Nuclear Angiotensin Receptors Induce Transcription of Renin and Angiotensinogen mRNA

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The observation that nuclei from hepatic tissue exhibit specific angiotensin II (Ang II) binding led us to explore whether Ang II modulates mRNA in general, mRNA specific for renin system components, or both. Nuclei from hepatic tissue exhibited a single high-affinity (K_d=0.4 nmol/L) Ang II–specific binding site, which was associated with increased RNA transcription. Whereas total RNA extracted from nuclei increased 1.5-fold in response to Ang II (10^{-9} mol/L), specific mRNA for renin and angiotensinogen increased 7.8- and 2.5-fold, respectively. Ang II binding and induced transcription showed parallel Ang II dose responses that were both inhibited by 10^{-9} mol/L DuP 753 or saralasin. Maximum Ang II binding and RNA transcription occurred at the same Ang II concentration (10^{-9} mol/L). Higher doses of Ang II resulted in a progressive decrease in RNA transcription. Together, these results demonstrate that hepatic nuclei have functional Ang II–specific receptors. It is concluded that Ang II may elicit responses at nuclear receptors, which herefore were associated only with Ang II receptors located on plasma membranes. However, the individual contribution of plasma and nuclear membrane Ang II receptors to the overall cellular Ang II transcriptional response and their possible interactions remain to be determined. (Hypertension. 1993;22:496-501.)

KEY WORDS • angiotensin II • renin-angiotensin system • renin • angiotensinogen • cell nucleus • RNA, messenger • transcription, genetic • receptors, angiotensin

Numerous studies have demonstrated that angiotensin target tissues contain the components required to sustain local angiotensin II (Ang II) generation (renin-angiotensin systems) independent of the peripheral circulating endocrine renin system.1-3 The potential importance of such tissue-renin systems, acting either in concert or independently of the endocrine-renin system, has recently been given wide attention.4-8 It has been hypothesized that intracellular angiotensin peptides, locally synthesized by tissue-renin systems, are secreted to act on specific plasma membrane receptors of the same or adjacent cells, thereby serving autocrine or paracrine functions or both. The recent reports that hepatic nuclei exhibit specific Ang II binding sites,9,10 which are associated with G protein activity,10 led us to explore whether these nuclei have the capacity to modulate gene transcription of renin system components via Ang II–specific receptors.

Methods

Nuclei were isolated from liver of the normal Wistar rat. The following steps were all performed at 4°C, and all solutions were prepared with diethylpyrocarbonate-treated distilled deionized water. Briefly, 3 to 5 g of fresh tissue was rinsed and then homogenized in a Potter Elvehjem extractor (Fisher Scientific Co, Pittsburgh, Pa) with 6 vol cold 0.5 mol/L sucrose-TEKSS buffer (50 mmol/L Tris-HCl, 1 mmol/L EDTA, 25 mmol/L KCl, 0.15 mmol/L spermidine, and 0.5 mmol/L spermine, pH 7.5) and filtered through a 100-mesh screen. The filtrate was centrifuged for 10 minutes at 2500g and the pellet suspended in 20 mL of the same buffer. The suspension was layered over 4 mL of 0.88 mol/L sucrose-TEKSS and centrifuged at 2500g for 10 minutes. The nuclei-containing pellet was resuspended in 20 mL of 2.1 mol/L sucrose-TEKSS, gently homogenized for 10 minutes, and centrifuged at 32 000g for 1 hour. This pellet was suspended in 20 mL of 0.5 mol/L sucrose-TEKSS, centrifuged for 10 minutes at 2500g, resuspended in 1.5 to 2 mL of 0.5 mol/L sucrose-TEKSS, and stored at ~20°C in 200-μL aliquots. Nuclear binding and transcriptional properties remained unaltered for at least 6 weeks.

Isolated nuclei were examined for integrity by phase-contrast microscopy, and nuclear protein concentrations were quantified by a modified Bradford assay using bovine serum albumin (Sigma Chemical Co, St Louis, Mo) as the standard.11 Unless otherwise indicated, 100-μg equivalents of nuclear protein (6 to 10 μL of suspended nuclei) were used in a final sample reaction volume of 100 μL for all experiments.

Angiotensin I, Ang II, and the Ang II antagonist saralasin were purchased from Peninsula Laboratories Inc, Belmont, Calif. The specific subtype 1 Ang II receptor antagonist DuP 753 was obtained from Sigma.
[125I]-labeled Ang II (specific activity, 2000 μCi/nmol) was prepared by the method of Hunter and Greenwood. The radiolabeled peptide was purified by ion-exchange chromatography on Bio-Rad 1x8, followed by reversed-phase high-performance liquid chromatography with a C18 column. Labeled Ang II was eluted isocratically with 20% acetonitrile in 0.05 mol/L sodium phosphate buffer (pH 7.4), and integrity of the peptide was verified by an Ang II–specific radioimmunoassay.

For measurement of saturation of Ang II binding to nuclei, nuclei were incubated with increasing concentrations of [125I]-labeled Ang II in the presence and absence of 10−5 mol/L unlabeled Ang II in a buffer consisting of 50 mmol/L Tris (pH 7.4), 100 mmol/L NaCl, 10 mmol/L MgCl2, 0.1% heat-denatured bovine serum albumin, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L 8-hydroxyquinoline hemisulfate for 30 minutes at 25°C according to the method of Tang et al. Incubated nuclei were washed on glass fiber filters (GF/B, Whatman Inc, Clifton, NJ) to separate bound and free peptide. Specific binding was defined as the difference between total binding and the binding observed in the presence of excess unlabeled Ang II. Specificity of binding was determined by incubating nuclei with 1 nmol/L [125I]-labeled Ang II in the presence and absence of Ang II antagonists (10−9 mol/L).

To ascertain whether Ang II binding to nuclei stimulated RNA transcription, isolated nuclei (100 μg of nuclear protein) were incubated with varying concentrations of Ang II in a total volume of 30 μL for 30 minutes at 25°C. These samples were then further incubated at 37°C for 1 hour with a transcription system consisting of 500 μmol/L ATP, GTP, and UTP; 250 μmol/L CTP; 250 μCi [α-32P]CTP (Amersham Corp, Arlington Heights, Ill; specific activity, 3000 Ci/mmol); 2 U/μL RNasin (Promega Corp, Madison, Wis); and 5 μL of 10X transcription buffer (Promega). Diethylpyrocarbonate-treated water was added to bring the total volume to 50 μL. RNA was then extracted and isolated with 4 mol/L guanidine isothiocyanate and phenol as previously described. After precipitation of RNA by addition of an equal volume of ice-cold 100% isopropanol and centrifugation, RNA was taken up in 50 μL diethylpyrocarbonate-treated water, aliquoted into 10-μL fractions, and stored at −70°C. Agarose gel electrophoresis and autoradiography indicated a major band at 1.6 to 1.8 kb and a very minor band at 5.1 kb (see Fig 4). To quantify newly synthesized RNA, RNA was precipitated with 10% trichloroacetic acid onto glass fiber filters, washed, and counted in an LS 5000TD scintillation counter (Beckman Instruments Inc, Fullerton, Calif). The intra-assay and interassay coefficients of variation for this nuclear transcription assay were 4.6±1.2% and 5.9±1.2%, respectively (n=4).

To determine whether renin or angiotensinogen mRNA was stimulated in response to Ang II binding, the same transcription and extraction procedure described above was performed with the exception that 500 μmol/L unlabeled CTP was used in place of 32P-labeled CTP. After RNA extraction, agarose gel electrophoresis in the presence of ethidium bromide revealed a band at 1.6 to 1.8 kb and smaller nucleotide chains reflecting active RNA synthesis (Fig 1). The ratio of 260 to 280 nm of all final RNA preparations exceeded 1.85. Specific mRNA for renin, angiotensinogen, and cytoskeletal β-actin (control) were quantified by slot blot hybridization with specific mRNA riboprobes under high stringency conditions. To further reduce cross homology, RNase digests were performed. Density measurements on the resulting autoradiographs were performed with a computerized optical scanner system (JAVA). The cDNA clones for both rat renin (1.4 kb) and angiotensinogen (1.6 kb) used in the riboprobe system were a gift from Dr K. Lynch. The specific activity of the synthesized probes did not differ significantly from each other. The cDNA clone for cytoskeletal β-actin was a gift from Dr K. Taylor.

Analysis of variance was used to ascertain statistical significance between samples exposed to various dose levels of Ang II or Ang II antagonists.

**Results**

Binding of Ang II to hepatic nuclei was saturable in the nanomolar range (Fig 2), and Scatchard plot analysis (Fig 2, inset) indicated the presence of a single high-affinity binding site with a $K_d$ of 0.4 nmol/L. The Ang II dose-dependent transcriptional response of hepatic nuclei is shown in Fig 3. Maximal stimulation of gene transcription was found between 10−10 and 10−8 mol/L Ang II. Comparison with Fig 2 indicates that the Ang II concentration that elicited maximal receptor saturation also induced maximal gene transcription. Further increases in the Ang II concentration significantly suppressed transcription. Corroborative evidence indicating that nanomolar Ang II concentrations induced maximal gene transcription is provided by the autora-
FIG 2. Representative plot shows angiotensin II (Ang II) binding in isolated hepatic nuclei (100 µg protein equivalents) with increasing doses of 125I-labeled Ang II. Ang II bound in parallel experiments with unlabeled excess Ang II (10^{-6} mol/L) was subtracted to obtain specifically bound Ang II. 

FIG 4. Autoradiogram shows extracted RNA after transcription in the presence of angiotensin II (Ang II) and electrophoresis on agarose gel. The same total RNA concentration was applied to all wells. Lanes 1 through 5 represent incubations with increasing doses of Ang II (control [no Ang II], 10^{-12}, 10^{-10}, 10^{-8}, and 10^{-5} mol/L Ang II, respectively). Maximum film density in the 1.8-kb band is observed with 10^{-9} mol/L Ang II.

Discussion

Our studies with hepatic nuclei confirm previous reports that these nuclei have high-affinity Ang II binding sites and increase gene transcription in response to this peptide hormone. The present study extends these observations to demonstrate that nuclear binding of Ang II and stimulation of RNA synthesis are both directly proportional to the Ang II concentration (up to 10^{-9} mol/L). Because supplementation of isolated hepatic nuclei with plasma membrane fractions

FIG 5. Bar graph shows incorporation of 32P-labeled CTP into RNA from isolated hepatic nuclei (100 µg protein equivalents, n=5) incubated in the presence and absence of 10^{-8} mol/L angiotensin II (AI). Angiotensin I (AI) and angiotensin II antagonists saralasin (saralasine) and DuP 753 were present in excess (10^{-5} mol/L). *Significant change from control, P<.05.
The Ang II–induced acceleration of gene transcription is not a general or nonspecific phenomenon related to peptides, because angiotensin I did not alter the rate of RNA transcription, and both specific Ang II antagonists, saralasin and DuP 753, inhibited Ang II–induced stimulation of RNA transcription (Fig 5). Furthermore, Ang II did not stimulate transcription of mRNA for cytoskeletal β-actin (Fig 7). These findings, in conjunction with the observations that Ang II binding to hepatic nuclei is saturable (Fig 2), reversible, and elicits a biologic response, support the conclusion that these nuclear binding sites represent functional hormone receptors. This conclusion is further supported by the observation that RNA polymerase II activity of hepatic nuclei increases in response to Ang II. Although the binding characteristics suggest that the nuclear Ang II receptors meet the criteria for angiotensin subtype I receptors, Ang II nuclear binding proteins have been shown to differ from those of the plasma membrane.

Saturation of Ang II binding sites results in a significant reduction in the overall rate of gene transcription (Figs 3 and 4) and may represent a negative feedback response to high Ang II concentrations. Such hormone concentration–dependent effects on transcription have recently been reviewed. However, further studies will have to be conducted to establish the dependency of transcription rates for individual mRNAs on the degree of Ang II saturation. As we have demonstrated, 10⁻¹⁰ and 10⁻⁹ mol/L Ang II were equally effective in the overall stimulation of gene transcription, but the latter concentration was required to obtain a significant increase in the transcription rate of renin- and angiotensinogen-specific mRNA (Figs 6 and 7). Although the increase in angiotensinogen mRNA (2.5-fold) was higher than the overall Ang II–induced RNA stimulation (1.5-fold), it was significantly less than the 7.8-fold increase in renin-specific mRNA. Based on the equal specific activity for both probes, the relative abundance of angiotensinogen mRNA is 10- to 15-fold greater than that of renin mRNA (Fig 7). The significance of these differences and their influence on local synthesis of renin and angiotensinogen will have to await future translation studies.

The finding that hepatic angiotensinogen mRNA is stimulated by Ang II is not unexpected, because the liver is the primary source of angiotensinogen and numerous studies have implicated this peptide in angiotensinogen synthesis. Several studies have also demonstrated the presence of renin mRNA in hepatic tissue, and physiological manipulations have been shown to alter the levels of hepatic renin mRNA. It appears that high stringency conditions and RNase digestion after hybridization, such as used in this study, are required to unequivocally detect renin mRNA.

At first glance it might appear that stimulation of renin mRNA by Ang II contradicts the well-known negative feedback loop between Ang II and circulating renin. However, if under normal physiological conditions tissue Ang II levels are on the order of 10⁻⁹ mol/L and higher levels of Ang II suppress renin gene transcription, as they do for the overall gene transcription (Figs 3 and 4), an increase of Ang II would result in suppression of renin gene transcription, supporting the concept of a negative feedback. Alternatively, the possibility cannot be ruled out that modulation of hepatic

**Fig 6.** Representative slot blot hybridization shows quantitation of angiotensinogen- and renin-specific mRNA from hepatic nuclei after incubation without or with 10⁻¹⁰ and 10⁻⁹ mol/L angiotensin II (AII). Hybridizations were performed with four concentrations of total RNA (2.5, 1.25, 0.65, and 0.032 µg). Within the linear range of the film, a linear correlation is evident between total RNA applied to the slot blot and specific mRNA.

**Fig 7.** Bar graph shows quantitation by densitometry of angiotensinogen, renin, and β-skeletal actin by slot blot hybridization (n=4). Significant change from control, P<.05. Cont, control; AII, angiotensin II.
renin mRNA by Ang II is guided by regulatory mechanisms other than those operative in the kidney. This point of view is supported by the recent finding that endothelins are potent stimulants of prorenin in cultured human decidua cells, whereas they suppress renin synthesis in the kidney. The presence of Ang II nuclear receptors with the ability to modulate angiotensinogen and renin mRNA transcription suggests that intracellular Ang II may have the potential to influence or autoregulate the local Ang II concentration by increasing the transcription rate of renin system components when the Ang II concentration is low and, conversely, suppressing transcription when Ang II is high. In view of the biphasic response of nuclei to Ang II and the low levels of this hormone in isolated nuclei, it is not surprising that low doses of Ang II stimulate both angiotensinogen and renin mRNA. Local regulation of Ang II, as proposed in the liver, may provide an answer to the enigma that plasma angiotensinogen concentrations are elevated after bilateral nephrectomy despite the fact that circulating Ang II is very low. The potential of intracellularly regulated Ang II concentrations and nuclear Ang II receptors may also provide a solution to the observation that the circulating Ang II concentration, under normal or even low circulating Ang II, is insufficient to induce the responses attributed to Ang II. In this context, the ability of intracellular Ang II to elicit gene transcription via nuclear binding does not appear to be a phenomenon restricted to hepatic nuclei. We have observed similar stimulation in isolated nuclei from several Ang II target tissues. In these tissues, representing the means of two separate pools of nuclei assayed in triplicate, rat kidney nuclei exhibited 13% Ang II specifically bound, with a concomitant increase of 38% in the transcription rate; for heart nuclei, 21% of the Ang II was specifically bound, with a 24% increase in transcription; and in adrenal nuclei, a 13% increase in transcription rate was noted in response to Ang II. However, nuclei from these tissues were derived from a wide variety of cell types, so conclusions regarding the nature of the binding and the transcriptional responses of individual cell types, which exhibit specific Ang II nuclear receptors, will have to await more detailed studies.

Although several tissues and cell lines have been shown to contain significant concentrations of immunoreactive Ang II-like peptides, the source, whether derived from the circulation or from local synthesis, remains to be fully explored. Because Ang II derived from plasma is rapidly degraded on entry into the cell, plasma-borne Ang II may not contribute significantly to the intracellular Ang II. However, Ang II synthesized in the vicinity of the nuclear receptor may well provide a mechanism to induce Ang II-mediated nuclear responses independent of the activity of the plasma-renin system. Local generation of Ang II, acting on nuclear receptors, may provide a solution to the enigma that converting enzyme inhibitors, in doses insufficient to lower blood pressure, can cause local effects, for example, regression of cardiac hypertrophy in states of normal or even low circulating Ang II. Although experiments with isolated limbs and organs already provide clear evidence that tissues are indeed capable of locally synthesizing Ang II, future studies on subcellular compartmentalization of renin system components and intracellular generation of Ang II in hepatic and other tissues need to be conducted to support these postulates.

In conclusion, we have demonstrated that hepatic nuclei exhibit Ang II-specific receptors that mediate transcription of mRNA, including specific mRNA for renin and angiotensinogen, but not cytoskeletal β-actin. Furthermore, the biphasic response of gene transcription to Ang II may potentially provide a means to locally autoregulate intracellular Ang II-dependent mechanisms. This response may be mediated by intracellularly generated Ang II and consequently independent of the circulating endocrine renin-angiotensin system.

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