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Angiotensin II Down-regulates the Vascular Smooth Muscle AT_1 Receptor by Transcriptional and Post-transcriptional Mechanisms: Evidence for Homologous and Heterologous Regulation

BERNARD LASSÈGUE, R. WAYNE ALEXANDER, GEORG NICKENIG, MIRJANA CLARK, T. J. MURPHY, and KATHY K. GRIENDLING

Division of Cardiology (B.L., R.W.A., M.C., K.K.G.) and Department of Pharmacology (G.N., T.J.M.), Emory University School of Medicine, Atlanta, Georgia 30322

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SUMMARY

The vascular angiotensin II (ANG II) receptor (AT_1) is a central component of the renin-angiotensin system; thus, regulation of its expression is likely to be important in cardiovascular responsiveness. We demonstrate that ANG II down-regulates its receptor in rat aortic vascular smooth muscle cells. Incubation for 4 hr with 100 nM ANG II decreased AT_1 mRNA and protein by 70% and 35%, respectively. This homologous down-regulation was concentration and time dependent and was blocked by the AT_1 antagonist losartan. It did not appear to be mediated by protein kinase C or other protein kinases but was dependent on the sustained signaling pathway sensitive to phenylarsine oxide. Heterologous down-regulation was observed with the agonists α-thrombin and ATP and the cAMP-increasing agent forskolin. ANG II inhibited transcription by 50% and destabilized the AT_1 mRNA. Down-regulation of AT_1 mRNA was blocked by transcription and translation inhibitors, suggesting that it required expression of a protein factor or factors. These results indicate that ANG II down-regulates its vascular receptor by both transcriptional and post-transcriptional mechanisms. Homologous and heterologous down-regulation of the AT_1 receptor may participate in the coordinated physiological adaptation of vascular tone to vasoactive hormones.

ANG II activates receptors in the adrenal cortex, blood vessels, kidney, and brain, thus contributing to arterial pressure, electrolyte and volume balance, and tissue perfusion control (for a review, see Ref. 1). In addition, ANG II has been implicated in pathological states such as hypertension and left ventricular hypertrophy (for a review, see Ref. 2). These effects of ANG II are mediated to a substantial extent by the AT_1 receptor subtype (1). Regulation of the responsivity of this receptor may therefore be a major factor in both normal and pathological cardiovascular control.

It is known that conditions that increase the activity of the renin-angiotensin system *in vivo* down-regulate ANG II receptors (except in adrenal cortex, in which receptors are regulated in the opposite direction) (3–7). Conversely, a decrease in the renin-angiotensin system activity *in vivo* induces up-regulation of ANG II receptors (3–6). These results have been interpreted as being a consequence of homologous regulation of ANG II receptors after prolonged agonist exposure or withdrawal, similar to that observed *in vitro* for other receptors (8). However, few studies have attempted to confirm ANG II receptor regulation *in vitro* and to explore its mechanism (9). Since the cloning of the vascular and adrenal AT_1 receptors (10, 11), it has been possible to study the mechanism of expression of the receptor at the mRNA level. Preliminary evidence indicated that ANG II may regulate the AT_1 mRNA in adrenal and kidney cells (12, 13), although the molecular mechanism of this effect has not been elucidated. Similarly, very few data concerning the possible signaling pathways involved in the regulation are available. Finally, the mechanisms that may confer specificity to homologous down-regulation of the ANG II receptor are unknown. In the present report, we provide evidence of homologous and heterologous down-regulation of the AT_1 receptor in VSMCs at the level of the mRNA and protein. We also show that AT_1 mRNA is down-regulated via both transcriptional and post-transcriptional mechanisms.

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ABBREVIATIONS: ANG II, angiotensin II; AT_1, angiotensin II receptor; VSMC, vascular smooth muscle cell; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSS, HEPES-buffered saline solution; GAPDH, glyceraldehyde phosphate dehydrogenase; PAO, phenylarsine oxide; SDS, sodium dodecyl sulfate; SSC, sodium chloride/sodium citrate buffer.

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Materials and Methods

Culture of VSMCs. VSMCs from Sprague-Dawley rat thoracic aorta were grown in Dulbecco's modified Eagle's medium with 10% calf serum as described previously (14). Cells between passages 7 and 20 were seeded into 100-mm dishes and used 1–2 days after confluence. Drugs and agonists were added to the culture medium with serum or, when indicated, in BSS of the following composition: 130 mM NaCl, 5 mM KCl, 1.5 mM$CaCl_2$, 1 mM $MgCl_2$, and 20 mM HEPES, buffered to pH 7.4 with Tris base.

RNA purification and Northern blotting. Total RNA was isolated according to the method of Chomczynski (15) or with the use of the single-step TRI reagent (Molecular Research Center) method. Both procedures gave identical experimental results.

Ten-microgram total RNA samples with 0.4 mg/ml ethidium bromide in loading buffer were separated by electrophoresis on 1% denaturing formaldehyde agarose minigels (16). The intensity of the ethidium staining of 28*S* and 18*S* ribosomal RNA was assessed by laser densitometry of a negative photograph and used to correct the loading of a second electrophoresis. RNA from consistently loaded gels was transferred to Magna NT nylon hybridization membranes and immobilized by UV cross-linking.

Blots were prehybridized at least 2 hr at 42° in the following solution: 1 M NaCl, 50 mM Tris·HCl, pH 7.4, 5× Denhardt's solution (0.1% acetylated bovine serum albumin, 0.1% ficoll, 0.1% polyvinylpyrrolidone), 50% formamide, 0.5% SDS, and 100 μg/ml sheared, denatured salmon sperm DNA. Identical conditions were used for overnight hybridization, except for the omission of the Denhardt's solution and the addition of denatured [^{32}P]labeled probe to the hybridization mixture. After hybridization, the blots were rinsed in 1× SSC (150 mM NaCl, 15 mM sodium citrate, pH 7) and washed four times for 15 min at 62° in 0.5× SSC, 0.1% SDS. The membranes were then autoradiographed 24–48 hr with Hyperfilm-MP at −80°.

The cDNA used to prepare the AT_{1A} probe was obtained by polymerase chain reaction amplification of an 825-basepair fragment of the AT_1 Ca18b clone (10) comprising the entire seven-transmembrane region. The polymerase chain reaction was performed with Vent exo(−)-DNA polymerase and the following primers: 5′-GTCATGATCCCTACCCTCTACAGC-3′ and 5′-CCGTAGAACAGAGGGTTCAGG-3′. The cDNA used to prepare the GAPDH probe was the full-length rat clone in pIBI 30 vector (17). Probes were prepared by random priming labeling of 12.5 ng cDNA per miniblot with 25 μCi [^{32}P]dCTP (Prime-it II kit, Stratagene). Unincorporated label was removed by filtration on a Biospin P30 column.

[^{3}H]Losartan binding assay. Binding of [^{3}H]losartan was performed in homogenized VSMCs as described previously for [^{125}I]ANG II (18). An aliquot of the homogenate was kept for protein determination according to the method of Lowry (19). At the end of the assay, filters retaining the radioactive ligand were transferred to scintillation vials, allowed to dry at room temperature overnight, and mixed with 10 ml Liquiscint for quantification by liquid scintillation spectrophotometry. Specific binding was defined as the difference between total binding and nonspecific binding measured in the presence of 1 μM unlabeled ANG II. Total and nonspecific binding were determined in duplicate for each concentration of radioligand. Saturation binding curve experiments were performed with five or six concentrations (2.5–60 nM) of [^{3}H]losartan, whereas single-point saturation binding was done with 60 nM radioligand. Receptor affinity for the ligand and number of binding sites per milligram of protein were determined by Scatchard analysis.

The following experiments were performed to check the validity of the binding procedure. [^{3}H]Losartan binding in homogenized VSMCs was saturable (typically 1500 fmol/mg protein). Nonspecific binding of [^{3}H]losartan increased from 15% of total binding at 5 nM to 45% at 60 nM ligand. Binding increased linearly with the amount of protein (6–200 μg) in the assay. The specific binding of labeled losartan was displaced by ANG II, with an IC_{50} of 10 nM. Association kinetic experiments showed that binding was essentially complete within 25 min; therefore, the 45-min incubation time used in further experiments was sufficient to reach association steady state. Association and dissociation kinetic experiments verified the affinity independently of Scatchard analysis. We found a K_d value of 14 nM, which is in good agreement with the value obtained by Scatchard analysis (12 ± 0.8 nM) and with the reported affinity of losartan for the AT_1 receptor (20).

Nuclear run-on assay. The assay was a modification of the method of Groudine *et al.* (21). VSMCs were harvested with trypsin EDTA and washed with culture medium with serum and with the following buffer: 150 mM KCl, 4 mM magnesium acetate, and 4 mM Tris·HCl, pH 7.4. Cells were pelleted at 4° and lysed for 10 min on ice in buffer with 0.5% Nonidet P-40. Lysates were layered over buffer containing 0.6 M sucrose and nuclei harvested by centrifugation at 1000 × *g* for 10 min at 4°. Nuclear pellets were resuspended in 40% glycerol, 50 mM Tris, 5 mM $MgCl_2$, and 0.1 mM EDTA and stored at −80°. Transcription reactions were carried out at 30° for 30 min with 1–3 × 10^7 nuclei in the same solution with 0.5 mM each of CTP, GTP, ATP and 100–200 μCi (0.2–0.3 μM) [α^{32}P]UTP (>3000 μCi/mmol). TRI reagent was used to terminate the reaction and purify the transcripts. Unincorporated label was removed by filtration on a Biospin P30 column.

DNA slot blots were prepared with 5 μg of AT_1 cDNA (Ca18b subcloned in pKS$^+$ Bluescript), the KS$^+$ Bluescript control vector without insert, and rat GAPDH cDNA in pIBI 30. Plasmids were linearized, denatured, blotted on nylon membrane, and immobilized by UV cross-linking. The blots were prehybridized for 2 hr at 42° in 100 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 0.3 M NaCl, 100 μg/ml *Escherichia coli* tRNA, and 5× Denhardt's solution. Hybridization was carried out with 5 × 10^6 to 2 × 10^7 cpm labeled RNA transcripts for 16 hr at 42° in the same buffer without Denhardt's solution. The blots were washed 10 min at room temperature in 2× SSC and 15 min at 50° in 2× SSC, 0.1% SDS. After a 12–48-hr autoradiography, results were quantified with laser densitometry.

Statistical Analysis. Comparisons were performed with the use of analysis of variance on initial data before expression as percentage of control.

Materials. All chemicals were of analytical grade or better. Molecular biology-grade salts and common chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), American Bioanalytical Co. (Natick, MA), Fisher (Pittsburgh, PA), or Boehringer Mannheim (Indianapolis, IN). Suramin was obtained from CB Chemicals (Woodberry, CT). Dulbecco's modified Eagle's medium with 25 mM HEPES and 4.5 g/l glucose and calf serum were purchased from Sigma. Glutamine, penicillin, and streptomycin were purchased from Whittaker Bioproducts (Walkersville, MD). Trypsin-EDTA was obtained from GIBCO (Grand Island, NY). Cell culture flasks, dishes, and sterile plasticware were obtained from Corning (Marietta, GA). Plastic cell scrapers were from Baxter Scientific Products (Stone Mountain, GA). Other materials and their sources were phenol, proteinase K, and DNAse I (GIBCO); ethanol (McCormick, Weston, MO); buffered phenol (United States Biochemical Corp., Cleveland, OH); TRI reagent (Molecular Research Center, Cincinnati, OH); agarose (FMC Bioproducts, Rockland, ME); Denhardt's solution (5′3′ Inc., West Chester, PA); Magna nylon membranes (Micron Separations Inc., Westboro, MA); Prime-it II kit and UV cross-linker (Stratagene, La Jolla, CA); Hyperfilm-MP and [^{32}P]dCTP (3000 Ci/mmol) (Amersham, Arlington Heights, IL); Vent (exo−)-DNA polymerase (New England Biolabs, Beverly, MA); [^{3}H]losartan and [α^{32}P]UTP (3000 Ci/mmol) (DuPont, Boston, MA); GF/F filters (Whatman, Hillsboro, OR); Biospin P30 columns (Bio-Rad Laboratories, New York, NY); and Liquiscint (National Diagnostics, Somerville, NJ). Other chemicals were purchased from Sigma.

Results

We first sought to determine whether ANG II is capable of affecting the expression of AT_1 mRNA in cultured rat

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VSMCs, a vascular model that abundantly expresses the AT_1 receptor subtype. VSMCs were incubated for several hours with a concentration of ANG II (100 nM) that we have previously found to be capable of maximally activating phospholipase-dependent second messenger systems (14, 22). AT_1 mRNA was measured by Northern blotting of total RNA extracts followed by hybridization with a labeled cDNA AT_1-specific probe.

The representative autoradiogram in Fig. 1 indicates that exposure to 100 nM ANG II for 4–8 hr markedly reduced the amount of AT_1 mRNA present in VSMCs. This effect was specific since the level of GAPDH message was slightly up-regulated during the same time frame (123 ± 19% control, n = 12). The bottom of the figure shows that fluorescence of ribosomal RNA after transfer to the membrane was the same in every lane, indicating that gel loading was consistent.

Fig. 2A shows that ANG II-induced AT_1 mRNA down-regulation was apparent after 2 hr of stimulation and reached a plateau of ~30% control at 4 hr that was stable until at least 8 hr. To determine whether ANG II also regulates the AT_1 receptor protein, we measured its expression by radioligand binding in VSMCs similarly exposed to the agonist. Because ANG II is known to induce internalization of its receptor (23), we measured total cellular receptor level in a broken cell assay. Binding of [^{3}H]losartan was specific and saturable. The linear Scatchard plots in the inset in Fig. 2A show that [^{3}H]losartan was bound to a single population of sites. In addition, exposure of VSMCs to ANG II did not affect the affinity of the sites for the ligand, as indicated by the identical slopes of the plots in control and ANG II-treated

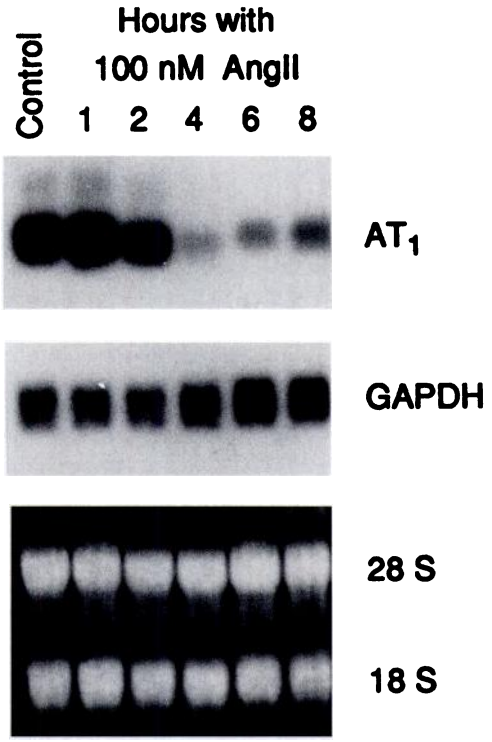

Fig. 1. Total cellular RNA blotting and hybridization. In this representative experiment, postconfluent VSMCs were incubated with 100 nM ANG II in culture medium for the indicated times. *Top*, autoradiogram from a Northern blot probed with AT_1 cDNA. *Middle*, autoradiogram from a Northern blot probed with GAPDH cDNA. *Bottom*, UV photograph of transfer membrane showing fluorescent 28*S* and 18*S* ribosomal RNAs in ethidium bromide-stained RNA samples.

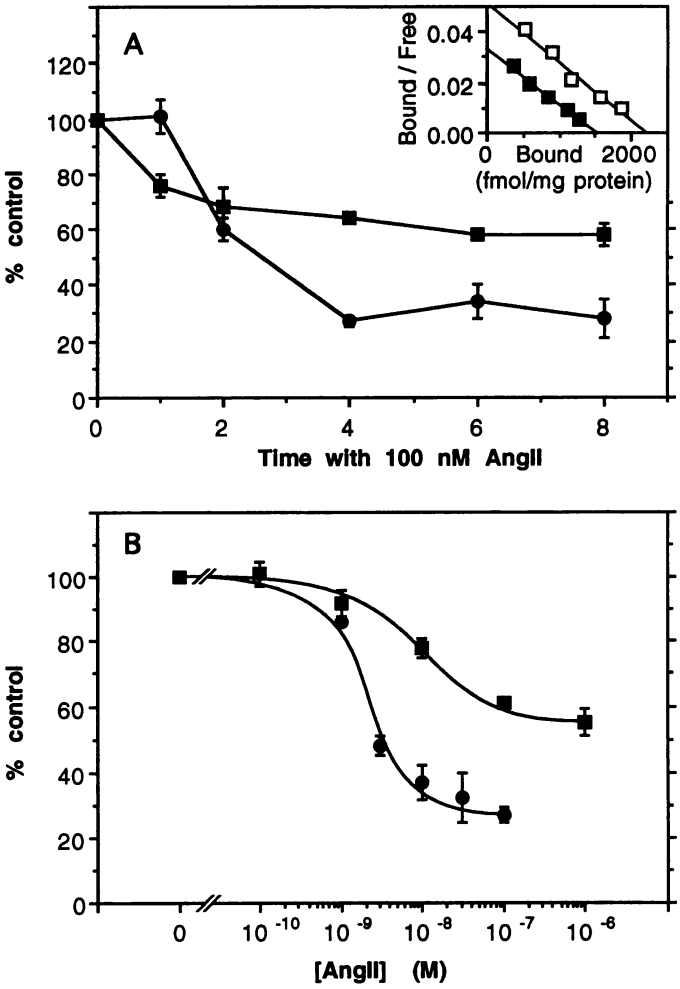

Fig. 2. Effects of ANG II on AT_1 mRNA and protein. A, Time courses of ANG II-induced down-regulation of AT_1 mRNA and protein. Postconfluent VSMCs were exposed to 100 nM ANG II in culture medium for the indicated times in hours. AT_1 mRNA (●) was measured by Northern blotting as described in Fig. 1. AT_1 protein (■) was assayed in broken cells by binding of [^{3}H]losartan. Data points represent the average ± standard error of values from 3 to 12 independent experiments expressed as percentage of the untreated control. *Inset*, Scatchard plots representative of eight experiments. VSMCs were preincubated in the absence (□) or presence (■) of 100 nM ANG II for 4 hours. Binding parameters were K_d = 8.1 nM and B_{max} = 2205 fmol/mg protein in control cells and K_d = 8.2 nM and B_{max} = 1534 fmol/mg protein in cells exposed to the agonist. B, Concentration-effect relationships of AT_1 mRNA (●) and protein (■), measured as in A, in VSMCs exposed for 4 hr to the indicated concentration of ANG II in culture medium. Data points represent the average ± standard error of values from two (RNA) and five (protein) independent experiments expressed as percentage of the untreated control.

cells. In contrast, at 4 hr, ANG II decreased the number of binding sites from 2205 to 1534 fmol/mg protein, as indicated by the shift in the Scatchard plots. The receptor protein was down-regulated to 60% control at 8 hr. Receptor down-regulation was maintained in cells that were challenged with a fresh dose of ANG II every 8 hr up to 24 hr, when it reached 37 ± 4% control (not shown).

To characterize ANG II-induced down-regulation, we determined the effect of ANG II concentration on AT_1 mRNA and protein. As can be seen in Fig. 2B, the EC_{50} values for down-regulation of the mRNA and protein were 2 and 9 nM, respectively. These values are in good agreement with those

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obtained for binding of [^{125}I]ANG II to its receptor (24) and for activation of phospholipases C (25) and D (14), suggesting that down-regulation is closely related to receptor activation. Disappearance of the protein after mRNA degradation may be subjected to additional regulation as suggested by its shallower concentration-response relationship.

We used the specific AT_1 antagonist losartan to determine whether the effect of ANG II is mediated by this receptor subtype. VSMCs were preincubated for 10 min with 1 μM losartan before exposure to 100 nM ANG II for 4 hr. As depicted in Fig. 3, losartan completely blocked ANG II-induced down-regulation of both AT_1 mRNA and protein, indicating that activation of the AT_1 receptor is a required first step in its down-regulation. This result suggests that hormonal stimulation triggers specific intracellular feedback mechanisms producing down-regulation of the AT_1 receptor, leading to reduced sensitivity to the stimulus.

To gain insight into the specificity of the response to ANG II, we challenged VSMCs with other agonists at concentrations previously found to be maximally effective in inducing calcium mobilization (26–28) before measuring AT_1 mRNA and protein. Fig. 3 indicates that a 4-hr exposure to 100 nM vasopressin did not significantly affect expression of the ANG II receptor. In contrast, both 140 nM α-thrombin and 100 μM ATP induced down-regulation of AT_1 mRNA and protein. This result indicates that regulation of the ANG II receptor expression is not strictly homologous.

Because ATP can be metabolized to adenosine at the cell surface, we attempted to determine which class of receptor (P_1 or P_2) mediates ATP-induced down-regulation of AT_1 mRNA. VSMCs were exposed to different purinergic agonists. As indicated in Table 1, the P_1 receptor agonist adenosine had no significant effect. In contrast, the P_2 receptor

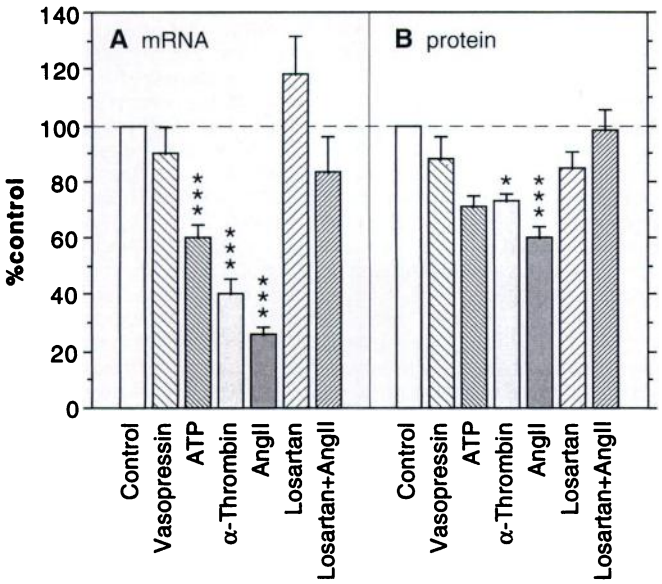

Fig. 3. Specificity of agonist-induced down-regulation of AT_1 mRNA and protein. AT_1 mRNA (A) and protein (B) were measured as in Fig. 2 in postconfluent VSMCs exposed in culture medium for 4 hr to either vehicle, 100 nM arginine-vasopressin, 100 μM ATP, 140 nM α-thrombin, 100 nM ANG II, or 1 μM losartan or preincubated for 10 min with 1 μM losartan before the addition of 100 nM ANG II. Bars represent the average ± standard error of values from two to four independent experiments expressed as percentage of control. Statistical difference from control: *, $p < 0.05$; ***, $p < 0.001$.

TABLE 1

Effect of purinergic agonists on AT_1 mRNA

AT_1 mRNA was measured by Northern blotting as in Fig. 1 in postconfluent VSMCs exposed to one of the indicated treatments for 4 hr. Cells were exposed to suramin for 10 min before the addition of ATP and the continued 4-hr incubation. Data represent the average ± standard error of mRNA values from two to eight independent experiments expressed as percentage of control.

Treatment	AT_1 mRNA
Control	100 ± 0
ANG II, 100 nM	26 ± 3[a]
ATP, 100 μM	60 ± 5[a]
ADP, 100 μM	62 ± 3[a]
UTP, 100 μM	58 ± 4[a]
α,β-Methylene-ATP, 100 μM	89 ± 9
Suramin, 1 mM	111 ± 12
Suramin + ATP	64 ± 18[b]
Adenosine, 100 μM	81 ± 5

[a] Statistically different from control, $p < 0.01$.
[b] Not significantly different from ATP alone, $p > 0.05$.

agonists ATP, ADP, and UTP were equally capable of inducing AT_1 mRNA down-regulation. The potent P_{2X} agonist α,β-methylene-ATP was ineffective and the P_{2X} and P_{2Y} antagonist suramin was unable to decrease the effect of ATP. Taken together, the results in Table 1 are consistent with the interpretation that ATP down-regulates AT_1 mRNA via activation of a receptor of the P_2, rather than the P_1 class, most likely P_{2U} (29), a receptor type known to be expressed in vascular smooth muscle (27, 29).

We then attempted to characterize the intracellular signaling pathways that may lead to receptor down-regulation. Because ANG II, ATP, and thrombin are coupled to phospholipase C and calcium mobilization, we incubated VSMCs for 4 hr with the calcium ionophore ionomycin (15 μM) to increase cytoplasmic calcium concentration (22). As indicated in Table 2, this treatment significantly reduced AT_1 mRNA levels. To test the effect of a decrease in cytoplasmic calcium concentration, VSMCs were preincubated in BSS with 2 mM EGTA and no added calcium for 10 min before exposure to ANG II for 4 hr. However, because the removal of extracellular calcium decreased basal AT_1 mRNA, no significant change in ANG II-induced down-regulation could be measured (Table 2). This effect on the baseline could reflect a role of calcium in transcription or stability of the message. It is also possible that a concomitant decrease in cellular magnesium concentration in these conditions may lead to a decrease in transcription by magnesium-dependent RNA polymerases.

In addition to raising cytoplasmic calcium concentration, ANG II induces a sustained increase in diacylglycerol that, together with calcium, activates protein kinase C (30). To determine whether this kinase participates in AT_1 mRNA regulation, we tested protein kinase C activators and inhibitors (Table 2). The protein kinase C activator phorbol myristate acetate induced a concentration-dependent down-regulation of AT_1 mRNA in the 1–100 nM range. The effect of 100 nM was time dependent, with a time course similar to that of ANG II (not shown). The 4α-isomer of phorbol myristate acetate, which does not stimulate protein kinase C, was inactive, suggesting that the observed effect was specific. We then attempted to block the effect of ANG II with the protein kinase C inhibitor GF-109203X. This compound did not antagonize the effect of ANG II (Table 2) in conditions that effectively inhibited PMA-induced activation of phospho-

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TABLE 2

Effects of intracellular signals on AT_1 mRNA

AT_1 mRNA was measured by Northern blotting as in Fig. 1 in postconfluent VSMCs exposed to one of the indicated treatments for 4 hr. Cells exposed to two agents were treated for 10 min with an inhibitor before the addition of ANG II and incubation for an additional 4 hr. The concentration of ANG II was 100 nM. Data represent the average ± standard error of mRNA values from three to five independent experiments expressed as percentage of control.

Signaling system	Treatment	AT_1 mRNA
	Control	100 ± 0
	ANG II, 100 nM	30 ± 4[c]
Calcium	Ionomycin, 15 μM	62 ± 4[c]
	No calcium-EGTA, 2 mM	38 ± 4[c]
	No calcium-EGTA + ANG II	47 ± 11[d]
Protein kinase C	Phorbol myristate acetate, 30 nM	30 ± 9[c]
	4-α-Phorbol myristate acetate, 100 nM	108 ± 10
	GF-109203X, 2 μM	51 ± 1[a]
	GF-109203X + ANG II	22 ± 1[d]
Protein phosphorylation	Sodium orthovanadate, 100 μM	70 ± 11[a]
	Orthovanadate + ANG II	58 ± 10[d]
	Okadaic acid, 30 nM	102 ± 10
	Okadaic acid + ANG II	19 ± 4[d]
	ST638, 10 μM	49 ± 16[b]
	ST638 + ANG II	39 ± 8[d]
Cytoplasmic cAMP	Forskolin, 10 μM	63 ± 11[a]
	1,9-Dideoxyforskolin, 10 μM	100 ± 14

Statistically different from control: [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$.
[d] Not significantly different from ANG II alone, $p > 0.05$.

lipase D in our system (82 ± 2% inhibition). We also tested the protein kinase C inhibitors staurosporine, chelerythrine, and calphostin C (not shown). All inhibitors decreased AT_1 mRNA baseline at higher concentrations (e.g., GF-109203X in Table 2), but none were able to alter the effect of ANG II. This depression of AT_1 message induced by high concentrations of protein kinase C inhibitors appears to be nonspecific because they also affected constitutive expression of GAPDH mRNA (56 ± 6% control with 300 nM staurosporine versus 110 ± 12% control with 100 nM PMA). Therefore, protein kinase C does not appear to mediate ANG II-induced down-regulation, although it may mimic the effect of the agonist when intensely stimulated by pharmacological agents.

We also tested the possible involvement of other protein phosphorylation pathways in AT_1 mRNA regulation (Table 2). Treatment for 4 hr with protein phosphatase inhibitors such as the tyrosine phosphatase inhibitor orthovanadate (100 μM) (31) or the serine/threonine phosphatase inhibitor okadaic acid (30 nM) (32) had little or no effect on AT_1 mRNA regulation by ANG II. Similarly, a 4-hr incubation with 10 μM ST638 (33), a tyrosine kinase inhibitor, decreased basal AT_1 mRNA but did not affect ANG II-induced down-regulation. We were thus unable to detect any apparent involvement of nonreceptor-specific protein kinases in ANG II-induced AT_1 mRNA down-regulation.

Because the promoter region of the AT_1 gene contains a cAMP responsive element (34) and the protein has a putative protein kinase A phosphorylation site (10), we also investigated the sensitivity of AT_1 mRNA expression to cAMP. VSMCs were incubated for 4 hr in the presence of 10 μM forskolin, conditions that increase the intracellular cAMP concentration at least 10-fold (35). As indicated in Table 2, this treatment decreased AT_1 mRNA. In contrast, the dideoxy inactive analogue of forskolin had no effect. Forskolin also specifically down-regulated the AT_1 protein to 78 ± 6% control at 4 hr, as measured by [^{3}H]losartan binding.

ANG II induces a biphasic phospholipase signaling sequence in vascular smooth muscle (22). The sustained second phase of ANG II signaling, characterized by activation of phospholipase D (14), is dependent on receptor internalization (14, 23). PAO can inhibit the internalization and sustained signaling mediated by various receptors, including AT_1 (23, 36–39), and inhibit phosphoprotein tyrosine phosphatases (40). It has been shown that activated thrombin receptors are internalized into coated pits (41, 42). P_{2U} purinergic receptors, which also belong to the G protein-coupled, seven-transmembrane-domain receptor family (29), are likely candidates for internalization. To determine whether down-regulation of the AT_1 receptor by these agonists may require sustained signaling, possibly dependent on receptor internalization, we tested its susceptibility to PAO. Table 3 indicates that 3 μM PAO effectively inhibited the down-regulation of AT_1 mRNA induced by a 4-hr stimulation with ANG II, α-thrombin, or ATP, suggesting that an event developing during the second phase of signaling may be mediating the effect of the three agonists.

Two mechanisms could account for ANG II-induced AT_1 mRNA down-regulation: a decrease in transcription of the gene or an accelerated degradation of the mRNA, i.e., a decrease in mRNA stability. To measure the influence of ANG II on transcription, we performed nuclear run-on assays. As shown in Fig. 4, transcription of the AT_1 message in

TABLE 3

Effects of phenylarsine oxide on AT_1 mRNA

Postconfluent VSMCs were exposed to 3 μM phenylarsine oxide for 10 min before the addition of the indicated agonist and the continued 4-hr incubation. AT_1 mRNA was measured by Northern blotting as in Fig. 1. Data represent the average ± standard error of mRNA values from three to seven independent experiments expressed as percentage of control.

Treatment	AT_1 mRNA
Control	100 ± 0
ANG II, 100 nM	22 ± 2
Phenylarsine oxide, 3 μM	93 ± 20
Phenylarsine oxide + ANG II	84 ± 13[a]
α-Thrombin, 140 nM	40 ± 6
Phenylarsine oxide + α-thrombin	101 ± 18[a]
ATP, 100 μM	57 ± 5
Phenylarsine oxide + ATP	120 ± 28[a]

[a] Statistically different from agonist alone, $p < 0.01$.

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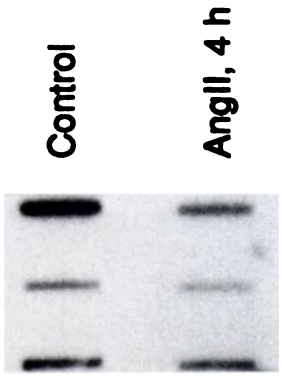

Fig. 4. Autoradiogram of a representative run-on assay showing the effect of ANG II on AT_1 mRNA transcription. VSMCs were exposed to either vehicle or 100 nM ANG II for 4 hr. Nuclei were isolated and transcription allowed to proceed in the presence of [^{32}P]UTP. Labeled transcripts were hybridized to the following denatured DNAs immobilized on a slot blot: AT_1 receptor cDNA in pKS$^+$BS vector, the vector without insert, and GAPDH cDNA. The autoradiograms were analyzed by densitometry, and AT_1 values were corrected for the change in vector signal. In three independent experiments, ANG II decreased AT_1 transcription to 50% of control and GAPDH to 95% of control.

cells that had been exposed to 100 nM ANG II for 4 hr was 50% control, after correction for the decreased signal observed with the vector alone. This was confirmed in three independent experiments. Transcription of GAPDH was not significantly affected by the treatment. This result indicates that ANG II-induced down-regulation of AT_1 mRNA may be due in part to an inhibition of transcription.

To measure the stability of the AT_1 mRNA, VSMCs were exposed to the transcription inhibitor actinomycin D. Fig. 5 indicates that the AT_1 mRNA half-life was ~9 hr, 3.5 times longer than the time it took ANG II to decrease the message by 50% (Fig. 2A). This result suggests that in addition to inhibiting transcription, ANG II can destabilize the AT_1 mRNA. To determine whether synthesis of a protein factor is necessary for ANG II to induce AT_1 down-regulation, VSMCs were exposed to transcription or translation inhibitors 10 min before addition of ANG II. Fig. 6 shows that both 5 μg/ml

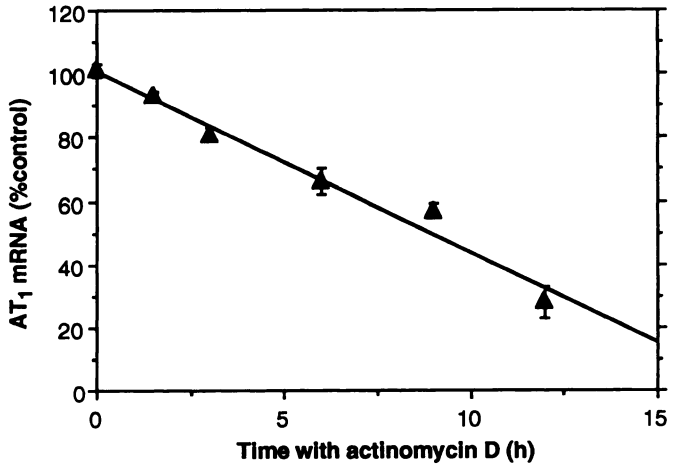

Fig. 5. Time course of AT_1 mRNA degradation. AT_1 mRNA was measured by Northern blot hybridization as in Fig. 1 in postconfluent VSMCs exposed to 5 μg/ml actinomycin D for the indicated time. Data points represent the average ± standard error of three to seven independent experiments expressed as percentage of control.

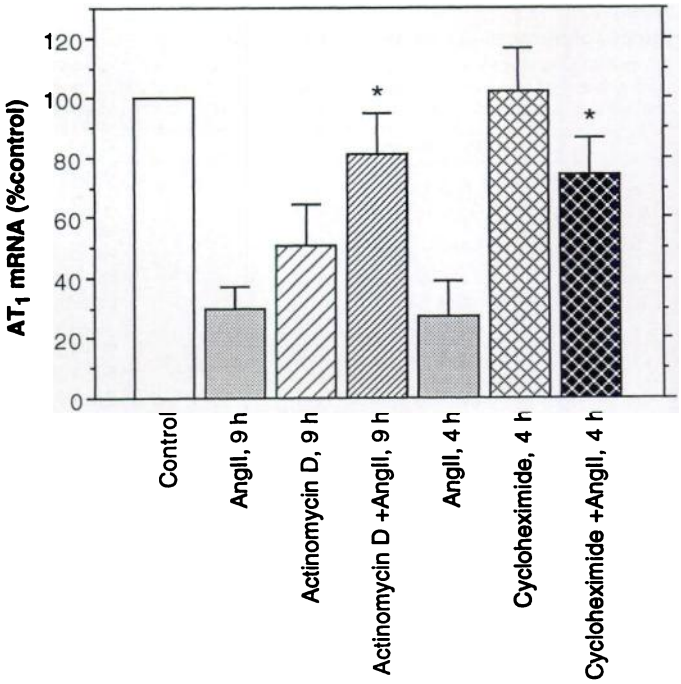

Fig. 6. Effects of transcription or translation inhibitors on ANG II-induced AT_1 mRNA down-regulation. AT_1 mRNA was measured as in Fig. 1 in postconfluent VSMCs exposed for 4 or 9 hr in culture medium to either vehicle, 100 nM ANG II, 5 μg/ml actinomycin D, or 10 μg/ml cycloheximide or preincubated with either drug for 10 min before the addition of agonist. Bars represent the average ± standard error of values from four or five independent experiments expressed as percentage of control. *, Significantly different from ANG II alone, $p < 0.05$.

actinomycin D and 10 μg/ml cycloheximide significantly inhibited ANG II-induced down-regulation. Thus, in addition to an effect on transcription, ANG II may also control AT_1 mRNA levels by an acceleration of degradation. This effect is likely to be mediated by transcription and translation of a protein that remains to be characterized. However, because some mRNAs appear to be destabilized by translation (43), part of the effect of cycloheximide could also directly result from the inhibition of AT_1 translation.

Discussion

In the present report, we show that specific activation of the AT_1 receptor induces its own down-regulation in VSMCs. Down-regulation of both mRNA and protein were significant after 2 hr and were sustained in the continuous presence of ANG II. A similar effect was also produced by the agonists α-thrombin and ATP. The effect of ANG II was correlated with the ability of the activated receptor to either internalize or induce prolonged phospholipid-dependent signaling. Furthermore, ANG II-induced mRNA down-regulation appeared to be mediated by a concomitant decrease in transcription and accelerated degradation of the message. This decreased mRNA stability may be dependent on the transcription and translation of unknown protein factor(s).

Our demonstration that ANG II down-regulates the AT_1 receptor protein in VSMCs provides a mechanistic explanation for previous reports indicating that activation of the renin-angiotensin system *in vivo* by chronic sodium deprivation or ANG II infusion reduces the number of specific bind-

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ing sites for ANG II in mesenteric vascular smooth muscle (4, 5, 7) as well as other tissues (3, 6). Our data are also in good agreement with the report that ANG II decreases its binding sites *in vitro* in cultured hepatocytes without affecting receptor affinity (9). The observation that ANG II also down-regulates AT_1 mRNA in VSMCs is consistent with previous reports in various tissues (12, 13, 44). The rapid decrease in receptor number after agonist exposure suggests that the protein half-life is short. Receptor degradation could be a direct consequence of stimulation since previous data indicate that agonist binding induces internalization of the receptor-ligand complex, followed by recycling of only ~25% of the receptor and degradation of the remainder (45, 46). Rapid turnover is a general feature of molecules whose concentration has to adapt to quickly changing conditions and would be expected of the ANG II receptor. Our results therefore support the interpretation that AT_1 down-regulation is responsible for the intermediate-term decreased sensitivity of the vascular system to ANG II. This is not in conflict with previous reports of an up-regulation of AT_1 protein and mRNA in the adrenal glomerulosa (3, 6, 47) because that tissue secretes aldosterone in response to ANG II, a function that is not attenuated by intermediate-term stimulation. It should also be noted that down-regulation that operates over a period of hours is distinct from receptor desensitization, a phenomenon occurring within minutes of exposure to high agonist concentrations that decreases sensitivity of the tissue to ANG II.

Down-regulation of AT_1 mRNA and protein by ANG II was blocked by losartan, attesting that this effect results from activation of the AT_1 receptor. The consequences of receptor activation that result in receptor down-regulation remain to be defined completely. Some of the signals involved may not be specific to the AT_1 receptor because other phospholipase C-coupled receptors, including those for ATP and α-thrombin, also down-regulated the AT_1 receptor protein and mRNA (Fig. 3). This heterologous down-regulation could not be associated definitively with any of the second messengers produced by receptor activation, although calcium mobilization may be involved. Heterologous regulation of the ANG II receptor has also been reported in neuronal cultures, where a 4–8-hour stimulation of α_1-adrenergic receptors with norepinephrine decreased the number of ANG II receptors with no change in affinity (48). Another example of receptor cross-talk is suggested by the experiments designed to increase cytoplasmic cAMP. The ability of forskolin to decrease AT_1 mRNA expands the range of heterologous ANG II receptor regulatory mechanisms. The existence of these heterologous regulatory mechanisms suggests an integrated control of the sensitivity of vascular smooth muscle to vasoactive agonists that may be more complicated than is generally assumed.

Homologous down-regulation by ANG II of the AT_1 receptor not unexpectedly appears to be a more powerful regulatory mechanism than the heterologous regulations noted. Selective down-regulation could result from specifically enhanced internalization and degradation of the agonist-occupied receptor. Agonist-induced phosphorylation of occupied receptors by kinases such as G protein-coupled receptor kinases provides a mechanism for specific modification of receptor function (8). Thus, homologous down-regulation may result from receptor-specific mechanisms as well as mechanisms shared by other receptor systems.

The fact that PAO was capable of blocking the down-regulation of AT_1 mRNA induced by three different agonists suggests that a common mechanism may be similarly altered in each case. PAO may inhibit agonist-induced receptor internalization and subsequent signaling. Although it is not known whether ATP receptors internalize upon activation or whether internalization of the thrombin receptor is important for transduction, these receptors belong to the same family as AT_1 and therefore are likely to also require internalization for sustained signaling (14, 23). The effect of PAO is consistent with the previous observation that the vascular ANG II receptor was up-regulated in potassium-depleted animals despite increased plasma renin and ANG II (49), because potassium depletion has also been shown to inhibit internalization and recycling of the ANG II receptor (50, 51). Because PAO does not affect the initial transient phospholipase C-mediated signaling in our system (23), it seems likely that a PAO-sensitive persistent signaling mechanism such as sustained phospholipid hydrolysis, e.g., by phospholipase D, may initiate receptor down-regulation. Another possibility is that PAO inhibits protein tyrosine phosphatases (40) and that the resulting increase in tyrosine phosphorylation of an unknown substrate may inhibit down-regulation of AT_1 by either agonist. This possibility appears to be less likely because the tyrosine phosphatase inhibitor vanadate had no effect (Table 2) and because down-regulation of AT_1 is correlated with an increase in receptor phosphorylation on tyrosine (52).

Unexpectedly, AT_1 down-regulation does not appear to be due to activation of protein kinase C by ANG II. Although an intense exogenous activation of protein kinase C by phorbol esters was capable of inducing receptor down-regulation, protein kinase C inhibitors failed to block the effect of the agonist. In recent studies (12, 44), H-7 was reported to reverse the down-regulation of AT_1 mRNA induced by ANG II. However, H-7 is notoriously nonspecific, and in these studies, no direct measurement of protein kinase C activity was made. Our results using a more specific protein kinase C inhibitor suggest that AT_1 down-regulation by ANG II is protein kinase C independent.

Our data indicate that ANG II was able to down-regulate AT_1 mRNA significantly faster than could be accounted for by interruption of transcription alone with actinomycin D, suggesting that the agonist specifically destabilizes the mRNA encoding its AT_1 receptor. This is similar to the decreased stability of β-adrenergic receptor mRNA observed after exposure to β-adrenergic agonists (53). Furthermore, the effect of ANG II was inhibited in cells exposed to actinomycin D or cycloheximide before ANG II stimulation. Although part of the effect of cycloheximide may result from its inhibition of AT_1 translation that could stabilize the mRNA (43), these observations suggest that ANG II induces the transcription and translation of a protein factor that is required for AT_1 mRNA destabilization. This factor may be homologous to the protein shown to destabilize the β-adrenergic receptor (54). It is also possible that the two AUUUA motifs present in the 3′ untranslated region of the rat AT_1 mRNA may be involved in the binding of destabilization factors, as is the case for several other labile mRNAs (for a review, see Ref. 43).

In summary, we presented evidence for homologous and heterologous down-regulation of the AT_1 receptor mRNA and

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protein. ANG II-induced down-regulation appears to be mediated by long term signaling events and the synthesis of protein factors. Down-regulation operates via both transcriptional and post-transcriptional mechanisms. These regulatory events most likely participate in an integrated control of vascular sensitivity to vasoactive agents and may be central to the physiology and pathology of the renin-angiotensin system.

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Send reprint requests to: Dr. Bernard Lassègue, Emory University, Department of Medicine, Division of Cardiology, P.O. Drawer LL, Atlanta, GA 30322.

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