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Effect of Hemorrhage on mRNA Expressions of Renin, Angiotensinogen and AT_1 Receptors in Rat Central and Peripheral Tissues

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In an attempt to investigate whether hemorrhage affects the gene expression of the renin-angioteusin system (RAS) components in the brain and peripheral angiotensin-generating tissues, changes in mRNA levels of the RAS components in response to hemorrhage were measured in conscious unrestrained rats. Wistar rats were bled at a rate of 3 ml/kg/min for 5 min, and then decapitated 7 h after hemorrhage. Levels of mRNA for renin, angiotensinogen and angiotensin II-AT_1 receptor subtypes (AT_{1A} and AT_{1B}) were determined with the methods of northern blot and reverse transcriptase-polymerase chain reaction (RT-PCR). Hemorrhage produced a profound hypotension with tachycardia, but blood pressure and heart rate recovered close to the basal level at 7 h. Plasma and renal renin levels were significantly increased at 7 h. Hemorrhage induced rapid upregulation of gene expression of both AT_{1A} and AT_{1B} receptor subtypes in the brainstem and hypothalamus, downregulation of them in the adrenal gland and liver. However, renin mRNA level increased in the brainstem, decreased in the liver, but was not changed in the hypothalamus, kidney and adrenals after hemorrhage. Angiotensinogen mRNA level was not significantly changed in any of the tissue except a slight increase in the liver. The kidney and liver did not show any significant change in gene expression of the RAS components. These results suggest that gene expression of the RAS in central and peripheral tissues are, at least in part, under independent control and the local RAS in each organ plays specific physiologic role.

Key Words: Northern blot, RT-PCR, Brain, Kidney, Liver, Adrenal

INTRODUCTION

The renin-angiotensin system (RAS) is one of the most important hormonal systems in the regulation of blood pressure and body fluid balance in the vertebrates (Ferrario et al, 1986; Campbell 1987; Phillips 1987). The peptide hormone angiotensin II (Ang II), the biologically active component of the RAS, exerts a wide variety of physiological effects on the cardiovascular, endocrine, and central and peripheral nervous systems. Ang II had long been thought as an endocrine, peripheral blood-borne hormone. There is now substantial evidence indicating that all of the components necessary for the local formation of Ang II coexist in numerous tissues including the brain, adrenal gland, kideny and heart (Dzau et al, 1988; Phillips et al, 1993). Ang II is formed independently of the plasma levels in response to various stimuli in each tissue.

Locally produced Ang II may exert a variety of paracrine/autocrine functions which may contribute to cardiorenal homeostasis. In the brain, Ang II stimulates thirst, alters sympathetic outflow and regulates the secretion of vasopressin (Phillips 1987; Ferguson & Wall 1992; Ahn et al, 1992). In the kidney, Ang II has multiple actions including regulation of blood flow, glomerular filtration, tubular reabsorption of electrolytes, and feedback inhibition of renin secretion (Blanz & Pelayo 1983; Harris 1992). Ang II in vascular smooth muscle and the adrenal gland mediates vasoconstriction (Skeggs et al, 1980) and secre-

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tion of aldosterone and catecholamines (Peter & Navar 1985), respectively.

All these effects are mediated by specific, high affinity receptors on the surface of target cells. Two Ang II receptors, AT_1 and AT_2, have been pharmacologically characterized using highly specific receptor antagonists (Bumpus et al, 1991). While AT_1 receptors are coupled to calcium-phospholipid signaling systems and appear to mediate the known actions of Ang II, the mechanism of action and physiological effects of AT_2 receptors remain to be elucidated (Catt et al, 1993). The AT_1 receptor was found to have A and B subtypes of 359 amino acids each that share 95% identity (Elton et al, 1992). Both subtypes could not be pharmacologically distinguished by their ligand binding properties (Chiu et al, 1993) and are coupled to the same signaling pathways (Martin et al, 1995). Despite these homologies, AT_{1A} and AT_{1B} receptor subtypes differ in their tissue distribution (Kakar et al, 1992) and regulation (Chansel et al, 1996), suggesting that these two subtypes could mediate different physiological functions.

Hemorrhage produces hypovolemia and hypotension which stimulate renal renin release and increase the circulating levels of Ang II (Fejes-Tóth et al, 1988). Ang II in various target organs produces multiple functions to restore blood volume and pressure (Hoffman et al, 1977; Phillips 1987). Recently, We demonstrated that endogenous Ang II in the brain primarily through AT_1 receptor plays an important physiological role in mediating rapid cardiovascular regulation and release of renin and vasopressin in response to hemorrhage in rats (Lee et al, 1995). The result suggests that hemorrhage may modulate gene expression of the brain as well as the peripheral RAS. Actually, Do et al. (1995) observed enhanced angiotensinogen mRNA levels after hemorrhage in both the brain and peripheral tissues. To our knowledge, there has been no extensive analysis of gene expressions of the RAS components in different tissues in hemorrhaged rats.

The purpose of the present study is to examine whether hemorrhage affects the gene expression of the RAS components in the brain and peripheral angiotensin-generating tissues in conscious unrestrained rats. Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) have been used to determine the changes in mRNA expression for renin, angiotensinogen, Ang II-AT_1 receptor subtypes (AT_{1A} and AT_{1B}) from the brainstem, hypothalamus, kideny, adrenals, and liver. To minimize the interexperimental variations, this study was performed to examine the effects of hemorrhage on gene expresion of the RAS components in brain and peripheral tissues from a single set of animals.

METHODS

Animals and experimental protocol

Ten-week old male Sprague-Dawley rats weighing 290-320g were used in this study. Rats were purchased from Korean Research Institute of Chemical Technology. An arterial catheter (PE-50, Clay Adams, Becton-Dickinson, N.J., U.S.A.) 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 ($20 \times 26 \times 13$ cm) and allowed to recover for overnight.

On the next morning, baseline mean arterial pressure (MAP) and heart rate (HR) were recorded on a polygraph (model 7E, Grass instruments Co., Quincy, Mass, U.S.A.) 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 5 min using a Harvard withdrawal pump. Rats were decapitated at 7 h after the hemorrhage and trunk blood samples were collected for determination of plasma renin concentration. The kidney, adrenal glands, liver, brainstem and hypothalamus were removed from each rat and snap frozen in liquid nitrogen, and then stored at $-80^{\circ}C$ until RNA extraction.

Total RNA isolation and Northern blot analysis

Total RNA was isolated according to the method described by Chomczynski & Sacchi (1987) with slight modifications in final step. The RNA pellet was dissolved in formamide and kept at $-20^{\circ}C$ until needed. Aliquots of total RNA (20~40 μg) were separated on a 1% agarose gel containing 0.66 M of formaldehyde in 1x MOPS buffer (0.02 M MOPS, pH 7.0; 5 mM sodium acetate; 1 mM EDTA, pH 8.0). RNA was transferred to a nitrocellulose membrane by capillary action in 20x SSC (1x SSC;

0.15 M sodium chloride, 0.015 M sodium citrate). The membrane was baked in a vacuum oven at 80°C for 2 hs and prehybridized for 4 h at 42°C in a solution containing 50% formamide, 6x SSC, 5x Denhardt's solution (0.1% each of Ficoll 400, polyvinylpyrrolidone and nuclease-free bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS) and 0.1 mg/ml salmon sperm DNA. [α-^{32}P] dCTP-labeled angiotensinogen or renin cDNA was added to the solution and the membrane was incubated for 18-20 h at 42°C. After hybridization, the membrane was sequentially washed in 2x SSC, 0.2% SDS for 5 min at room temperature, for 30 min at 42°C. The membrane was washed thereafter in 0.1x SSC, 0.1% SDS at 60°C until the signal and background were distinguishable. The resulting membrane was exposed to X-ray film for 1-4 days at −70°C. After autoradiogram, the membrane was deprobed in 0.5% SDS by boiling, and was rehybridized with rat GAPDH cDNA probe for loading control.

Reverse transcriptase-PCR (RT-PCR)

The nucleotide sequence of the primers and references are presented in Table I. Total RNA (20 μg) was primed with oligo (dT) primers, and the first strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, U.S.A.) in a 30 μl reaction volume for 90 min at 37°C. With 1~2 μl (equivalent to 330-660 ng of total RNA) of this reaction mixture, PCR was carried out in a 25 μl reaction mixture containing 0.1% formamide. PCR was conducted in a DNA Thermal Cycler (Perkin-Elmer, Cetus) and cyclic amplification profiles were as follows: 94°C for 1 min, 56°C for 1 min, 72°C for 1.5 min. In a preliminary experiment, we conducted PCR with serial dilutions of reverse-transcribed RNA from different tissues. PCR cycles and the amount of template were carefully selected according to the relative abundance of mRNAs in different tissues.

Densitometric analysis

The autoradiogram and polaroid film were scanned using an Epson (ES-600) scanner with a resolution of 70 DPI and the maximum gray scale unit of 256. In order to achieve a linear response curve, the brightness and the contrast were finely tuned to the point where the gray scale unit of the background was within 20-40 and that of the darkest band was within 200-240. The resulting image was analyzed using NIH-Image analysis program (NIH, Betheda, M.D.). The scale of each band was expressed by multiplying the mean density and the total area of the band. The resulting scale was used to quantify each band.

Plasma and renal renin levels

For the measurement of plasma renin concentration (PRC), blood sample was centrifuged at 2,500 rpm for 20 min at 4°C, then 50 μl of plasma was transferred to a tube containing 50 μg of EDTA. The plasma sample was incubated with excess exogenous angiotensinogen at 37°C, pH 7.4, in the presence of

Table 1. Primer sequences for AT_{1A}, AT_{1B}, renin and GAPDH used in this study

Gene	Primer sequence	Position	Product size	Reference
AT_{1A}				Kitami et al, 1992
(s)	GCCTGCAAGTGAAGTGATTT	1369-1388	388bp	
(as)	TTTAACAGTGGCTTTGCTCC	1737-1756		
AT_{1B}				Kitami et al, 1992
(s)	GCACACTGGCAATGTAATGC	1181-1200	204bp	
(as)	GTTGAACAGAACAAGTGACC	1365-1384		
renin				Burnham et al, 1987
(s)	AGGCAGTGACCCTCAACATTACCAG	935-959	362bp	
(as)	CCAGTATGCACAGGTCATCGTTCCT	1271-1296		
GAPDH				Tso et al, 1985
(s)	ATCAAATGGGGTGATGCTGGTGCTG	318-342	504bp	
(as)	CAGGTTTCTCCAGGCGGCATGTCAG	798-821		

protease inhibitors [3.4 mM 8-hydroxyquinolone sulfate, 0.25 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1.6 mM dimercaprol, and 5 mM sodium tetrathionate] for 1 h. The source of angiotensinogen was renin-free plasma from rats nephrectomized 48 h prior to experiment. The Angiotensin I (Ang I) generated was measured by radioimmunoassay described earlier (Cho et al, 1987).

The kidney renin level was similarly determined. Cortical slices (0.4 mm thick) were made in parallel to the renal surface, and the initial slice was discarded. Two slices, one from each renal hemisphere, were weighed and homogenized in 100 ml volume of cold 0.1 M Tris buffer, pH 7.4. The homogenate was centrifuged at 3,000 rpm for 30 min at 40°C and the supernatant was stored at −20°C until assay. The supernatant, 10 μl, was incubated with excess angiotensinogen in the presence of protease inhibitors for 10 min at 37°C. Then a 20 μl aliquot was taken for radioimmunoassay of generated Ang I. Tissue renin content was expressed as ng Ang I/gm protein/h.

Statistical Analysis

All data are presented as mean±SE. Unpaired t-test was used for the comparison of means of different groups. Differences were considered significant if $P < 0.05$.

RESULT

Cardiovascular and renin responses to hemorrhage are shown in Table 2. Mean arterial pressure was reduced from 110±2 to 102±2 mmHg (P<0.05), but heart rate was slightly increased from 342±9 to 361 ±11 beats/min 7 h after hemorrhage. Hemorrhage produced approximately 1.75- and 1.2-fold increase in plasma (P<0.01) and renal (P<0.01) renin contents, respectively.

Table 2. Mean arterial pressure (MAP), heart rate (HR), plasma osmolality (Posm), plasma and renal renin levels (PRC, RRC) before and 7 h after hemorrhage in Sprague-Dawley rats

	Control	Hemorrhage
MAP, mmHg	110±2.3	102±1.7*
HR, beat/min	342±9.0	361±11
Posm, mOsm/kg	295±1.0	293±1.0
PRC, ng/ml/h	2.0±0.14	3.5±0.25**
RRC, ng/g protein/h	3.8±0.11	4.4±0.16**

Values are mean±SE. of 8 rats.
*P <0.05, **P <0.01, vs. control.

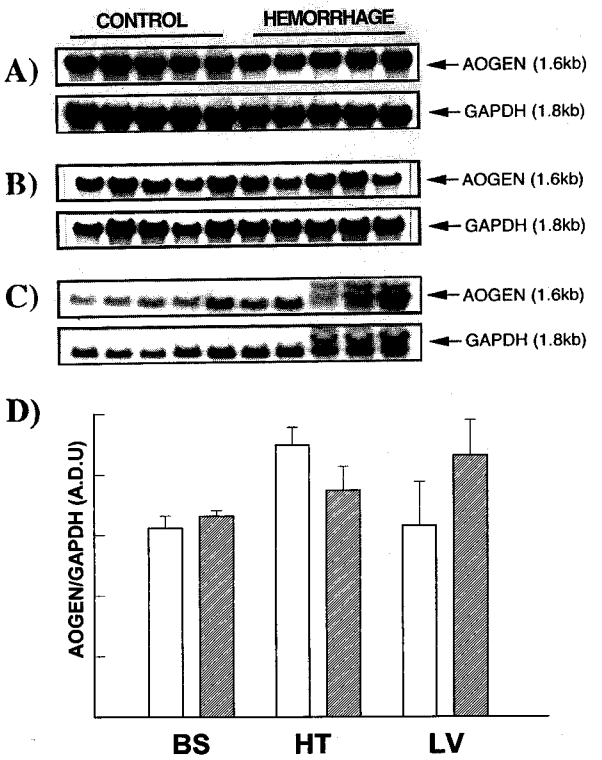

Fig. 1. Northern blot analysis of angiotensinogen (Aogen) mRNA in the brainstem (A), hypothalamus (B), and liver (C) from the control (n=5) and hemorrhaged rats (n=5). Each lane represents 1 rat and the amount of RNA used was 20 μg for brainstem and hypothalamus, 15 μg for liver. The membrane was exposed to X-ray film at -70°C for 1~2 days to yield optimum autoradiogram for densitometric analysis. After autoradiogram, the membrane was deprobed in 0.5% SDS at 95°C for 1 h, and rehybridized with rat GAPDH cDNA for loading control. Densitometric analysis of autoradiograms (D) for angiotensinogen mRNA in the brainstem (BS), hypothalamus (HT), and liver (LV) from the control (open bar) and hemorrhaged rats (hatched bar). The values were expressed in arbitrary densitometric unit (A.D.U) corrected for GAPDH mRNA.

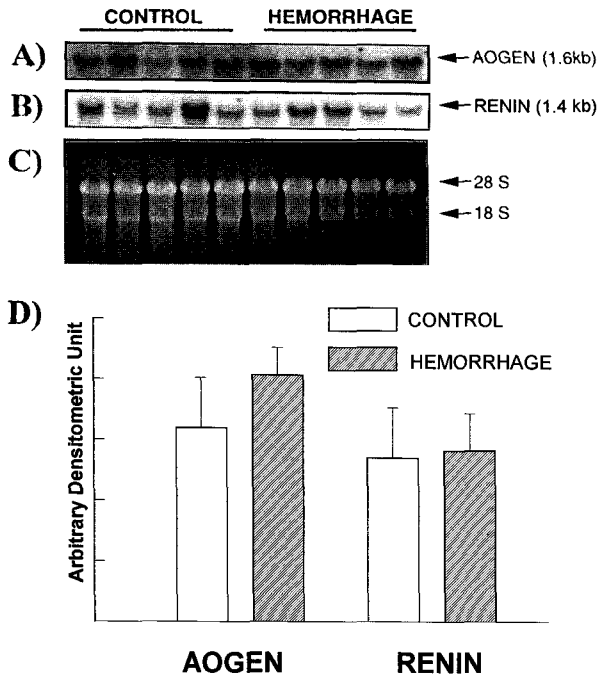

Fig. 2. Northern blot analysis of angiotensinogen (A) and renin (B) mRNAs in the kidney from the control (n=5) and hemorrhaged rats (n=5). Each lane represents 1 rat and the amount of RNA used was 40 μg. The membrane was first hybridized with angiotensiongen cDNA, then deprobed and rehybridized with renin cDNA probe. The membrane was exposed to X-ray film at $-70^{\circ}C$ for 2~4 days to yield optimum autoradiogram for densitometric analysis. Ethidium bromide-stained RNA gel (C) was presented to show RNA integrity and loading control. Densitometric analysis of autoradiograms (D) for angiotensionogen and renin mRNAs in the kidney from the control and hemorrhaged rats. The values were expressed in arbitrary densitometric unit corrected for 28S ribosomal RNA.

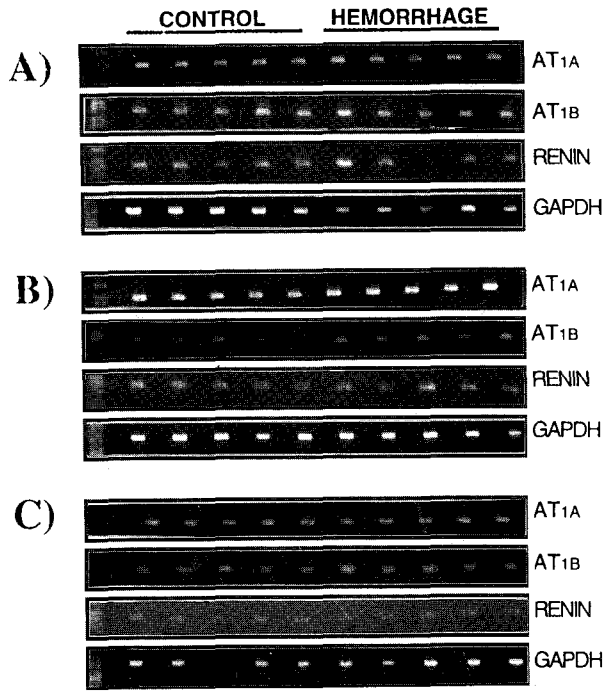

Fig. 3. Ethidium bromide-stained gel of PCR products for Ang II receptor subtypes and renin mRNAs in the brainstem (A), hypothalamus (B), and liver (C) from the control (n=5) and hemorrhaged rats (n=5). Twenty μg of total RNA from each tissue were reverse transcribed in a 30 μl reaction mixture. Small aliquots (1~2 μl) of this mixture were amplified with primer sets designed for each gene. Rat GAPDH mRNA was amplified in parallel for internal control.

Effects of hemorrhage on gene expression of tissue RAS components

Angiotensinogen mRNA levels in the brainstem, hypothalamus, liver, and kidney tissues of the control and hemorrhaged rats were examined by Nothern blot analysis. Hemorrhage produced no conceivable changes, although slightly increased in the liver and kidney, in gene expression of angiotensionogen (Fig. 1, 2). Kidney renin mRNA level was not affected by hemorrhage, either (Fig. 2).

It has been reported that AT_1 receptor subtypes (AT_{1A}, AT_{1B}) and renin mRNA levels are very low in the brain and peripheral tissues. And as AT_{1A} and AT_{1B} subtypes share more than 95 % homology in coding region, it would be very difficult to distinguish one subtype from the other by Nothern blot method. Thus, we employed RT-PCR techniques to examine changes in gene expression of AT_{1A}, AT_{1B}, and renin mRNAs in the brain and peripheral tissues. The use of RT-PCR techniques enabled us to easily detect these genes in all the tissues examined (Fig. 3, 5). Hemorrhage increased both AT_{1A} and AT_{1B} receptor subtype mRNA levels about 2~3 fold in the brainstem (Fig. 3A, 4) and hypothalamus (Fig. 3B, 4). However, hemorrahge decreased them approximately 50% in the adrenal gland (Fig. 5A, 6), whereas it had no effects on these gene expression in the liver (Fig. 3C, 4) and kidney (Fig. 5A, 6).

Renin mRNA level was not affected by hemor-

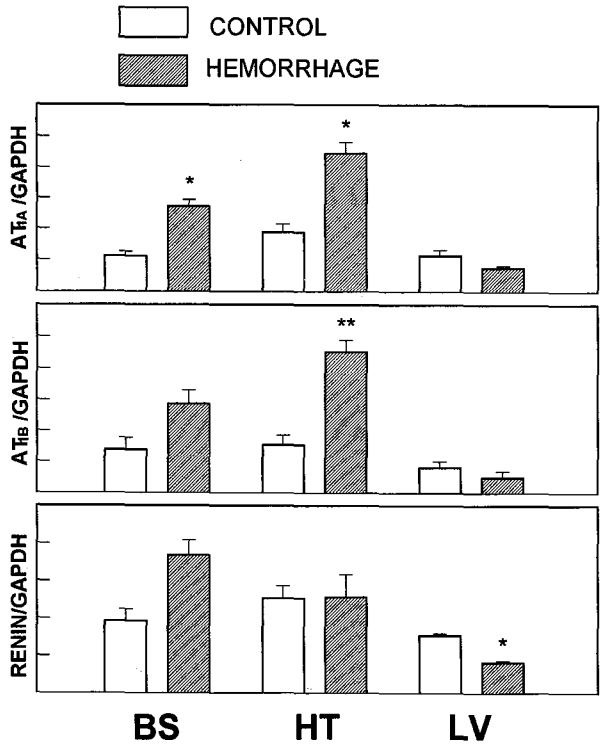

Fig. 4. Densitometric analysis of the PCR products for AT_{1A} (A), AT_{1B} (B), and renin (C) mRNAs in the brainstem (BS), hypothalamus (TH), and liver (LV) from the control (n=5) and hemorrhaged rats (n=5). The values were expressed in arbitrary densitometric unit corrected for GAPDH mRNA. $*P < 0.05$, $**P < 0.01$, vs. control group.

rhage in all the tissues except the liver in which it decreased about 50% (Fig. 3, 4).

DISCUSSION

In an attempt to compare gene expressions of central and peripheral RAS, changes in mRNA levels of the RAS components in response to hemorrhage were investigated. Hemorrhage has been shown to stimulate both the central (Cameron et al, 1986; Lee et al, 1995) and peripheral RAS (Fejes-Toth et al, 1988). The present study demonstrates that hemorrhage enhanced gene expression of both AT_1 receptor subtypes (AT_{1A} and AT_{1B}) in the brainstem and hypothalamus, while suppressed them in the adrenal gland. Renin mRNA level was increased in the brainstem, decreased in the liver, but was not changed in

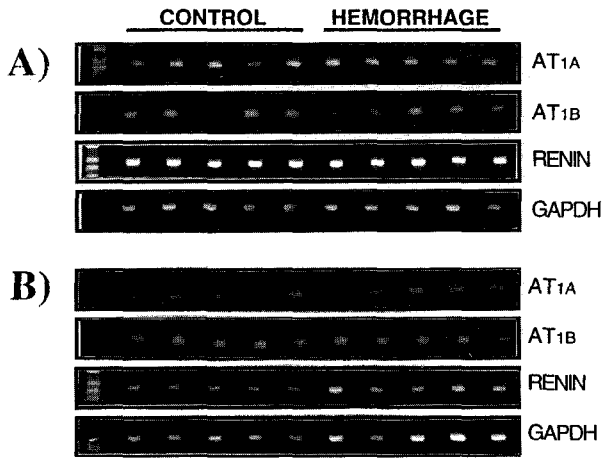

Fig. 5. Ethidium bromide-stained gel of PCR products for AT_{1A}, AT_{1B}, and renin mRNAs in the kidney (A) and adrenal gland (B) from the control (n=5) and hemorrhaged rats (n=5). Twenty μg of total RNA from each tissue were reverse transcribed in a 30 μl reaction mixture. Small aliquots (1~2 μl) of this mixture were amplified with primer sets designed for each gene. Rat GAPDH mRNA was amplified in parallel for internal control.

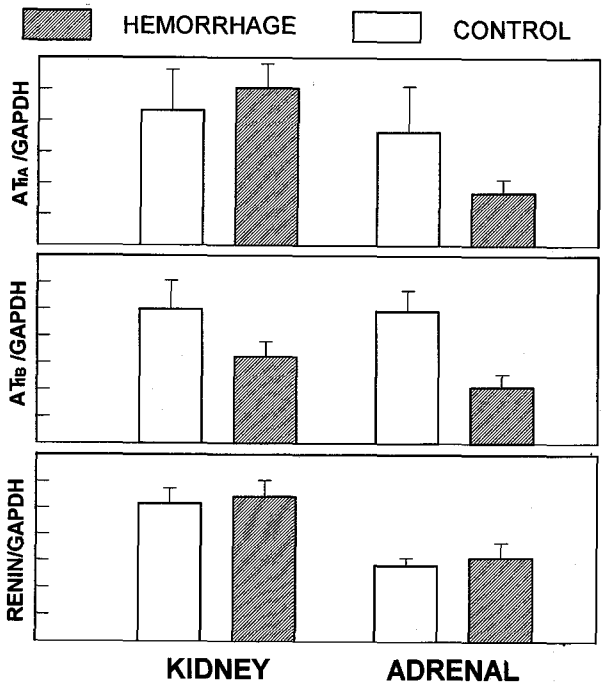

Fig. 6. Densitometric analysis of the PCR products for AT_{1A}, AT_{1B}, and renin mRNAs in the kidney and adrenal gland from the control (n=5) and hemorrhaged rats (n=5). The values were expressed in arbitrary densitometric unit corrected for GAPDH mRNA.

other tissues. Hemorrhage did not affect angiotensinogen mRNA level in any of the tissues examined.

The presence of mRNAs for all the RAS components in various tissues has been demonstrated (Phillips et al, 1993; Jo et al, 1996). Changes in gene expression of the RAS components in brain and peripheral tissues have been demonstrated under physiological stresses such as hemorrhage (Do et al, 1995), dehydration (Lee, 1995) and sodium depletion (Du et al, 1995; Kitami et al, 1992; Jo et al, 1996; Pratt et al, 1989; Sandberg et al, 1994). More studies have been conducted on the effects of salt intake on gene regulation of the RAS, but in most studies one or two mRNAs of the RAS components were measured from the brain or peripehral tissues. Furthermore, the results showed some inconsistencies between the experiments, which are due, at least in part, to differences in the duration and magnitude of the sodium intervention, the cDNA probe used, and the species and age of the animals studied (Du et al, 1995). There are only limited data available on effects of hemorrhage (Do et al, 1995) and dehydration (Lee et al, 1995) on gene expression of the RAS components in different tissues.

Recently, Do et al. (1995) in our laboratory observed that hemorrhage enhances angiotensinogen mRNA level in the brainstem, hypothalamus and liver. However, in the present study angiotensinogen mRNA levels were not significantly altered in brain and peripheral tissues after hemorrhage. The reason for the discrepant results is not clear. The only difference in the experimental protocol is that hemorrhage performed was 3 ml · kg^{-1} min^{-1} for 7 min in the previous experiment and for 5 min in the present study. Since there is no other results to compare, further studies are required to elucidate effects of hemorrhage on angiotensinogen mRNA expression in various tissues.

In the present study, hemorrhage increased mRNA levels of both AT_{1A} and AT_{1B} receptors in the brainstem and hypothalamus, but decreased them in the adrenal glands and liver. In sodium depleted rats, both receptor subtype mRNAs were also changed to the same direction (Jo et al, 1996). Sodium deprivation enhanced expression of AT_{1A} and AT_{1B} receptor mRNAs in the hypothalamus, kidney and adrenals, but suppressed them in the brainstem. Under certain conditions, however, regulation of receptor expression apprears to be different for the two AT_1 receptor subtypes. Bilateral nephrectomy decreased expression of liver AT_{1A} receptor mRNA but increased adrenal AT_{1B} mRNA levels (Iwai & Inagami 1992). In renovascular hypertension, adrenal AT_{1B} receptor mRNA levels decreased, whereas AT_{1A} levels did not change (Llorens-Cortes et al, 1994). AT_{1A} receptor mRNA was increased in rat ventricles with myocardial infarction, whereas the AT_{1B} receptor mRNA level was unaffected (Nio et al, 1995). Further studies are required to elucidate the regulatory mechanisms for gene expression of AT_1 receptor subtypes in different tissues.

Renin mRNA level after hemorrhage increased approximately two fold in the brainstem, but decreased half in the liver. Other tissues did not show any change in renin mRNA level after hemorrhage. It has been demonstrated that the brain expresses renin mRNA, but the levels are low and the distribution is dissimilar to the distribution of angiotensinogen mRNA (Dzau et al, 1986; Ekker et al, 1989). With a Northern blot analysis, it has been found that renin mRNA expressed in the brainstem in very low abundance but was not detected in the hypothalamus. With RT-PCR method, we could detect renin mRNA both in the brainstem and hypothalamus, and found increased renin mRNA level in the brainstem but no change in the hypothalamus after hemorrhage. In the peripheral tissues, hemorrhage-indcued stimulation of renin gene expression was also organ specific. Plasma and renal renin content was significantly increased after hemorrhage, whereas changes in renal gene expressions of renin, angiotensinogen and AT_1 receptor subtypes were not detected in the present study. It is hard to interpret the results. Recently, Boudy et al, (1996) reported region-specific distribution of AT_{1A} and AT_{1B} receptors in the different zones of the kideny. In the present study, 0.4 g kidney was used to extract RNA and thus it is possible different zones of the kideny were used in each case. This may give the result of no change in mRNA levels of the renal RAS components.

In summary, hemorrhage had different effects on gene expression of central and peripheral RAS components, suggesting the existence of different regulatory mechanisms in each tissue. Further studies are necessary to determine whether the change in mRNAs of the RAS components corresponds with that of proteins and to elucidate the physiological functions

of the hormone in each organ.

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