Angiotensin II regulates activation of Bim via Rb/E2F1 during apoptosis: involvement of interaction between AMPKβ1/2 and Cdk4

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Kim Y-C, Day RM. Angiotensin II regulates activation of Bim via Rb/E2F1 during apoptosis: involvement of interaction between AMPKβ1/2 and Cdk4. Am J Physiol Lung Cell Mol Physiol 303: L228–L238, 2012. First published June 1, 2012; doi:10.1152/ajplung.00087.2012.—Apoptotic cell death is essential for mammalian development and tissue homeostasis. Dysregulation of apoptosis has been identified in pathologies including in pulmonary fibrotic remodeling. We previously reported that a key proapoptotic factor in fibrosis, angiotensin II (Ang II), mediates apoptosis in primary pulmonary artery endothelial cells (PAEC) via the AT2 receptor and requires activation of AMP-activated protein kinase (AMPK). We now demonstrate that Ang II induces E2F1 transcription factor binding to and activation of the promoter for the Bcl-2 homology 3 (BH3)-only protein Bim. In PAEC, Ang II treatment induced cyclin-dependent kinase 4 (Cdk4)-mediated hyperphosphorylation of retinoblastoma protein (Rb) and its disassociation from E2F1, a key step in facilitating E2F1-directed transcriptional activity. Indeed, ectopic expression of a dominant negative Cdk4 mutant inhibited Ang II-mediated hyperphosphorylation of Rb and Bim promoter activation. Our data also show that the β-subunit of AMPK was constitutively associated with Cdk4 in PAEC and that Ang II treatment induced AMPKβ phosphorylation and subsequent disassociation of this complex. Both Ang II-induced Rb hyperphosphorylation and Cdk4-AMPK disassociation were blocked by the AMPK inhibitor compound C. Together these findings illuminate a novel proapoptotic signaling pathway in endothelial cells, whereby Ang II triggers E2F1-mediated transcriptional upregulation of Bim via activation of AMPKβ1/2 and Cdk4.

The peptide hormone angiotensin II (Ang II) is well known for its role in inducing vasoconstriction in vascular smooth muscle cells for the regulation of systemic blood pressure. However, more recent research has revealed that Ang II may induce cellular proliferation, growth arrest, or apoptosis, in a cell type- and tissue type-specific manner. The cellular responses to Ang II underlie a broad spectrum of normal and pathological biological functions of Ang II ranging from tissue homeostasis, fertility, inflammation, immune function, tissue repair, and tissue aging and degeneration (3, 31, 49, 52). Accumulating evidence indicates that receptor-mediated apoptotic signaling by Ang II is also involved in pulmonary fibrotic remodeling (41, 54).

Most of the biological activities of Ang II are mediated by two specific receptors: angiotensin type 1 (AT1) and type 2 (AT2), both of which belong to the seven transmembrane G protein-coupled receptor family (38, 44). Cellular responses to Ang II are receptor subtype and cell type specific. Either AT1 or AT2 activation can induce proliferation or apoptosis (33).

Activation of AT2 receptors inhibits cellular proliferation and induces apoptosis in a variety of cell types including epithelial and endothelial cells (22, 33, 51). The signaling pathways leading to apoptosis downstream of AT2 activation appear to vary with cell type (22, 33, 48).

A key step in the intrinsic apoptosis pathway is the permeabilization of the outer mitochondrial membrane for the release of cytochrome c to induce formation of the apoptosome and caspase 9 activation (58). The B cell lymphoma 2 (Bcl-2) proteins are critical regulators of mitochondrial membrane permeability and cytochrome c release. Bcl-2-associated X protein (Bax) and the Bcl-2 homologous antagonist killer (Bak) protein are essential proapoptotic members of the Bcl-2 family that oligomerize to form pores in the outer mitochondrial membrane (29, 61). Antiapoptotic members of the Bcl-2 family, including Bcl-2 and Bcl-xL, bind Bak and Bax and prevent their oligomerization (58). A third group of proteins of the Bcl-2 family, the Bcl-2 homology 3 (BH3)-only proteins, including Bim, Noxa, Puma, and Bad, function to release Bax and Bak from binding to the antiapoptotic Bcl-2 proteins and may activate Bax or Bak directly (11, 34, 61). Regulation of specific BH3-only proteins is cell type specific and apoptosis signal specific (21).

Our laboratory recently demonstrated that Ang II-induced intrinsic apoptosis in pulmonary artery endothelial cells (PAEC) is associated with the downregulation of the antiapoptotic Bcl-2 family protein Bcl-xL, and that Ang II-induced apoptosis was also blocked by inhibition of Bax (33). To further understand apoptosis signaling induced by Ang II, we examined the regulation of BH3-only proteins. We now demonstrate that Ang II regulates the association of AMPKβ1/2 with Cdk4, leading to the hyperphosphorylation of Rb and induction of E2F1-dependent Bim gene activation.

METHODS

Reagents and antibodies. Ang II was purchased from Bachem (Torrance, CA). Compound C [6-[4-(2-piperidin-1-ylethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]-pyrimidine], LY294002, wortmannin, and chelerythrine chloride were purchased from Sigma-Aldrich (St. Louis, MO). Cdk4 inhibitor II was purchased from Calbiochem (San Diego, CA). The AMPKβ1/2 (no. 4150), phospho-Ser108 AMPKβ (no. 4181), phospho-Ser473 Akt (no. 9271), Akt (no. 9272), Bim (no. 2933), and Rb (no. 9309) antibodies were from Cell Signaling Technology (Danvers, MA). Antibodies against anti-AMPKβ2 (sc-20164), Cdk4 (sc-260), GFP (sc-8334 and sc-9996), phospho-Ser780 Bax (sc-12901), Bax (sc-7480), Noxa (sc-30209), AT2 (sc-7420), E2F1 (sc-193), α-tubulin (sc-8035) and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-Cdk4 (MAB8879), anti-E2F1 (05–379) monoclonal antibody for chromatin immunoprecipitation (ChIP) assay, rabbit anti-AMPKβ1 (03–324) was purchased from Millipore (Billerica, MA). Anti-mouse hemagglutinin (HA) antibody was purchased from Roche Applied Science (Indianapo-
Anti-rabbit HA antibody was purchased from Bethyl Laboratory (Montgomery, TX). Anti-phospho-Ser795 Rb antibody was from Sigma-Aldrich (St. Louis, MO). Anti-phospho Ser826 Rb antibody was from US Biological (Swampscott, MA). Anti-phospho-Ser807/811 Rb (J112-906) and anti-Rb (G99-549 for immunoblotting) antibodies were from BD Biosciences (San Jose, CA). Cytochrome c and caspase-9 were from BioVision (Milpitas, CA) and MBL International (Woburn, MA), respectively.

Fig. 1. Bcl-2 homology 3 (BH3)-only Bim protein is involved in E2F1-mediated apoptosis signal. Pulmonary artery endothelial cells (PAEC) were transfected for 48 h and subsequently treated with angiotensin II (Ang II; 1 μM) for an additional 24 h prior to harvesting the cells. A: cells were transfected with empty vector or hemagglutinin (HA)-tagged wild-type E2F1 (E2F1). Total cell lysates were loaded for Western blots with the indicated antibodies against Bax, Noxa, and Bim antibodies. The membranes were stripped and reblotted with anti-HA or β-actin antibodies for loading control. Bar graph indicates normalized band densitometry compared with control. Data show means ± SD. *Significant differences, vs. respective control transfection with no Ang II, P < 0.05, n = 3. The normalized band density of untreated, empty vector-transfected cells was set at 1. The relative band densities of other treatment groups were quantified as ratios of that control. B: cells were transfected with empty vector, HA-tagged wild-type (wt) E2F1 (E2F1), or mutant E2F1 (E132) for 48 h. Total cell lysates were loaded for Western blots with the indicated antibodies against Bim and HA. The membranes were stripped and reblotted with anti-HA or β-actin antibodies for loading control. Bar graph indicates normalized band densitometry compared with control. Data show means ± SD. *Significant difference vs. empty vector control with no Ang II, P < 0.05; †significant difference from empty vector control with Ang II treatment, P < 0.05, n = 3. The normalized band density of untreated, empty vector-transfected cells was set at 1. The relative band densities of other treatment groups were quantified as ratios of that control. C: cells were transfected with empty vector, HA-tagged wt E2F1 (E2F1), or mutant E2F1 (E132) for 48 h, then subsequently treated with Ang II (1 μM) for an additional 24 h prior to harvesting the cells. Total cell lysates were loaded for Western blots with the indicated antibodies against cytochrome c and with caspase-9 antibodies. The membranes were stripped and reblotted with anti-HA or β-actin antibodies for loading control.
**Cell culture and transfection.** Bovine PAEC were cultured as reported previously (33) in RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS, Gemini Bioproducts, Woodland, CA) with penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified 5% CO₂ incubator at 37°C. Cos7 cells were grown in DMEM. The medium was supplemented with 10% FBS with penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified 5% CO₂ incubator at 37°C. GFP-Cdk4, HA-Cdk4, HA-wild type (wt) E2F1, and the HA-DNA binding deficient mutant of E2F1 [E2F1(E132)] were the gifts of Dr. S. G. Rane (National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD) (25). HA-AMPKβ1 and HA-AMPKβ2 was kindly provided by Dr. Y.-H. Feng (Department of Pharmacology, Uniformed Services University of the Health Sciences). A constitutively active form of AKT was provided from Dr. X. Zhou (Department of Medicine, Uniformed Services University of the Health Sciences).

The dominant-negative mutant of Cdk4 (DN-Cdk4) was kindly received from Dr. S. G. Rane. PAEC were grown to 70% confluence for transfection. Plasmids were transfected into PAEC and Cos7 cells by using FuGENE 6 according to the manufacturer’s recommendation (Roche Applied Science, Indianapolis, IN). After 48-h incubation, cell lysates were prepared for immunoblotting and coimmunoprecipitation assay. To test the effect of compound C or Cdk4 inhibitor, cells were pretreated for 20 min or 1 h, respectively, before harvesting of the cells.

**Immunoblotting and coimmunoprecipitation.** PAEC were grown to 80% confluence and were treated at indicated time points with Ang II and/or inhibitors. Immunoblotting (27) and coimmunoprecipitation (28) were performed as described previously.

**Luciferase assay.** To assess reporter gene expression, 0.2 μg/well of the Bin reporter gene plasmids (6) were introduced in PAEC in a 24-well plate by using FuGENE 6 transfection reagent according to the manufacturer’s instructions (Roche Applied Science). After a 6-h incubation, cells were untreated (control) or Ang II (10 μM) was added. To control for transfection efficiency, Renilla luciferase gene expression plasmid was cotransfected (pRL-TK, 150 ng/well). Luciferase and Renilla activities were measured 24 h after treatment by using a dual luciferase assay kit according to the manufacturer’s instructions (Promega, Madison, WI).

**ChIP assay.** The ChIP assays were performed according to the manufacturer’s recommendation (Active Motif, CA), by using protocols described previously (28) with anti-mouse E2F1 antibodies (Millipore, Billerica, MA) and normal mouse IgG (Santa Cruz Biotechnology). Primers were designed according to the Genomatix Software (Munich, Germany). The immunoprecipitated DNA was then used to detect the bovine Bin promoter by PCR, with the following primers: Forward, 5'-TGT ACC TCG GAG AAG TTC -3'; Reverse, 5'-AAG TCT GGG GAA GGT TTG GCA A-3'. The PCR conditions were 5 min at 94°C and then 1 min at 94°C, 30 s at 55°C, 30 s at 72°C for 40 cycles, followed by final extension was for 10 min at 72°C. The PCR products were analyzed on 2% agarose gels in TAE buffer.

**Statistical analysis.** Statistically significant differences between two groups were determined by the Student’s t-test. For three or more groups, statistical analysis was performed by one-way ANOVA, followed by the Bonferroni post hoc analysis, as appropriate. P values were calculated by means ± SD, with P < 0.05 considered to be a statistically significant difference.

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**Fig. 2.** E2F1 occupies the Bin gene promoter and regulates transcriptional activation of Bin gene. A: schematic representation of murine, human, and bovine Bin promoter containing putative E2F1 binding sites (top). Chromatin immunoprecipitation (ChIP) assay was performed with predicted bovine Bin gene promoter region. PAEC were treated with Ang II (1 μM) for 6 h (bottom). Input lane shows binding of E2F1 with Bin gene promoter as positive control. Normal mouse IgG was used for negative control. B: PAEC were transfected with empty vector (pGL-basic) or the p3.6-luciferase vector of murine Bin gene promoter (6). To control for the efficiency of transfection, Renilla luciferase gene expression was normalized by pRL-TK cotransfection. After 6-h incubation, cells were treated with or without Ang II (10 μM). Luciferase activity was analyzed 24 h later. Bar graphs show the relative induction of Bin promoter-driven luciferase activity normalized to Renilla luciferase activity. Data show means ± SD. *Significant differences, P < 0.05 vs. the respective plasmid control, n = 3. The normalized luciferase activity in untreated, empty vector-transfected cells was set at 1. The luciferase induction in other treatment groups was quantified as the ratio of this control. C: PAEC were transfected with empty vector, wt E2F1, or DNA binding mutant of E2F1 (E132), cotransfected with the p3.6 luciferase-linked murine Bin gene vector and the pRL-TK vector. Transfected cells treated with or without Ang II (10 μM). Bar graphs show the relative luciferase induction normalized to Renilla luciferase activity. Data show means ± SD. *Significant differences, P < 0.05 vs. control and n = 3. The normalized luciferase activity in control, untreated empty vector-transfected cells was set at 1. The luciferase induction in other treatment groups was quantified as the ratio of this control.
RESULTS

BH3-only protein Bim is regulated by E2F1 in Ang II-induced apoptosis. Our laboratory previously showed in PAEC that Ang II-induced apoptosis requires the downregulation of Bcl-xL protein, an antiapoptotic member of the Bcl-2 protein family (33). We were interested in determining the effects of Ang II on the expression of proapoptotic members of the Bcl-2 family of proteins. Using immunoblotting, we found that the BH3-only protein Bim was increased 2.5-fold in treatment with Ang II for 24 h in PAEC transfected with an empty vector (Fig. 1A). In contrast, the levels of two other BH3-only proteins, Bax and Noxa, were not changed (Fig. 1A). Previous studies showed that the E2F transcription factors, especially E2F1, upregulate the expression of BH3-only proteins for the induction of apoptosis in response to specific apoptotic signals (4, 18, 21). We investigated the potential role of E2F1 in Bim regulation. Overexpression of wt E2F1 caused an additional 2.5-fold increase in the level of Bim protein induced by Ang II for 24 h in PAEC compared with Ang II treatment with cotransfection of an empty vector (Fig. 1, A and B). In contrast, in PAEC transfected with an E2F1 DNA binding mutant (E132), Ang II-induced expression of Bim was significantly inhibited (Fig. 1B).

We next examined whether E2F1 overexpression enhances Ang II-induced apoptosis and whether mutant E2F1 (E132) attenuates it. In our previous study, we demonstrated that cytochrome c release and caspase-9 activity were activated by Ang II in PAEC (33). Therefore, we examined these same determinants in PAEC overexpressing either HA-E2F1 or E2F1(E132). Our data show that cytochrome c release and caspase-9 activation were significantly increased by Ang II in the presence of wt E2F1 but were blocked by the E132 mutant (Fig. 1C).

To further elucidate the regulatory role of E2F1 for Bim gene activation, we tested the binding of E2F1 to the bovine Bim promoter regions using ChIP assay. The region of the Bim promoter amplified for ChIP was selected based on the E2F1-binding region previously identified in the murine and human Bim promoter sequences and the predicted binding site for E2F1 (4). The sheared chromatin from PAEC treated with or without Ang II for 6 h was specifically pulled down with anti-E2F1 antibody for the detection of the Bim gene promoter. Low levels of E2F1 binding to the Bim promoter were detected in untreated PAEC (Fig. 2A, bottom). We observed twofold increased binding of E2F1 to the bovine Bim promoter region containing the predicted E2F1 binding site after treatment with Ang II compared with the untreated control (Fig. 2A, bottom).

We next investigated Ang II regulation of a Bim-luciferase reporter construct in PAEC using a 3.6-kb fragment of the Bim gene promoter containing the putative E2F1 binding sites (6). The control reporter construct, containing the pGL-basic promoter, was modestly increased 50% in the presence of Ang II (Fig. 2B). However, the activity of the 3.6-kb Bim promoter-luciferase construct was enhanced ~3.5-fold by Ang II treatment relative to the untreated control (Fig. 2B). We examined the effect of coexpression of the 3.6 kb of Bim-luciferase reporter plasmid with empty vector, wt E2F1, or an E2F1 DNA-binding mutant (E132) (15). In the presence of wt E2F1, the reporter activity was significantly increased compared with empty vector cotransfectants (Fig. 2C). In contrast, coexpression of the DNA binding mutant E2F1(E132) markedly blocked Ang II-induced expression (Fig. 2C). We observed some increase in basal level expression of the Bim promoter in the presence of E2F1(E132); this increase may be due to “derepression” effects of the mutant, releasing endogenous E2F1 from the Rb complex. Together, the results suggest that E2F1 is a target for Ang II-mediated transcriptional activation of Bim in PAEC.

Ang II-mediated hyperphosphorylation of Rb and Bim expression require Cdk4. Rb suppresses the transcriptional activity of E2F1 by directly binding E2F1s. E2F binding to Rb depends predominantly on the phosphorylation status of intact Rb, and hyperphosphorylation of Rb renders the release of E2Fs from Rb (2, 19, 56). Rb is directly phosphorylated by cyclin D or Cdk4/6, triggering dissociation of Rb from E2F1 (26). We investigated the effect of Ang II on Rb phosphorylation and the role of Cdk4 in this process. Ang II treatment for 4 h induced hyperphosphorylation of Rb at serines 780, 795, 807/811, and at threonine 826 in PAEC transfected with an empty control vector (~2- to 3-fold over untreated control for all sites examined, Fig. 3A). In the presence of wt Cdk4 overexpression, increased phosphorylation of Rb occurred in the absence of Ang II treatment and was further enhanced upon Ang II stimulation (Fig. 3A). In contrast, Ang II-induced Rb phosphorylation was inhibited to near basal levels with the overexpression of DN-Cdk4 (Fig. 3A).

We next examined the association of Rb and E2F1 and the effects of Ang II on this association. We determined that E2F1 coimmunoprecipitated with Rb (Fig. 3B, left) and that Rb reciprocally coimmunoprecipitated with E2F1 in control PAEC (Fig. 3B, right). Treatment with Ang II significantly reduced Rb-E2F1 association within 2 h in both immunoprecipitation experiments (Fig. 3B). Interestingly, the Rb-E2F1 interaction was completely restored after Ang II treatment for 24 h (data not shown). This suggests that transient activation of E2F1 is sufficient to induce Bim gene expression. To confirm the requirement of Cdk4 activation for Bim promoter activity downstream of Ang II, we examined the effect of wt Cdk4 or DN-Cdk4 overexpression on activation of the Bim-luciferase reporter. Whereas ectopic expression of wt Cdk4 increased the activation of the Bim promoter upon Ang II stimulation, overexpression of DN-Cdk4 blocked this effect (Fig. 3C). An

Ang II augments phosphorylation of AMPKβ1 and decreases the association of AMPKβ1/2 with Cdk4 in PAEC. Our laboratory previously demonstrated that Ang II induces the phosphorylation of AMPK in the kinase domain (T172) of the α-subunit (10). Phosphorylation at this site is associated with activation of kinase activity (53). We examined the effect of Ang II treatment on the phosphorylation of the AMPKβ1 subunit, which contains domains that interact with the AMPK α- and γ-subunits as well other proteins (40, 62). Western blot analysis showed dose-dependent Ang II-induced phosphorylation of AMPKβ1 on Ser108 within 2 h (Fig. 4A). We next investigated the association between AT2 and AMPKβ1/2. Our data indicate that AT2 and AMPKβ1/2 coimmunoprecipitate in PAEC (Fig. 4B). This association was slightly reduced following Ang II treatment for 2 h, but this was not statistically significant (Fig. 4B). We previously demonstrated that Ang II-induced loss of the antiapoptotic protein Bcl-xL (33). Others have shown that antiapoptotic Bcl-2 family members can block the proapoptotic activity of BH3 family members (1). We therefore investigated whether there is regulatory cross talk...
between Bcl-xL and Bim regulation. As shown in Fig. 4C, inhibition of Bcl-xL with chelerythrine chloride for 1 h greatly enhanced Bim activation by Ang II at 24 h. This suggests that apoptotic process by Ang II may be reciprocally determined through the balance between endogenous Bcl-xL and Bim.

Previous studies demonstrated that AMPK can phosphorylate Rb (8) and that AMPK can mediate Bim gene activation for apoptosis (7). However, others have shown that Cdk4 is a primary regulator of Rb/E2F1 (59). Thus we investigated whether a direct interaction exists between AMPK and Cdk4 for apoptosis signaling by Ang II. Immunoprecipitation with anti-AMPKβ1/2 or anti-Cdk4 antibodies resulted in the coprecipitation of endogenous Cdk4 and AMPKβ1/2 in PAEC, respectively (Fig. 5, A and B). We also performed coimmuno-
precipitation using ectopic overexpression of AMPKβ1/2 and Cdk4 in Cos7 cells. Consistent with Fig. 5, A and B, the binding was also detected in the Cos7 cells exogenously overexpressing Cdk4 and AMPKβ1/2 (Fig. 5C).

To understand the role of Ang II in regulating the AMPKβ1/2-Cdk4 interaction in PAEC, we investigated changes in this association in the presence of Ang II or pharmacological kinase inhibitors of Cdk4 or AMPK. Treatment with Ang II for 2 h resulted in a decrease of AMPKβ1/2-Cdk4 association levels in PAEC (Fig. 5D). Interestingly, we observed that compound C blocked the Ang II-mediated dissociation of the AMPKβ1/2-Cdk4 interaction, whereas the presence of Cdk4 inhibitor did not (Fig. 5D), suggesting that Ang II-induced uncoupling of AMPKβ1/2 and Cdk4 in PAEC is dependent on AMPK activity, but not on Cdk4 activation. In addition, compound C significantly blocked Ang II-induced phosphorylation of Rb at all phosphorylation sites (Fig. 5E), confirming AMPK is required for Ang II-induced Rb phosphorylation.

Ang II treatment suppresses AKT phosphorylation in PAEC. In studies of apoptosis in neurons, phosphatidylinositol 3-kinase (PI3K)/AKT signaling was demonstrated to suppress E2F1-mediated apoptosis, inhibit BH3-only protein expression, and promote cell survival (16). We investigated whether the PI3K/AKT pathway is active during Ang II-induced apoptosis in PAEC. Western blots showed that Ang II dramatically reduced phosphorylation of AKT at Ser473 within 1 min (Fig. 6A). To address the functional role of AKT in apoptosis signaling in PAEC, we tested Ang II-mediated Bim gene regulation by the coexpression of wt E2F1, with or without a control vector or constitutive active form of AKT (CA AKT).

Our data showed that reporter gene expression was increased ~2.5-fold by Ang II treatment in the presence of E2F1. In
contrast, the Ang II-induced Bim gene activation and E2F1 Bim gene activation were remarkably suppressed by the overexpression of CA AKT (Fig. 6B). Consistent with the observation, pretreatment with PI3K inhibitors, LY 294002 or wortmannin, slightly increased the Ang II-induced activation of Bim gene with overexpression of E2F1 (Fig. 6C). Taken together, the data suggest that AKT negatively regulates Ang II-mediated transcriptional activation of Bim in PAEC for apoptosis.

**DISCUSSION**

Ang II-induced apoptosis occurs in both normal and pathological processes (30, 54). In the present study, we demonstrate that Ang II activation of AMPK leads to transcriptional regulation of the proapoptotic BH3-only protein Bim in PAEC. Ang II-induced phosphorylation of AMPK β1/2 causes dissociation of AMPK from Cdk4, upstream of Cdk4 hyperphosphorylation of Rb and its subsequent dissociation from E2F1.
Fig. 5. AMPKβ1/2 association with Cdk4 is regulated by Ang II. A: endogenous Cdk4 proteins from PAEC were immunoprecipitated with anti-Cdk4 antibodies (MAB8879, Millipore) followed by immunoblotting with antibodies to AMPKβ1/2 (no. 4150, Cell Signaling Technology). Membranes were stripped and rebotted with anti-Cdk4 (sc-260, Santa Cruz Biotechnology) as a loading control. Normal IgG antibodies were used for immunoprecipitation as a negative control. Input lysates serve as a positive control for AMPKβ1/2 detection in PAEC. B: protein lysates from PAEC were immunoprecipitated with anti-AMPKβ1 or AMPKβ2 antibodies followed by immunoblotting with anti-Cdk4 antibodies to detect endogenous AMPKβ1/2-Cdk4 complexes. The membranes were stripped and rebotted with anti-AMPKβ1/2 for loading control. C: protein lysates were prepared from Cos7 cells mock transfected (mock) or overexpressing either empty vector (empty) or GFP-Cdk4 with HA-AMPKβ1 or HA-AMPKβ2. Whole cell lysates were immunoprecipitated with anti-rabbit GFP antibodies (sc-8334, Santa Cruz Biotechnology) followed by immunoblotting with anti-rabbit HA-antibody. GFP antibodies were used for blotting as a control for expression. D: PAEC were pretreated with Cdk4 inhibitor (0.4 μM) for 1 h or with compound C (40 μM) for 20 min prior to treatment with Ang II (1 μM) for 2 h followed by preparation of whole cell lysates. Immunoblot analysis was performed to detect AMPKβ1/2-Cdk4 interaction. Protein lysates were immunoprecipitated with an anti-Cdk4 antibody (MAB8879, Millipore) followed by Western blots with AMPKβ1/2 antibodies (no. 4150, Cell Signaling Technology). Input lane shows total AKT as a loading control (bottom). The membrane was stripped and rebotted with total AKT as a loading control (bottom). Bar graph indicates normalized band densitometry compared with control. Data show means ± SD. *Significant differences, P < 0.05 vs. control, n = 3.

Fig. 6. Ang II reduces phosphorylation of AKT and AKT negatively regulates transcriptional activation of Bim gene. A: phosphorylation level of AKT at Ser473 and total expression of AKT in PAEC. PAEC were treated with the indicated times of 10 μM Ang II. Total cell lysates were prepared and equal amounts of protein were loaded for Western blotting for phosphorylated AKT (p-AKT-ser473, top). The membrane was stripped and rebotted with total AKT as a loading control (bottom). Bar graph indicates normalized band densitometry compared with control. Data show means ± SD. *Significant differences, P < 0.05 vs. control, n = 3. B: PAEC were transfected with empty vector, wt E2F1, or constitutively activated form of AKT (CA AKT), cotransfected with the p3.6 luciferase-linked murine Bim gene vector and the pRL-TK vector. Transfected cells treated with or without Ang II (10 μM). Bar graphs show the relative luciferase induction normalized to Renilla luciferase activity. Data show means ± SD. *Significant differences, P < 0.05 vs. control and n = 3. The normalized luciferase activity in control, untreated cells was set at 1. The luciferase induction in other treatment groups was quantified as the ratio to controls. C: PAEC were transfected with empty vector, or wt E2F1, cotransfected with the p3.6 lucerase-linked murine Bim gene vector and the pRL-TK vector. After 6-h incubation, cells were pretreated with PI3K/AKT inhibitors LY294002 (5 μM) or wortmannin (1 μM) for 1 h prior to treatment with Ang II (10 μM). Bar graphs show the relative lucerase induction normalized to Renilla luciferase activity. Data show means ± SD. *Significant differences, P < 0.05 vs. control and n = 3. The normalized luciferase activity in control, untreated cells was set at 1. The luciferase induction in other treatment groups was quantified as the ratio to controls.
thus allowing E2F1-mediated increased expression of Bim (Fig. 7). We previously demonstrated that Ang II activation of AMPK is required for the downregulation of Bcl-xL in PAEC via SHP-2 activation, nucleolin dephosphorylation, and destabilization of Bcl-xL mRNA (33). Together our findings suggest that, in Ang II-induced intrinsic apoptosis, AMPK can coordinate signaling to regulate the levels of both pro- and antiapoptotic members of the Bcl-2 family of proteins through independent mechanisms.

AMPK, a heterotrimeric kinase composed of an α-catalytic subunit and regulatory β- and γ-subunits, is regulated by the cellular energy balance and can be activated either by high consumption of ATP or by an increased AMP/ATP ratio (20, 47). AMPK activity has been studied primarily with regard to maintenance of cellular metabolic homeostasis, but recent studies have demonstrated that AMPK signaling can also regulate cell growth, apoptosis, mitochondrial biogenesis, autophagy, polarity, and gene transcription (37). Our laboratory recently demonstrated that Ang II-induced AT2 receptor-dependent phosphorylation of the α-subunit mediates activation of AMPK for energy-dependent and -independent signaling for apoptosis (10). We now demonstrate that Ang II also induces phosphorylation of AMPKβ1 at Ser108 in PAEC. The β-subunit of AMPK has been demonstrated to act as scaffolding partner for the AMPK heterotrimer. We hypothesize that the interaction AT2 with AMPKβ subunit can bring the other two subunits (α and γ) of the heterotrimers into proximity with the receptor. It is possible that an adaptor protein such as Grb2, or another kinase, such as Cdk4, may participate in the coupling of AT2 with AMPK for downstream signaling (14, 45). The AT2 and AMPK domains important for this protein-protein interaction are currently under investigation.

Our data also demonstrate for the first time that AMPK directly interacts with the cyclin-dependent kinase Cdk4 for apoptotic signaling. Although Cdk4 has been primarily studied for its role in cell growth and proliferation via cell cycle regulation, Cdk4 can have antiproliferative effects, activating apoptosis and senescence (9, 16). Others have shown that Cdk4 is necessary for neuronal apoptosis in vivo and in vitro (24, 46). In response to specific apoptotic stimuli, Cdk4 activity leads to the loss of repression of proapoptotic genes through the release of E2Fs from Rb complexes (5, 26, 39, 46, 55). Several studies showed that the proapoptotic activity of Cdk4 in neurons required the suppression of PI3K/Akt activity (16, 17). Phosphorylation and activation of AKT generally promote cell survival owing to AKT inactivation of proapoptotic effectors such as Bad and Bim (12, 50). AKT phosphorylation also induces the interaction of TopBP1 with E2F1, which suppresses E2F1-mediated apoptosis (36). Consistent with these studies, our data indicate that Ang II suppresses basal AKT activity during proapoptotic Cdk4 activation in primary endothelial cells. Furthermore, our data demonstrate that inhibition of PI3K/AKT enhances Ang II activation of the Bim promoter. We interpret our data to indicate that the AKT/PI3K signaling pathway results in increased cell survival decisions that block apoptotic signaling by Ang II (16).

The E2F transcription factors function in the regulation of genes for cell proliferation, differentiation, and apoptosis. In response to proliferative signaling, E2F activation regulates cell cycle genes to advance the cell to S phase entry for DNA synthesis, but in apoptotic signaling E2F activates gene expression of the BH3-only proteins (23). Our data indicate that overexpression of E2F1 was not sufficient to activate the Bim promoter in our luciferase reporter construct. There are several potential reasons that E2F1 is not sufficient for the activation of the Bim promoter: 1) epigenetic events such as acetylation of E2F1 have been demonstrated to modulate E2F1-mediated apoptotic target gene regulation (63); 2) E2Fs may associate with endogenous corepressors such as KAP1 or TopBP1, which suppress E2F proapoptotic activity (36, 60); 3) activation of coactivators, such as p300, modulate the apoptotic activity of E2F1 (32, 43). It is possible that a combination of...

Fig. 7. Proposed models of Ang II-induced apoptosis signaling in PAEC. Schematic of the physiological AMPKβ1/2-Cdk4 complex for apoptotic signaling upon Ang II stimulation. Ang II binds to the G protein-coupled receptor (GPCR) AT2 receptor to transduce downstream signaling pathway for cell growth/differentiation, inflammation, and apoptosis (44). In untreated cells, AMPKβ1/2 is associated with Cdk4. Upon treatment with Ang II treatment, Cdk4 is dissociated from AMPKβ1/2, coincidentally with AMPKβ1 phosphorylation. Cdk4-mediated hyperphosphorylation of Rb renders release of E2F1 from Rb. Free E2F1 binds to the promoter region of the proapoptotic Bim protein for transcriptional activation. At the same time, Ang II treatment suppresses antiapoptotic signaling by the Akt pathway by suppressing basal Akt phosphorylation. MOMP, mitochondrial outer membrane permeabilization.
E2F1 regulatory events prevent the activation of proapoptotic gene transcription in the absence of Ang II signal transduction. Consistent with previous reports (4, 21), our study indicates that Bim protein is a direct target of E2F1-dependent apoptosis signaling. Interestingly, we did not detect the regulation of other BH3-only protein family members such as Noxa or Bax by Ang II. Findings in the literature suggest that BH3-only proteins may be differentially regulated in a cell type-specific and signaling-specific manner (13, 42, 57). For instance, Bim is a key regulator in apoptosis mediated by IL-7 cytokine deprivation (35). Our present data suggest that regulation of Bim is specific in Ang II-induced intrinsic apoptosis in primary endothelial cells.

In conclusion, we have demonstrated that a functional interaction between AMPKβ1/2 and Cdk4 for Ang II-induced intrinsic apoptotic signaling, which is required for Cdk4-induced phosphorylation of Rb and activation of the E2F1 transcription factor. This Cdk4/Rb/E2F1 signaling pathway directly targets enhanced transcription of the proapoptotic protein Bim. Because of its role in normal and pathological processes, understanding of the signaling pathways activated in Ang II-mediated apoptosis may lead to the development of therapeutic targets for the protection of endothelial cells in pathogenic processes such as fibrotic remodeling.

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DISCLOSURES
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ANG II-INDUCED E2F1 REGULATES BIM IN APOPTOSIS