Hypertensive End-Organ Damage and Premature Mortality Are p38 Mitogen-Activated Protein Kinase–Dependent in a Rat Model of Cardiac Hypertrophy and Dysfunction

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Background—Numerous pathological mediators of cardiac hypertrophy (eg, neurohormones, cytokines, and stretch) have been shown to activate p38 MAPK. The purpose of the present study was to examine p38 MAPK activation and the effects of its long-term inhibition in a model of hypertensive cardiac hypertrophy/dysfunction and end-organ damage.

Methods and Results—in spontaneously hypertensive stroke-prone (SP) rats receiving a high-salt/high-fat diet (SFD), myocardial p38 MAPK was activated persistently during the development of cardiac hypertrophy and inactivated during decompensation. Long-term oral treatment of SFD-SP rats with a selective p38 MAPK inhibitor (SB239063) significantly enhanced survival over an 18-week period compared with the untreated group (100% versus 50%). Periodic echocardiographic analysis revealed a significant reduction in LV hypertrophy and dysfunction in the SB239063-treatment groups. Little or no difference in blood pressure was noted in the treatment or vehicle groups. Basal and stimulated (lipopolysaccharide) plasma tumor necrosis factor-α concentrations were reduced in the SB239063-treatment groups. In vitro vasoreactivity studies demonstrated a significant preservation of endothelium-dependent relaxation in animals treated with the p38 MAPK inhibitor without effects on contraction or NO-mediated vasorelaxation. Proteinuria and the incidence of stroke (53% versus 7%) were also reduced significantly in the SB239063–treated groups.

Conclusions—These results demonstrate a crucial role for p38 MAPK in hypertensive cardiac hypertrophy and end-organ damage. Interrupting its function with a specific p38 MAPK inhibitor halts clinical deterioration. (Circulation. 2001; 104:1292-1298.)

Key Words: heart failure ■ hypertension ■ signal transduction ■ stroke ■ hypertrophy

Cardiac remodeling associated with hypertension and the decrease in cardiac performance observed in the failing heart involve alterations in specific signaling molecules and their respective downstream pathways in individual myocytes.1-4 Through the use of cardiomyocyte cell culture and animal models, neurohormones, growth factors, cytokines, and mechanical stress have been implicated as mediators of cardiac hypertrophy and failure. These hypertrophic stimuli effect the necessary alterations in gene expression by receptor-mediated or non–receptor-mediated (stretch) activation of downstream kinase cascades (reviewed by Sugden and Bogoyevitch4,5). In particular, Gq-dependent pathways of cardiac hypertrophy and failure that are activated by neurohormones, such as angiotensin II,6 endothelin-1,7 or norepinephrine,8 stimulate mitogen-activated protein kinases (MAPK), including p38 MAPK.4,9,10 In addition, cytokines, such as tumor necrosis factor (TNF)-α, interleukin-1β, or transforming growth factor-β, can also induce myocardial changes similar to those observed in chronic heart failure, eg, myocyte hypertrophy,11 myocyte apoptosis,12 interstitial matrix alterations,13 and contractile depression.14,15 The mechanisms of cytokine actions in the myocardium are unclear; however, all have been shown to activate p38 MAPK pathways.16 In addition, TNF-α and interleukin-1β can be generated via p38 MAPK–dependent pathways.17 Finally, mechanical stress, through direct and/or indirect actions, can activate MAPK cascades.18,19

Thus, p38 MAPK can be activated by diverse hypertrophic stimuli, and it may be a common pathway mediating pathological stimuli in the myocardium. We therefore postulate a...
central role for p38 MAPK in the development of cardiac hypertrophy and failure. In the present study, we examined myocardial p38 MAPK activity in a hypertensive rat model of cardiac hypertrophy, decompensation, and end-organ damage. In addition, the effects of long-term treatment with a selective p38 MAPK inhibitor (SB239063) were investigated.

Methods

Animals
Spontaneously hypertensive stroke-prone rats (SHR-SP), obtained from the National Institutes of Health (NIH; Bethesda, Md), were bred in the Department of Laboratory Animal Science at GlaxoSmithKline (King of Prussia, Pa). Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23).

Time-Course Study
Male SHR-SP, 10 weeks old, were divided into 2 groups, placed in individual cages, and fed either standard rat NIH-07 diet (n=15; Na+, 0.33%; K+, 0.80%) with tap water ad libitum or a high-salt/high-fat diet (SFD; n=35; 1% NaCl in drinking water ad libitum and 24.5% fat) as described previously. Matched Wistar-Kyoto normotensive rats (n=4) were included in initial myocardial p38 MAPK activity comparisons.

Treatment Study
A colony of 80 male SHR-SP, 9 to 12 weeks old, was divided and assigned to 1 of 4 groups of 20 rats each. All animals were first placed on the normal chow diet (ND; NIH-07 diet) while baseline/entry monitoring was completed and then started on the SFD. The 2 treatment groups received 1200 or 2000 ppm SB239063 ([trans-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl methoxy)pyridimidin-4-yl)imidazole], a highly selective p38 MAPK inhibitor, added to the diet for the duration of the study (18 weeks). One SP group was maintained on the ND throughout the study. Mean age and body weight were consistent in all groups. SFD-SPs commonly become moribund and were promptly euthanized when failure to thrive signs were noted. These may include piloerection, lack of grooming, hypersensitivity to sound or touch, loss of appetite in the setting of terminal weight loss of 25% or greater, and sedation.

Echocardiography and Hemodynamic Monitoring
Transthoracic echocardiograms (GE/Vingmed System V) were performed under isoflurane anesthesia (1.5%) before treatment, at 8 and 16 weeks after initiation of the SFD treatment, and in moribund rats as possible. By use of the leading-edge method, systolic and diastolic thicknesses of the anterior and posterior walls and the left ventricular (LV) diameter were measured. Relative wall thickness (RWT) was calculated as RWT= (AWd+PWd)/LVDd, where AWd is diastolic anterior wall thickness, PWd is diastolic posterior wall thickness, and EDV and EDS are LV end diastolic and LV end systolic volume, respectively. Stroke volume and cardiac output were also determined by a modified Simpson’s method. Systolic blood pressure and heart rate were measured by a noninvasive tail-cuff method (HTC Life Science Apollo Analyzer, model 179BP).

Magnetic Resonance Imaging
For MRI, the animals were intubated for respiratory gating and maintained on isoflurane anesthesia (1.5%). All data acquisitions were performed on a 4.7-T/40-cm Bruker BIOSPEC imaging system. Diffusion-weighted imaging (DWI) data (SE: TR/TE=1500/45 ms; 128×128; FOV=3×3 cm; slice thickness=2 mm; B factor=1550 s/mm²) and T2 data (same parameters as DWI except that TR/TE=2000/65 ms and diffusion gradients were turned off) were collected from treated (n=14) and untreated (n=15) SFD-SP rats at the end of the study (17 to 18 weeks) or when moribund. All MR images were evaluated with an SGI UNIX workstation and image analysis software (AnalyzeDirect.com).

Lipopolysaccharide Stimulation of TNF-α
An in vitro whole-blood assay for TNF-α was developed. Blood samples collected in EDTA were obtained randomly from a subset (n=6 per group) of treated and untreated SFD-SP animals at the 6-week time point. Blood samples (250 μL) were treated with 0.1 μg of lipopolysaccharide (LPS) in 100 μL of DMEM. The tubes were incubated open at 37°C with 95% O₂ and 5% CO₂ for 3 hours, then centrifuged. Plasma TNF-α was determined after protein extraction according to the manufacturer’s instructions (Endogen).

Protein Extraction and Western Blot Analysis
Protein was extracted by pulverization of heart tissues (LV + septum) under liquid nitrogen, followed by incubation with chilled extraction buffer (1 mL buffer/400 mg tissue: in mmol/L: HEPES 20 [pH 7.4], α-glycerophosphate 20, NaVO₃ 0.1, PMSF 0.4, EDTA 0.1, dithiothreitol 0.5, MgCl₂, 2.5, and NaCl 75, plus 0.1% Triton, and protease inhibitors [Roche Diagnostic]) for 30 minutes. Samples were centrifuged at 14 000 rpm at 4°C for 10 minutes to remove tissue debris. The supernatant was reconstituted at 10 000 rpm (4°C) for 30 seconds to prepare aliquots for analysis. The protein concentration was determined with the Biorad DC Protein Assay. Samples were stored at −80°C until use. Total protein (30 to 60 μg) was separated by SDS-PAGE (10% ready gels; Bio-Rad). Proteins were transferred overnight to polyvinylidene difluoride membrane at 23 V (4°C). Membranes were incubated with rabbit polyclonal p38 MAPK or phospho-specific p38 MAPK antibody (Cell Signaling) and then incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Biorad). Immunoreactive bands were detected via chemiluminescence (Amersham International).

Myocardial p38 MAPK and MAPKAPK-2 Activity Assays
Myocardial p38 MAPK activity was determined with a commercially available assay performed according to the manufacturer’s instructions (p38 MAPKine Assay Kit; Cell Signaling Technology). Myocardial protein samples (200 μg) were immunoprecipitated overnight. The activity was determined by Western blot analysis using phospho-ATF-2 antibody (Cell Signaling Technology). Myocardial MAPKAPK-2 activity was determined after overnight immunoprecipitation of total protein of 100 μg using a phospho-specific assay (Upstate Biotechnology).

Urinary Protein Excretion
Subsets (n=8) of each group were selected randomly for the determination of 24-hour urinary protein excretion. Animals were placed in metabolism cages for the collection of urine at entry (baseline) and at 12 and 18 weeks. Urinary protein concentrations were determined and normalized to the 24-hour urine volume.
Statistical Analysis

All summary values are expressed as mean±SEM. A $\chi^2$ test was used for quantal analysis of survival data, a Fisher’s exact test was used for the comparison stroke incidence, and the Kruskal-Wallis test followed by Dunn’s multiple comparison test was used for the nonparametric analysis of TNF-α levels. All other multiple comparisons were made by ANOVA followed by post hoc analysis with the Bonferroni correction for multiple comparisons. All statistical analyses were done with InStat (GraphPad Software), and a value of $P<0.05$ was considered to be significant.

Results

Time Course of Myocardial p38 MAPK Phosphorylation

SP rats were maintained on an SFD to accelerate end-organ damage and subsequent premature mortality. Another group of SPs were maintained on ND. In the initial studies, myocardial p38 MAPK activity (ATF-2 phosphorylation) was examined in age-matched (16-week-old) SFD-SPs (n=6), ND-SPs (n=3), and normotensive Wistar-Kyoto rats (n=3). Myocardial p38 MAPK activity was consistently greatest in the SFD-SP group (Figure 1).

The time course of myocardial p38 MAPK phosphorylation was examined in the SFD-SP group before the start of the diet and at 3, 6, and 9 weeks. Myocardial p38 MAPK phosphorylation was increased at 3 weeks and remained elevated at 6 and 9 weeks in surviving animals (Figure 2). Myocardial p38 MAPK phosphorylation in decompensated (moribund) SFD-SPs (8.7±1.3 weeks), however, was not statistically different from that in the basal condition. Downstream MAPKAPK-2 activity showed a similar phosphorylation profile but remained elevated even in the moribund group (Figure 2).

Long-Term p38 MAPK Inhibition

To examine the functional significance of this persistent activation of myocardial p38 MAPK, SFD-SPs were treated long-term with an orally active and highly selective p38 MAPK inhibitor, SB239063. An additional study group of SPs, composed of age-matched littermates, received a SFD and served as untreated controls (SFD-SP). Pilot studies established a dietary dosing regimen for SB239063 that was sufficient to achieve steady-state plasma levels ≥400 ng/mL (≈1 μmol/L). Long-term treatment with SB239063 (1200 and 2000 ppm in the SFD), which was initiated at 12 weeks of age in the SFD-SPs (study week 0), abolished the prema-

Figure 1. Myocardial p38 MAPK activity (ATF-2 phosphorylation), extracted from LV, was assessed in age-matched (16 weeks) normotensive Wistar-Kyoto (WKY) rats, SP rats, and SDF-SP rats. These 2 sets of blots are representative of results obtained in 4 to 7 animals from each group.

Figure 2. Activation of p38 MAPK pathway in SFD-SP myocardium. a, Phosphorylation of myocardial p38 MAPK (top row) increased at 3 weeks, remained elevated throughout hypertrophic phase, and decreased with decompensation. MAPKAPK-2 activity (phosphorylation of hsp27; second row) had a similar profile. p38 MAPK protein (third row) remained constant. b, Histograms of ratio of p38 MAPK phosphorylation and MAPKAPK-2 activity to p38 MAPK protein for different time points. Error bars represent SEM for 3 different animals per time point ("P<0.05 vs baseline).
ure mortality observed in the untreated littermates on the SFD (Figure 3). No signs of morbidity/mortality were observed in any of the 40 high- and low-dose animals treated with SB239063. In contrast, 50% of the untreated SFD-SPs died within 17 weeks.

TNF-α Generation

Whole-blood TNF-α was examined in SB239063–treated and untreated SFD-SP groups. After 18 weeks of treatment with SB239063, basal levels of TNF-α in whole blood were significantly decreased compared with age-matched untreated SFD-SPs (Figure 4a). In addition, stimulation with LPS (0.1 µg/mL) produced an increase in TNF-α generation in whole blood obtained from the untreated group. In contrast, LPS failed to increase TNF-α generation significantly in whole blood obtained from animals treated long-term with the p38 MAPK inhibitor (Figure 4b).

Structural and Functional Assessment of the Heart

Echocardiography and hemodynamic monitoring were performed at entry and at 8 and 16 weeks of study in the treated and untreated SFD-SP groups. In decompensated animals (observed only in the untreated SFD-SP group), echocardiography was performed when signs of morbidity were noted. The study duration in the decompensated group was 11.3±1.4 weeks. Surviving untreated SFD-SP animals (compensated) exhibited marked LV hypertrophy, determined as anterior, posterior, and relative wall thickness, from 8 weeks onward compared with baseline and the treatment groups. LV hypertrophy was even more pronounced in the decompensated SFD-SP animals. These echocardiographic findings were consistent with the heart weight taken at the time of death (Table) and the reduction in both serum pro–atrial natriuretic peptide (ANF)11-67 and LV pro–ANF11-30 concentrations in SB239063-treated groups (data not shown). The decompensated group also showed a significant decrease in stroke volume, cardiac output, and cardiac index (Table). In contrast, treatment with the p38 MAPK inhibitor (SB239063) prevented cardiac hypertrophy and preserved cardiac function throughout the study period. The increase in systolic blood pressure was attenuated only at week 8 in SFD-SPs receiving the high dose (2000 ppm) of SB239063.

Vasoreactivity and Endothelial Function

Studies were performed to compare adrenergic vasoconstriction as well as endothelium-dependent and endothelium-independent vasorelaxation in the various study groups. Norepinephrine was a more potent and efficacious spasmogen in thoracic aortas obtained from the untreated SFD-SP than in thoracic aortas obtained from either of the SB239063-treated groups (Figure 5). The EC50 for norepinephrine in aortas from the untreated SFD-SP control group was 9.4±3.4 nmol/L, compared with 21.9±2.9 nmol/L and 20.2±1.6 nmol/L in the 1200- and 2000-ppm treatment groups, respectively (P<0.05). Endothelium-dependent vasorelaxation induced by carbachol was significantly compromised in the aortas obtained from the SFD-SP control group. Both the potency and efficacy of carbachol were reduced significantly compared with the SB239063-treated groups, eg, EC50=1574±607 nmol/L versus 66±10 nmol/L and 106±25 nmol/L in the 1200- and 2000-ppm treatment groups, respectively (P<0.05). Endothelium-independent relaxation induced by SNAP (1 µmol/L), however, was equivalent in all aortas. These results suggest that SB239063 preserved endothelial function in the SFD-SP.

Protection Against Stroke

The incidence of stroke was assessed with MRI (T2 and DWI) of the brain in treated and untreated SFD-SPs. MRI scans were performed at the 18-week time point or when animals became moribund. Evidence of stroke was prevalent in the untreated control SFD-SP group, especially in the moribund subset of animals (n=7). Of the 15 animals scanned in this group, 8 had evidence of stroke, 7 of which were moribund. The nature of the injury varied in type (MRI patterns indicative of hemorrhagic and ischemic stroke) and location (striatum versus cortex). In contrast, evidence of cerebral injury was rare in SFD-SPs treated with SB239063 (P=0.014 versus untreated SFD-SP group). Only 1 animal in the high-dose group (n=7) and none in the lower-dose group (n=7) had evidence of stroke (Figure 6).

Assessment of Renal Dysfunction

Renal dysfunction was evaluated in treated and untreated groups by examination of 24-hour total urinary protein...
Hemodynamic and Echocardiographic Analysis

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<td>HW, g</td>
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<td>1.45±0.03*</td>
<td>1.43±0.02†</td>
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BW indicates body weight; SBP, systolic blood pressure; HR, heart rate; SV, stroke volume; CO, cardiac output; CI, cardiac index; EDV, end-diastolic volume; ESV, end-systolic volume; AW, anterior wall thickness; PW, posterior wall thickness; and HW, heart weight. Values are mean±SEM.

*P<0.05 vs SFD-SP; †P<0.05 vs Decompensation.

Discussion

There is general agreement that MAPK cascades, activated by G protein–coupled receptor agonists, cytokines, and stretch, are involved in features of cardiac hypertrophy (reviewed by Sugden and Clerk). Recent data in transgenic animals underscore the activation of MAPK during cardiac hypertrophy, and in particular p38 MAPK. The present results extend previous findings by demonstrating (1) sustained myocardial p38 MAPK activation during hypertensive cardiac hypertrophy in SFD-SPs and (2) that long-term treatment with a selective p38 MAPK inhibitor, SB239063, reduces mortality, cardiac hypertrophy, and hypertensive end-organ damage.

Stimuli responsible for sustained p38 MAPK activation in the heart may include neurohormones, which are upregulated in this model; wall stress, which is increased because of the increased afterload; and proinflammatory cytokines.

Figure 5. Vasoreactivity studies were performed in isolated segments of thoracic aortas obtained at study week 18 from SFD-SP rats in control group (n=4) and SFD-SP rats in SB239063 treatment groups (1200 ppm, n=4, and 2000 ppm, n=4). Arteries obtained from untreated SFD-SP group were more sensitive to contractile effects of norepinephrine (a) and less sensitive to carbachol-induced relaxation (b) than SFD-SP rats treated with SB239063 (see text for EC⁵₀ and IC⁵₀). Maximal relaxation elicited by SNAP (1 μmol/L) was similar in all groups (c).

Figure 6. Cerebral damage (a) assessed by MRI (T2 and DWI) and incidence of stroke (b) in treated (1200 ppm, n=7, 2000 ppm, n=7, all at 18 weeks) and untreated SFD-SP rats (n=7 decompensated and n=8 at 18 weeks). T2 and DWI patterns indicative of hemorrhagic stroke (a) and ischemic stroke (b). Incidence of stroke was significantly reduced in SB239063 group (pooled data; *P=0.014, Fisher’s exact test).
With regard to some cytokines, p38 MAPK is known to regulate their production and actions, e.g., TNF-α and interleukin-1β. In the present study, plasma levels of TNF-α and LPS-induced TNF-α generation were reduced in groups receiving SB239063. Thus, it is tempting to suggest that the protective effects of SB239063 are related to inhibition of proinflammatory cytokines. It is noteworthy that phosphorylated p38 MAPK was reduced in moribund/decompensated animals. A similar downregulation of p38 MAPK activity was reported in the failing human heart.

The most striking finding in the present study was that long-term treatment with a specific second-generation p38α MAPK inhibitor, SB239063, abolished morbidity and mortality throughout the 18-week study period. Numerous p38 MAPK–dependent actions may contribute to this effect. Most notably, inhibition of p38 MAPK prevented cardiac hypertrophy and dysfuction. It has been shown in cultured cardiomyocytes that activated p38 MAPK can augment cell size and induce natriuretic peptides and that these effects can be inhibited pharmacologically by inhibiting p38 MAPK. In the present study, cardiac hypertrophy was greatly attenuated in SFD-SPs treated with SB239063. Surrogate markers of cardiac hypertrophy, plasma pro-ANP and LV pro-ANP were also reduced in animals treated with the p38 MAPK inhibitor. These effects on cardiac remodeling appear to be direct actions to inhibit cardiomyocyte hypertrophy and not secondary to reductions in blood pressure.

The association of hypertensive renal damage with endothelial dysfunction has been described in both the clinical and experimental settings and is manifested mainly as a decrease in endothelial function. The association of endothelial dysfunction, p38 MAPK, and stroke, however, remains speculative.

Finally, hypertensive renal dysfunction/damage, based on increases in 24-hour protein excretion, was markedly reduced in groups receiving SB239063. The present results suggest that p38 MAPK may play a role in transducing the deleterious effects of stretch and/or angiotensin II in the kidney. Beneficial effects mediated by the preservation of endothelial function may also contribute to the preservation of renal function.

Collectively, the data strongly suggest that sustained activation of myocardial p38 MAPK plays a critical role in the development of hypertensive cardiac hypertrophy and subsequent dysfunction. Long-term treatment with a selective p38 MAPK inhibitor, SB239063, provided a remarkable reduction in morbidity, mortality, and hypertensive end-organ damage and dysfunction. The efficacy observed with long-term p38 MAPK inhibition may represent a common signaling pathway approach to the treatment of heart failure that differs fundamentally from the traditional concept of blocking specific neurohormone receptors.

References


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