Tissue-specific Regulation of Angiotensinogen mRNA Accumulation by Dexamethasone*

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The regulation of angiotensinogen gene expression in response to adrenalectomy and dexamethasone treatment was examined in multiple rat tissues. Angiotensinogen mRNA as quantitated by slot blot hybridization utilizing an angiotensinogen cRNA probe was most abundant in the liver with levels in the brain, kidney, and adrenal of 50, 25, and 10%, respectively. No angiotensinogen mRNA was detected in testes or heart. Although no change in the quantity of angiotensinogen mRNA was found following adrenalectomy and maintenance on 0.9% saline, dexamethasone treatment resulted in a time-dependent and tissue-specific accumulation of angiotensinogen mRNA. In normal animals, the hepatic response to treatment was a 4.5-fold increase in angiotensinogen mRNA by 8 h which remained 2.4-fold above basal levels by 24 h. Angiotensinogen mRNA levels in the brains of normal rats treated with dexamethasone increased only 60% by 6 h and returned to basal levels by 24 h. In contrast to the increases seen in brain and liver, angiotensinogen mRNA derived from kidney did not significantly change following dexamethasone treatment. In adrenalectomized animals, the hepatic response to dexamethasone was similar to normal animals with a 3.7-fold increase by 6 h. The accumulation in brain was greater in these animals compared to normals and increased 3-fold by 8 h. Finally, dexamethasone did not significantly increase levels in the kidney. These results clearly demonstrate glucocorticoid regulation of angiotensinogen mRNA levels in liver and brain. In contrast, the kidney, an organ known to contain glucocorticoid receptors, does not respond with increased angiotensinogen mRNA levels following glucocorticoid stimulation. These studies provide the first evidence for tissue-specific differences in the control of angiotensinogen mRNA.

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**EXPERIMENTAL PROCEDURES**

Animals—Adult (140-160 g, body weight) male Wistar rats (Charles Rivers) used in these studies were maintained on ad libitum rat chow (1.7 meq Na/g) and tap water. Adrenalectomy was performed subsequently converted to the biologically active peptide angiotensin II by angiotensin-converting enzyme (1). In plasma, the concentration of circulating angiotensin I is less than that of angiotensinogen, the precursor in this enzymatic cascade (2). The commonly accepted $K_{m}$ for the renin reaction clearly indicates that approximately 10-fold more circulating angiotensinogen than is physiologically available is required to produce a zero order renin-angiotensinogen reaction (2). Thus, angiotensinogen concentration is rate-limiting in the renin reaction, and ultimately important in the regulation of the angiotensin II concentration.

It is well known that angiotensin II, acting through specific receptors on vascular smooth muscle and the adrenal gland, mediates vasoconstriction (3) and aldosterone and catecholamine secretion (4), respectively. Studies have also demonstrated angiotensin II receptors in other target tissues such as the brain and kidney. In the brain it has been demonstrated that angiotensin II stimulates thirst (5), alters sympathetic outflow (6), and regulates pituitary hormone secretion (7, 8). In the kidney, angiotensin II stimulates both sodium and water reabsorption and decreases the glomerular filtration rate (9).

The liver has been shown to be the major source of plasma angiotensinogen (10) but more recent studies have now demonstrated angiotensinogen mRNA in several angiotensin II target tissues (11) suggesting local angiotensin II-generating systems. This is further supported by the localization of renin- and angiotensinogen-converting enzyme activities in these same tissues (12). Additionally, recent studies have demonstrated that the plasma concentration of angiotensin II is in the picomolar range (13), while the binding affinity of its receptor is in the nanomolar range (14, 15). Taken together these data suggest that the tissue-specific production of angiotensin II may be acting as either a paracrine or an autocrine with activities that are independent and/or complementary to those of the circulating hormone.

Physiologic alterations such as volume depletion and low sodium diet, as well as endotoxic shock, insulin, estrogen, and glucocorticoid treatment increase the circulating levels of angiotensinogen in the rat (16, 17). The mechanism of this increase is unknown. Thus, we have undertaken the present study to characterize local angiotensinogen-generating tissues and accurately assess the regulation of the angiotensinogen gene. Utilizing our rat angiotensinogen cRNA probe, we have demonstrated for the first time the tissue-specific regulation of angiotensinogen mRNA accumulation by dexamethasone treatment.
under chloral hydrate (6.25%, 0.5 ml/100 g) anesthesia. Adrenalectomized animals were maintained on ad libitum 0.9% saline instead of tap water and used 2 weeks after surgery. Animals were killed by decapitation. No residual adrenal tissue was detected in adrenalectomized animals at the time of killing. Dexamethasone (Elkins-Sinn, Inc., Cherry Hill, NJ) (7 mg/kg) was administered to animals by intraperitoneal injection.

RNA Isolation—Total RNA from the tissues specified was isolated by a modification of the guanidine thiocyanate method of Chirgwin (18) as previously described (19). Experiments were performed in duplicate on paired animals. All tissues were processed and analyzed separately. The results represent average values.

Synthesis of an Angiotensinogen cRNA Probe—The cRNA used in these experiments was derived from the 3′ end of a rat angiotensinogen double-stranded cDNA clone. The angiotensinogen recombinant plasmid was digested with PstI and the 3′ fragment ligated into pGem-3 vector (Fromega Biotech). This pGem-angiotensinogen plasmid was transfected into Escherichia coli HB101 utilizing standard techniques (20). An angiotensinogen cRNA probe was synthesized in a 20-μl transcription reaction containing: 1 μg of pGem-angiotensinogen DNA digested with PstI, 40 mM Tris-HCl, pH 7.9, 6 mM MgCl2, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0.5 mM each of ATP, GTP, UTP and 12 μM CTP with 50 μCi of [α-32P]CTP, 20 units of RNasin, and 7 units of Sp6 polymerase. The reaction mixture was incubated at 37 °C for 60 min. The DNA template was removed by digestion with 2 units of RNase-free DNase for 15 min at 37 °C. Yeast rRNA (40 μg) was added, the mixture was extracted with phenol-chloroform (1:1) and the [32P]RNA angiotensinogen probe was recovered by ethanol precipitation. The specific activity of the probe was typically 1.4 × 106 dpm/μg.

Northern Blot Hybridization—Total RNA was denatured with 1 M glyoxal, 50% dimethyl sulfoxide (20), electrophoresed on a horizontal 1.2% agarose gel, and transferred to nylon membrane (Hybond-N, Amersham Corp.) (21, 22). The filter was prehybridized for 24 h at 60 °C in an identical solution containing 0.1% SSC and 0.5% sodium dodecyl sulfate, and 200 ng of denatured salmon sperm DNA. Hybridization was for 24 h at 60 °C in an identical solution containing 1 × 106 cpm/ml of [32P]cRNA angiotensinogen probe. The filter was washed with 0.1 × SSC and 0.1% sodium dodecyl sulfate at 60 °C, which eliminated background binding of the cRNA probe to ribosomal RNA. The autoradiograph was obtained by placing the filter in a cassette with an immobilized 0.1-μm nitrocellulose filters (Schleicher & Schuell). The sample was quantitated by slot blot hybridization. Total RNA was serially diluted (4.0–0.5 μg of RNA/100 μl of H2O), mixed with 300 μl of 6.15 M formaldehyde, 10 × SSC, and incubated at 65 °C for 15 min. The denatured RNA solution was applied and slowly filtered through the nitrocellulose filter. The wells were rinsed with 400 μl of 10 × SSC, and the filter was baked at 80 °C in a vacuum oven for 2 h. The conditions for hybridization are described above. Autoradiographs of the filters were scanned with a laser densitometer (LKB 2202 Ultra Scan) to quantify probe hybridization to angiotensinogen mRNA. Control experiments confirmed quantitative recovery, and several different exposure lengths were obtained to ensure that the autoradiographs were within the linear scanning range which was determined utilizing serial dilutions of mRNA.

RESULTS

Size and Tissue Quantification of Angiotensinogen mRNA—Total RNA derived from tissues of normal male rats was denatured and examined by gel electrophoresis and hybridization with α-32P-labeled angiotensinogen-cRNA probe. As shown in Fig. 1, a single band of 1800 bases corresponding to angiotensinogen mRNA was identified in liver, brain, kidney, and adrenal gland. Evaluation of poly(A)-enriched mRNA from these normal tissues, total RNA was denatured and hybridized with an Angiotensinogen cRNA probe. The autoradiographs revealed that angiotensinogen mRNA was most abundant in liver, brain, and kidney, and 10 times more abundant than in adrenal gland (A) derived from liver (L), heart (H), brain (B), kidney (K), testes (T), and adrenal gland (A) of normal rats that had been maintained on 0.9% saline. The amount of angiotensinogen mRNA in liver, brain, and kidney was not altered by this treatment.

To determine the relative amounts of angiotensinogen mRNA in these normal tissues, total RNA was denatured and analyzed by slot blot hybridization to the cRNA probe. Den sitometric scanning (LKB 2202 Ultra-Scan) of the autoradiographs revealed that angiotensinogen mRNA was most abundant in liver with levels 2–3 times more abundant than in brain, 3–4 times more abundant than kidney, and 10 times more abundant than adrenal angiotensinogen mRNA (Fig. 2). Fig. 2 also shows the results of angiotensinogen mRNA measurements in tissues derived from adrenalectomized animals that had been maintained on 0.9% saline. The amount of angiotensinogen mRNA in liver, brain, and kidney was not altered by this treatment.

Tissue-specific Regulation by Dexamethasone—Although adrenalectomy followed by maintenance on 0.9% saline did not alter angiotensinogen mRNA levels in the tissues tested,
it remained a possibility that a glucocorticoid effect on the regulation of angiotensinogen synthesis was masked by the effects of other regulatory hormones. Therefore, we tested the effect dexamethasone treatment had in both normal and adrenalectomized animals. The experiments described below were done in duplicate utilizing paired animals. The RNA isolation and angiotensinogen mRNA quantitation was done on pooled RNA samples in the adrenalectomized animals and on individual RNA samples in the control rats. The range of variation between paired samples was less than 10% for the liver and brain and less than 30% for kidney.

Fig. 3 shows the results of the mRNA measurements taken at various times after treatment of the normal animals. The hepatic response to dexamethasone was a 4.5-fold increase in accumulation of angiotensinogen mRNA peaking at 8 h and remaining over 2-fold above basal levels at 24 h. The brain was much less responsive showing only a 60% increase in angiotensinogen mRNA at 6 h which returned to basal levels by 24 h. Kidney angiotensinogen mRNA did not change significantly, although there was a suggestion of an increase at 8 h.

In order to determine if basal glucocorticoid stimulation altered dexamethasone responsiveness, similar experiments utilizing rats which had been adrenalectomized and maintained on 0.9% saline for 14 days prior to receiving dexamethasone treatment were performed (Fig. 4). In these experiments, the liver showed a similar response with a 3.7-fold increase in hepatic angiotensinogen mRNA accumulation at 6 h. The accumulation in the brain of 3-fold at 8 h was more dramatic than that seen in normal animals. The kidney again showed no significant change although there was a suggestion of decreasing levels at 48 h. Although we did not study in detail the kinetics of the return to basal levels, tissue-specific differences in the duration of the effect were noted. In normal (control) animals, liver angiotensinogen mRNA remained approximately 2-fold above initial levels at 24 h while the angiotensinogen mRNA in the brain returned to basal levels by 24 h.

**DISCUSSION**

The data presented demonstrates both the existence and the tissue-specific glucocorticoid regulation of angiotensinogen mRNA synthesis in hepatic and extrahepatic tissues. Both Northern and slot blot hybridizations demonstrate the presence of significant amounts of angiotensinogen mRNA in the liver, brain, kidney, and adrenal gland, known target
tissues for angiotensin II action. Angiotensinogen mRNA was most abundant in the liver with levels in the brain, kidney, and adrenal of 50, 25, and 10%, respectively. These relative levels are all slightly higher than those reported by Ozkubo et al. (11) and Campbell and Habener (23) and may represent species variation, minor differences in the diet or age of the animals, or the use of cRNA probes. We utilized a cRNA probe in these studies because Cox et al. demonstrated a 10-fold increase in the sensitivity of detection of complementary mRNA sequences when cRNA rather than cDNA probes were utilized in situ hybridizations. In spite of the use of this probe, no angiotensinogen mRNA was detected in heart or testes although both tissues contain angiotensin II receptors. These data, in combination with the identification of the other components of the renin-angiotensinogen system in the brain (12, 25) and the kidney (12, 26), suggest a role for the local generation and action of angiotensinogen II in the regulation of thirst, sympathetic discharge, pituitary hormone release, renal blood flow, electrolyte, and ultimately blood pressure homeostasis.

If this hypothesis is correct, differential angiotensinogen gene expression in the liver, the major contributor to circulating angiotensinogen and other generating tissues, may exist. In an attempt to characterize these local systems further, we focused on glucocorticoid modulation of angiotensinogen mRNA levels since glucocorticoids have been shown to alter serum levels of angiotensinogen in rats (16) and humans (27). In order to estimate the effects of endogenous adrenal steroids, angiotensinogen mRNA accumulation was measured in liver, brain, and kidney from adrenalectomized animals. No change in accumulation was noted in any tissue compared to normal controls. This result is in agreement with that of Clausen et al. (16) who found that rat hepatic angiotensinogen production did not decrease with adrenalectomy and extends the tissues studied to include the brain and renal generating systems. These investigators, however, did find an increase in plasma renin activity. Our studies in cultured Reuber H35 (H4EII) rat hepatoma cells indicate that glucocorticoid deficiency decreases angiotensinogen mRNA. This, in combination with in vivo data, suggests that other factors, possibly renin or vasopressin, which is known to increase with adrenalectomy (28), or angiotensinogen II may act in a compensatory fashion to form a positive feedback loop.

We next investigated the role of glucocorticoid treatment in the regulation of angiotensinogen mRNA derived from tissues of normal and adrenalectomized rats. The liver was the most dexamethasone-responsive tissue in normal rats by virtue of a 4.5-fold increase in the quantity of angiotensinogen mRNA at 8 h. The brain was less responsive with a 60% increase, while in the kidney no change was noted following dexamethasone treatment. In adrenalectomized animals, dexamethasone treatment resulted in a 3-fold increase in angiotensinogen levels in brain and a 3.7-fold increase in the liver at 8 h with no change in mRNA levels in the kidney. It is of interest that the time course of dexamethasone induction of angiotensinogen mRNA is similar to the induction of hepatic angiotensinogen mRNA following lipopolysaccharide treatment (17). In addition to differences in the tissue-specific maximal responsiveness to dexamethasone, differences were also observed in the duration of the effect. In normal animals, elevated levels of angiotensinogen mRNA in the brain returned to basal levels by 24 h after dexamethasone stimulation while the levels in the liver continued to be 2-fold above basal levels. In contrast, the difference in duration of effect was not seen in tissues derived from adrenalectomized animals. More detailed studies are in progress.

A possible explanation for the differences in tissue responsiveness could be differences in angiotensin II receptor number. The liver contains approximately 10 times the number of angiotensin II receptors as the brain or kidney (14), and Nasjletti et al. (31) and Sernia et al. (32) have suggested that angiotensin II may be involved in a positive feedback loop which increases angiotensinogen synthesis and release from the liver. Thus, the glucocorticoid stimulation of angiotensinogen synthesis in the liver may be enhanced by an increase in circulating angiotensin II. Although angiotensin II receptors have been localized in the brain and kidney (33), it remains to be determined if these same cells are also responsible for the synthesis of angiotensinogen and whether they are involved in angiotensin II feedback regulation of angiotensinogen.

Modulators other than hormones have recently been shown to alter angiotensinogen mRNA levels. Kageyama et al. (17) noted an induction in angiotensinogen mRNA in rat liver but not brain following acute inflammation, and Ingelfinger et al. (34) have reported that salt depletion induces a more dramatic increase in renal angiotensinogen mRNA compared to liver. Fried and Simpson (35) demonstrated that uninephrectomy reduces angiotensinogen mRNA levels in the remaining kidney.

Our studies demonstrate that angiotensinogen mRNA accumulation in some tissues (liver and brain) is modulated by the glucocorticoid dexamethasone. This accumulation in mRNA could result from a direct steroid-receptor-induced increase in transcription rate, alterations in post-transcriptional processes such as mRNA degradation, or from a combination of these mechanisms. All three alternatives have been demonstrated in other steroid-responsive genes. For example, glucocorticoid induces tryptophan oxygenase mRNA (36), the human metallothionein-IIa mRNA (37), and the mouse mammary tumor virus mRNA (38) by increasing the transcription rate of these genes. Studies on the regulation of vitellogenin (39) and apo-VLDL II (very low density apolipoprotein II) (40) have demonstrated that the estrogen-induced increase in mRNA is secondary to both an increased rate of transcription and a prolongation in the mRNA half-life. The regulation of the phosphoenolpyruvate carboxykinase gene has been extensively studied and is likely under complex transcriptional and post-transcriptional control by glucocorticoids, cAMP and insulin (41). These mechanisms could all contribute to the dexamethasone-induced increase in angiotsensinogen mRNA seen in brain and liver.

In addition, angiotensinogen mRNA could respond indirectly to dexamethasone as a consequence of the translational product coded by the mRNA of a separate target gene. This mechanism has been shown to account for the several hundredfold increase in α1 acid glycoprotein (43) mRNA that results from glucocorticoid stimulation (42) and for the slow induction of the rat mRNA S11 by T3 or dexamethasone (43). The induction of both are inhibited by cycloheximide, thus
suggesting the need for ongoing synthesis of a glucocorticoid-responsive regulatory protein.

Dexamethasone induced the accumulation of angiotensinogen mRNA at the earliest time tested (2 h) in the rat liver and brain and as early as 30 min in our hepatoma cell culture system.1 Within the limits of the systems used, this suggests that the early response is not secondary to the induction of another regulatory factor. It seems likely then that glucocorticoids increase angiotensinogen accumulation at least in part by altering transcription rate directly. The role of mRNA stabilization and post-transcriptional events is under investigation in both brain and liver.

Our results indicate significant tissue-specific induction of angiotensinogen mRNA levels following dexamethasone treatment. These results strongly suggest that the angiotensinogen-generating systems in liver, brain, and kidney are, at least in part, under independent control. The hepatic and central nervous system generating systems are more sensitive to glucocorticoid stimulation, suggesting that they subserve functions related to changes in hormonal milieu. In contrast, the renal angiotensinogen-generating system may be of major importance in the regulation of renal blood flow and may thus be more powerfully regulated by changes in salt and water balance.

Thus, the presence of angiotensinogen mRNA and, more importantly, its modulation in multiple extrahepatic tissues provides strong evidence for a physiologic role for its local production. In these tissues of multiple cell types, further studies will be required to determine both the anatomic distribution of cells synthesizing angiotensinogen mRNA and their response to various physiologic perturbations. These studies should provide a better understanding of the function of the local synthesis of angiotensinogen and whether it is independent and/or complementary to the function of the circulating angiotensinogen.

REFERENCES