Location and Regulation of Rat Angiotensinogen Messenger RNA

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SUMMARY The presence of angiotensinogen messenger RNA (mRNA) was detected in rat vascular and adipose tissue. Angiotensinogen mRNA in rat aorta was localized in the adventitia and surrounding adipose tissue, and not in the vascular smooth muscle. Freshly dispersed and cultured endothelial and aortic smooth muscle cells did not contain detectable amounts of angiotensinogen mRNA. In addition to periaortic adipose tissue, angiotensinogen mRNA was present in other fat depots of both brown and white types. To examine regulation of angiotensinogen gene expression, Sprague-Dawley rats were treated with angiotensin converting enzyme inhibitor or underwent bilateral nephrectomy. Relative levels of angiotensinogen mRNA in brown adipose tissues increased dramatically by 48 hours after bilateral nephrectomy. However, only one source of brown adipose tissue showed increased angiotensinogen mRNA levels after animals were treated for 5 days with converting enzyme inhibitor. In addition, angiotensinogen was released into the medium from incubated adipose tissues with levels increasing over a 2-hour period. These results demonstrate that angiotensinogen is synthesized by adipose tissue in the rat and may play a role in the function of this tissue.

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KEY WORDS • angiotensinogen • messenger RNA • adipose • aorta

THERE is considerable evidence that peripheral tissues represent major sites of production of angiotensin II, a peptide that plays a dominant role in blood pressure, fluid, and electrolyte homeostasis. Local synthesis of angiotensin is dependent on the availability of the only known precursor of the angiotensin peptides, angiotensinogen.1 Angiotensinogen synthesis has been demonstrated on the basis of messenger RNA (mRNA) or inferred by immunocytochemistry in a variety of tissues, including liver, brain, kidney, adrenal gland, and blood vessels.2-4 However, the precise cell types responsible for angiotensinogen gene expression have not been identified in extrahepatic tissues.

One of the demonstrated sites of angiotensinogen gene expression is in the vasculature of the rat.2 There are three layers of a blood vessel wall: endothelial cells lining the lumen of the vessel, the medial smooth muscle cell, and the adventitia (outside the muscle mass), which is composed of a variety of cell types (such as nerve endings, adipocytes, mast cells, and fibroblasts). In addition, adipose tissue commonly encases major muscular arteries.3 In this study, in addition to the initial determination of the presence of angiotensinogen mRNA in various rat blood vessels, we have attempted to determine which vascular wall layer(s) or cell type(s) is responsible for angiotensinogen gene expression. Based on preliminary results, we also have examined perivascular and nonvascular adipose tissues in our attempt to determine predominant sites for angiotensinogen gene expression.

Regulation of angiotensinogen production appears to differ depending on the tissue type and endocrine factors involved.2,6 Two methods that interfere with the renin-angiotensin system were chosen to examine regulation of angiotensinogen gene expression. The levels of angiotensinogen mRNA in arteries and adipose tissue were examined in animals 48 hours after bilateral nephrectomy or after administration of a converting enzyme inhibitor for 5 days.

Materials and Methods

Tissue Isolation and Vessel Wall Separation

Male Sprague-Dawley rats (weight, 250–325 g; Dominion Laboratories, Dublin, VA, USA) were used for most experiments. Rat thoracic aorta was removed and processed for RNA extraction either after the vessel...
was dissected free of fat or with adhering periaortic fat still attached. For separation of vessel wall elements, rat thoracic aortas were freed of periaortic fat and incubated in a solution containing collagenase (1 mg/ml; Type II, Lot 4457082, Worthington Biochemicals, Freehold, NJ, USA) and elastase (0.25 mg/ml; Type I, Lot 64N6760, Worthington) at 37°C for 20 minutes. With the use of a dissecting microscope, the adventitia was then carefully peeled from the remaining muscle layers and each tissue (adventitia and muscle) processed separately.

The following adipose sources \((n = 6)\) were removed and RNA was extracted: periaortic fat (pooled tissue from three rats), interscapular fat, perirenal fat, mesenteric fat (adjacent to the mesenteric artery), and epididymal fat (pooled tissue from three rats). In addition to adipose tissues and thoracic aorta, RNA also was isolated from freshly dispersed rat aortic smooth muscle cells and bovine aortic endothelium, cultured smooth muscle cells, and cultured bovine pulmonary artery and bovine endothelial cells. Cells were prepared and cultured as previously described. The rat angiotensinogen complementary DNA (cDNA) cross-hybridizes with bovine mRNA for angiotensinogen (K.R. Lynch, personal communication, 1987).

**RNA Extraction and Gel Electrophoresis**

RNA was extracted from 1.0 g of vascular or adipose tissues according to the method of Chirgwin et al. Gel electrophoresis, cDNA labeling, blotting, and hybridization conditions were performed by standard techniques previously described. A full-length, 1650-base pair rat angiotensinogen cDNA (0.1 µg in the labeling reaction) was used to detect angiotensinogen mRNA sequences.

**Bilateral Nephrectomy**

For nephrectomy experiments, 600-g male Sprague-Dawley rats \((n = 6)\) were anesthetized with pentobarbital, 8.3 mg/kg, a midline dorsal incision was made, and both kidneys were removed. Animals were killed 48 hours after nephrectomy, and blood vessels and fatty tissues removed for mRNA analysis.

**Converting Enzyme Inhibitors**

Enalapril maleate (27 mg/kg; Merck Sharp & Dohme) was given to 10 male Sprague-Dawley rats (weight, 300 g) for 5 days in their drinking water. To ensure converting enzyme inhibition, levels of converting enzyme activity were measured in rat plasma collected on the day of death from treated and control rats using a Ventrex kit (Portland, ME, USA). Appropriate tissues were removed, and RNA was extracted.

**Angiotensinogen Concentration**

Interscapular fat, epididymal fat pad, periaortic fat, and the thoracic aorta (2 cm in length) were incubated in 2 ml of Krebs-Ringer solution (95% \(O_2\), 5% \(CO_2\)) at 37°C for 2 hours. Then, 100-µl aliquots of media were removed at 30, 60, and 120 minutes and placed in 500 µl of 0.05 M phosphate buffer (pH 6.8) and an excess of hog renin (EC 3.4.99.19; 0.002 unit of which will liberate 200 ng of angiotensin I in 1 hour at 37°C; Sigma Chemical, St. Louis, MO, USA). The following protease inhibitors were present: 10 mM phenylmethylsulfonfl fluoride, 5 mM EDTA, 1 mM 8-hydroxyquinoline, and teprotide, 10 µg/ml. At the end of 1 hour, the reaction was stopped by heating at 100°C for 10 minutes. Precipitated protein was removed by centrifugation at 2000 g for 10 minutes. Angiotensin I in the supernatant was measured by radioimmunoassay.

**Densitometric Analysis**

For densitometric analysis of angiotensinogen mRNA levels, three autoradiograms (at three different levels of exposure) were transferred onto an Eikonix image digitizer (Bedford, MA, USA). The digitizer was then used to obtain an integrated density value. Values from each series of experiments were then averaged \((± SEM)\) and compared either with values obtained from rat liver or as the x-fold increase over control. Densitometric analysis was only performed on dot blots to compare angiotensinogen mRNA levels.

**Results**

Total RNA from aorta, caudal and mesenteric arteries was examined for the presence of angiotensinogen mRNA (Figure 1). Angiotensinogen mRNA was detected in all arteries. Angiotensinogen mRNA levels in rat aorta, mesenteric artery, and caudal artery were 30, 18, and 13%, respectively, compared with hepatic angiotensinogen mRNA levels per total RNA.

To determine the location of the angiotensinogen mRNA in the vessel wall, we separated the adventitia from the smooth muscle of rat aorta. When these layers were examined separately, a very small amount of angiotensinogen mRNA was present in the adventitia, with no detectable levels found in the muscle (Figure 2, right panel). In addition, neither cultured rat aortic vascular smooth muscle, cultured bovine aortic endothelial cells, nor freshly dispersed bovine aortic endothelial cells contained detectable amounts of angiotensinogen mRNA (Figure 2, left panel). However, these freshly dispersed or cultured cells did contain actin mRNA (data not shown). In Figure 2, the source of RNA for rat aorta is Wistar-Kyoto rats (WKY), in contrast to Sprague-Dawley aorta used in all other experiments. Thus, species differences do not exist in expression of the angiotensinogen gene in rat aorta.

Since aorta had a much larger amount of angiotensinogen mRNA than was found in adventitial plus medial layers, a further attempt was made to comprehensively examine the rat aorta, and we readily established the presence of angiotensinogen mRNA in periaortic adipose tissue. An abundant amount of angiotensinogen mRNA was detected in this adipose tissue, as well as in several other nonvascular sources of fat (Figure 3, right panel). Among the adipose sources examined, angiotensinogen mRNA levels were greatest (mRNA levels per total RNA) in perirenal fat, followed by the epididymal fat pad, with similar levels found in peri-
When animals were treated with a converting enzyme inhibitor, there were no significant differences between angiotensinogen mRNA levels from treated versus control rats in most adipose tissues examined (see Figure 3, right panel). However, angiotensinogen mRNA levels were increased in interscapular fat pads from enalapril-treated rats (see Figure 3, right panel). Plasma angiotensin converting enzyme activity (1 unit = nmol/min/ml) was measured in control (30–40 units) and enalapril-treated (levels were below the detection limit of the assay) rats, substantiating the actions of enalapril over the time frame examined. In Figure 3, left panel, the effects of both nephrectomy and converting enzyme inhibition on angiotensinogen mRNA levels in the rat aorta are depicted. Aortas that were processed with periaortic fat still attached always contained greater levels of angiotensinogen mRNA than did aortas dissected free of fat. Even though periaortic fat alone showed no significant changes in angiotensinogen mRNA levels after converting enzyme inhibition, greater angiotensinogen mRNA levels were present in aorta with attached periaortic fat from enalapril-treated rats than in the corresponding controls (see Figure 3, left panel, Lanes 1 and 2).

Levels of angiotensinogen (as measured by angiotensin I generation following the addition of hog renin) released from the aortas and periaortic, interscapular, and epididymal fat were determined. Over a 2-hour period, angiotensinogen levels detected in the incubation media from all fat sources increased in a linear fashion (Figure 5). However, in rat aorta cleaned of periaortic fat, the amount of angiotensinogen released was less than that from adipose tissues and either decreased or remained the same over the time frame examined.

**Discussion**

In an attempt to determine the cell types in rat blood vessels involved in angiotensinogen gene expression, we have found angiotensinogen mRNA in aortic, interscapular, and mesenteric fat and the lowest levels found in mesenteric fat.

In tissues taken from rats 48 hours after bilateral nephrectomy, levels of angiotensinogen mRNA in periaortic and interscapular fat increased 4.4-fold and 10.4-fold, respectively, with no significant changes in whole aortic angiotensinogen mRNA levels (Figure 4, right panel). Angiotensinogen mRNA levels in periaortic and interscapular fat from nephrectomized rats increased to levels greater than those based on total RNA from livers of control rats.
FIGURE 3. Relative levels of angiotensinogen mRNA in tissues from animals treated with enalapril (MK421). Dot blot analysis of total RNA (0.1–1.00 μg) from aorta (Ao) and various adipose tissues are shown. Left panel: Lane 1, aorta with periaortic fat from enalapril-treated rats; Lane 2, aorta with periaortic fat from control rats; Lane 3, aorta from nephrectomized (Nx) rats; Lane 4, aorta from enalapril-treated rats; Lane 5, muscle; Lane 6, liver. Angiotensinogen cDNA (100 ng) was labeled to a specific activity of $6.98 \times 10^5$ dpm/μg. Right panel: Lanes 1 and 2, liver from treated and control rats; Lanes 3 and 4, interscapular fat (ISF) from treated and control rats; Lanes 5 and 6, periaortic fat (PAF) from treated and control rats; Lanes 7 and 8, epididymal fat (EF) from treated and control rats; Lanes 9 and 10, mesenteric fat (MF) from treated and control rats; Lanes 11 and 12, perirenal fat (PRF) from treated and control rats. Angiotensinogen cDNA (100 ng) was labeled to a specific activity of $2.72 \times 10^5$ dpm/μg. Kodak XRP film was exposed for 10 days in the presence of an image-intensifying screen.

FIGURE 4. Angiotensinogen mRNA in tissues from control rats and animals 48 hours after bilateral nephrectomy. Left panel: Northern gel analysis of total RNA (10 μg/lane except for liver, 2 μg) and hybridization with an angiotensinogen cDNA labeled to a specific activity of $3.2 \times 10^6$ dpm/μg. Lanes 1 and 2, periaortic fat (PAF) from nephrectomized (Nx) and control rats; Lanes 3 and 4, interscapular fat (ISF) from nephrectomized and control rats; Lanes 5 and 6, aorta (Ao) from nephrectomized and control rats; Lane 7, liver (Liv); and in the next lane, RNA standard depicting 7.5 and 2.4 kb. Right panel: Relative levels of angiotensinogen mRNA in tissues from control rats and animals following bilateral nephrectomy. Dot blot analysis of total RNA (0.1–1.00 μg) from tissues is shown. Angiotensinogen cDNA (100 ng) was labeled to a specific activity of $6.4 \times 10^5$ dpm/μg.
ventitia, in adipose tissue surrounding major blood vessels, as well as in other sources of fat. The presence of angiotensinogen mRNA in periaortic adipose tissue, with very small levels detected in the adventitia and none in the muscle plus endothelium, suggested that the adipocytes present within the adventitia and periaortic adipose tissue may be the only cell element responsible for angiotensinogen gene expression in rat aorta. Clearly, freshly dispersed or cultured aortic vascular smooth muscle and endothelium did not contain detectable levels of angiotensinogen mRNA. After determining that angiotensinogen mRNA was present in periaortic fat, all subsequent aortas used in these studies were exhaustively cleaned of adipose tissue. This step resulted in much smaller levels of angiotensinogen mRNA detected in rat aorta cleaned of fat (see Figure 3, left panel, Lane 1 vs 4). However, besides adipocytes, several other cell types are present in perivascular adipose tissue, and this precludes the definitive identification of the adipocyte as the cell responsible for angiotensinogen gene expression.

In most species, adipose tissue can be categorized into two metabolically distinct types: brown and white. Brown adipose tissue, which encases most major blood vessels of the rat, is used for thermogenesis, while white fat is a metabolic source. In addition to periaortic fat, two other brown adipose tissues in the rat were examined for the presence of angiotensinogen mRNA. Perirenal fat and interscapular fat pad were shown to express the angiotensinogen gene. To determine whether angiotensinogen gene expression was limited to brown adipose tissue, the epididymal fat pad of the rat, a classic white adipose tissue, also was examined. Interestingly, this adipose tissue contained even larger quantities of angiotensinogen mRNA.

Angiotensinogen secretion from adipose tissue (interscapular, periaortic, and epididymal fat) increased in a linear fashion over a 2-hour period. Thus, angiotensinogen mRNA is translated into measurable protein that is exported or released by the tissue. These results suggest continued synthesis and release of the protein, rather than a leaching of protein sequestered from the circulation. The physiological importance of the presence of angiotensinogen in adipose tissue is unknown.

Angiotensin converting enzyme inhibitor and bilateral nephrectomy were used to examine angiotensinogen gene regulation. These methods were chosen since both interfere with the renin-angiotensin system. However, bilateral nephrectomy is a much more radical manipulation and, as such, results in a variety of changes besides a decrease in plasma renin activity and angiotensin levels, including changes in various endocrine factors, which in turn may modulate angiotensin gene expression. In contrast, converting enzyme inhibitors specifically interfere with the renin-angiotensin and kallikrein-kinin systems.

Bilateral nephrectomy resulted in a dramatic increase in angiotensinogen mRNA levels in all brown adipose tissues examined, to levels greater than those seen in rat liver. Following bilateral nephrectomy, Campbell and Habener found slight increases in liver angiotensinogen mRNA but decreases in angiotensinogen mRNA levels in a variety of other tissues examined, including rat aorta. We also found slight decreases in angiotensinogen mRNA levels in rat aorta after nephrectomy; however, changes in aortic angiotensinogen mRNA levels were not significant. The specific mediator responsible for the dramatic increases in adipose angiotensinogen mRNA levels after nephrectomy remains to be determined.

In contrast to changes seen with nephrectomy, converting enzyme inhibition increased angiotensinogen mRNA levels only in interscapular adipose tissue. Therefore, increases in angiotensinogen mRNA levels in adipose tissue after nephrectomy may not be related to parameters that are altered by converting enzyme inhibitors.

In conclusion, brown and white adipose tissues in the rat are capable of angiotensinogen gene expression and production of the protein. Angiotensinogen mRNA in rat aorta may be restricted to adipocytes in the adventitia and periaortic fat since no detectable levels were present in the muscle. The presence of angiotensinogen mRNA in adipose tissue encasing major arteries implies local angiotensin synthesis in the vicinity of sympathetic innervation to the artery, with the potential for enhanced sympathetic neurotransmission. Finally, the tremendous regulation of adipose angiotensinogen mRNA levels after bilateral nephrectomy suggests that this local tissue renin-angiotensin system may play an important pathologic role in a variety of disease states. Adipose angiotensinogen production may well provide insights into the relationship between obesity and hypertension or the metabolic disturbances in adipose tissue and vascular lesions such as those seen in diabetes.
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