ASK1 Regulates Cardiomyocyte Death but Not Hypertrophy in Transgenic Mice

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Rationale: Apoptosis signal-regulating kinase (ASK)1 is a central upstream kinase in the greater mitogen-activated protein kinase cascade that mediates growth and death decisions in cardiac myocytes in response to diverse pathological stimuli.

Objective: However, the role that ASK1 plays in regulating the cardiac hypertrophic response in vivo remains controversial.

Methods and Results: Here, we generated mice with cardiac-specific and inducible overexpression of ASK1 in the heart to assess its gain-of-function effect. ASK1 transgenic mice exhibited no induction of cardiac hypertrophy or pathology at 3 and 12 months of age, and these mice showed an identical hypertrophic response to controls following 2 weeks of pressure-overload stimulation or isoproterenol infusion. Although ASK1 overexpression did not alter the cardiac hypertrophic response, it promoted cardiomyopathy and greater TUNEL following pressure-overload stimulation and myocardial infarction. Indeed, ASK1 transgenic mice showed a greater than 2-fold increase in ischemia reperfusion-induced injury to the heart compared with controls. Examination of downstream signaling showed a prominent activation of mitogen-activated protein kinase kinase 4/6 and c-Jun NH2-terminal kinase (JNK)1/2 (but not p38 or extracellular signal-regulated kinases [ERKs]), inhibition of calcineurin-NFAT (nuclear factor of activated T cells), and induction of Bax in the hearts of ASK1 transgenic mice following 1 and 8 weeks of pressure-overload stimulation. Mechanistically, cardiomyopathy associated with ASK1 overexpression after 8 weeks of pressure overload was significantly reduced in the calcineurin Aβ–null (CnAβ–/–) background.

Conclusions: These results indicate that ASK1 does not directly regulate the cardiac hypertrophic response in vivo, but it does alter cell death and propensity to cardiomyopathy, in part, through a calcineurin-dependent mechanism. (Circ Res. 2009;105:1110-1117.)

Key Words: myocardial infarction ■ apoptosis ■ hypertrophy ■ mitogen-activated protein kinase ■ signaling
tutively active mutant of ASK1 in a wide variety of cell types induced apoptosis, whereas expression of dominant negative ASK1 blocked tumor necrosis factor (TNF)-α-, oxidative stress-, anticancer agent-, and growth factor withdrawal–induced cell death.8–13 Mouse embryonic fibroblasts generated from Ask1−/− mice were also resistant to TNFα- and H2O2-induced cell death.14 In the heart, ASK1 has been implicated as a mediator of cellular remodeling and apoptotic and necrotic cell death. Indeed, Ask1−/− mice exhibited reduced ventricular remodeling in response to angiotensin II infusion, myocardial infarction (MI), and pressure-overload stimulation.15,16 Cardiomyocytes generated from Ask1−/− mice were also resistant to H2O2-induced apoptosis.16 In vivo, Ask1−/− mice showed less apoptotic and necrotic cell death following ischemia-reperfusion (I/R) injury to the heart.17

Although ASK1 is prominently activated by hypertrophic stimuli in vitro and in vivo, such as pressure overload, I/R, and agonist treatments,16–18 its mechanistic involvement in directing the cardiac growth program has become an area of controversy in the literature. For example, Ask1−/− mice showed reduced hypertrophy following angiotensin II infusion, suggesting that ASK1 positively regulates cardiac growth,15 whereas studies in cultured neonatal myocytes with activated and dominant negative ASK1 suggested a growth inhibitory effect.19 c-Raf-I−/null mice showed abundant ASK1 activity in the heart without corresponding hypertrophy,20 and pressure overload induced by transverse aortic constriction (TAC) did not result in less cellular hypertrophy in Ask−/− mice compared with wild-type (WT) mice.16 More recently, Ask1−/− mice were even reported to develop more cardiac hypertrophy following 4 weeks of swimming exercise, suggesting that it is normally antihypertrophic to this form of physiological stimulation.21 Thus, the role of ASK1 as a hypertrophic regulator is far from settled, despite its prominent effect in controlling p38 and JNK signaling in the heart.

Methods

Animals
A tetracycline-responsive binary α-myosin heavy chain transgene system permitted temporally regulated expression of ASK1 in myocytes of the heart.22 TAC was performed as described previously.22 Transthoracic echocardiography to measure cardiac dimensions and pressure gradients across the aortic constriction was performed as described previously.24 Alzet 1002 osmotic minipumps (Cupertino, Calif) either filled with isoproterenol (60 mg/kg per day in PBS) or PBS were implanted under the skin for 2 weeks with a routine surgical procedure. The surgical procedure for I/R or MI injury in the mouse and analysis of injury area have been described previously.25 Luciferase assays from NFAT-luciferase reporter mice were performed as described previously.23 Calcineurin Aβ−/− mice have been described previously.26

Histological Analysis, TUNEL, Western Blotting, and Kinase Assays
Assessment of TUNEL from paraffin sections was performed with TMR Red In Situ Death Detection Kit (Roche Diagnostics) according to the instructions of the manufacturer (Roche Diagnostics).24 Protein extraction from mouse heart and subsequent Western blotting followed by enhanced chemiluminescence detection was performed as described previously.23,25 ASK1 activity assays with recombinant MKK6 has been described previously.19

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Generation of ASK1-Inducible Transgenic Mice
To further examine the function of ASK1 in vivo, we generated inducible, cardiac-specific transgenic mice expressing mouse ASK1. Heart-specific and -inducible expression was achieved with a binary α-myosin heavy chain promoter–based transgene strategy (Figure 1A).22 Two independent responder transgenic lines were generated (4.1 and 11.5) that each permitted expression of ASK1 in the heart only in the presence of the driver transgene encoding the tetracycline transactivator (tTA) protein (double transgenic [DTG]) when Dox is absent (Figure 1B). Administration of Dox completely eliminated expression of ASK1 protein in DTG mice in both lines (Figure 1C). Most subsequent experiments were performed with Dox administration for the first 3 weeks of life, which blocks developmental expression, followed by removal of Dox thereafter to selectively permit transgene expression beginning only in young adulthood and thereafter (Figure 1C). Despite relatively high levels of ASK1 protein overexpression in the adult heart, no hypertrophy or reductions in cardiac ventricular performance were noted at 3 and 12 months of age (Figure 1D through 1G), nor were signs of histopathology observed (data not shown). These results indicate that ASK1 overexpression in the heart is without appreciable effect.

ASK1 Overexpression Does Not Enhance Stimuli-Induced Hypertrophy
ASK1 is not normally activated in unstimulated hearts; hence, overexpression of this protein might require costimulation to achieve activity given exquisite silencing by coregulatory proteins in vivo. Because ASK1 is strongly induced by pressure overload,16 we predicted that TAC stimulation would unmask silencing of ASK1, permitting alteration of the
hypertrophic response as a result of its overexpression. However, 2 weeks of TAC stimulation in ASK1 DTG mice at 12 weeks of age showed an identical hypertrophic response to WT or tTA control mice, with equivalent pressure gradients across the aortic constriction (Figure 2A and 2B). Measurement of cell surface area also showed no difference in hypertrophy between the 3 groups of mice, nor was ventricular performance significantly altered after 2 weeks of TAC stimulation (Figure 2C and 2D). Isoproterenol was used as another hypertrophic stimuli to extend and confirm the pressure overload results. Once again, ASK1 DTG mice showed no difference in cardiac hypertrophic growth at the whole organ or cellular level compared with WT or tTA control mice (Figure 2E and 2F). Thus, greater ASK1 expression did not alter the baseline growth of the heart or significantly alter stimulus-induced hypertrophy.

**ASK1 DTG Mice Are More Susceptible to Ischemic Injury**

Although ASK1 overexpression had no effect on the hypertrophic response, it did alter myocyte death following ischemic injury, indicating that the transgene is functional and that ASK1 can have an important biological effect in the heart. Specifically, ASK1 DTG mice showed worse ventricular performance 2 and 3 weeks after MI compared with WT and tTA control groups, although sham mice were unaffected (Figure 3A and 3B). This greater reduction in ventricular performance in ASK1 DTG mice was associated with more expansive and thinner scars in the left ventricle, as assessed from histological sections (Figure 3C). More importantly, careful quantitation of TUNEL in the peri-infarct region from histological sections 21 days after MI showed nearly a 2-fold increase in DTG mice versus control mice (mixture of WT and tTA) (Figure 3D). At this same 21-day time point, DTG mice showed a much greater induction of ASK1 activity, as measured with a direct kinase assay in vitro (Figure 3E). The MI model was performed by permanent ligation of the left coronary artery to analyze secondary remodeling attributable to enhanced ASK1 activity over extended periods of time. We also performed an acute cell death model involving I/R injury by coronary artery occlusion for 60 minutes followed by 24 hours of reperfusion. ASK1 DTG mice showed a 2-fold increase in total area of infarction in this acute assay, whereas the area at risk was not different from WT or tTA controls (Figure 3F and 3G). Taken together, these results indicate that ASK1 can directly enhance myocyte death following MI or I/R injury.

**ASK1 DTG Mice Exhibit Cardiomyopathy With Long-Term Pressure Overload**

Whereas ventricular performance in ASK1 DTG mice was unaffected after 2 weeks of TAC stimulation, 8 weeks of...
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cineurin is required to activate ASK1 in cultured cardiomyocytes. Thus, ASK1 overexpression in cardiomyocytes.19 Thus, it was critical to investigate what

TAC uncovered a greater propensity toward heart failure and cell death. ASK1 DTG mice showed significantly greater increases in heart weight normalized to body weight compared with WT and tTA mice after 8 weeks of TAC (Figure 4A). However, careful assessment of cardiomyocyte cross-sectional surface areas showed no greater cellular hypertrophy compared with controls, suggesting that the greater increase in heart weight was attributable to dilation and failure (Figure 4B). Consistent with this interpretation, ASK1 DTG mice exhibited a significant reduction in ventricular fractional shortening and chamber dilation in systole and diastole (Figure 4C through 4E). Hearts from ASK1 DTG mice also developed significant fibrosis after TAC, as assessed by Masson’s trichrome staining of histological sections, but not in WT and tTA hearts (Figure 4F). This increase in fibrosis was also associated with significantly greater levels of TUNEL after TAC stimulation in hearts of DTG mice (Figure 4G). Thus, increased ASK1 expression predisposed the heart to cardiomyopathy associated with greater levels of TUNEL and fibrosis after 8 weeks of pressure-overload stimulation, but not more cellular hypertrophy.

**ASK1 Regulates JNK and Calcineurin Signaling in the Heart**

In addition to regulating JNK and p38, we previously demonstrated that ASK1 could alter calcineurin-NFAT activation in cardiomyocytes.19 Thus, it was critical to investigate what signaling effectors might be altered by ASK1 overexpression in the heart to gain insight into its mechanism of action. Consistent with the lack of a baseline phenotype, ASK1 overexpression in unstimulated hearts had no effect on activation of any MAPKK or MAPK factor at baseline, nor was calcineurin activity altered in NFAT-luciferase transgenic mice crossed to contain the ASK1-inducible transgenes (Figure 5A and 5C). We also performed direct ASK1 kinase assays with MKK6 protein as an in vitro substrate, and, once again, ASK1 overexpression did not induce activity at baseline (Figure 5B). However, TAC stimulation showed substantial induction of ASK1 kinase activity at 1 and 8 weeks and much greater phosphorylation of MKK4 and JNK1/2 in the hearts of ASK1 DTG mice compared with controls (Figure 5A and 5B). No activation was observed at 10 minutes, 60 minutes, 6 hours, or 1 day of TAC, nor was p38 or ERK1/2 appreciably activated by ASK1 overexpression at any time point investigated (Figure 5A). Proteins that alter the cellular apoptotic response were also investigated, revealing a significant increase in Bax protein levels in ASK1 DTG mice at 1 and 8 weeks of TAC, but no substantial alteration in Bak, Bcl-2, or Bcl-xl (Figure 5A). Thioredoxin protein levels were not changed by ASK1 overexpression at baseline or after TAC (Figure 5A). However, ASK1 overexpression did significantly reduce the increase in NFAT-luciferase activity following TAC stimulation (Figure 5C), consistent with our previous observations that ASK1 can antagonize calcineurin-NFAT signaling in cardiomyocytes.19 Thus, ASK1 overexpression selectively increases MKK4/6 and JNK1/2 activity in the heart after at least 1 week of TAC stimulation, as well as antagonizes calcineurin-NFAT signaling and leads to increased Bax protein levels.

**ASK1 Functions, in Part, Through Calcineurin in the Heart**

Calcineurin is a Ca2+-activated protein phosphatase that directly dephosphorylates members of the NFAT transcription factor family in the cytoplasm, promoting their translocation into the nucleus, where they participate in the transcriptional induction of various genes with specific inducible functions.28 The calcineurin-NFAT signaling circuit has been shown to play a central role in regulating the hypertrophic growth response and survival versus apoptotic decisions of cardiomyocytes.29 We previously demonstrated that calcineurin is required to activate ASK1 in cultured cardiomyocytes by dephosphorylation of serine 967, leading to dissociation of 14-3-3 proteins.19

Here, we crossed the ASK1-inducible transgenes into the CnAβ/−/− background to examine the hypothesis that reductions in calcineurin activity would render ASK1 less functional in overexpressing DTG mice. Indeed, when crossed into the CnAβ/−/− background, ASK1 overexpression no
longer promoted greater increases in heart weights normalized to body weight after 8 weeks of TAC stimulation, whereas DTG control mice from the same cross that had WT CnA/H9252 alleles showed proportionately greater increases in heart weight normalized to body weight (Figure 6A and 6B). Deletion of CnA/H9252 also partially protected ASK1 DTG mice from loss of ventricular performance and chamber dilation compared with DTG mice in the CnA/H9252/H11001 background (Figure 6C through 6F). The increase in myocardial fibrosis following 8 weeks of TAC in ASK1 DTG mice was also significantly less in the CnA/H9252/H11002 background (Figure 6G and 6H). Finally, we also measured ASK1 kinase activity in hearts, which, again, showed increases only in ASK1 DTG mice after TAC stimulation in the CnA/H9252/H11001 background and a substantial reduction in the CnA/H9252/H11002 background (Figure 6I). The results suggest that calcineurin participates in controlling the effectiveness of ASK1 signaling in vivo, consistent with our previous in vitro results.19

Discussion

ASK1 has emerged as a kinase of central importance in cardiac myocytes given its dominant role in regulating MAPK signaling and subsequent control of cell death. To gain additional insight into the full range of ASK1 function in the heart, we generated inducible transgenic mice to overexpress this protein in the adult heart. Although overexpression approaches in the mouse heart can produce nonphysiologic effects, numerous kinases and phosphatases have been overexpressed as a means of gaining functional insight. For example, overexpression of activated calcineurin or activated MEK1 in the mouse heart provided important mechanistic data regarding the function of these signaling factors in regulating the cardiac hypertrophic response.29,30 Also noteworthy was that not all overexpressed signaling proteins in the heart produce a phenotype, because ERK2 overexpression with the /-myosin heavy chain promoter was without effect, nor did it enhance pressure overload hypertrophy.30 In a similar manner, ASK1 transgenic mice had no discernable phenotype up to 12 months of age, despite very high levels of overexpression.
ASK1 overexpression in the heart was likely without baseline effect given the many levels of regulation imposed on this kinase. ASK1 is uniquely activated by phosphorylation of threonine 845, whereas dephosphorylation of serine 83, serine 967, and serine 1034 results in activation.\textsuperscript{27} We previously determined that calcineurin enhances ASK1 activation through direct dephosphorylation of serine 967 and an indirect increase in threonine 845 phosphorylation.\textsuperscript{19} ASK1 is also bound to and inhibited by thioredoxin and glutaredoxin, which after oxidative stress, are released allowing ASK1 activation.\textsuperscript{12,31} Even though we failed to identify increases in thioredoxin protein in the hearts of ASK1 DTG mice at baseline or after TAC, endogenous levels of thioredoxin may be in excess and fully capable of silencing overexpressed ASK1.

The most prominent biological effect observed in ASK1 overexpressing mice was a sensitization to cell death following stimulation. ASK1 transgenic mice exhibited greater cardiac TUNEL after MI and following 8 weeks of pressure-overload stimulation. ASK1 DTG mice also exhibited a more than 2-fold increase in I/R injury compared with control mice. The I/R model is particularly relevant given ROS generation during reperfusion, which should liberate ASK1 from thioredoxin and glutaredoxin. Previous work showed that \( Ask^{+/−} \) mice had reduced cardiomyocyte apoptosis and TUNEL in response to angiotensin II infusion\textsuperscript{15} or in response to 4 weeks of pressure-overload stimulation or 4 weeks after MI.\textsuperscript{16} Cardiomyocytes generated from \( Ask^{+/−} \) mice were also resistant to \( \text{H}_2\text{O}_2 \) and calcium overload-induced apoptosis.\textsuperscript{16,17} Finally, deletion of \( Ask \) in the mouse rescued cardiomyopathy and the increase in cardiac apoptosis associated with a cardiac-specific deletion of the \( c-Raf-1 \) gene.\textsuperscript{20} Thus, our results in ASK1 overexpressing TG mice are consistent with data obtained in \( Ask^{+/−} \) mice, together indicating that ASK1 plays a critical role in regulating cardiomyocyte death, possibly because of increased JNK1/2 activity and upregulated Bax.

In contrast to the cell death observations, ASK1 transgenic mice showed no increase in myocyte or whole organ hypertrophy following TAC or isoproterenol stimulation for 2 weeks compared with controls. Even after 8 weeks of TAC, ASK1 transgenic mice showed no increase in myocyte cross-sectional areas compared with controls. Importantly, pressure-overload stimulation is known to potently activate ASK1 in the adult mouse heart\textsuperscript{16}; therefore, if ASK1 truly functioned as a hypertrophic regulator, the overexpressing mice should have shown enhancement in this process. Ironically, we previously observed increased cardiomyocyte hypertrophy by adenoviral infection with a dominant negative ASK1 mutant, whereas overexpression of WT ASK1 suppressed hypertrophy caused by calcineurin, phenylephrine, and FBS stimulation.\textsuperscript{19} Not surprisingly, results in cultured neonatal myocytes are sometimes at odds with results obtained in genetically modified mouse models, in part because of the variability in the culture model itself. Indeed, other studies in cultured myocytes suggest that overexpression of activated ASK1 actually induced cardiomyocyte hypertrophy in culture, whereas overexpression of dominant negative ASK1 attenuated hypertrophy.\textsuperscript{18}

Previous results in genetically modified mouse models are similarly unclear in defining the role of ASK1 in regulating cardiac hypertrophy. For example, \( Ask^{+/−} \) mice showed reduced hypertrophy following angiotensin II infusion, suggesting that ASK1 could positively regulate cardiac growth in vivo.\textsuperscript{15} However, TAC stimulation did not result in less cellular hypertrophy in \( Ask^{+/−} \) mice compared with WT mice, suggesting that ASK1 is not required in vivo for successful pressure overload hypertrophy.\textsuperscript{16} More recently, \( Ask^{+/−} \) mice were actually shown to have enhanced physi-
ological hypertrophy following swimming exercise, suggesting that ASK1 antagonizes the adaptive growth response. Our results in ASK1 overexpressing TG mice suggest that ASK1 is not a central regulator of the pathological hypertrophy response. The discordance in results from the various studies in Ask1−/− mice and in cultured myocytes may reflect secondary effects associated with increased cell death or greater propensity toward cardiomyopathy. Indeed, 8 weeks of TAC stimulation produced increased heart weights normalized to body weight in ASK1 transgenic mice, although more careful inspection of these mice revealed greater ventricular dilation as the causative factor in affecting total heart weights. It is even more complicated when one attempts to invoke an underlying mechanism for an effect on hypertrophy, because ASK1 transgenic mice showed inhibition of NFAT activity following TAC stimulation, but hypertrophy was not inhibited. To explain this effect, it is likely that ASK1 regulates other growth effecting pathways that might counteract this antihypertrophic effect, such as alterations in MAPK signaling. Second, ASK1 overexpression induces greater cell death with some degree of cardiomyopathy that likely secondarily enhances the cardiac hypertrophic response through greater neuroendocrine dysfunction. Thus, ASK1 is likely a disease-modifying kinase that can secondarily impacts cardiac hypertrophy and heart failure through a primary mechanism involving cell death, ventricular remodeling, and other uncharacterized effects.

We previously observed that ASK1 overexpression in neonatal cardiomyocytes induced activation of p38 and JNK, as well as inhibition of calcineurin-NFAT signaling. Otsu and colleagues similarly observed that TAC stimulation in Ask1−/− mice resulted in defective cardiac JNK activation. We failed to observe an increase in p38 activation in the heart with ASK1 overexpression after TAC stimulation, although we did observe enhanced activation of MKK4/6 and JNK1/2. It is possible that p38 was not induced in the hearts of ASK1 DTG mice because stimuli other than TAC are needed to induce coupling between ASK1 and the p38 branch of the MAPK cascade when ASK1 is in abundance. Indeed, ASK1 might serve a scaffold function, such that its overexpression affects p38 signaling in a different manner from that observed in Ask1−/− mice. However, ASK1 overexpression did show coupling to the JNK1/2 signaling branch in the heart after TAC stimulation, an effect known to alter the cell death response.

The interconnectivity between calcineurin-NFAT and ASK1 signaling circuits is even more intricate, because calcineurin appears to be required for ASK1 activation through dephosphorylation of serine 967 in cultured cardiomyocytes. Here, we extended this latter observation in our transgenic mice. Specifically, we crossed the ASK1 transgene into the CnAβ−/− background as a way of reducing total calcineurin activity in the heart, as we have previously characterized. Consistent with our in vitro loss-of-function experiments, the CnAβ−/− background attenuated the ability of ASK1 to promote cardiomyopathy on pressure-overload stimulation. Calcineurin is known to play a critical role in altering the decision of death versus survival of cardiomyocytes in response to stress stimulation. Thus, ASK1 is a highly interconnected signaling effector that holds potential therapeutic relevance, such that inhibitors against this kinase might be cardioprotective in response to diverse disease stimuli that result in cardiomyopathy.

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Disclosures

None.

References


Supplemental Materials and Methods
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Animal Models and Procedures.

A tetracycline-responsive binary α-MHC transgene system was used to allow temporally regulated expression of ASK1 in cardiomyocytes of the heart.1 Dox was administered in the food with a special diet formulated by Purina (625 mg/kg in pellets). In all experiments that required ASK1 protein induction, Dox was removed from the food at weaning, resulting in induced expression of ASK1 a few weeks later in young adulthood. All experimental procedures with animals were approved by the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Medical Center.

Transverse aortic constriction (TAC) of the transverse aorta was performed as previously described.2 Transthoracic echocardiography to measure cardiac dimensions and pressure gradients across the aortic constriction was performed as described previously.3 Pressure gradients were calculated as \(4 \times V_{\text{max}}^2\) (m/s) where \(V_{\text{max}}\) is the velocity of the blood flow across the aortic constriction measured by Doppler. Fractional shortening (FS) from echocardiographic measurements was calculated using left ventricle dimensions in end of systole and diastole (LVES and LVED, respectively) according to the formula: \(\text{FS} = \frac{[(\text{LVED} - \text{LVES})/\text{LVED}]}{\times 100\text{\%}}\).

Azlet 1002 osmotic minipumps (Cupertino, CA) either filled with isoproterenol (60 mg/kg/day in phosphate-buffered saline) or phosphate-buffered saline were implanted under the skin for 2 weeks following a routine surgical procedure.

The surgical procedure for ischemia-reperfusion (I/R) injury and myocardial infarction (MI) injury in the mouse were described previously.4 Briefly, a suture was tied with a slip-knot around the left coronary artery, and mice were revived by removal from anesthetic during 60 min of ischemia, after which the knot was released and the heart was reperfused for 24 h (I/R). The MI procedure was identical except that the ligature was permanent with no reperfusion. Mice were sacrificed by CO\(_2\) asphyxiation, and hearts were analyzed as previously described using 2% triphenyltetrazolium chloride in saline and 2% Evan's blue dye infusion to identify area at risk, infarct area, and area of perfusion for the I/R procedure.4

Histological Analysis, Cell Size Measurement, and TUNEL.
For histological analysis, adult hearts were fixed in 10% formalin/phosphate-buffered saline and dehydrated for paraffin embedding. Fibrosis was detected with Masson's Trichrome staining on 5-µm paraffin sections. Blue collagen staining was quantified using Metamorph software. For cell surface area measurements, membranes were stained with TRITC- or FITC-labeled lectin from *Triticum vulgaris* (Sigma), and nuclei were labeled with TO-PRO 3 iodine (Molecular Probes, Carlsbad, CA). Cellular areas were quantified with ImageJ 1.33 software (Scion Corp., Frederick, MD). Assessment of TUNEL from paraffin sections was performed with TMR Red In Situ Death Detection Kit (Roche Diagnostics) according to the manufacturer's instructions (Roche Diagnostics).³

*Luciferase reporter assays in mouse hearts*

The NFAT-luciferase reporter mouse was described previously.² In brief, ASK1 transgenic mice were crossbred with NFAT-luciferase reporter mice and ventricles were excised and stored at –70°C. The frozen hearts were homogenized in 1 ml luciferase assay buffer (100 mM KH₂PO₄, pH 7.8, 0.5% Nonidet P-40, and 1 mM DTT). Homogenates were centrifuged at 3,000 g for 10 min at 4°C and the supernatants assayed for luciferase activity as described previously.²

*Western Blotting*

Protein extraction from mouse heart and subsequent Western blotting followed by enhanced chemiluminescence detection was performed as previously described.²⁴ Antibodies including anti-JNK1/2, anti-phospho-JNK, anti-p38, anti-phospho-p38, anti-ERK1/2, anti-phospho-ERK1/2, anti-MKK4, anti-phospho-MKK4, and anti ASK1 (Thr 845) were obtained from Cell Signaling Biotechnology (Beverly, MA). Anti-ASK1 antibody and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA).

*Statistical Analysis*

Results are shown as means ± SEM. Paired data were evaluated by Student’s *t* test. A one-way or two-way ANOVA with the Bonferroni’s post hoc test or repeated-measures ANOVA was used for multiple comparisons. *p* < 0.05 was considered significant.

*References*

