caspase 12, which activates caspase 9 and caspase 3, resulting in apoptosis (16). Calreticulin (CRT) is a key component of calcium homeostasis. As molecular chaperones in ER, both GRP78 and CRT are up-regulated in the setting of ERS to modulate calcium homeostasis and stop the ERS response (17).

ERS is associated with the pathophysiology of many diseases, including CVDs, mental illnesses, neurodegenerative diseases, and cancers (18). ERS-related apoptosis represents a recently identified apoptotic pathway. There

![FIGURE 1. A simplified diagram of the adaptive and apoptotic pathways of ERS. The initial phase of ERS is an adaptive response: the activated cytosolic domain of PERK phosphorylates the eIF2α, inhibiting translation and resulting in cell cycle arrest; the activated cytosolic domain of IRE1α cleaves the 252-bp intron from its substrate XBP1, facilitating its translation to form the transcription factor XBP1; the activated ATF6 translocates to the Golgi, cleaved by proteases to form an active 50-kDa fragment (ATF6p50). ATF6p50 and XBP1 produce up-regulation of the proteins involved in the adaptive response. In conditions of prolonged stress, proteins downstream of all three transmembrane protein pathways have been identified as having proapoptotic roles. All the three transmembrane proteins could induce expression of CHOP protein ultimately. And CHOP represses the expression of the antiapoptotic protein Bcl-2 and up-regulates the proapoptotic proteins Bax and CHOP. By binding with the protein TRAF, IRE1 activates a JNK signaling pathway and the downstream caspases to cause apoptosis. ERS = endoplasmic reticulum stress; PERK = protein kinase RNA-like ER kinase; ATF6 = activating transcription factor 6; CHOP = CCAAT/enhancer-binding protein-homologous protein; TRAF = tumor necrosis factor receptor-associated factor; JNK = c-Jun N-terminal kinase; ER = endoplasmic reticulum. Color image is available only in online version (www.psychosomaticmedicine.org).]
exists compelling evidence that ERS-related apoptosis plays a fundamental role in both the development and the progression of CVD, including heart failure, ischemic heart disease, and atherosclerosis (19). Previous studies have demonstrated that ERS-related apoptosis is involved in myocardial injury induced by hypoxia/reoxygenation, pressure overload, and acute myocardial infarction (20–23). PTSD may also induce the ERS response. Human studies showed that elevated systemic expression of ERS-related genes (GRP78 and ER degradation enhancer mannosidase alpha-like 1) were associated with past month PTSD among community-dwelling individuals (24). Research based on animal experiments demonstrated that single prolonged stress (SPS) induced changes in the expression levels of GRP78, CRT, and caspase 12 in the hippocampus and the prefrontal cortex of PTSD rats, indicating that the ER pathway may be involved in PTSD-induced apoptosis, and the hippocampal apoptosis could be one of the pathological mechanisms related to the memory disorders in PTSD (25–27). However, whether ERS is involved in the molecular mechanisms underlying PTSD-induced myocardial injury is unclear. We hypothesized that PTSD causes myocardial injury by inducing ERS-related apoptosis through PERK/CHOP, JNK, and caspase 12 pathways.

SPS rats could mimic the pathophysiological abnormalities and behavioral characteristics of PTSD, and the face and construct validity of the SPS model supported it as a model for PTSD (28). Therefore, in this study, we investigated the myocardial damage by detecting apoptosis and ultrastructure in a rat model simulating PTSD induced

**FIGURE 2.** The effect of simulated PTSD on the cardiomyocyte apoptotic rate as determined via TUNEL staining (×400, bar = 36 μm) and myocardial tissue morphology as determined via hematoxylin and eosin staining (×200, bar = 50 μm). A, Representative images of TUNEL-stained cardiomyocytes. Blue (left panels) represents DAPI-stained nuclei; green (middle panels), fragmented DNA. B, The apoptotic rate was quantified as the ratio of TUNEL-positive cells to the total number of cardiomyocytes. n = 6, *p < .05 versus control. The error bars represent the standard error. PTSD = posttraumatic stress disorder; TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling; HE = hematoxylin and eosin. Color image is available only in online version (www.psychosomaticmedicine.org).
by SPS. We subsequently studied alterations of ERS signaling to determine the mechanisms underlying ERS-related apoptosis in PTSD-induced myocardial injury.

MATERIALS AND METHODS

Animals
A total of 48 male Sprague-Dawley rats (180–220 g), from the Animal experimental Center of Chinese PLA General Hospital, were housed in an air-conditioned room (22–24°C and 50%–60% humidity) on a 12-hour light/dark cycle and given standard food and tap water. After 7-day acclimation period, rats were randomly divided into the following two groups: control group (n = 18) and PTSD group (n = 30). The rats in the PTSD group were subjected to the SPS procedure. The study protocol conformed to the “Principles of Laboratory Animal Care” (National Institutes of Health publication no. 86-23, revised 1985) and was approved by the Institutional Animal Care and Use committee of the Chinese PLA General Hospital. The data were collected between January 2014 and July 2014.

PTSD Model Preparation
The SPS procedure was performed as previously described (28,29). Briefly, the animals were restricted for 2 hours before being forced to swim for 20 minutes immediately after release. The forced swim was performed using six rats at a time in an plastic tub (60 cm long, 40 cm wide, and 50 cm high) filled two-thirds full with water (24°C). After 15 minutes of recuperation, the rats were exposed to ethyl ether until rendered unconscious, and then they were left undisturbed in cages (6 rats per cage, 20 minutes at 22°C and 50% humidity) on a 12-hour light/dark cycle and given standard food and tap water. After 12-hour light/dark cycle and given standard food and tap water. After 7-day acclimation period, rats were randomly divided into the following two groups: control group (n = 18) and PTSD group (n = 30). The rats in the PTSD group were subjected to the SPS procedure. The study protocol conformed to the “Principles of Laboratory Animal Care” (National Institutes of Health publication no. 86-23, revised 1985) and was approved by the Institutional Animal Care and Use committee of the Chinese PLA General Hospital. The data were collected between January 2014 and July 2014.

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Open-Field Testing
On the 14th day after SPS, all rats were tested in an open field (OF; square field, 80 cm × 80 cm × 50 cm). Briefly, the rats were placed in the center of the OF and allowed to stay for 5 minutes. The numbers of crossing and rearing of rats were measured by two individuals blinded to the experiment.

Transmission Electron Microscopy
Under anesthesia (1.5 g/kg urethane, Intraperitoneal), hearts were removed (n = 3, randomly selected from each group), immediately cut into blocks (1 mm × 1 mm × 1 mm) on ice, and immersed in precooled 2.5% glutaraldehyde in 0.1 M phosphate-buffered solution (PBS) overnight at 4°C. After sufficient washing with 0.1 M PBS, the blocks were postfixed in 1% osmium tetroxide for 30 minutes at 4°C. They were subsequently rinsed thrice with 0.1 M PBS, dehydrated in a graded series (30%–100%) of acetone, embedded with acetone–Epon 812 (1:1) overnight at room temperature, and embedded in pure Epon 812. The ultrathin sections were prepared, stained with both uranyl acetate and lead citrate, and subsequently observed and photographed using a transmission electron microscope (JEM 1230; JEOL, Tokyo, Japan).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick-End Labeling
The hearts were fixed in 4% formaldehyde, transected at the midventricular level, routinely processed, and embedded in paraffin. A terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay was performed using an in situ cell death detection kit according to the manufacturer's instructions (Promega; n = 6, randomly selected from each group). Staining was observed under an Olympus laser confocal microscope, and 1000 cardiomyocytes were counted in each section. Apoptosis rate was expressed as the percentage of TUNEL-positive cardiomyocytes.

Western Blot Analysis
The hearts were removed and the left ventricles were isolated rapidly on ice (n = 3, randomly selected from each group). Equal amounts of protein were extracted from the left ventricle (100 μg/lane as estimated via a bicinchoninic acid assay) and separated using 10% sodium dodecyl sulfate–polyacrylamide gels (30). After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose membranes blocked with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 at room temperature for 40 minutes. The membranes were then incubated overnight at 4°C with primary antibodies against Bel-2 (1:500, #2876S; CST), G1 (1:500, #2272; CST), GRP78 (1:500, SPA-826; Stressgen), CRT (1:500, SPA-600; Stressgen), CHOP (1:500, #2895S; CST), JNK (1:500, #9252; CST), p-JNK (1:200, sc-6254; Santa Cruz), caspase-12 (1:500, ab18677; Abcam), ATF6α (1:200, sc-22799; Santa Cruz), PERK (1:500, #3192S; CST), p-PERK (1:200, sc-32577; Santa Cruz), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:200, sc-25778; Santa Cruz). The antibody-tagged membranes were probed with a secondary antibody solution consisting of either a 1:1000 dilution of HRP-conjugated goat antimouse IgG (for CHOP and p-JNK) or a 1:1000 dilution of HRP-conjugated goat antirabbit IgG (for GRP78, CRT, Bel-2, Bax, PERK, p-PERK, caspase 12, ATF6α, and GAPDH). An enhanced chemiluminescent detection system (sc-2048; Santa Cruz) was used to detect the immunoblot protein. The optical density of the bands (measured in arbitrary densitometry units) was determined using Image-Pro Plus, and the densitometry of the immunoblot was normalized against GAPDH.

Statistical Analysis
The results are expressed as the means ± SEMs. The differences between groups were analyzed using Student’s t test. Pearson r correlation coefficients were calculated to determine the specific relations between behavioral variables and cardiomyocyte apoptosis in the PTSD group. For all statistical analyses, a value of p < .05 was considered statistically significant (SPSS Software; Chicago, IL).

RESULTS

The General Conditions of and Behavioral Changes Observed in the Rats
The SPS rats were dispirited, curled up, and less active but resisted when captured. Their hairs stood on end and appeared dry without gloss. However, the rats in the control group exercised normally, and their hair appeared smooth and shiny.

The OF test reflected both the spontaneous activity (activity and exploration) and the anxiety noted in the rats. Compared with the control rats, the number of crossing and rearing decreased by 23.8% (p < .001) and 31.2% (p < .001), respectively, in the SPS rats. These findings indicated that the PTSD models were successfully reproduced.

The Effect of Simulated PTSD on Cardiomyocyte Apoptosis in Rats

The Ultrastructure of the Myocardium
The cardiomyocytes in the control rats exhibited normal morphology: the nucleus is large and round, and the density of chromatin is uniform. The cardiomyocytes of the SPS rats exhibited changes characteristic of apoptosis, including

475 mm × 350 mm × 200 mm for 14 days.

20 minutes immediately after release. The forced swim was performed using six rats at a time in an plastic tub (60 cm long, 40 cm wide, and 50 cm high) filled two-thirds full with water (24°C). After 15 minutes of recuperation, the rats were exposed to ethyl ether until rendered unconscious, and then they were left undisturbed in cages (6 rats per cage, 475 mm × 350 mm × 200 mm) for 14 days.

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FIGURE 3. The effects of simulated PTSD on the phosphorylation of PERK and JNK, the protein levels of Bcl-2, Bax, GRP78, CRT, and CHOP, and the activation of caspase 12 and ATF6α in the myocardial tissues of the SPS rats. A, Representative Western blotting images for Bcl-2 and Bax. GAPDH served as loading control. B-E, The statistic column chart under the same conditions as in panel A. Each condition was performed in triplicate ($n=3$). $^p<.05$ versus control. The error bars represented the standard error. PTSD = posttraumatic stress disorder; PERK = protein kinase RNA–like ER kinase; JNK = c-Jun N-terminal kinase; GRP78 = glucose-regulated protein 78; CRT = calreticulin; CHOP = CCAAT/enhancer-binding protein-homologous protein; SPS = single prolonged stress; GAPDH = glyceraldehydes-3-phosphate dehydrogenase.
chromatin pyknosis and clotting around the nuclear membrane or dispersal in the nucleoplasm.

**TUNEL Assay**
Hematoxylin and eosin staining demonstrated that the myocardial fibers of the control rats were arranged in neat rows, whereas the myocardial fibers of the SPS rats were ruptured, disorganized, and infiltrated by inflammatory cells. The apoptosis rate was (7.26% ± 0.53%) in the control group. Compared with the control group, the apoptosis rate increased by 3.2-fold in the SPS rats ($p < .001$; Fig. 2).

**The Effect of Simulated PTSD on ERS Molecule Levels in the Myocardial Tissues of the Rats**

**ERS Molecule Expression**
Compared with the control group, the protein levels of GRP78 and CRT in the myocardial tissues of the SPS rats increased by 1.50- and 1.53-fold, respectively ($p = .017$ and $p = .040$); the protein level of CHOP in the myocardial tissues of the SPS rats increased by 1.23-fold ($p = .047$; Fig. 3A and C). There was no significant difference in the phosphorylation of JNK ($p = .790$) and no significant change in procaspase 12 levels in the myocardial tissues of the SPS rats ($p = .165$), whereas the cleaved caspase 12 levels increased significantly ($p = .003$), and the ratio of cleaved caspase 12 to procaspase 12 increased by 4.46-fold ($p < .001$; Fig. 3A and D), compared with the control rats. The phosphorylation of PERK in the myocardial tissues of the SPS rats increased by 6.29-fold ($p = .003$; Fig. 3A and B), compared with the control rats. An increasing trend in the activation of ATF6 in the SPS rats was noted, although the difference was not statistically significant ($p = .118$).

**Bcl-2 and Bax Protein Expression**
Both Bcl-2 and Bax protein expressions were measured via Western blotting. Compared with the control group, the ratio of Bcl-2 to Bax protein decreased by 49.93% in the SPS rats ($p = .005$; Fig. 3A and E).

**Behavior-Apoptosis Rate of Cardiomyocyte Correlations**
The animal responses to the OF test were predictably correlated with the cardiomyocyte apoptosis 14 days after the SPS. In the PTSD group, numbers of crossing and rearing were both negatively correlated with the apoptosis rate of cardiomyocyte (Fig. 4). Because of the small sample sizes, statistical significance of the correlations was not computed here.

**DISCUSSION**
In this study, we reproduced a rat model simulating PTSD using the SPS method. Compared with the control group, the numbers of rearing and crossing of the SPS rats were significantly lower based on the results of the OF test. This result suggested that the SPS rats exhibit significant behavioral changes, which provided evidence for the successful replication of the PTSD model. However, it is a limitation that we did not conduct the reliability analysis for the ratings for the OF testing. TUNEL staining and transmission electron microscopy both demonstrated typical apoptotic features in the myocardial tissues of the SPS rats, suggesting that cardiomyocyte apoptosis may contribute to myocardial injury in the SPS rats. We observed a tremendous ERS response in the myocardial tissues of SPS rats via a Western blotting analysis, findings supported by the up-regulation of the protein levels of the ERS molecular chaperones, GRP78 and CRT; the ERS-related apoptosis molecule, CHOP; and the activation of caspase 12, suggesting that ERS-related apoptosis may be associated with the molecular mechanisms underlying myocardial injury in the SPS rats.

There is compelling evidence that psychological stress increases the risk of CVD through enhancing coagulation...
factor level (31,32), leading to endothelial dysfunction (33), increasing platelet activity (34), and elevating blood lipid levels (35). These studies suggested that PTSD may result in inflammatory injury via the overactivation of the hypothalamic-pituitary-adrenal axis (36,37); systemic inflammatory responses were prevalent in the patients with PTSD (38). These findings indicated that the inflammatory responses induced via hypothalamic-pituitary-adrenal axis activation may be one of the mechanisms underlying PTSD-related myocardial injury. PTSD may induce sympathoadrenomedullary dysfunction, as indicated by the elevated catecholamine levels noted in the periphery (39). These catecholamine changes affected both the heart and blood vessels, resulting in myocardial injury and contributing to heart failure (40,41).

In this study, the number of apoptotic cardiomyocytes was significantly increased in the SPS rats as determined via the TUNEL assay. Apoptotic morphologic changes were observed in the cardiomyocytes of the SPS rats, including cell shrinkage, chromatin condensation, and nuclear pyknosis accumulation around the nuclear membrane and dispersal within the nucleoplasm. These observations indicated that simulated PTSD induces cardiomyocyte apoptosis, which may cause myocardial injury. The behavioral changes seemed to be directly related to the degree of cardiomyocyte apoptosis in the SPS rats; correlational analyses indicated that a greater degree of apoptosis was related to lower behavioral level 14 days after the SPS. Although the small sample sizes in the current study do not warrant statistical analyses to determine the significant correlation coefficients, the findings suggest that behavioral changes are predictably related to cardiomyocyte apoptosis in the PTSD group, and highlight the need for studying potential individual differences in the behavioral and cardiovascular responses to environmental stressors in the SPS model.

The elevated circulating catecholamine levels may act directly on cardiomyocytes, destroying the ER steady state and inducing ERS-related apoptosis. This mechanism may be tied to both the occurrence and the development of myocardial injury among patients with PTSD. In this study, we observed the up-regulation of the ERS molecule chaperones, GRP78 and CRT; the ERS-related apoptosis molecule, CHOP; and the activation of caspase 12, as determined via Western blotting. We also observed the increased phosphorylation of PERK, a nonsignificant change in the phosphorylation of JNK, and the activation of ATF6 in the hearts of the SPS rats. The ratio of Bel-2 to Bax was significantly decreased in the SPS rats as determined via Western blotting. These results suggested that the ERS-related apoptosis mediated by the PERK/CHOP and caspase 12 pathways may be associated with the molecular mechanisms underlying myocardial injury in the SPS rats.

In summary, this study demonstrated that the simulated PTSD might cause myocardial injury in rats; its underlying molecular mechanism may be associated with ERS related apoptosis, which manifests via the up-regulated expression of the ERS molecules, GRP78 and CRT; the ERS apoptotic pathway molecule, CHOP; and the activation of caspase 12. These results could advance our understanding of how PTSD contributes to cardiovascular injury on a molecular level, and provide us new potential therapeutic targets for the prevention of cardiovascular complication in patients with PTSD.

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Stress-Induced Myocardial Injury

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