Role of IκB Kinase-β in the Growth-Promoting Effects of Angiotensin II In Vitro and In Vivo

Priscilla Doyon,* Wendy J. van Zuylen,* Marc J. Servant

Objective—Angiotensin II (Ang II) is implicated in processes underlying the development of arterial wall remodeling events, including cellular hypertrophy and inflammation. We previously documented the activation of IκB kinase-β (IKKβ) in Ang II–treated cells, a kinase involved in inflammatory reactions. In light of a study suggesting a role of IKKβ in angiogenesis through its effect on the tuberous sclerosis (TSC) 1/2–mammalian target of rapamycin complex 1 pathway in cancer cells, we hypothesized that targeting IKKβ could reduce arterial remodeling events by affecting both the inflammatory and the growth-promoting response of Ang II.

Approach and Results—Treatment of aortic vascular smooth muscle cells with Ang II induced the rapid and sustained phosphorylation of TSC1 on Ser511, which paralleled the activation of effectors of the mammalian target of rapamycin complex 1 pathway. Furthermore, we show that Ser511 of TSC1 acted as a phosphoaecceptor site for Ang II–activated IKKβ. Consistent with this, the use of different short hairpin RNA constructs targeting IKKβ reduced Ang II–induced TSC1, S6 kinase, and 4EBP1 phosphorylation and the rate of protein synthesis. Overexpression of TSC1 lacking Ser511 in vascular smooth muscle cells also exerted detrimental effects on the hypertrophic effect of Ang II. Furthermore, the selective IKKβ inhibitor N-(6-chloro-7-methoxy-9H-β-carbolin-8-yl)-2 methyl nicotinamide reduced the inflammatory response and dose-dependently diminished Ang II–induced TSC1 phosphorylation and effectors of the mammalian target of rapamycin complex 1 pathway, leading to inhibition of protein synthesis in vitro and in rat arteries in vivo.

Conclusions—Our findings provide new insights into the molecular understanding of the pathological role of Ang II and assist in identifying the beneficial effects of IKKβ inhibition for the treatment of cardiovascular diseases. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: angiotensin II m I-kappa B kinase m protein synthesis m tuberous sclerosis 1

As the effector of the renin–angiotensin system, the hormone angiotensin II (Ang II) plays a critical role in controlling cardiovascular homeostasis. By binding to the Ang II receptor (Ang II type I receptor or Ang II type II receptor), it regulates salt/water homeostasis and vasoconstriction and modulates cellular responses in a paracrine and autocrine manner. However, deregulated production of Ang II in the vessel wall has been implicated in cardiovascular diseases associated with vascular smooth muscle cell (VSMC) hypertrophy and inflammation, including hypertension and atherosclerosis.1–4

We and others have shown that this inflammatory response mediated by Ang II, involving nuclear factor-κB (NF-κB) transcription factors, required activation of IκB kinase-β (IKKβ) in VSMC.5–7

In the classical NF-κB pathway, IKKβ forms the IκB kinase (IKK) complex with IKKα and the scaffold protein NEMO. In this complex, the IKKβ subunit can modulate the activity of the NF-κB pathway by phosphorylating IκBα at Ser32 and Ser36, which leads to its polyubiquitination and degradation, resulting in release and nuclear accumulation of NF-κB to induce gene expression. Interestingly, in addition to IκBα, many other substrates of IKKβ have been identified, including β-catenin,6 p65,6 SRC-3,6 FOXO3α,6 CYLD,12 DOK1,13 and, more recently, tuberous sclerosis (TSC) 1.14

The tumor suppressor gene TSC1 (hamartin) acts in a complex with TSC2 (tuberin) and prevents its ubiquitination and degradation.15 The hamartin–tuberin complex suppresses the activation of the small GTPase Ras homolog enriched in brain (RHEB) via a GTPase-activating domain near the C terminus of tuberin.16 Active GTP-bound RHEB activates the mammalian target of rapamycin complex 1 (mTORC1), which results in phosphorylation of eukaryotic initiation factor 4E–binding protein 1 (4E-BP1) and S6 kinases (S6K1 and S6K2),17 to increase protein translation and cell growth. Hence, when functional hamartin or tuberin is lost, increased levels of active RHEB constitutively activate mTORC1, which in turn leads to increased cell growth.

Diverse environmental cues, including growth factors, energy status, oxygen, and amino acids, promote mTORC1 signaling. Particularly in response to growth factors (eg,
insulin, insulin-like growth factor 1, and epidermal growth factor), TSC2 is phosphorylated by several kinases, including AKT (on Ser939, Ser981, and Thr1462), extracellular signal–regulated kinases (ERK; on Ser664), and p90 ribosomal S6 kinase (on Ser1798), which leads to inhibition of the GTPase-activating protein function of TSC2 toward RHEB.18,19 Conversely, AMPK and GSK3 phosphorylation of TSC2 (on Ser1345 and Ser1341 plus Ser1337, respectively) positively regulates its GTPase-activating protein function toward RHEB.20 Interestingly, in cancer cells activated IKKβ was demonstrated to phosphorylate TSC1 on Ser511, which results in the inhibition of the TSC1–TSC2 complex and, consequently, the activation of mTORC1, leading to inflammation-mediated tumor angiogenesis.14

In addition to mediating vascular inflammation,5 Ang II also acts as a growth factor that stimulates protein synthesis and induces cellular hypertrophy in VSMC.21 Through the Ang II type I receptor, Ang II induces phosphorylation of tyrosine kinases, including c-Src, Janus family kinases, focal adhesion kinase, PYK2, and phosphatidylinositol 3-kinase to stimulate cell growth.22,23 In addition, Ang II activates mitogen-activated protein kinases, including ERK1/2 and mTOR, which are both involved in the growth properties of the peptide.21,24 Despite these investigations, the molecular mechanisms by which Ang II mediates VSMC growth are not fully elucidated. We, therefore, hypothesized that Ang II–activated IKKβ is not only involved in the inflammatory response but is also involved in the signal transduction underlying the hypertrophic response. Thus, the present study was undertaken to examine this possible new role of IKKβ in the phenotypic responses of VSMC.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Angiotsensin II Induces the Phosphorylation of TSC1 at Ser511, Which Parallels the Activation of mTORC1 Effectors

We first interrogated whether Ang II had the ability to induce the phosphorylation of TSC1 on Ser511, a proposed IKKβ consensus site.14 Using primary human aortic VSMC exposed to Ang II, we detected a significant increase in the Ser511 phospho-signal (Figure 1A and 1B). This signal occurred rapidly, was sustained, and, importantly, correlated with the presence of the mTOR Ser2481 phosphosignal, which directly monitors intrinsic mTORC-specific catalytic activity.25 We, therefore, investigated whether the observed increase in the phosphorylation of TSC1 at Ser511 and mTOR activation correlated with the activation of mTORC1 effectors, namely S6K1 and 4E-BP1. As previously reported,26,27 an increase in the phospho-signal of S6K1 at Thr389 (pS6K1(T389)) and 4E-BP1 at Ser65 (p4E-BP1(S65)) on addition of Ang II was observed (Figure 1C and 1D). Interestingly, these perfectly paralleled phosphorylation of TSC1 on Ser511. Similar results were obtained in quiescent rat aortic smooth muscle cells stimulated with 100 nmol/L angiotensin II (Ang II) during the indicated time course. Whole cell extracts were prepared and subjected to immunoblot analysis using indicated antibodies. One of the 3 independent experiments with similar results is shown. B and D, Densitometric analysis of the data presented in A and C, respectively. Data are mean±SD of the 3 pooled experiments. Asterisks indicate significantly above corresponding conditions without Ang II treatment: *P<0.05, **2 symbols indicate P<0.01, ***P<0.001.

IKKβ Is Required for the Phosphorylation of TSC1 on Ser511 and Activation of mTORC1 Effectors in Ang II–treated VSMC

We have previously demonstrated that Ang II induces a rapid and sustained activation of IKKβ in primary VSMC.5 Consequently, we assessed its phosphotransferase activity toward recombinant TSC1 using an in vitro kinase assay. We
observed that IKKβ strongly phosphorylated TSC1 on Ang II activation. This phosphorylation signal was similar in intensity to that of IκBα, the canonical substrate of IKKβ. Interestingly, IKKβ was no longer able to induce the phosphorylation of TSC1 when the IKKβ consensus site (Ser511) was mutated to an alanine residue (Figure 2A and 2B). Next, we examined the requirement of IKKβ in the Ang II–induced phosphorylation of TSC1 and mTORC1 effectors in primary cultured human VSMC. Reducing the expression level of IKKβ with 2 different short hairpin RNA constructs significantly affected the ability of Ang II to induce the phosphorylation of TSC1 at Ser511 and the phosphorylation of S6K1 and 4E-BP1 (Figure 2C and 2D).

Taken together, these results demonstrate the requirement of IKKβ in the phosphorylation of TSC1 at Ser511 and the activation of the mTORC1 pathway in response to Ang II.

**Inhibition of Ang II–Induced Activation of the mTORC1 Pathway by ML120B**

In addition to the short hairpin RNA knockdown approach, we used a pharmacological approach. N-(6-chloro-7-methoxy-9H-β-carbolin-8-yl)-2 methylnicotinamide (ML120B) is a potent and selective ATP-competitive inhibitor of IKKβ. ML120B has been reported to inhibit tumor necrosis factor α–induced nuclear translocation of p65 and inhibit the growth of multiple myeloma cell lines in vitro and in vivo. In our hands, this inhibitor was potent in inhibiting NF-κB activation because it antagonized tumor necrosis factor α–induced IκBα degradation, with maximal efficacy occurring at 30 µmol/L in human aortic VSMC (Figure IA in the online-only Data Supplement). Importantly, this concentration also blunts the ability of Ang II to induce the expression of the inflammatory marker vascular cell adhesion molecule 1 (VCAM-1) in cultured VSMC (Figure IB in the online-only Data Supplement). This inhibitor was next used to investigate the involvement of IKKβ in Ang II–induced hypertrophy. Quiescent human aortic VSMC were incubated with ML120B added at a dose range of 3 to 70 µmol/L before stimulating with Ang II. Interestingly, inhibition of IKKβ with increased amounts of ML120B correlated with a reduction in Ang II–induced phosphorylation of TSC1 on Ser511 and the autophosphorylation of mTOR and phosphorylation of the downstream mTORC1 effectors S6K1 and 4E-BP1 (Figure 3). Similar results were obtained in rat aortic VSMC stimulated with Ang II (Figure IIA in the online-only Data Supplement). The effect of the specific IKKβ inhibitor was also tested on Ang II stimulation during a time course. Pretreatment with 30 µmol/L ML120B compromised the ability of Ang II to activate S6K1 and inhibit 4E-BP1 at all time points examined, whereas Ang II–induced ERK1/2 phosphorylation (pERK1/2 (T185/Y187)) was not (Figure IIB in the online-only Data Supplement). Thus, the kinase activity of IKKβ is required for Ang II to stimulate effectors of the translational machinery.

**IKKβ–TSC1 Signaling Cascade Is Required for the Hypertrophic Effect of Ang II in VSMC**

We next addressed the role of IKKβ and TSC1 in the ability of Ang II to increase the rate of protein synthesis in cultured VSMC. Importantly, use of the IKKβ inhibitor ML120B was found to concentration-dependently inhibit Ang II–induced protein synthesis with an IC50 of 10.6 µmol/L (Figure 4A). It is noteworthy that this concentration also abolished ≈50% of the quantified TSC1 and S6K1 phosphorylation (Figure 3B and 3D). Consistent with the effect of the pharmacological inhibitor, reduction in the expression level of IKKβ with distinct short hairpin RNA constructs in quiescent primary human VSMC inhibited Ang II–induced protein synthesis (Figure 4B). Same observation was made in rat VSMC.
exposed to 2 different siRNA duplexes targeting IKKβ (data not shown). Remarkably, this remodeling event is likely under the specific control of IKKβ over the growth suppressor gene TSC1. Although transient overexpression of TSC1wt had only marginal effect on the ability of Ang II to induce [3H]-leucine incorporation, the use of a mutant form of TSC1 (S511A) lacking the IKKβ consensus site (and, therefore, not phosphorylated by IKKβ; see Figure 2A) severely compromised the hypertrophic effect of Ang II (Figure 4C). However, expression of the NF-kB super-repressor (IκBα-2Ndelta4) in VSMC, which was previously shown by our group to abrogate Ang II–induced NF-kB activation and expression of MCP-1 and interleukin-6, did not alter the trophic effect of the peptide (Figure III in the online-only Data Supplement). Taken together, our results demonstrate that both the kinase activity of IKKβ and the phosphorylation of TSC1 at Ser511 are required for the activation of effectors of the translational machinery and the hypertrophic effect of Ang II.

Additive Effect of ML120B and UO126 on Ang II–Stimulated Protein Synthesis

We have previously shown that activation of the MEK1/2–ERK1/2 pathway is necessary but not sufficient for the
Inhibition of IKKβ Reduces Ang II–Induced Protein Synthesis In Vivo

As the effect of ML120B on Ang II signaling events was identical in both cultured human and rat VSMC (Figure 3 and Figure II in the online-only Data Supplement), we, therefore, well positioned to investigate the role of IKKβ in Ang II–induced hypertrophy in vivo using a previously reported rat model that allows the measurement of vascular protein synthesis.24,33 Although the dose of Ang II used in this protocol does not affect the mean blood pressure (Table), it clearly induces vascular remodeling as observed by the enhanced rate of protein synthesis in rat aorta and rat mesenteric arteries (Figure 5A and 5B). It is worth mentioning that inhibition of IKKβ with ML120B resulted in an important reduction in the remodeling effect of the peptide without affecting the mean blood pressure, ruling out an indirect effect of this hemodynamic parameter. As expected, the use of the IKKβ inhibitor also reduced Ang II–induced VCAM-1 expression in aorta (Figure 5C and 5D). Taken together, these results demonstrate the physiological relevance of IKKβ requirement in both inflammatory and hypertrophic actions of Ang II.

Discussion

The vascular wall is an active organ, which consists of endothelial cells, VSMC, and fibroblasts. In the intact arterial media, VSMC and matrix are responsible for vessel wall contraction/relaxation, growth, development, remodeling, and repair, whereas abnormal growth of VSMC plays a major role in the development of vascular diseases, including hypertension and atherosclerosis.3,4 Deregulated production of the octapeptide Ang II mediates VSMC hypertrophy and inflammation.2,34 Therefore, understanding the signal transduction processes underlying Ang II–mediated cell growth is essential in identifying new therapeutic strategies to combat vascular diseases.

Previous studies have identified 2 major signaling cascades that are directly linked to Ang II–mediated protein synthesis in VSMC (Figure 6, right). Selectively blocking MEK1 and MEK2 by PD98059 and consequently ERK1/2 and MNK1 reduced Ang II–induced protein synthesis in VSMC and rat aorta,2,13,35 implicating the Ras–Raf–MEK1/2–ERK1/2–MNK1 signaling cascade in the increased rate of protein translation. Through the effect on TSC2, other studies also suggest that the ERK substrate mitogen-activated protein kinase–activated protein kinase p90 ribosomal S6 kinase is also required for full mTOR activation.36,37 The other major signaling cascade is the phosphatidylinositol 3-kinase–PDK1–AKT module. Treatment of human SMC with phosphatidylinositol 3-kinase inhibitor wortmannin reduced both Ang II–mediated protein synthesis and dissociation of 4E-BP1 with eIF4E,26 whereas expression of a dominant-negative mutant of AKT in VSMC reduced Ang II–induced protein synthesis.38 Interestingly, reactive oxygen species generation by Ang II also leads to the activation of AKT in a PDK1- and p38-dependent mechanism.39 Therefore, in addition to the activation of MEK1/2–ERK1/2 modules, the phosphatidylinositol 3-kinase–AKT cascade is involved in Ang II–mediated protein synthesis in VSMC. Despite these detailed investigations, the molecular mechanisms by which Ang II mediates VSMC growth are not fully elucidated. In the present study, we examined the involvement of IKKβ in Ang II–induced VSMC growth. We have previously reported that Ang II induces TRAF6–TAK1 and ERK1/2–ribosomal S6 kinase intracellular pathways, independently and sequentially, which lead to T-loop phosphorylation of IKKβ.4 This phosphorylation of IKKβ was observed as early as 15 seconds and plateaued within 5 to 10 minutes. Here, we demonstrate that on Ang II treatment, the detected phospho-signal of TSC1 at the IKKβ consensus site Ser511 increased after 5 minutes of stimulation, suggesting that IKKβ acts upstream of TSC1 (Figure 1A). Supporting this proposition is the fact that IKKβ was no longer able to phosphorylate a recombinant TSC1 lacking the IKKβ consensus site (Figure 2A). In addition, reducing the expression level of IKKβ using short hairpin RNA or blocking its phosphotransferase activity using ML120B significantly reduced the phospho-signal of TSC1 on Ser511 (Figures 2C, 3A, and 3B and Figure II in the online-only Data Supplement). These results indicate that IKKβ phosphorylates TSC1 on Ser511 in response to Ang II in primary rat and human VSMC.

Ang II modulates the expression of proinflammatory molecules in the vessel wall, such as VCAM-1, which stimulates recruitment of mononuclear leucocytes into the vessel media.40 In VSMC, the IKKβ inhibitor ML120B abrogated this Ang II–induced VCAM-1 expression (Figure 1B in the online-only Data Supplement). Interestingly, ML120B also dose-dependently reduced Ang II–induced autophosphorylation of mTOR (Figure 3A and 3B) and downstream effectors of the mTORC1 pathway (Figure 3C and 3D). In fact, at the maximal concentration of 30 μmol/L, ML120B totally prevented mTOR activation and dramatically affected S6K1 and 4E-BP1 phosphorylation, correlating with a novel function of

Table. Hemodynamic Parameters Measured in Freely Moving Rats

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Values are mean±SEM. Ang II indicates angiotensin II; DBP, diastolic blood pressure; MBP, mean blood pressure; ML120B, N-(β-carbolin-8-yl)-2-methylnicotinamide; n, number of rats per group in which hemodynamic measurements were made; and SBP, systolic blood pressure.
IKKβ in the hypertrophic effect of Ang II (Figure 4A and 4B). Mechanistically, treatment with the MEK inhibitor UO126 plus ML120B had an additive effect on the inhibition of Ang II–induced protein synthesis (Figure IV in the online-only Data Supplement) strengthening the hypothesis that Ang II–activated IKKβ increases the rate of protein synthesis mainly via an MEK1/2–ERK1/2–MNK1 independent pathway. The inhibitory behavior of the mutant of TSC1 lacking the IKKβ consensus site (TSC1 S511A) in human VSMC (Figure 4C) further demonstrates the importance of the IKKβ–TSC1 signaling module governing the hypertrophic effect of Ang II (Figure 6, left). We would like to assert that this novel signaling

Figure 5. Inhibition of IκB kinase-β (IKKβ) abrogates angiotensin II (Ang II)–induced protein synthesis in vivo. Two groups of rats were infused with Ang II for 26 hours, whereas 2 groups did not receive Ang II. A, After 22 hours, the animals that were infused with Ang II received the vehicle (n=9) or N-(6-chloro-7-methoxy-9H-β-carbolin-8-yl)-2 methylnicotinamide (ML120B; n=7) as an intravenous bolus dose of 0.25 mg/kg (equivalent to 10 µmol/L plasma concentration). In addition, after 22 hours, the animals that were not infused with Ang II also received the vehicle (n=6) or ML120B (n=4). The rate of protein synthesis was measured by [3H]–leucine incorporation into the aorta. Data are displayed as mean±SEM. **P<0.005 vs Ang II. B, After 22 hours, the animals that were infused with Ang II received the vehicle (n=13) or ML120B (n=5) as an intravenous bolus dose of 0.25 mg/kg. In addition, after 22 hours, the animals that were not infused with Ang II also received the vehicle (n=12) or ML120B (n=4). The rate of protein synthesis was measured by [3H]–leucine incorporation into the mesenteric arteries. Data are displayed as mean±SEM. *P<0.05 vs Ang II. C, Where indicated, rats were infused with Ang II for 6 hours. After 2 hours of stimulation, vehicle (n=3) or ML120B (n=3) was administrated as described in A. Control rats were sham operated. Protein lysates were prepared from aorta and subjected to immunoblot analysis. D, Densitometric analysis of vascular cell adhesion molecule 1 (VCAM-1) expression relative to β-actin expression presented in C.

Figure 6. Proposed model demonstrating the involvement of IκB kinase-β (IKKβ) in the hypertrophic effect of angiotensin II (Ang II). After binding to the Ang II type I receptor, a TRAF6–TAK1 complex leads to T-loop phosphorylation and activation of IKKβ, which is also under the control of an MEK1/2–ERK1/2 pathway. Activated IKKβ then phosphorylates p65 to induce an inflammatory response (not shown). In addition, our study reveals a novel signal transduction pathway whereby activated IKKβ phosphorylates and inhibits tuberous sclerosis (TSC) 1 to induce mammalian target of rapamycin (mTOR) activation, which leads to an increase in the rate of protein synthesis. Through their effect on TSC2, the MEK1/2–ERK1/2–p90RSK and phosphatidylinositol 3-kinase (PI3K)–PDK1–AKT–p38 signaling modules are also involved in controlling the overall rate of protein synthesis. The protein kinase MNK1, which is activated by ERK1/2, is also implicated in cap-dependent translation. 4E-BP indicates eukaryotic initiation factor 4E–binding protein 1; MAPAPK, mitogen-activated protein kinase–activated protein kinase; RHEB, Ras homolog enriched in brain; RSK, p90 ribosomal S6 kinase; and S6K, S6 kinase.
module does not necessary lessen the contribution of previously characterized signaling cascades or second messenger molecules involved in the growth properties of Ang II. Rather, as observed in the generation of reactive oxygen species and protein kinases such as c-Src, which were previously shown to be part of upstream signaling cascades governing the activation of IKKβ, we propose that specific vascular hypertrophic signaling pathways engaged by the Ang II type I receptor (eg, c-Src, generation of reactive oxygen species) might as well converge to the activation of IKKβ. Another scenario could also be considered, in which phosphorylation of TSC2 by the AKT and ERK1/2 pathways is involved but not sufficient to fully activate mTOR and increase the rate of protein synthesis in Ang II–treated cells. Notably, although TSC2 phosphorylation by ERK1/2 and AKT is proposed to inhibit its GTPase-activating protein function toward RHEB, phosphorylation of TSC1 on Ser511 was recently shown to be an upstream signaling event affecting its association with TSC2, altering TSC2 membrane localization and activation of the mTOR pathway.

In our in vivo studies, the IKKβ inhibitor ML120B was administered as an intravenous bolus to deliver a precise dose of ML120B into the vascular system. Because the pharmacokinetic profile of ML120B on intravenous administration was unknown, the dosage was selected from previous reports demonstrating that ML120B at a plasma concentration of 10 μmol/L is effective at inhibiting IKKβ-dependent NF-κB activation in vivo. IKKβ inhibition at this concentration was confirmed by the observation that Ang II–mediated VCAM-1 expression was reduced in aorta (Figure 5C and 5D). More importantly, IKKβ inhibition clearly reduced the rate of protein synthesis mediated by Ang II in both aorta and mesenteric vessels (Figure 5A and 5B). Taken together, the presented pharmacological and molecular data are most consistent with the involvement of IKKβ in the ability of Ang II to suppress the mTOR inhibitor TSC1, leading to activation of S6K1 and inhibition of 4E-BP1 and resulting in increased protein synthesis.

A previous study identified a link between the innate immune protein kinase IKKβ and TSC1 in cancer cells, which was proposed to be critical for inflammation-mediated tumor angiogenesis. However, this study did not address whether this molecular link could become a potential target in cardiovascular diseases involving cellular processes associated with both growth and inflammatory events. Our study goes further to suggest that IKKβ is responsible for coupling the Ang II type I receptor not only to the activation of NF-κB transcription factors leading to a proinflammatory response but also to the direct activation of the mTORC1 pathway. We, therefore, propose that IKKβ could become an attractive target for drug development to treat pathological conditions, including hypertension and atherosclerosis.

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Disclosures

None.

References


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Material and methods

Materials
Ang II was purchased from Sigma (Saint Louis, MO) and [3H]-Leucine from MP Biomedicals (Santa Ana, CA). The IKKβ inhibitor N-(6-chloro-7-methoxy-9H-β-carbolin-8-yl)-2-methylnicotinamide (ML120B) was a kind gift from Millennium: The Takeda Oncology Company (Cambridge, MA). The MEK1/2 inhibitor UO126 was purchased from Calbiochem (Gibbstown, NJ). Both drug inhibitors were resuspended in dimethyl sulfoxide (DMSO) and stored at -80°C until used. Primary human aortic VSMC were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured according to manufacturer recommendations. The pGEX6P-1-GST-TSC1 and pGEX6P-1-GST-TSC1(S511A) constructs were kindly donated by Dr. Mien-Chie Hung (University of Texas, HOU). pcDNA3.1 myc-TSC1 (#12133) was obtained from Dr. Cheryl Walker through Addgene and subcloned into the lentiviral bicistronic pLVX-IRES-Puro expression vector (Clontech). Mutation of Ser511 of TSC1 into Ala residue was accomplished using the QuikChange Lightning kit (Agilent Technologies). The commercial antibodies were purchased from the following suppliers: anti-IKKα/β (SC-7607) from Santa Cruz Biotechnology; phospho-mTOR (Ser2481, #2974), mTOR (#2983), phospho-S6K1 (Thr389, #9205), S6K1 (#2708), phospho-4EBP1 (Ser65, #9451), 4EBP1 (#9452), phospho-ERK1/2 (Thr202-Tyr204, #9101), and anti-ERK1/2 (#9102) from Cell Signaling Technology (Beverly, MA); phospho-TSC1 (Ser511), and TSC1 from Bethyl Laboratories (Montgomery, TX); anti-β-actin clone AC-74 (A5216) from Sigma (Saint Louis, MO).

Cell culture
Primary human and rat aortic VSMC were cultured and synchronized in the quiescent state as described previously 1, 2. For experiments with pharmacological inhibitors, the cells were treated with vehicle alone or with the indicated inhibitor concentrations for 30 min prior to addition of Ang II.

Preparation of cellular extracts and immunoblot analysis
Accomplished as described previously 1, 2. In some Western blot experiments, cellular extracts were equally divided (µg) and used in parallel on the same SDS-gel. Briefly, VSMC were washed twice with ice-cold phosphate-buffered saline (PBS), and whole cell extracts were prepared using 1% Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% Na Deoxycholate, 0.1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonfyl fluoride, 10-6M leupeptin, 10-6M pepstatin A, 10-6M aprotinin) for 30 min at 4°C. Lysates were clarified by centrifugation at 13000 g for 10 min, and equal amounts of protein (30-60 µg) were subjected to electrophoresis on 10% or 12% acrylamide gels. Proteins were electrophoretically transferred to Hybond-C nitrocellulose membranes (Amersham) in 25 mM Tris and 192 mM glycine. Immunoblot analysis for each antibody was carried out according to manufacturer’s instructions.
In vitro Kinase assay
The phosphotransferase activity of the IKKβ was measured as described previously\(^1\). Briefly, quiescent VSMC were stimulated with Ang II for 0 or 10 min. 500 µg of whole cell extracts were incubated for 4 h at 4 °C with a specific antibody to IKKβ (SC-7607) preabsorbed to protein A Sepharose beads. The immune complexes were washed twice with lysis buffer and twice with kinase buffer (20 mM Hepes, pH 7.4, 25 mM NaCl, 1 mM EGTA, 20 mM MgCl₂, 1 mM dithiothreitol (DTT), 5 mM p-nitrophenyl phosphate, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10⁻⁴ M phenylmethylsulfonyl fluoride). IKKβ activity was assayed by resuspending the beads in 40 µl of kinase buffer containing, 20 µM ATP, 20 µCi of [γ⁻³²P]ATP and 2 µg GST-IκBα (amino acids 1–54), 2 µg GST-TSC1 (amino acids 501–610) or 2 µg GST-TSC1 S511A (amino acids 501–610). The reactions were incubated at 30 °C for 60 min and stopped by the addition of 5X Laemmli's sample buffer. The samples were analyzed by SDS-gel electrophoresis. Following Coomassie staining, the gels were dried and exposed to a gel documentation device (Typhoon scanner 9410, Amersham Biosciences) for imaging and quantification.

Lentiviruses Production and Transduction
The production and transduction of lentiviral particles were accomplished as described previously\(^3\). Briefly, 293T cells were transfected using lipofectamine 2000 with 1.5 µg pMDLg/pRRE, 1.5 µg pRSV-REV and 3 µg pVSV-G as previously described\(^4\) whether with 6 µg of pLKO.1-puro encoding shRNA targeting IKKβ (TRCN0000018918 (#4) and TRCN0000018919 (#5)) or shRNA non-target (NT, Sigma), or with pLVX-IRES-puro encoding TSC1-Myc or TSC1 S511A-Myc. After 24 hours, mediums containing lentiviral particles were harvested and cellular debris removed by filtration (0.45 µm). For shRNA strategy, human aortic VSMC were infected at a MOI of 1.0 in complete medium containing 10 µg/ml of polybrene. After 16 hours, the medium was changed and two days after the cells were split in presence of 1 µg/ml of puromycine. Four days later the cells were synchronized for 48 hours, stimulated and than harvested and analyzed by immunoblotting or used for \[^3^H\]-Leucine incorporation. For overexpression strategy, human aortic VSMC were directly overlaid with lentiviruses in complete medium containing 10 µg/ml of polybrene. After 16 hours, the medium was changed and 24 hours later the cells were synchronized 48 hours and than used in \[^3^H\]-Leucine incorporation assays.

Measurement of protein synthesis in vitro
The rate of protein synthesis using \[^3^H\]-Leucine was measured as described previously\(^5\). Briefly, quiescent rat VSMC in triplicate wells of 24-well plates were stimulated with 100 nM Ang II in serum-free medium containing 0.5 µCi/ml \[^3^H\]-Leucine. After 24 h of stimulation, the medium was aspirated and the cells were incubated for 30 minutes in ice-cold 5% trichloroacetic acid. The wells were then washed once with 5% trichloroacetic acid and three times with ice-cold tap water. The radioactivity was measured by liquid scintillation counting after solubilization in 0.1 M NaOH. For experiments with pharmacological inhibitors, the cells were treated with the indicated inhibitor for 30 minutes and stimulated for 24 h in the continuous presence of the inhibitor.
**Measurement of protein synthesis in vivo**

The rate of protein synthesis using [3H]-Leucine infusion in vivo was measured as described previously. Briefly, each male Sprague-Dawley rats weighing 275-300 g (Charles River Laboratories, QC, Canada) received an injection of buprenorphine (0.03 mg/kg s.c.) and were anesthetized with isoflurane (2-3%) for insertion of polyethylene catheter in the left femoral vein and artery. After surgery, each rat was housed in individual cage. Monitoring was performed regularly (each 120 minutes). A second dose of buprenorphine was administrated 12 hours after the beginning of the surgery. Rats were free to move and have access to food and water. Following the infusion of [3H]-Leucine, rats were euthanized with pentobarbital i.v. Prior to undertaking the studies described, approval for all experiments using rats was obtained from the Animal Ethics Committee of the Université de Montréal (protocol #12-025) and was conformed to the Guide for the Care and Use of Laboratory Animals.

To study Ang II-induced vascular protein synthesis, osmotic pumps (model 1003D Alzet) releasing a constant dose of Ang II (400 ng/kg per min) were implanted subcutaneously in two groups of animals (Ang II and Ang II plus ML120B, Figure 5). Two additional groups of animals did not receive Ang II (Control and ML120B only, Figure 5). The dosage of Ang II was selected from previous results demonstrating the induction of protein synthesis without affecting blood pressure. Rats were allowed to recover unrestrained and had free access to food and water. 22 h after the implantation of the osmotic pumps, a saline solution containing [3H]-Leucine was infused intravenously for the last 4 hours at a rate of 12 µCi/hour to allow quantification of protein synthesis. In addition, one hour before [3H]-Leucine infusion, two groups of animals (ML120B only and Ang II plus ML120B, Figure 5) received an intravenous bolus of 0.25 mg/kg ML120B suspended in 0.2% carboxymethyl cellulose (10 µM plasma concentration), whereas the other two groups of animals (Control and Ang II, Figure 5) received an intravenous bolus 0.2% carboxymethyl cellulose (vehicle). The arterial pressure and heart rate were recorded during drug administration and [3H]-Leucine infusion utilizing the arterial catheter in freely moving rats. At the end of the treatments, aorta and small mesenteric arteries (arteries branching from, but excluding the superior mesenteric artery) were harvested, freed from surrounding tissue, frozen, and powdered in liquid nitrogen. Tissues were solubilized in 1 M KOH at 45°C for 4 h. For each tissue, 5 µl was used to measure protein concentration and 500 µl to measure incorporation of [3H]-Leucine in newly synthesized proteins by liquid scintillation counting. Data are expressed as counts per minute per milligram of protein, which represents the rate of vascular protein synthesis over a 4 hours-period. For immunoblot analysis, tissue extracts were prepared using 1% Triton X-100 lysis buffer containing 0.1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 10⁻⁶M leupeptin, 10⁻⁶M pepstatin A, and 10⁻⁶M aprotinin.

**Statistical analysis**

In vitro and in vivo data are expressed as mean +/- SD and SEM respectively. Statistical analysis was performed using GraphPad Prism version 5.0 for Mac (GraphPad Software, San Diego, CA). Comparison of two groups was carried out using a two-tailed unpaired t test, and comparison of more than two groups was carried out with one-way analysis of variance and a Bonferroni post-test. Statistical significance was accepted at a p value below 0.05. The number of independent experiments is denoted by n.
References

1. Doyon P, Servant MJ. Tumor necrosis factor receptor-associated factor-6 and ribosomal s6 kinase intracellular pathways link the angiotensin ii at1 receptor to the phosphorylation and activation of the ikappab kinase complex in vascular smooth muscle cells. J Biol Chem. 2010;285:30708-30718


Supplemental Figure I. ML120B inhibits the proinflammatory actions of TNF-α and Ang II in cultured VSMC. A) Quiescent human aortic VSMC were pretreated with increasing concentrations of ML120B as indicated or the vehicle dimethyl sulfoxide (DMSO) 30 minutes before stimulation with 10 ng/ml TNF-α for 10 minutes. Whole cell extracts were prepared and subjected to immunoblot analysis. Data are representative of 2 independent experiments. B) Quiescent rat VSMC were pretreated with vehicle (DMSO) or 30 µM ML120B for 30 minutes before stimulation with 100 nM Ang II for 24 hours in the continuous presence of the drug or vehicle. Whole cell extracts were prepared and subjected to immunoblot analysis. Data are representative of 2 independent experiments.
Supplemental Figure II. ML120B inhibits the Ang II-induced activation of effectors of the mTORC1 pathway in rat VSMC. A) Quiescent rat VSMC were pretreated for 30 minutes with vehicle (DMSO) or increasing concentrations of ML120B. The cells were then stimulated with Ang II (100 nM) for 10 min. Whole cell extracts were prepared and subjected to immunoblot analysis. One of three independent experiments with similar results is shown. B) Quiescent rat VSMC were pretreated for 30 min with vehicle (DMSO) alone or 30 µM ML120B, followed by stimulation with 100 nM Ang II over the indicated time-course. Whole cell extracts were prepared and subjected to immunoblot analysis. One of three independent experiments with similar results is shown.
Supplemental Figure III. The classical IκBα/NF-κB transcription factors signaling cascade is not involved in the hypertrophic effect of Ang II. Quiescent rat VSMC populations stably expressing the NF-κB super repressor (IκBα-2Ndelta4) or encoding for the neomycin cassette only (Neo), were stimulated with Ang II for 24 h. Protein synthesis was measured by [3H]-Leucine incorporation. Each value represents the mean ± SD of triplicate determinations.
Supplemental Figure IV. Additive effect of ML120B and UO126 on Ang II-stimulated protein synthesis in VSMC. Quiescent rat VSMC were pretreated with vehicle or 30 µmol/L ML120B and/or 10 µmol/L UO126 for 30 min. The cells were then stimulated for 24 h with 100 nmol/L Ang II in the continuous presence of vehicle or inhibitor drugs. Protein synthesis was measured by [3H]-Leucine incorporation and each value represent the mean ± S.D. of triplicate determinations.