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DIFFERENTIAL REGULATION OF RAT AT1a AND AT1b RECEPTOR mRNA

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Summary: Rat type 1 angiotensin II receptor has two subtypes, namely type 1a and type 1b. The regulation of the expressions of these two subtype receptor mRNAs was studied by using a competitive polymerase chain reaction method. The expression of the type 1a mRNA in the liver was negatively and that of the type 1b mRNA in the adrenal was positively modulated by bilateral nephrectomy. In the ventricle of 16 week old Spontaneously hypertensive rat, the expression level of the AT1b receptor mRNA was higher than that in the ventricle of the age-matched Wistar-Kyoto rat, while the expresion levels of the AT1a mRNA in the ventricle were almost similar between the two strains at this age. Although type 1a and type 1b have almost similar functional properties, the expressions of their mRNAs were differentially regulated.

Angiotensin II(AII) is a multifunctional peptide that elicits variety of actions in its diverse target cells (1). Recent introduction of new AII antagonist has identified two major subtypes in this receptor, namely type 1 and type 2 (2). Type 1 angiotensin II receptor(s) seem to mediate all of the known functions of angiotensin II (2). Recently, cDNAs for the type 1 angiotensin II receptors (AT1R) have been cloned in bovine (3), rat (4,5,6), human (7), and mouse (8). Unexpectedly, rat and mouse have two subtypes in AT1R, while human and bovine seem to have only one subtype in the type1 angiotensin II receptor. These two subtype AT1Rs in rat have highly homologous sequences, similar binding and functional characteristics (5,6). The AT1R which is predominantly expressed in adrenal gland is termed as AT1b-R, and that which is predominantly expressed in kidney is termed as AT1a-R (6). We have reported the regulation of the expression of the AT1a-R mRNA in rat adrenal(9). However, it is recently disclosed that the AT1b-R is the predominant AT1-R in rat adrenal gland, and in other various tissues (6). In the present study, we have established a competitive polymerase chain reaction method for quantitation for the AT1a-R and AT1b-R mRNAs and the regulation of the expressions of these subtype receptor mRNAs was investigated in the present study.

Materials and Methods

Competitive polymerase chain reaction

Quantitation of the expression levels of AT1b-R and AT1a-R mRNA were performed by using a competitive polymerase chain reaction method (10). The plasmid pSPORT-1 which contained

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a full length cDNA fragment for the AT1b-R (6) was digested by a restriction enzyme Msc I and was then self-ligated. The resultant plasmid contained a cDNA fragment which lacked a region encompassing between the nucleotide number 187 and 475. And the deletion mutated cRNA for the AT1b-R mRNA was synthesized from this plasmid by using SP6 RNA polymerase after linealizing the plasmid by a restriction enzyme Hind III. Template plasmid DNA was completely digested by DNase I, and degradated DNA and free nucleotides were removed by repeated washing in the Centricon 30. Sample RNAs and known amounts of the deletion mutated cRNA were combined and were reverse transcribed using random primers as a primer. The resultant cDNA mixtures were amplified by the polymerase chain reaction method using the following primers:

Primer-1: 5'-GGAAACAGCTTGGTGGTG-3' (133-150)
Primer-2: 5'-GCACAATCGCCATTATCC-3' (739-719).

These two primers correspond to the regions where no sequence divergence is noted between AT1a-R and AT1b-R. One micrograms of sample RNAs combined with known amounts of the deletion-mutated cRNA were reverse-transcribed, and the resultant cDNA mixtures were amplified by PCR using the primers described above. Native AT1a-R and AT1b-R mRNA should give 607bp DNA fragment and the deletion mutated cRNA should give 419bp DNA fragment. The reaction profile included 35 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 60 seconds, and polymerization at 74°C for 120 seconds. Contamination of genomic DNA in sample RNAs were neglected by subjecting the sample RNAs directly to PCR amplification without a step of reverse transcription, in which no significant product was visible by 35 cycles. A trace amount of [α^{32}P]dCTP was included in PCR reaction for assessment of molar ratio of the 607bp fragment to the 419bp fragment. PCR products were resolved on 1.5-2.0 % agarose gel for visual check, and on 5-6% polyacrylamide gel for precise quantitation. PCR fragments were cut out from polyacrylamide gel and radioactivity was measured by Cerenkov counting.

RNA blot hybridization analysis

Total RNA and poly(A)+RNA were isolated as described previously (5). RNA hybridization analysis were carried out as described previously (5).

Animals

Male Sprague-Dawley(SD) rats (200-225 g), Spontaneously hypertensive rats (SHR), and Wistar-Kyoto rats (WKY) were obtained from Charles River Laboratories Inc. (Atsugi, Japan). SD rats were sacrificed 48hours after bilateral nephrectomy. Six adrenals and three livers were pooled to isolate RNAs.

Results and Discussion

Validity of the method

One microgram of adrenal total RNA combined with 0 (lane 6), 0.5 (5), 4.0 (4), or 32 pg(3) of the deletion-mutated cRNA was reverse-transcribed, and the resultant cDNA mixtures were amplified by PCR. As shown in the Fig 1A., the molar ratios of the 607bp DNA fragment to the 419bp fragment correlate well with the input amounts of the deletion-mutated cRNA. The deletion mutated cRNA (one microgram) was directly subjected without the prior step of reverse transcription to the amplification by PCR without a step of reverse transcription (lane 1 and 2). Seventy cycles were necessary to visualize 419bp fragment. This verified that contamination of the template plasmid DNA in the deletion mutated cRNA preparation was neglegiable. To assess the sensitivity of this method, we have prepared two kinds of RNA samples. One (Samle-A) is the total RNA isolated from rat adrenal gland, and the other (Sample-B) is comprised of 33% Cos 7 cell total RNA and 67% rat adenal total RNA. The expression level of the AT1-R mRNA in the sample-A is expected to be 1.5 fold higher than that in the sample-B. One microgram of the sample-A or sample-B combined with 70fg, 125fg, 250fg, or 500fg of the deletion mutated cRNA

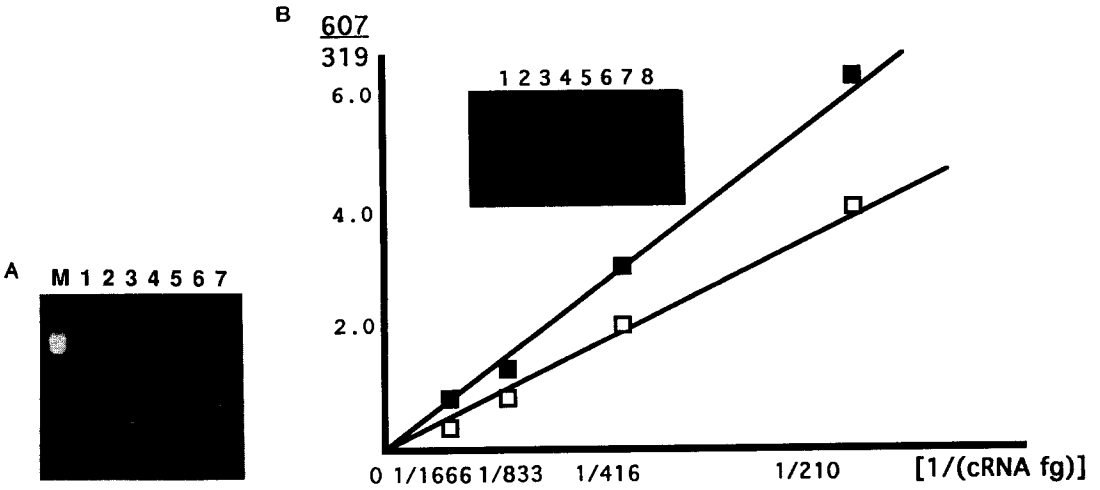

Fig.1. Establishment of the competitive PCR method.
A) Lane1, 2. One microgram of the deletion mutated cRNA was directly subjected to the amplification. Cycle number was 70 in lane1, and 35 in lane2. One microgram of the adrenal total RNA was combined with 32pg (lane3) 4.0pg (lane4), 0.5pg (lane5), and 0pg (lane6) of the deletion mutated cRNA. Those mixtures of RNAs were reverse-transcribed, and the resultant cDNA mixtures were subjected to the amplification by PCR.
B) One microgram of the sample-A (see text) was combined with 210fg (lane1), 416fg (lane3), 833fg (lane5), and 1666fg (lane7). One microgram of the sample-B was also combined with 210fg (lane2), 416fg (lane4), 833fg (lane6), and 1666fg (lane8). Those mixtures of RNAs were reverse-transcribed, and the resultant cDNA mixtures were amplified by PCR. The ratios of the radioactivity of the 607bp fragment to that of the 419bp fragment were plotted against the amounts of the deletion mutated cRNA included in the samples.

was reverse transcribed, and the resultant cDNA mixtures were amplified by polymerase chain reaction method. The ratios of the radioactivity of the 607 bp fragment to that of the 419bp fragment were plotted against the amount of the cRNA combined with the samples. The ratio of the slope obtained from the sample-A to that from tha sample-B was 1.5, which agrees well with the expected value 1.5 (Fig. 1B). This also verifies that 1.5 fold difference in the concentration can be discernible by this method

Assessment of the ratio of the AT1a-R mRNA to the AT1b-R mRNA

The ratios of the expression level of the AT1a-R mRNA to that of the AT1b-R mRNA were determined as described previously (6). In the livers of the SD rats employed in the present study, AT1a-R mRNA was almost exclusively expressed (Fig. 2). On the other hand, in the adrenals of the SD rats employed in the present study, AT1b-R mRNA was predominantly expressed (Fig.2).

Effects of the bilateral nephrectomy on the expression of the AT1-R mRNA

One microgram of the adrenal total RNAs obtained from the bilaterally nephrectomized rats or from sham operated rats was combined with 210, 416, 833, or 1666 fg of the deletion mutated cRNA. Those mixtures of the RNAs were reverse transcribed, and the resultant cDNA mixtures were amplified by PCR. The ratio of the slope obtained from the nephrectomized adrenals to that from the sham operated adrenals was 1.6 (Fig. 3A). In another set of the experiment, 1.5 was obtained (data not shown).

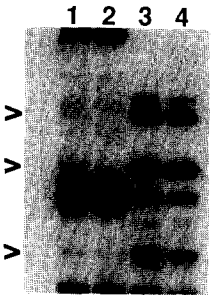

Fig.2. Assessment of ratios of AT1a-R mRNA / AT1b-R mRNA.
One microgram of the total RNA isolated from the control liver (lane1), the nephrectomised liver (lane2), the control adrenal (lane3), and the nephrectomised adrenal (lane4) was reverse transcribed and the resultant cDNA mixtures were amplified by PCR. Those PCR products were digested by a restriction enzyme Hae III and were resolved on 5% polyacrylamide gel.

One micrograms of the liver total RNAs obtained from the bilaterally nephrectomised rats and from the sham operated rats were combined with 210, 416, 833, and 1666 fg of the deletion mutated cRNA. Those mixtures of the RNAs were reverse-transcribed, and the resultant cDNA

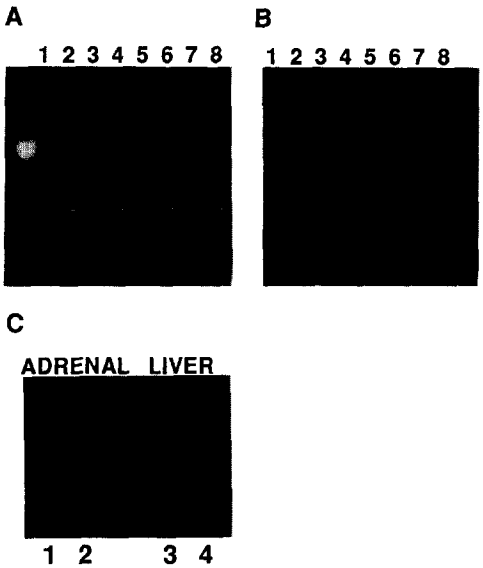

Fig.3. Effects of nephrectomy on the expression of AT1-R mRNAs.
A) One microgram of the total RNA isolated from the control adrenals was combined with 210fg (lane1), 416fg (lane3), 833fg (lane5), and 1666fg (lane7) of the deletion mutated cRNA. One microgram of the total RNA from the nephrectomised adrenals was also combined with 210fg (lane2), 416fg (lane4), 833fg (lane6), and 1666fg (lane8) of the deletion mutated cRNA. Those mixtures of RNA were reverse transcribed and the resultant cDNA mixtures were amolified by PCR.
B) One microgram of the total RNA from the nephrectomised livers was combined with 210fg (lane1), 416fg (lane3), 833fg (lane5), and 1666fg (lane7). That from the control livers was also combined with 210fg (lane2), 416fg (lane4), 833fg (lane6), and 1666fg (lane8).
C) The quality of the total RNA used in Figs. 3A and 3B was checked by ethidium bromide staining. Lane 1, Five micrograms of the control adrenal RNA; lane 2, Five micrograms of the nephrectomised adrenal RNA; lane3, Twenty micrograms of the control liver RNA; lane4, Twenty micrograms of the nephrectomised liver RNA.

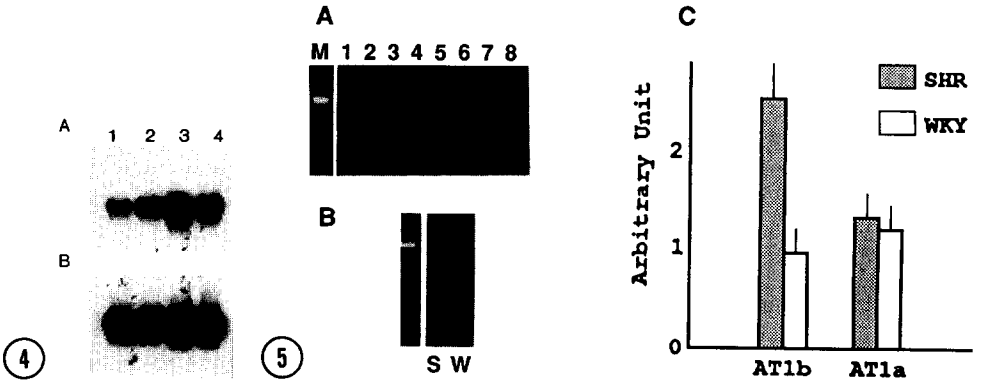

Fig.4. Nothern blot analysis.
Five micrograms of the poly(A)+ RNA isolated from the control adrenals (lane1), nephrectomised adrenals (lane2), control livers (lane3), and the nephrectomised livers (lane4) was electrophoresed in glyoxal/DMSO system. Two filters were prepared. One was hybridized to the full length cDNA fragment of the rat AT1b-R (A), and the other was hybridized to the Pst I fragment of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (13).

Fig.5. Expression levels in the ventricles.
A) The total RNA of one microgram isolated from the ventricle of 16 week old SHR (lane 5-8) or 16 week old WKY (lane 1-4) combined with 32 (lane 1,5), 64 (2,6), 128 (3,7), or 256 (4,8) fg of the deletion mutated cRNA was reverse transcribed and the resultant cDNA mixtures were amplified by PCR.
B) The total RNA of one microgram isolated from the ventricle of the 16 week old SHR or 16 week old WKY was reverse transcribed and the resultant cDNA mixtures were amplified by PCR. The PCR products were digested by a restriction enzyme Hae III and were resolved on 2.0% agarose gel. The ratio of the expression level of AT1a to AT1b was calculated as (the radioactivity of the 151 bp fragment /the radioactivity of the 263 bp fragment) X (108[GC content of 263 bp fragment]/57[GC content of 151 bp fragment]).
C) The calculated expression levels of the AT1a and AT1b receptor mRNA in the ventricles of 16 week old SHR and WKY are shown (n=3).

mixtures were amplified by PCR (Fig. 3B). The ratio of the slope obtained from the nephrectomized livers to that from the sham operated livers was 0.69. In another set of the experiment, 0.65 was obtained (data not shopwn). Quality of the RNAs were assessed by ethidium bromide staining (Fig. 3C). The results obtained from the competitive PCR method agrees well with the results obtained by a conventional method, Nothern blot analysis (Fig. 4).

Effects of hypertension on the expression of AT1-R mRNA

The involvement of renin-angiotensin system in the pathogenesis of hypertension in Spontaneously hypertensive rat(SHR) has been long recognised (11). We have previously assessed the expression levels of AT1a-R mRNA between SHR and its normotensive control Wistar-Kyoto rat(WKY), and no significant difference in the expresion levels was found (5). The expression levels of the AT1a and AT1b-R mRNAs in various tissues of SHR and WKY were reassessed in the present study. Fig. 5A and B shows the expression levels of the AT1a-R and AT1b-R mRNA in the ventricles of Spontaneously hypertensive rat and the age-matched Wistar-Kyoto rat. In the 16th week of age, the expression level of the AT1-R mRNA in SHR was 1.8 fold higher than that in WKY (Fig. 5A). The ratio of the expression level of the AT1a-R mRNA to that of AT1b-R mRNA was 0.5 in SHR and 1.2 in WKY (Fig 5B). These values indicated

that the increment of the expression level of the AT1-R mRNA in the ventricle of the 16 week old SHR compared to that of the age-matched WKY was due to 2.7 fold increase of the expression of the AT1b mRNA, and no significant difference in the expression of the AT1a mRNA was detected (Fig.5C).

In the present study, we have demonstrated that, in the rat, the expresion of the AT1a-R mRNA and the expression of the AT1b-R mRNA are differentially regulated. The expression of the AT1a-R mRNA in the liver was negatively and the expression of the AT1b-R mRNA in the adrenals was positively regulated by nephrectomy. We have previously reported that the expression of the AT1a-R mRNA in the adrenal was negatively regulated by nephrectomy (9). Although the expresion level of the AT1a-R mRNA in the adrenals was low compared to that of the AT1b-R mRNA in the SD rats employed in the present study, our previous result was recomfirmed in the Fig. 2, in which the DNA fragment derived from the AT1a-R mRNA was markedly decreased by nephrectomy (indicated by the arrow).

To examine a possible involvement of the AT1-R in the pathogenesis of hypertension in SHR, the expression levels of the AT1a and AT1b receptor mRNA were compared between SHR and its normotensive control WKY. And interestingly, the augmented expression of the AT1b-R mRNA but not the AT1a-R mRNA was found in the ventricles of 16 week old SHR. Angiotensin II has been recently recognised as a growth factor, and a blockade of the renin angiotensin system is known to reduce the hypertrophic change of the ventricles of SHR (12). However, futher studies will be necessary whether this augmented expression of the AT1b-R mRNA might contribute to the hypertrophic change of the ventricle of SHR.

While the AT1a-R and AT1b-R have highly homologous sequences, almost similar biding characteristics, and functional properties (5,6), the present study has demonstrated that the expressions of these two subtypes of the rat AT1-R are regulated differentially.

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