MAPK-Activated Protein Kinase-2 in Cardiac Hypertrophy and Cyclooxygenase-2 Regulation in Heart

John M. Streicher, Shuxun Ren, Harvey Herschman, Yibin Wang

Rationale: Activation of p38 mitogen-activated protein kinase (MAPK) has a significant impact on cardiac gene expression, contractility, extracellular matrix remodeling, and inflammatory response in heart. The p38 kinase pathway also has a controversial role in cardiac hypertrophy. MAPK-activated protein kinase-2 (MK2) is a well-established p38 downstream kinase, yet its contribution to p38-mediated pathological response in heart has not been investigated.

Objective: We examined the specific contribution of MK2 to the pathological remodeling induced by p38.

Methods and Results: We used a cardiomyocyte specific and inducible transgenic approach to determine the functional and molecular impact of acute activation of the p38 pathway in heart in either a MK2 wild-type or a MK2-null background. p38 activation in wild-type mice led to a rapid onset of lethal cardiomyopathy associated with cardiomyocyte hypertrophy, interstitial fibrosis, and contractile dysfunction. Inactivation of MK2 partially but significantly reduced cardiomyocyte hypertrophy, improved contractile performance, and prevented early lethality. MK2 inactivation had no effect on the mRNA levels of hypertrophic marker genes or the proinflammatory gene cyclooxygenase (COX)-2. However, MK2 had a major role in COX-2 protein synthesis without affecting the mRNA level or protein stability.

Conclusions: p38 activity in adult myocytes can contribute to pathological hypertrophy and remodeling in adult heart and that MK2 is an important downstream molecule responsible for specific features of p38-induced cardiac pathology. (Circ Res. 2010;106:00-00.)

Key Words: MK2 ■ p38 MAPK ■ hypertrophy ■ COX-2 ■ Heart Failure

Heart failure, a disease with high prevalence and mortality rates, can be induced by external stressors such as increased hemodynamic load, ischemia or reperfusion injury. A complex network of signaling molecules has been implicated in mediating the pathological effects of these extrinsic stressors, including p38 Mitogen Activated Protein Kinases (MAPK); a branch of the conserved MAP kinase family mostly implicated in stress signaling and regulation.

In cultured cardiomyocytes, p38 activation induces myocyte hypertrophy and apoptosis and promotes fetal gene expression and cytokine production. However, p38 chronic activation in intact mouse hearts did not result in cardiac hypertrophy but instead caused restrictive cardiomyopathy associated with extracellular matrix remodeling and contractile dysfunction. In addition, elevated p38 activity suppresses hypertrophy via the negative regulation of NFAT (nuclear factor of activated T cells) activity in culture. Furthermore, cardiomyocyte-specific genetic knockout of p38α results in a normal hypertrophic response subsequent to pressure overload, but is accompanied by accelerated heart failure, elevated myocyte death and interstitial fibrosis. Mice expressing a dominant negative cardiac specific p38α displayed normal hypertrophy but are resistant to fibrosis. Thus, the p38 pathway is generally considered not to be a major component of the hypertrophy pathway in heart, but is thought to be important for both adaptive and pathological remodeling. However, these in vivo studies used chronic activation or inactivation approaches to characterize p38 function in heart. In vivo, p38 activity is mostly induced in a transient fashion under stressed conditions. Therefore, the impact of short-term activation of p38 activity in adult heart has not been investigated using genetic models.

MAPK-activated protein kinase-2 (MK2) is a major downstream p38 MAPK substrate that plays an important role in p38-mediated inflammatory regulation in immune cells. The MK2-deficient mouse develops normally, but has a significantly increased resistance to endotoxic shock with impaired cytokine induction. MK2 is an important...
regulator of mRNA stability for COX-2, interleukin-6, tumor necrosis factor (TNF)α, and MIP (macrophage inflammatory protein), and its known substrates include serum response factor, CREB (cAMP response element binding protein), and ATF-1 (activating transcription factor-1). Shiroto et al also demonstrated that MK2 deficiency protects the heart against ischemia/reperfusion injury. However, the specific contribution of MK2 in p38-mediated pathological responses in heart has not been characterized.

In this report, we describe a new transgenic mouse model with conditional and cardiomyocyte-specific expression of a constitutively activated p38 specific upstream activator MKK3bE and explore its biology in both wild-type and MK2-null mice. Unexpectedly, conditional activation of p38 in adult heart led to the rapid onset of cardiac hypertrophy accompanied by interstitial fibrosis and contractile dysfunction. MK2 loss significantly reduced MKK3bE induced hypertrophy, improved the contractile performance, and rescued lethality. However, MK2 inactivation did not affect the mRNA levels of hypertrophic marker genes or proinflammatory molecules. In contrast, MK2 inactivation had a major impact on COX-2 protein synthesis, without a significant effect on the COX-2 mRNA level or protein stability. These data are the first in vivo evidence demonstrating that acute p38 activation in adult heart is sufficient to induce lethal cardiac hypertrophy and other pathological heart remodeling. We also reveal a previously uncharacterized MK2 dependent regulation of COX-2 protein synthesis in heart and find that MK2 activity plays an important role in p38-mediated cardiac hypertrophy and heart failure.

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

Transgenic Animals
The conditional αMHC-flox-MKK3bE (MKK3) mouse line, the tamoxifen inducible Mer-Cre-Mer mouse line (Cre, a kind gift from Dr Molkentin, Cincinnati), and the MK2-null mouse line (kindly provided by Dr Huiping Jiang, Boehringer Ingelheim Pharmaceutical Inc) were already established as described. Details of crossbreeding and tamoxifen induction are described in the Online Data Supplement.

Statistical Analysis
Comparisons between more than 2 groups were accomplished using a factorial ANOVA with Fisher’s protected least significant difference post hoc test. Comparisons between pairs of survival curves was accomplished by a χ² test. Analysis was carried out using the StatView program (Abacus Concepts, Berkeley, Calif). In all cases, a significant result was defined as P<0.05.

A detailed description of all methods used is available in the Online Data Supplement.

Results
The Effect of MK2 Inactivation on p38 MAPK Activation in Adult Mouse Heart
In an earlier study, we demonstrated, using a tamoxifen-inducible Cre combined with a floxed MKK3bE transgenic line, that we can induce MKK3bE expression and specific p38 activation in adult mouse heart. Using 4- to 6-month-old adult mice with the 5 different genotypes of wild-type, Mer-Cre-Mer (Cre), MK3/Cre, MK2−/−, and MK3/Cre/MK2−/− (Figure 1B and Methods), we observed that loss of MK2 significantly reduced the total p38 protein in heart, similar to the previous observations made in other tissues (Figure 1C). However, phospho-p38/total p38 ratios remained elevated in the tamoxifen-treated MKK3/Cre/MK2−/− hearts relative to other controls at a comparable level as observed in the tamoxifen-treated MKK3/Cre hearts (Figure 1C). Therefore, loss of MK2 significantly reduced the total p38 protein but did not affect the upstream MKK3-dependent p38 phosphorylation/activation. As expected, phosphorylation of Hsp27, a well-established downstream target of the p38/MK2 pathway, was readily detected in the tamoxifen-treated MKK3/Cre hearts but was completely abolished in the tamoxifen-treated MKK3/Cre/MK2−/− hearts, suggesting the loss of MK2 downstream signaling in MK2-null hearts.

The Impact of MK2 Inactivation on MK3-Induced Cardiac Hypertrophy and Dysfunction
At 8 days after induction, MKK3bE expression resulted in significant changes in the gross morphology of the heart, including ventricular hypertrophy and enlargement of the left atria with a prominent presence of thrombotic clot, indicating a state of hypertrophic cardiomyopathy (Figure 2). The status of ventricular hypertrophy was supported quantitatively by the average myocyte cross-sectional area measured from histological sections (Figure 2B and 2C), end-systolic posterior wall thickness of the left ventricle measured from echocardiography (Figure 3), and left ventricular weight versus body weight ratio in tamoxifen-treated MKK3/Cre mice comparing to other control groups (Online Table II). In the MK2-null background, the extent of cardiac hypertrophy was partially but significantly ameliorated as determined from cross-sectional area (Figure 2C) and posterior wall thickness (Figure 3) and a strong trend of reduction in left ventricular weight (Online Table II).

Also using echocardiography, we determined that MKK3 induction in adult hearts led to a significant increase in fractional shortening and ejection fraction,
supporting again the status of restrictive and concentric hypertrophy (Figure 3). However, impaired cardiac function manifested in an increased mitral valve Doppler E/A ratio (Figure 3), suggesting elevated stiffness of the myocardium. Contractile dysfunction was further measured from a pressure catheter placed directly into the left ventricular chamber (Online Table I), which showed a significant reduction of left ventricular developed pressure, an increase in end-diastolic pressure and tau following p38 activation (Online Table I). In the MK2-null background, MKK3-induced loss of left ventricle developed pressure was partially reversed (Figure 2D), whereas changes in E/A Doppler ratio and tau were not affected. Therefore, MK2 activity appears to contribute to specific aspects of contractile dysfunction in MKK3-induced hearts.

Cardiac fibrosis as determined by Masson’s Trichrome staining was induced in tamoxifen-treated MKK3/Cre hearts in higher level in WT than in the MK2-null background (Figure 2B). Considering the potential deleterious impact of tamoxifen treatment on cardiac function as reported by Koitabashi et al., we also examined the function of tamoxifen-treated Mer-Cre-Mer transgenic mice (Cre+TX). Under our treatment regimen, the MCM (Cre) mice showed no cardiomyopathy, as indicated by echocardiography (Figure 3), heart mass (Online Table II), or gene expression, in agreement with the earlier observations made by Sohal et al. and Petrich et al.

The Role of MK2 in MKK3-Induced Lethal Heart Failure and Gene Expression

Acute tamoxifen induction of MKK3 activity in adult mice led to significant lethality with 38% survival at day 8 (Figure 4). By longitudinal telemetric monitoring, we observed that MKK3-induced mice developed progressive bradycardia accompanied by a gradual lowering of the body temperature as they approach death, a common observation made in end-stage heart failure (Online Figure I). Inactivation of MK2 significantly reduced the mortality observed in MKK3-induced animals. These data support the notion that MK2 inactivation in MKK3/Cre mice significantly reduced lethal heart failure. Therefore, MK2 contributes to MKK3-induced contractile dysfunction and the development of heart failure.

Despite a significant impact on myocyte hypertrophy and cardiac function, MK2 inactivation did not reduce
MKK3-induced expression of hypertrophic marker genes, including atrial natriuretic factor and β-myosin heavy chain (βMHC) (Figure 5). In addition, collagen I induction was also not affected. Lastly, MK2 inactivation did not affect COX-2 mRNA induction in the MKK3 expressing hearts. In a previous report, MK2-null mice were found to have a normal or increased induction of TNFα mRNA but impaired TNFα protein biosynthesis. This observation raised the question whether MK2 might also regulate COX-2 expression at a post-transcriptional level in p38 activated mouse heart.

MK2 Regulates COX-2 Protein Expression

In Vitro

COX-2 induction is associated with hypertrophic cardiomyopathy (Streicher, JMCC, in press). To further characterize MK2-mediated COX-2 expression at the post-transcriptional level in cardiomyocytes, we infected neonatal rat ventricular myocytes with adenoviral constructs encoding constitutively active MK2 (caMK2), dominant negative MK2 (dnMK2), and constitutively active MKK3 (caMKK3), which specifically activates p38. Both caMK2 and caMKK3 robustly induce COX-2 expression over the control adenovirus (LacZ), whereas the dnMK2 was able to significantly antagonize COX-2 protein induction by caMKK3 (Figure 6A). Both caMK2 and caMKK3 also robustly induced Hsp27 phosphorylation, a downstream target of MK2, validating the functionality of these vectors. These data indicate that MK2 activation is able to induce COX-2 protein expression in cardiomyocytes and that MK2 activity is necessary for MKK3 mediated COX-2 induction.

The role of MK2 in COX-2 protein regulation was also observed in mouse embryonic fibroblasts (MEFs). In MK2−/− MEF cells, COX-2 protein was significantly reduced both at basal levels and following p38 activation (Figure 6B). In contrast, quantitative PCR analysis revealed no difference in COX-2 mRNA levels between MK2+/+ and MK2−/− MEFs at the basal condition (Figure 6C), and p38-induced COX-2 mRNA was only partially...
reduced in MK2−/− MEFs. This finding supports the notion that MK2 has a role in regulating COX-2 protein in mammalian cells.

**MK2 Promotes COX-2 Protein Induction in Mouse Heart**

To determine whether MK2 has any functional role in COX-2 regulation in intact heart, we stimulated COX-2 induction in mouse heart by treating MK2+/+ and MK2−/− mice with lipopolysaccharide (LPS) for 6 hours. LPS treatment led to a robust COX-2 protein induction in MK2+/+ hearts. In contrast, this induction was completely abolished in MK2−/− mice (Figure 6D). However, cardiac COX-2 mRNA levels were robustly induced in both MK2+/+ and MK2−/− hearts (Figure 6E). Thus, MK2 inactivation had no impact on the COX-2 mRNA induction in response to LPS but completely abolished COX-2 protein induction. We confirmed that the LPS treatment stimulates an inflammatory cardiac response in MK2−/− mice by showing a similarly robust stimulation of TNFα mRNA accumulation in both wild-type and MK2−/− hearts (Figure 6F). Therefore, both in vitro and in vivo observations strongly suggest that MK2 activity plays an essential role in the expression of COX-2 at the protein level in response to inflammatory stimulation.

**MK2 Regulates COX-2 Protein Synthesis Without Affecting Its Degradation**

Steady state protein levels can be regulated by either (or both) the rate of synthesis and the rate of degradation. To determine the mechanism of MK2-mediated COX-2 protein regulation, we metabolically labeled MK2+/+ and MK2−/− MEF cells with 35S-methionine (see Supplementary Methods). By continuously labeling cells with radioactive methionine over time, we were able to measure the accumulation rate of newly synthesized COX-2 protein.
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mined the COX-2 protein degradation rates in MK2
result of protein synthesis and degradation, we also deter-

Figure 7E). However, we detected no differences in
the luciferase signal in response to LPS, as expected
between 2 hours and 4 hours post labeling, the newly
synthesized COX-2 protein accumulation rate (K) in wild-
type MEFs was nearly 2.4 fold faster than in MK2+/−
MEFs (0.56 versus 0.23 h⁻¹) (Figure 7A).

Because the rate of protein accumulation is a combined
result of protein synthesis and degradation, we also deter-

mined the COX-2 protein degradation rates in MK2+/−
and MK2−/− MEF cells, using a pulse-chase labeling
method.²⁶ As shown in Figure 7B and 7C, COX-2 protein
turn-over rates (τ) were the same for MK2+/− and MK2−/−
cells (τCOX-2=1.5 hours). The global synthesis and degra-
dation protein profiles were also obtained in the same
samples as described in the Supplementary Methods and
were found to be the same for the two cell types (τtotal=2.4
hours) (Online Figures II and III). This finding thus
suggests that MK2 promotes COX-2 protein expression
most likely by enhancing its translational rate rather than
affecting COX-2 protein stability or turn-over.

One potential site for MK2 to regulate COX-2 translation
is through the COX-2 mRNA 3′-untranslated region
(3′UTR), which has been shown to bind translational
regulators like TIA-1 and tristetraprolin/tis-11.²⁵,²⁷ To test
this possibility, we transfected a reporter containing a
constitutive promoter driving luciferase cDNA followed
by the full-length COX-2 3′UTR (Figure 7D) into MK2+/−
and MK2−/− MEF cells. We then stimulated these cells
with LPS, which is reported to stabilize the COX-2 mRNA
translation protein profiles were also obtained in the same
data indicated that the loss of COX-2 protein
seen in the MK2−/− cells is most likely via a mechanism
independent of the COX-2 3′UTR.

Discussion
In this study, we have established an inducible, heart
specific model of MKK3 induction that permits the exam-
ine of acute p38 MAPK activation. By breeding the
inducible transgene alleles into the MK2−/− background,
we examined the role of MK2 in acute p38 activation in
adult heart. Induction of MKK3 led to cardiomyocyte
hypertrophy, cardiac remodeling, contractile dysfunction,
pathological gene activation, and lethal bradycardic heart
failure. Removal of the MK2 gene rescued some aspects of
this pathological response, including partially ameliorated
hypertrophy and contractile dysfunction, and prevention of
early lethality. Thus, MK2 has an important role in
mediating some aspects of p38-induced heart failure. Its
contribution to p38 signaling can be mediated by either
affecting the total p38 protein level and signaling intensity
(Figure 1) or by specifically blocking the MK2 dependent
downstream signaling (as demonstrated by Hsp27 phos-
phorylation in Figure 1). From both in vitro and in vivo
analysis, we also established a previously uncharacterized
role for MK2 in COX-2 protein synthesis regulation.
Therefore, our study not only demonstrates a specific
contribution of MK2 in p38-induced cardiomyopathy but
also reveals a new mechanistic link between p38 and
inflammatory gene expression in heart.

The finding that p38 activation was sufficient to induce
ventricular myocyte hypertrophy is supported by wall
thickness measured in vivo by echocardiography, normal-
ized ventricular mass and myocyte cross-sectional area
from histological sections. The finding that p38 induces
hypertrophy in adult heart is in accordance with in vitro
myocyte experiments.³ However, most in vivo models of
p38 activation have not shown myocyte hypertrophy.⁶,⁷
One potential explanation for this discrepancy lies in the
timing and duration of p38 activation. Earlier in vivo
studies used constitutively activated promoters to achieve
p38 activation in an unregulated manner at earlier develop-
mental stages.⁶,⁷ In contrast, our present study used a
tamoxifen dependent Cre gene to activate the p38 pathway
in adult heart (see Methods). This may eliminate the
potential confounding factors involving p38-mediated reg-
ulation of cardiomyocyte proliferation and differentiation
as implicated in other reports,²⁸ or secondary compensa-
tory changes. However, we cannot exclude other compli-
cating factors such as contribution of tamoxifen treatment and green fluorescent protein expression. Although we have different control groups to support the specificity of the observed phenotype in MKK3b-expressing hearts.

Considering the fact that p38 is usually activated in a transient fashion in response to acute stress stimuli, our model may be more relevant to pathological induction of the p38 pathway in heart. Our data also shows that MK2 had a major role in p38-mediated cardiac hypertrophy. MK2 inactivation partially reduced LV weight, cross-sectional area and LV wall thickness induced by p38 activation. In short, our data suggests that activation of the p38 pathway in adult heart may still have a significant contribution to cardiomyocyte hypertrophy in addition to other aspects of pathological remodeling. Furthermore, MK2 is a significant downstream signaling component in p38-induced hypertrophy.

In addition to hypertrophy, we also showed that p38 MAPK activation is linked to cardiac remodeling and contractile defects as supported by histology, gene expression and functional data, in good agreement with previous findings. MK2 inactivation significantly improved cardiac contractility in MKK3 transgenic hearts.
based on hemodynamic measurements. However, MK2 did not rescue the observed myocardial stiffness or fibrotic remodeling. One possibility is that MK2 acts directly downstream of p38 MAPK to mediate negative inotropic signaling on the contractile apparatus. However, the underlying molecular mechanism needs to be further investigated.

In this study, we demonstrate that MK2 has a previously uncharacterized role in regulating COX-2 protein expression. Our data suggests that MK2 modulates the rate of COX-2 protein synthesis without affecting its degradation. We also suggest that the MK2-mediated modulation of COX-2 protein synthesis does not involve the COX-2 mRNA 3’UTR. This observation differs from previous reports that implicates p38 and MK2 in COX-2 induction through mRNA stabilization. MK2-mediated regulation of COX-2 protein synthesis is an important addition to the regulatory mechanisms linking stress stimuli to inflammatory responses in heart cells.

COX-2 protein induction has been found in human patients with myocardial infarction or heart failure, and prostaglandins such as PGF2α are induced in heart failure patients as well. COX-2 inhibition protects the heart from endotoxin and lipoteichoic acid treatment. COX-2 inhibition also protects the heart from ischemia related damage in coronary artery ligated rat and mouse heart. However, a number of other studies have reported no effect, or the opposite effect of COX-2 inhibition in myocarditis and ischemia (reviewed elsewhere). Evidence from our laboratory suggests that COX-2 expression in heart is sufficient to induce cardiac hypertrophy and fetal gene expression. Clearly, more investigation will be needed to establish the specific function of COX-2 in MK2-mediated regulation of cardiac pathology and to uncover the underlying molecular basis by which MK2 regulates COX-2 protein synthesis.

The MK2 KO mouse appears to tolerate the loss of MK2 protein well without adverse effects. Therefore, MK2 inhibition may serve as an alternative means of preventing cardiac hypertrophy, heart failure and inhibiting inflammatory response. Indeed, specific MK2 inhibitors are already being evaluated for rheumatoid arthritis and their efficacy in treating heart failure remains to be examined. Nevertheless, the in vivo evidence and molecular data presented in this study suggest that MK2 is a potential target of intervention for cardiomyopathy and inflammation.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Chronic activation of a stress inducible p38 mitogen activated protein kinase (MAPK) pathway can lead to pathological changes in the heart, including gene expression, contractile dysfunction, extracellular remodeling, and myocyte death. However, its specific contribution to cardiac hypertrophy remains controversial.
- MAPK activated protein kinase-2 (MK2) is a major downstream protein kinase of p38 MAPK; however, its role in p38-induced cardiac pathology is unknown.
- Cyclooxygenase (COX)2 is a known downstream target of p38 signaling and has a role in the development of hypertrophy. However, the significance and mechanism of MK2-mediated pathway in COX-2 expression in heart is unknown.

What New Information Does This Article Contribute?

- Inducible activation of p38 pathway in adult heart led to hypertrophy and pathological remodeling, a phenotype different from the one that resulted from chronic activation of the p38 pathway.
- MK2 inactivation has a significant but selective impact on the pathological effect of p38 activation in the intact heart.
- MK2 is a critical signal molecule for stress-induced COX-2 expression in heart and other cells. The underlying mechanism involves protein synthesis instead of transcriptional regulation, mRNA stability, or protein degradation.

Chronic activation of p38 MAPK can lead to pathological changes in gene expression, contractility, extracellular matrix remodeling, and inflammatory response in the heart. However, the specific role of the p38 kinase pathway in cardiac hypertrophy is controversial. MK2 is a well established p38 downstream kinase, yet its contribution to p38-mediated pathological response in heart has not been investigated. In this report, we developed new transgenic mouse models with cardiomyocyte specific and inducible activation of the p38 pathway in the heart in either an MK2 wild-type or an MK2-null background. Through phenotypic and molecular characterizations, we have established that acute p38 activation in the adult mouse heart causes rapid onset of lethal cardiomyopathy associated with cardiomyocyte hypertrophy, different from the phenotype resulting from chronic p38 activation. Our study also demonstrates for the first time the selective contribution of MK2 to p38-induced pathological changes, including a major role in the post-transcriptional regulation of the proinflammatory protein COX-2. This study provides a new understanding of the functional significance of a major stress-response signaling pathway in the heart and of the downstream signaling mechanisms.
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Regulation in Heart

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Supplemental Information

I. Detailed Experimental Methods

Generation and Treatment of Transgenic Animals

All animal handling and procedures were carried out in compliance with UCLA guidelines following IACUC approved protocols. The conditional αMHC-flox-MKK3bE (MKK3) mouse line 1, the tamoxifen inducible Mer-Cre-Mer mouse line (Cre, a kind gift from Dr. Molkentin, Cincinnati) 2, 3, and the MK2 null mouse line (kindly provided by Dr. Huiping Jiang, Borehinger Ingelheim Pharmaceutical Inc.) 4 were already established as described.

Experimental animals were littermates from crossbreeding between MKK3 and Cre lines either in C57BL6 widltype (WT) background or in C57BL6-MK2 null (MK2-/-) background. The genotypes of WT, MKK3/Cre, MK2-/-, and MKK3/Cre/MK2-/- were used in this experiment between 4-5 months of age. All groups of animals were treated with 20 mg/kg of tamoxifen (TX) (Sigma) in 30% ethanol/PBS injected intraperitoneally for three consecutive days. MKK3/Cre and MKK3/Cre/MK2-/- animals were also treated with 30% ethanol/PBS as an additional control (Veh). Following tamoxifen or vehicle injections, all mice were analyzed by echocardiography on days 0 and 7 post-induction and used for terminal experiments or sacrificed on day 8.

Echocardiography

Except where indicated, treated mice were imaged non-invasively for heart function and morphology by echocardiography on days 0 and 7 post tamoxifen treatment (injections from days 1-3). The mice were anesthetized and maintained with 2% isofluorane in 95% oxygen. A Vevo 770 (VisualSonics, Toronto, Canada) echocardiography system with a 30mHz scanhead was used to acquire the data. A parasternal short axis view was used to obtain M-mode images for analysis of fractional shortening, while a parasternal long axis view was used to obtain power doppler traces of
mitral valve filling for the calculation of the E/A ratio. The heart rate was calculated directly from the short axis view of heart contraction.

**Pressure Catheterization**

A subset of treated mice had their heart function measured by invasive pressure catheterization on day 8 (injections from day 1-3). The mice were anesthetized with 2g/kg urethane and maintained on a ventilator with 95% O₂, 5% CO₂. The chest cavity was opened, and an SPR-1000 micro-tip catheter pressure transducer (Millar Instruments, Houston, TX) was introduced through the apex into the center of the left ventricle. An MPCU-2000 pressure/volume conduction system (Millar Instruments) was used to control the catheter, and the data was acquired using Chart 5 (AD Instruments, Colorado Springs, CO). The resultant data was analyzed and functional parameters obtained using MPress (Millar Instruments). After the end of the experiment, the hearts were perfused with 10% formalin and used for histological analysis. None of these hearts were used for protein or mRNA analysis.

**Histology**

A subset of treated mice had their hearts collected for histological analysis on day 8, generally after pressure catheterization. The mouse hearts were perfused and fixed with 10% formalin prior to embedding in paraffin. For display purposes, selected hearts from each treatment group were embedded and sliced in a coronal orientation (i.e. all four chambers visible). The hearts used for analysis were embedded in a cross section orientation, and all slices were cross sections of the heart. All slices were taken from the midpoint of the ventricle. 4μm slices were deparaffinized and rehydrated prior to staining with Masson’s Trichrome stain. Whole mount and trichrome stained images
(40X objective) were collected using a SPOT digital camera system (Diagnostic Instruments, Sterling Heights, MI). For the purpose of calculating myocyte cross sectional area (CSA), 10 images were acquired from each heart from the epicardial region. In each image, 10 cells were quantified for their CSA using the SPOT Advanced software (Diagnostic Instruments). The resultant areas of 100 cells were all averaged to give the CSA of each heart, which counted as a sample size of 1 for statistical analysis.

**ECG Telemetry**

In a separate experiment, WT and MKK3/Cre mice were used for continuous ECG monitoring by a telemetry system. These mice were not imaged by echocardiography or otherwise disturbed in order to facilitate stable ECG monitoring. The heart tissue from these mice was also not used for analysis, either histological or biochemical.

The telemetry system described below was obtained from Data Sciences International (St. Paul, MN). The mice were surgically implanted with an ETA-F20 model transmitter according to the manufacturer’s recommendations. The mice were then allowed to recover for 7 days before tamoxifen injection. Upon tamoxifen induction as described above, the mice were singly housed in cages on top of a RPC-1 model PhysioTel Receiver for data acquisition. The mice were left undisturbed as much as possible. The data was routed through a Data Exchange Matrix, and acquired, processed and analyzed using Dataquest A.R.T. The mice were continuously monitored for temperature, activity, and ECG waveform. The mice were allowed to die naturally, and the data analyzed for the periods prior to and just after tamoxifen induction, as well as during pathological development and at the time of death.
Cell Culture

NRVMs were isolated from P1-P3 day old Sprague-Dawley rat pups of mixed gender as described in Lu et al. \(^6\). The NRVMs were incubated in plating medium overnight after isolation, then infected with adenoviruses and incubated for an additional 48 hours in serum free DMEM medium supplemented with ITS (Invitrogen, Carlsbad, CA). In one set of experiments, a selective p38 inhibitor SB203580 (10μM, Calbiochem, San Diego, CA) was applied concurrently with the adenovirus to the cells, in serum free medium.

MEF cells were isolated from embryonic day 13 MK2 WT and KO embryos in a procedure adapted from Hogan et al. \(^7\). The resulting MEF cells were cultured in DMEM + 10% FBS. All experiments were performed between passages 3 and 6. For adenoviral transfection, MEF cells were infected with adenovirus constructs in DMEM + 2% FBS, and maintained for 48 hours before harvesting. For transient transfection, MEF cells were treated with plasmids and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol, and maintained in serum free DMEM + ITS for 48 hours before harvest.

Adenoviral Constructs

The various adenoviral constructs were cloned, propagated and purified using the AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Final adenoviral titers were applied as multiplicities of infection (MOI) and were empirically determined for each construct.

The constitutively active (ca) and dominant negative (dn) MK2 mutants were generated by \textit{in vitro} mutagenesis of the thr222 and thr334 activation residues to
glutamate (for ca mutant) and alanine (for dn mutant), respectively. The constitutively activated caMKK3 was described previously.

*Western Blotting and Antibodies*

A subset of treated mice were sacrificed by cervical dislocation on day 8 for mRNA and protein analysis of the hearts. These mice were not used for pressure catheterization or other experiments. Hearts were removed, sectioned by chamber and snap frozen in liquid nitrogen prior to extraction of RNA or protein.

Heart total soluble protein was extracted from left ventricle pieces homogenized with a glass-glass homogenizer in a protein lysis buffer. Cell lysates were prepared in the same buffer and then sonicated. This buffer contained 20mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton-X100, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM sodium orthovanadate, 1mM PMSF, 10mM NaF and a complete mini protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The lysates were also spun down in order to separate the insoluble components. Protein concentration was determined by BCA Protein Assay (Thermo Scientific, Rockford, IL). 30μg of protein was separated onto 12% bis-tris polyacrylamide gels (Invitrogen) and transferred to Hybond-ECL membrane (Amersham Biosciences, Piscataway, NJ). An anti-rabbit-HRP or anti-mouse-HRP secondary antibody (Cell Signaling, Danvers, MA) with ECL detection (Thermo Scientific) was used.

The primary antibodies used were a mouse monoclonal p-p38, and polyclonal p38, p-MK2/MK2, p-ERK/ERK, GFP, p-hsp27 (all from Cell Signaling) and COX-2 (from Cayman Chemical, Ann Arbor, MI). An anti-HA antibody from Sigma and an anti α-actin antibody from Santa Cruz Biotechnology (Santa Cruz, CA) were also used. To
successfully blot for COX-2 in heart tissue, it was necessary to perform a COX-2 immunoprecipitation in these samples before immunoblotting. This assay was performed using a standard protocol (Cell Signaling) with Protein G beads and a polyclonal goat anti-COX-2 antibody (Santa Cruz). The resultant precipitate was then blotted using the COX-2 antibody from Cayman Chemical as described above.

Quantitative Real-Time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Left ventricle pieces were homogenized in the TRIzol using a glass-glass homogenizer. Cell lysates were prepared directly from the culture plate. 0.5μg of the resultant total RNA was reverse transcribed into cDNA using the SuperScript II RT system (Invitrogen) with Oligo dT primers according to the manufacturer’s instructions.

mRNA levels of selected genes were determined by quantitative PCR from the generated cDNA. 50μl reactions were used with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 250 nM of each primer and 1μl of cDNA. The reactions were run in a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad) and the data was collected using the Bio-Rad iQ5 software (Bio-Rad). The cycler program used was an initial denaturation at 95C for 5min, 40 cycles of 45 seconds each of 95C, 60C, and 72C, another denaturation at 95C for 5min, and a final product melting curve. Data was normalized to the GAPDH mRNA levels which were run for each sample with every reaction. The threshold cycle count for each data point was subtracted from the GAPDH cycle count, and then used as in the formula x=1/2^cycle in order to facilitate data analysis. Primers were also designed to flank intronic sequences, which would reveal the
presence of genomic DNA contamination after the reaction had completed. The products were run on agarose gels to check for this contamination for every cDNA sample. The primer sequences used were as follows: GAPDH F 5’- TCC TGC ACC ACC AAC TGC TTA G -3’; GAPDH R 5’- GAT GAC CTT GCC CAC AGC CTT G -3’; COX-2 F 5’- CCA GAT GCT ATC TTT GGG GA -3’; COX-2 R 5’- CGC CTT TTG ATT AGT ACT GTA G -3’; ANF F 5’- CTG ATG GAT TTC AAG AAC CTG CT -3’; ANF R 5’- CTC TGG GCT CCA ATC CTG TC -3’; βMHC F 5’- CTC AAC TGG GAA GAG CAT CCA -3’; βMHC R 5’- CCT TCA GCA AAC TCT GGA GGC –3’; Collagen I F 5’- GAC GCC ATC AAG GTC TAC TG -3’; Collagen I R 5’- GAA TCC ATC GGT CAT GCT CT -3’; TNFα F 5’- CTC TTC AAG GGA CAA GGC TG -3’; TNFα R 5’- TGG AAG ACT CCT CCC AGG TA -3’.

Induction of COX-2 In Vivo by LPS

MK2 WT and KO mice were treated with LPS in order to induce COX-2 expression in the heart. LPS (Sigma) from S. typhosa was dissolved in sterile saline and injected intraperitoneally at a dose of 5 mg/kg. Six hours post injection, mice were sacrificed by rapid cervical dislocation. Hearts were removed and snap frozen in liquid nitrogen. COX-2 protein and mRNA levels were determined as described above.

Metabolic Labeling of COX-2 in MEF Cells

Cells were first pre-incubated for 48 hours in DMEM + 2% FBS followed by incubation for 15 minutes in methionine free DMEM with 25mM HEPES and 2% dialyzed FBS (Invitrogen). The cells were labeled with 35S-methionine (0.1 mCi/ml, Perkin Elmer, Boston, MA). For the protein translation assay, the cells were labeled with the radioactive medium for a time course from 2 to 4 hours before harvesting. For the
protein stability (pulse/chase) assay, the cells were pulse labeled for 30 minutes, and then chased with medium containing 15 mg/L of non-radioactive methionine for an additional 0 to 8 hours before harvesting.

Labeled cells were harvested for protein preparation as described above, and the protein concentration quantified by a BCA assay (Thermo Scientific). For each experiment, a constant amount of protein from each lysate was subjected to immunoprecipitation for COX-2 using a standard protocol (Cell Signaling), Protein G agarose beads, and a goat anti-COX-2 polyclonal antibody (Santa Cruz Biotechnology). The immunoprecipitates were separated on an SDS-PAGE gel and an autoradiogram of the gel was recorded on film after exposure for 7 to 21 days. The signals were quantified using Scion Image (Scion Corporation, Frederick, MD) and the density data averaged for each time point and fit with either a linear (translation experiments) or negative exponential curve (degradation experiments) using Excel (Microsoft, Redmond, WA)\textsuperscript{10}. The protein synthesis rates (K) and time constant of degradation (τ) were derived from the fitted line (y\textsubscript{t} = K\textsubscript{t}+C) or curve(y\textsubscript{t} = C e\textsuperscript{t/τ}).

The total levels of metabolic labeling for each sample were also determined as an experimental control for global protein degradation. A constant amount of protein from each sample was precipitated by 20% trichloroacetic acid (TCA) and counted for radioactivity in a liquid scintillation counter. The resultant values were fitted to an exponential curve as described above to calculate the global protein degradation time constant.

\textit{COX-2 mRNA 3’UTR Luciferase Reporter}
The COX-2 mRNA 3’UTR reporter used an SV40 promoter to drive constitutive expression of a destabilized *renilla* luciferase. The full length COX-2 3’UTR followed the luciferase, and thus alterations in luciferase expression should be due to the activity of the 3’UTR. The reporter was transiently transfected as described above into WT and KO MEF cells, and the cells were incubated for 48 hours in serum free DMEM + ITS followed by treatment with either medium or medium plus 5 μg/ml of *S. typhosa* LPS (Sigma) for 6 hours. A Luciferase Kit (Promega, Madison, WI) was used to measure the *renilla* luciferase activities from protein extracts prepared from the cells according to manufacturer’s instructions. A Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) was used to measure the signal with a 10 second integration time. Each sample was performed in triplicate, and the average signal was then normalized to the protein concentration of that sample. At least three sets of experimental samples were analyzed for each condition.

**Statistical Analysis**

Comparisons between more than two groups were accomplished using a factorial analysis of variance (ANOVA) with Fisher’s Protected Least Significant Difference post-hoc test. Comparisons between two groups were accomplished using an unpaired two-tailed T-test. Comparisons between the survival curves were accomplished using a chi-square test. Analysis was carried out using the StatView program (Abacus Concepts, Berkeley, CA). In all cases a significant result was defined as p<0.05.
Online Figure I: Continuous ECG monitoring of transgenic mice. MKK3/Cre mice were implanted with a continuous ECG monitoring telemetry device, and induced with tamoxifen. Examples of heart rate and body temperature data are shown here before (Day 0) (A, D) and after tamoxifen induction at Day 4 (B, E) and Day 5 (C, F, G) with representative ECG traces illustrated for the time point indicated in the boxes (D-G).
Online Figure II: Global degradation rates in MEFs. MEFs were labeled for 30 mins. with $^{35}$S-methionine, then transferred to non-radioactive medium. At the time shown the radioactivity remaining in the TCA precipitable protein for wildtype (● solid line) or MK2$^{-/-}$ (△ dashed line) MEFs was measured. Degradation rates were calculated based on curve fitting as described in the Supplementary Methods.
Online Figure III: Global synthesis rates in MEFs. MK2 WT and MK2\(^{-/-}\) cells were labeled with \(^{35}\)S-methionine for the indicated time course. Total protein was harvested. At the time shown the radioactivity remaining in the TCA precipitable protein for wildtype (● solid line) or MK2\(^{-/-}\) (△ dashed line) MEFs was measured. Synthesis rates were calculated based on linear curve fitting as described in the Supplementary Methods.
**Online Table I: Hemodynamic parameters of mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>WT + TX</th>
<th>MKK3/Cre + TX</th>
<th>MK2−/− + TX</th>
<th>MKK3/Cre/MK2−/− + TX</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>597.8 ± 49.5</td>
<td>537.7 ± 39.3</td>
<td>482.0 ± 86.7</td>
<td>582.3 ± 56.9</td>
</tr>
<tr>
<td>LVDP</td>
<td>84.1 ± 3.7</td>
<td>52.6 ± 3.6*</td>
<td>90.6 ± 3.1</td>
<td>95.3 ± 14.0 &amp;</td>
</tr>
<tr>
<td>Pmax</td>
<td>87.2 ± 3.3</td>
<td>62.7 ± 4.9*</td>
<td>114.2 ± 15.2†</td>
<td>102.8 ± 12.5 &amp;</td>
</tr>
<tr>
<td>Ped</td>
<td>4.9 ± 0.9</td>
<td>13.3 ± 2.8*</td>
<td>4.1 ± 1.6</td>
<td>9.6 ± 3.6</td>
</tr>
<tr>
<td>dP/dT_max</td>
<td>9307 ± 1596</td>
<td>6291 ± 574</td>
<td>6550 ± 222</td>
<td>9264 ± 2737</td>
</tr>
<tr>
<td>dP/dT_min</td>
<td>-6964 ± 1126</td>
<td>-4182 ± 624</td>
<td>-6572 ± 72</td>
<td>-7502 ± 2015</td>
</tr>
<tr>
<td>Tau</td>
<td>5.52 ± 0.44</td>
<td>8.43 ± 1.02*</td>
<td>3.85 ± 0.27</td>
<td>8.59 ± 0.82**</td>
</tr>
</tbody>
</table>

Data reported as mean ± standard error. HR, heart rate (beats/min); LVDP, left ventricle developed pressure (mmHg); Pmax, maximum pressure (mmHg); Ped, end diastolic pressure (mmHg); dP/dT, rate of pressure change (mmHg/sec); Tau, time constant of relaxation. * p<0.05 vs. WT+TX, ** p<0.05 vs. MK2−/− + TX, † p<0.05 vs. WT + TX; &, p<0.05 vs. MKK3/Cre +TX

**Online Table II: Heart and lung mass in mice**

<table>
<thead>
<tr>
<th>Groups</th>
<th>WT + TX</th>
<th>Cre + TX</th>
<th>MKK3/Cre + Veh</th>
<th>MKK3/Cre + TX</th>
<th>MK2−/− + Veh</th>
<th>MKK3/Cre/MK2−/− + Veh</th>
<th>MKK3/Cre/MK2−/− +TX</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVW/BW</td>
<td>3.11 ±0.05</td>
<td>3.56 ±0.10</td>
<td>3.27 ±0.14</td>
<td>4.52 ±0.45*</td>
<td>3.24 ±0.23</td>
<td>3.28 ±0.07</td>
<td>3.82 ±0.23*</td>
</tr>
<tr>
<td>LAW/BW</td>
<td>0.07 ±0.01</td>
<td>0.12 ±0.01</td>
<td>1.18 ±0.90</td>
<td>2.19 ±0.71*</td>
<td>0.10 ±0.02</td>
<td>0.16 ±0.01</td>
<td>0.54 ±0.15#</td>
</tr>
<tr>
<td>LuW/BW</td>
<td>5.75 ±0.78</td>
<td>5.89 ±0.26</td>
<td>7.39 ±1.14</td>
<td>12.25 ±2.41*</td>
<td>4.26 ±0.24</td>
<td>4.14 ±0.15</td>
<td>9.47 ±1.50*</td>
</tr>
</tbody>
</table>

Data reported as mean ± standard error. LVW/BW, ratio of left ventricle weight over body weight (mg/g); LAW/BW, ratio of left atrial weight normalized to body weight (mg/g); LuW/BW, ratio of lung weight normalized to body weight (mg/g). * p<0.05 vs. WT + TX; & p=0.054, vs. MKK3/Cre +TX; # p < 0.05 vs. MKK3/Cre+TX
III. Supplementary References


