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Mitogen-Activated Protein Kinase–Activated Protein Kinase 2 in Angiotensin II–Induced Inflammation and Hypertension:

Regulation of Oxidative Stress

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Abstract

Vascular oxidative stress and inflammation play an important role in angiotensin II–induced hypertension, and mitogen-activated protein kinases participate in these processes. We questioned whether mitogen-activated protein kinase–activated protein kinase 2 (MK2), a downstream target of p38 mitogen-activated protein kinase, is involved in angiotensin II–induced vascular responses. In vivo experiments were performed in wild-type and *Mk2* knockout mice infused intravenously with angiotensin II. Angiotensin II induced a 30 mm Hg increase in mean blood pressure in wild-type that was delayed in *Mk2* knockout mice. Angiotensin II increased superoxide production and vascular cell adhesion molecule-1 in blood vessels of wild-type but not in *Mk2* knockout mice. *Mk2* knockdown by small interfering RNA in mouse mesenteric vascular smooth muscle cells caused a 42% reduction in MK2 protein and blunted the angiotensin II–induced 40% increase of MK2 expression. *Mk2* knockdown blunted angiotensin II–induced doubling of intracellular adhesion molecule-1 expression, 2.4-fold increase of nuclear p65, and 1.4-fold increase in Ets-1. *Mk2* knockdown abrogated the angiotensin II–induced 4.7-fold and 1.3-fold increase of monocyte chemoattractant protein-1 mRNA and protein. Angiotensin II enhanced reactive oxygen species levels (by 29%) and nicotinamide adenine dinucleotide phosphate oxidase activity (by 48%), both abolished by *Mk2* knockdown. Reduction of MK2 blocked angiotensin II–induced p47phox translocation to the membrane, associated with a 53% enhanced catalase expression. Angiotensin II–induced increase of MK2 was prevented by the nicotinamide adenine dinucleotide phosphate oxidase inhibitor Nox2ds-tat. *Mk2* small interfering RNA prevented the angiotensin II–induced 30% increase of proliferation. In conclusion, MK2 plays a critical role in angiotensin II signaling, leading to hypertension, oxidative stress via activation of p47phox and inhibition of antioxidants, and vascular inflammation and proliferation.

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Disclosures

None.

Keywords

MK2; angiotensin II; oxidative stress; vascular inflammation; hypertension; arterial

Vascular wall inflammation plays a key role in the pathogenesis of hypertension, atherosclerosis, and other forms of cardiovascular disease. Angiotensin II (Ang II) induces vascular injury by modulating release of inflammatory chemokines such as monocyte chemoattractant protein-1 (MCP-1)¹ and the nuclear action of proinflammatory transcription factors such as nuclear factor- κ B (NF- κ B). The latter, in turn, regulates expression of vascular cell adhesion molecule-1 (VCAM-1)² and intracellular adhesion molecule-1 (ICAM-1). These events induce inflammation within the vascular wall, deposition of extracellular matrix and hypertrophy or hyperplasia of vascular smooth muscle cells (VSMCs). Ang II actions in the vascular wall are mediated in large measure through increase in the generation of reactive oxygen species (ROS).³ The major source of ROS in the vessel wall is reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.⁴ Upon stimulation by Ang II or other agents, the cytosolic subunits are translocated to the membrane and assemble with the membrane subunits to form the active NADPH oxidase capable of producing superoxide anion ($\cdot\text{O}_2^-$). Redox-sensitive pathways are important in Ang II-stimulated growth and differentiation of cells, probably through activation of mitogen-activated protein kinases (MAPKs).⁵

p38 MAPK is markedly activated in cardiovascular cells by a variety of stimuli, including Ang II, and typically mediates apoptosis and inflammatory responses. Ang II rapidly stimulates the phosphorylation of p38 MAPK in cultured VSMCs⁶ as well as in cardiac and vascular tissues.⁷ Some in vitro studies suggest that Ang II-induced activation of NADPH oxidase results in upregulation of p38 MAPK.^{8,9} In addition, p38 MAPK plays an important role in endothelial inflammation and dysfunction associated with salt-sensitive hypertension.¹⁰ Treatment with SB202190, a p38 MAPK inhibitor, resulted in marked reductions in blood pressure in a diabetic rat model, which underlines the role of p38 MAPK in hemodynamic control.¹¹ Upregulation of p38 MAPK induces early response gene expression and cell growth.^{12,13} Finally, in VSMCs from spontaneously hypertensive rat, p38 MAPK has been shown to influence Ang II-induced collagen production.¹⁴

p38 MAPK activates many protein kinases, such as MAPK-activated protein kinase 2 (MK2),¹⁵ MK3, MAPK-interacting kinases 1¹⁶ and 2, and p38-regulated/activated protein.¹⁷ Phosphorylation of MK2 by p38 MAPK unmasks a nuclear export signal in the C-terminal part of the molecule.^{18,19} Several substrates have been described for MK2, including mRNA-binding proteins such as tristetraproline, transcription factors such as heat shock protein-1, proteins interacting with the cytoskeleton such as heat shock protein-25, as well as regulators of the cell cycle and apoptosis such as Cdc25B/C.²⁰⁻²⁴ Interaction of p38 MAPK with the preexisting complex of heat shock protein-27 and Akt is mediated by MK2.²⁵ MK3, an isoform of MK2, was shown by Ronkina et al using a gene targeting approach to be coexpressed with MK2 in cells and tissues and to share similar physiological functions.²⁶ More recently, *Mk2* knockout (KO) mice were demonstrated to be more resistant to

lipopolysaccharide-induced endotoxic shock as a consequence of a reduction of tumor necrosis factor- α production.²⁷

In the present study, we investigated in vivo and in vitro whether MK2, one of the downstream targets of p38 MAPK, is involved in the pathway that mediates Ang II-induced inflammation, oxidative stress, and proliferation in VSMCs from mesenteric arteries, and in Ang II-induced hypertension. In vivo experiments were performed examining the effects of Ang II infusion into *Mk2* KO mice. In vitro, Ang II action was tested on VSMCs in which *Mk2* knockdown was achieved using small interfering RNA (siRNA).

Methods

An expanded Methods section can be found in an online data supplement available at <http://hyper.ahajournals.org>.

Animals and Blood Pressure Determination

Ten-week-old male C57BL/6 mice (Harlan Laboratories; Indianapolis, IN) were killed humanely by exsanguination under isoflurane anesthesia. Mesenteric arteries were dissected and used for the isolation of VSMCs. Fourteen- to 17-week-old male *Mk2* KO and wild-type (WT) littermates were anesthetized with isoflurane, surgically instrumented with PA-C10 radiotelemetry transmitters as recommended by the manufacturer (Data Sciences International; St. Paul, MN), allowed to recover for 10 days, and then blood pressure was determined every 5 minutes for 10 s for 3 consecutive days. Thereafter, mice were infused with Ang II (400 ng/kg/min; Calbiochem; EMD Biosciences Inc.) or vehicle (Veh) using Alzet osmotic mini-pumps (DURECT Corp.) for 14 days. Blood pressure was determined as above during the 2-week treatment successfully in 5 WT+Veh, 3 WT+Ang II, 3 *Mk2*+Veh, and 4 *Mk2*+Ang II. At the end of the experiment, mice were killed as above, and the mesenteric vascular bed and aorta were dissected, frozen, and stored at -80°C for VCAM-1 and superoxide determination.

Cell Culture and Stimulation

VSMCs were isolated from mesenteric arteries as described previously²⁸ and were used after 3 to 6 passages. They were transfected with siRNA (5 nmol/L; Qiagen; Mississauga, ON, Canada) targeting *Mk2* (siMK2) or *luciferase* used as a negative control for 30 hours, or with a control fluorescein isothiocyanate (FITC)-coupled siRNA (10 $\mu\text{mol/L}$; Santa Cruz Biotechnology; Santa Cruz, CA) used to determine the efficiency of transfection. VSMCs were serum starved for 18 hours and treated with vehicle or Ang II (100 nmol/L) for 10 minutes to 24 hours, depending on the experiments. In some experiments, cells were also pre-exposed for 30 minutes to 10 $\mu\text{mol/L}$ Nox2ds-tat (NADPH oxidase inhibitor) or to scrambled peptide used as negative control before stimulation with Ang II.²⁹ For the efficiency of transfection after being transfected with FITC-coupled siRNA, VSMCs were stained with 4',6-diamidino-2-phenylindole (Sigma Chemicals). Fluorescence was visualized with a DM2000 microscope with an FITC filter (488 nm; Leica Microsystems).

Nuclear Extract Preparation

VSMC nuclear extracts were prepared as described previously³⁰ (details in the online supplement).

Western Blotting

Total or fractioned proteins (15 to 20 μ g) were extracted from VSMCs, separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated overnight at 4°C with antibodies (1:1000) against the following: MK2, p38 MAPK, and NF- κ B p65 subunit (from Cell Signaling Technology), and ICAM-1, VCAM-1, Ets-1, and p47phox (from Santa Cruz Biotechnology). After incubation with secondary antibodies, signals were revealed by chemiluminescence (SuperSignal West Pico chemiluminescent signal; Thermo Scientific), with the Molecular Imager Chemidoc XRS system (Bio-Rad), and quantified by densitometry using Quantity One software (Bio-Rad). Membranes were subsequently stripped and reprobbed with anti- β -actin antibody (Sigma Chemicals) to verify equal loading. VCAM-1 was also determined in protein extracts of mesenteric arteries from *Mk2* KO and WT mice.

Quantitative RT-PCR

MCP-1 mRNA expression levels were determined by quantitative real-time PCR. RNA was extracted from VSMCs using the TRIzol reagent (Invitrogen). One microgram of total RNA was reversed transcribed with a Quantitect RT kit (Qiagen). Quantitative real-time PCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen) with the Mx3005P real-time PCR cycler (Stratagene). Quantitative real-time PCR results were normalized with ribosomal protein S16 and expressed as fold change of negative-control siRNA-treated samples. Primers were designed using Primer3³¹ and are presented in the online data supplement.

MCP-1 Assay

MCP-1 levels were measured in the supernatant of VSMCs using a simplex microbead-based immunoassay kit on a Bio-Plex 200 (Bio-Rad) according to the manufacturer protocol.

ROS Levels

Intracellular ROS levels were evaluated using 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl- ester mixed isomers (CM-H₂DCFDA; Invitrogen), a chloromethyl derivative of dichlorodihydrofluorescein diacetate that becomes fluorescent when oxidized. Dihydroethidium (DHE) was used to evaluate in situ production of superoxide ($\cdot\text{O}_2^-$)³² (online supplement).

NADPH Oxidase Activity

The lucigenin-enhanced chemiluminescence assay was used to determine NADPH oxidase activity in VSMC homogenates as described previously.³³ VSMCs were transfected with siMK2 or siRNA targeting *luciferase*, serum starved, and stimulated or not with Ang II for 5 minutes as above (online supplement).

Proliferation Assay

Ang II–stimulated DNA synthesis was determined by measuring incorporation of [³H]thymidine into DNA³⁴ (online supplement).

Statistical Analysis

Experiments were repeated 3 to 6 times in duplicate. Results are presented as means±SEM and were compared by analysis of variance followed by a Student Newman–Keuls post hoc test. $P<0.05$ was considered significant.

Results

***Mk2* Deletion Delays and Blunts the Ang II–Induced Rise in Mean Blood Pressure**

To establish whether MK2 participates in Ang II–induced blood pressure rise in vivo, we measured mean blood pressure (MBP) by radiotelemetry in WT and *Mk2* KO mice infused or not with Ang II for 14 days. No significant difference in day and night MBP was observed between *Mk2* KO and WT mice at baseline and during the 14-day infusion with the vehicle (Figure 1). Ang II caused a 30- to 35-mm Hg increase in day and night MBP from day 2, which was maintained up to the end of the protocol in WT mice. *Mk2* deletion delayed and blunted the rise in day and night MBP, respectively. Day MBP of Ang II–infused *Mk2* KO mice increased significantly only at day 11 to day 14, whereas night MBP only tended to increase during the last few days of the protocol.

***Mk2* KO Reduces Ang II–Induced Vascular Superoxide Generation**

$\cdot\text{O}_2^-$ production was evaluated by DHE fluorescence in aorta sections of WT and *Mk2* KO mice infused or not with Ang II for 14 days. Basal production of $\cdot\text{O}_2^-$ was significantly enhanced by *Mk2* deletion (Figure 2A). Ang II increased $\cdot\text{O}_2^-$ production in WT mice, whereas this change was blunted in *Mk2* KO mice.

***Mk2* Deletion Blunts Ang II–Induced Inflammatory Responses**

The levels of the proinflammatory molecule VCAM-1 were determined in mesenteric arteries of WT and *Mk2* KO mice infused or not with Ang II for 14 days. Basal levels of VCAM-1 were increased 1.7-fold in *Mk2* KO mice compared with WT mice (Figure 2B). Ang II infusion increased 1.4-fold the levels of VCAM-1 in WT mice and had no effect in *Mk2* KO mice.

Ang II Upregulates p38 MAPK and MK2

Ang II increased p38 MAPK phosphorylation by 45% (Figure 3A) as well as MK2 phosphorylation and total expression in VSMCs by 29% and 39%, respectively (Figure 3B and 3D). For gene silencing experiments, the efficiency of VSMCs siRNA transfection was determined using a control FITC-coupled siRNA. We observed an efficiency of 56% (Figure 3C). siMK2 decreased MK2 protein by 42% and blunted the Ang II–induced increase of MK2 expression (Figure 3D). The expression of p38 MAPK was not significantly affected by MK2 knockdown (data not shown).

MK2 Is Involved in Ang II–Induced Increase of ICAM-1, NF- κ B, Ets-1, and MCP-1

VSMC *Mk2* knockdown did not significantly alter protein levels of ICAM-1, VCAM-1, and Ets-1, but resulted in a 1.5-fold increase of the nuclear level of the p65 subunit of NF- κ B (Figure 4). Ang II increased ICAM-1 and nuclear p65 2-fold and Ets-1 1.4-fold without altering the levels of VCAM-1. siMK2 blunted these Ang II responses. siMK2 did not alter the basal expression of MCP-1, but abrogated the Ang II–induced 4.7- and 1.3-fold increase of MCP-1 at mRNA and protein levels, respectively (Figure 5). These results demonstrate the involvement of MK2 activation in some of Ang II–induced inflammatory pathways.

MK2 Mediates Ang II–Induced ROS Generation Via NADPH Oxidase

Intracellular ROS levels and in situ production of $\cdot\text{O}_2^-$ were evaluated respectively in VSMCs by dichlorodihydrofluorescein diacetate and by DHE fluorescence. *Mk2* knockdown did not affect the basal levels of intracellular ROS or generation of $\cdot\text{O}_2^-$ in VSMCs (Figure 6A and 6B). Ang II increased intracellular ROS levels (by 29%) and production of $\cdot\text{O}_2^-$ revealed by DHE staining in VSMCs, both abrogated by *MK2* knockdown. Ang II increased NADPH oxidase activity (48%), the main vascular source of $\cdot\text{O}_2^-$ (Figure 6C). *Mk2* knockdown abolished the increase in NADPH oxidase activity induced by Ang II without altering basal levels. These results suggest that Ang II–induced NADPH oxidase activation involves MK2.

Ang II/MK2–Induced NADPH Oxidase Involves p47phox Translocation

To further understand the molecular pathways of activation of NADPH oxidase by MK2, we examined the translocation of p47phox, one of the main cytosolic subunits of NADPH oxidase, the translocation of which from the cytosol to the membrane is essential for the assembling of the active NADPH oxidase in the cell membrane. Ang II increased p47phox translocation to the VSMC membrane 2-fold, an effect that was prevented by *Mk2* knockdown (Figure 7A). Basal p47phox membrane/cytosolic ratio was not altered by *Mk2* knockdown.

Ang II–Induced NADPH Oxidase Upregulates MK2 Expression

To determine whether activation of NADPH oxidase affects the expression of MK2, VSMCs were pretreated with the NADPH oxidase inhibitor Nox2ds-tat and then stimulated or not with Ang II. Nox2ds-tat is a small peptide that was designed to inhibit assembly of p47phox with gp91-phox (Nox2) and thus inhibit NADPH oxidase (Nox2) activation. An Ang II–induced increase of MK2 protein levels was prevented by ds-tat but not by the ds-tat scrambled peptide (Figure 7B). This result suggests that Ang II–induced NADPH oxidase-driven superoxide generation induces a positive feedback loop by increasing the expression of MK2.

Downregulation of MK2 Caused Ang II to Upregulate Catalase in VSMCs

We then evaluated the effects of modulation of MK2 on expression of the antioxidant catalase in VSMCs. Neither Ang II nor *MK2* knockdown had any effect on the basal levels of catalase (Figure 8). However, in presence of siMK2, Ang II increased catalase expression

by 53%. These results suggest that MK2 has an inhibitory effect on catalase in oxidative conditions, which further increases ROS production.

Downregulation of MK2 Prevents Ang II–Induced VSMC Proliferation

To determine whether MK2 mediates Ang II–induced vascular remodeling, VSMC proliferation was evaluated using [³H]thymidine incorporation. MK2 knockdown decreased by 50% [³H]thymidine VSMC incorporation under basal conditions (Figure 9). Ang II increased [³H]thymidine incorporation into VSMCs by 30%, which was abolished by MK2 knockdown, suggesting that MK2 is a mediator of Ang II–induced proliferation.

Discussion

The present study demonstrates the following novel findings. In vivo, *Mk2* plays a key role in Ang II–induced rise in blood pressure and vascular superoxide production and inflammation. Similarly, in vitro MK2, one of the direct downstream targets of p38 MAPK, mediates Ang II–induced increase of inflammatory mediators as well as superoxide production from NADPH oxidase, through regulation of the p47phox subunit of NADPH oxidase and the antioxidant enzyme catalase, and VSMC proliferation.

To investigate the role of MK2 in Ang II–induced hypertension; we performed in vivo experiments using *Mk2* KO mice. There are contradictory results on the effect of p38 MAPK pharmacological inhibitors on hypertension in different experimental models.^{10,35,36} However, our results showed for the first time that the absence of MK2, which is downstream of p38 MAPK, delayed Ang II–induced hypertension. In addition, our in vivo data showed a decrease of Ang II–induced oxidative stress and inflammation in *Mk2* KO mice, demonstrating a crucial role for MK2 in Ang II signaling, leading to increases in blood pressure. To determine the molecular mechanisms involved, we performed in vitro experiments by knocking down MK2 in mesenteric VSMCs. Vascular MK2 in mice in our study was upregulated by Ang II. Previous in vitro studies reported that Ang II activates p38 MAPK in VSMCs from resistance and conduit arteries.³⁷ In addition, acute and chronic infusion of Ang II upregulated p38 MAPK in hypertensive models.³⁸ Indeed, activated MK2 is cotransported with active p38 MAPK to the cytoplasm.¹⁸ siMK2 blunted the Ang II–induced increase of MK2 but did not affect p38 MAPK expression. This result is in agreement with a previous study on *MK2* KO mice embryonic fibroblasts that showed that p38 MAPK activity was unchanged by Ang II.²⁷ p38 MAPK is involved in Ang II–induced inflammatory responses.³⁹ Accordingly, we examined the effect of MK2 downregulation on Ang II–induced increase of vascular inflammatory mediators. MK2 is involved in Ang II–induced increase of inflammatory adhesion molecule such as ICAM-1, as well as the proinflammatory chemokine MCP-1, and the proinflammatory transcription factor NF- κ B. Previous experiments have shown that SB203580, an inhibitor of p38 MAPK, blocks the synthesis of proinflammatory cytokines at the transcriptional level.⁴⁰ Moreover, mice lacking *MK2* gene showed a defect in lipopolysaccharide-induced biosynthesis of inflammatory cytokines.²⁷ Recently, it was reported that Ang II stimulates MCP-1 expression via distinct p38 MAPK- and NF- κ B–dependent pathways in VSMCs.⁴¹ Thus, it appears that Ang II–induced upregulation of MCP-1 occurs through a coactivation of MK2

and NF- κ B, with MK2 influencing as well the NF- κ B pathway. Further, it has been reported that p38 MAPK is involved in monocyte/macrophage survival regulated by the macrophage colony-stimulating factor.⁴² Recently, a novel role for transcription factor Ets-1 has been described as a transcriptional mediator of vascular inflammation and remodeling.⁴³ The induction of MCP-1 by Ang II is largely dependent on Ets-1. We have shown here that MK2 downregulation led to a marked reduction in the induction of Ets-1 by Ang II. Further experiments are needed to determine which inflammatory factor is initially upregulated by Ang II-activated MK2.

A central mechanism by which Ang II promotes inflammation is via the generation of ROS.⁴⁴ In fact, p38 MAPK is a redox-sensitive kinase.^{6,45} Ang II-induced MK2 upregulation was significantly reduced by NADPH oxidase inhibition, suggesting that ROS produced by NADPH oxidase act as second messengers in Ang II-triggered MK2 upregulation (Figure 10). Some in vitro studies suggest an important influence of NADPH oxidase in p38 MAPK activation.^{9,33} We found that downregulation of MK2 abolished Ang II-induced increase of ROS production and NADPH oxidase activation. This suggests that MK2 plays an important role in NADPH oxidase activation by Ang II and is in agreement with our in vivo data as well as with what has been reported previously for p38 MAPK. p38 MAPK activates NADPH oxidase in human endothelial cells by enhancing phosphorylation and assembly of its subunits.⁸ SB239063, a p38 MAPK inhibitor, blunted Ang II-induced increase of NADPH oxidase subunits and restored endothelium-dependent relaxation in stroke-prone spontaneously hypertensive rats fed a high-salt/high-fat diet.³⁸ Our results suggest that MK2 is one of the most important downstream targets of p38 MAPK and is involved directly in Ang II-induced redox signaling. This suggests that upregulation of MK2 by Ang II activates proinflammatory molecules that could regulate NADPH oxidase activation and ROS generation. Our data also appear to suggest the involvement of Nox2 NADPH oxidase (also known as, Nox2 oxidase). Activation of Nox2 oxidase, which has been shown to be present in resistance artery smooth muscle,⁴⁶ is a multistep process that is initiated by serine phosphorylation of the cytosolic regulatory subunit p47phox. In the canonical Nox2 oxidase system, the activated p47phox subunit translocates to the membrane along with other essential cytosolic subunits and associates with the membrane-bound Nox2 (also known as gp91-phox) and p22phox subunits to produce active NADPH oxidase. We were able to demonstrate that MK2 plays a critical role in Ang II-induced p47phox translocation. Further, we demonstrated that the Nox2 oxidase inhibitor Nox2ds-tat, which targets p47phox and interferes with subunit assembly, blunted Ang II-induced MK2, indicating that MK2 is in fact part of a positive-feedback loop. Ang II increased NADPH oxidase activity through MK2, and NADPH oxidase activation induced MK2. Interestingly, a recent study reported that Ets-1 is a critical transcriptional mediator of ROS generation by Ang II by regulating the expression of p47phox subunit.⁴⁷ Because we have shown that MK2 plays a role in Ang II-induced Ets-1 upregulation, we can hypothesize that one of the mediators involved in the regulation of p47phox by MK2 could be Ets-1. Moreover, we found that only in the absence of MK2, Ang II induced a slight increase of catalase, a major antioxidant enzyme expressed in the vasculature.⁴⁸ In hypertensive animal models, H₂O₂ levels are higher compared with normotensive animals.⁴⁹ In addition, H₂O₂ is specifically involved in the blood pressure-induced enhancement of responses to Ang II.⁵⁰ The

generation of H₂O₂ after Ang II stimulation is a critical step in arterial wall hypertrophy.⁵¹ This could explain in part the regulation of catalase by MK2 in hypertensive models and suggests that active MK2 regulates cellular ROS levels in part by inhibiting antioxidant enzymes.

Vascular inflammation is closely related to vascular cell proliferation. Thus, to further understand the functional effect of MK2 activation by Ang II, we investigated VSMC proliferation and demonstrated that MK2 plays an important role in Ang II–induced cell growth. p38 MAPK is activated by G-protein–coupled receptors, such as Ang II type 1 receptors, and plays an important role in the induction of proliferative responses in susceptible cells.¹² However, in rat VSMCs, Ang II–induced growth may be p38 MAPK independent.⁵² It was reported recently that endothelin-1 drives proliferation of VSMCs through stimulation of extracellular signal-regulated kinase and p38 MAPK.⁵³ Moreover, very recently, using a cardiomyocyte-specific and inducible transgenic approach, MK2 activity was shown to play an important role in p38 MAPK–mediated cardiac hypertrophy and heart failure.⁵⁴ Our findings suggest that MK2 activation by Ang II may contribute to vascular remodeling by promoting cell growth.

Our *in vivo* data on blood pressure support clearly the implication of MK2 in Ang II–induced hypertension, and the potential molecular mechanism was demonstrated in VSMCs. However, additional *in vivo* experiments will be performed to confirm the proposed signaling pathway, and experiments with shorter time of Ang II infusion may be needed to determine whether the observed Ang II effects involving MK2 are related to blood pressure increase.

In summary, the data reported here demonstrate the novel findings that MK2 is involved in Ang II–induced hypertension and is upregulated by Ang II in VSMCs through generation of ROS via NADPH oxidase, likely Nox2 oxidase. Activation of MK2 upregulates proinflammatory molecules that further activate ROS generation, via effects on the p47phox subunit of NADPH oxidase as well as on antioxidant enzymes. Upregulation of MK2 appears to be part of a vicious circle that potentiates inflammatory responses to Ang II, leading as well to an increase in VSMC proliferation (Figure 10).

Perspectives

Clinical trials with p38 MAPK pharmacological inhibitors as anti-inflammatory therapies have been shown to be ineffective, attributable in part to the influence of p38 MAPK on diverse cellular pathways. The present results show for the first time that MK2 is an essential component of Ang II–induced hypertension and vascular oxidative stress and inflammation and could be a promising target for specific cardiovascular anti-inflammatory therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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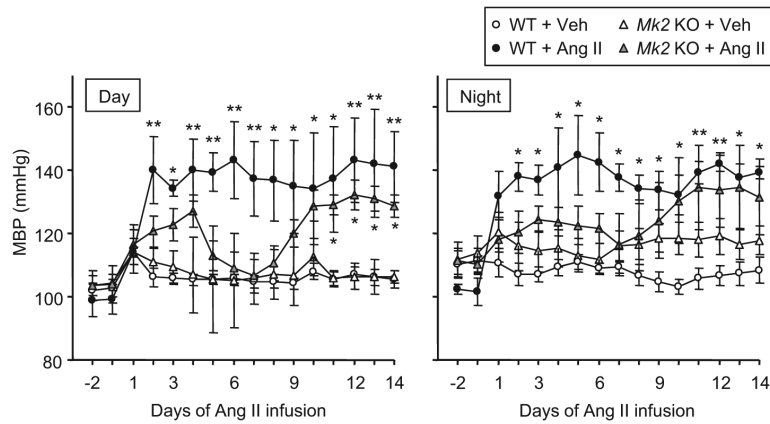


Figure 1.

Mk2 deletion delays and blunts, respectively, the Ang II–induced day and night blood pressure rise in mice. MBP was determined by radiotelemetry in WT and *Mk2* KO mice at baseline and during the infusion with vehicle (Veh) or Ang II (400 ng/kg/min) for 14 days. The day (6 AM to 8 PM) and night (8 PM to 6 AM) average MBPs are presented. Results are means \pm SEM; * P <0.05 and ** P <0.01 vs respective Veh, with $n=5$ for WT+Veh, $n=3$ for WT+Ang II, $n=3$ for *Mk2*+Veh, and $n=4$ for *Mk2*+Ang II.

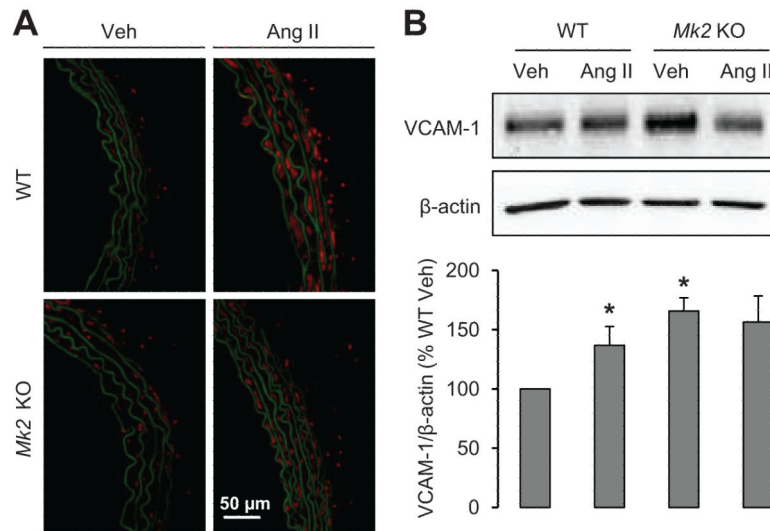


Figure 2. Absence of MK2 prevents Ang II–induced superoxide and proinflammatory VCAM-1 production. In situ detection of superoxide generation in the aorta using DHE staining (A) and mesenteric artery protein level of VCAM-1 using Western blot (B) were determined in WT and *Mk2* KO mice infused with vehicle (Veh) or Ang II (400 ng/kg/min) for 14 days. Representative Western blots of VCAM-1 and bar graphs representing VCAM-1 expression in different conditions are presented. Results are means±SEM; n=3 to 4; * $P<0.05$ vs respective WT+Veh.

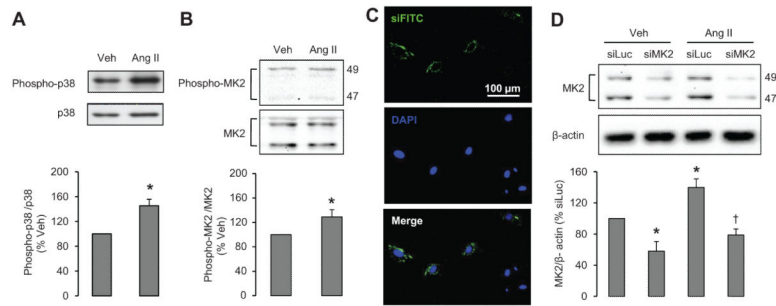


Figure 3.

A and B, Ang II increases p38 MAPK and MK2 phosphorylation levels. VSMCs were stimulated with vehicle (Veh) or Ang II for 10 minutes. Representative Western blots of phosphorylated and total p38 MAPK (A) and MK2 (B) and corresponding bar graphs are represented. C, VSMCs were transfected with a nontargeting control FITC-siRNA (siFITC) for 24 hours. Representative siFITC and 4',6-diamidino-2-phenylindole (DAPI) images are presented. D, *MK2* siRNA transfection blunts Ang II-induced MK2 expression. VSMCs were transfected with siMK2 or negative control siRNA targeting *luciferase* (siLuc) and treated with Veh or Ang II for 24 hours. Representative Western blots of MK2 and bar graphs representing MK2 expression in different conditions are depicted. Results are means \pm SEM; n=4 to 5; * P <0.05 vs Veh for A and B and vs siLuc+Veh for D, and † P <0.01 vs siLuc+Ang II for D.

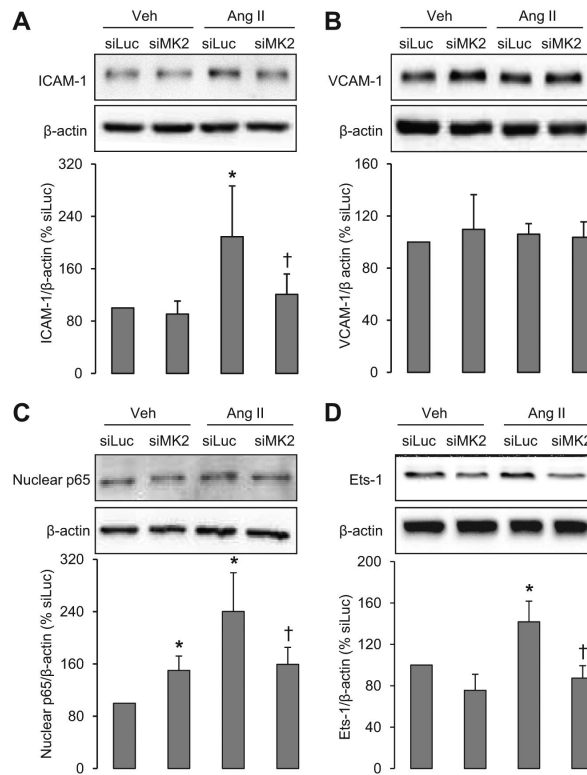


Figure 4.

Downregulation of MK2 prevents Ang II–induced proinflammatory molecules. The protein levels of ICAM-1 (A), VCAM-1 (B), NF- κ B p65 subunit (C), and Ets-1 (D) were determined in VSMCs transfected with siMK2 or with the *luciferase* siRNA (siLuc) used as a negative control and treated with vehicle (Veh) or Ang II for 24 hours. Representative Western blots of proinflammatory mediators and bar graphs representing proinflammatory molecule expression in different conditions are shown. Results are means \pm SEM; n=4 to 5; * P <0.05 vs siLuc+Veh and † P <0.05 vs siLuc+Ang II.

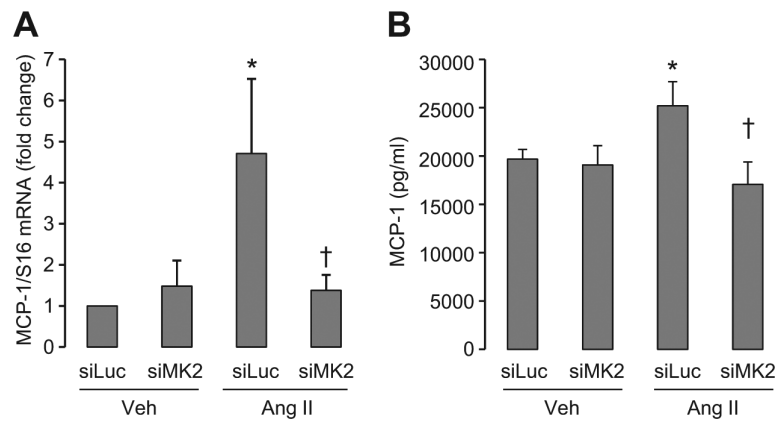


Figure 5. Downregulation of MK2 prevents Ang II–induced increase of MCP-1. The mRNA (A) and protein (B) levels of MCP-1 were determined in VSMCs transfected with siMK2 or with the *luciferase* siRNA (siLuc) used as a negative control and treated with vehicle (Veh) or Ang II for 24 hours. Results are means±SEM; n=4; * $P < 0.05$ vs siLuc+Veh and † $P < 0.05$ vs siLuc+Ang II.

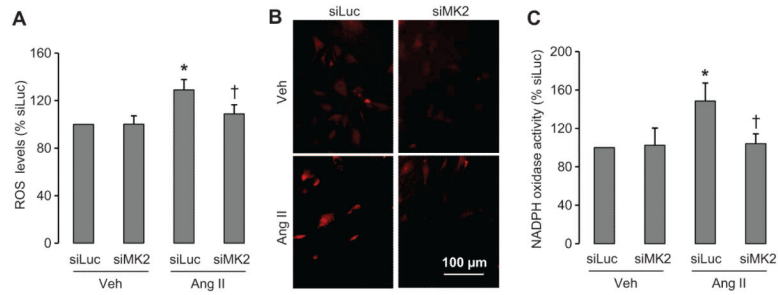
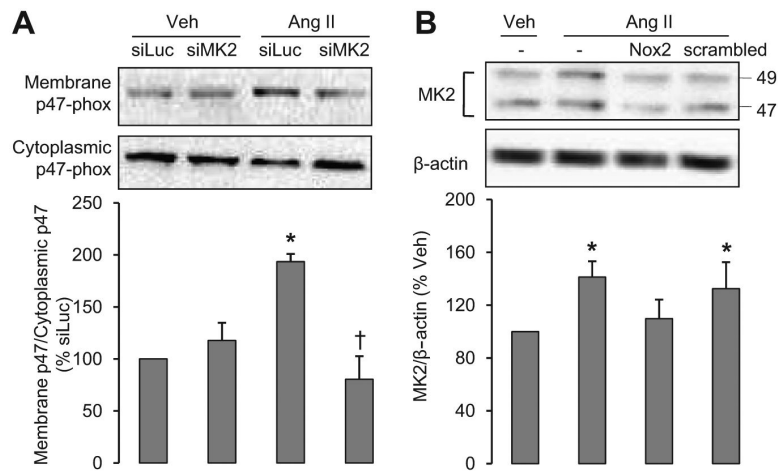


Figure 6.

Downregulation of MK2 abolished Ang II–induced increase of oxidative stress. ROS were measured by DCF-DA fluorescence (A), superoxide generation was detected in situ with DHE staining (B), and NADPH oxidase activity was determined by lucigenin chemiluminescence (C) in VSMCs transfected with siMK2 or with the *luciferase* siRNA (siLuc) used as a negative control and treated with vehicle (Veh) or Ang II for 10 minutes. Results are means±SEM; n=5; * P <0.05 vs siLuc+Veh and † P <0.05 vs siLuc+Ang II.

**Figure 7.**

MK2 is involved in an Ang II–induced NADPH oxidase activation positive feedback loop. A, p47phox translocation from cytoplasm to the membrane was demonstrated in VSMCs transfected with siMK2 or with the *luciferase* siRNA (siLuc) used as a negative control and treated with vehicle (Veh) or Ang II for 5 minutes. B, MK2 protein level was determined in VSMCs exposed or not to the NADPH oxidase inhibitor Nox2ds-tat (Nox2) or a scrambled peptide and treated with vehicle (Veh) or Ang II for 24 hours. Representative Western blots of p47phox, MK2, and bar graphs representing membrane and cytoplasmic p47phox and MK2 expression in different conditions are depicted. Results are means±SEM; n=4, * $P < 0.01$ vs siLuc+Veh and † $P < 0.05$ vs siLuc+Ang II for A and n=5, * $P < 0.01$ vs Veh for B.

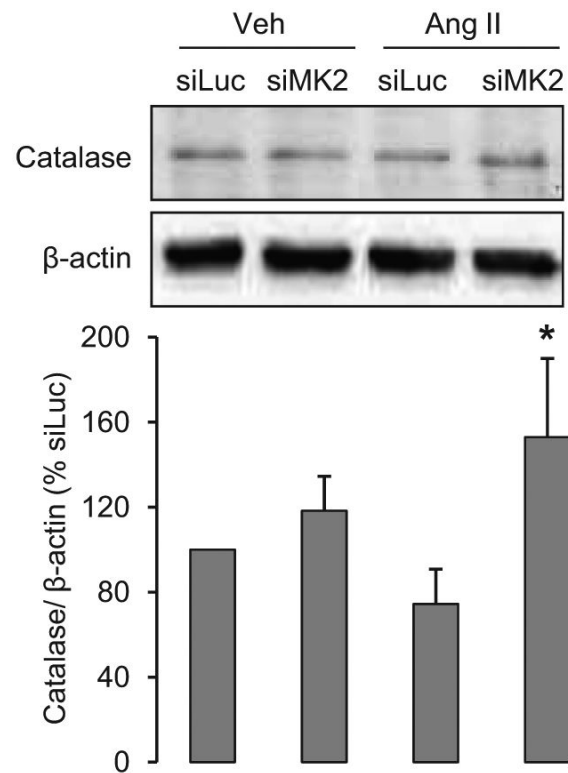


Figure 8.

MK2 knockdown causes Ang II to upregulate catalase in VSMCs. Catalase protein levels were determined in VSMCs transfected with siMK2 or with the *luciferase* siRNA (siLuc) used as a negative control and treated with vehicle (Veh) or Ang II for 24 hours.

Representative Western blots of catalase and bar graphs representing catalase expression in different conditions are presented. Results are means±SEM; n=4; * $P<0.05$ vs siLuc+Veh.

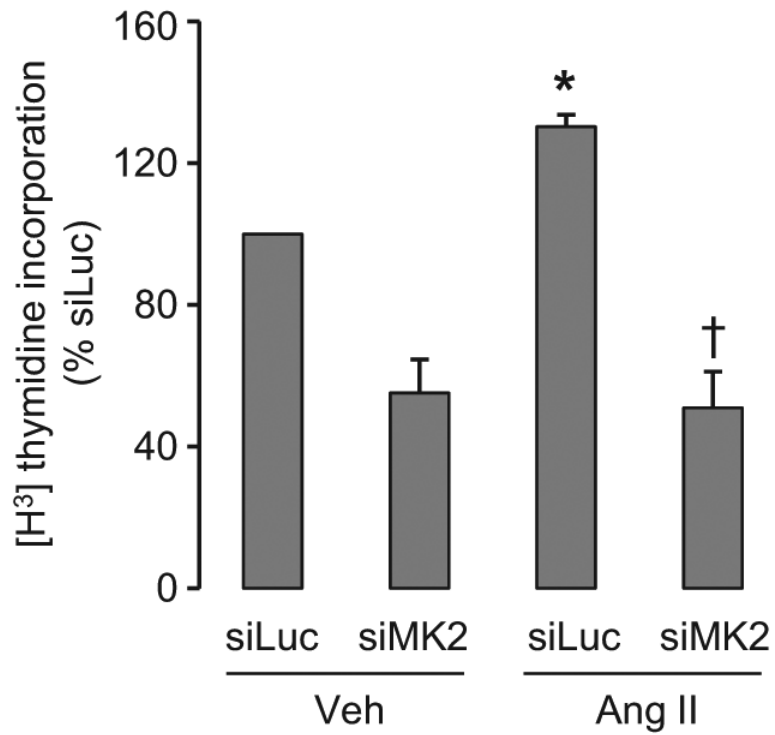


Figure 9.

Downregulation of MK2 blocks Ang II–induced VSMC proliferation. [³H]thymidine incorporation was determined in VSMCs transfected with siMK2 or with the *luciferase* siRNA (siLuc) used as a negative control and treated with vehicle (Veh) or Ang II for 24 hours. Results are means±SEM; n=5; * P <0.05 vs siLuc+Veh and † P <0.05 vs siLuc+Ang II.

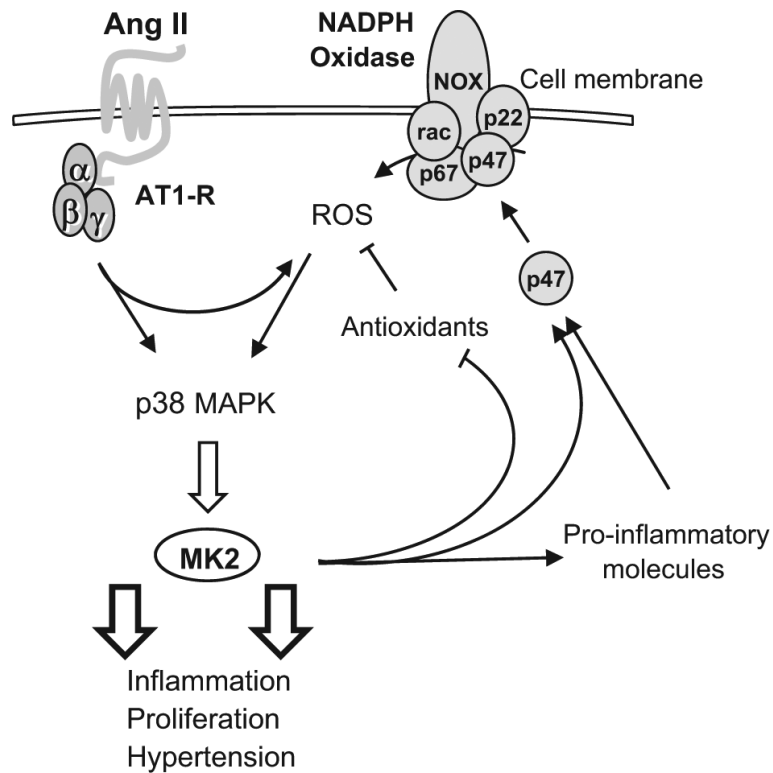


Figure 10.

Proposed role for MK2 in Ang II signaling in mesenteric VSMCs. Ang II activates the NADPH oxidase through angiotensin receptor type 1 (AT1R). The increased production of ROS activates p38 MAPK, which leads to the upregulation of MK2. Activated MK2 in turn activates proinflammatory molecules, inhibits antioxidants, and potentiates NADPH oxidase activation by regulating the translocation of the p47 phox (p47) subunit to the membrane. These effects lead to vascular inflammation, cell proliferation, and hypertension. NOX, rac, p67, p47, and p22 are NADPH oxidase subunits.