**Brief Review**

**Molecular Biology of Angiotensinogen**

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Angiotensinogen is a moderately abundant 55,000–60,000 Da serum glycoprotein that is the precursor to the angiotensin peptides and is the only known naturally occurring renin substrate. It is synthesized by a variety of cells, most prominently hepatocytes, adipocytes, and astrocytes. Most angiotensinogen is extracellular (it is constitutively secreted); thus, there is apparently no way that an organism can orchestrate rapid changes in angiotensinogen concentration. The angiotensinogen gene is regulated by several hormones (e.g., glucocorticoid and estrogen), and angiotensinogen is an acute-phase protein. Angiotensinogen is a member of the serpin superfamily, but there is little reason to suppose that this protein is a serine protease inhibitor. The available evidence indicates that angiotensinogen functions solely as an extracellular reservoir of angiotensin peptides. In this article, we attempt a critical review of the literature on the biology of this protein, with an emphasis on recent molecular biological studies. We recommend other recent reviews, for example, those of Tewksbury or Campbell, for alternative views of this subject matter.

**Gene Structure and Promoter Function**

The rat, mouse, and human angiotensinogen genes have been isolated and characterized. All are present as a single copy; the rat gene has been mapped to chromosome 19, the human gene to chromosome 8. The rat gene is the most thoroughly characterized, but the structure and function of the mouse and human genes, so far as is now known, are not significantly different. The rat angiotensinogen gene is roughly 12 kilobase pairs (kbp) long and consists of five exons separated by four introns. The first exon encodes only a portion of the 5' untranslated region of the angiotensinogen messenger RNA (mRNA) and is separated from remaining exons by a 5 kbp intron. The intron/exon boundaries of the gene conform to the consensus sequence of pre-mRNA introns and are perfectly conserved among all three genes. The region immediately flanking the structural portion of the rat gene contains several identifiable (i.e., by sequence comparison) promoter elements, including a “TATA” element at position -30 bp (relative to the start site of transcription) and two putative glucocorticoid responsive elements (5'-AGAACA-3') at positions -586 bp and -477 bp.

Habener and coworkers have reported a series of studies characterizing the promoter of the rat angiotensinogen gene. Stretches of DNA that lie 5' to the transcription start site (cap site) of the rat angiotensinogen gene were fused to a firefly luciferase reporter gene and transiently expressed in placental JEG-3 cells and hepatoma HepG2 cells. Progressive deletions of the angiotensinogen gene flanking DNA identified a minimal promoter region of 688 bp that supported expression of the fusion gene in both cell types. This fragment contains six transcriptionally active elements (as defined by DNase I footprinting), including an enhancer element. The latter element acts to silence transcription in its natural position (-108 to -60 nucleotides where +1 is the start site of transcription) but functions as a transcriptional enhancer when moved further away from the structural portion of the fusion gene. This enhancer element also functions to suppress the transcriptional activity of a viral (SV-40 early) promoter. In another study, a cis-acting element at -545 bp, which was identified by DNase I footprinting and is similar to the NFkB transcription factor binding site, was found to function in the acute-phase response (i.e., transcriptional activation in response to cytokines). This element, when fused upstream of a cytokine-unresponsive gene, confers a cytokine-responsive phenotype on that gene. The element binds NFkB as well as another, unidentified, transcription factor. A third study showed that glucocorticoids were necessary for the acute-phase-responsive phenotype in the H35 rat hepatoma cell line. This result confirms an earlier study with H35 cells that showed cytokine interleukin-6 is ineffective in inducing increased angiotensinogen mRNA accumulation in the absence of glucocorticoid. The human HepG2 hepatoma line, which is deficient in glucocorticoid receptor, does not support interleukin-1 induction of angiotensinogen gene expression unless glucocorticoid receptor is also introduced (via transfection of a functional glucocorticoid receptor gene). One (of two) glucocorticoid response element in this region overlaps the acute-phase response element.
Clouston et al. identified what is nearly the same minimal promoter region (750 bp from the immediate 5' flanking region of the mouse angiotensinogen gene). This region was sufficient to confer glucocorticoid, estrogen, and bacterial endotoxin (the latter induces an acute-phase response) inducibility on a minitransgene (i.e., a mouse angiotensinogen gene that contains an internal deletion of much of its coding region) introduced into the genome of Swiss mice. This transgene was also expressed in a tissue-specific manner in the recipient mice. Fukamizu et al. analyzed the promoter function of the 5' flanking region (-1,222 to +44) of the human angiotensinogen gene by fusing this region to a chloramphenicol acetyl transferase reporter gene and then tested this fusion gene in the human hepatoma cell line HepG2 and in the human astrocytoma line T98G. This construct was active only in the hepatoma cell line. The conclusion that can be drawn from these studies is that the angiotensinogen gene contains multiple cis-acting transcriptional elements within a kilobase of the major start site of transcription and that these elements include those ultimately responsive to glucocorticoids, estrogens, and cytokines. This region also contains an enhancer element and confers tissue specificity on angiotensinogen gene expression.

**Messenger RNA Structure**

Rat angiotensinogen mRNA is roughly 1,800 nucleotides long, consisting of 61, 1,431, and 200–400 nucleotides of 5' untranslated, coding, and 3' untranslated sequence, respectively. The size heterogeneity of this mRNA is largely because at least four different polyadenylation sites are used. These sites, which were identified by S1 nuclease mapping, are closely spaced (at 1,650, 1,785, 1,800, and 1,840 nucleotides). Further size heterogeneity of the rat angiotensinogen mRNA is a result of an unusual feature of its gene, that is, treatment with glucocorticoids increases liver angiotensinogen synthesis (see below) and elicits the use of two additional, upstream transcription start sites (at -386 and -328 nucleotides). Although this phenomenon is interesting as regards mechanisms of glucocorticoid action, these extended angiotensinogen mRNAs apparently have the same coding potential as the predominant mRNA species and therefore have no obvious bearing on the functioning of the renin-angiotensin system. There is no evidence for alternate splicing of the angiotensinogen gene RNA transcript. A potentially important report predicting different forms of angiotensinogen mRNA based on electrophoretic analysis of their in vitro translation products has not been independently verified (e.g., by subsequent analyses of complementary DNA [cDNA] clones).

**Protein Structure**

Analyses of cDNA and genomic clones have shown convincingly that the angiotensinogen gene encodes a single protein product. This protein (477 amino acids in the rat and mouse; 485 amino acids in the human) is synthesized as a preform. The cotranslational removal of the leader peptide reveals the mature protein (452 amino acids in humans, 453 in rodents) with the (invariant) angiotensin I (Ang I) decapeptide at the amino terminus. The amino acid sequence immediately following this decapeptide is dissimilar in rodent and human angiotensinogens. This difference suggested an explanation for the observed low activity of primate renin when acting on nonprimate angiotensinogen. This suggestion was confirmed by Quinn and Burton, who used synthetic tetradecapeptides to show that the amino acid residues immediately following the scissile peptide bond were responsible for this substrate selectivity.

Angiotensinogen contains several potential sites for N-linked glycosylation (Asn-X-Ser/Thr), and different glycosylation patterns are apparently responsible for the isoelectric point and size variants of circulating protein. Some angiotensinogen also circulates as a high molecular weight variant; although this form is normally only a minor component of the total plasma angiotensinogen, it exists at higher levels during the last trimester of pregnancy and is occasionally the predominate form in the sera of hypertensive, pregnant women. This higher molecular weight angiotensinogen is an effective renin substrate and consists of angiotensinogen that is noncovalently linked to another serum protein. The physiological, or pathophysiological, relevance of these multiple forms of angiotensinogen, if any, is not known.

Rat and mouse angiotensinogens are 87% identical, whereas the rodent and human angiotensinogens are 60% and 64% identical, a level of similarity that is perhaps surprisingly low. Soon after the rat angiotensinogen amino acid sequence was published by Nakanishi and colleagues, Doolittle pointed out that angiotensinogen is faintly, but significantly, related to α1-antitrypsin, antithrombin, and ovalbumin and thus is a member of the serpin gene superfamily.

**Sites of Angiotensinogen Synthesis**

Most angiotensinogen is extracellular, and therefore its site of synthesis is most readily determined by detecting its mRNA. Angiotensinogen RNA sequences accumulate in liver, fat, and brain and have been detected, albeit in small amounts, in a variety of tissues including lung, kidney, ovary, adrenal gland, heart, spinal cord, and testes. It may be that the source of angiotensinogen in these tissues is not parenchymal cells but rather adherent fat or fibroblasts. The latter cell type has been identified tentatively as containing angiotensinogen mRNA in sections of rat mesenteric vessels and in clonal lines of mouse 3T3 fibroblasts. However, all low level angiotensinogen mRNA accumulation cannot be attributed to fibroblasts or adipocytes; for example, this mRNA has been localized to the proximal convoluted tubule. Finally, angiotensinogen may be synthesized in the bloodstream since its mRNA has been
detected in leukocytes (Dr. A. Gomez, personal communication).

The predominant source of circulating angiotensinogen in the adult animal is probably the liver, although the variety of fat depots that have been shown to synthesize angiotensinogen also presumably contribute to the blood angiotensinogen levels. The detection of angiotensinogen mRNA in liver confirmed earlier studies that showed renin substrate secretion from perfused liver and dispersed hepatocytes. The unexpected occurrence of angiotensinogen RNA sequences in adipocytes was first detected in periaortic adipocytes by in situ hybridization performed on rat aorta sections and later noted in both brown and white fat including perirenal, epididymal, and interscapular depots. Intact white and brown fat pads isolated from rats also have been shown to secrete this protein. Angiotensinogen RNA sequences also accumulate in cultured Swiss mouse 3T3-L1 fibroblasts, and the level of this mRNA increases as these cells differentiate to adipocytes. The reason for angiotensinogen synthesis (and secretion) by adipocytes remains a mystery; the authors suggested that it serves as a local source for angiotensin peptides functioning to enhance norepinephrine release at sympathetic nerve terminals.

Protoplasmic astrocytes (i.e., astrocytes in gray matter) synthesize angiotensinogen in brain. This fact was shown by several methods. For example, combined in situ hybridization and immunocytochemistry performed on rat brain stem and hypothalamic sections revealed a striking congruence of silver grains (labeled oligonucleotide probe) and glial fibrillary acidic protein (GFAP, a standard astrocyte marker) but not with microtubule-associated protein II, a neuronal marker. The astrocytes that contain detectable angiotensinogen mRNA are clustered in about three dozen nuclei found in the hypothalamus, midbrain, and brain stem. Quantitative (i.e., grain counting) analysis of many slices from these brain regions shows that individual brain nuclei differ in their proportion of angiotensinogen mRNA- and angiotensinogen RNA-sequence accumulation. The nuclei that are most active in angiotensinogen synthesis (i.e., mRNA accumulation) are generally those that also exhibit high affinity angiotensin binding. (The brain regions that show a disparity between angiotensin binding and angiotensinogen mRNA levels, that is, high binding, low mRNA, generally lie outside the blood–brain barrier, for example, the area postrema.) Angiotensinogen secretion from astrocytes is apparently the source of the protein in cerebrospinal fluid (where it makes up 2–3% of all protein) since blood-borne angiotensinogen does not gain access to the brain parenchyma.

The astrocytic origin of angiotensinogen is perhaps difficult to reconcile with the immunohistochemical detection of angiotensin II in presynaptic nerve terminals. Disregarding the trivial explanation that the neuronal immuno–cross-reactive material is not angiotensin II, the simplest explanation for this discrepancy is that a low level of neuronal angiotensinogen mRNA, and thus synthesis, has escaped detection by the combined in situ hybridization/immunocytochemistry technique. Another explanation is that the immunoreactive material is angiotensin II taken up by receptor-mediated endocytosis, as has been shown in the adrenal gland. The alternatives of extraneuronal processing of angiotensinogen followed by uptake of angiotensin II and controlled release at the synapse or uptake of the prohormone by neurons with subsequent processing are without precedent.

Control of Angiotensinogen Gene Expression

Some of the impetus for studies of angiotensinogen gene promoter activity described previously was provided by the body of literature describing developmental and hormonal influences on angiotensinogen gene expression. Angiotensinogen RNA sequences were detected at low levels on embryogenesis day 11 (E11) in rat fetuses (bodies) and yolk sacs, and these RNA levels increased from day E13 to E17,
whereas the increase in angiotensinogen RNA in rat fetus heads did not occur until days E20–E21. However, angiotensinogen RNA is barely detectable in fetal (E15–E20) rat liver, suggesting that the bulk of fetal angiotensinogen RNA is from nonhepatic sources, perhaps kidney and fat depots. Liver angiotensinogen RNA levels rise dramatically soon after birth and achieve adult (i.e., 90-day-old) levels within 24 hours postpartum. During phenotypic modulation of 3T3 fibroblasts (3T3-L1 and 3T3-F442A) to adipocytes, angiotensinogen mRNA accumulation increases dramatically (Reference 31 and M.J. Peach, unpublished data).

The most studied regulatory phenomenon regarding angiotensinogen is that exerted by glucocorticoids. In addition to the unmasking of additional transcription start sites upstream of the angiotensinogen gene and exerting a permissive effect with acute-phase response mediators (see above), glucocorticoids increase the level of angiotensinogen gene expression. This augmentation is seen clinically as an increase in serum renin substrate levels during hypercorticism and as a decrease in these levels during adrenal insufficiency. Increased angiotensinogen synthesis also has been shown in perfused liver and liver slice preparations from animals pretreated with glucocorticoids, and in a rat hepatoma cell line. Furthermore, adrenalectomy results in diminished liver angiotensinogen synthesis, and this deficit is reversed on glucocorticoid administration.

There also is increased liver angiotensinogen RNA sequence accumulation in response to glucocorticoids applied to intact animals, dispersed hepatocytes, and cultured cell lines. The increase in angiotensinogen RNA accumulation occurs rapidly, is insensitive to protein synthesis inhibitors, and is blocked by the glucocorticoid antagonist RU486. These observations, along with the existence of glucocorticoid response elements upstream of the angiotensinogen gene (see above), suggest that the activated glucocorticoid receptor interacts directly with this gene.

Angiotensinogen gene expression is also positively affected by sex steroids. Estradiol treatment of intact rats results in elevated plasma renin substrate levels and increased accumulation of hepatic angiotensinogen RNA. Estradiol treatment of a rat liver slice preparation also elicits increased angiotensinogen production, but this hormone failed to increase angiotensinogen RNA sequences in a rat hepatocyte preparation. The estradiol-induced increase in angiotensinogen gene expression is reflected in elevated plasma renin substrate levels seen in women taking oral contraceptives. Androgen treatment of female rats or castrated male rats has been reported to increase kidney angiotensinogen RNA levels.

Angiotensin II also has been shown to increase angiotensinogen release in perfused livers when the peptide was infused continuously over a period of several hours, but attempts to reproduce this stimulation in hepatocytes have produced mixed results. The regulation of angiotensinogen gene expression by insulin also has been reported in studies on cultured cells where this hormone exerts an inhibitory effect. Finally, angiotensinogen is an acute-phase response protein, and its synthesis by cultured rat hepatoma cells is increased by treatment with interleukin-6 but only when this cytokine is presented to the cells with glucocorticoids. This synergism is reminiscent of that observed in dispersed hepatocytes treated with a cyclic AMP analogue (S-cyclic AMP) and glucocorticoids.

Less information is available regarding hormonal control of extrahepatic angiotensinogen synthesis. Given the ubiquity of the glucocorticoid receptor, one would expect that glucocorticoid agonists would increase expression of the angiotensinogen gene in most tissues where the gene is expressed. Treatment of rats with a combination of the glucocorticoid agonist dexamethasone, estradiol, and thyroid hormone elicited increased accumulation of angiotensinogen RNA sequences. Likewise, a single dose of estradiol reportedly increased liver angiotensinogen RNA accumulation, increased this RNA in the brain to a lesser extent, but had no effect on the heart angiotensinogen RNA accumulation.

Functions of Angiotensinogen

The function of angiotensinogen is to act as a circulating renin substrate, and the products of the reaction are the amino terminal derived decapeptide Ang I and des(Ang I)-angiotensinogen. There is debate regarding other possible functions of angiotensinogen. An oddity of the angiotensin system that must be considered in debates of the functioning of the system is the extracellular nature of angiotensinogen. Angiotensinogen is a ubiquitous, moderately abundant protein with plasma and cerebrospinal fluid concentrations of approximately 1 μM and 0.2 μM, respectively. It is present at low levels intracellularly; for example, immunocytochemical detection of the hepatic protein is difficult without prior colchicine treatment. Brain angiotensinogen is also mostly present in the extracellular space. None of the numerically prominent cell types that have been shown to synthesize and secrete angiotensinogen (i.e., hepatocytes, adipocytes, or astrocytes) possess a concentrating secretory compartment but rather constitutively secrete proteins. Furthermore, Descheppe and Reudelhuber reported that angiotensinogen (in contrast to renin) is not directed to secretory granules when expressed in cultured AtT-20 cells but rather is released constitutively.

These facts bear on the functioning of putative local angiotensin-generating systems in that, so far as is known, there is no way that an organism can
orchestrate rapid changes in angiotensinogen concentration and there is no obvious route for intracellular processing of this protein. Local angiotensin systems might consist of a locally released (or activated) renin (or reninlike) enzyme that cleaves the angiotensinogen present in most extracellular fluids. Moffet et al. presented a cogent argument in support of this type of pathway in the processing of brain angiotensinogen. Currently, the only physiologically relevant processing enzyme recognized is renin; however, several serine proteases such as tonin and cathepsin G are capable of cleaving angiotensin II directly from angiotensinogen, at least in vitro.

If angiotensinogen is simply an extracellular reservoir of angiotensin peptides as we contend, then a question arises as to why the angiotensinogen output of the liver is not sufficient to supply all (non-central nervous system) tissues. A plausible explanation suggested by Campbell is that individual angiotensinogen-secreting tissues would have higher levels of angiotensinogen than those that receive this protein only from circulation. Depending on the concentration and activity of renin (or reninlike) enzymes, the elevated angiotensinogen could result in higher levels of angiotensin. It is worth noting that the local concentrations of renin-angiotensin system components are not known in any tissue angiotensin system.

Finally, functions of angiotensinogen unrelated to its role as a prohormone need to be considered. The large ratio of the size of precursor (452-3 amino acids) to product (10 amino acids) encourages a teleologic argument regarding alternate functions of angiotensinogen. The hypothesis that angiotensinogen is a precursor to erythropoietin, which was largely based on cross-reacting antisera, is apparently incorrect since erythropoietin has subsequently been described by molecular cloning and is unrelated to angiotensinogen. The suggestion that des(Ang 1)-angiotensinogen is a renin inhibitor also has not been confirmed experimentally. Angiotensinogen is distantly, but significantly, related to several serine protease inhibitors and therefore has been assigned to the serpin gene family. This family is diverse; although it is named for its protease inhibitor members, there are noninhibitor members as well, and thus, membership in this superfamily is insufficient evidence to assign a protease inhibitor activity to angiotensinogen. Furthermore, angiotensinogen contains an amino terminal extension relative to other serpins, and the putative active sites of the most closely related serpins are not conserved between human and rodent angiotensinogens. Regarding the latter point, the relatively high degree of sequence divergence (more than 35%) between rodent and human angiotensinogens is evidence that there is little pressure to conserve much of the amino acid sequence of this protein. In addition, proteolytic cleavage of angiotensinogen at a site analogous to the reactive center loop of α1-antitrypsin fails to generate a form with the increased heat stability associated with the conversion of the stressed to the relaxed form observed in classical serpins. Finally, no experimental evidence showing that angiotensinogen is a serine protease inhibitor has been reported.

In summary, the existing data support only a limited role for angiotensinogen, that is, as an extracellular reservoir of angiotensin peptides. Hepatocytes, astrocytes, adipocytes, and a variety of (numerically) less prominent cell types elaborate angiotensinogen. The reason for synthesis of this protein by a wide variety of tissues is unknown; perhaps angiotensinogen concentrations are significantly higher in the extracellular spaces of these tissues. Although expression of the angiotensinogen gene is controlled by several hormones, changes in angiotensinogen concentration are sluggish (as compared with those of renin, for example) and the physiological relevance of these control mechanisms is not obvious. There is apparently no direct evidence that angiotensinogen is processed intracellularly, whereas a coherent body of evidence suggests angiotensinogen is constitutively released and mostly extracellular. Review articles often close with a promise of many exciting things to come with further study; we wonder whether angiotensinogen and its gene have already yielded their innermost secrets.

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