Selective inhibition of p38α MAPK improves cardiac function and reduces myocardial apoptosis in rat model of myocardial injury

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Selective inhibition of p38α MAPK improves cardiac function and reduces myocardial apoptosis in rat model of myocardial injury. The beneficial effects of p38 MAPK inhibition were evaluated by the improvement of cardiac function and the reduction of myocardial damage and deterioration of cardiac function. In a rat model of myocardial injury, we have investigated cardioprotective effects of the inhibition of p38 MAPK using a novel, orally available p38α MAPK inhibitor. Rats were treated with an Nω-nitro-L-arginine methyl ester (L-NAME, 40 mg·kg⁻¹·day⁻¹) in drinking water plus 1% salt for 14 days and ANG II (0.5 mg·kg⁻¹·day⁻¹) for 3 days. A selective p38α MAPK inhibitor, SD-282 (60 mg·kg⁻¹), was administrated orally, twice a day for 4 days, starting 1 day before ANG II administration. The cardioprotective effects of p38α MAPK inhibition were evaluated by improvement of cardiac function, reduction of inflammatory cell infiltration, and cardiomyocyte apoptosis. SD-282 significantly improved cardiac function indicated by increasing stroke volume, cardiac output, ejection fraction, and stroke work and significantly decreasing arterial elastance. SD-282 also significantly reduced macrophage infiltration as judged by reduction of a specific marker, ED-1-positive staining cells (P < 0.05) in the myocardium. Furthermore, cardiomyocyte apoptosis as indicated by caspase-3 immunohistochemical staining was abolished by SD-282, and this effect may contribute to the reduction of myocardial damage evaluated by imaging analysis (P < 0.05 in both cases). Data suggest that p38α MAPK may play a critical role in the pathogenesis of cardiac dysfunction. Inhibition of p38α MAPK may be used as a novel cardioprotective strategy in attenuation of inflammatory response and deterioration of cardiac function that occurs in acute cardiovascular disease such as myocardial infarction.

In the family of mitogen-activated protein kinases, there are three major enzymes that have been well characterized, including the extracellular signal-regulated kinases (ERK or p42/44 MAPK), the c-Jun NH2-terminal kinases, and the p38 MAPK (20). p38 MAPK, a serine-threonine kinase, is activated in response to a variety of environmental stimuli, including ischemia and cellular stress (1, 2, 19). Four p38 MAPK isoforms, including α, β, δ, and γ, have been characterized. In human heart cDNA libraries, the levels of α- and β-transcripts are higher than those of δ- and γ-isoforms (6).

It has been demonstrated that p38α MAPK may play an important role in cardiovascular diseases. Activation of p38 MAPK in the heart may be associated with cardiac myocyte hypertrophy and apoptosis (21). In heart failure secondary to ischemic heart disease, p38 MAPK is activated in the heart tissue (5). However, the role of p38 MAPK in executing pathophysiological responses to cardiac dysfunction during heart failure is still not fully understood. There is conflicting literature as to whether p38 MAPK is cardioprotective during ischemic heart disease. In the studies of ischemic preconditioning, the enhanced activation of p38 MAPK during the sustained period of ischemia seems to be the major contributor of cardioprotection. The cardioprotective effects of preconditioning can be abolished by SB-203580, an inhibitor of p38 MAPK (15, 17, 22). In contrast, it is also reported that activation of p38α/β MAPK during sustained ischemia-reperfusion has been associated with myocardial necrosis/apoptosis. Inhibition of p38 MAPK decreases ischemia-induced necrosis and apoptosis and improves posts ischemic cardiac function (12, 13, 18). Thus to understand whether the inhibition of p38α MAPK results in cardioprotective effects during ischemic heart disease is necessary.

In the present study, a novel small-molecule inhibitor of p38α MAPK, SD-282, was used to investigate the cardioprotective effects of p38α MAPK inhibition in a rat model of acute myocardial injury. The beneficial effects of p38 MAPK inhibition were evaluated by the improvement of cardiac function and the reduction of myocardial apoptosis.

MATERIALS AND METHODS

Animals and reagents. Male Wistar rats weighing 250–300 g were obtained from Charles River Breeding Laboratories (Hollister, CA). Animals were fed with normal rat chow and water ad libitum and kept on a 12:12-h light-dark cycle. Rats were allowed to acclimate for 1 wk before being randomly divided into various groups for the experiment. All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85–23, Revised 1996). The protocol of the study was approved by the Institutional Animal Care and Use Committee of Scios, Inc.

Nω-nitro-L-arginine methyl ester (L-NAME) and ANG II were purchased from Sigma (St. Louis, MO). The L-NAME-containing water was prepared daily with distilled water. ANG II dosing solution was prepared under sterile conditions with a vehicle containing 1% polyethylene glycol (PEG) in saline.
**Protocol.** The myocardial injury was pharmacologically induced by L-NAME/ANG II/NaCl, described by Martinez et al. (14), with a slight modification. Briefly, L-NAME (40 mg·kg\(^{-1}\)·day\(^{-1}\)) was administered in drinking water with 1% NaCl from day 0 to day 14. ANG II, 0.5 mg·kg\(^{-1}\)·day\(^{-1}\), was administered subcutaneously via Alza osmotic minipumps (model 2001, Alza, Mountain View, CA) during the last 3 days of L-NAME/NaCl treatment.

To assess the effect of p38 MAPK inhibition, we administered a synthetic p38 MAPK inhibitor, SD-282, 1 day before ANG II treatment (n = 16) via gastric gavage at a dose of 60 mg/kg, twice daily for 4 days. A group of rats (n = 16) was treated with 1% PEG, a vehicle solution for SD-282. Thirteen rats without any treatment were used as control. At day 14 of the experiment, all animals were instrumented for blood pressure and cardiac function measurement. Hearts were dissected and weighed before fixation for histological and immunohistochemical assessment.

**Measurement of blood pressure and cardiac function.** The systolic blood pressure was measured before the termination of the experiment by using a tail-cuff technique. Rats were placed in plastic restrainers and allowed to acclimate to the environment for 10 min before the systolic blood pressure was measured. The systolic blood pressure in conscious rats was recorded by a computerized blood pressure recording system (model 31, IITC/Life Science Instruments, Woodland Hills, CA).

For cardiac function measurement, rats were instrumented with a VetEquip rodent anesthesia system (VetEquip, Pleasanton, CA) and anesthetized by inhalation of isoflurane-oxygen. A 1.4-Fr catheter equipped with electrodes and a micromanometer (SPR-719, Millar Instruments, Austin, TX) was inserted into the left ventricular cavity through the right carotid artery. The ARIA Pressure-Volume Conductance System (Millar Instruments) was used to monitor and record the pressure-volume loops. The system was set with the excitation frequency at 20 kHz, the low-pass cutoff frequency at 50 Hz, and the full-scale current selection at 30 μA.

After 10-min stabilization, the steady-state pressure-volume loops were acquired with the computer data-acquisition system. A series of pressure-volume loops (20–30 loops) were randomly recorded and analyzed by Millar PVAN 2.9 software. Parameters for cardiac function, including heart rate (HR), stroke volume (SV), maximum and minimum volume of the first derivative of left ventricular pressure against time (+dP/dt and −dP/dt), ejection fraction (EF, stroke volume divided by volume at maximum dP/dt), cardiac output (CO; SV times HR and divided by 1,000), stroke work (SW, area covered by pressure-volume loops), and arterial elastance (Ea, calculated by the steady-state end-systolic pressure-to-SV ratio), were measured.

**Histopathology and immunohistochemistry.** Rats were perfused with saline followed by 4% neutralized formalin through a needle inserted in the circulation system for in situ perfusion fixation. Hearts were collected and postfixed in 10% neutralized formalin. To visualize and semiquantify the myocardial lesion at different levels, the heart segments were embedded in paraffin, and 4-μm cross sections were cut from each segment. Sections were stained for hematoxylin and eosin and Masson’s trichrome stain before histopathological evaluation of myocardial injury was performed.

For immunohistochemical staining, slides were deparaffined and rehydrated in PBS followed by blocking the endogenous peroxidase with 3% hydrogen peroxide. To avoid nonspecific reaction with primary antibody, slides were pretreated with 15% normal goat serum before incubation with primary antibodies. The primary antibodies used in this study were mouse anti-rat ED-1 monoclonal antibody at the concentration of 1:300 (Serotec, cat. no. MCA311R) and rabbit affinity-purified polyclonal for caspase-3 at the concentration of 1:400 (R&D Systems, cat. no. AF853). A goat anti-mouse biotinylated IgG (Chemicon International, cat. no. AP187B) for caspase-3 were used as secondary antibodies. Normal mouse IgG was used as a negative control. The immunoreactivities were visualized by ABC reagents (Vector, Burlingame, CA) and diaminobenzidine (Research Genetic, cat. no. 750118) followed by counterstaining with hematoxylin.

The image analysis was performed by using a Nikon E600 light microscope equipped with Spot digital camera. An Image Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD) was used for image analysis of myocardial injury, macrophage infiltration, and myocardial apoptosis. Myocardial injury was determined by the percentage of necrosis/fibrosis area against the total area of myocardium. The number of macrophage or apoptotic cells was counted in 10 fields per slide and normalized by the number of covered fields. The image analysis of myocardial injury and the number of macrophages and apoptotic cells was blinded to the person who performed the histopathological evaluation.

**Statistical analysis.** Data were analyzed by one-way ANOVA followed by Bonferroni multiple group comparison test (Instat V3.0, GraphPad, San Diego, CA). A P value <0.05 was accepted as statistically significant.

**RESULTS**

**Systolic blood pressure and heart-to-body weight ratio.** The effect of p38α MAPK inhibitor on blood pressure was evaluated by measuring the systolic blood pressure in conscious rats by using a tail-cuff technique. Figure 1A shows the changes in systolic blood pressure measured by tail-cuff technique in naive rats and the rats receiving L-NAME/ANG II, with or without SD-282. The systolic blood pressure of the rats receiving L-NAME/ANG II plus vehicle was 174 ± 11 mmHg, −1.4-fold higher than that of control rats (127 ± 9 mmHg). SD-282 treatment had no effect on the increased systolic blood pressure (173 ± 13 mmHg) in rats with L-NAME/ANG II-induced myocardial injury. The heart-to-body weight ratio, as calculated by weight 

![Fig. 1. Effects of SD-282 on systolic blood pressure (SBP; A) and heart-to-body weight (H/BW) ratio (B). N= nitro-l-arginine methyl ester (L-NAME)/ANG II induced increase in systolic blood pressure. SD-282 has no effect on SBP (P > 0.05, n = 9). SD-282 significantly reduced the heart-to-body weight ratio that has been enhanced by L-NAME/ANG II (P < 0.01, n = 9).](http://ajpheart.physiology.org/)

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an indicator of cardiac hypertrophy, was remarkably increased in the rats treated by l-NAME/ANG II plus vehicle (>140%). However, the increase in heart-to-body weight ratio was significantly (P < 0.01) reduced by administration of SD-282 (Fig. 1B).

**Improvement of cardiac function.** As summarized in Table 1, cardiac function deteriorated with the treatment of l-NAME/ANG II, indicated by a significant reduction of CO, EF, and cardiac index. Furthermore, the cardiac contractility as judged by +dp/dt and -dp/dt was also reduced by l-NAME/ANG II treatment. The deterioration of cardiac function induced by l-NAME/ANG II was significantly improved by administration of SD-282. Compared with l-NAME/ANG II plus vehicle treatment, the improvement of cardiac function with l-NAME/ANG II plus SD-282 was demonstrated by an increase in SV (205%, P < 0.01), EF (149.8%, P < 0.01), CO (238.9%, P < 0.001), and SW (198.9%, P < 0.05) and a decrease in $E_a$ (46.7%, P < 0.01). Furthermore, the p38 MAPK inhibitor SD-282 demonstrated an inotropic effect as indicated by increases in +dp/dt (129.0% vs. l-NAME/ANG II + vehicle treated, P = 0.07) and -dp/dt (132.9% vs. l-NAME/ANG II + vehicle treated, P < 0.05). It was noticed that the HR was significantly reduced by the l-NAME/ANG II, and SD-282 has no effect on HR. There was no notable effect on cardiac function when SD-282 was given to naïve rats (data not shown).

**Histopathology and immunohistochemistry.** The myocardial injury induced by l-NAME/ANG II was characterized by multiple foci of necrosis/fibrosis with some infiltration of leukocytes (Fig. 2). Myocardial lesions were likely distributed to the entire layer of myocardium in both left and right ventricles except the atrium. Image analysis of the hearts collected from l-NAME/ANG II plus vehicle-treated rats showed myocardial damage with the necrosis/fibrosis area of 11.3 ± 2.5%. The extent of cardiac damage was significantly reduced in the rats receiving l-NAME/ANG II plus SD-282 treatment (7.5 ± 2.2%, P < 0.05). However, there was no significant difference in the diameter of cardiomyocyte, an indicator of cardiac hypertrophy, in l-NAME/ANG II plus vehicle-treated and l-NAME/ANG II plus SD-282-treated rats.

The infiltrated macrophages visualized by positive immunostaining with ED-1 antibody were observed within the myocardial lesions as well as in the myocardium adjacent to the lesion (Fig. 3). Although macrophages were observed in the lesions in both l-NAME/ANG II plus vehicle-treated and l-NAME/ANG II plus SD-282-treated rats, the number of macrophages was significantly reduced by administration of SD-282 (64.3 ± 16.8/field in l-NAME/ANG II plus vehicle-treated vs. 46.9 ± 13.0/field in l-NAME/ANG II plus SD-282 groups, P < 0.05). Furthermore, it has been noticed that the number of macrophages was well associated with the area of myocardial lesions. The decreased number of macrophages in SD-282-treated rats might be a result of the reduction of myocardial damage. Apoptotic cells highlighted by caspase-3 immunostaining were observed in the myocardium surrounding the necrosis/fibrosis area in both l-NAME/ANG II plus vehicle-treated and l-NAME/ANG II plus SD-282-treated rats (Fig. 4). However, the number of apoptotic cells in the rats receiving SD-282 treatment was significantly less than those in vehicle-treated rats (17.4 ± 8.9/field in l-NAME/ANG II plus vehicle-treated group vs. 9.3 ± 5.7/field in l-NAME/ANG II plus SD-282-treated group, P < 0.05). The significant reduction of apoptosis in l-NAME/ANG II plus SD-282-treated rats vs. l-NAME/ANG II plus vehicle-treated rats was also confirmed by deoxyribonucleotide transferase-mediated dUTP nick end-labeling (TUNEL) staining (Fig. 5). The visualization of striation in the most apoptotic cells indicated that they were of cardiomyocyte origin.

**DISCUSSION**

l-NAME/ANG II-induced myocardial damage was characterized by cardiac myocyte necrosis, fibrosis, and apoptosis along with macrophage infiltration and connective tissue accumulation. The myocardial damage affected the entire layer of myocardium and impaired cardiac function. In the rats treated with l-NAME/ANG II + SD-282, the infiltration of macrophages was significantly reduced, which implied the inhibition of inflammatory response induced by l-NAME/ANG II stimulation. Although the decreased macrophage infiltration resulted from decreasing myocardial necrosis/fibrosis, less inflammatory response in the myocardium may play a critical role in protection of the heart from further deterioration of its function.
function. These findings are consistent with our previous study in which inhibition of p38α MAPK was cardioprotective in a rat model of myocardial injury (9). The cardioprotective effects of p38α MAPK inhibition were closely associated with inhibition of inflammatory response indicated by reduction of macrophage infiltration in the myocardium during acute myocardial injury.

Administration of l-NAME/ANG II significantly increased systolic blood pressure, which was the major contributor to induce myocardial hypertrophy and necrosis/fibrosis. SD-282 has no effect on systolic blood pressure, indicating that the beneficial effects of SD-282 were not associated with the regulation of blood pressure. Likewise, although cardiac hypertrophy is closely related to the pressure overload, SD-282-induced reduction of heart-to-body weight ratio was independent of the regulation of blood pressure as well. In addition, the reduction of heart-to-body weight ratio in SD-282-treated rats might be from the inhibition of inflammatory responses, such as edema in the myocardium, because there was no difference in the diameter of cardiomyocyte measured from l-NAME/ANG II plus vehicle-treated and l-NAME/ANG II plus SD-282-treated rats.

Fig. 2. Histopathology of control (A), l-NAME/ANG II + vehicle (B), and l-NAME/ANG II + SD-282-treated hearts (C; Masson’s trichrome stain, ×200). l-NAME/ANG II induced patchy style necrosis/fibrosis in the myocardium. The necrosis/fibrosis area was reduced by the treatment of p38α MAPK inhibitor, SD-282 (D). Bars represent means ± SE of 5 (control) and 7 rats (l-NAME/ANG II + vehicle-treated and l-NAME/ANG II + SD-282-treated groups, P < 0.05).

Fig. 3. ED-1 immunohistochemical (IHC) staining for macrophages in the heart. Compared with control (A), the number of macrophages was increased in the heart after the treatment with l-NAME/ANG II + vehicle (B); this increase was reduced in rats administered l-NAME/ANG II + SD-282 (C). D: imaging analysis of macrophages in the heart. Bars represent means ± SE of 5 (control) and 7 rats (l-NAME/ANG II + vehicle-treated and l-NAME/ANG II + SD-282-treated groups, P < 0.05).
Apoptosis or programmed cell death occurring during cardiac remodeling in a failing heart may play a critical role in the transition from compensated hypertrophy to heart failure. Apoptotic cell death may contribute to the reduction in the relative myofibrillar mass, resulting in cardiac dysfunction during the transition from compensation to heart failure (4, 8). A recent study demonstrated that p38α MAPK, as a mediator, may promote cardiomyocyte apoptosis through Bcl-X(L) deamidation and is responsible for cardiac remodeling after myocardial infarction (16). In addition, other studies have shown that inhibition of p38 MAPK decreased cardiac myocyte apoptosis, inhibited cardiac remodeling, and provided significant cardioprotection in a mouse model of heart failure (11, 12). In the present study, it has been demonstrated that p38α MAPK inhibition by SD-282 reduced the number of apoptotic cells in the myocardium. The reduction of cardiomyocyte death by apoptosis may partially contribute to the cardioprotective effects of p38α MAPK inhibition.

The present study has demonstrated that in vivo administration of the p38α MAPK inhibitor SD-282 not only significantly...
reduced myocardial injury but also markedly improved cardiac function that has deteriorated by the treatment of L-NAME/ANG II. Although Liao and colleagues (10) have reported that in a culture system, p38 MAPK has negative inotropic effects in cardiac myocytes, in an in vivo environment, p38α MAPK inhibition by SD-282 resulted in a clear trend toward increasing contractility as judged by enhancement of maximal and minimal dP/dt. In addition to the enhancement of contractility, SD-282 significantly increased the SV, and this increase in consequence contributed to the improvement in EF, CO, and cardiac work. Moreover, it should be noted that the improvement in cardiac function induced by SD-282 was well correlated to the reduction of myocardial injury. These observations have led us to hypothesize that the improvement in cardiac function might result from the preservation of myocardium during myocardial damage induced by L-NAME/ANG II. This hypothesis was well supported by an ex vivo study that demonstrated that a reduction of cardiomyocyte apoptosis as a result of treatment with SB-203580, a p38 MAPK inhibitor, was able to improve the cardiac function during ischemia-reperfusion injury (12).

In summary, we have demonstrated that administration of L-NAME plus ANG II in rats induces an acute myocardial injury with deterioration of cardiac function. The L-NAME/ANG II-induced myocardial lesions were associated with an infiltration of macrophages and apoptosis. A specific p38α MAPK inhibitor, SD-282, reduces inflammatory response and apoptosis, resulting in a reduction of myocardial damage, which, in turn, improves cardiac function. Therefore, this study provides evidence that p38α MAPK may play a critical role in the pathogenesis of myocardial injury. Inhibition of this enzyme may be a potential therapeutic strategy for pharmacological management of acute myocardial injury that occurs in ischemic heart diseases such as myocardial infarction in humans.

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REFERENCES