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Article

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A novel role for the cyclin-dependent kinase inhibitor p27^{Kip1} in angiotensin II–stimulated vascular smooth muscle cell hypertrophy

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Angiotensin II (Ang II) has been shown to stimulate either hypertrophy or hyperplasia. We postulated that the differential response of vascular smooth muscle cells (VSMCs) to Ang II is mediated by the cyclin-dependent kinase (Cdk) inhibitor p27^{Kip1}, which is abundant in quiescent cells and drops after serum stimulation. Ang II treatment (100 nM) of quiescent VSMCs led to upregulation of the cell-cycle regulatory proteins cyclin D1, Cdk2, proliferating cell nuclear antigen, and Cdk1. p27^{Kip1} levels, however, remained high, and the activation of the G1-phase Cdk2 was inhibited as the cells underwent hypertrophy. Overexpression of p27^{Kip1} cDNA inhibited serum-stimulated [^{3}H]thymidine incorporation compared with control-transfected cells. This cell-cycle inhibition was associated with cellular hypertrophy, as reflected by an increase in the [^{3}H]leucine/[^{3}H]thymidine incorporation ratio and by an increase in forward-angle light scatter during flow cytometry at 48 hours after transfection. The role of p27^{Kip1} in modulating the hypertrophic response of VSMCs to Ang II was further tested by antisense oligodeoxynucleotide (ODN) inhibition of p27^{Kip1} expression. Ang II stimulated an increase in [^{3}H]thymidine incorporation and the percentage of S-phase cells in antisense ODN–transfected cells but not in control ODN–transfected cells. We conclude that p27^{Kip1} plays a role in mediating VSMC hypertrophy. Ang II stimulation of quiescent cells in which p27^{Kip1} levels are high results in hypertrophy but promotes hyperplasia when levels of p27^{Kip1} are low, as in the presence of other growth factors.

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Introduction

Angiotensin II (Ang II) is known to stimulate vascular smooth muscle cell (VSMC) growth (1–3). This effect is associated with increased expression of the proto-oncogenes c-*fos*, c-*myc*, and c-*jun* and autocrine growth factors PDGF-AA and bFGF (4, 5). Yet, despite activation of these proto-oncogenes and mitogenes, Ang II does not induce hyperplasia but, rather, causes hypertrophy of confluent quiescent VSMCs in serum-free media. The molecular determination of cellular hypertrophy versus hyperplasia could be mediated at the level of the molecular machinery regulating cell-cycle progression (6).

Cell-cycle entry and progression, the final common pathway of cell-growth response, depends on the carefully regulated expression and activation of certain enzymes, termed cyclin-dependent kinases (Cdk's), and their regulatory subunits, the cyclins (6, 7). For G1-phase, D-type cyclins (D1, D2, D3), cyclin E, and cyclin A play an important role (8). D-type cyclins complex with Cdk4/Cdk6 and regulate G1-phase progression; cyclin E/Cdk2 or cyclin A/Cdk2 is essential for the G1/S transition; and cyclin A/Cdk1 or cyclin B/Cdk1 (Cdc2) initiates mitosis (9). Proliferating cell nuclear antigen (PCNA) also associates with the G1-phase cyclin/Cdk complexes and stimulates the processivity of DNA-polymerase-δ (10). Cdk's are activated through phosphorylation and dephosphorylation at specific sites, and this activation is controlled by Cdk inhibitors that bind to and inhibit the activation of the Cdk/cyclin complexes (11).

We postulated that in Ang II–induced VSMC hypertrophy, cell-cycle entry takes place, but progression through cell-cycle toward DNA synthesis and mitosis is blocked. The recently discovered Cdk inhibitor p27^{Kip1} has been shown to play an important role in cell-cycle regulation. High levels of p27^{Kip1}, present in quiescent (G0) cells, have been shown to decline upon mitogen induction (11). This decrease in p27^{Kip1} appears to be critical in enabling the cells to enter the cell cycle. In this study, we explored the role of p27^{Kip1} and other cell-cycle proteins in mediating Ang II–induced VSMC hypertrophy. We also compared the influence of hypertrophic Ang II stimulation to hyperplastic stimulation with serum or PDGF on VSMC p27^{Kip1} levels and on Cdk/cyclin activation. Finally, we studied the effect of inhibition of p27^{Kip1} expression in Ang II–treated cells using antisense oligodeoxynucleotides (ODNs), and of overexpression of p27^{Kip1} in mitogen-stimulated VSMCs by gene transfer, to test our hypothesis that the response of VSMCs to Ang II stimulation is determined, at least in part, by the intracellular activity of this Cdk inhibitor.

Methods

Materials. The following primary antibodies were used in the study: mouse monoclonal anti-Cdk1, mouse monoclonal anti-PCNA (PC10), rabbit polyclonal anti-Cdk2 (M2), mouse monoclonal anti-$p27^{Kip1}$ (F8), mouse monoclonal anti-cyclin D1 (HD11), and rabbit polyclonal anti-Cdk4 (C-22) (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA).

Cell culture. Primary cultures of VSMCs were initiated by enzymatic dissociation from the aortae of 7- to 8-week-old male Sprague-Dawley rats (Charles River Breeding Laboratories, Kingston, New York, USA) by the method of Owens et al. (12). VSMCs were maintained in a 1:1 mixture of DMEM and Ham's F12 (DMEM/F12; GIBCO BRL, Rockville, Maryland, USA), 10% heat-inactivated FBS (GIBCO BRL), penicillin (100 U/mL), streptomycin (100 mg/mL), 25 mM HEPES buffer, glutamine, and glucose. Cells were incubated at 37°C in a humidified atmosphere of 95% O_2/5% CO_2. Using this technique, VSMCs exhibit typical, spindle-shaped morphology and a multilayered hill-and-valley growth pattern. Expression of α-actin was demonstrated by immunohistochemical staining with a smooth muscle–specific anti–α-actin antibody (Sigma Chemical Co., St. Louis, Missouri, USA). Studies were conducted on subconfluent VSMCs (passages 7–12) or after achieving confluence for 2 days in 10% FBS/DMEM/F12, followed by serum withdrawal for 2 days to induce quiescence. Ang II (Sigma Chemical Co.) and PDGF-BB (R&D Systems Inc., Minneapolis, Minnesota, USA) were used at a concentration of 100 nM and 20 ng/mL, respectively.

Transfection procedures. We overexpressed a plasmid containing the human $p27^{Kip1}$ cDNA (pACCMVplpa vector; kindly donated by P.D. Nisen, University of Texas Southwestern Medical Center, Dallas, Texas, USA) in VSMCs by transfection, using the lipid formulation Fugene (Boehringer Mannheim Biochemicals Inc., Indianapolis, Indiana, USA). Plasmid DNA was purified using the QIAGEN Anion-Exchange Resin (QIAGEN Inc., Valencia, California, USA) according to the manufacturer's instructions. For a 35-mm dish, 6 μL liposomes was added to 100 μL Opti-MEM (GIBCO BRL) and mixed with 2 μg DNA. After an incubation period of 15 minutes, the mixture was carefully added to the cells. The medium was changed 24 hours later and left until the cells were harvested. These levels of DNA and liposomes proved to yield a 30–40% transfection efficiency when cells were transfected with a cytomegalovirus (CMV) promoter–driven control reporter plasmid.

For $p27^{Kip1}$ antisense ODN experiments, phosphorothioate oligonucleotides were modified by the addition of a propynyl group to the pyrimidine base cytidine, which is thought to enhance base stacking and facilitate the sense-antisense interaction (13). The antisense ODN sequence used in the experiments was 5′-CACTCTCACGTTTGACAT-3′ (targets bp 1–18 of rat $p27^{Kip1}$); reverse ODN sequence was 5′-TACAGTTTGCACTCTCAC-3′; and sense ODN sequence was 5′-ATGTCAAACGTGAGAGTG. For the lipofection procedure, 20 nM ODN was mixed with GS2888 cytofectin (kindly donated by M. Flanagan, Gilead Sciences, Foster City, California, USA) (14) in serum-free medium (Opti-MEM) and incubated for 10 minutes. VSMCs were rinsed once in serum-free medium and refed with ODN-cytofectin solution in medium containing 0.2% serum. Cells were then incubated for 24 hours and harvested by trypsinization for FACS® analysis, Western blot, and RT-PCR, or were pulsed with tritiated thymidine or leucine to determine DNA and protein synthesis, respectively. FITC-labeled ODN showed a nuclear uptake in up to 90% of cells using this technique.

Metabolic labeling studies and determination of cell number. DNA synthesis was assessed by administering [^{3}H]thymidine (2 μCi/mL, specific activity 70–90 Ci/mmol; NEN Life Science Products, Boston, Massachusetts, USA) for a period of 2 hours before the cells were harvested. For measurement of protein synthesis, the cells were incubated the same way with [^{3}H]leucine. At the end of the incubation period, the medium was removed, the cells were washed twice with PBS, then washed once with 10% TCA, followed by incubation with 10% TCA for 30 minutes at 4°C. The TCA insoluble material was washed twice with ethanol, solubilized in 0.25 M NaOH, and then buffered to neutral pH. [^{3}H]thymidine and leucine incorporation was determined by liquid scintillation spectrometry.

For determination of cell number, the cells were trypsinized, resuspended in PBS, and counted with a Coulter Counter ZN (Coulter Electronics Ltd., Hialeah, Florida, USA).

Flow cytometry. For measurement of cellular DNA and forward-angle light scatter (FALS), cells were harvested by trypsinization, fixed overnight with 75% methanol, washed, and incubated with 100 μg/mL RNase (Oncogene Research Products, Cambridge, Massachusetts, USA) and 10 μg/mL propidium iodide (PI) in PBS for 1 hour at 37°C. Samples were analyzed using standard methods on a Coulter Epics XL-MCL flow cytometer. Data were computer analyzed with Multiple Option Cell Cycle Fitting software (version 2.50; Phoenix Flow Systems Inc., San Diego, California, USA).

When cells were analyzed for $p27^{Kip1}$, fixed VSMCs were washed once with PBS and incubated for 1 hour at 37°C in PBS containing 100 μg/mL RNase, 10 μg/mL PI, 3% FBS, and 5 μg/mL mouse anti-$p27^{Kip1}$ antibody (mouse nonimmune IgG was used as control). After 2 washes in PI-PBS, a secondary FITC-labeled goat anti-mouse antibody in PI-PBS was applied (1:1,000; Caltag Laboratories Inc., Burlingame, California, USA) for 1 hour at 37°C. After another 2 washes, the cells were resuspended in 500 μL PI-PBS and analyzed for DNA content (PI) and FALS of the FITC ($p27^{Kip1}$)–positive cells. Cells expressing the green fluorescent protein (GFP) were processed as already described and were directly analyzed for DNA content and FALS.

Preparation of cellular lysates and Western immunoblot analysis. Cells were rinsed with PBS and lysed in ice-cold RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/mL PMSF, 30 μg/mL aprotinin, 1 mol/L sodium orthovanadate). Lysates were centrifuged at 4°C for 15 minutes in a microfuge at maximum speed, and the supernatant was taken for Western immunoblot analysis. Thirty micrograms of protein was separated on denaturing SDS/10% polyacrylamide gels and then blotted onto nitrocellulose (Hybond-ECL; Amersham Life Sciences Inc., Arlington

Heights, Illinois, USA) by wet electroblotting. Blots were blocked overnight at 4°C with 5% nonfat dry milk in TBS-T (20 mmol/L Tris-HCl [pH 7.6], 137 mM NaCl, 0.1% Tween-20) and then incubated with the first antibody (dilution 1:200 for anti-PCNA, cyclin D1, Cdk1, Cdk2, $p27^{Kip1}$) for 1 hour at room temperature. After washing in TBS-T, the blots were incubated with the second antibody (horseradish peroxidase–conjugated goat anti-mouse [cyclin D1, Cdk1, PCNA] or anti-rabbit immunoglobulin [Cdk2, $p27^{Kip1}$]; 1:3,000). Specific proteins were detected by enhanced chemiluminescence (ECL; Amersham Life Sciences Inc.) according to the manufacturer's instructions.

For immunoprecipitation, cells were rinsed with PBS and lysed in 500 μL immunoprecipitation buffer (IP-buffer) (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 100 μg/mL phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 30 μL/mL aprotinin). After incubation on ice for 20 minutes, the cellular debris were pelleted by centrifugation, and the supernatant was transferred to a new tube. One milligram of antibody (anti-Cdk1 or anti-Cdk2) was incubated with 150 μg of lysate for 4 hours at 4°C, after which 20 μL of 50% protein A/G-agarose (Oncogene Research Products) was added and incubation was continued for another 2 hours. Precipitates were then washed 4 times with IP buffer; the recovered pellets were suspended in SDS-electrophoresis buffer and boiled for 5 minutes; and the protein was separated on denaturing SDS/10% polyacrylamide gels. Blotting onto nitrocellulose and immunodetection was performed as already described here.

Histone H1 kinase assays. VSMCs were washed with PBS and lysed for 30 minutes at 4°C in RIPA buffer. Lysates were microcentrifuged for 15 minutes, and 150 μg of protein was incubated for 4 hours at 4°C with 1 μg of anti-Cdk1 or anti-Cdk2 antibody with rocking. Twenty microliters of 50% protein A/G-agarose (Oncogene Research Products) was added, and incubation was continued for 2 hours more. Immune complexes, bound to the beds, were washed twice in buffer A (20 mM Tris-HCl [pH 8], 250 mM NaCl, 1 mM EDTA, 0.5% NP-40), twice in the same buffer containing 100 mM NaCl, and then once in kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT). The kinase activity in the immunoprecipitates was assayed by addition of kinase buffer, 5 μg of histone H1 (Boehringer Mannheim Biochemicals Inc.), 1 μM ATP, and 5 μCi [γ-^{32}P]ATP (3,000 Ci/mmol; NEN Life Science Products Inc.) to a final volume of 50 μL, and was incubated for 30 minutes at 30°C. The samples were then boiled for 5 minutes, electrophoresed through a 12% SDS-polyacrylamide gel, dried, and exposed to x-ray film. Quantification was done densitometrically using the BioDoc II and the ScanPack 3.0 software (both from Biometra, Goettingen, Germany).

RT-PCR. RNA was extracted from VSMCs using the Ultraspec RNA Isolation System (Biotecx Laboratories Inc., Houston, Texas, USA). RT was carried out by incubation of 0.25 μg of total RNA in a reaction buffer containing 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 μM of each oligonucleotide, 10 U/mL RNase inhibitor (Perkin-Elmer Corp., Norwalk, Connecticut, USA), 2.5 μM random hexamers, and 26 U of avian myeloblastosis virus RT (GIBCO BRL) for 1 hour at 42°C. Specific primers were directly added to the RT-reaction product, and PCR was carried out with 2.5 U *Taq* polymerase (Perkin-Elmer Corp.) in a total volume of 50 μL. Thirty-two cycles were used, with cycle times of 94°C for 1 minute, 54°C for 1 minute, and 72°C for 1 minute. Twenty milliliters of the PCR product was electrophoresed in a 1.6% agarose gel and viewed with ethidium bromide. The following oligonucleotide primers were used: $p27^{Kip1}$, 5′-CGTGAGAGTGTCTAACGGGAG-3′ (sense), 5′-TCTTCTGTTCTGTTGGCCCT-3′ (antisense); GAPDH, 5′-AGACAGCCGCATCTTCTTGT (sense), 5′-CCACAGTCTTCTGAGTGGCA (antisense).The resultant PCR products were 594 bp ($p27^{Kip1}$) and 607 bp (GAPDH).

Quantitative analysis of apoptosis by fluorescence microscopy. Fluorescent DNA-binding dyes were used to define nuclear chromatin morphology as a quantitative index of apoptosis. Cells to be analyzed were stained with HOECHST 33342 (5 μg/mL) and were added to the culture medium for 20 minutes at 37°C. The media and the PBS rinses were collected, and the cells were trypsinized. Media, PBS, and trypsinized cells were pooled and collected by centrifugation, 5 minutes at 4°C. Cell pellets were resuspended in a small volume (50 μL) of serum-containing medium with 1 μg/mL HOECHST 33342 and 5 μg/mL PI. An aliquot (25 μL) was placed on a glass slide, covered with a glass coverslip, and viewed under fluorescence microscopy. Individual nuclei were viewed at ×400 to distinguish the normal uniform nuclear pattern from the characteristic condensed, coalesced chromatin pattern of apoptotic cells.

Statistical analysis. Data are given as mean ± SEM. Statistical analysis was performed by ANOVA. Post-test multiple comparison was performed by the method of Bonferroni. All experiments, including the immunoblots, were independently repeated at least 3 times.

Results

Ang II induces cell-cycle entry but not further progression of VSMCs. Confluent, quiescent VSMCs underwent hypertrophy when stimulated with Ang II (100 nM) in the absence of serum. Figure 1a shows that [^{3}H]leucine incorporation, a measure of protein synthesis, increased by 52% after 24 hours and by 85% after 48 hours of Ang II stimulation, in comparison with cells held serum-free. [^{3}H]thymidine incorporation, an indicator of DNA synthesis, increased only by 14% after 24 hours and by 20% after 48 hours. As a consequence, the ratio of [^{3}H]leucine/[^{3}H]thymidine incorporation, an indicator of cellular hypertrophy, increased from 1.15 in quiescent control cells to 1.53 after 24 hours and to 1.78 after 48 hours of Ang II stimulation. In parallel experiments, mitogenic stimulation with 10% FBS resulted in a decrease of this ratio to 0.5; cell number, which did not change during Ang II treatment, doubled within 48 hours (data not shown). The hypertrophic response of VSMCs to Ang II was demonstrated further by a rightward shift of the FALS, a relative measure of cell size on flow cytometry (Figure 1b).

Cell-cycle entry was documented via increases in the protein levels of critical cell-cycle proteins, as measured by immunoblot. Cyclin D1, PCNA, and Cdk2 were cho-

Table 1
Analysis of apoptotic nuclei after $p27^{Kip1}$ and GFP overexpression

	Apoptotic nuclei ± SEM (%)		
	Untransfected	GFP	$p27^{kip1}$
24 hours	0.6 ± 0.35	3.4 ± 5.8*	1.9 ± 0.25*
48 hours	0.2 ± 0.10	8.2 ± 0.29*	3.6 ± 0.58*
72 hours	1.5 ± 0.43	4.4 ± 0.20*	4.5 ± 0.20*

$P < 0.05$ vs. untransfected.

sen because they are important for G1-phase progression (8). In addition, we measured Cdk1 (Cdc2) because the protein level rises in G1 phase, although it does not gain kinase activity until complex formation with cyclin B in G2 phase (9). Figure 2a demonstrates that during Ang II treatment of quiescent VSMCs, cyclin D1, PCNA, Cdk2, and Cdk1 protein accumulated as it did during FBS stimulation. However, treatment with 10% FBS seemed slightly more potent in inducing these cell-cycle proteins. No effect was seen on the protein levels of Cdk4, which do not change during mitogenic stimulation; it was, therefore, used as control.

In contrast to serum, however, Ang II failed to activate either Cdk2 or Cdk1, as demonstrated by histone H1 kinase assay with subsequent viewing on a polyacrylamide gel. A quantification of the kinase activity is shown for the 24-hour time point in Figure 2b. The lack of Cdk2 activation correlated with the failure of Ang II–treated cells to progress significantly into S phase, as indicated by its inability to induce [^{3}H]thymidine incorporation. Because cells did not reach G2 phase, Cdk1 did not gain kinase activity. We further explored the role of the Cdk inhibitor $p27^{Kip1}$ in this inhibition of cell-cycle progression after Ang II stimulation. A high level of $p27^{Kip1}$ protein was measured by immunoblot in quiescent, confluent VSMCs (Figure 3a). Although $p27^{Kip1}$ level dropped sharply upon serum-stimulated cell-cycle entry, treatment with Ang II was not accompanied by a downregulation of $p27^{Kip1}$ protein. Instead, immunoprecipitation indicated that $p27^{Kip1}$ was bound to the upregulated but inactive Cdk2 (Figure 3b).

Cellular hypertrophy: a novel function of the Cdk inhibitor $p27^{Kip1}$. We first tested whether the hypertrophic response to Ang II could be mimicked by the maintenance of high levels of $p27^{Kip1}$ during cell-cycle entry induced by an otherwise hyperplastic stimulus. A CMV-driven plasmid containing the cDNA of human $p27^{Kip1}$ was transfected into subconfluent, serum-stimulated VSMCs using the lipid formulation Fugene, and $p27^{Kip1}$-transfected cells were compared with untransfected cells and cells transfected with a control plasmid encoding GFP. Cells were harvested 48 hours after transfection and analyzed for successful cell-cycle arrest. Increases in cell number and [^{3}H]thymidine incorporation were inhibited 26% and 58% in $p27^{Kip1}$-overexpressing cells, respectively ($P < 0.001$) (Figure 4, a and b). Despite the fact that cell number was diminished, [^{3}H]leucine incorporation in $p27^{Kip1}$-transfected VSMCs was similar to control-transfected cells. Consequently, the ratio of protein/DNA synthesis increased from 0.18:1 in proliferating GFP-transfected cells to 0.42:1 in $p27^{Kip1}$-transfected cells, indicating cellular hypertrophy. Hypertrophy in $p27^{Kip1}$-overexpressing cells was also confirmed by FACS analysis. Cells were labeled by fluorescent immunocyto-

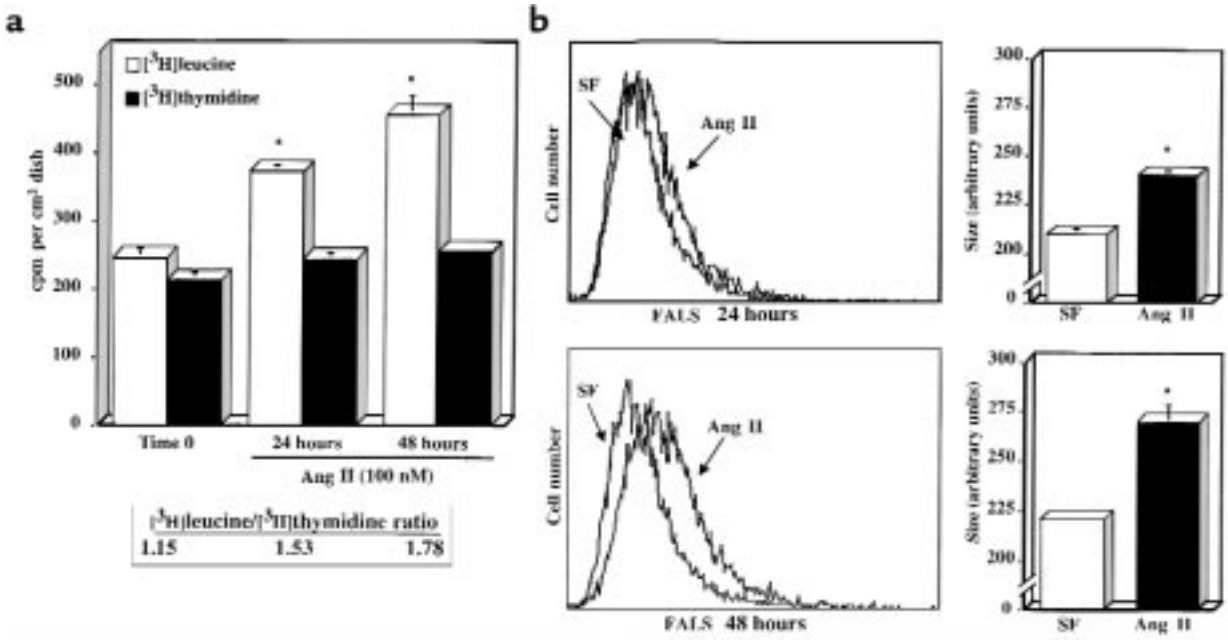


Figure 1
Ang II induces hypertrophy in VSMCs. Confluent, quiescent VSMCs (time 0) were incubated with Ang II (100 nM) for 24 and 48 hours. (**a**) The cells were incubated with [^{3}H]thymidine or [^{3}H]leucine for 2 hours, and radioactive incorporation was counted ($n = 4$; $^*P < 0.001$). The table demonstrates the increase of protein/DNA ratio, an indicator of cellular hypertrophy. (**b**) Relative cell size was also determined by FALS during flow cytometry ($n = 3$; $^*P < 0.001$). The histogram depicts FALS of representative samples comparing VSMCs held serum-free (SF) or incubated with Ang II for 24 or 48 hours.

Table 2
Flow cytometric analysis of VSMCs after treatment with $p27^{Kip1}$ antisense ODN

	Percent cells in		
	G0/G1	S	G2/M
Serum withdrawal	88.7 ± 0.6	6.8 ± 0.3	4.5 ± 0.4
Reverse antisense ODN	85.1 ± 0.4	9.5 ± 0.2	5.4 ± 0.3
+ Ang II (10 nM)	84.9 ± 0.8	9.9 ± 0.6	5.2 ± 0.2
Sense ODN	86.0 ± 0.8	7.3 ± 0.7	6.7 ± 0.4
+ Ang II (10 nM)	87.6 ± 0.8	7.6 ± 0.6	4.8 ± 0.4
Antisense ODN	73.1 ± 0.5[A]	19.1 ± 0.6[A]	7.8 ± 0.3
+ Ang II (10 nM)	68.9 ± 0.7[A,B]	25.5 ± 0.8[A,B]	5.6 ± 0.6
PDGF-BB (20 ng/mL)	61.7 ± 1.0[A]	24.8 ± 1.0[A]	13.5 ± 0.6[A]

[A]$P < 0.0001$ vs. serum withdrawal. [B]$P < 0.001$ vs. antisense ODN.

chemical staining for $p27^{Kip1}$. $p27^{Kip1}$ levels in successfully transfected VSMCs were about 13-fold higher than endogenous $p27^{Kip1}$ levels. These cells were gated and were compared with cells from the same flow cytometric analysis that were untransfected and in which the endogenous $p27^{Kip1}$ protein levels remained low. Measurement of FALS demonstrated an increase in size of the $p27^{Kip1}$-overexpressing cells by 11% at 24 hours after transfection, 46% at 48 hours, and 53% at 72 hours (Figure 5). Cells overexpressing GFP did not significantly change size during this period.

Because $p27^{Kip1}$ has been found to induce apoptosis in certain tumor and fibroblast cell lines (15, 16), we evaluated the influence of $p27^{Kip1}$ overexpression on VSMC programmed cell death by fluorescence microscopy of nuclear chromatin morphology after HOECHST 33342/PI staining (Table 1). In our VSMC primary cultures, transfection of either $p27^{Kip1}$ or GFP was accompanied by a small but significant increase in apoptotic nuclei compared with untransfected cells. $p27^{Kip1}$ overexpression, however, did not add to this nonspecific induction of apoptosis by transfection.

$p27^{Kip1}$ protein levels and Ang II's effect on VSMC growth. The results of $p27^{Kip1}$ overexpression studies suggested that this Cdk inhibitor can, in fact, play a role in mediating VSMC hypertrophy in response to stimuli inducing cell-cycle entry. To test whether the VSMC hypertrophy seen after Ang II–induced cell-cycle entry is, at least in part, caused by a failure to adequately lower cellular levels of $p27^{Kip1}$, VSMCs were transfected with antisense ODN to $p27^{Kip1}$ (20 nM). Evidence of successful decreases of both $p27^{Kip1}$ mRNA and protein levels 24 hours after ODN transfection was seen on RT-PCR and immunoblot analyses, respectively, compared with both untransfected and control ODN–transfected cells (Figure 6).

Subconfluent VSMCs were exposed to $p27^{Kip1}$ antisense ODN or control ODN (reverse antisense ODN or sense ODN) and then transferred for 24 hours to medium lacking serum mitogens. [^{3}H]thymidine incorporation was about 41% higher in $p27^{Kip1}$ antisense ODN–treated cells than in untreated or control ODN–treated cells (Figure 7), underscoring its importance in regulation of the G1- to S-phase transition. Addition of Ang II (100 nM) had no significant effect on DNA synthesis in untreated or control ODN–treated cells in which $p27^{Kip1}$ levels remained high. However, antisense ODN–treated VSMCs experienced an additional, significant increase in [^{3}H]thymidine incorporation when stimulated with Ang II, to 208% of levels in untreated cells and to 173% and 189% in reverse antisense and sense ODN–treated controls, respectively. In comparison, [^{3}H]thymidine incorporation increased by 194% in response to the potent VSMC mitogen PDGF-BB (20 ng/mL) and by 255% when stimulated with PDGF-BB and Ang II. Simultaneous determination of the [^{3}H]leucine/[^{3}H]thymidine ratio further suggested that Ang II promoted hyperplasia in cells treated with $p27^{Kip1}$ antisense ODN. Finally, flow cytometry of PI-stained cells revealed a shift of cells from G0/G1 phase to S or G2/M phase in $p27^{Kip1}$ antisense ODN–treated VSMCs compared with controls ($P < 0.0001$) (Table 2). Ang II treatment resulted in an additional shift of these antisense ODN–treated cells from G0/G1 phase to S phase ($P < 0.001$). This pattern of Ang II–augmented hyperplasia was also observed after

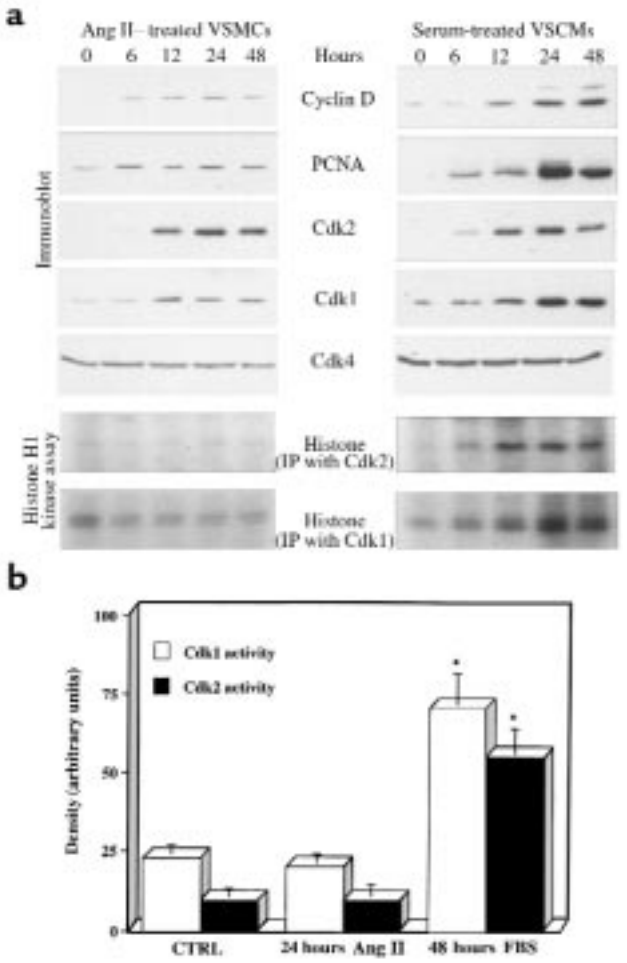


Figure 2
Ang II induces cell-cycle protein upregulation but not activation of Cdk's. Confluent, quiescent VSMCs (time 0) were either stimulated with Ang II (100 nM) or 10% serum for up to 48 hours. (**a**) Cell-cycle protein expression was determined by immunoblot of whole-cell lysates. Histone H1 kinase assay was performed after immunoprecipitation (IP) with either anti-Cdk2 or anti-Cdk1, and phosphorylated histone H1 was viewed on a 12% SDS-PAGE. All blots were independently repeated at least 3 times. (**b**) Quantification of histone H1 kinase assay, for the 24-hour time point ($n = 4$; $^*P < 0.002$).

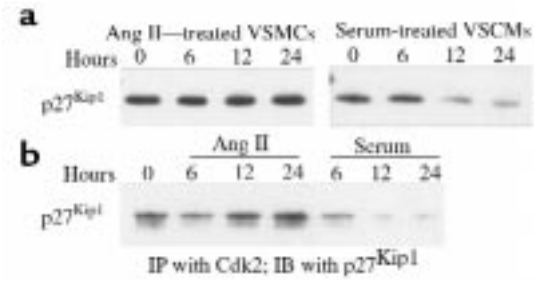


Figure 3
Ang II fails to downregulate the Cdk inhibitor $p27^{Kip1}$, which binds to and inhibits Cdk2. Confluent, quiescent VSMCs (time 0) were either stimulated with Ang II (100 nM) or 10% serum for up to 24 hours. Immunoblots (IB) for $p27^{Kip1}$ were done of whole-cell lysates (**a**) or after immunoprecipitation (IP) with anti-Cdk2 (**b**).

treatment with the potent mitogen PDGF-BB, in which a drop of $p27^{Kip1}$ levels is known to occur.

Discussion

It has been suggested that Ang II acts as a bifunctional modulator of VSMC growth (17), promoting hyperplasia in the presence of serum (18), but inducing hypertrophy in the absence of other growth factors (1, 2). We postulated that the decision of a cell to undergo hyperplasia or hypertrophy in response to Ang II could be mediated at the level of the molecular machinery regulating cell-cycle progression. In this study, we hypothesized that VSMC hypertrophy is induced when cells are stimulated to enter the cell cycle but do not downregulate the key Cdk inhibitor $p27^{Kip1}$. G1-phase cell-cycle proteins were, in fact, upregulated by Ang II treatment of serum-deprived, quiescent VSMCs, although to a smaller degree than that seen after stimulation with the complex mixture of potent mitogens found in FBS. However, levels of $p27^{Kip1}$ remained high after Ang II treatment; the activation of G1-phase Cdk's, such as Cdk2, was inhibited; and the cells underwent hypertrophy rather than hyperplasia. We subsequently tested $p27^{Kip1}$'s role in mediating cellular hypertrophy using a gain-of-function/loss-of-function approach. $p27^{Kip1}$ overexpression succeeded in converting the response of VSMCs to serum stimulation from hyperplasia to hypertrophy. Conversely, treatment with $p27^{Kip1}$ antisense ODN resulted in cell-cycle progression in serum-deprived VSMCs in a sequence-specific manner. Furthermore, in the presence of $p27^{Kip1}$ antisense ODN, but not control ODN, Ang II augmented cellular hyperplasia, implicating that the VSMC response to Ang II is regulated by the level of $p27^{Kip1}$ protein/activity. The results of this study suggest that the Cdk inhibitor $p27^{Kip1}$ plays a key role in the G1-phase determination toward cellular hypertrophy or hyperplasia.

Growth factors induce entry into G1 phase, which is characterized by the synthesis of proteins needed for increased cellular metabolism and for further progression through the DNA synthesis and mitosis phases of the cell cycle (8, 19, 20). The importance of protein synthesis in G1 phase is reflected in the rapid activation of the translational machinery upon growth factor stimulation (21, 22).

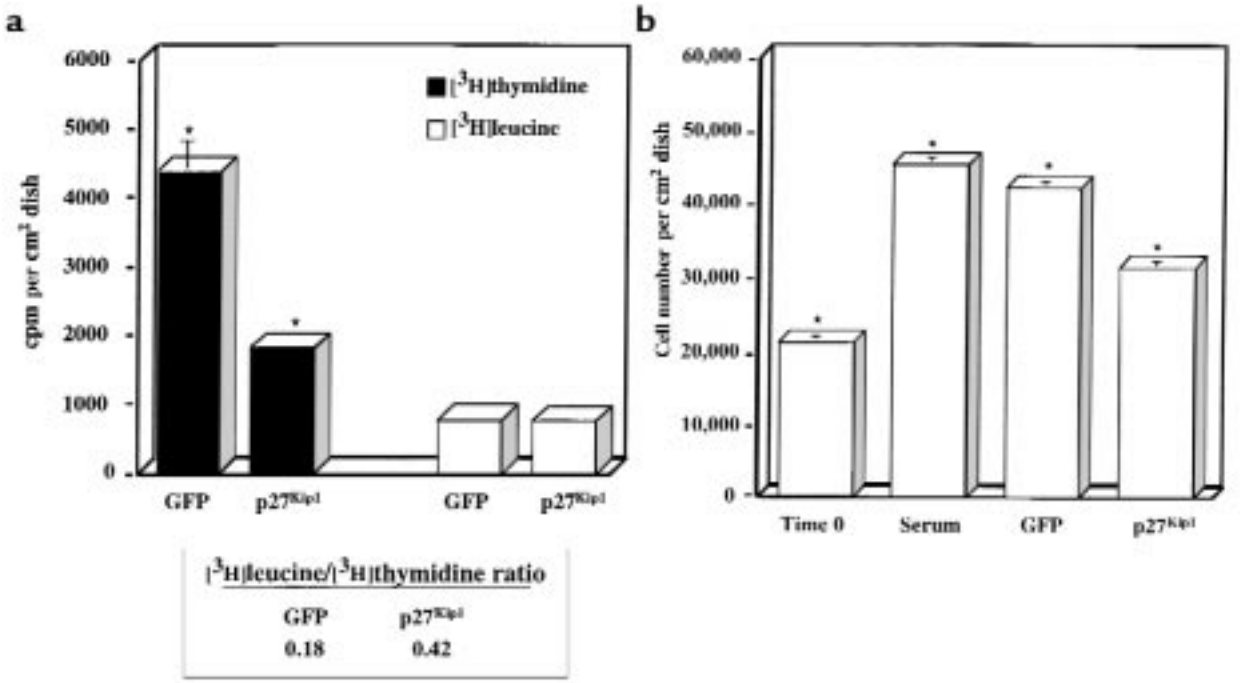
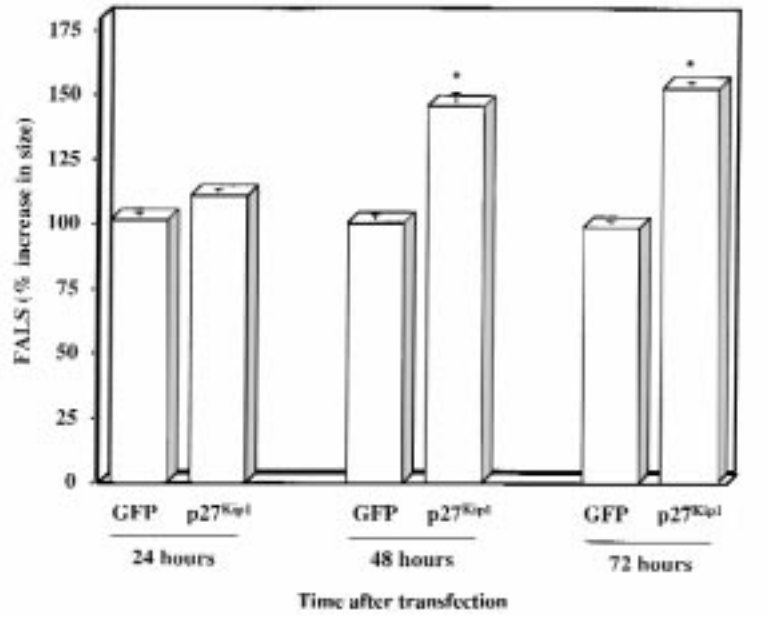


Figure 4
$p27^{Kip1}$ overexpression inhibits proliferation and induces cellular hypertrophy in VSMCs. Subconfluent, serum-stimulated VSMCs were transfected with cDNA for either $p27^{Kip1}$ or GFP (as a control). After 48 hours, cell number was determined (**a**) and [^{3}H]thymidine and [^{3}H]leucine incorporation was measured (**b**). The table in **b** demonstrates the increase of the protein/DNA ratio after $p27^{Kip1}$ transfection, an indicator of cellular hypertrophy. ($n = 4$, $^*P < 0.001$.)

Figure 5
FALS indicates cellular hypertrophy in VSMCs transfected with cDNA for $p27^{Kip1}$. Subconfluent, serum-stimulated VSMCs were transfected with cDNA for either $p27^{Kip1}$ or GFP, harvested after 24, 48, and 72 hours, and subjected to flow cytometry. Hypertrophy was measured as FALS. Cells with high levels of $p27^{Kip1}$ protein after gene transfer were identified by intensity of fluorescent signal from immunocytochemical staining for $p27^{Kip1}$. GFP-transfected control cells were similarly identified by fluorescent signal. FALS in these cells is expressed as a percentage of FALS in cells from the same flow cytometric analysis that demonstrated low levels of either $p27^{Kip1}$ immunofluorescence or GFP fluorescence ($n = 4$; $^*P < 0.001$).



Ang II has been shown to trigger the activation of enzymes important for translation. In myocytes, for example, Ang II directly influences the activity of $p70^{S6}$ kinase (23), and we have previously demonstrated that cell-cycle regulatory protein expression and cell-cycle progression in VSMCs depend on the early activation of the mTOR/$p70^{S6}$ kinase signal-transduction pathway (24). Sustained activation of this enzyme has been shown to induce cellular hypertrophy in fibroblasts (25). As in this study, Ang II was found to upregulate cell-cycle regulatory proteins in a human adrenal cell line (26) and in myocytes, where it also induced hypertrophy (27). These data support our hypothesis that Ang II induces cell-cycle entry but does not trigger an adequate drop of $p27^{Kip1}$ protein levels to allow progression beyond G1. Reduction of $p27^{Kip1}$ levels through antisense ODN treatment did allow Ang II to augment cellular hyperplasia further. The antisense approach used in this study was limited, particularly because $p27^{Kip1}$ antisense ODN treatment, when used alone, resulted in a substantial increase in DNA synthesis, even in the absence of Ang II. The addition of Ang II, therefore, resulted only in a modest, but statistically significant, further increment in [^{3}H]thymidine incorporation. This significant increase in cell-cycle progression was not seen after Ang II stimulation of cells that had been treated with either of 2 control ODN sequences. In the presence of PDGF-BB, when $p27^{Kip1}$ levels were also found to be low (data not shown), Ang II promoted hyperplasia as well, corroborating the results of the more artificial model of $p27^{Kip1}$ reduction through antisense ODN.

$p27^{Kip1}$ has been isolated, cloned, and characterized (29, 30). Its overexpression causes G1-phase arrest and prevents entry of cells into S phase through inhibition of G1-phase cyclin/Cdk complexes. In contrast to other cell-cycle inhibitory proteins, such as $p21^{Cip1}$, $p27^{Kip1}$ is expressed at high levels in confluent, quiescent cells, and protein levels drop rapidly upon mitogen stimulation (8). This pattern of expression is also found in VSMCs in vitro and in vivo after vascular injury (31, 32). The ability of specific mitogens to induce transit through the restriction point, a position in G1 phase at which the cell commits itself to another round of DNA replication, parallels their ability to downregulate $p27^{Kip1}$, and antisense ODN inhibition of $p27^{Kip1}$ expression has prevented cell-cycle arrest in response to mitogen depletion (13). Recently, disruption of the $p27^{Kip1}$ gene was reported to enhance the growth of mice through an increase in cell number of all organs examined. Actual cell sizes, however, were found to be smaller in $p27^{Kip1}$ knockout animals (33, 34). This strongly implied a coupling between cellular hypertrophy and proliferation. To

Figure 6
Suppression of $p27^{Kip1}$ mRNA and protein with antisense ODN treatment of VSMCs. $p27^{Kip1}$ mRNA and protein levels from lysates of subconfluent VSMCs were analyzed by RT-PCR and immunoblot (IB), respectively. (**a**) Untransfected cells after 24 hours of serum withdrawal. (**b**) Cells transfected with reverse antisense ODN before 24 hours of serum withdrawal. (**c**) Cells transfected with sense ODN before 24 hours of serum withdrawal. (**d**) Cells transfected with antisense ODN against $p27^{Kip1}$ before 24 hours of serum withdrawal. (**e**) Untransfected cells, serum-stimulated.

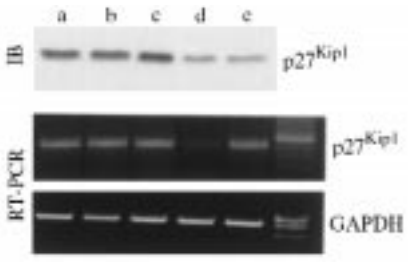
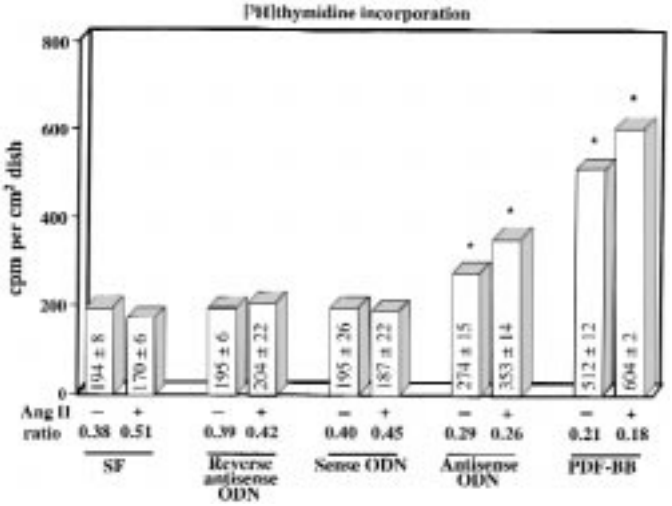


Figure 7
Ang II promotes hyperplasia in the absence of $p27^{Kip1}$. Subconfluent VSMCs were exposed to $p27^{Kip1}$ antisense ODN, reverse antisense ODN, or sense ODN (20 nM). After transfection, Ang II (100 nM) was added as indicated and cells were subjected to 24 hours of serum withdrawal. Thereafter, [^{3}H]thymidine incorporation and [^{3}H]leucine/[^{3}H]thymidine ratio were determined and compared with cells left untransfected but subjected to serum withdrawal (SF), and with cells treated for 24 hours with PDGF-BB (20 ng/mL) ($n = 4$; $*P < 0.001$).



our knowledge, our study provides the first evidence for a link between $p27^{Kip1}$ levels and regulation of cellular hypertrophy. $p27^{Kip1}$ has also been demonstrated to induce programmed cell death in certain tumor and fibroblast cell lines (15, 16). In our primary culture of rat aortic VSMCs, however, $p27^{Kip1}$ overexpression resulted in G1-phase arrest, but not in an increased rate of apoptosis.

It has previously been shown that Ang II's effects on VSMC growth are linked to an induction of TGF-β expression (2). TGF-β's antimitogenic effect has been linked to $p27^{Kip1}$ in mink lung epithelial cells (29, 35). Although $p27^{Kip1}$ was not actively upregulated by TGF-β in these cells, TGF-β indirectly yielded an increase in the level of $p27^{Kip1}$ available to bind and inhibit Cdk2 (36). In B-cell lines, TGF-β has been demonstrated to actively upregulate $p27^{Kip1}$ (37). Nothing is known about TGF-β's effect on $p27^{Kip1}$ levels in vascular cells. It is possible that Ang II induces TGF-β in VSMCs, which in turn induces an increase in $p27^{Kip1}$ protein levels. Further studies are required to elucidate whether the maintained $p27^{Kip1}$ levels after Ang II stimulation reflect a relative reduction in degradation or a relative increase in new protein synthesis compared with mitogen stimulation.

Our study directly relates the level of $p27^{Kip1}$ protein expression to cellular hypertrophy. It is interesting to speculate that G1-phase entry coupled with subsequent cell-cycle arrest may be a general mechanism for cellular hypertrophy. Studies in our laboratory and others have recently suggested that G1 phase of the cell cycle may represent a state in which responses to a variety of stimuli that influence cellular fate can be modulated. Not only may decisions regarding hyperplasia versus hypertrophy be made during G1 phase, but the response to stimuli of cellular activation (38) and programmed cell death may also be affected by early cell-cycle entry (39, 40). Such an influence of cell-cycle stage on cell phenotype may have implications for the future design of therapeutic interventions in a wide range of disease processes.

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