p38 MAP kinase inhibitor reverses stress-induced cardiac myocyte dysfunction

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A GROWING BODY OF LITERATURE supports an association between stress and adverse outcomes from medical illnesses (5, 28). The relationship between stress and adverse cardiovascular outcomes is particularly well defined (9). There is now compelling data demonstrating that the diagnosis of clinical depression after a myocardial infarction confers a severalfold increase in the risk of subsequent cardiac death (7, 12). Proposed mechanisms through which stress increases cardiovascular mortality include enhanced platelet aggregation and autonomic dysfunction (5, 9, 12). A direct effect of stress on cardiac myocyte function has not been adequately explored.

We developed an animal model to elucidate mechanisms by which stress can directly affect the heart. Prenatal stress (PS) among third trimester pregnant mammals (including humans) has been repeatedly shown to produce offspring with altered behavioral and physiological characteristics similar to patients with posttraumatic stress disorder and clinical depression (21, 30). We developed a model of prenatal stress resulting from saline injections and handling of pregnant rats (30). This model reproducibly gives rise to male offspring with biochemical, physiological, and behavioral characteristics observed in humans with posttraumatic stress disorder and/or clinical depression. We proposed to test the hypothesis that the altered neurohumoral activation characteristic of prenatal stress would result in myocardial dysfunction after subsequent exposure to postnatal stress.

We have previously proposed that common intracellular stress-signaling pathways contribute to the reversible myocardial dysfunction and β-adrenergic desensitization seen in diverse clinical and experimental conditions (14, 15). We recently reported that the human immunodeficiency virus coat protein gp120 could reversibly depress cardiac myocyte contractility through a stress-signaling pathway involving p38 mitogen-activated protein (MAP) kinase (16). p38 MAP kinase has also been shown to mediate myocardial dysfunction in response to other cellular stresses as well (3, 4, 18, 19, 27, 29). Interestingly, restraint stress (R) has selective regional effects on MAP kinases in the brain (22). We explored the effects of R on myocardial function and intracellular stress signaling in the offspring of “normal” rats (control) and the offspring of mothers who were exposed to third trimester stress (PS). We report that R alone is sufficient to reversibly depress cardiac myocyte function through p38 MAP kinase phosphorylation in this model of PS.

**MATERIALS AND METHODS**

**Materials.** All reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Adult male (250–300 g) and female (225–250 g) Sprague-Dawley rats were purchased from Hilltop Lab Animals (Scottsdale, PA) and housed in a room specifically dedicated for rats in the Animal Care Facility of the Robert C. Byrd Health Sciences Center of West Virginia University. Strict adherence to the protocol approved by the West Virginia University Animal Care and Use Committee was maintained throughout.

**Statistical methods.** In vivo echocardiographic data represent means ± SE of values obtained from 12 different rats in each group. In vitro adult rat ventricular myocyte (ARVM) physiological data represent means ± SE derived from 12–24 different determinations from 12–24 individual cardiac myocytes from 6–8 different ARVM preparations derived from 6–8 different rats. The nature of the physiological experiments resulted in the use of two to four ARVM per preparation (i.e., 2–4 ARVM/rat heart). Student’s t-test was used.

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http://www.jap.org
for paired comparisons (see Figs. 3–5 and 7). Analysis of variance was used for multigroup comparisons (see Figs. 6 and 8). Average values of the fractional shortening (FS) were computed across cells for each animal per dose combination. Those averages were used as the response variables in the ensuing statistical analyses. The statistical methodology involved an analysis of variance appropriate to the split-plot (or repeated measures) nature of the experimental design. In those analyses, the factor of primary interest was the interaction term. Bonferroni-corrected orthogonal contrasts were used when significance in the interaction term was observed to compare the experimental (PS + R) and control (control + R) groups at each dosage level. Values of $P < 0.05$ were considered statistically significant.

JMP statistical software was used for all statistical procedures (SAS Institute). Western analyses were performed on the same ARVM used for the physiological experiments with identical results using at least three different heart preparations derived from at least three different rats in each group.

**Stress paradigm.** Offspring were obtained by breeding male and female Sprague-Dawley rats. Maternal stress was induced by daily saline injections (0.9%, 1 ml sc) at different times of the day and moving from cage to cage from gestational day 14 to day 21, as our laboratory previously reported (30). Male offspring from stressed (PS) or control dams were restrained for 2 h at 25°C in plastic tubes under normal room light, followed by open-field exposure for 10 min at 42 and again at 49 days of age. During restraint, the animal’s head was exposed, but the animal was unable to back up or turn and had limited lateral mobility.

**Echocardiography.** Left ventricular (LV) FS (a measurement of LV systolic function) was determined by measuring the LV chamber dimensions with an echocardiographic system with integral Doppler capabilities (25). M-mode imaging of the vertical excursion of cardiac structures over time at a single line through the heart was performed after two-dimensional imaging. Imaging was performed with a 12-MHz oscillating single-crystal mechanical transducer. LV FS was calculated from the M-mode LV dimensions using the equation

$$FS = \frac{100 \times \text{LVEDD} - \text{LVESD}}{\text{LVEDD}},$$

where LVEDD is LV end-diastolic volume and LVESD is LV end-systolic volume.

**ARVM.** Cells were isolated from the hearts of adult male Sprague-Dawley rats, as we previously reported (16). Rats were anesthetized with pentobarbital sodium, and the hearts were removed rapidly and perfused with Krebs-Hensleit bicarbonate buffer (KHB) containing (in mM) 118.1 NaCl, 3.0 KCl, 1.8 CaCl$_2$, 1.2 MgSO$_4$, 1.0 KH$_2$PO$_4$, 27.3 NaHCO$_3$, 10.0 glucose, and 2.5 pyruvic acid, pH 7.4, according to the method of Langendorff at a constant rate of 8 ml/min using a peristaltic pump. All buffer and enzyme solutions used during cell isolation were maintained at 37°C and preequilibrated with 95%O$_2$-5%CO$_2$. Hearts were perfused with KHB for 15 min, following by change to low-Ca$^{2+}$ KHB containing (in mM) 105.1 NaCl, 3.0, 0.01 CaCl$_2$, 1.2 MgSO$_4$, 1.0 KH$_2$PO$_4$, 20.0 NaHCO$_3$, 10.0 glucose, 5.0 pyruvic acid, 10.0 taurine, and 5.0 mannitol, pH 7.4, for an additional 10 min. Hearts were then immersed in recirculating KHB with low Ca$^{2+}$ containing collagenase B (1.25 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) for 40 min. The ventricles were minced and placed into a 50-ml centrifuge tube, adjusted to 25 ml with low-Ca$^{2+}$ KHB and centrifuged at 50 g for 2 min. The supernatant was aspirated, and the concentration of Ca$^{2+}$ in KHB was increased in four increments (0.08, 0.6, 1.2, and 1.8 mM). Finally, the mixture was passed through 225-μm nylon mesh and centrifuged at 50 g for 2 min. The centrifuge procedure was repeated until the preparation was composed of at least 80% viable LV myocytes. The myocytes exhibited a typical striated and rod-shaped appearance when viewed by light microscope. Only those myocytes that were rod shaped, with striations, no blebs and not spontaneously contracting, were included for analyses (16). Physiological experiments were conducted with continuous superfusion of 95%O$_2$-5%CO$_2$ KHB. Myocytes typically retained their baseline FS for at least 4 h, and only freshly isolated cells were used for physiological experiments.

**MAP kinase phosphorylation assay.** p38 MAP kinase phosphorylation was determined using phospho-p38 MAP kinase (Thr180/Tyr182) antibody according to the manufacturer’s recommendations, as we previously reported (16). The quantity of each sample loaded was determined by using nonphosphoprotein antibodies on the same blots. To reuse the blot, the blot was left in the stripping buffer (7 M guanidine-HCl, 2.5 M glycine, 0.05 mM EDTA, 0.1 M KCl, 20 mM mercaptoethanol) for 15 min, and the blot was washed using distilled water.

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**DIAGRAM OF EXPERIMENTAL PROTOCOL**

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![Fig. 1. Flow diagram illustrating the steps involved in generating this animal model of stress-induced reversible myocardial dysfunction. There were no significant differences in myocardial function determined by echocardiography in control alone (Con) or the prenatal stress (PS) alone groups of animals. Differences were only observed between PS and Con after postnatal restraint (PS + R vs. Con + R).](http://jap.physiology.org/)
LVESD is left ventricular end-systolic diameter.

LVEDD

M-mode LV dimensions using the equation FS (%) = [(LVEDD - LVESD)/LVEDD] × 100, where LVEDD is left ventricular end-diastolic diameter and LVESD is left ventricular end-systolic diameter.

Fig. 2. Representative M-mode echocardiogram of left ventricular (LV) dimensions from control (Control + R) and prenatally stressed (PS + R) rats subjected to restraint and open-field stresses at 42 and again and 49 days of postnatal age. M-mode imaging of the vertical excursion of cardiac structures over time at a single line through the heart was performed after 2-dimensional imaging. Values are left ventricular end-systolic (2.69 vs. 3.58) and end-diastolic (6.57 vs. 6.64) dimensions in millimeters from Control and PS + R, respectively. LV fractional shortening (FS) was calculated from the diastolic dimensions in millimeters from Control (6.57 vs. 6.64) and end-systolic (2.69 vs. 3.58) dimensions in millimeters from Control imaging. Values are left ventricular end-systolic and end-diastolic dimensions at a single line through the heart was performed after 2-dimensional imaging.

water and reblootted by different antibodies. Blots were detected by using the Amersham enhanced chemiluminescence system. Cells were lysed by adding 100 μl of lysis buffer and immediately scraping cells off the 30-mm dish and transferring the extract to a microtube to keep on ice. This was followed by sonicating for 2 s and centrifuging at 10,000 g for 15 min at 4°C. The supernatant was transferred to a new centrifuge tube. Sample buffer was added to protein samples at a ratio of 2:1 and microcentrifuged for 30 s followed by loading 20 μg of protein onto SDS PAGE.

RESULTS

Maternal stress was induced by daily subcutaneous saline injections into skinfolds behind the neck (0.9%, 1 ml sc) at different times of day and moving from cage to cage from gestational day 14 through day 21, as we previously reported (30). Echocardiograms performed on the male offspring of the stressed dams and age-matched controls at 6 wk of postnatal age revealed no significant difference in LV FS (%FS; a measurement of LV systolic function) between the control animals (56 ± 0.9%); n = 12) and the PS offspring of the stressed dams (53 ± 1.1%; n = 12).

Male offspring from maternally stressed (i.e., PS offspring) and control dams (i.e., control offspring) were subsequently subjected to a previously established and defined postnatal stress paradigm at 42 and again at 49 days of postnatal age (Fig. 1) (30). Stress was produced by a combination of restraint and open-field exposure. Rats were gently restrained for 2 h at room temperature (25°C) in plastic tubes under normal room light. The animals’ heads were exposed, but the animals were unable to back up or turn and had limited lateral mobility. The rats were subsequently transferred from the plastic tubes and exposed to an open field (100 cm²) for 10 min under enhanced fluorescent lighting (2,300 lx). Figure 2 is a representative M-mode echocardiographic image obtained from age- and sex-matched control (control + R) and PS (PS + R) rats after restraint and open-field stress at 42 and again at 49 days of age. The PS + R rats consistently revealed a decrease in %FS (45.8 ± 3.9 vs. 61.9 ± 2.4%; n = 12 for each group; P < 0.01) that persisted for at least several weeks after both of the postnatal stresses (Fig. 3). The systolic dysfunction observed in the PS + R rats was characterized by a predominant increase in end-systolic dimension, with little change in end-diastolic dimensions (Fig. 4).

Possible mechanisms responsible for the decrease in myocardial function observed in the PS + R rats were subsequently explored using isolated ARVM. PS + R revealed diminished (Fig. 5).
%FS compared with control + R (8.2 ± 1.3 vs. 14.1 ± 2.9%, respectively; n = 12) (Fig. 5).

An impaired response to β-adrenergic stimulation is a common characteristic of chronic heart failure from both idiopathic as well as ischemic etiologies (15). A blunted contractile response to the β-adrenergic agonist isoproterenol was consistently observed in the PS + R compared with control + R rats (n = 12, P < 0.01) (Fig. 6). This was true whether comparisons were made on the basis of percentage of baseline or absolute %FS.

p38 MAP kinase is a central component of signaling pathways activated in response to infectious and ischemic stress (13, 26). The addition of a p38 MAP kinase inhibitor, SB 203580 (10 μM), reversed the depression in %FS seen in ARVM from PS + R rats (8.2 ± 1.3 vs. 13.3 ± 1.7%, n = 12) (Fig. 7). Pretreatment of ARVM from PS + R rats with the p38 MAP kinase inhibitor SB-203580 also significantly reversed the blunted adrenergic response (n = 12; P < 0.01) (Fig. 8). SB-203580 had no effect on baseline %FS in control + R, as we previously reported in untreated controls (16). The phosphorylation of p38 MAP kinase by stress alone and its inhibition by SB-203580 was confirmed by Western blot analysis (Fig. 9).

DISCUSSION

These data demonstrate that a combination of prenatal and postnatal stresses alone was sufficient to result in LV dysfunction in vivo associated with isolated cardiac myocyte depression, β-adrenergic desensitization, and phosphorylation of p38 MAP kinase (Figs. 1–9). It is important to note that control rats were subjected to identical postnatal stresses but did not develop myocardial dysfunction. It is equally noteworthy that PS alone also did not result in myocardial dysfunction. Rather, the combination of third trimester PS along with postnatal stress resulted in myocardial dysfunction (PS + R; Figs. 1–8).

We provide novel evidence implicating p38 MAP kinase in the reversible myocardial depression associated with stress (PS + R) (Figs. 7–9). p38 MAP kinase is a member of a class of intracellular enzymes that phosphorylate proteins in response to inflammatory mediators (e.g., cytokines) and stress.

Fig. 6. Concentration-response curve depicting the effect of restraint and open-field stress at 42 and 49 days of postnatal age on β-adrenergic responsiveness as reflected in respective changes from %FS by ARVM from Control + R compared with PS + R in response to the continuous superfusion of cumulatively increasing concentrations of the β-adrenergic agonist isoproterenol (Iso). Values are means ± SE; n = 24 individual cardiac myocytes isolated from 8 separate myocyte preparations from 8 different rats in each group. *Statistically significant difference (P < 0.01) between the main effects of treatment (i.e., Control + R vs. PS + R) and interactions between individual means at the same concentration of isoproterenol, as determined by the post hoc Bonferroni test.

Fig. 7. The P38 MAP kinase inhibitor SB-203580 (SB) reversed the myocardial depressant effects of stress on PS + R (8.2 ± 1.3 vs. 13.3 ± 1.7) with no effect on the Control + R. Values are means ± SE; n = 12 individual cardiac myocytes isolated from 6 separate myocyte preparations from 6 different rats in each group. *P < 0.01, Student’s t-test.

Fig. 8. The p38 MAP kinase inhibitor SB partially reversed the blunted inotropic response to the continuous superfusion of cumulatively increasing concentrations of isoproterenol by ARVM isolated from PS + R. As noted previously, SB increased %FS from baseline in PS + R but had no effect on %FS from baseline in Control + R (0 value on x-axis). Values are means ± SE; n = 24 individual cardiac myocytes isolated from 8 separate myocyte preparations from 8 different rats in each group. *Statistically significant difference (P < 0.01) between the main effects of treatment (i.e., Control + R vs. PS + R), which is completely eliminated by the addition of SB alone in the absence of isoproterenol.

Fig. 9. Representative Western blot depicting the effect of restraint and open-field stresses at 42 and 49 days of postnatal age on p38 MAP kinase activation in ARVM from Control + R vs. PS + R. SB, a p38 MAP kinase inhibitor, inhibited p38 MAP kinase activation in the ARVM isolated from PS + R. The quantity of each sample loaded was determined by using nonphosphoprotein antibodies on the same blots. This experiment was replicated at least 3 times using at least 3 different myocyte preparations from at least 3 different rats from each group with identical results using phospho-p38 MAP kinase (Thr180/Tyr182) antibody according to the manufacturer’s recommendations.
(e.g., ischemia) (27, 29). Evidence is rapidly accumulating supporting a pathogenic role for p38 MAP kinase in myocardial dysfunction in animal models and humans (4, 17). p38 MAP kinase activation has been implicated in ischemia, hypertrophy, apoptosis, and adrenergic signaling in cardiac myocytes (18, 19, 22, 24, 31, 32). Continuous activation of p38 MAP kinase was required in our system since SB-203580 was effective in reversing the myocardial depression (Figs. 7 and 8). The requirement for continuous activation suggests an inherently simple mechanism for reversibility of myocardial depression by p38 MAP kinase. It has yet to be determined what role p38 MAP kinase phosphorylation plays in other clinical and experimental conditions associated with reversible myocardial dysfunction (14, 15).

Cardiac myocytes (ARVM) isolated from PS + R demonstrated a blunted inotropic response to continuous superfusion with the β-adrenergic agonist isoproterenol (Figs. 6 and 8). A physiological role for p38 MAP kinase in adrenergic signaling in cardiac myocytes has been suggested by a study in a transgenic mouse model lacking β₁-adrenergic receptors (32). Inhibition of p38 MAP kinase activation with SB-203580 was found to enhance the positive inotropic effect of the β-adrenergic agonist isoproterenol through the β₂-adrenergic receptor. A physiologically relevant role for p38 MAP kinase in adrenergic signaling in cardiac myocytes was further supported by studies in avian cardiac myocytes (20). Magne et al. (20) provided strong evidence for the regulation of β₂-adrenergic signaling through a MAP kinase-cytosolic phospholipase A₂ pathway. Human heart failure is associated with a relative decrease in β₁- and an increase in β₂-adrenergic receptors (2, 11). Thus activation of p38 MAP kinase by stress would lead to blunted autonomic responses typical of human cardiomyopathies and chronic heart failure.

A negative inotropic effect of p38 MAP kinase has been reported by Liao et al. (17). Activation of p38 MAP kinase was achieved by adenoviral gene transfer of an activated mutant of its upstream kinase MKK3bE. The authors reported an intracellular Ca²⁺ concentration-independent negative inotropic effect of the activated MKK3bE mutant. Liao et al. concluded that troponin I phosphorylation was not responsible for the negative inotropic effect of p38 MAP kinase. Their conclusion was based on the results of their experiments, which did not reveal direct phosphorylation of purified troponin I protein by p38MAP kinase in vitro. These observations would suggest that p38 MAP kinase does not directly phosphorylate troponin I. However, p38 MAP kinase could still participate in an upstream process that results in troponin I phosphorylation.

We have previously shown that stressing pregnant rats with handling, daily saline injection, and exposure to a novel environment during the last week of gestation (gestational weeks 14–21) produces offspring that are more susceptible to the behavioral and neuroendocrine effects of stress (PS) (10, 30). To our knowledge, there have been no previous studies conducted to explore direct myocardial effects of PS alone and/or in combination with postnatal stress (PS + R) in animal models or humans. The clinical relevance of our observations is further supported by anecdotal reports of emergency room presentations for unexplained episodes of reversible heart failure after stress (1). Studying the signaling pathways activated in animal models after stress provides a unique opportunity to derive important mechanistic insights of considerable potential diagnostic and therapeutic relevance for idiopathic cardiomyopathies and heart failure in humans.

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