Mechanism by Which Angiotensin II Stabilizes Messenger RNA for Angiotensinogen

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Abstract
The most important specific regulatory mechanism for hepatic angiotensinogen synthesis and secretion is its stimulation by angiotensin II, the effector peptide of the renin-angiotensin system. In the circulating system, this octapeptide is thought to stimulate hepatic angiotensinogen synthesis through a positive feedback loop. In the present study, we have identified the intracellular mechanisms leading to an increase in angiotensinogen messenger RNA (mRNA) and secretion. In a [3H]uridine-dependent pulse and chase system as well as in hepatocytes in which de novo synthesis of mRNA has been blocked by actinomycin D or 5,6-dichlorobenzimidazole riboside, angiotensin II significantly increased the half-life of angiotensinogen mRNA. In contrast, no effect of angiotensin II on the transcription of angiotensinogen mRNA could be observed in a nuclear run-on assay with nuclei from pretreated hepatocytes, whereas dexamethasone, as a positive control, increased the transcription fivefold to sevenfold. We have isolated a 12-kD protein from the polysomal fraction of isolated hepatocytes, which has an affinity to the nontranslated 3' tail of angiotensinogen mRNA. For in vitro transcription of this mRNA fragment, the DNA sequence coding for the nontranslated 3' tail was excised from the vector pRAG 16 and cloned into the transcription vector pGEM 5zf+. Molecular weight and isoelectric point of the mRNA-binding protein correspond to the parameters of a cytosolic protein that becomes phosphorylated by decreased cyclic AMP concentrations as analyzed in [32P]orthophosphate-loaded hepatocytes. In a cytosolic incubation system in which the polysomal fraction was integrated, the mRNA-binding protein increased the half-life of angiotensinogen mRNA significantly. Thus, there is evidence that the angiotensin II-induced stimulation of hepatic angiotensinogen synthesis depends on mRNA stabilization. (Hypertension. 1994;23[suppl I]:I-120-I-125.)

Key Words • angiotensinogen • transcription, genetic • RNA, messenger • angiotensin II

Angiotensinogen is an important component of the systemic renin-angiotensin system (RAS) and serves as a substrate for the subsequent formation of angiotensin I and angiotensin II (Ang II) by renin and converting enzyme.1 The formation rate of the effector peptide Ang II is also controlled by the circulating amount of substrate because the plasma concentrations of angiotensinogen correspond to the K_M value of the enzyme substrate reaction. Ang II is an important mediator for the regulation of the tone of vascular smooth muscle and for renal reabsorption of NaCl, controlling peripheral vessel resistance and systemic blood pressure by multiple actions.1,2 A pathogenic role of angiotensinogen has been discussed for certain forms of hypertension, and aberrations of the angiotensinogen gene appear to cosegregate with the hypertension phenotype.3 The synthesis of angiotensinogen is of a constitutive nature.4,5 In a circulating system in which the polysomal fraction was integrated, the mRNA-binding protein increased the half-life of angiotensinogen mRNA significantly. Thus, there is evidence that the angiotensin II-induced stimulation of hepatic angiotensinogen synthesis depends on mRNA stabilization.

Involvement of angiotensinogen during acute or chronic inflammation and the physiological consequences are not clear.10 However, regulatory functions of these hormones cannot be interpreted as specific for the synthesis of angiotensinogen because these mediators control the synthesis of a number of other hepatic proteins not functionally related to the circulating RAS. A more specific regulatory function was expected from the components of the RAS themselves, and Ang II was reported to stimulate the synthesis and secretion of angiotensinogen in a positive feedback loop. This effect of Ang II has been reported on primary cultures11 or on hepatocytes in suspension.7 It has been further shown that the stimulation of the secretion rate of Ang II is related to an increase in intracellular angiotensinogen mRNA concentrations, suggesting that the secretion of angiotensinogen is of a constitutive nature.7,12 In a previous report, we have identified the inhibition of adenyl cyclase but not the stimulation of phosphodiesterase C with the subsequent increase in inositol 1,4,5-trisphosphate, diacylglycerol, and intracellular calcium concentrations as the major pathway by which Ang II stimulates angiotensinogen synthesis and secretion.7 In the present study, we demonstrate that the incorporation of angiotensinogen mRNA is not dependent on transcriptional stimulation but rather on stabilization of mRNA. With band-shift and cross-linking analyses as detection systems, we were able to isolate an intracellular hepatic protein that selectively binds to the 3' nontranslated region of angiotensinogen mRNA. The increase in angiotensinogen mRNA half-life was also observed in a cell-free incubation system after addition of this protein. We further describe experiments that suggest a

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link between the Ang II–dependent decrease in cyclic AMP (cAMP) and the mRNA-stabilizing protein, indicating that phosphorylation is an important factor in the activity of the isolated mRNA-binding protein.

Methods
Isolation of Hepatocytes and Hepatic Nuclei

Hepatocytes were prepared from livers of male Sprague-Dawley rats weighing approximately 200 g (Ivanovas, Kisslegg, Germany) by the collagenase technique and subsequent Percoll density gradient centrifugation as described previously. At a density of 1 to 3×10^6 cells/mL, cells were resuspended in a modified minimal essential medium (MEM, Serva, Heidelberg, Germany) and incubated in a shaking water bath (CO/stokes/min, 37ºC, 95% O2/5% CO2) for various times. After a pretreatment period of 2 hours in the presence or absence of dexamethasone (10 µmol/L) or angiotensinogen (90 nmol/L steady-state concentrations during continuous infusion), nuclei were isolated under hypotonic conditions (10 mmol/L HEPES [pH 7.5], 3 mmol/L MgCl2, 40 mmol/L KCl, 5 mmol/L glucose, and 1 mmol/L dithiothreitol [DTT, Serva]) as described by Greenberg and Ziff and were resuspended in 10 mmol/L Tris-HCl, 5 mmol/L MgCl2, 40% glycerol, and 1 mmol/L EDTA.

Nuclear Run-on Assay

Aliquots of 10^6 (100 µL) nuclei were substituted with 100 µL transcription buffer (10 mmol/L Tris-HCl [pH 8], 5 mmol/L MgCl2, 300 mmol/L KCl, and 5 mmol/L DTT); 0.5 mmol/L ATP, GTP, and CTP; and 100 µCi [32P]UTP (760 Ci/mmol) for one-half hour to allow synthesis of radioactively labeled mRNA at 37ºC. Total RNA was isolated after a DNase I (0.6 µg) and a subsequent proteinase K (0.2 mg) digest according to Greenberg and Ziff. The RNA preparations, angiotensinogen mRNA concentrations were analyzed either by liquid hybridization using a nonradioactive 716-nucleotide cRNA transcript derived from vector pRAG3 or by the dot-blot technique with a denatured, EcoRI-linearized, pRAG16 vector as cRNA, which contained nearly the complete DNA sequence coding for angiotensinogen.

Pulse and Chase Analysis

For the determination of angiotensinogen mRNA half-life in hepatocytes, 30-ml suspension aliquots were pulse labeled with 1 mCi [3H]uridine (46 mCi/µmol) for 1 hour; then, the cells were separated by gentle centrifugation (35g), washed, and resuspended in fresh MEM containing a 1500-fold excess of cold uridine. Subsequently, cells were chased during an incubation period of 2 hours in the presence (90 mmol/L) or absence of angiotensinogen. At the beginning of this chase period, the total amount of [3H]uridine uptake was analyzed in two 1-mL suspension aliquots. At 0, 30, 60, 90, 120, and 150 minutes, triplicate samples of 1.5 mL were taken, and the cell pellets were processed for the isolation of RNA. The decay of a specific amount of radioactively labeled angiotensinogen mRNA was analyzed by liquid hybridization with a nonradioactive pRAN2 cRNA transcript.

Isolation of Polysomal Proteins

The polysomal fractions from either control or Ang II–treated hepatocytes were isolated from cytosolic extracts obtained under hypotonic conditions (see "Isolation of Hepatocytes and Hepatic Nuclei"). After removal of the nuclei (centrifugation at 10 000g for 1 to 2 minutes), polysomes were collected from the supernatants in the presence of 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF, Serva) by centrifugation at 210 000g for 3 hours. From the polysomal fractions, binding proteins were dissolved in the presence of 0.5 mol/L KCl according to the method of Müller et al. Dissolved proteins were then isolated by centrifugation and fractionated ammonium sulfate precipitation. The 40% to 50% precipitate, which contained most of the binding material, was dissolved, dialyzed against 100 mmol/L Tris-HCl (pH 7.6), substituted with 14 mmol/L mercaptoethanol, 10% glycerol, 1 mmol/L DTT, and 0.1 mmol/L EDTA, and chromatographed on two columns in series (0.94x25 cm each) of Zorbax GF-250 with 0.2 mol/L Na-phosphate (pH 7.4) as eluant. Aliquots of the eluate were assayed in band-shift or cross-link assays for protein binding to the 3′UTR of angiotensinogen mRNA.

Estimation of Protein Binding at the 3′UTR of Angiotensinogen mRNA

For analysis of protein binding to the 3′UTR of angiotensinogen mRNA, the DNA sequence coding for the nontranslated part of angiotensinogen mRNA was excised from the vector pRAG16 by Nco I and Nde I and inserted into the polylinker site of the transcription vector pGEM 3zf+. Flanked by RNA polymerase promoters SP6 and T7. Restriction with Ava II of the newly constructed plasmid pRAG3 and transcription with T7 polymerase resulted in an mRNA of 269 nucleotides representing the nearly complete 3′UTR of angiotensinogen mRNA. Protein binding was assessed by band-shift or cross-linking analysis as described by Schwemmel et al. Using a [32P]UTP-labeled 3′UTR fragment for band-shift and a [32P]UTP, [32P]ATP, double-labeled fragment for cross-linking analysis. In the band-shift assay, RNA was incubated (30°C, 10 minutes) in the presence of proteins (approximately 10 µg). A complex formation between RNA and proteins inhibited the subsequent gentle RNase T1 digest (10 U, 10 minutes), and larger RNA fragments were identified on a 4% nondenaturating polyacrylamide gel with subsequent autoradiography. For cross-linking analysis, protein-RNA complexes were covalently linked by irradiation at 254 nm. After complete RNase digest (RNase A, 20 U; RNase T1, 20 µg, 1 hour), radioactively labeled proteins were identified by autoradiography subsequent to separation on a 12% polyacrylamide gel.

Identification of Intracellular Phosphorylated Proteins

Phosphorylated proteins were identified in [32P]orthophosphate–loaded hepatocytes. For this purpose, 1-ml aliquots of hepatocytes (2.5 to 3.0×10^6 cells/mL) were loaded with [32P]orthophosphate (500 µCi) for 2 hours. Subsequently, cells were exposed for 10 minutes to a specific cAMP antagonist (Rp-cAMP, 50 µmol/L), guanfacine (10 µmol/L), or angiotensinogen (50 nmol/L), all of which are known to inhibit the activity of protein kinase A, the cAMP antagonist by a direct mechanism, angiotensinogen, or guanfacine by an inhibition of adenyl cyclase activity. Immediately thereafter, cells were collected, and the cytosolic fractions were obtained by detergent permeabilization. Cytosolic extracts were run on a two-dimensional gel electrophoretic system as described by O′Farrell (first dimension: isoelectric point, pH 3 to 10; second dimension: sodium dodecyl sulfate [SDS]–polyacrylamide gel electrophoresis [PAGE] [10% to 15% gel]). Phosphorylated proteins were identified by autoradiography.

Cell-Free Incubation System

To analyze and modulate the degradation of RNA in vitro, we established a cell-free incubation system without nuclei. Cytosolic extracts were obtained under hypotonic conditions, and aliquots of 300 µL were diluted with 450 µL of 20 mmol/L Tris-HCl (pH 7.6), 0.1 mmol/L EDTA, 2 mmol/L DTT, and 0.1 mmol/L PMSF containing 80 U RNase-inhibitor (RNasin, Boehringer Mannheim, Germany) and the following protease inhibitors: 0.7 mmol/L N-[N-(L-3-mercaptooxiran-2-carboxyl]-L-lysyl]-2-lycdyl-

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isolated from 140 μL for the determination of angiotensinogen mRNA and β-actin mRNA.

Determination of Angiotensinogen mRNA and β-Actin mRNA

Angiotensinogen mRNA and β-actin mRNA concentrations were determined by liquid hybridization as described recently. Liquid hybridization was performed at 62°C for 18 hours. After digestion by RNase A (37 μg/mL) and T1 (1.8 μg/mL) and precipitation by trichloroacetic acid (10%), the nondigested RNA hybrids were transferred to a nitrocellulose membrane and counted by liquid scintillation. The amount of specific RNA was calculated from a standard calibration curve established by unlabeled sense transcripts ranging from 2 to 30 pg.

Results

Identification of the Intracellular Site of Action of Ang II

In a previous study, we have demonstrated that the Ang II–induced stimulation of angiotensinogen secretion is related to an increase in the mRNA coding for angiotensinogen and that this increase depends on a transient decrease in cAMP. In the presence of Ang II (3 to 90 nmol/L), angiotensinogen mRNA increased rapidly during the first hour and more slowly in the subsequent incubation period of 3 hours. In the present study, we have first focused on the question of whether the elevation of angiotensinogen mRNA is related to an increased rate of transcription or to the activation of other posttranscriptional mechanisms.

Modulation of Transcription Rate

To analyze whether Ang II stimulates angiotensinogen synthesis by increasing its transcription rate, nuclei were isolated from hepatocytes pretreated with Ang II (90 nmol/L) for 2 hours and incubated for 30 minutes for the synthesis of [32P]UTP–labeled mRNA. The amount of de novo synthesized angiotensinogen mRNA was analyzed by either liquid hybridization or dot-blot analysis. Fig 1A summarizes the results obtained by β-counting of the isolated hybrids (n=5). For comparison, the effect of dexamethasone is also shown because dexamethasone is a standard stimulus for the synthesis and secretion of angiotensinogen. In the presence of this glucocorticoid (10 μmol/L), a sixfold to ninefold stimulation of the transcription of angiotensinogen was determined independent of the hybridization technique used (P<.005). In contrast, angiotensinogen does not significantly alter the transcription rate of angiotensinogen. In the following experiments, we therefore analyzed whether angiotensinogen increases the half-life of angiotensinogen mRNA.

Half-life Measurement in Hepatocytes During Transcription Blockage

In these experiments (n=4), three experimental aliquots were prestimulated for 2 hours in the presence of dexamethasone (10 μmol/L), leading to a twofold to threefold increase in angiotensinogen mRNA (from 5.1±0.3 to 13.2±0.8 pg/μg RNA, P<.005). At 2 hours in one experimental group, the de novo synthesis of mRNA decreased with a half-life of approximately 90 minutes (5.8±0.4 pg/mg RNA at 4 hours). When, however, Ang II (90 nmol/L) was added simultaneously with actinomycin D, this decrease was significantly attenuated (12.9±0.7 pg/μg RNA, P<.005 to the actinomycin D group). Essentially the same results were obtained with 5,6-dichlorobenzimidazole riboside (50 μmol/L), a transcription inhibitor structurally unrelated to actinomycin D.

Half-life Measurement in Hepatocytes Using a Pulse and Chase System

The aspect of mRNA stabilization was examined in more detail by directly measuring the half-life of angiotensinogen mRNA in a pulse and chase experiment. A representative experiment is shown in Fig 2B. Hepatocytes were first pulse labeled in the presence of [3H]uridine and then chased in the presence of a 1500-fold excess of unlabeled uridine. Under these chase conditions, [3H]UTP–labeled angiotensinogen mRNA decreased in control hepatocytes with a half-life of 83 minutes, whereas the half-life of angiotensinogen mRNA was significantly increased to 191 minutes in hepatocytes exposed to angiotensinogen. This effect was confirmed in three additional experiments, in which Ang II increased the half-life of angiotensinogen mRNA between 2.1- and 2.6-fold.

Isolation and Characterization of mRNA-Binding Proteins

When total cytosolic extracts containing the complete pattern of intracellular compartments except nuclei were probed with the 3′UTR mRNA fragment in a gel mobility shift experiment, a large shift occurred that was more evident in extracts isolated from Ang II–treated hepatocytes than in controls. A proteinase K digest before electrophoretic separation completely abolished the shift,
suggesting that the band-shift signal was related to a protein-mRNA and not to a mRNA-mRNA interaction. Further subfractionation of the extracts revealed that most of the mRNA-binding activity was localized in the polysomal fractions, from which proteins were dissolved by a potassium chloride (0.5 mol/L) wash and further subjected to a fractionated ammonium sulfate precipitation. The 0% to 40% fraction contained most of the binding activity and, at this step of purification binding activity, was higher in the fraction derived from Ang II–preincubated cells. For high-performance liquid chromatographic separation, the 0% to 40% fraction was applied to two Zorbax GF-250 columns in series, and the eluted proteins were monitored by measuring their binding capacity to the 3'UTR probe in a band-shift assay. According to this separation protocol, we were able to isolate two proteins with a molecular weight of 12 or 45 kD that each had an activity to the 3'UTR of angiotensinogen mRNA. However, biochemical characterization by SDS-PAGE performed under reducing or nonreducing conditions showed that the high molecular weight protein might be an oligomeric precursor of the low molecular weight protein, which is formed preferentially under nonreductive conditions. Cross-linking analysis of both proteins under reductive and nonreductive conditions demonstrates that only the low molecular weight protein had an affinity to the 3'UTR. The specificity of the binding to the [3P]UTP-labeled 3'UTR of angiotensinogen mRNA was estimated by competition with an unlabeled 3'UTR fragment compared with competition using an unrelated, unlabeled mRNA fragment of similar length from the translated region of renin mRNA. Fig 2A shows that the binding of the low molecular weight protein to the 3'UTR fragment was displaced in a dose-dependent fashion by unlabeled 3'UTR mRNA but not by a 100-fold excess of unlabeled renin mRNA. Biochemical characterization of the low molecular weight protein by two-dimensional gel electrophoresis confirmed the low molecular weight of 12 kD and revealed an isoelectric
Identification of Phosphorylated Proteins in Response to Decreased cAMP Concentrations

Because most of the Ang II–induced increase in angiotensinogen mRNA occurs during the first 30 minutes of exposure and because this effect depends on a transient inhibition of adenyl cyclase activity, it appeared likely that the stabilization of angiotensinogen mRNA is related to a modification of an already present protein, possibly by phosphorylation or dephosphorylation, rather than to de novo synthesis of mRNA-binding factors. To examine this question, we analyzed the phosphorylation pattern of [32P]orthophosphate–loaded hepatocytes in response to cAMP-lowering agents, e.g., guanfacine or Ang II, or in response to Rp-cAMP, a cAMP antagonist that directly inhibits the activity of protein kinase A, subsequent to an incubation period of 10 minutes. Cytosolic extracts were harvested by digitonin permeabilization, and phosphorylated proteins were identified by two-dimensional gel electrophoresis and subsequent autoradiography. Quantification of the phosphorylation state was performed by densitometric analysis. Fig 2C shows the results for cytosolic extracts isolated from Ang II–treated cells (right panel) compared with the phosphorylation pattern in control cells (left panel). When the same experiment was repeated with Rp-cAMP or guanfacine, the phosphorylation pattern of cytosolic proteins was less complex; however, some of the newly phosphorylated proteins are identical to those observed in the presence of Ang II (these are marked by arrows). Interestingly, a consistent finding for all cAMP-lowering agents was the phosphorylation of a 12-kD protein with an isoelectric point of approximately 5.0. It thus appears possible that this protein is identical to the 12-kD polysomal protein that specifically binds to the 3'UTR fragment of angiotensinogen mRNA.

Modulation of Angiotensinogen mRNA Half-life in a Cell-Free Incubation System

To examine the angiotensinogen mRNA–stabilizing effect of the isolated 12-kD protein in vitro as a functional correlate to its mRNA-binding properties, we established a cytosolic incubation system that allowed analysis of the decay of angiotensinogen mRNA in vitro and its modulation by the addition of either the 12-kD protein or acid phosphatase. As a control, the decay of total RNA or β-actin mRNA was determined. At the beginning of incubation, angiotensinogen mRNA concentrations of 6 pg/µL RNA were determined. In control extracts, angiotensinogen mRNA had a half-life of approximately 30 minutes. In the presence of the 12-kD protein (200 ng/µL), the half-life significantly increased (15% decay in 2 hours), whereas in the presence of acid phosphatase (1 U/µL), the decay of angiotensinogen mRNA was significantly accelerated at 30 and 60 minutes. Acid phosphatase also attenuated the mRNA-stabilizing properties of the 12-kD protein (angiotensinogen mRNA half-life of approximately 2 hours). In control extracts, total RNA (3.8 µg/µL) remained stable during the entire incubation period, and β-actin mRNA decreased between 15% and 29%. Neither parameter was significantly altered by the 12-kD protein or by acid phosphatase treatment.

Discussion

Ang II has been shown to stimulate angiotensinogen synthesis and secretion in the liver.7,11 The mechanism, however, by which Ang II stimulates angiotensinogen mRNA appears to be different from those of other well-characterized stimuli of the synthesis of angiotensinogen. For example, glucocorticoids, estrogens, and some mediators of acute inflammatory processes have been identified to modulate angiotensinogen gene expression by control of the transcription rate.4,4 For glucocorticoids, we could confirm this mechanism because dexamethasone treatment of hepatocytes stimulates the rate of transcription several-fold, as shown in a nuclear run-on system, in which the rate of [32P]UTP incorporation into newly synthesized angiotensinogen mRNA was assessed. This observation is consistent with the presence of glucocorticoid-responsive elements in the promoter region of the angiotensinogen gene.5 In contrast, Ang II treatment of hepatocytes has only a negligible, if any, effect on the rate of transcription in the nuclear run-on assay. Therefore, we have examined the possibility that the observed increase in angiotensinogen mRNA induced by Ang II in intact cells results from stabilization of the mRNA. This appears to be the case, as suggested by experiments in which transcription was blocked by actinomycin D or 5,6-dichlorobenzimidazole riboside. In dexamethasone-stimulated hepatocytes, actinomycin D causes a sharp decline in angiotensinogen mRNA concentrations, which could be significantly attenuated by the simultaneous addition of Ang II. Direct evidence for stabilization of angiotensinogen mRNA is provided by the demonstration that Ang II prolongs the half-life of angiotensinogen mRNA from 83 to 191 minutes. Although the overriding control of gene expression is at the transcriptional level, modulation of the life span of mRNAs has been identified as an important regulatory mechanism in the overall expression of several genes, in addition to changes in translational efficiency and posttranslational events. For example, RNA stabilization plays a significant role in the regulation of the half-life of phosphoenolpyruvate mRNA, vitellogenin mRNA, and transferrin receptor mRNA.21 One specific mechanism for mRNA stabilization, which may also be pertinent to the presently studied mRNA, is the binding of protein(s) to the 3'UTR of the mRNA upstream of the poly-A tail, both of which are important structures in the determination of mRNA half-life. In the present study, we were able to isolate a polysomal protein that binds to the 3'UTR fragment of angiotensinogen mRNA but not to an unrelated mRNA fragment. The binding activity of this protein was increased after exposure of hepatocytes to Ang II. Furthermore, in a cytosolic incubation system, the mRNA-binding protein protects full-length angiotensinogen mRNA from nucleolytic degradation. The identity of the 12-kD protein is not known. However, it may be related to the changes in protein phosphorylation pattern induced by Ang II in hepatocytes. Analysis of the phosphorylation pattern of cytosolic proteins obtained in cells incubated with Ang II revealed, among other changes, a dramatic increase in the phosphoryl-
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tion of a 12-kD protein with an IP of approximately 5.0. Interestingly, the biochemical parameters of the RNA-binding protein are in good agreement with the parameters of the adenosine-uridine-binding factor, an RNA-binding protein described by Malter and Hong22 that stabilizes the mRNA of c-fos, v-myc, interleukin-3, or interferon in response to a protein phosphorylation that is induced by phorbol esters or calcium ionophores. However, the amino acid sequence is not available for adenosine-uridine-binding factor or for the 12-kD protein; therefore, determination of a possible relation between these proteins awaits further research.

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