Role of p38 MAPK and MAPKAPK-2 in angiotensin II-induced Akt activation in vascular smooth muscle cells


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Taniyama, Yoshihiro, Masuko Ushio-Fukai, Hirofumi Hitomi, Petra Rocic, Michael J. Kingsley, Chun Pfahnl, David S. Weber, R. Wayne Alexander, and Kathy K. Griendling. Role of p38 MAPK and MAPKAPK-2 in angiotensin II-induced Akt activation in vascular smooth muscle cells. Am J Physiol Cell Physiol 287: C494–C499, 2004—Angiotensin II activates a variety of signaling pathways in vascular smooth muscle cells (VSMCs), including the MAPKs and Akt, both of which are required for hypertrophy. However, little is known about the relationship between these kinases or about the upstream activators of Akt. In this study, we tested the hypothesis that the reactive oxygen species (ROS)-sensitive kinase p38 MAPK and its substrate MAPKAPK-2 mediate Akt activation in VSMCs. In unstimulated VSMCs, Akt and p38 MAPK are constitutively associated and remain so after angiotensin II stimulation. Inhibition of p38 MAPK activity with SB-203580 dose-dependently inhibits Akt phosphorylation on Ser473, but not Thr308. Angiotensin II-induced phosphorylation of MAPKAPK-2 is also attenuated by SB-203580, as well as by inhibitors of ROS. In addition, angiotensin II stimulates the recruitment of MAPKAPK-2 to p38 MAPK complex, and an in vitro kinase assay shows that MAPKAPK-2 immunoprecipitates of VSMC lysates phosphorylates recombinant Akt in an angiotensin II-inducible manner. Finally, intracellular delivery of a MAPKAPK-2 peptide inhibitor blocks Akt phosphorylation on Ser473. These results suggest that the p38 MAPK-MAPKAPK-2 pathway mediates Akt activation by angiotensin II in these cells by recruiting active MAPKAPK-2 to a signaling complex that includes both Akt and p38 MAPK. Through this mechanism, p38 MAPK confers ROS sensitivity to Akt and facilitates downstream signaling. These results provide evidence for a novel signaling complex that integrates diverse upstream signals contributing to VSMC hypertrophy.

MATERIALS AND METHODS

Materials. Anti-phospho-Akt1/PKB-α (Thr308) was obtained from Upstate Biotechnology (Lake Placid, NY). Phospho-MAPKAPK-2 (Thr222) antibody, phospho-Akt (Ser473), Akt antibody, phospho-p38 MAPK antibody, and p38 MAPK antibody were from Cell Signaling (Danvers, MA). Chariot delivery reagent was from Active Motif (Carlsbad, CA). All other chemicals and reagents, including DMEM with 25 mM HEPES and 4.5 g/l glucose, were from Sigma (St. Louis, MO).

Cell culture. VSMCs were isolated from male Harlan Sprague-Dawley rat thoracic aortas by enzymatic digestion, as described previously (10). Cells were grown in DMEM supplemented with 10% calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells between passages 6 and 17 were used for experiments.

Although it has been clearly shown that activation of Akt requires phosphatidylinositol 3-kinase (PI3-K) activity and NAD(P)H oxidase-derived production of ROS (6, 26), the full complement of upstream mediators of Akt activation by ANG II is not yet fully understood. Akt activation requires phosphorylation of two amino acid residues by upstream kinases: threonine 308 (Thr308) by 3-phosphoinositide-dependent protein kinase (PDK)-1 and serine 473 (Ser473) by a “so-called” PDK2, whose identity is controversial (4). To date, five kinases have been proposed as candidates for PDK2: Akt itself, PDK1, integrin-linked kinase (ILK), conventional PKC isoforms, and MAPKAPK-2 (15).

In VSMCs, the shared ROS sensitivity of p38 MAPK and Akt suggests that they are in the same signaling axis leading to hypertrophy. In the present study, we tested the hypothesis that p38 MAPK mediates Akt activation via MAPKAPK-2 because MAPKAPK-2 is a known substrate of p38 MAPK (19) and phosphorylates Akt in vitro (1). We found not only that p38 MAPK is required for ANG II-induced Akt activation, but also that ANG II stimulates the recruitment of MAPKAPK-2 to p38 MAPK-Akt complexes, where MAPKAPK-2 can phosphorylate Akt on Ser473. Thus the redox sensitivity of Akt is conferred via the upstream ROS-sensitive kinase p38 MAPK. These results provide evidence for a novel signaling complex that integrates diverse upstream signals contributing to VSMC hypertrophy.

Address for reprint requests and other correspondence: K. K. Griendling, Emory Univ., Division of Cardiology, 319 WMB, 1639 Pierce Dr., Atlanta, GA 30322 (E-mail: kgriend@emory.edu). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
For MAPKAPK-2 peptide inhibitor experiments, the MAPKAPK-2 inhibitor was first incubated with the peptide delivery agent Chariot for 30 min (1:1 vol/vol). VSMCs at 40–50% confluence were incubated in serum-free medium with the Chariot-peptide complex for 1 h at 37°C and allowed to recover in 0.1% calf serum containing DMEM for 1 h before treatment with ANG II.

**Western blotting.** VSMCs at 80–90% confluence in 100-mm dishes were made quiescent by incubation with DMEM containing 0.1% calf serum for 24 h. Cells were stimulated with agonist at 37°C in serum-free DMEM for the specified durations. Some cells were preincubated with various inhibitors, as indicated. After treatment, cells were lysed with 500 µl of ice-cold lysis buffer, pH 7.4 (in mM: 50 HEPES, 5 EDTA, 100 NaCl), 1% Triton X-100, protease inhibitors (10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin), and phosphatase inhibitors (in mM: 50 sodium fluoride, 1 sodium orthovanadate, 10 sodium pyrophosphate, 0.001 microcystin). Solubilized proteins were centrifuged at 27,000 × g at 4°C for 15 min, and supernatant protein was quantified by the Bradford assay. Proteins were separated by using 9% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked at room temperature with PBS containing 5% nonfat dry milk and 0.1% Tween 20 for 1 h. Blots were incubated with the indicated primary antibodies overnight, and the immunocomplex was detected as early as 1 min and peaked at 5 min. Similar, Akt was phosphorylated on Thr222 was dose-dependently inhibited by SB-203580, with a peak at 5 min (2.87 ± 0.6-fold at 5 min; 2.94 ± 0.2-fold at 10 min; n = 3) (Fig. 4). Thus MAPKAPK-2 and Akt are phosphorylated concurrently in VSMCs. As shown in Fig. 3, p38 MAPK phosphorylation (data not shown). These data suggest that p38 MAPK is an upstream mediator for the phosphorylation of Akt on Ser473 but not Thr308.

**Interaction of p38 and Akt.** It has been shown that p38 MAPK and Akt are able to associate with each other in human neutrophils (18). To determine whether such an association might facilitate Akt phosphorylation by p38 MAPK-dependent mechanisms in VSMCs, we performed immunoprecipitation followed by immunoblotting to investigate a possible interaction of these two kinases in VSMCs. As shown in Fig. 3, p38 MAPK and Akt coimmunoprecipitate even under basal conditions, and this association continues after both are phosphorylated (see band shift of phosphorylated p38 MAPK at 5 min). These results suggest that p38 MAPK and Akt are constitutively associated in VSMCs.

**Statistical analysis.** Results are expressed as means ± SE. Statistical significance was assessed by ANOVA, followed by Bonferroni’s test. A value of P < 0.05 was considered to be statistically significant.

**RESULTS**

**Time course of p38 MAPK and Akt phosphorylation.** Our laboratory has previously shown that ANG II activates both Akt and p38 MAPK in VSMCs (25, 26). To examine in more detail the relative time courses of phosphorylation of these two kinases, we performed Western analysis with phospho-specific antibodies. As shown in Fig. 1, ANG II induced a rapid phosphorylation of p38 MAPK, which was detected as early as 1 min and peaked at 5 min. Similarly, Akt was phosphorylated on Ser473 by ANG II, beginning at 2 min and reaching a maximum at 5 min. These time courses are consistent with the hypothesis that p38 MAPK is upstream of Akt.

**Effect of p38 MAPK inhibition on ANG II-induced Akt phosphorylation.** To determine whether p38 MAPK mediates activation of Akt, we tested the effects of a specific p38 MAPK inhibitor, SB-203580, on the ANG II-induced phosphorylation of Akt at Ser473 and Thr308, sites critical for full activation of Akt. Our laboratory has previously shown that 10 µM SB-203580 completely inhibits p38 MAPK activation in VSMCs (25). As shown in Fig. 2A, SB-203580 dose-dependently inhibited ANG II-induced phosphorylation of Ser473 (maximal inhibition: 64 ± 2%). Similar results were found with a pharmacologically distinct p38 MAPK inhibitor, PD-169316 (10 µM, data not shown). Inhibition of ERK1/2 with PD-98059 (30 µM) had no effect on Ser473 phosphorylation by ANG II (data not shown). In contrast to Ser473 phosphorylation, as shown in Fig. 2B, SB-203580 had little effect on the phosphorylation of Thr308 by ANG II. Finally, the converse experiment, that is, inhibition of Akt using the PI3-K inhibitor wortmannin (0.1–1 µM) or LY-293002 (10–30 µM), had no effect on p38 MAPK phosphorylation (data not shown). These data suggest that p38 MAPK is an upstream mediator for the phosphorylation of Akt on Ser473 but not Thr308.

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a maximum inhibition of 46 ± 7% at 10 μM SB-203580. It has been shown that phosphorylation of MAPKAPK-2 on Thr222 is required for kinase activity (3), suggesting that ANG II activates MAPKAPK-2 in VSMCs in a p38 MAPK-dependent manner.

Because p38 MAPK is ROS sensitive (25), we also tested the effects of inhibitors of ROS on ANG II-induced MAPKAPK-2 phosphorylation. As shown in Fig. 6, both the flavin oxidase inhibitor diphenylene iodonium and N-acetylcysteine, at concentrations previously shown to inhibit ROS generation in VSMCs (20), completely blocked the ability of ANG II to phosphorylate MAPKAPK-2. This is consistent with the finding that p38 MAPK is upstream of MAPKAPK-2.

**Functional interaction of Akt and MAPKAPK-2.** To test the hypothesis that MAPKAPK-2 is a PDK2 in VSMCs, we took three approaches. First, we investigated whether MAPKAPK-2 and Akt form a complex in VSMCs by using immunoprecipitation. As shown in Fig. 7, Akt was present in MAPKAPK-2 immunoprecipitates. Furthermore, ANG II treatment (100 nM, 2 min) significantly increased the association of these kinases. Together, with the results in Fig. 2, these data suggest that MAPKAPK-2 is recruited to the Akt-p38 MAPK complex on ANG II activation.

To investigate whether VSMC MAPKAPK-2 can phosphorylate Akt on Ser473, we immunoprecipitated MAPKAPK-2 from VSMC lysates and performed an in vitro kinase assay using recombinant Akt as a substrate. As shown in Fig. 8, Akt at Ser473 was phosphorylated by the MAPKAPK-2 immunocomplex to a small extent in control cells. ANG II (100 nM; 5 min) significantly increased MAPKAPK-2-dependent phosphorylation of Akt.
Finally, to confirm that MAPKAPK-2 is upstream of Akt in intact cells, we used a specific peptide inhibitor of MAPKAPK-2 and investigated its effect on ANG II-induced Akt phosphorylation on Ser473. As shown in Fig. 9, the MAPKAPK-2 inhibitor dose-dependently inhibited Akt phosphorylation on Ser473.

DISCUSSION

In this study, we provide insight into the upstream signaling pathways that mediate Akt activation by ANG II in VSMCs. Although many different kinases have been proposed to be the elusive PDK2, little is known about the mechanisms responsible for Akt phosphorylation in smooth muscle. Our data clearly demonstrate that the p38 MAPK-MAPKAPK-2 pathway mediates Akt activation by ANG II in these cells, by recruiting active MAPKAPK-2 to a signaling complex that includes both...
Akt and p38 MAPK. By this mechanism, p38 MAPK confers ROS sensitivity to Akt and facilitates downstream signaling. These results provide evidence for a novel signaling complex that may help to spatially organize hypertrophy-related, ROS-sensitive signaling in VSMCs.

Candidates for PDK2 other than MAPKAPK-2 include Akt itself, PDK1, ILK, and conventional PKC isoforms. In vitro evidence exists supporting most of these kinases, but their in vivo role seems to be cell-type specific. Although Akt can autophosphorylate on Ser 473 in vitro (23), kinase-inactive Akt can still be phosphorylated at Ser 473 in human embryonic kidney (HEK)-293 cells (1), calling into question the importance of autophosphorylation in vivo. PDK1 also phosphorylates Ser 473 in vitro (2); however, in PDK1−/− embryonic stem cells, Akt is still phosphorylated at Ser 473 in response to epidermal growth factor (27), suggesting that other mechanisms exist. ILK is a viable candidate because depletion of ILK in HEK-293 cells with the use of siRNA almost completely inhibits Ser 473 phosphorylation (24), although it is presently unknown whether ILK exerts these effects directly or via an intermediate kinase. Finally, in platelets, activation of PKC can phosphorylate Akt on Ser 473 in a PI3-K-independent manner (14). This is unlikely to be the case in VSMCs, however, because ANG II-induced activation of Akt is PI3-K-dependent (26), as it is for most other agonists and cell types (15).

The data presented here strongly suggest that the p38 MAPK-MAPKAPK-2 pathway regulates phosphorylation of Akt on Ser 473 in ANG II-stimulated VSMCs. MAPKAPK-2 is a known substrate of p38 MAPK that is activated in response to cellular stresses (19). It has been shown to phosphorylate 27-kDa heat shock protein (21), the light chain of myosin II (12), and vimentin (5) and to be involved in cytoskeletal reorganization and migration (13). Furthermore, MAPKAPK-2 phosphorylates Akt on Ser 473 in vitro (1), and a peptide inhibitor of MAPKAPK-2 blocks Ser 473 phosphorylation in neutrophils stimulated with f-Met-Leu-Phe (18). Our data extend these in vitro observations to VSMCs and provide a mechanism by which the activation of MAPKAPK-2 and its interaction with Akt occur. We found that ANG II stimulates a dynamic association of MAPKAPK-2 with the constitutive Akt-p38 MAPK complex (Figs. 2 and 7). Moreover, MAPKAPK-2 immunocomplexes phosphorylate Ser 473 on recombinant Akt in an ANG II-inducible manner (Fig. 8), and a MAPKAPK-2 inhibitor blocks Akt phosphorylation in intact cells (Fig. 9). Because inhibition of p38 MAPK attenuates both MAPKAPK-2 and Ser 473 Akt phosphorylation (Figs. 3B and 5), these data suggest that p38 MAPK initiates the interaction and activation of the other kinases. The specificity of this pathway is evident from both the lack of effect of SB-230580 on Thr 308 Akt phosphorylation (Fig. 3A) and the inability of the ERK1/2 pathway inhibitor PD-98059 to block Ser 473 phosphorylation. Taken together, our results indicate that p38 MAPK phosphorylates MAPKAPK-2 and recruits it to the constitutively present p38 MAPK-Akt complex, permitting phosphorylation of Akt on Ser 473.

It is noteworthy that activation of Akt occurs on recruitment of MAPKAPK-2 to the signaling complex. There is some evidence in other cell types that this occurs in the nucleus (17), placing this complex in an ideal location to mediate gene transcription. All three proteins phosphorylate specific transcription factors, and their presence in a complex is likely to provide a coordinated regulation of gene expression. Clearly, further experiments are necessary to evaluate the functional consequences of these protein-protein interactions.

Although previous work has shown that ANG II activates p38 MAPK (16, 25), MAPKAPK-2 (16), and Akt (26) in VSMCs, the present study provides a framework with which to integrate these signaling molecules and to understand the relationship between ROS-sensitive kinases important in growth (Fig. 10). ANG II stimulates NAD(P)H oxidases to increase the production of ROS (9), which, in turn, mediates

![Fig. 9. Inhibition of ANG II-induced Akt phosphorylation by a peptide MAPKAPK-2 inhibitor. VSMCs were pretreated with the MAPKAPK-2 inhibitor peptide at the indicated amount for 1 h before addition of 100 nM ANG II for 5 min, as indicated in MATERIALS AND METHODS. Cells were lysed and prepared for Western analysis with the use of anti-phospho-Ser 473 Akt antibodies. Bar graph represents averaged data quantified by densitometry of immunoblots, expressed as fold increases in phosphorylation, in which the phosphorylation observed in unstimulated cells was defined as 1.0. Values are means ± SE for 4 independent experiments. *P < 0.01 vs. control; **P < 0.01 vs. ANG II alone.](http://ajpcell.physiology.org/)
both p38 MAPK and Akt activation (25, 26). We now show that p38 MAPK is upstream of Akt (Fig. 3) and that MAPKAPK-2 serves as an intermediate, ROS-sensitive kinase in this signaling pathway. The constitutive association of p38 MAPK and Akt provides a basis for their mutual ROS sensitivity and importance in hypertrophy, and the recruitment of MAPKAPK-2 to this complex supplies the mechanism by which p38 MAPK activates Akt.

In summary, we have found that Akt activation is mediated by the p38 MAPK-MAPKAPK-2 pathway in VSMCs stimulated with ANG II. These data support the concept that, at least in VSMCs, MAPKAPK-2 can function as a PDK2 to phosphorylate Ser\(^{733}\) of Akt. This pathway is critically important in VSMC hypertrophy and may provide a specific therapeutic target for vascular remodeling in disease.

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REFERENCES