Reactive Oxygen Species Mediate the Activation of Akt/Protein Kinase B by Angiotensin II in Vascular Smooth Muscle Cells*

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Angiotensin II, a hypertrophic/auto-antipotic hormone, utilizes reactive oxygen species (ROS) as growth-related signaling molecules in vascular smooth muscle cells (VSMCs). Recently, the cell survival protein kinase Akt/protein kinase B (PKB) was proposed to be involved in protein synthesis. Here we show that angiotensin II causes rapid phosphorylation of Akt/PKB (6-±0.4-fold increase). Exogenous H2O2 (50–200 μM) also stimulates Akt/PKB phosphorylation (maximal 8-±0.2-fold increase), suggesting that Akt/PKB activation is redox-sensitive. Both angiotensin II and H2O2 stimulation of Akt/PKB are abrogated by the phosphatidylinositol 3-kinase (PI3-K) inhibitors wortmannin and LY294002 (2-(4-morpholinyl)-8-phenyl-4

-1-benzopyran-4-one), suggesting that PI3-K is an upstream mediator of Akt/PKB activation in VSMCs. Furthermore, diphenylene iodonium, an inhibitor of flavin-containing oxidases, or overexpression of catalase to block angiotensin II-induced intracellular H2O2 production significantly inhibits angiotensin II-induced Akt/PKB phosphorylation, indicating a role for ROS in agonist-induced Akt/PKB activation. In VSMCs infected with dominant-negative Akt/PKB, angiotensin II-stimulated [3H]leucine incorporation is attenuated. Thus, our studies indicate that Akt/PKB is part of the remarkable spectrum of angiotensin II signaling pathways and provide insight into the highly organized signaling mechanisms coordinated by ROS, which mediate the hypertrophic response to angiotensin II in VSMCs.

Recently, reactive oxygen species (ROS) such as H2O2 and superoxide have gained acceptance as modulators of receptor-mediated signal transduction in a variety of cell types (1).
anisms coordinated by ROS that mediate the hypertrophic response to Ang II in VSMCs.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP (3000 Ci/mmol) was from NEN Life Science Products (Wilmington, DE). Phospho-Akt (Ser473) and glycogen synthase kinase-3 (GSK-3)α/β (Ser21/9) antibodies and GSK-3 antibody were from New England Biolabs, Inc. (Beverly, MA). Sheep anti-human Akt/PKB antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Protein G Plus-agarose was from Santa Cruz Biotechnology (Santa Cruz, CA). Histone 2B was purchased from Roche Molecular Biochemicals. 2', 7'-Dichlorofluorescein diacetate (DCF-DA) was obtained from Acros (Pittsburgh, PA). Wortmannin and LY294002 were from Alexis Corp. (San Diego, CA). Diphenylene iodonium (DPI) was from Toronto Research Chemicals (Downsview, Ontario, Canada). All other chemicals and reagents, including Dulbecco's modified Eagle's medium (DMEM) with 25 mM Hepes and 4.5 g/liter glucose, were from Sigma.

**Cell Culture**—VSMCs were isolated from male Harlan Sprague-Dawley rat thoracic aortas by enzymatic digestion as described previously (15). Cells were grown in DMEM supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and were passed twice a week by harvesting with trypsin:EDTA and seeding into 75-cm² flasks. For experiments, cells between passages 6 and 19 were used at confluence.

In some experiments, we used VSMCs that had been stably transfected with human catalase. In these cells, catalase mRNA and protein expression were significantly increased (4, 6). Transfected cells were maintained in selection medium until they were plated into 35- or 100-mm dishes for experiments.

**Detection of Akt/PKB Phosphorylation by Immunoblotting**—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 specified durations. After treatment, cells were lysed with 500 μl of ice-cold lysis buffer, pH 7.4 (10 mM HEPES, 5 EDTA, 50 mM NaCl, 1% Triton X-100, protease inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, and phosphatase inhibitors) for 30 min at 4 °C. Supernatant protein was quantified by the Bradford assay. Proteins (25 μg) were separated using 9% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked overnight at room temperature with phosphate-buffered saline containing 6% nonfat dry milk and 0.1% Tween 20. Blots were incubated with primary rabbit polyclonal phosphospecific Akt/PKB antibody (detects Akt/PKB only when activated by phosphorylation on Ser473) at 1:1000. After incubation with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit antibody, 1:2000), phosphorylated forms of proteins were detected by enhanced chemiluminescence. Band intensity was quantified by densitometry of immunoblots using NIH Image, version 1.61. Phosphorylation of Akt/PKB on Ser473 is required for activation (16); therefore, phosphorylation at this site was routinely taken as a measure of Akt/PKB enzymatic activity. In some experiments, Akt/PKB activity was assessed directly in Akt/PKB immunoprecipitates.

**Immunoprecipitation and Akt/PKB Activity Assay**—VSMCs lysates were prepared as described above for Akt/PKB phosphorylation assays. For immunoprecipitation, cell lysates (400 μg) were incubated with sheep anti-human Akt/PKB antibody (4 μg)/25 μg of protein G-agarose beads complex for 2 h at 4 °C with gentle rocking. The beads were washed three times with 500 μl of lysis buffer containing 500 instead of 50 mM NaCl, twice with 500 μl of washing buffer (50 mM Tris-HCl (pH 7.5), 0.03% (w/v) Brij-35, 0.1 mM EDTA, and 0.1% 2-mercaptoethanol), and once with 100 μl of kinase buffer (10 mM Tris-HCl, 7.5 mM β-glycerophosphate, 1 dithiothreitol, 1 mM sodium orthovanadate). The kinase reaction was carried out by incubating the beads in 50 μl of kinase buffer containing 10 μCi [γ-32P]ATP, 50 μM ATP, 7.5 mM MgCl2, and 2 μg of histone 2B for 30 min at 30 °C. Anti-Akt/PKB immunoprecipitates were subjected to 15% SDS-polyacrylamide gel electrophoresis, and 32P-labeled histone 2B was detected using a Phospho-Imager and quantified by densitometry using NIH Image, version 1.61. In some experiments, GSK-3 fusion protein (1 μg) was used as the substrate. Radiolabeled ATP was omitted from the reaction, and anti-phospho-GSK-3 antibody was used to detect phosphorylated GSK-3.

**Measurement of Intracellular H2O2 Levels**—H2O2 levels were measured using the peroxide-sensitive fluorescent DCF-DA (5 μM) as described previously (4, 6). Although DCF-DA is oxidized by H2O2 as well as other peroxides, the complete inhibition of fluorescence in Ang II-stimulated cells by the addition of catalase (350 units/ml) (data not shown) and by catalase overexpression (4) indicates that the fluorescence signal evoked by Ang II was derived predominantly from H2O2.

**Construction of Dominant-Negative Akt/PKB Adenovirus and Infection of VSMCs**—pcDNA HA-Akt(AA) was a kind gift from Dr. J. R. Testa (Fox Chase Cancer Center). HA-Akt(AA) is a cDNA encoding mouse Akt/PKB containing alanine mutations in the regulatory site (Thr308 and Ser473) fused to the hemagglutinin (HA) epitope (16). HA-Akt(AA) was inserted into the EcorRI/XbaI site of the pACCMVpLpA plasmid. pACCMVpLpA-HA-Akt(AA) was cotransfected into 293 cells with a vector modified from the Ad5 genome, which has a 4.3-kilobase PBSr insert and confers resistance to tetracyclin and ampicillin (pJM17). The resulting replication-defective recombinant adenoviruses were purified from isolated plaques and amplified in 293 cells. Viral preparations were purified by two CsCl gradient centrifugations as described previously (17). The control virus, Ad-β-Gal, contains the bacterial β-galactosidase gene downstream from the cytomegalovirus promoter/enhancer (17). Multiplicity of infection (MOI) was determined spectrophotometrically. For experiments, VSMCs were incubated with...
Redox-sensitive Activation of Akt/PKB by Angiotensin II in VSMC

Fig. 2. Effects of Ang II on Akt/PKB phosphorylation in VSMCs. A, time course of Akt/PKB phosphorylation by Ang II. VSMCs were stimulated with 100 nM Ang II for the indicated times. B, dose response of Akt/PKB phosphorylation by Ang II. VSMCs were stimulated with various concentrations of Ang II (1–100 nM) for 5 min. The top panels are representative immunoblots of Ang II-induced phosphorylation of Akt/PKB. The bottom panels represent averaged data quantified by densitometry of images, expressed as fold increase in phosphorylation, in which the phosphorylation observed in cells at time 0 (A) or in unstimulated cells (B) was defined as 1.0 (control). Values are the means ± S.E. for three independent experiments. *p < 0.05 versus control.

Fig. 3. Effects of H2O2 and Ang II on Akt/PKB activity in VSMCs. VSMCs were treated with 200 μM H2O2 or 100 nM Ang II for the indicated times. Akt/PKB immunoprecipitates were incubated with GSK-3 or histone 2B, and phosphorylation of the substrate was assessed. The upper panel is a representative image of GSK-3 phosphorylation by H2O2 and Ang II. The lower panel represents averaged data quantified by densitometry of images, expressed as the fold increase in phosphorylation, in which the phosphorylation observed in cells at time 0 was defined as 1.0 (control). Values are the means ± S.E. for four independent experiments. *p < 0.05 versus control.

RESULTS

Effect of Exogenous H2O2 and Ang II on Akt/PKB Activation—Because ambient ROS are required for VSMC survival and growth (4, 7), we examined whether Akt/PKB is activated by H2O2. H2O2 (200 μM) induced a rapid activation of Akt/PKB, with a peak occurring 15 min after H2O2 addition (8–10.2-fold increase) (Fig. 1A). Akt/PKB activation was still detectable at 60 min. H2O2-induced Akt/PKB phosphorylation was dose-de-

Various MOI of either Ad-β-Gal or Ad-HA-Akt(AA) in the presence of 0.1% calf serum for 48 h before measurement of hypertrophy.

1H]Leucine Incorporation—To measure hypertrophy of VSMCs, cells were quiesced for 48 h in DMEM containing 0.1% calf serum. Cells were incubated with [3H]leucine (0.5 μCi/ml) in the presence or absence of 100 nM Ang II for an additional 24 h, and [3H]leucine incorporation was measured as described previously (6).

Statistical Analysis—Results are expressed as mean ± S.E. Statistical significance was assessed by Student’s unpaired two-tailed t test on untransformed data. A p value of <0.05 was considered to be statistically significant.

Role of PI3-K in H2O2- and Ang II-induced Akt/PKB Activation—As noted above, both PI3-K-dependent and PI3-K-independent pathways have been shown to be involved in Akt/PKB activation in other cell types (13, 19). To assess the role of PI3-K in H2O2- and Ang II-induced Akt/PKB phosphorylation, VSMCs were pretreated with the PI3-K inhibitors wortmannin (0.001–0.1 μM) and LY294002 (0.1–10 μM). These concentrations have been previously shown to effectively abrogate PI3-K activity (14, 20). As shown in Fig. 4, Akt/PKB phosphorylation by either H2O2 or Ang II was dramatically reduced by both inhibitors in a dose-dependent manner, suggesting that PI3-K is an upstream mediator of Akt/PKB activation in VSMCs.

Role of Intracellular H2O2 in Ang II-induced Akt/PKB Activation—The stimulation of Akt/PKB by exogenous H2O2 suggests that intracellular H2O2 ([H2O2]i) may mediate the effects of Ang II-induced Akt/PKB activation (4). To assess this possi-
Akt/PKB phosphorylation by Ang II was dramatically inhibited by 75% (6). Similar results were obtained with a second line of catalase-overexpressing cells. This effect was not due to differences in AT1 receptor expression or nonspecific inhibition of signaling pathways caused by overexpression of catalase, because vector- and catalase-transfected cells matched for receptor number, and p42/44 MAPK activation by Ang II was unaffected (4, 6). These results strongly suggest that Ang II-induced Akt/PKB activation is mediated by intracellularly produced H2O2.

Role of Akt/PKB in Ang II-induced Hypertrophy—We have previously shown that ROS play an important role in Ang II-induced hypertrophy (4, 6, 22), raising the possibility that Akt/PKB is also involved in this response. To assess the role of Akt/PKB in hypertrophy, we tested the effect of dominant-negative Akt/PKB (HA-Akt(AA)) on Ang II-stimulated Akt/PKB activity, as demonstrated by its ability to inhibit insulin-induced Akt/PKB activity in CHO cells (10). As shown in Fig. 7, infection of VSMCs with adenovirus encoding dominant-negative Akt/PKB (Ad-HA-Akt(AA)) inhibited Ang II-induced [3H]leucine incorporation. This Akt/PKB mutant effectively inhibits endogenous Akt/PKB activity, as demonstrated by its ability to inhibit insulin-induced Akt/PKB activity in CHO cells (10). The extension of inhibition paralleled the expression of HA-Akt(AA), as determined by Western analysis (data not shown). The inhibitory effects of Ad-HA-Akt(AA) are not caused by nonspecific or toxic effect of viral infection, because infection of the cells with a control virus containing the β-galactosidase gene had no effect on the hypertrophic response up to 600 MOI (Fig. 7), and Ad-HA-Akt(AA) did not affect p38MAPK phosphorylation (data not shown). Furthermore, the trypan blue exclusion test for cell viability indicated that cells infected with Ad-HA-Akt(AA) were >95% viable up to 600 MOI. These results suggest that Akt/PKB contributes to Ang II-induced hypertrophy.

**DISCUSSION**

It has become apparent that ROS play important roles as modulators of Ang II signal transduction in VSMCs (4, 6, 21, 22). We have previously found that generation of ROS is required for Ang II-induced hypertrophy and that one of the
molecular targets of ROS involved in this response is p38MAPK. Here we extend these observations to demonstrate that Ang II activates the pivotal cell survival kinase Akt/PKB in a PI3-K-dependent manner. Importantly, Ang II-induced Akt/PKB phosphorylation is mediated by intracellular H$_2$O$_2$, indicating that Akt/PKB is part of a redox-sensitive signaling pathway. Our studies also demonstrate a previously unappreciated role for Akt/PKB in Ang II-induced hypertrophy of VSMCs.

Akt/PKB has been shown to be activated by various growth factors and by cellular stresses such as heat shock and hyperosmolarity (8, 13). Consistent with our findings, the Akt/PKB pathway can also be activated by G protein-coupled receptor agonists, including thrombin in human platelets (23), isoproterenol in rat epididymal fat cells (24), and fMet-Leu-Phe in human neutrophils (25). More recently, Murga et al. (26) reported that stimulation of M$_1$ or M$_2$ muscarinic receptors transfected into COS-7 cells induces Akt/PKB activation. The upstream signaling mechanisms responsible for Akt/PKB activation by these various agonists have not been fully elucidated.

We have previously shown that Ang II stimulates superoxide generation in VSMCs by activating an NADH/NADPH oxidase (21, 22). Superoxide is rapidly dismutated to H$_2$O$_2$, which may be the ROS that is most important in modulating biological responses (6). Indeed, Ang II-induced H$_2$O$_2$ formation is detectable as early as 1 min after agonist stimulation (4), suggesting that it may mediate subsequent early signaling events. Previous experiments with catalase-overexpressing cells have demonstrated that intracellularly produced H$_2$O$_2$ mediates activation of p38MAPK and the hypertrophic response induced by Ang II (4, 6), emphasizing the critical role of ROS as signaling molecules. In this study, we demonstrate that Ang II-induced Akt/PKB phosphorylation is significantly inhibited both by the NADH/NADPH oxidase inhibitor DPI and by overexpression of catalase (Figs. 5 and 6), suggesting that ROS act as potential signal transducers linking the AT$_1$ receptor to the Akt/PKB pathway in VSMCs. The redox sensitivity of Akt/PKB is further confirmed by the observation that exogenous H$_2$O$_2$ stimulates Akt/PKB phosphorylation (Fig. 1). A similar effect of H$_2$O$_2$ was found in COS-7 cells transfected with Akt/PKB, but the role of ROS in agonist-mediated Akt/PKB phosphorylation was not assessed (27).

Growing evidence suggests that PI3-K is involved in the activation of Akt/PKB by mitogens in various systems (28). PI3-K-independent mechanisms have also been documented; however, Akt/PKB activation by $\beta_2$-adrenergic receptor (24), cyclic AMP (29), and cellular stress such as heat shock and hyperosmolarity (13) are all mediated by pathways insensitive to PI3-K blockers. In this study, we found that PI3-K is a crucial upstream mediator for Ang II-induced Akt/PKB activation, because two structurally unrelated, specific PI3-K inhibitors, wortmannin and LY294002, dose-dependently blocked Akt/PKB phosphorylation (Fig. 4). These agents abrogated H$_2$O$_2$-induced Akt/PKB activation as well, suggesting that PI3-K may be involved in coupling Ang II-induced H$_2$O$_2$ forma-
tion to the Akt/PKB pathway. The differential sensitivity of Ang II- and H2O2-induced Akt/PKB activation to LY294002 presumably results from the involvement of additional signaling mechanisms stimulated by Ang II but not H2O2. This is supported by the fact that neither DPI nor catalase completely inhibited Ang II activation of Akt/PKB.

Although PI3-K appears to be both necessary and sufficient for Akt/PKB activation, our present data clearly indicate that Akt/PKB activation by Ang II is redox-sensitive. The molecular target of ROS involved in agonist-induced Akt/PKB phosphorylation remains to be defined. It has been reported that the PI3-K products phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate interact with the pleckstrin homology domain of Akt/PKB resulting in the translocation of Akt/PKB to the plasma membrane, where it is activated by phosphorylation on Thr308 and Ser473 (16). Although phosphatidylinositol-dependent kinase-1 (PDK1) has been shown to phosphorylate Thr308 (30), the kinase responsible for Ser473 phosphorylation has not been molecularly identified but is referred to as PDK2 (31). Either of these kinases could be redox-sensitive, or another upstream step might be sensitive to ROS, including PI3-K itself. In these kinases could be redox-sensitive, or another upstream

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