p38 MAP Kinase Mediates Inflammatory Cytokine Induction in Cardiomyocytes and Extracellular Matrix Remodeling in Heart

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Background—Increasing evidence suggests that development of heart failure involves activation of stress-response inflammatory cytokines, including tumor necrosis factor-α and interleukin-6. Yet, the myocyte contribution to their induction in failing hearts and the underlying regulatory mechanism in stressed myocardium remain unclear.

Methods and Results—In cultured cardiac myocytes, specific activation of stress-activated mitogen-activated protein kinase, p38, by upstream activator MKK6bE led to significant induction of tumor necrosis factor-α and interleukin-6 secretion, whereas treating cells with a selective p38 inhibitor (SB239068) significantly blocked the cytokine secretion from myocytes and increased their intracellular accumulation. Targeted expression of MKK6bE in transgenic hearts also resulted in a marked elevation in plasma tumor necrosis factor-α and interleukin-6; oral administration of SB239068 resulted in a significant reduction in their plasma levels but an increase in intracardiac accumulation of both cytokines. MKK6bE transgenic hearts developed marked interstitial fibrosis with increased matrix metalloproteinase abundance and selective induction of tissue inhibitor of matrix metalloproteinase-1; this extracellular matrix remodeling was also significantly attenuated by p38 inhibition. Along with cytokine induction and extracellular remodeling, MKK6bE transgenic animals displayed impaired hemodynamic function, whereas p38 inhibition improved the cardiac performance and prolonged the survival of the animals.

Conclusions—Stress-activated p38 kinase is a critical regulator of inflammatory response in cardiomyocytes with significant contribution to pathological remodeling in stressed myocardium. Inhibition of p38 may represent a useful therapeutic avenue to ameliorate cardiac pathology and heart failure evolution. (Circulation. 2005;111:2494-2502.)

Key Words: inflammation ■ cardiomyopathy ■ signal transduction ■ heart failure

Heart failure is one of the most common causes of morbidity and mortality among the adult population, affecting >5 million individuals in the United States alone.1 Whether caused by increased pressure load (eg, hypertension), ischemic injury, or infection, common features are observed, particularly at the late stage of disease, including contractile dysfunction and extracellular matrix (ECM) remodeling.2 Interventions to blunt or reverse such remodeling are thought to have a significant impact on ameliorating the progression of heart failure.3

The underlying regulatory mechanism for ECM remodeling has been the subject of intense study. In both human failing hearts and experimental animal models, chronic induction of inflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) is associated with the onset and progression of cardiac remodeling.4–8 Indeed, targeted expression of TNFα and augmenting IL-6 signaling in the heart result in lethal cardiomyopathy associated with extensive ECM remodeling.9–14 Inflammatory cytokines were originally thought to be produced by circulating lymphocytes such as macrophages induced by endotoxin or other stress stimulation.15 However, recent evidence suggests a direct role of cardiomyocytes in inflammatory gene induction.5,16 A key regulatory pathway for TNFα and IL-6 synthesis in macrophages and T cells involves a highly conserved family of stress-activated mitogen-activated protein (MAP) kinase, p38.17 Phosphorylation of transcription factors by p38 MAP kinases, including ATF2, NF-κB, and MEK-2, triggers transcriptional activation of TNFα.17 Other regulatory steps in TNFα synthesis also involve mRNA stabilization and membrane shedding via TNFα converting enzyme (TACE) activities,18–22 although the specific role of p38 in these processes has not been well established. Similarly, p38 activity has been implicated in the induction of IL-6 in cardiomyocytes in

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culture, although its regulatory function in vivo has not been demonstrated. Proinflammatory response has long been recognized in stressed myocardium during pathological remodeling, however, the molecular nature of stress signaling pathways involved in their induction is unclear.

We and others have reported that p38 is activated in hearts under pressure overload, ischemia, and other pathological conditions. Using targeted transgenesis, we showed that p38 activation in ventricular myocytes was sufficient to induce restrictive cardiomyopathy associated with marked interstitial fibrosis. In the present study, using both genetic and pharmacological approaches, we have provided in vitro and in vivo evidence that activation of p38 MAP kinase in cardiomyocytes is sufficient to induce IL-6 and TNFα expression and release. The p38-mediated inflammatory induction is directly associated with cardiac ECM remodeling and contractile dysfunction, and p38 activity plays a novel role in regulating IL-6 and TNFα protein secretion in cardiac myocytes. These data demonstrate that modulation of p38 activity can potently affect cardiac chamber remodeling and contractile dysfunction involving proinflammatory cytokine induction. Therefore, p38 as a key stress-inducible signaling pathway represents a potential target of therapy for heart failure.

Methods

Animals and Treatment Procedure

MKK6bE transgenic mice were generated by breeding floxed-MKK6bE mice with the Cre recombinase transgenic mice controlled under myosin light chain-2v promoter (MLC-2v/Cre) as described previously. The double-transgenic offspring of floxed-MKK6bE and MLC-2v/Cre (designated MKK6bE mice) were screened by PCR. Therefore, p38 as a key stress-inducible signaling pathway represents a potential target of therapy for heart failure.

Cardiomyocyte Culture and Adenovirus Infection

Neonatal rat ventricular cardiomyocytes were prepared using collagenase-protease digestion from 1-day-old Sprague-Dawley rats as reported previously. Isolated cardiomyocytes were grown overnight in medium containing DMEM/199 (4:1) supplemented with 10% horse serum, 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mmol/L glutamine, followed by γ-irradiation (35 Gy) to limit the proliferation of noncardiomyocytes and to maintain cardiomyocyte levels at >95%. Cells were then incubated in serum-free DMEM and infected with adenovirus at a multiplicity of infection of 100 particles per cell 2 days before harvesting and analysis. Then, 20 μmol/L SB239068 (Calbiochem) or 70 μmol/L TACE inhibitor (579050, TAPI-0, Calbiochem) was added when adenovirus infection was applied. For TNFα treatment, recombinant TNFα (RD Systems) was added in serum-free medium with a final concentration of 50 ng/mL for 10, 30, 60, 90, and 120 minutes of incubation before analysis.

Immunoblotting and Kinase Assay

Snap-frozen left ventricle was smashed on dry ice and homogenized in lysis buffer containing 20 mmol/L Tris (ph 7.4), 150 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 μg/ml leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Protein from cultured neonatal cardiomyocytes was isolated in the same lysis buffer combined with brief sonication and centrifugation. Protein concentration was determined with a BCA protein assay kit (Pierce, Inc.). Equal amounts of protein were separated on NuPAGE 4% to 12% Bis-Tris Gel (Invitrogen) and electroblotted onto Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech). Polyclonal antibodies for p38, phospho-p38 MAP kinase (Cell Signaling, Inc.), TNFα, and tissue inhibitor of matrix metalloproteinase (TIMP)-1/2/3 (Santa Cruz Biotechnology) were used according to manufacturers’ recommendations. Horseradish peroxidase–conjugated goat anti-rabbit IgG was used as secondary antibodies (Sigma). The reactions were developed with SuperSignal West Pico chemiluminescent substrate (Pierce, Inc.) and exposure to autoradiographic film. Signal intensity was quantified from scanned films with Scion NIH Image software (Scion). Kinase assay for p38 was performed with a p38 MAP kinase assay kit (Cell Signaling, Inc.) according to manufacturer’s protocol using recombinant ATF-2 as substrate.

RNA Preparation and Real-Time Reverse-Transcriptase PCR

Total RNA was extracted from snap-frozen left ventricle tissues with Trizol (Invitrogen) following manufacturer’s instructions. The first-strand cDNA was reverse transcribed (RT) with oligo(dT) primer using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time RT-PCR was carried out in a DNA Engine Opticon System (MJ Research) with SYBR Green I (Molecular Probes, Inc) as fluorescent dye. Primer pairs for TNFα and GAPDH were selected according to Overbergh et al. PCR thermal cycling conditions were as follows: 10 seconds at 96°C, 20 seconds at 60°C, and 20 seconds at 72°C, with total of 45 cycles. Expression level of TNFα and COX-2 was presented as fold induction over controls after normalization against GAPDH signals.

MMP Assay

Matrix metalloproteinase (MMP) abundance in cardiac tissue was measured by gelatin zymography (Invitrogen). Whole-heart extract was prepared following the method by Brower and Janicki. Briefly, 20 to 25 mg frozen heart tissues was smashed and agitated for 48 hours at 4°C in 0.5 mL ice-cold extraction buffer containing 10 mmol/L cacodylic acid, 150 mmol/L NaCl, 1 mmol/L ZnCl2, 20 mmol/L CaCl2, 3 mmol/L Na2S, and 0.01% Triton X-100. The supernatant was collected after mixing with 100 μL of 0.1 mol/L Tris-HCl buffer (ph 7.4) and centrifugation at 13 000g for 4°C for 15 minutes. Equal amounts of protein from each sample were mixed with Tris-glycline SDS sample buffer without reducing agents and separated in gelatin zymography gel by electrophoresis. Subsequently, the gel was incubated in 2.5% Triton X-100 for 30 minutes, followed by incubation in 10 mmol/L Tris-HCl (ph 7.4), 0.2 mol/L NaCl, 5 mmol/L CaCl2, and 0.02% Brij 35. The processed gel, stained with 0.5% Coomassie blue R250 and destained in 30% MeOH and 10% acetic acid to reveal the lytic bands, was scanned into digital images.

TNFα and IL-6 ELISA

Mouse blood was collected into a plasma separation tube (Becton Dickinson) and immediately centrifuged. The 24-hour conditioned medium from cultured neonatal rat cardiomyocytes was collected 48 hours after adenovirus infection with or without SB239068 or TACE inhibitor treatment. Cell pellets were sonicated in lysis buffer containing 50 mmol/L Tris-HCl (ph 7.5), 75 mmol/L NaCl, and 1 mmol/L PMSF. Cell lysates were centrifuged at 10 000g at 4°C for 15 minutes. IL-6 and TNFα concentrations were measured by the Cytokine Core Laboratory at the University of Maryland (Baltimore) using standard 2-antibody ELISA with commercial antibody pairs and recombinant standards from Endogen.

TACE Activity Assay in Heart Tissues

TACE activity was measured by the ability to cleave the internally quenched fluorescent peptide according to published procedure. Briefly, heart tissues were homogenized in buffer containing...
10 mmol/L sodium phosphate buffer (pH 7.4), 1 mmol/L MgCl₂, 30 mmol/L NaCl, 0.02% NaN₃, 0.2 μg/ml DNase, and 1 μmol/L PMSF. The homogenates were centrifuged for 20 minutes at 12 000 rpm and 4°C. Pellets were dissolved in buffer with 10 mmol/L Tris HCl (pH 8.0), 1 mmol/L MgCl₂, 30 mmol/L NaCl, and 1% octylglucoside and rotated for 1 hour at 4°C. Supernatant was collected by centrifuging lysates for 20 minutes at 12 000 rpm and 4°C. TACE activity assay was performed with 20 μmol/L fluorescent peptide (Biomol) and 40 μg heart tissues extracts for 1 hour at 37°C. The final volume was adjusted to 100 μL with the TACE assay buffer containing 50 mmol/L Tris-HCl (pH 7.4), 5 mmol/L CaCl₂, 0.002% NaN₃, and 0.002% Brij35. Background control was set up by adding 20 μmol/L fluorescent peptide to the TACE assay buffer. TACE inhibitor (579050, TAPI-0, Calbiochem) was added as control for assay specificity. Fluorescent density was measured with the Perkin Elmer VICTOR III Multilabel Counter with a 340-nm excitation filter and 430-nm emission filter. Sample fluorescence density was normalized by subtracting background from original readings.

**Histological Analysis**

Heart was excised and fixed in 4% paraformaldehyde PBS overnight, followed by dehydration, paraffin embedding, and sectioning at 5-μm thickness. Picrosirius red staining was performed by sequential treatment of 0.2% phosphomolybdic acid for 2 minutes, 0.1% Sirius red solution for 90 minutes, and 0.01N HCl for 2 minutes. Collagen fibers were identified by red fluorescent signal under confocal microscopy with a Rhodamine filter set.

**Hemodynamic Measurements**

Hemodynamic measurements were performed as previously described.27 Briefly, anesthetized animals were ventilated, the chest was opened to expose the ventricular apex, and a miniature pressure-volume catheter (SPR-839, Millar Instruments) was inserted into the heart with the distal end placed in the aortic root. Pressure-volume data were digitized at 2 kHz and calibrated as described27; hemodynamic function was assessed from 6 to 10 digitally averaged cycles with custom software.

**Statistical Analysis**

Results are expressed as mean±SD. Statistical significance was evaluated with Student’s t test or ANOVA, with values of P<0.05 considered significantly different.

**Results**

p38-Mediated Regulation of Inflammatory Genes in Cardiac Myocytes

To investigate the functional role of p38 activity in inflammatory gene regulation in cardiomyocytes, we first tested whether p38 activation is sufficient to induce inflammatory genes in cardiomyocytes. Neonatal rat cardiomyocytes were transfected with MKK6bE-expressing adenoviruses shown to activate p38 without affecting other MAP kinase pathways (Figure 1A).33 ELISA analysis of the conditioned media detected dramatically elevated levels of TNFα (Figure 1B) from cells infected with adv/MKK6bE vector compared with untreated controls. Cotreatment with the p38 inhibitor SB239068 or TACE inhibitor TAPI-0 blocked 72% or 97% of MKK6bE-induced TNFα secretion, respectively (Figure 1B). Similarly, the IL-6 level in conditioned media detected only in MKK6bE-expressing myocytes was also completely blocked by treatment with SB239068 (Figure 1C). This suggests that p38 activation in cardiomyocytes is sufficient to induce high levels of secretion of proinflammatory cytokines.

Interestingly, inhibition of p38 activity in MKK6bE-expressing cells by SB239068 treatment also significantly increased the intracellular contents of TNFα as detected from cell pellets in a manner similar to that of cells treated with TACE inhibitor (Figure 1D). Similar intracellular accumulation of IL-6 on SB239068 treatment was also observed (Figure 1E).

**Figure 2.** Modulation of p38 activities in intact heart. A, p38 activity was measured from immunoprecipitated phosphor-p38 with recombinant ATF-2 as substrate. Phosphorylation of ATF-2 at Thr71 was detected by immunoblotting with anti–phospho-ATF-2 antibody (arrow). B, Quantification of kinase activity showing that p38 activity is significantly increased in MKK6bE transgenic heart, which was then attenuated on SB239068 treatment. *P<0.05, **P<0.01 vs control; #P<0.05, ##P<0.01 vs MKK6bE-infected cells.
p38-Mediated Inflammatory Gene Induction in Intact Hearts

To further investigate whether p38 activation in cardiomyocytes was sufficient to induce inflammatory genes in intact hearts, MKK6bE was targeted in ventricular myocytes with a gene-switch transgenic approach as reported.27 As shown before, these transgenic hearts had significantly elevated p38 activity27 that can be attenuated by oral treatment with the p38 inhibitor SB239068 (Figure 2). Correlating with this activity, TNFα mRNA level increased in the transgenic hearts 3-fold over control animals based on quantitative real-time RT-PCR (11.38±0.42 versus 3.75±0.86 arbitrary units; \( P<0.01 \)), and SB239068 treatment reversed TNFα mRNA to nearly baseline levels (5.67±1.45; \( P<0.01 \) versus transgenic, \( P=NS \) versus controls). Along with elevated p38 activity, high levels of secreted TNFα and IL-6 protein were detected in plasma from MKK6bE transgenic mice compared with their basal levels in control animals (Figure 3A and 3C). Correlated with the increase of cytokines in serum, the intramyocardial level of pro-TNFα was increased as determined by immunoblot (Figure 3B), whereas the IL-6 level was decreased as measured by ELISA (Figure 3D), suggesting an activate state of cytokine production and secretion. Similar to our in vitro observations, oral SB239068 treatment in the transgenic animals significantly reduced TNFα plasma levels by 41% and IL-6 plasma levels by 46% (\( P<0.05 \) versus both control and MKK6bE) (Figure 3A and 3C). On the other hand, intracardiac levels of pro-TNFα and IL-6 were increased in SB239068-treated animals (Figure 3B and 3D). However, activation of p38 did not have any significant effect on total TACE-dependent protease activities in heart (Figure 3E). All these data provide the first clear in vivo evidence that activation of p38 in ventricular myocytes is sufficient to induce inflammatory cytokines, including IL-6 and TNFα, and that p38 activity has a critical role in cytokine processing and secretion in myocytes independently of TACE regulation.

TNFα-Induced p38 Activation in Cardiomyocytes

To further investigate the potential interaction between p38 activity and TNFα, we determined the effect of TNFα treatment on p38 kinase activity in cardiomyocytes. As shown in Figure 4, adding 50 ng/mL recombinant TNFα in cultured cardiomyocytes elicited strong p38 activation that lasted >90 minutes. Combined with the above observation regarding p38-mediated TNFα expression, these data suggest a potential positive feedback loop between p38 MAP kinase activation and TNFα induction.

p38-Mediated ECM Remodeling

Previous studies have demonstrated that chronic activation of TNFα expression in heart leads to severe cardiomyopathy characterized by interstitial fibrosis and increased MMP abundance and activity and TIMP-1 expression,10,12 whereas enhanced IL-6 signaling in heart led to cardiomyopathy also associated with extensive remodeling. MKK6bE transgenic hearts demonstrated marked interstitial fibrosis (Figure 5),
which was substantially blunted by SB239068 treatment. MMP abundance/activity increased in MKK6bE heart as revealed by in vitro zymography, most notably in the kilodalton range, consistent with MMP2 (Figure 6A and 6B). However, TIMP-1 expression was also selectively induced in MKK6bE transgenic hearts and reversed to normal levels after SB239068 treatment (Figure 6C). Therefore, the induced fibrosis in MKK6bE transgenic heart may be contributed by increased TIMP-1 expression, which leads to reduced net MMP activity. Reduced TIMP-1 expression after SB239068 treatment would release MMP activity to reduce fibrosis. These data suggest that p38-mediated cardiac remodeling involves induced interstitial fibrosis with altered ECM biosynthesis and degradation.

Effects of p38 Activity on Chamber Remodeling and Cardiac Function

Cardiac function and remodeling were studied by in vivo pressure-volume relationships (Figure 7A and the Table). MKK6bE transgenic hearts exhibited impaired contractility (eg, reduced end-systolic elastance with leftward shift of the end-systolic pressure-volume relation, reduced preload-recruitable stroke work, ventricular peak power index; all P<0.01). In addition, there was profound diastolic stiffening, with increased end-diastolic pressure and chamber elastic stiffness, as well as relaxation delay. The net result was reduced filling and ejection, resulting in a marked fall in cardiac output. SB239068 treatment reduced end-diastolic and end-systolic chamber stiffening, likely a consequence of the decline in fibrosis, but had little direct effect on contractile dysfunction. Importantly, net chamber filling and output were restored toward baseline despite persistently depressed systolic function. As shown in Figure 7B, this was accompanied by a prolonged life span, with 50% survival at 16 weeks of age versus 0% survival in untreated MKK6bE animals. Together, these results support the notion that cardiac ECM remodeling plays an important role in cardiac dysfunction and long-term prognosis of heart failure.

Discussion

Inflammation, particularly induction of TNFα, is an important stress response in various cell types, and elevated plasma levels of inflammatory cytokines are linked to heart failure in clinical and animal studies. Recent transgenic models have provided compelling evidence that TNFα and IL-6 induction is important to the pathological heart remodeling, particularly in the ECM. However, it has been unclear whether cardiomyocytes themselves can significantly contribute to cytokine induction and, if so, what signaling pathways/mechanisms contribute to this process. The present study provides direct in vivo and in vitro evidence that p38 activation is sufficient to induce inflammatory cytokine expression in cardiomyocytes and that p38 activity has a critical role in cytokine secretion from cardio-
TNFα induction is observed in many different forms of stressed myocardium and may play a protective role in response to acute myocardial injury when the induction is self-limiting and transient as in innate immune responses. However, prolonged induction of TNFα in failing hearts is believed to exert a detrimental effect by triggering a spectrum of pathological changes, including loss of contractility, apoptosis, contractile dysfunction, and ECM remodeling. The underlying regulatory mechanism that governs the induction pattern of TNFα in heart has not been clearly defined. Interestingly, induction of p38 activity is also associated with a wide spectrum of cardiac pathologies overlapping extensively that observed for TNFα.

More importantly, p38 activation has been implicated as both protective and detrimental in response to cardiac injury (reviewed elsewhere). Our data showed that TNFα but not TGF-β is a predominant cytokine induced by p38, whereas TNFα also induced sustained p38 activation. Therefore, the interplay between p38 and TNFα in cardiomyocytes as reported in this study may represent a physiologically significant signaling mechanism linking their induction and dysregulation in compensatory and pathological responses.

In hearts failing from different diseases, IL-6 level is highly correlated with the severity of cardiomyopathy. Cardiomyocytes express very little IL-6 receptor, but circulating IL-6 can bind to secreted IL-6 receptor to induce gp130 signaling in cardiomyocytes. The downstream signaling of gp130, including JAK and STAT3, has been largely implicated in cardiac protection and hypertrophic remodeling in hearts. Although it is conceivable that elevated IL-6 and gp130 signaling contributes to lack of apoptosis in p38 activated hearts, the specific role of IL-6 in extracellular remodeling of the heart is still unknown, and further studies are needed.

Both IL-6 and TNFα expression are regulated at multiple levels, from modulation of transcriptional activity and mRNA stability to a proteolytic step from membrane-bound 26-kDa pro-TNFα protein to 17-kDa secreted form via TACE. Our data from MKK6bE transgenic hearts showed that activation of p38 MAP kinase resulted in a ~3-fold increase in cardiac TNFα mRNA. Several transcription factors involved in TNFα transcriptional regulation are reported as downstream substrates of p38, including ATF5, MEF-2, and C/EBP. Tristetraprolin is also a p38-regulated protein involved in stabilizing TNFα mRNA. Further studies are needed to determine whether and how much the induction of TNFα mRNA by p38 in cardiomyocytes is due to transcriptional induction or to an increase in mRNA stability. On the other hand, accumulation of pro-TNFα protein in heart and cultured myocytes after p38 inhibition was an unexpected result, implying that p38 activity has an additional regulatory role in TNFα protein processing and secretion. Because TACE activity assay did not detect any changes in all experimental hearts regardless of their p38 activity and
may have physiological significance because secreted and membrane-bound pro-TNFα are shown to have distinct biological consequences in cardiac remodeling. Recent studies showed that 17-kDa secreted TNFα induces ECM remodeling and chamber dilation, whereas 26-kDa membrane-bound pro-TNFα triggers concentric hypertrophy without interstitial remodeling. It is possible that p38 activity is part of the regulatory mechanism that determines the overall ratio of pro-TNFα to secreted TNFα in stressed myocardium that leads to a differential pattern of remodeling. Finally, although several studies have illustrated the beneficial effect of p38 inhibition in stressed heart related to inflammatory cytokine regulation, our study provides the first direct in vivo and in vitro evidence to implicate p38-mediated TNFα secretion and ECM remodeling as potential downstream mechanisms. Further studies will help to determine both the underlying molecular nature and potential benefit in targeting p38 pathway as a new venue to ameliorate the progression of heart failure.

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