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Enhanced Expression of Angiotensinogen mRNA in Rat Central and Peripheral Tissues Following Hemorrhage

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=ABSTRACT=

The renin-angiotensin system plays an important role in the regulation of blood pressure and in body fluid homeostasis. There is increasing evidence for generation of endogenous angiotensin II in many organs and for its role in paracrine functions. Studies were designed to investigate whether hemorrhage produces rapid changes in the gene expression of angiotensinogen in peripheral and brain tissues. Wistar rats received saline drinking water for 7 days, were bled at a rate of 3 ml·kg^{-1}· min^{-1} for 7 min, and then decapitated 0, 2, 4, 8, or 24 hr after hemorrhage.

Hemorrhage produced a profound hypotension with tachycardia at 2~8 hr, but blood pressure and heart rate had not fully recovered to the basal level at 24 hr. Plasma renin concentration was significantly increased at 2, 4, and 8 hr (maximum sixfold increase at 4 hr) and had returned to the basal level at 24 hr. Renal renin content was significantly increased only at 4 hr after hemorrhage. Angiotensinogen mRNA in both the kidney and liver were stimulated at 2 to 8 hrs, but recovered to the basal level at 24 hr. On the other hand, angiotensinogen mRNA levels in the hypothalamus and brainstem were continuously increased from 2 to 24 hrs.

The present study demonstrates the presence of angiotensinogen mRNA in both hepatic and extrahepatic tissues, and more importantly, their up-regulation after hemorrhage. These results suggest that the angiotensinogen-generating systems in the liver, kideny and brain are, at least in part, under independent control and play a local physiological role.

Key Words: Angiotensinogen mRNA, Hemorrhage, Brain, Kidney, Liver

INTRODUCTION

Regulation of blood pressure and body fluid balance in the vertebrate is accomplished by a combination of neurological, hormonal and neuroendocrine mechanisms. The renin-angiotensin system is one of the most important regulators (Campbell 1987; Phillips 1987). It is well known that angiotensin II (Ang II), acting on specific receptors in various organs, mediates a variety of effects. In the brain, Ang II stimulates thirst, alters sympathetic outflow, and regulates the secretion of pituitary hormones (Phillips 1987; Ferguson & Wall 1992).

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In the kidney, Ang II stimulates both sodium and water reabsorption and affects renal hemodynamics (Hall et al, 1995). Ang II in vascular smooth muscle and the adrenal gland mediates vasoconstriction (Skeggs et al, 1980), and secretion of aldosterone and catecholamines (Peter & Navar 1985) respectively.

Ang II has long been thought of as an endocrine, peripheral blood-borne hormone. The liver and kidney have been shown to be the major source of plasma angiotensinogen (Nasjletti & Masson 1972) and renin (Davis & Freeman 1976), respectively. However, accumulating evidence has raised a question over the concept of the renin-angiotensin system as an endocrine system alone. The cloning of renin, angiotensinogen, converting enzyme and angiotensin receptor genes has confirmed the widespread distribution of the renin-angiotensin system in many organs including brain, kidney, heart, liver, blood vessels and sex organs (Dzau et al, 1988; Phillips et al, 1993). Additionally, it has been demonstrated that the plasma concentration of Ang II is in the picomolar range (Bailie et al, 1971), while the binding affinity of its receptor is in the nanomolar range (Glossmann et al, 1985). Taken together, these results suggest that a locally expressed renin-angiotensin system may be involved in the regulation of individual tissue functions that are dependent on and/or complementary to those of the circulating hormone. Thus, Ang II in the brain and various peripheral tissues expresses possible paracrine/autocrine functions which may contribute to cardiovascular and renal homeostasis.

Hemorrhage-induced hypovolemia and hypotension stimulate renal renin release and increase the circulating levels of Ang II (Fejes-Tóth et al, 1988). Ang II in many peripheral tissues produces multiple effects to restore blood volume and pressure (Hoffman et al, 1977; Phillips 1987). Recently, we demonstrated that brain Ang II plays an important physiological role, primarily through AT1 receptor, in mediating rapid cardiovascular regulation and vasopressin release in response to hemorrhage in rats (Lee et al, 1995). Therefore, it can be hypothesized that hemorrhage stimulates gene expression in the brain as well as the peripheral renin-angiotensin system. The purpose of the present study is to examine whether hemorrhage affects expression of angiotensinogen mRNA in the brain and peripheral angiotensin-generating tissues in conscious unrestrained rats.

METHODS

Animals and experimental protocol

Experiments were performed on male Wistar rats weighing 350~450 g (The National Institute of Safety Research of Korea). Rats were given 0.9% saline as drinking water for a week to suppress the renin-angiotensin system: a small change in mRNA level superimposed on a high baseline may not be detectable, whereas the same change may be more easily recognized if mRNA baseline levels are low. On the fifth day of the saline drinking, an arterial catheter (PE-50, Clay Adams, Becton-dickinson, NJ, USA) was implanted into the abdominal aorta through a femoral artery under pentobarbital anesthesia (40 mg/kg, i.p.) and penicillin was administered (25 mg/kg, i.m.). The rats were then individually housed in cages (20×26×13 cm) and allowed to recover for two days.

In the morning of the experiments, baseline mean arterial pressure (MAP) and heart rate (HR) were recorded on a polygraph (model 7E, Grass instruments Co., Quincy, MA, USA) while the rat remained calm in a home cage. Then, hemorrhage was induced: arterial blood was withdrawn through the arterial catheter into an empty syringe at a rate of 3 ml/kg/min for 7 min using a Harvard withdrawal pump. Rats were decapitated at 0, 2, 4, 8, or 24 hr after the hemorrhage and trunk blood samples were collected for determination of plasma renin concentration. Then the liver, kidneys, hypothalamus and lower brainstem were collected. The brainstem was

removed from the rest of the brain by a coronal cut at the level of the superior colliculus. To isolate the hypothalamic-thalamic-septal block, coronal cuts were done rostral to the optic chiasm and caudal to the mammary bodies. Organs were snap-frozen in liquid nitrogen within 5 min and stored at -80℃ until RNA extraction. The angiotensinogen mRNA level was measured in these organs using the Northern blot method.

Isolation of total RNA

Total cellular RNA was extracted by the method of Chomczynski and Sacchi (1987). The tissues were homogenized in tenfold tissue volume of RNA isolation buffer composed of 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% N-lauryl sarcosine, and 0.1 M mercaptoethanol. Sequentially, 0.1 volume of 2 M sodium acetate, pH 4.0, and 1 volume of chloroform-isoamylalcohol mixture (49 : 1) were added to the homogenate, with thorough mixing by inversion, and the homogenate was then cooled on ice for 15 min. Samples were centrifuged at 10,000 g for 15 min at 4℃. After centrifugation, the aqueous phase was transferred to a fresh tube, and extraction was repeated. The aqueous upper phase was mixed with an equal volume of isopropanol and then placed at -20℃ overnight to precipitate RNA. After centrifugation at 10,000 g for 15 min, the RNA pellet was resuspended in 75% ethanol, sedimented and dried at room temperature. RNA was resuspended in DEPC-treated water and stored at - 70℃. The concentration of total cellular RNA was determined by measuring the absorbance at 260 nm. The quality of the extracted RNA was confirmed by the ratio of 260 nm per 280 nm optical density and the electrophoretic pattern of the 28S and 18S rRNA bands.

Northern blot hybridization analysis

For Northern Blot analysis, aliquoted RNA was size-separated by electrophoresis using a 1% formaldehyde-agarose gel. Comparison of relative mRNA levels were made by the rRNA band intensities appearing in ethidium bromide stained gel, in reference to the same amount of total RNA applied per lane. In studies of angiotensinogen mRNA, the amounts of total RNA run on the agarose gel were 20 μg for the liver, 50 μg for the brainstem and hypothalamus, and 75 μg for the kidney.

After electrophoresis, RNAs were transferred to nylon filters (Amersham, Buckinghamshire, England) by capillary action with 20xstandard saline citrate (SSC) (1 × SSC equals 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) for 24 hr. Efficiency of transfer was confirmed by ethidium bromide staining of the remaining gels. The blots were prehybridized for 2~4 hr at 42℃, and hybridized for 16~24 hr in a buffer to which α-^{32}P-labeled cDNA probes were added.

To remove the unhybridized probe, blots were washed in 1 × SSC with 0.1% sodium dodecyl sulfate (SDS) at room temperature, then three times in 0.2 × SSC with 0.1% SDS at 65℃ (10 min each). The autoradiograph was obtained by placing the membrane in a cassette with an intensifying screen (Cronex, E.I. du Pont de Nemours, Wilmington, DE, USA) and Kodak XAR-5 x-ray film for several days at - 70℃. Several exposoures were obtained to achieve optimal autoradiography signals that could be well visualized. To minimize the interassay variability, one set of serial samples over the time course was extracted simultaneously and assayed at the same time.

cDNA clones and preparation of α-32P-labeled probes

The angiotensinogen probe (pRange3) used was a full-length rat liver angiotensinogen cDNA cloned by Lynch et al, (1986) into the Sma I site of pGEM4. Probe for angiotensinogen was a gift of Dr. Kevin Lynch (University of Virginia). Double-stranded DNA hybridization probes were labeled with α-^{32}P]dATP, using a nick-translation kit from Amer-

sham, to a specific activity of 1-3 × 10^9 dpm/μg of cDNA.

Determination of renal and plasma renin levels

For plasma renin concentration (PRC) assay, 50 μl plasma was incubated at 37℃ for one hr with excess exogenous substrate (pH 7.4) with protease inhibitors [3.4 mM 8-hydroxyquinolone sulfate, 0.25 mM EDTA, 0.1 mM PMSF, 1.6 mM dimercaprol, and 5 mM sodium tetrathionate]. The source of the substrate was renin-free plasma (diluted 1 : 3) from rat nephrectomized 48 h before plasma collection. The angiotensin I generated was measured by radio-immuno-assay (Cho et al, 1987).

Kidney renin concentration was similarly determined. Cortical slices 0.4 mm thick were made parallel to the renal surface, and the initial slice was discarded. Two slices (one from each renal hemisphere) were weighed and homogenized in 100 volume of cold 0.1 M Tris buffer (pH 7.4). The homogenate was centrifuged at 3000 rpm for 30 min at 4℃ and the supernatant was stored at -20℃ until assay. The supernatant (10 μl) was incubated with excess angiotensinogen in the presence of protease inhibitors for 10 min at 37℃. A 20 μl aliquot was then taken for radioimmunoassay of generated angiotensin I (Ang I). Tissue protein level was measured by the Folin-Ciocalteu phenol method (Lowry et al, 1951). Tissue renin content was expressed as ng Ang I/gm protein·hr.

Statistical analysis

Data are expressed by mean ± SE. Values were analyzed with a one-way ANOVA followed by a subsequent Newman-Keuls test.

RESULT

Cardiovascular and renin responses to hemorrhage

Time course changes in MAP and HR after

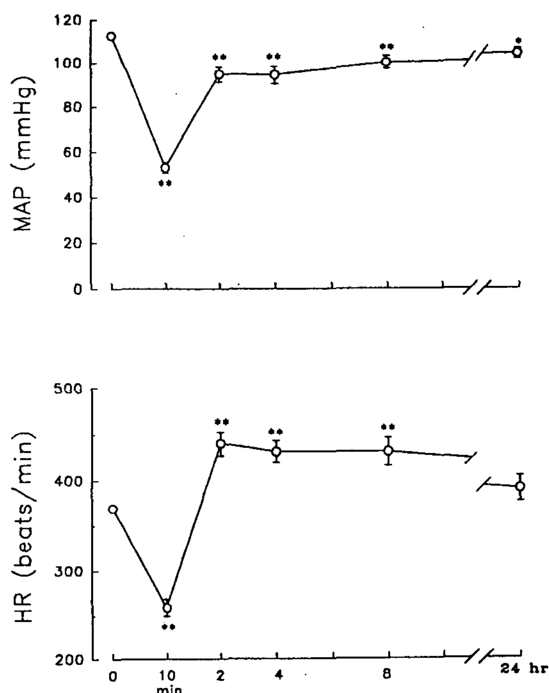

Fig. 1. Time course of the changes in mean arterial pressure (MAP) and heart rate (HR) before and after hemorrhage (21 ml/kg) in rats having drunk 0.9% saline for 7 days.

hemorrhage are presented in Fig. 1. Immediately following the 7-min hemorrhage, MAP and HR were reduced from 112±2 to 53±2 mmHg, and from 379±7 to 259±9 beats/min, respectively. At 2, 4, and 8 hours after the hemorrhage, MAP was significantly lower, but HR was higher than the initial values. At 24 hr, MAP was still significantly lower than the basal level, but HR had been restored to the basal level.

Plasma renin concentration (PRC) and renal renin contents (RRC) are shown in Fig. 2. Rats fed 0.9% saline for 7 days showed significantly lower PRC and RRC than those fed tap water. Hemorrhage produced an almost sevenfold increase in PRC at 10 min. A high PRC level was maintained for 8 hr, being restored close to the basal level at 24 hrs. Changes in RRC and PRC after hemorrhages were

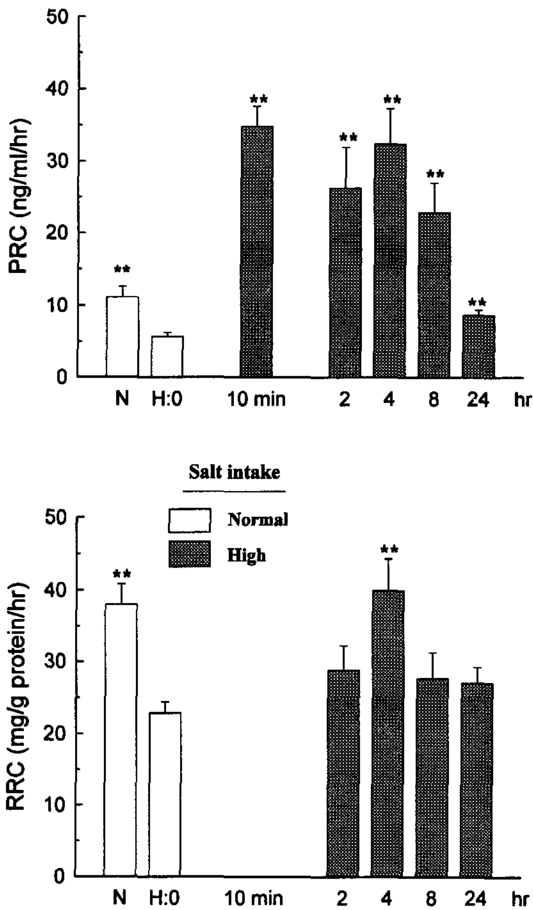

Fig. 2. Time course of the changes in plasma renin concentration (PRC) and renal renin content (RRC) before and after hemorrhage (21 ml/kg) in rats having drunk 0.9% saline (high salt) or tap water (normal salt) for 7 days.
**P<0.05, **P<0.01, compared with high salt intake.*

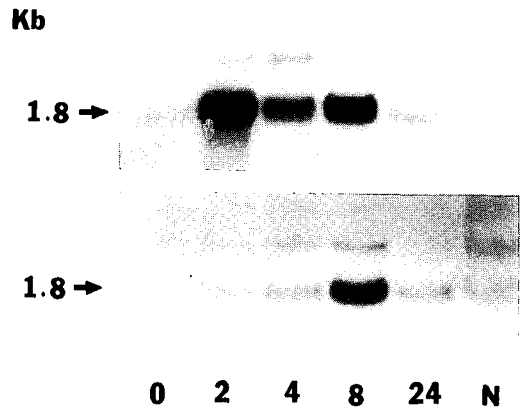

Fig. 3. Representative examples of Northern blot analyses of hepatic and renal angiotensinogen mRNA from rats killed 0, 2, 4, 8 or 24 hr after hemorrhage (21 ml/kg). Rats were given 0.9% saline as drinking water for 7 days and then hemorrhage was induced. N represents rats which drank tap water. Amount of renal and hepaptic RNA applied for electrophoresis was 75 and 20 μg and autoradiographies were performed for 15 days and 1 day, respectively. Sizes of RNA markers are indicated.

not in parallel. RRC tended to increase at 2 hr, was significantly increased at 4 hr and decreased thereafter.

Effect of salt intake on gene expression

Representative autoradiograms of Northern blot analysis of angiotensinogen mRNA in the liver and kidney from individual rats in the series are shown in Fig. 3, and those in the brain are shown in Fig. 4. Based on signals generated in the Northern blot

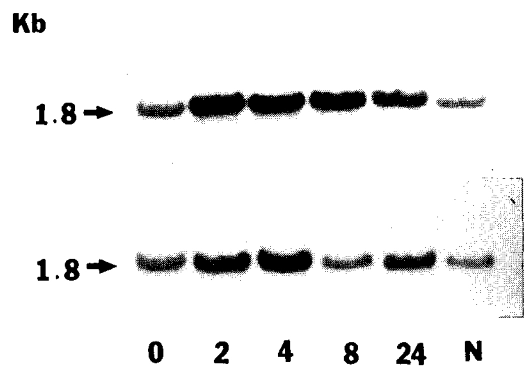

Fig. 4. Representative examples of Northern blot analyses of hypothalamus and brainstem angiotensinogen mRNA from rats killed 0, 2, 4, 8 or 24 hr after hemorrhage (21 ml/kg). Rats were given 0.9% saline as drinking water for 7 days and then hemorrhage was induced. N represents rats which drank tap water. Amount of RNA applied for electrophoresis was 50 μg and autoradiographies were performed for 2 days. Sizes of RNA markers are indicated.

analysis, renal angiotensinogen mRNA was lower in animals given 0.9% saline compared to those given tap water. However, angiotensinogen gene expression in the liver, brainstem and hypothalamus was not influenced by salt intake.

Angiotensinogen mRNAs in the brain, liver and kidney were indistinguishable by size according to their migration in agarose gels. The size of angiotensinogen mRNA migrated on agarose gel electrophoresis was approximately 1.8 kb. These hybridization patterns and sizes of mRNA species agree with those reported previously (Dzau et al, 1986; Kohara et al, 1992). Angiotensinogen mRNA was found in the following tissues in descending order of abundance: the liver, hypothalamus, brainstem and kidney.

Tissue-specific gene expression after hemorrhage

On this Northern blot, hemorrhage produced a clear-cut stimulation of angiotensinogen gene expression in the liver, kidney, hypothalamus and brainstem. In comparison with the band intensity, hepatic angiotensinogen mRNA was remarkably upregulated at 2, 4, and 8 hrs after hemorrhage, then was lowered at 24 hr, but was still higher than the pre-hemorrhagic level. Renal angiotensinogen mRNA increased after hemorrhage, peaking at 8 hr, but decreased at 24 hr. On the ohter hand, the expressions of angiotensinogen gene in both the hypothalamus and brainstem were continuously increased at 2 to 24 hrs after hemorrhage. The hypothalamus was more responsive to hemorrhage than the brainstem.

DISCUSSION

The present study demonstrates both the existence of angiotensinogen mRNA and its upregulation after hemorrhage in the brain and peripheral tissues. As observed by others (Dzau et al, 1986; Kalinyak & Perlman 1987), angiotensinogen mRNA was most abundant in the liver and, in descending order, in the hypothalamus, brainstem and kidney, known target tissues for Ang II action. The present data, in combination with the identification of the other components of the renin-angiotensin system such as angiotensin-converting enzyme, and angiotensin receptors in the brain (Lanzillo et al, 1985) and the kidney (Rosenberg et al, 1990), suggest a role for the local generation and paracrine action of Ang II in various tissues.

In an attempt to characterize the local renin-angiotensin system, we focused on effects of hemorrhage on angiotensinogen mRNA synthesis in various tissues, since hemorrhage has been shown to stimulate the central (Cameron et al, 1986; Lee et al, 1995) and peripheral renin-angiotensin system (Fejes-Tóth et al, 1988). The present study provides the first demonstration that hemorrhage-induced hypovolemia and hypotension is a potent stimulus for the expression of the angiotensinogen gene in both the periphery and the brain. The duration of the angiotensinogen mRNA response was not the same in different tissues. The liver and kidney showed a marked increase in angiotensinogen mRNA level at 2 to 8 hr but a decrease at 24 hr after hemorrhage. However, the hypothalamus and brainstem showed a continuous increase in angiotensinogen mRNA until 24 hr after hemorrhage. This result indicates the tissue-specific regulation of angiotensinogen mRNA synthesis in the brain and peripheral tissues. The mechanism by which hemorrhage induces angiotensinogen gene expression is poorly understood. The liver is the main source of circulating angiotensinogen (Clauser et al, 1989), but in both hepatic and extrahepatic tissues angiotensinogen mRNA synthesis is sensitive to hemorrhage. The liver contains approximately 10 times the number of Ang II receptors as the brain or kidney (Glossmann et al, 1985). It has been suggested that Ang II may be involved in a positive feedback loop which increases angiotensinogen synthesis and release from the liver (Nasjletti & Masson 1973; Sernia et al, 1985). Intravenous infusion of Ang II increased the level of liver angiotensinogen mRNA (Kohara et al,

1992). Thus, hemorrhage-induced increase in angiotensinogen mRNA level in the liver may be due, at least in part, to increased circulating Ang II. Modulators other than Ang II have recently been shown to alter angiotensinogen mRNA levels. Angiotensinogen mRNA in rat liver but not brain was increased following acute inflammation (Kageyama et al, 1985). Uninephrectomy reduces angiotensinogen mRNA levels in the remaining kidney (Fried & Simpson 1986). Salt depletion induces a dramatic increase in the expression of angiotensinogen mRNA in the kidney, but not in the liver (Ingelfinger et al, 1986). The present results also show that salt state influences angiotensinogen mRNA in the kidney, but not in the liver or brain. The brain angiotensinogen gene is probably under more complex regulation than liver angiotensinogen mRNA, but limited data are available to elucidate the mechanism. Previous studies showed that brain angiotensinogen mRNA may be stimulated by cortisol (Deschepper & Flaxman 1990; Kalinyak & Perlman 1987), catecholamines, or both (Ferrario et al, 1986; Hoffman et al, 1977). Although the mechanism for hemorrhage-induced upregulation of central angiotensinogen gene expression is not clear, the finding raises questions as to its function. Lynch et al, (1987) showed that the hypothalamus and the medulla oblongata are rich in angiotensinogen mRNA. Several lines of evidence have suggested that these areas of the brain contain the pathways that participate in the central control of sympathetic discharge (Faber & Brody 1984), thirst (Gregory & Printz 1983), pituitary hormone release (Keil et al, 1975; Hoffman et al, 1977), renal blood flow, electrolytes (Myers et al, 1975), and ultimately blood pressure and body fluid homeostasis (Ferguson & Wall 1992; Phillips 1987). Recently, we observed that intracerebroventricular administration of Ang II receptor antagonists impairs both cardiovascular regulation and vasopressin release in response to hemorrhage in rats (Lee et al, 1995). This demonstrates the physiological role of brain Ang II in the control of blood pressure and vasopressin release. Taken together, above evidence suggests that increased central angiotensinogen gene expression after hemorrhage may be involved in compensating for hypovolemia and hypotension.

Hemorrhage increased renal angiotensinogen mRNA as well as plasma and renal renin levels. The mechanism of synthesis and release of renal renin has been well documented, but not that of renal angiotensinogen. Synthesis and release of renal renin is regulated by mechanisms that include the intrarenal baroreceptor (Davis & Freeman 1976), sympathetic nerves (Keeton & Campbell 1981), and sodium intake (Ingelfinger et al, 1986). A mild hemorrhage stimulates sympathetic activities, whereas a severe hemorrhage inhibits them with hypotension (Brizzee et al, 1991) as observed in the present study. Plasma concentrations of renin and Ang II are markedly increased after hemorrhage. However, intravenous administration of Ang II has been shown to exert negative effects on kidney renin mRNA (Johns et al, 1990; Kohara et al, 1992). Thus, after hemorrhage sympathetic nerve and circulating Ang II may not exert positive actions on intrarenal renin gene expression and renin production. Although the mechanisms remain unclear, hemorrhage may exert effects on gene expression of the renal renin-angiotensin system by indirect mechanisms, such as the intrarenal baroreceptor or the macula densa. Both of these mechanisms are known to be the primary regulators for renin release (Davis & Freeman 1976).

In summary, the present study demonstrates the presence of angiotensinogen mRNA in multiple extrahepatic tissues and, more importantly, its tissue-specific upregulation after hemorrhage. These results strongly suggest that the angiotensinogen-generating systems in the liver, brain and kideny are, at least in part, under independent control and play a physiological role in its local production. Further studies are required for a better understanding of the function of the local synthesis of angiotensinogen

and whether it is independent and/or complementary to the function of circulating angiotensinogen.

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