Inhibitor κB Kinase 2 Is a Myosin Light Chain Kinase in Vascular Smooth Muscle

Zhekang Ying, Jussara M. do Carmo, Lusha Xiang, Alexandre A. da Silva, Minjie Chen, Michael J. Ryan, Michael Ostrowski, Sanjay Rajagopalan, John E. Hall

Rationale: Myosin light chain (MLC) phosphorylation determines vascular contractile status. In addition to the classic Ca\(^{2+}\)-dependent MLC kinase (MLCK), another unidentified kinase(s) also contributes to MLC phosphorylation in living cells. Inhibitor κB kinase 2 (IKK2)–deficient mouse embryonic fibroblasts demonstrate abnormal morphology and migration, suggesting that IKK2 may be involved in MLC phosphorylation.

Objective: Therefore, we tested whether IKK2 is an MLCK in living cells and the role of IKK2 in mediating vasoconstriction and blood pressure regulation.

Methods and Results: In the present study, we showed that recombinant IKK2–phosphorylated MLC and intact myosin in vitro, and the kinetic parameters were comparable with those of the classic MLCK. Overexpression of IKK2 increased cellular MLC phosphorylation level, and pharmacological inhibition of IKK2 markedly decreased vascular smooth muscle cell MLC phosphorylation, suggesting that IKK2 is an MLCK in living cells. IKK2 inhibitors dose- and time-dependently attenuated vasoconstriction elicited by diverse agonists, suggesting the physiological importance of IKK2 as an MLCK. Vascular smooth muscle cell-specific IKK2-deficient mice had decreased aortic contractile responses, and reduced hypertensive responses to several vasoconstrictors, compared with wild-type mice, confirming the physiological importance of IKK2 as an MLCK.

Conclusions: Our data provide a novel mechanism whereby IKK2 regulates MLC phosphorylation as an MLCK and, thus, vascular function and blood pressure.

Key Words: blood pressure ■ blood vessels ■ hypertension ■ IKK2 ■ MLC ■ muscle, smooth, vascular ■ myosins ■ phosphorylation

Initiation of vascular smooth muscle contraction is primarily regulated at the level of myosin light chain (MLC) Ser19 phosphorylation, which is essential for interaction of actin and myosin and activation of actin-activated myosin ATPase. The relative activity of MLC kinases (MLCKs) versus MLC phosphatase (MLCP) ultimately determines the phosphorylation level of MLC Ser19 and, thus, vascular contractile responses.

Previous studies have demonstrated that, in addition to the classic Ca\(^{2+}\)-dependent MLCK, another unidentified kinase(s) also contributes to MLC phosphorylation in living cells. For example, basal MLC phosphorylation in vascular smooth muscle cells (VSMCs) is resistant to MLCK inhibitors ML-7 and ML-9. MLCP inhibition with calycin A or microcystin-LR induces potent vasoconstriction; these contractile responses are also resistant to MLCK inhibitors, indicating that a kinase(s) other than the classic MLCK is primarily responsible for MLC phosphorylation. The most direct evidence comes from studies in MLCK-deficient mice, showing that blood vessels from these mice remain responsive to elevated [Ca\(^{2+}\)], strongly suggesting that a kinase(s) other than MLCK phosphorylate MLC in VSMCs.

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Thus far, several kinases other than MLCK have been shown to phosphorylate MLC Ser19 in vitro, including Rho-associated protein kinase, integrin-linked kinase (ILK), p21-activated kinase (PAK), and zipper-interacting protein kinase (ZIPK). However, it remains controversial whether these kinases phosphorylate MLC in living cells. The Rho-Kinase inhibitor Y-27632 has no effect on calycin A–induced vasoconstriction and MLC phosphorylation, suggesting that Rho-Kinase may not be an important MLCK in VSMCs. Two potent ZIPK inhibitors, AV25 and SM1, do not relax MLCP inhibition-induced vasoconstriction, ruling out the possibility that ZIPK phosphorylates MLC in this context. PAK1, one of the most intensively studied PAKs, has been shown to phosphorylate MLC Ser19 in vitro, and the interaction of PAK1 with FAK has been demonstrated in vivo. The role of PAK1 in vascular smooth muscle contraction is not yet fully understood.

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contributes to MLC phosphorylation in neurons, breast carcinoma cells, Hela cells, and pulmonary endothelial cells. However, dominant-negative PAK or shRNA induces myoepithelial cell contraction, in contrast to the expected relaxation. In guinea pig VSMCs, PAK1 also induces relaxation through MLC phosphorylation and subsequent inhibition of MLCK, raising doubt on the role of PAKs’ MLCK activity in VSMCs. Therefore, we tested the hypothesis that IKK2 regulates MLC phosphorylation in VSMCs, and that IKK2 is important in mediating vascular contractile responses to several agonists as well as in blood pressure regulation.

Methods

An expanded Methods section can be found in the Online Data Supplement.

Generation of Smooth Muscle–Specific IKK2-Deficient Mice

IKK2flox/flox mice were generated as previously described. SM22-Cre mice were purchased from the Jackson Laboratory. SM22-Cre IKK2flox/flox (IKK2smKo) mice were generated by crossing TNFR1−/−SM22-Cre−/−IKK2flox/flox and TNFR1−/−SM22-Cre−/−IKK2flox/flox.

Blood Pressure Measurements

The 20-week-old IKK2flox mice (n=7) and littermate control mice (n=5) were implanted with radiotelemeter probes as previously described (model TA11PA-C10; Data Sciences International). Ten days after surgery, we began monitoring 24-hour mean arterial pressure and heart rate for 5 consecutive days. We also measured blood pressure in younger mice by the tail cuff method. IKK2flox mice (n=16), and littermate WT control mice (n=16) were trained beginning at the age of 6 weeks for 2 weeks (daily, 5 d/wk), and then systolic blood pressure was recorded beginning at the age of 8 weeks for 2 weeks (daily, 5 d/wk). The average systolic blood pressure during this period was recorded.

To measure 24-hour mean arterial blood pressure in TNFR1−/−IKK2smKo, TNFR1−/−IKK2smKo and control littermate mice (n=6/group) were implanted with radiotelemeter probes as previously described. After 8 days of recovery, mean arterial pressure and heart rate were recorded for 4 consecutive days. For analyzing the acute effects of angiotensin II (0.3 µg/kg), phenylephrine (PE; 10 µg/kg), thromboxane (30 µg/kg), and sodium nitroprusside (32 µg/kg), those mice were also implanted with catheter inserted into the jugular vein for drug infusion. After a 30-minute stabilizing period, bolus injections of vasoactive drugs were administered at 30-minute intervals.

Cell Culture

A human aortic VSMC line (HVSMCs, ATCC#: CRL-1999) was purchased from ATCC. The cells were maintained in DMEM supplemented with 10% fetal bovine serum. Treatments were applied to cells ∼90% confluent in 60 mm dishes.

Transfection

Lipofectamine 2000 transfection reagent was used to perform transfection with plasmids, according to manufacturer instruction. Briefly, HVSMCs (105 cells per 60 mm dish) were seeded 1 day before transfection. Transfection complex was prepared at DNA (µg): Lipofectamine 2000 (µL) ratio of 1:1.

Western Blotting

Western blotting was performed using standard techniques as previously reported with primary antibodies as follows: mouse antiphospho-MLC-2 (Ser19, Cell Signaling Technology), mouse anti-MLC (Sigma), rabbit anti-1xBr (Cell Signaling Technology), monoclonal anti-β-actin (Sigma), rabbit anti-IKK2 (Sigma), rabbit anti-IKK1 (Sigma), mouse antiphospho-1xBr (Ser32/36, Cell Signaling Technology), mouse anti-myosin phosphate target subunit 1 (BD Transduction Laboratories), and rabbit antiphospho-myosin phosphate target subunit 1 (Thermo, Upstate). Signals were detected by chemiluminescence and analyzed by densitometry.

Vascular Reactivity

Reactivity of rat and mouse aortic rings to several vasoactive agonists was assessed as previously described. Iw1 kinase-1 peptide inhibitor, NF-kB Activation Inhibitor II (ISH-23), and SC-514 were purchased from EMD Chemicals. All other materials were purchased from Sigma. The experimental protocols for this study were approved by...
the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center and were performed according to both the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and the guidelines of the Animal Welfare Act. All the mice were housed 2 to 3 animals per cage at 22°C (12-hour light-dark cycle) with free access to food and water.

Sprague-Dawley rats (16 weeks old; Taconic) or mice (8–18 weeks) were anesthetized with pentobarbital sodium, and the thoracic aorta was quickly removed and cleaned in physiological salt solution, containing (mmol/L) the following: NaCl, 130; NaHCO3, 14.9; KCl, 4.7; KH2PO4, 1.18; MgSO4·7H2O, 1.18; CaCl2·2H2O, 1.56; EDTA, 0.026; glucose 5.5. The aorta was cut into 2-mm rings, and the endothelium was mechanically removed by gently rubbing the intimal surface with a stainless steel wire. The aortic rings were then mounted in a muscle bath containing physiological salt solution at 37°C and bubbled with a mixture of 95% O2 and 5% CO2. The resting tension of 30 mN (rats) or 4 mN (mice) was applied.

A stainless steel wire was mechanically removed by gently rubbing the intimal surface with a stainless steel wire. The aortic rings were then mounted in a muscle bath containing physiological salt solution at 37°C and bubbled with a mixture of 95% O2 and 5% CO2. The resting tension of 30 mN (rats) or 4 mN (mice) was applied.

To determine whether the MLCK activity of IKK2 in vitro is biologically relevant, we performed kinetic analyses. Figure 1I–1D demonstrate that the MLCK activity of IKK2 may be physiologically significant.

Further supporting the in vitro MLCK activity of IKK2, Online Figures IV and V show that SC-514, a IKK2 inhibitor, decreased ML phosphorylation by IKK2 but not other MLCKs, including classic MLCK, Zip-kinase, and Rho-associated protein kinase 1. More importantly, the reaction was also inhibited by IκBα, a substrate peptide derived from IκBα (Figure 1G and 1H), and the calculated Ki,MLC was comparable with Ki,IκBα (Online Table I). Notably, although IKK1 and IKK2 share the IKK activity, IKK1 did not phosphorylate MLC in vitro (Online Figure VII).

To determine whether the MLCK activity of IKK2 is biologically relevant, we performed kinetic analyses. Figure II and IJ demonstrate that the kinetics of ML phosphorylation by IKK2 were similar to those of IκBα phosphorylation (Vmax: 3.7 and 2 μmol/mg per minute; Ki,MLC: 2.7 and 0.5 μmol/L; Kpeptide: 1.7 and 1.4 μmol/L; MLCK and IκBα, respectively), indicating that the MLCK activity of IKK2 may be biologically important.

IKK2 Phosphorylates MLC in Living Cells

To examine whether IKK2 is an MLCK in living VSMCs, we overexpressed IKK2 in human VSMCs (HVSMS). Consistent with our in vitro results, IKK2 overexpression significantly increased the phosphorylation level of MLCK Ser19 but not the regulatory subunit of MLCP myosin phosphatase target subunit 1 Thr696 (Figure 2A and 2B).
We next analyzed whether endogenous IKK2 is an MLCK in living cells. Figure 2C and 2D and Online Figure VIII show that SC-514 treatment markedly attenuated the basal MLCK phosphorylation and MLCK phosphorylation in response to angiotensin II, U-46619 (a thromboxane mimetic), or ionomycin in HVSMCs. Notably, if compared with the basal level, those agonists markedly increased MLCK phosphorylation even in the presence of SC-514 (Figure 2C and 2D), suggesting that IKK2 mainly contributes to the basal but not agonist-induced MLCK phosphorylation. We also tested the MLCK phosphorylation effect of a cell-permeant IKK2 inhibitory peptide.38,39

Consistent with the SC-514 results, the IKK2 peptide inhibited markedly decreased the basal MLCK phosphorylation in HVSMCs (Figure 2E and 2F).
To exclude the possibility that IKK2 inhibitors decrease MLC phosphorylation through nonspecifications, we analyzed the MLC phosphorylation effect of SC-514 in IKK2-deficient mouse embryonic fibroblasts. Figure 2G and 2H show that SC-514 markedly decreased MLC phosphorylation levels in WT but not in IKK2−/− mouse embryonic fibroblasts, strongly supporting that SC-514 decreases MLC phosphorylation via targeting IKK2. Notably, consistent with its proposed role in MLC phosphorylation, IKK2−/− mouse embryonic fibroblasts had lower basal MLC phosphorylation level (Figure 2G and 2H).

**IKK2 Is Implicated in Vasoconstriction**

To assess the physiological importance of the MLCK activity of IKK2, we analyzed the role of IKK2 in vasoconstriction, an MLC phosphorylation-dependent physiological process. Figure 3A shows that SC-514 caused relaxation in endothelium-denuded rat aortic rings precontracted with PE, KCl, U-46619 (a thromboxane mimetic), or calyculin A (MLCP inhibitor). To establish a high level of intracellular Ca2+, we treated endothelium-denuded aortic rings with the Ca2+ ionophore, ionomycin. This contraction was markedly relaxed by SC-514 (Figure 3B), suggesting that SC-514 does not cause relaxation by lowering intracellular Ca2+. Next, we used SC-514 to further document the role of IKK2 in vasoconstriction. Figure 3C reveals that SC-514 relaxed PE-induced contraction with a half maximal inhibitory concentration (IC50) comparable with that of inhibiting IKK2 in vitro. Consistent with the effect of SC-514, IKK2 inhibitory peptide, but not control peptide, significantly relaxed PE-induced contraction (Figure 3D and 3E), confirming the involvement of IKK2 in vasoconstriction. In contrast, a classic MLCK inhibitor ML-9 potently relaxed PE-induced endothelium-denuded rat aortic ring contraction, but had little effect on calyculin A–induced contraction (11±7% of precontraction; n=4; Figure 3F), suggesting that SC-514 did not primarily target the classic MLCK for its vasodilator actions at least in calyculin A–induced contraction. However, we cannot completely exclude the possibility that SC-514 relaxed PE-induced contraction through inhibition of MLCK.

IKK2 inhibition induces apoptosis in diverse cell types. However, the vasodilator action of IKK2 inhibitors is unlikely because of decreased viability of smooth muscle cells because the IKK2 inhibitory peptide-induced decrease in vasoconstriction recovered in hours (Figure 3D), and the vascular effect of 30 µmol/L SC-514 was completely reversible by washing (Online Figure IX). In addition, although NF-κB is the best known downstream molecule from IKK2, it is not involved in the vasodilator action of IKK2 inhibitors because the action was observed within seconds, and JSH-23, an NF-κB antagonist

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**Figure 3. Inhibitor κB kinase 2 (IKK2) contributes to diverse vascular contractile responses.** A, Endothelium-denuded rat aortic rings were contracted by the indicated vasoconstrictors (1 µmol/L phenylephrine [PE], 120 µmol/L potassium chloride [KCl], 1 µmol/L U-46619, a thromboxane A2 agonist; 1 µmol/L calyculin A, a MLCP inhibitor), and then SC-514 (100 µmol/L) or vehicle was added. A representative result from 4 independent experiments is presented. B, Endothelium-denuded rat aortic rings were treated with ionomycin (1.5 µmol/L) in calcium-free physiological salt solution for 30 minutes, and contracted with CaCl2 (0.4 mmol/L), and the IKK2 inhibitor SC-514 (100 µmol/L) or vehicle was added. A representative result from 3 independent experiments is presented. C, Endothelium-denuded rat aortic rings were contracted by PE (1 µmol/L), and the IKK2 inhibitor SC-514 was added in an accumulative manner. Results were expressed as percentage of precontraction. D and E, Phenylephrine (PE; 1 µmol/L) precontracted endothelium-denuded rat aortic rings were treated with IKK2 inhibitory or control peptide (the same peptides as in Figure 2E and 2F; 50 µg/mL). The representative recordings (C) and the quantification data (D) are presented. n=3. *P<0.05 vs control; Student t test. F, Endothelium-denuded rat aortic rings were contracted with calyculin A, followed by adding ML-9 (100 µmol/L). After washing, the rings were contracted with PE again and followed by ML-9 (100 µmol/L). A representative result from 3 independent experiments is presented.
that inhibits NF-κB binding to DNA, but not IKK2 activation,\(^{42}\) did not have a similar vasodilator action (Online Figure X).

To confirm the role of IKK2 in vasoconstriction, we generated smooth muscle–specific IKK2-deficient mice (IKK2\(^{mko}\)). Western blot analysis demonstrated that IKK2\(^{mko}\) mice had a 58% decrease in aortic IKK2 protein expression (Figure 4A and 4B). In agreement with the proposed role of IKK2 in vasoconstriction, aortic MLC phosphorylation in IKK2\(^{mko}\) mice was decreased by 41% (Figure 4A and 4B). In contrast, IKK2\(^{mko}\) mice had comparable IKK2 expression and MLC phosphorylation in the kidney (Figure 4A and 4B). Further supporting the importance of IKK2 in vasoconstriction, IKK2 deficiency significantly reduced aortic vasoconstrictor responses to KCl or PE (Online Figure XIA and XIB), and SC-514 had markedly decreased vasodilator action in IKK2-deficient mouse aortic rings (Online Figure XIC). Given the important role of resistant arteries in blood pressure regulation, we further documented the vascular contractile responses in mesenteric arteries. Figure 4C–4E revealed that IKK2 deficiency in VSMCs markedly reduced contraction of mesenteric arteries induced by KCl, PE, or U-46619. Notably, mesenteric arteries from IKK2\(^{mko}\) mice had significantly decreased relaxation responses to acetylcholine and SC-514 (Figure 4F and 4G).

**IKK2 Deficiency Decreases Vascular Contractile Response and Basal Blood Pressure**

To investigate the physiological role of IKK2, we measured 24-hour mean arterial blood pressure of IKK2\(^{mko}\) mice.
using telemetry as previously described.34 Unexpectedly, no significant change in mean arterial blood pressure was observed (WT versus IKK2smKo: 99±1 vs 102±6 mm Hg; n=5 or 7/group; P=24.6% Student t test). However, 20-week-old IKK2smKo mice developed severe dermatitis (data not shown), which may have tended to increase blood pressure as a consequence of inflammation33,34 and, thus, oppose the blood pressure lowering effect of smooth muscle–specific IKK2 deficiency. Supporting this concept, we observed lower systolic blood pressure in 8- to 10-week-old IKK2smKo mice (WT versus IKK2 smKo: 105±9 versus 98±10 mm Hg by the tail cuff method; n=16/group; P<0.05, Student t test).

IKK2 deficiency–induced dermatitis is TNFR1 dependent.45 To further test the role of IKK2 in blood pressure regulation, we generated a mouse model with whole body TNFR1 deficiency and smooth muscle–specific IKK2 deficiency (TNFR1−/−IKK2smKo). As expected, these mice did not develop dermatitis. Supporting the important role of IKK2 in blood pressure regulation, TNFR1−/−IKK2smKo mice had lower 24-hour mean blood pressure as measured by telemetry (Figure 5A). To verify that IKK2 regulates blood pressure through regulating MLC phosphorylation, we assessed the acute blood pressure responses to 3 vasoconstrictors (thromboxane, PE, and angiotensin II) each with a different mechanism of action. Figure 5B–5D demonstrates that TNFR1−/−IKK2smKo mice had markedly reduced hypertensive responses to those vasoconstrictors. In contrast, the vasodilator sodium nitroprusside, a nitric oxide donor, produced similar decreases in blood pressure in TNFR1−/− and TNFR1−/−IKK2smKo mice (Figure 5E).

Discussion

In the present study, we demonstrate that IKK2 is an important MLCK in VSMCs, as evidenced by the following results: (1) IKK2 phosphorylates MLC and intact myosin in vitro with kinetics comparable with that by the classic MLCK; (2) overexpression of IKK2 increases cellular MLC phosphorylation level, whereas inhibition of IKK2 reduces cellular MLC phosphorylation level; (3) the effects of IKK2 inhibitors on vascular MLC phosphorylation and contractile responses are not mediated by the classic MLCK and MLCP; (4) VSMC–specific IKK2 deficiency decreases aortic MLC phosphorylation and contractile responses to several agonists, lowers basal blood pressure, and reduces the hypertensive responses to vasoconstrictors.

Previous studies have provided compelling evidence for the existence of an MLCK(s) other than the classic MLCK in living cells.3–5 In addition to the classic MLCK, several kinases, including Rho-Kinase,10 ILK,11 PAK,12 and ZIPK,13 can phosphorylate MLC in vitro. However, except for ILK, the MLCK activity of those kinases has been shown not to be of physiological significance.14,15 To our knowledge, this is the first study demonstrating that a kinase other than the classic MLCK regulates VSMC MLC phosphorylation as an MLCK, contributes to vascular contractile responses, and, is important in blood pressure regulation.

Previous studies have shown that the basal MLC phosphorylation is independent of the classic MLCK because its inhibitors have no effect on the basal MLC phosphorylation.3–5 However, the factors that contribute to basal MLC phosphorylation in VSMCs have not been previously elucidated. Our data demonstrate that IKK2 contributes importantly to basal MLC phosphorylation.
phosphorylation in VSMCs. Given that various vasoconstrictor agonists induce further MLC phosphorylation, in excess of basal MLC phosphorylation, IKK2 may be implicated in diverse vascular contractile responses. This conclusion is consistent with our results demonstrating that IKK2 inhibition markedly reduced basal MLC phosphorylation and vasoconstrictor responses to all tested agonists. However, the mechanisms whereby IKK2 regulates cellular MLC phosphorylation in different physiological and pathophysiological conditions remain to be determined.

Although our results indicate that IKK2 plays a key role in basal MLC phosphorylation, its role in stimulating further MLC phosphorylation in response to vasoconstrictors is less clear. We found that vasoconstrictors increased MLC phosphorylation even in the presence of an IKK2 inhibitor, suggesting that IKK2 may not be essential for agonist-induced MLC phosphorylation. In addition, because IKK2 inhibition relaxed all tested contractions (agonist-induced MLC phosphorylation can be through diverse pathways and thus less likely to be inhibited by a single inhibitor, whereas basal MLC phosphorylation definitely contributes to all contractions), it is more likely that the contribution of IKK2 to agonist-induced MLC phosphorylation may be trivial. However, this remains to be determined. Unfortunately, the methods commonly used to determine the phosphorylation level of cellular MLC, such as the one used in the present study and urea-glycerol gel, are only semiquantitative. It may be necessary to assess the contribution of IKK2 to agonist-induced MLC phosphorylation with more quantitative methods, such as isolate tracers, in the future. Our finding that pharmacological blockade of IKK2 or VSMC-specific genetic deficiency of IKK2 markedly attenuates vasoconstrictor responses to several agonists with different modes of action and reduces blood pressure suggests that IKK2 may be important in agonist-induced as well as basal MLC phosphorylation in VSMCs.

Another interesting aspect of IKK2 as an MLCK is that in contrast to the Ca\textsuperscript{2+}-dependent activation of the classic MLCK, the MLCK activity of IKK2 seems to be Ca\textsuperscript{2+} independent because the Ca\textsuperscript{2+} chelator ethylene glycol tetraacetic acid does not have an inhibitory effect on the IKK2-induced MLC phosphorylation in vitro. This is consistent with our results, demonstrating that IKK2 plays an important role in cellular basal MLC phosphorylation. Notably, although the MLCK activity of IKK2 is Ca\textsuperscript{2+} independent, it could theoretically enable Ca\textsuperscript{2+}-dependent MLCK phosphorylation and contraction in the absence of classic MLCK. The MLCK activity of IKK2 is antagonized by the active MLCP in the absence of agonists, which maintains MLC phosphorylation at a low level. As agonists increase [Ca\textsuperscript{2+}], and subsequently inhibit the antagonizing MLCP through recently identified Ca\textsuperscript{2+}-dependent signaling pathways,\textsuperscript{46} IKK2 then can increase MLC phosphorylation through its MLCK activity and consequently induce contraction. Therefore, it will be particularly interesting to determine whether the vasoconstriction of MLCK-deficient mice is dependent on the MLCK activity of IKK2 in future studies.

Consistent with the proposed role of IKK2 in the regulation of MLC phosphorylation, our data also show that VSMC-specific IKK2 deficiency on the genetic background of TNFRF1 deficiency results in a significant reduction in blood pressure. Furthermore, mice with VSMC-specific IKK2 deficiency had reduced hypertensive responses to all vasoconstrictor agonists tested. These observations suggest that IKK2 may be a potential target for antihypertensive treatment. However, further studies are needed to assess the blood pressure lowering effect of IKK2 inhibitors in diverse hypertensive animal models.

In conclusion, our study reveals that IKK2 is an important MLCK in living cells and is critical in vascular smooth muscle contractile regulation both in vitro and in vivo. Therefore, our observations provide a novel pathway for physiological regulation of vascular smooth muscle contraction that may be an important target for antihypertensive therapy.

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**Disclosures**

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20. MLC phosphorylation regulates diverse myosin II–dependent cellular responses, and the classic MLCK is the only kinase identified to date that can phosphorylate MLC in living cells. Here, we report that IKK2 acts as an intracellular MLCK. These findings reveal a novel pathway for the physiological regulation of vascular smooth muscle mediated by IKK2. Although our studies were focused on vasoconstriction, a broader implication of our work is that IKK2 may also regulate other cellular responses, such as vascular cell motility and morphology, independently of its effects on nuclear factor κB activity.

Because abnormal contractility of smooth muscle has been implicated in various diseases, such as hypertension and asthma, these findings suggest that IKK2 may be a novel target for regulating smooth muscle contractility.

What Is Known?

- Phosphorylation of the myosin light chain (MLC) plays a central role in regulating vascular smooth muscle contractile responses.
- In vascular smooth muscle cells, MLC is phosphorylated by the classical MLCK (MLCK), although MLC could be phosphorylated by other, not yet identified, kinase(s) as well.

What New Information Does This Article Contribute?

- MLC is phosphorylated by inhibitor s8 kinase 2 (IKK2).
- IKK2 is a vascular smooth muscle cell MLCK.
- IKK2 regulates vascular tone and blood pressure through its MLCK activity.

MLC phosphorylation regulates diverse myosin II–dependent cellular responses, and the classic MLCK is the only kinase identified to date that can phosphorylate MLC in living cells. Here, we report that IKK2 acts as an intracellular MLCK. These findings reveal a novel pathway for the physiological regulation of vascular smooth muscle mediated by IKK2. Although our studies were focused on vasoconstriction, a broader implication of our work is that IKK2 may also regulate other cellular responses, such as vascular cell motility and morphology, independently of its effects on nuclear factor κB. Because abnormal contractility of smooth muscle has been implicated in various diseases, such as hypertension and asthma, these findings suggest that IKK2 may be a novel target for regulating smooth muscle contractility.
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Methods:

Ethics Statement: The experimental protocols for this study were approved by the Institutional Animal Care and Use Committee at the Ohio State University and were carried out according to both the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and the guidelines of the Animal Welfare Act.

Generation of smooth muscle-specific IKK2 deficient mice: IKK2^flox/flox^ mice were generated as previously described. SM22-Cre^+/+^ mice were purchased from the Jackson Laboratory. SM22-Cre^+/+^IKK2^flox/flox^ (IKK2^smKo^) mice were generated by crossing SM22-Cre^+/+^IKK2^flox/flox^ and IKK2^flox/flox^, and the IKK2^flox/flox^ littermates were used as wild-type (Wt) control. In preliminary studies we found that IKK2^smKo^ mice had dermatitis. To prevent dermatitis in IKK2^smKo^ mice, we crossed these mice with TNFR1^-/-^ mice obtained from the Jackson Laboratory. Littermate TNFR1^-/-^SM22-Cre^-/-^IKK2^flox/flox^ control mice (hereafter presented as TNFR1^-/-^ mice) and TNFR1^-/-^IKK2^smKo^ mice were generated by crossing TNFR1^-/-^SM22-Cre^-/-^IKK2^flox/flox^ and TNFR1^-/-^SM22-Cre^-/-^IKK2^flox/flox^.

Blood pressure measurements: The 20-week-old IKK2^smKo^ mice (n = 7) and littermate control mice (n = 5) were implanted with radiotelemeter probes as previously described (model TA11PA-C10; Data Sciences International). Ten days after surgery, we began monitoring 24-hour mean arterial pressure (MAP) and heart rate (HR) for 5 consecutive days.

We also measured blood pressure in younger mice by the tail cuff method. IKK2^smKo^ mice (n = 16) and littermate Wt control mice (n = 16) were trained beginning at the age of 6 weeks for two weeks (daily, 5 day/week), and then systolic blood pressure...
was recorded beginning at the age of 8 weeks for two weeks (daily, 5 days/week). The average systolic blood pressure during this period was recorded.

To measure 24 hour mean arterial blood pressure in TNFR1\(^{-/-}\)IKK2\(^{smKo}\), TNFR1\(^{-/-}\)IKK2\(^{smKo}\) and control littermate mice (n=6/group) were implanted with radiotelemeter probes as previously described\(^{34}\). After 8 days of recovery, mean arterial pressure and heart rate were recorded for 4 consecutive days. For analyzing the acute effects of angiotensin II (0.3 μg/kg), phenylephrine (10 μg/kg), thromboxane (30 μg/kg) and sodium nitroprusside (32 μg/kg), those mice were also implanted with catheter inserted into the jugular vein for drug infusion. After a 30-min stabilizing period, bolus injections of vasoactive drugs were administered at 30 min intervals.

**Cell culture:** A human aortic vascular smooth muscle cell line (HVSMCs, ATCC#: CRL-1999) was purchased from ATCC. The cells were maintained in DMEM supplemented with 10% fetal bovine serum. Treatments were applied to cells approximately 90% confluent in 60 mm dishes.

To measure MLC phosphorylation, cells were serum-starved for two hours and then treated with the indicated compounds followed by washing with ice-cold PBS once. The cell lysates were then prepared with RIBA buffer supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Pierce) on ice. The MLC phosphorylation levels were determined by western blot analysis with mouse anti-Phospho-Myosin Light Chain 2 (Ser19, Cell Signaling Technology) and mouse anti-MLC (Sigma).

**Transfection:** Lipofectoamine™ 2000 transfection reagent was used to perform transfection with plasmids, according to manufacturer’s instruction. Briefly, HVSMCs
(10^6 cells/ 60 mm dish) were seeded one day before transfection. Transfection complex was prepared at DNA (μg): Lipofectamine™ 2000 (μl) ratio of 1:1.

**Western blotting:** Western blotting was performed using standard techniques as previously reported with primary antibodies as follows: mouse anti-Phospho-Myosin Light Chain 2 (Ser19, Cell Signaling Technology), mouse anti-MLC (Sigma), rabbit anti-IkBα (Cell Signaling Technology), monoclonal anti-β-actin (Sigma), rabbit anti-IKK2 (Sigma), rabbit anti-IKK1 (Sigma), mouse anti-phospho-IkBα (Ser32/36, Cell Signaling Technology), mouse anti-MYPT1 (BD Transduction Laboratories), and rabbit anti-phospho-MYPT1 (Thr696, Upstate). Signals were detected by chemiluminescence and analyzed by densitometry.

**Vascular reactivity:** Reactivity of rat and mouse aortic rings to several vasoactive agonists was assessed as previously described. IkB Kinase Inhibitor Peptide, NF-κB Activation Inhibitor II (JSH-23), SC-514 was purchased from EMD Chemicals. All other materials were purchased from Sigma. The experimental protocols for this study were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center and were carried out according to both the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and the guidelines of the Animal Welfare Act. All the mice were housed 2-3 animals per cage at 22 °C (12-h light-dark cycle) with free access to food and water.

Briefly, Sprague Dawley rats (16 weeks old, Taconic) or mice (8-18 weeks) were anesthetized with pentobarbital sodium, and the thoracic aorta was quickly removed and cleaned in physiological salt solution (PSS), containing (mM): NaCl, 130; NaHCO₃, 14.9; KCl, 4.7; KH₂PO₄, 1.18; MgSO₄•7H₂O 1.18; CaCl₂•2H₂O, 1.56; EDTA, 0.026;
glucose 5.5. The aorta was cut into 2-mm rings, and the endothelium was mechanically removed by gently rubbing the intimal surface with a stainless steel wire. The aortic rings were then mounted in a muscle bath containing PSS at 37°C and bubbled with 95% O₂-5% CO₂. Isometric force generation was recorded with either a Multi Myograph System (Danish Myo Technology A/S) or organ chamber. A resting tension of 30 mN (rats) or 4 mN (mice) was imposed on each ring, and the rings were allowed to equilibrate for 1 hour. Arterial integrity was assessed first by stimulation of vessels with 80 mM KCl. Endothelium-integrity was assessed by measuring the dilatory response to ACh (10 µM) in PE-contracted vessels (3 µM). The failure of ACh to relax denuded aortic rings was considered proof of endothelium disruption.

To analyze the responses of mesenteric arteries, male WT and IKK2smKo mice (8-10 weeks old) were killed with an overdose of sodium pentobarbital (120 mg/kg i.p.). The mesentery was rapidly removed and placed in ice-cold gassed (95% O₂/5% CO₂) PSS. The second-order mesenteric arteries were dissected free of adipose and connective tissue and immediately were mounted in a wire myograph (Danish MyoTechnology, Aarhus, Denmark). After an initial 30-min equilibration, vessel wall tension and diameter were normalized in a standardized procedure and stabilized for 1 h. Wire myography was used to assess the KCl-, PE- or U-46619-induced contractile responses of arteries.

**In vitro phosphorylation:** The assays were established based on HTScan kinase assay kit (Cell signaling technology). 100 ng IKK2 in the kit or other kinases (MLCK and Zip kinase from Upstate and ROCK1 from Abcam) were incubated with 0.75 µM MLC (Calbiochem) or 0.75 µM IkBα (Biomol) at 37°C in a total of 50 µl buffer containing 60 mM HEPES-NaOH (pH 7.5), 3 mM MgCl₂, 3 mM MnCl₂, 3 µM Na-orthovanadate, 1.2
mM DTT, 10 µM ATP. MLCK phosphorylated MLC in total of 50 µl buffer containing
60 mM HEPES-NaOH (pH 7.5), 3 mM MgCl₂, 3 mM MnCl₂, 3 µM Na-orthovanadate,
1.2 mM DTT, 10 µM ATP, 0.1 mM CaCl₂, and 1 µM calmodulin. Reaction was stopped
by adding 50 µl SDS-PAGE sample buffer and boiling for 5 minutes.

**Kinetic analyses:** Assays were performed by ProQinase. Recombinant, insect cell
expressed IKK2 protein was used as full-length, GST-tagged fusion protein (ProQinase
#0258-00001 LOT008). Substrate peptide MLC-tide (KKRPQRATSNVFS-amide) was
supplied by Penninsula Laboratories. Radiometric Filter Binding Assay was used to
determine kinetics. In brief, recombinant IKK2 (50ng/well) was incubated with its
substrate MLC-tide (various amounts) and co-substrate ATP (various amounts)
containing radioactive 33Pγ- ATP as a tracer in a constant ratio. Phosphorylated peptide
were bound to MSPH-Filter plates (Millipore #MSPHN0B10), washed, and bound
radioactivity was detected by liquid scintillation counting. In detail, assay components
were added to a final volume of 50 µl in a 96 well PP V-bottom plate in assay mix (70
mM HEPES pH7.5; 3 mM MgCl₂; 3 mM MnCl₂; 3 µM Naorthovanadate; 1,2 mM DTT;
50 µg/ml PEG20,000; ATP and MLC-tide in various amounts; IKK-beta 50 ng/well) in
the following order: Assay buffer, MLC-tide, IKK2 and ATP. The assay was mixed and
incubated for 20 min at 30°C. The reaction was stopped by addition of 20 µl 10% H₃PO₄.
The MSPH filter plate was washed with 20 µl 100% ethanol followed by 200 µl 150 mM
H₃PO₄, the reaction mixture was transferred to the filter plate and incubated for 30 min at
room temperature. The mixture was passed through the filter by application of vacuum,
washed 3 times with 200 µl 150 mM H₃PO₄ and once with 20 µl 100% ethanol. After
drying the plate, 50 μl/well of liquid scintillator were added and bound radioactivity
determined by measurement in a liquid scintillation counter.

*Statistical analysis:* Probability values less than 0.05 were considered significant.
Student’s t test or ANOVA were used, as appropriate, for statistical analyses with
Graphpad Instat 5 software (Graphpad Instat Software, San Diego, CA).
Online Table I. Calculated $K_{MLC}$. $K_{MLC}$ was calculated based on the information of Figure 1H and according to the formula $V = V_{max}(1+[MLC]/([MLC]+K_{MLC}^{app}))$ and $K_{MLC}^{app} = K_{MLC} \times (K_{IkBa}+[IkBa])/K_{IkBa}$. $V/V_0$, MLC phosphorylation in the presence of $IkBa$; MLC phosphorylation in the absence of $IkBa$. *, Li J. *et al* J Biol Chem. 1998;273(46):30736-41.
Online Figure I. MLC was phosphorylated by IKK2 (25 nM, Cell Signaling Technology) or MLCK (25 nM, Upstate) for the indicated time. Phosphorylated MLC at Ser^{19} in the products was analyzed by western blotting, and total MLC was visualized by ponceau’s solution.
Online Figure II. IKK2 is not contaminated with MLCK. A, MLC was phosphorylated by IKK2 (25 nM, Cell Signaling Technology) or MLCK (25 nM, Upstate) for 1 hour in the presence of 1 mM EGTA or vehicle. B, MLC was phosphorylated by IKK2 in the presence of SC-514 (30 µM) or ML-9 (100 µM). Phosphorylated of MLC at Ser\textsuperscript{19} in the products was analyzed by western blotting, and total MLC was visualized by ponceau s solution.
Online Figure III. IKK2 phosphorylates myosin. Myosin II (0.075 µM, Cytoskeleton) or MLC (0.15 µM, RayBiotech) was phosphorylated by IKK2 (25 nM, Cell Signaling Technology) for the indicated time. Phosphorylated MLC at Ser¹⁹ and total MLC in the products was analyzed by western blotting.
Online Figure IV. SC-514 inhibits MLC phosphorylation by IKK2. MLC (0.75 μM) and IKK2 (25 nM) were incubated at 37°C for one hour in the presence of 30 μM SC-514 or vehicle. Phosphorylated MLC was measured by western blotting with anti-phospho-myosin light chain 2 (Ser¹⁹). MLC was visualized by ponceau s solution. A representative picture (A) and the summary (B) are presented. n≥3. *P<0.05 vs vehicle; #P<0.05 vs IKK2. Student’s t test.
Online Figure V. SC-514 did not inhibit MLCK, zip-kinase, and ROCK1. MLC (0.75 μM) was phosphorylated by MLCK (Upstate, A), zip-kinase (Upstate, B), and ROCK1 (Abcam, C) at 37°C for one hour in the presence of inhibitor or vehicle. Phosphorylated MLC was measured by western blotting with anti-phospho-myosin light chain 2 (Ser19). MLC was visualized by ponceau s solution. A representative picture of at least two independent experiments is presented.
Online Figure VI. IKK2 but not ROCK inhibitor markedly decreases vascular smooth muscle cell basal MLC phosphorylation. After starvation, HVSMCs were treated with the indicated inhibitors for 30 minutes, and then harvested for analysis of MLC phosphorylation as described in Method section.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concn (M)</th>
<th>BMS-345541 (30 M)</th>
<th>SC-514 (30 M)</th>
<th>Y-27632 (10 μM)</th>
</tr>
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<tbody>
<tr>
<td></td>
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### Table A

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>w/o</td>
<td>bio-Ahx-4x(LRRWSLG)</td>
</tr>
<tr>
<td>IKBa</td>
<td>DDRHDSGLDSMKDKKK</td>
</tr>
<tr>
<td>IRS</td>
<td>KKHTDDGYMPMSGVA</td>
</tr>
<tr>
<td>4xChocktide</td>
<td>bio-Ahx-4x(LRRWSLG) RFARKGSLRQKV</td>
</tr>
<tr>
<td>3xChocktide</td>
<td>bio-Ahx-3x(LRRWSLG)</td>
</tr>
<tr>
<td>S6P-2</td>
<td>bio-Ahx-RRRRRRRRRR-IARKRRLLRRASTSSKESSQQK</td>
</tr>
<tr>
<td>Zip tide</td>
<td>KKLNRRTLSFAEPG</td>
</tr>
<tr>
<td>Jerini A-G02</td>
<td>bio-Ahx-SRLRRASQLKIT</td>
</tr>
<tr>
<td>MLC</td>
<td>KKRPRQATSNVFS</td>
</tr>
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</table>

### Online Figure VII.

**MLC is the substrate of IKK2 but not IKK1.** Several potential substrate peptides (1μM; sequences are present in A) were tested for phosphorylation by the kinase IKK2 (B; 50 ng/50 μl; ProQinase) and IKK1 (C; 50 ng/50 μl; ProQinase) in duplicate in a radiometric filter binding assay. Peptides were incubated in 50 μl assay mix (70 mM HEPES pH7.5; 3 mM MgCl2; 3 mM MnCl2; 3 M aorthovanadate; 1,2 mM DTT; 50 μg/ml PEG20,000; 1 M ATP (883,096 cpm 33Pγ-ATP as a tracer). After 60 min incubation at 30°C the reaction was terminated by addition of 10% H3PO4 and transferred to a MSPH filterplate (Millipore #MSPHNB010). Excess ATP was removed and peptide bound radioactivity determined by measurement in a scintillation counter. Autophosphorylation of IKK1 or IKK2 was measured in the absence of any substrate peptide (w/o).
Online Figure VIII. SC-514 dose-dependently inhibits basal MLC phosphorylation in HVSMCs. after incubation with the indicated concentration of SC-514 for 15 minutes, MLC phosphorylation in HVSMCs was analyzed by western blotting.
Online Figure IX. The vascular effect of SC-514 is reversible. Endothelium denuded rat aortic rings pre-contracted by PE (1 μM) were relaxed by the indicated concentration of SC-514. After washing, the rings were re-contracted by PE (1 μM). n=3.
Online Figure X. NF-κB activation inhibitor JSH-23 does not relax pre-contracted rat aortic rings. A, JSH-23 (4-methyl-N1-(3-phenylpropyl)benzene-1,2-diamine) does not inhibit IκBα degradation induced by TNFα. Human vascular smooth muscle cells were treated with TNFα (10 ng/ml) for 5 minutes in the presence of the indicated inhibitor (20 μM), and then the IκBα expression level was analyzed by western blotting. B, JSH-23 inhibits induction of COX2 by TNFα. Human vascular smooth muscle cells were treated with 10 ng/ml TNFα for 16 hours in the presence of the indicated inhibitor (20 μM). The COX2 expression level was then analyzed by western blotting. C, JSH-23 does not relax contraction by KCl. Endothelium-denuded rat aortic rings were contracted by 80 mM KCl and then 20 μM NF-κB activation inhibitor was added.
Online Figure XI. IKK2 deficiency reduces aortic contractile responses. A and B, responses of aortic rings from IKK2<sup>smKo</sup> and their littermate WT controls to KCl (A. 80 mM; n=8/group; *P<0.05 vs. Wt; student t-test.) or PE (B. n=4/group; *P<0.05 vs. Wt; Two way ANOVA). C, aortic rings from IKK2<sup>smKo</sup> and their littermate controls were contracted by PE (1 µM) and then relaxed by adding SC-514. n=5-6/group. *P<0.05 vs. Wt; Two way ANOVA. D, After equilibration at a tension of 4 mN, aortic ringes were treated with SNP (1 µM). n=4/group. Student’s t test.
Online Figure XII. Analysis of aorta from WT and smKo mice. A, Male IKK2<sup>flox/flox</sup> (Wt, n=4) and SM22-Cre IKK2<sup>flox/flox</sup> (smKo, n=4) were sacrificed and the expression of the indicated genes in the ascending aorta were assessed by real time RT-PCR. *p<0.05, student’s t test. B, Male smKo and littermate wt mice (n=3/group) were sacrificed and the ascending aorta in each group were pooled and the expression of the indicated genes were analyzed by western blot.