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# Enhanced Angiotensin Receptor Type 1 mRNA Degradation and Induction of Polyribosomal mRNA Binding Proteins by Angiotensin II in Vascular Smooth Muscle Cells

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**SUMMARY**

Stimulation of cultured rat thoracic aorta vascular smooth muscle cells (VSMCs) with 100 nM angiotensin II (Ang II) reduces angiotensin receptor type 1 ($AT_1$-R) gene expression. mRNA levels are reduced to ~30% of control levels 4 hr after the addition of Ang II to the culture medium. The loss of mRNA remains sustained for up to 24 hr after the addition of Ang II. The half-life of the $AT_1$-R mRNA is ~2 hr in cells treated with a single dose of 100 nM Ang II. This represents a 3-fold reduction from its half-life of 6 hr in nonstimulated cells, as assessed by treatment with 5,6-dichlorobenzimidazole or actinomycin D to block transcription. Thus, the $AT_1$-R mRNA is moderately unstable in VSMC and destabilized further by treatment with Ang II. Ang II-induced $AT_1$-R mRNA destabilization is prevented by pretreatment with transcriptional inhibitors or the protein synthesis inhibitor cycloheximide, suggesting that Ang II-induced $AT_1$-R mRNA destabilization requires the induction of an unknown factor or factors that are postulated to mediate this effect. $AT_1$-R mRNA levels decrease more rapidly *in vitro* from a polyribosomal fraction isolated from VSMC exposed for 2 hr to 100 nM Ang II compared with that from vehicle-treated cells, suggesting that polyribosomal-associated $AT_1$-R mRNA is at least one site of action for the mRNA destabilization effect of Ang II. Ang II stimulation induces a complex of polyribosomal proteins that bind specifically in the distal 350 bases of the $AT_1$-R mRNA. Regulation of mRNA stability accounts in part for modulation of $AT_1$-R gene expression by Ang II in VSMCs, and Ang II-induced $AT_1$-R mRNA polyribosomal binding proteins are associated with this phenomenon.

The principal physiological role of the octapeptide hormone Ang II is to enhance vascular smooth muscle contractility and body fluid retention through activation of G protein-coupled $AT_1$-Rs (1). Factors that influence $AT_1$-R gene expression can thus have marked effects on the cardiovascular status of animals. Down-regulation of G protein-coupled receptors is thought to represent an adaptation of these receptors to sustained or skewed stimuli (2, 3). The molecular mechanisms involved in this longer term adaptation are not well understood but likely involve cellular processes superimposed on better-understood, more-immediate events such as desensitization and sequestration of cell surface receptors. Included among these longer term adaptive processes, as first shown for the β-adrenergic receptors, are the induction of putative mRNA destabilization factors, which may participate in enhancing the rate of decay of receptor coding mRNAs (4, 5)

Several studies demonstrated diminished $AT_1$-R expression after extended exposure to Ang II or by manipulations that might be expected to increase circulating levels of Ang II *in vivo* (6–8). Recent studies in cultured VSMCs suggest that $AT_1$-R mRNA synthesis and stability are modulated by a group of compounds representative of a diverse array of extracellular signals (9–11). Thus, in smooth muscle cells, it appears that $AT_1$-R gene expression is regulated by numerous extrinsic factors, perhaps through multiple mechanisms. To begin to unravel the complexity of this system, this report focuses specifically on regulation of VSMC $AT_1$-R mRNA stability by Ang II.

Modification of mRNA stability is an established mechanism of gene expression control and has been implicated in, for example, the transient alteration of expression for several cytokines, transcription factors, and immediate-response genes (12). Specific sequences of mRNA, polydeadenylation, and nucleolytic processes in concert with cytosolic and polyribosomal-associated mRNA-binding proteins have been im-

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**ABBREVIATIONS:** Ang II, [Ile$^5$]angiotensin II; VSMC, vascular smooth muscle cell; $AT_1$-R, angiotensin receptor type 1; PMSF, phenylmethylsulfonyl fluoride; GAPDH, glyceraldehyde phosphate dehydrogenase; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

plicated in these pathways (13). The unstable RNAs for many of these genes share the characteristics of transient induction and rapid mRNA turnover, which differs from that for the $AT_1$-R mRNA in VSMCs, in which its synthesis is matched by its degradation before activation of VSMCs by extracellular signals (current report and Refs. 9 and 11). Generally, the mechanisms relating mRNA-binding proteins to mRNA stabilization or destabilization are understood incompletely. Thus, elucidation of molecular mechanisms involved in the control of VSMC $AT_1$-R mRNA stability can specifically deepen our understanding of how $AT_1$-R gene expression is regulated in normal and pathological conditions and may more generally broaden our understanding of general mechanisms regulating mRNA stability. In this report, we demonstrate that the $AT_1$-R is moderately unstable in intact VSMCs and destabilized further in a transcriptionally and translationally coupled manner in response to Ang II treatment. Ang II-induced $AT_1$-R mRNA instability is retained in a cell-free VSMC polyribosomal extract and is associated with the induction of polyribosomal proteins that bind within the distal 3′-untranslated region of the $AT_1$-R mRNA. These Ang II-induced $AT_1$-R mRNA-binding proteins may represent candidate factors that regulate $AT_1$-R mRNA stability in VSMCs.

## Experimental Procedures

**Materials.** Cycloheximide, ATP, creatine phosphokinase, GTP, spermine tetrahydrochloride aprotinin, PMSF, DTT, 5,6-dichlorobenzimidazole riboside, salts, and buffers were purchased from Sigma Chemical (St. Louis, MO). Antibiotics, actinomycin D, serum, and cell culture media were from Life Technologies (Gaithersburg, MD). Hybond N membranes, [$^{32}$P]UTP, [$^{32}$P]CTP, and [$^{32}$P]dCTP (each at 3000 Ci/mmol) were from Amersham (Arlington Heights, IL). TRI reagent was from Molecular Research Center (Cincinnati, OH), and angiotensin peptides were from Bachem (Torrance, CA) or Sigma Chemical. Oligonucleotides were synthesized using Millipore chemicals on a Millipore Cyclone Plus automated DNA synthesizer (Bedford, MA). RNasin and amino acids were purchased from Promega (Madison, WI), and creatine phosphate was from Calbiochem (San Diego, CA).

**Cell culture.** Rat thoracic aorta VSMCs (14) were grown at 37° in a 5% $CO_2$ atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Experimental cells (10–20 passes from primary culture) were grown to confluence and then deprived of serum for 24 hr before treatments by incubation in serum-free medium (Dulbecco's modified Eagle's medium, 100 units/ml penicillin, 100 μg/ml streptomycin). All drugs were diluted from sterile, 100-fold concentrated stocks into the culture media to initiate treatments.

**RNA isolation and Northern blot analysis.** RNA samples were extracted from VSMCs grown on 6-cm-diameter dishes after experimental treatments with TRI reagent, and Northern blot hybridizations were conducted identically as described previously using $AT_1$-R and GAPDH cDNA probes (9). A photographic record was obtained of the ethidium staining pattern on these gels and blots before and after UV light cross-linking to ensure uniform nucleic acid transfer. Those blots for which this was not evident were excluded from further study. A volume integration protocol was used to quantify the autoradiographic hybridization signals with the Personal Densitometer and the ImageQuant program from Molecular Dynamics (Santa Clara, CA). Hybridization signal borders of scanned autoradiograms were marked manually by rectangulation, and the total pixel values within a signal area were summed and subtracted with pixel values within an identically sized rectangular background area on the same film. For comparisons of treatments and presentation, the integrated volumes obtained in this manner were normalized as a percentage of the hybridization values from control, vehicle-treated cells.

**Preparation of polyribosomes.** Confluent VSMCs grown on 150-mm-culture dishes were serum deprived for 24 hr and then incubated an additional 2 hr in the absence or presence of 100 nM Ang II. Cells were washed with phosphate-buffered saline, and polyribosomes were isolated with modifications of described protocols (15). VSMC monolayers were harvested with 0.25% trypsin/versine, pelleted, and washed once in buffer A (1 mM potassium acetate, 10 mM Tris·HCl, pH 7.6, 1.5 mM magnesium acetate). The cell pellet was resuspended in 2.5 ml of buffer B (buffer A plus 2 mM DTT, 1 mM PMSF, 0.023 TIU/ml aprotinin, 2 units RNasin/ml) and incubated for 10 min on ice; then, they were homogenized with 15 vigorous strokes using a small hand-held glass tissue grinder (Kontes Glass, Vineland, NJ; clearance, 0.1 mm). The homogenates were centrifuged at 3000 × *g* for 15 min at 4°, and this supernatant was overlaid onto 1.5 ml of a 30% sucrose in buffer B. The samples were centrifuged at 130,000 × *g* at 4° for 2.5 hr using a Beckman SW 60 rotor. The pellet containing the polyribosomes were washed twice carefully with buffer A and resuspended in buffer A containing 2 mM DTT, 1 mM PMSF, and 2 units/ml RNasin. The polyribosomes were used immediately or aliquots were quickly frozen in a methanol/dry ice bath, stored at −80°, and thawed only once before use.

***In vitro* mRNA stability assays.** These assays were essentially performed according to published protocols (15). Each 30-μl reaction was mixed on ice and contained 8–15 μg of polyribosome RNA (assessed by absorbency at 260 nm), 100 mM potassium acetate, 5 mM magnesium acetate, 10 mM Tris·HCl, pH 7.6, 10 μg of yeast tRNA, 30 μM concentration of each amino acid, 10 mM creatine phosphate, 1 μg of creatine phosphokinase, 0.2 mM GTP, 0.1 mM spermine tetrahydrochloride, 2 mM ATP, 2 mM DTT, and 25 units of RNasin. The samples were incubated for 20 min on ice before increasing the temperature to 37° for the indicated times. Reactions were terminated by the addition of 0.8 ml of TRI reagent. RNA isolation was performed as described above, and 5–10 μg was loaded onto formaldehyde gels. Northern blot hybridization was used to assess the quantity of $AT_1$-R mRNA in the samples as described above.

**Preparation of nuclear extracts.** Nuclei were isolated from cultured VSMCs as described previously (9). Protein extracts of these were prepared according to established protocols (16) except additional protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml antipain, and 0.023 TIU/ml aprotinin) were added to each of the hypotonic, low-salt, and high-salt buffers. Aliquots were stored at −80° and thawed only once before use. Protein concentration was determined by the Bradford method using a BioRad kit (Hercules, CA) and IgG as standard according to the manufacturer's recommendations.

**Plasmid constructs and *in vitro* transcription.** The rat vascular smooth muscle cell $AT_1$-R cDNA from pCa18b (17), including flanking pCDM8 vector remnant, was excised with *Hin*dIII and *Not*I and cloned into those sites of pBluescript II (Stratagene, La Jolla, CA). Full-length (bases 1–2213) sense riboprobes were transcribed *in vitro* by linearizing this plasmid with *Not*I and using T3 RNA polymerase. A 1–400-base RNA was transcribed from the same construct by linearization with *Ssp*I. To transcribe the 3′-untranslated region of the $AT_1$-R cDNA, a 1446-bp *Hin*cII/*Sau*I fragment was excised and discarded, and the plasmid was recircularized by blunt end ligation. Linearization with *Not*I and *in vitro* transcription with T3 RNA polymerase generated a 767-base riboprobe from bases 1446–2213 of the RNA. A 2.3-kb *Xba*I fragment of the pCa18b $AT_1$-R cDNA, with pCDM8 vector remnant, was subcloned in the *Xba*I site of pBluescript II to create pDJ1, placing the 5′ end of the cDNA next to the T7 RNA promoter and used with T7 RNA polymerase to transcribe sense RNA from the following pDJ1 derivatives. A *Sac*I site was introduced at 1627 bp by oligonucleotide-directed mutagenesis to create pGN8. A 1627-bp pGN8 fragment was excised by *Sac*I diges-

tion and discarded, and the plasmid was recircularized to create pGN9. Linearization of this with *Hin*dIII was used to transcribe a 1627–2213-base RNA, and linearization with *Rsa*I was used to generate a 1627–1905-base transcript. The complete cDNA in pGN8 was excised with *Not*I, and after an additional *Sau*I digestion, the cDNA insert was cut at 1446 bp, leaving 767-bp downstream and 1446-bp upstream fragments. After Klenow flushing, the 767-bp fragment encoding bases 1446–2213 of the mRNA was re-ligated into the blunted *Not*I sites of the plasmid to create pGN11. Linearization with *Sac*I (followed by additional flushing of the 5′ recessive end) was used to transcribe a 1446–1627-base RNA. A *Sac*I site was inserted in pDJ1 at 1864 bp with oligonucleotide-directed mutagenesis to create pGN10. A 1864-bp fragment was excised from pGN10 with *Sac*I and discarded, and the plasmid was recircularized. Linearization of pGN10 with *Hin*dIII was used to generate a 1864–2213-base transcript.

Before *in vitro* transcription, the linearized DNA templates were digested for 30 min at 56° with 200 μg/ml proteinase K, extracted with phenol/chloroform, ethanol precipitated, and dissolved in RNase-free water. Transcription reactions were performed according to the manufacturer's instructions, using a Maxiscript Kit (Ambion, Austin, TX). For radioactive riboprobe synthesis, each reaction contained ~1 μg of DNA template, 80 μCi of [$^{32}$P]UTP (or [$^{32}$P]CTP in some experiments) (3000 μCi/mmol), 1 mM GTP, and 10 mM $m^7$G(5′)ppp(5′)G-cap analog (Ambion). After a 1-hr incubation at 20°, the transcription reactions were exposed to DNase I and extracted with phenol/chloroform. The free nucleotides were removed using a Bio-Spin 30 chromatography column (BioRad). Riboprobes were ethanol precipitated and dissolved in RNase-free water. The specific activity (typically 1–3 × $10^5$ cpm/pmol) was determined following the manufacturer's protocol (Ambion) using a trichloroacetic acid precipitation method. In some experiments, larger riboprobes were gel purified on a 5% acrylamide/5.8 M urea gels and eluted. Probes were stored at −80° and used within 48 hr. Nonradioactive, capped RNA transcripts were synthesized following the manufacturer's protocol using a Message Machine *in vitro* Transcription Kit (Ambion). Size, integrity, and quantity of the transcripts were verified by inspection of samples subjected to agarose/formaldehyde gel electrophoresis and ethidium staining.

**mRNA-protein binding assays.** These assays were modifications of published protocols (18). Polysomal protein (10 μg) was mixed on ice with 4–10 pmol of [$^{32}$P]UTP-labeled RNA transcripts (4 × $10^5$–$10^6$ cpm) in a volume of 10 μl containing 5 μg of yeast tRNA, 2 mM DTT, 1 mM PMSF, 2 units of RNasin, 10 mM HEPES, pH 7.9, 40 mM potassium chloride, 3 mM magnesium chloride, and 5% glycerol in the absence or presence of the indicated amounts of competitor RNAs. Binding reactions were incubated for 15 min at room temperature followed by the addition of 0.25 unit of heparin. After 5 min of additional incubation at room temperature, the samples were exposed for 30 min to UV light on ice in 96 multiwell plates using a Stratalinker 2400 (Stratagene). After this, 0.5 μl (2.5 μg) of RNase A and 0.5 μl (2.5 units) of RNase T1 were added, and the samples were incubated for 30 min at 37°. An equal volume of a 2× SDS-gel loading buffer was added, and after a 10-min incubation at 70°, the samples were electrophoresed through a 12% SDS-polyacrylamide Laemmli gel at 200 V for 5 hr at 4°. The gels were dried and exposed to film at −80° for 12–24 hr.

## Results

Ang II was added to VSMCs to a concentration of 100 nM Ang II at time zero, and the relative abundance of $AT_1$-R mRNA was determined by Northern blot hybridization analysis at various later time points (Fig. 1). The probe hybridizes to two alternatively processed transcripts at 2.2 and 3.2 kb (17). Only the autoradiographic signals generated from hybridization to the considerably more abundant 2.2-kb transcript were quantified and compared. However, the expression of both transcripts seem to be regulated similarly by Ang II. After autoradiography, the $AT_1$-R cDNA probe was stripped from the same blot, which was subsequently hybridized with a GAPDH probe as described previously (7) to quantify the GAPDH mRNA for each sample.

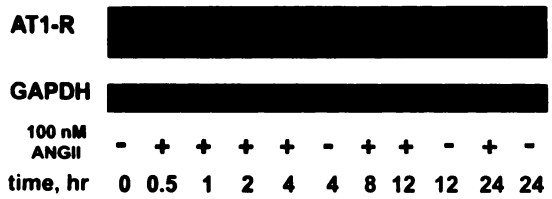


**Fig. 1.** Representative Northern blot hybridization autoradiograms. Hybridization of an $AT_1$-R (*AT1-R*) cDNA probe to Northern blots of 10 μg of total RNA extracted from VSMCs treated for the indicated times with Ang II and hybridization of a GAPDH cDNA probe to the same blot stripped of the $AT_1$-R cDNA probe. RNA was extracted from cells after single-dose treatment in the absence (−) or presence (+) of 100 nM Ang II (*ANGII*) for the indicated times. Results are representative of three separate experiments.

These autoradiograms reveal that $AT_1$-R activation induces a significant time-dependent attenuation of the $AT_1$-R transcript levels. The quantified results of several experiments are shown in Fig. 2. $AT_1$-R mRNA levels are reduced by 50% after 2 hr of exposure to Ang II and maximally to 32 ±

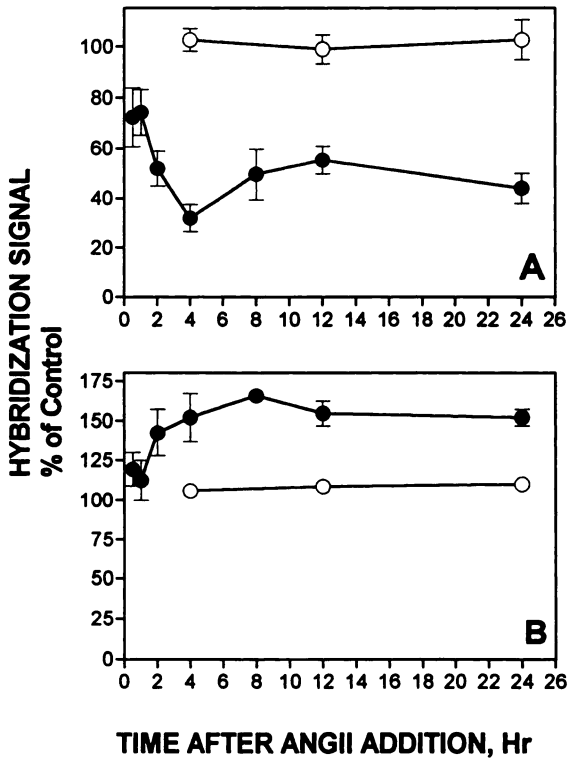


**Fig. 2.** Quantification of Northern blot hybridization signal intensity. A, Time course of the $AT_1$-R mRNA response in the absence (○) or presence (●) of 100 nM Ang II. B, Time course of the GAPDH mRNA response in the absence (○) or presence (●) of 100 nM Ang II. RNA was extracted from VSMCs grown on 6-cm dishes at the indicated times after initial exposure to either vehicle or 100 nM Ang II (*ANGII*). After Northern transfer, hybridizations were performed as described in Experimental Procedures on 10-μg samples of total RNA. *Points*, relative hybridization signal (mean ± standard error) normalized to the zero-hour treatment with vehicle (100%) from three independent experiments.

9% (mean ± standard error, $n$ = 3) of the level in vehicle-treated control cells 4 hr after exposure to 100 nM Ang II. Although a reduction of mRNA levels is sustained for up to 24 hr, a tendency toward a rebound to 50% of control levels is apparent. At various time points over the 24-hr experimental period, $AT_1$-R mRNA levels were determined from vehicle-treated cells and are shown to remain constant relative to the mRNA levels at the zero hour time point, demonstrating the steady state stability of the mRNA over the experimental time frame in response to vehicle. Because the GAPDH mRNA is induced by Ang II up to 50% of that in vehicle-treated control cells, no attempt was made to use these for normalization of the $AT_1$-R hybridization signals.

The stability of the $AT_1$-R mRNA was assessed after blocking VSMC gene transcription through preincubation of the cells for 30 min with 20 μg/ml actinomycin D. Under these conditions, there is a complete (>99%) inhibition relative to vehicle control of [$^3$H]uridine incorporation during a 4-hr chase into poly(A)$^+$ RNA fractions isolated from VSMCs (results not shown). The $AT_1$-R mRNA decays to ~50% of the vehicle-treated control levels after 6 hr of inhibitor treatment (Fig. 3, *top*). This rate of decay is not distinguishable from that in cells pretreated with actinomycin D for 30 min before subsequent addition of Ang II. The hybridization signals derived from the early time points after Ang II treatment (shown in Fig. 2) are also replotted on Fig. 3 and show that the rate of $AT_1$-R mRNA decay in response to Ang II is accelerated ~3-fold relative to that observed by transcriptional inhibition alone. Together, these data demonstrate that the $AT_1$-R mRNA is moderately unstable in unstimulated VSMCs but destabilized further in response to Ang II and that this effect of Ang II requires ongoing transcription. In contrast, the levels of GAPDH mRNA remain stable over this period of time in the presence of transcriptional inhibitors, and GAPDH mRNA induction by Ang II is blocked by actinomycin D (Fig. 3, *bottom*).

Cycloheximide (15 μg/ml) pretreatment was used to block mRNA translation in VSMC, followed by a 4-hr incubation in the absence or presence of 100 nM Ang II (Fig. 4). Although cycloheximide treatment alone enhances the levels of $AT_1$-R mRNA, it also blocks the ability of Ang II to attenuate $AT_1$-R mRNA levels. The simplest interpretation of these experiments with broad inhibitors of gene expression is that enhanced degradation of $AT_1$-R mRNA by Ang II likely involves the induction and synthesis of a factor or factors that mediate this effect.

The stability of the $AT_1$-R mRNA *in vitro* was thus examined in enriched polyribosomal fractions isolated from VSMC subsequent to preincubation of the cells for 2 hr in the absence or presence of 100 nM Ang II. Aliquots of polyribosomes were incubated in a translationally competent regenerating

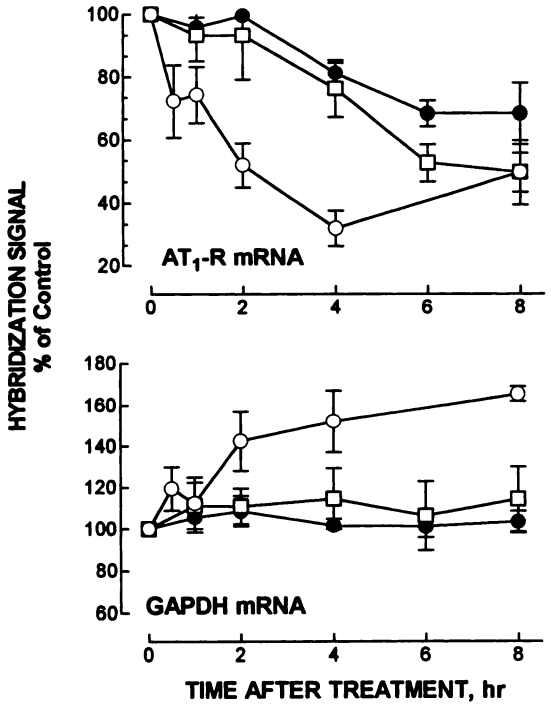


**Fig. 3.** Effect of transcriptional blockade with actinomycin D on VSMC mRNA levels. *Top*, $AT_1$-R mRNA levels. *Bottom*, GAPDH mRNA levels. VSMCs on 6-cm dishes were pretreated for 30 min with 20 μg/ml actinomycin D and then exposed for the indicated times to vehicle (□) or 100 nM Ang II (●) before extraction of RNA. The effect of Ang II in the absence of actinomycin D is also shown (replotted from Fig. 2, ○). After Northern blot hybridization with the $AT_1$-R cDNA probe, the blots were stripped and rehybridized with a GAPDH cDNA probe. *Points*, relative hybridization signal (mean ± standard error of three independent experiments) normalized to the mRNA hybridization signal obtained from cells that had been treated for 30 min with either actinomycin D alone (□, ●) or vehicle alone (○).

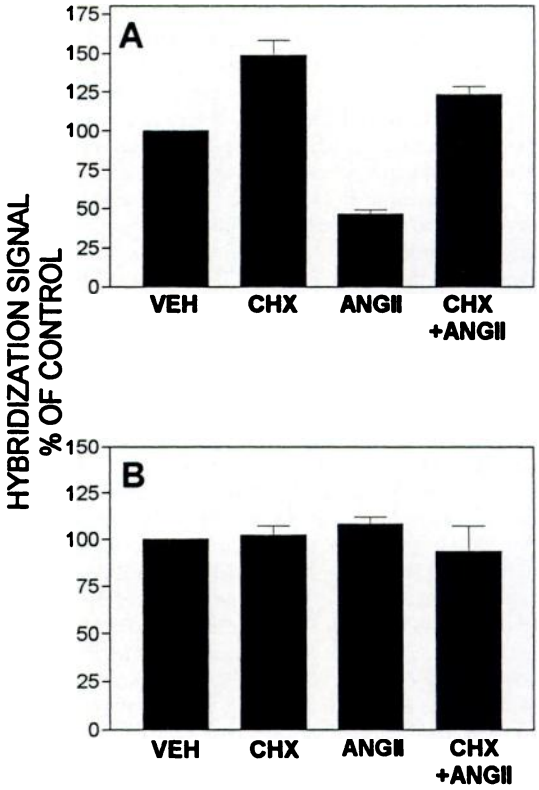


**Fig. 4.** Effect of cycloheximide on Ang II-mediated changes in VSMC mRNA. A, Relative hybridization to the $AT_1$-R mRNA. B, Relative hybridization to the GAPDH mRNA. VSMCs were incubated for 4 hr with vehicle (basal; *VEH*), 15 μg/ml cycloheximide (*CHX*), 100 nM Ang II (*ANGII*), or 15 μg/ml cycloheximide in the presence of 100 nM Ang II (*CHX+ANGII*). After hybridization to the $AT_1$-R cDNA probe, the blots were stripped and rehybridized with a GAPDH cDNA probe. The data are expressed as a percentage of the hybridization signal from RNA from vehicle-treated cells. *Bar*, mean ± standard deviation of three separate experiments.

system at 37° for increasing periods of time up to 60 min, followed by RNA extraction and examination of $AT_1$-R mRNA content by Northern blot hybridization analysis (Fig. 5). Ethidium bromide-stained gels of these samples revealed intact 28*S* and 18*S* ribosomal RNAs over the duration of the *in vitro* incubation period, attesting to the general stability of RNA in the *in vitro* system. This was further confirmed by the results of hybridization with a GAPDH cDNA probe, which showed no significant decay of the GAPDH mRNA over a 60-min incubation *in vitro*, although Ang II treatment again shows GAPDH mRNA induction. The $AT_1$-R mRNA hybridization signals at the 0-min control time points in these experiments are markedly reduced in polyribosomes isolated from VSMCs exposed to 100 nM Ang II for 2 hr compared with that in polyribosomes from vehicle-treated cells. This is not unexpected, given that steady state $AT_1$-R mRNA levels in intact VSMCs are reduced to 50% of control levels after a 2-hr treatment with the agonist. However, the *in vitro* rate of decay of the $AT_1$-R mRNA signal in polyribosomes from Ang II-stimulated cells is accelerated relative to that in vehicle-treated cells, which is evident by the densitometric analysis of three separate experiments (Fig. 6). The simplest interpretation of this cell-free analysis of $AT_1$-R mRNA stability suggests that polyribosomal-associated factors induced or activated by Ang II enhance $AT_1$-R mRNA degradation.

We postulated that putative polyribosomal mRNA destabilizing factors regulated by Ang II may involve direct interactions of polyribosomal proteins with the $AT_1$-R mRNA. Binding assays were performed in which enriched VSMCs polyribosomal fractions were mixed with *in vitro* transcribed, [$^{32}$P]UTP-labeled mRNAs prepared from $AT_1$-R cDNA templates. Subsequent exposure to UV light cross-links proteins in close proximity to the labeled mRNAs. These bound proteins are then detectable by separation on SDS-PAGE and autoradiography. In this way, we were able to first demonstrate induction by Ang II of polyribosomal protein binding to a full-length labeled mRNA probe (bases 1–2213), which was completely inhibited by an unlabeled RNA encoding the entire 3′-untranslated region of the $AT_1$-R mRNA (bases 1146–2213) but not by an unlabeled $AT_1$-R RNA encoding the 5′-untranslated and coding regions up to base 1445 ($n$ = 3; data not shown). For this reason, we focused on interactions of these mRNA-binding proteins using probes from the 3′-untranslated region of the $AT_1$-R mRNA.

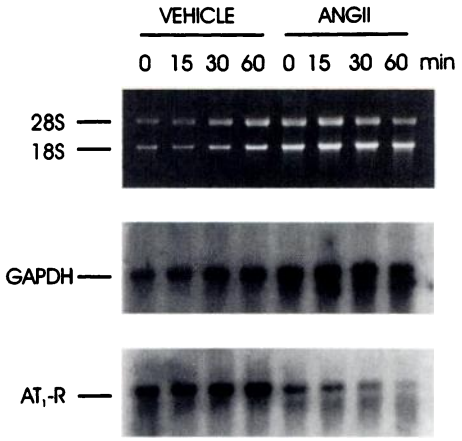


**Fig. 5.** Time course of mRNA levels in polyribosomal extracts of VSMC incubated *in vitro*. *Top*, representative ethidium bromide-stained formaldehyde agarose gel showing intact 28 S and 18 S ribosomal RNA (*arrows*) in polyribosomal extracts incubated for 0–60 min at 37° *in vitro*. *Middle*, representative autoradiogram of a GAPDH mRNA hybridization signal (*arrow*) using a GAPDH cDNA probe to a blot of the same gel. *Bottom*, representative autoradiogram of an $AT_1$-mRNA hybridization signal using an $AT_1$-R cDNA probe on the same blot. Polyribosomes (~8 μg of RNA) were extracted from VSMCs that had been incubated for 2 hr with either vehicle (*left four lanes*) or 100 nM Ang II (*ANGII*) (*right four lanes*) and subsequently incubated *in vitro* at 37° for 0, 15, 30, or 60 min at 37° as described in the Experimental Procedures. After this, the RNA was extracted from the samples and electrophoretically separated on a formaldehyde agarose gel, blotted to a nylon membrane, and subsequently iteratively hybridized. Data are representative of three experiments.

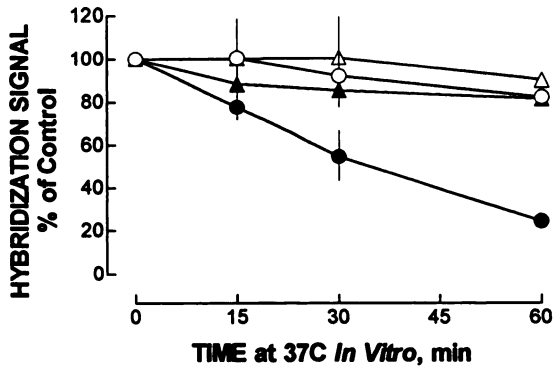


**Fig. 6.** Differential mRNA decay in polyribosomes isolated from VSMCs. Densitometric quantification of autoradiographic signals from hybridizations to Northern blots of polyribosomal RNAs incubated *in vitro* at 37° for the indicated times. $AT_1$-R mRNA levels in polyribosomes isolated from VSMCs treated for 2 hr in the absence (○) or presence (●) of 100 nM Ang II. GAPDH mRNA levels in polyribosomes isolated from VSMCs treated for 2 hr in the absence (△) or presence (▲) of 100 nM Ang II. *Points*, hybridization signal expressed as a percentage of the respective hybridization signal obtained from polyribosomes incubated at 37° for 0 min (mean ± standard error of three experiments).

When the aforementioned 3′-untranslated region competitor from bases 1446–2213 of the mRNA was used as a labeled probe, the time-dependent induction by Ang II of a complex pattern VSMC polyribosomal mRNA-binding proteins was evident (Fig. 7). In analyses of several such experiments, we conclude that at least six proteins bind to these probes specifically with relative molecular masses of ~86,000, ~82,000, ~63,000, ~51,000, ~45,000, and ~28,000 (termed p86, p82, p63, p51, p45, and p28), respectively. Binding of several of these proteins is evident in unstimulated cells but seem to be significantly and maximally induced in cultured VSMCs at 2–4 hr after VSMC treatment with 100 nM Ang II, perhaps with the exception of p86 (Fig. 7). To map further the location of polyribosomal binding on the mRNA, competition binding studies were performed using polyribosomal protein extracts prepared from VSMCs stimulated for 2 hr with 100 nM Ang II. Binding of these proteins to the larger [$^{32}$P]UTP-labeled probe encoding bases 1446–2213 of the transcript is competed for effectively and in a dose-dependent manner only by an unlabeled RNA representing the most distal bases (1864–2213) of the transcript and not by unlabeled RNAs encoding bases 1146–1624 or bases 1627–1905 of the transcript (Fig. 8). A 400-base competitor mRNA prepared from the 5′ end of the $AT_1$-R mRNA, sized similarly to the 1864–2213 base competitor, failed to inhibit polyribosomal protein binding to the 446-2213-base riboprobe ($n$ = 3,

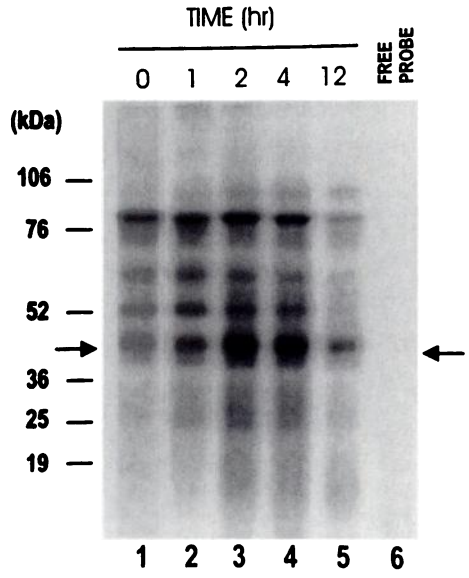


**Fig. 7.** Time course for Ang II-induced polysomal protein binding to the 3′-untranslated region of the $AT_1$-R mRNA. Cultured VSMCs were stimulated without (*lane 1*) or with (*lanes 2–5*) a single dose of 100 nM Ang II for the indicated times. After extraction, 10 $\mu$g of polyribosomal protein was incubated with a capped, [$^{32}$P]UTP radiolabeled 3′-untranslated region $AT_1$-R mRNA probe (bases 1446–2213; ~6 pmol; 1 × $10^6$ cpm). *Lane 6*, probe incubated in the absence of polyribosomal protein yet otherwise treated like the other samples. The samples were then cross-linked with UV light before treatment with RNAases and electrophoretic resolution by SDS-PAGE. Autoradiogram is representative of three experiments. *Arrows*, p45 protein; *markers*, sizes (in kDa) of the relative mobilities of a protein molecular mass standard.

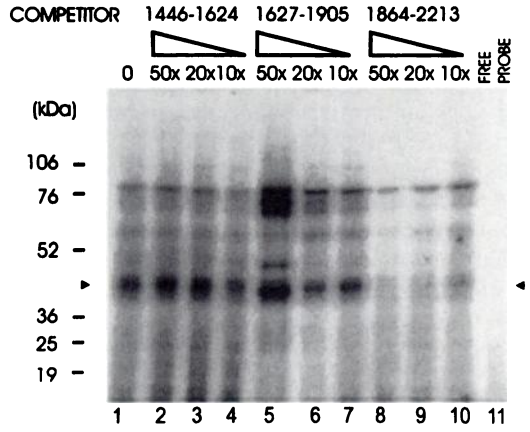


**Fig. 8.** Competition mapping polyribosomal protein binding activity to the most distal 349 bases of the 3′-untranslated region in the $AT_1$-R mRNA. Cultured VSMCs were stimulated for 4 hr with 100 nM Ang II before isolation of polysomal extracts. Protein (10 $\mu$g) aliquots of these were incubated with ~6 pmol (1 × $10^6$ cpm) of a [$^{32}$P]UTP-labeled $AT_1$-R mRNA probe composed of bases 1446–2213 in the absence (*lane 1*) or presence (*lanes 2–10*) of a molar excess of unlabeled competitor RNAs encoding regions of the $AT_1$-R mRNA 3′-untranslated region. Unlabeled RNA competitors: *lanes 2–4*, bases 1446–1624; *lanes 5–7*, bases 1627–1905; *lanes 8–10*, bases 1864–2213. *Lane 11*, labeled probe treated like all other samples except polyribosomal protein is lacking. *Arrows*, position of the p45-labeled protein. Autoradiogram is representative of three experiments.

data not shown). This series of experiments indicates that Ang II induces in VSMCs several polyribosomal mRNA-binding proteins that bind to the 3′-untranslated region of the $AT_1$-R mRNA and that the most distal 349 bases of the transcript are necessary for these interactions. The most

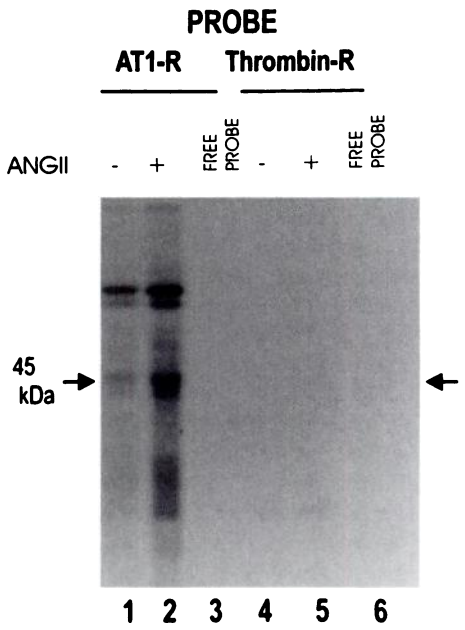


**Fig. 9.** Ang II (*ANGII*)-induced up-regulation of polyribosomal p45 protein binding to the $AT_1$-R mRNA but not the thrombin receptor mRNA. Cultured VSMCs were stimulated for 4 hr with vehicle (*lanes 1 and 4*) or 100 nM Ang II (*lanes 2 and 5*) before extraction of polyribosomes. Protein (10 $\mu$g) were incubated with ~6 pmol (1 × $10^6$ cpm) of a [$^{32}$P]UTP-labeled $AT_1$-R mRNA probe (bases 1864–2213) or a full-length (bases 1–3418) rat VSMC thrombin receptor riboprobe (~6 pmol; 3 × $10^6$ cpm) of the thrombin receptor mRNA. The samples were exposed to UV light, exposed to RNAases, and separated by electrophoresis on denaturing 12% SDS-PAGE gels. *Lanes 3 and 6*, probes incubated like the other samples except protein was lacking. *Arrows*, p45 mRNA-binding protein. Autoradiogram is representative of three separate experiments.

distal 3′ 349 bases of the $AT_1$-R mRNA, when now used as a probe rather than as a competitor, seem to be also sufficient for Ang II-induced binding of the p86, p82, p45, and p28 proteins, whereas binding of the p63 and p51 proteins is markedly reduced (Fig. 9). Interestingly, as Fig. 9 shows, these same proteins do not bind as robustly to a full-length [$^{32}$P]UTP-labeled thrombin receptor mRNA probe, although longer exposures of the autoradiograms such as shown do reveal cross-linking of VSMC polyribosomal proteins to it. The thrombin receptor mRNA expressed in these VSMCs, however, differs from that for the $AT_1$-R in that it is not destabilized by Ang II treatment under these conditions.[1]

Subcellular distribution studies were performed to determine whether $AT_1$-R mRNA receptor mRNA-binding proteins are present elsewhere within VSMCs stimulated for 2 hr with 100 nM Ang II, again using a labeled probe encoding bases 1146–2213 of the $AT_1$-R mRNA transcript. Detectable binding is absent in S130 cytosolic supernatant (Fig. 10, *lane 1*), but the addition of S130 aliquot to enriched polyribosomes seems to enhance the level of binding observed in polyribosomes alone (Fig. 10, compare *lanes 4 and 3*). Binding is evident in a postnuclear supernatant termed cytoplasm, which includes cytosol and polyribosomes (*lane 3*), and a high degree of binding is present in VSMC nuclear extracts. Some of these nuclear proteins migrate coincidentally with polyri-

[1] G. Nickenig, X. F. Wang, and T. J. Murphy, Differential regulation of thrombin and serotonin $5HT_2$ receptor mRNA in vascular smooth muscle cells, manuscript in preparation.

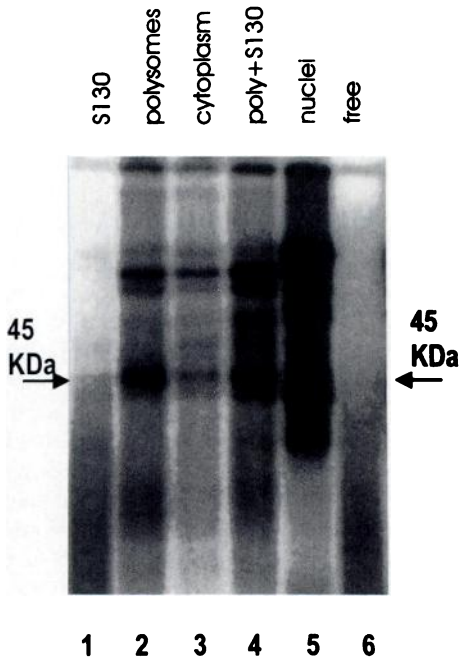


**Fig. 10.** Subcellular distribution of protein that bind to the $AT_1$-R mRNA 3′-untranslated region. VSMCs were stimulated for 4 hr with 100 nM Ang II before homogenization and isolation of the indicated fractions. Proteins (10 $\mu$g) from each fraction were incubated with 6 pmol ($1 \times 10^6$ cpm) of a [$^{32}$P]UTP-labeled $AT_1$-R mRNA 3′-untranslated region riboprobe (bases 1446–2213). *Lane 1*, 130,000 × *g* cytosolic supernatant; *lane 2*, enriched polyribosomes; *lane 3*, crude, post-nuclear cytoplasmic fraction; *lane 4*, 130,000 × *g* cytosolic supernatant (10 $\mu$g of protein) plus enriched polyribosomes (10 $\mu$g of protein); *lane 5*, nuclear protein extract; *lane 6*, probe not exposed to extract. *Arrows*, p45 mRNA-binding protein.

bosomal proteins, including p82, p51, and p45. We have not characterized these nuclear mRNA-binding proteins further, but they may represent either different factors, factors that shuttle between the nucleus and polyribosomes, or proteins associated with polyribosomes that remain physically associated with the nucleus after cellular fractionation. Additional experiments will test these possibilities.

## Discussion

A 4-hr treatment of VSMCs with Ang II attenuates nuclear $AT_1$-R mRNA synthesis partially (~50%) (9). However, $AT_1$-R mRNA levels within intact VSMCs are reduced at a faster rate after Ang II treatment than what occurs using more effective general transcriptional inhibitors alone. This suggests that Ang II induces an additional post-transcriptional process, superimposed on transcriptional inhibition, that likely involves enhanced mRNA degradation and is defined here as destabilization. Further support for a role for an inductive process in Ang II-mediated $AT_1$-R mRNA destabilization are the observations that (1) pretreatment with transcriptional and translational inhibitors block the agonist effect and (2) polyribosomal $AT_1$-R mRNA disappears more rapidly *in vitro* after prior Ang II stimulation of the cells compared with that in vehicle-treated cells. Importantly, this latter *in vitro* experiment removes the $AT_1$-R mRNA from the source of its synthesis and thus directly assesses $AT_1$-R mRNA stability post-transcriptionally. Here, we observe the $AT_1$-R mRNA is perhaps more unstable after Ang II stimulation than what might be predicted from measurements of mRNA levels of intact cells. Taken together, our present observations and previous studies suggest that an Ang II-inductive mRNA destabilization process is superimposed on transcriptional attenuation of the $AT_1$-R gene in VSMCs. Both mechanisms, and perhaps others, are ultimately responsible for attenuated $AT_1$-R gene expression in VSMCs. We argue, however, that because the mRNA disappears at a rate faster than is seen using transcriptional inhibitors alone, destabilization likely plays a more significant role than the transcriptional attenuation within the first few hours of stimulation by Ang II.

Because Ang II-elicited $AT_1$-R mRNA destabilization is transcriptionally inductive, this suggests that latent mRNA destabilizing factors, under the control of $AT_1$-R-stimulated signaling networks, alone are not sufficient for this response. Although cytoplasmic $AT_1$-R mRNA is moderately unstable in unstimulated cells, clearly something else must be induced for the more robust destabilization elicited by Ang II. Whether the putative inductive factor or factors enhance the activity of the latent machinery responsible for $AT_1$-R mRNA instability in the unstimulated conditions or is itself a destabilizing factor absent within unstimulated VSMCs is a matter for continued exploration. The experiments shown in this report do not test these possibilities. We have shown previously that growth factor receptor activation in VSMCs also results in attenuated $AT_1$-R mRNA synthesis and enhanced destabilization (7). As for the response to Ang II, this destabilization effect of growth factors is also transcriptionally and translationally coupled (7). However, those studies show the $AT_1$-R mRNA is stabilized modestly by growth factor receptor activation in the presence of transcriptional inhibitors, unlike what is shown in the present report in response to Ang II. This suggests that latent factors that participate in the control of $AT_1$-R mRNA stability likely exist in VSMCs, may be modulated by certain extracellular signals independent of new transcription, and may have opposing effects relative to the transcriptionally coupled destabilizing factors. The failure to observe $AT_1$-R mRNA stabilization by Ang II during transcriptional blockade, unlike that observed during growth factor receptor activation, might imply that these factors differ. Another possibility is that the presumably divergent signaling networks activated by either Ang II or the growth factors converge on a common set of mRNA regulatory factors, with subtly distinct regulatory outcomes. It will be of interest, therefore, to determine whether the same destabilizing factors induced and regulated by growth factors are also controlled by Ang II through $AT_1$-R activation.

Although the observation that cycloheximide can prevent Ang II-induced degradation of $AT_1$-R mRNA is consistent with the synthesis of an inducible regulatory protein or proteins, it is also possible that blockade of Ang II-induced destabilization of $AT_1$-R mRNA by cycloheximide reflects a requirement for $AT_1$-R mRNA translation to precede its degradation. The literature suggests that destabilization of mRNAs is in general a translationally coupled process, although such findings to date have eluded satisfactory experimental evidence for mechanistic explanations (11). Consistent with this are data shown here revealing higher $AT_1$-R mRNA levels after cycloheximide treatment, in the absence of agonist. Perhaps this result reflects the failure of the $AT_1$-R mRNA to form a translationally dependent structure compliant for subsequent degradation or the possibility that

cycloheximide-arrested ribosomes are unable to read cryptic arrest signals and subsequently recruit destabilization factors to the mRNA. This and other certain testable predictions related to this issue are one focus of ongoing studies.

The accelerated loss of $AT_1$-R mRNA from polyribosomal extracts of Ang II-stimulated VSMCs suggests that important processes related ultimately to $AT_1$-R mRNA degradation occur here but do not indicate that the mRNA is degraded on polyribosomes, *per mean*. Nevertheless, as an initial approach to further explore the molecular basis for these processes, the hypothesis was tested that Ang II stimulates the association of polyribosomal mRNA-binding proteins with the $AT_1$-R mRNA. The results presented in this report clearly support this staged hypothesis because we identified several factors that bind in an apparent specific context to the $AT_1$-R mRNA. We mapped the interactions of these proteins on the $AT_1$-R mRNA to its most distal 349 bases in the 3′-untranslated region, and no fewer than six $AT_1$-R mRNA-binding proteins are identified clearly by the cross-linking approach that we used. Because each of these proteins has been identified by virtue of their ability to be labeled with $^{32}P$ supplied in the assay on the $\alpha$ phosphate of UTP, it is reasonable to conclude that each of their mRNA recognition properties involves close apposition to uracil bases on the mRNA. We argue that each protein likely interacts with distinct uridine-containing elements or structures within the mRNA. Whether these proteins interact coordinately to form a complex is a matter for ongoing studies. If so, this suggests that experiments that focus on identification of a restricted RNA element at its recognition factor may trivialize the complexity of these interactions. In preliminary fractionation experiments of postnuclear VSMC cytosol,[2] we obtained evidence that as early as 30 min after stimulation with Ang II, the VSMC $AT_1$-R mRNA seems to disassemble from heavier polysomes and moves to a sucrose gradient fraction slightly lighter than the 40*S* ribosome peak. We speculate that this peak may represent an intermediate Ang II-induced protein complex that may function to participate in $AT_1$-R mRNA degradation.

Several well-characterized RNA-binding proteins have been shown to recognize AU-rich sequences and/or to bind specifically to AUUUA repeats in essentially unstable mRNAs (19–23). The 349-base sequence between bases 1864 and 2213 of the $AT_1$-R mRNA contains a single AUUUA pentamer, which is coincidentally immediately upstream of a site for alternate splicing and polyadenylation in the $AT_{1a}$-R gene (24). Furthermore, adenosine and uridine residues predominate (87%) in the most 3′ distal 100 bases of the $AT_1$-R transcript. It is thus possible that a member of the AU-repeat mRNA-binding protein family is among those shown here to interact with the $AT_1$-R mRNA. A 35-kDa mRNA-binding protein, termed βARB, is induced by β-adrenergic agonists in DDT-MF1 vas deferens smooth muscle cells and has been implicated in agonist-induced destabilization of the β-adrenergic receptor mRNA (25, 26). Also, an apparently related protein may also participate in down-regulation of thrombin receptor mRNA levels (27). It has been suggested that this factor may be involved in recognition and regulation of transcripts encoding several G protein-coupled receptors (25–27). Both the β-adrenergic receptor and the $AT_1$-R mRNAs are down-regulated by their cognate agonists, and mRNA degradation is likely an important pathway for both systems. However, the properties of the $AT_1$-R and the β-adrenergic receptor mRNA-binding proteins seem to differ. Not only do they differ in size, but the time course of appearance for the 35-kDa β-adrenergic receptor mRNA-binding protein is substantially slower (12–48 hr) than that for the $AT_1$-R mRNA-binding proteins in VSMCs (1–4 hr). Furthermore, the 35-kDa β-adrenergic receptor mRNA-binding protein has been only identified in soluble cytosolic extracts, whereas $AT_1$-R mRNA-binding proteins are not detectable in this fraction in the VSMCs used in this study. Thus, the relationships between the factors that seem to be involved in controlling the expression of the two receptors are uncertain at this time.

It will be of interest to determine the extent to which these findings are relevant in the larger context of mechanisms controlling gene expression in VSMCs in pathological settings. VSMCs are capable of dedifferentiating from a contractile to a proliferative phenotype in response to a diverse array of extracellular signals (28, 29). This phenotypic switch is accompanied by down-regulation of several genes associated with contractile function, such as the $AT_1$-R. It is attractive to speculate that mRNA-destabilizing mechanisms controlling VSMC $AT_1$-R gene expression might reflect more generalized processes used in VSMCs to coordinate its phenotypic characteristics in response to persistent or skewed exposure to extracellular signals.

[2] X. F. Wang and T. J. Murphy, unpublished observations.

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