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QUANTITATIVE MEASUREMENT OF EXTRA-RENAL RENIN mRNA BY POLYMERASE CHAIN REACTION[1]

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SUMMARY : We established a simplified method of the quantitative measurement of extra-renal renin messenger RNA using polymerase chain reaction system. We could detect the renin messenger RNA in the kidney, heart, aorta and adrenal gland from a single RNA sample obtained from a Wistar-Kyoto rat. In the kidney, heart, aorta and adrenal gland, the contents of renin messenger RNA were found to be 55.8 ± 17.8, 0.15 ± 0.05, 0.11 ± 0.03 and 0.16 ± 0.07 pg/μg of total RNA (n=5, mean ± s.d.), respectively. The present method is very useful to study the extra-renal renin-angiotensin system.

The renin-angiotensin system (RAS) plays an important role in maintaining cardiovascular homeostasis. Circulating renin is mainly produced by juxtaglomerular cells of the kidney. Besides a circulating RAS, there exist tissue or local renin systems in different organ sites (1-4). The function of extra-renal tissue renin systems is yet to be clarified.

Measurement of renin messenger RNA (mRNA) indicates that the renin gene is expressed in the tissues including the adrenal gland, testis, brain and heart (5-9). However, a large amount of RNA samples are necessary to measure extra-renal mRNA. Polymerase chain reaction (PCR) is generally used for the detection of a small

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amount of DNA populations. The technique of labeled primer PCR (LP-PCR) enables us to measure low levels of mRNA populations.

We established, in the present study, a method for the quantitative measurement of extra-renal renin mRNA using LP-PCR and studied the expression of renin gene in the extra-renal tissues in Wistar-Kyoto rats (WKY).

MATERIALS AND METHODS

Animals: Male WKY were purchased from Chales River Japan Inc. (Atsugi, Japan) and were 16 weeks old at the time of experiment. Animals (n=5) were fed a standard laboratory diet and given tap water. They were anesthetized with ether and killed by decapitation. The kidney, heart, aorta and adrenal gland were removed from each animal, immediately snap-frozen in liquid nitrogen and stored at -80 °C until the isolation of RNA.

Isolation of tissue total RNA: Total RNA was extracted from each organ by the method of LeMeur et al. (10). Snap-frozen tissues were homogenized with a Waring blender in 20 volumes (W/V) of 3 M LiCl, 6 M urea, 200 μg/ml of heparin, 0.1% sodium dodecylsulfate and 10 mM sodium acetate (pH 5.0). The resulting homogenate was left overnight at 4 °C. The RNA was pelleted by centrifugation at 15,000g for 20 min at 4 °C, washed twice with 4 M LiCl and 8 M urea, and dissolved in ribonuclease (RNase)-free water. After phenolization, the RNA was ethanol-precipitated, washed twice with 70% ethanol and dried. The pellet of RNA was finally dissolved in RNase-free water and was quantified by measuring the absorbance at 260 nm.

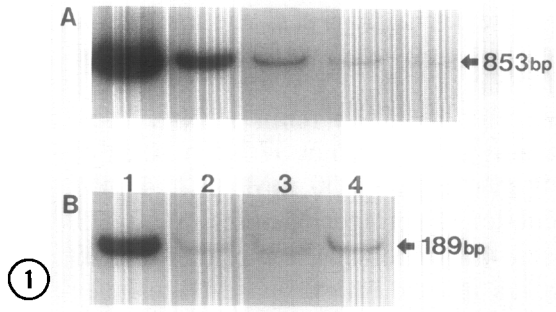
Oligonucleotide primers and internal standard: Two sets of oligonucleotide primers were synthesized by an automatic DNA synthesizer (Gene Assembler Plus; Pharmacia LKB Biotetechnology, Uppsala, Sweden). The primers used for amplification of rat renin cDNA occupied equivalent positions in the unique rat renin gene (11). Sequences of the oligonucleotides used in this study were as follows : rat renin exon 8 primer (EX 8), CTCAGCAACATGGACTATGT ; rat renin exon 9 primer (EX 9), TTAGCGGGCCAAGGCGAACC. An 853 base pair (bp) fragment of genomic rat renin DNA was subcloned into a polylinker site of plasmid pT3T7 lac (Boeringer Mannheim, Mannheim, Germany). This fragment contained the equivalent sequences for amplification by using the primers of EX 8 and EX 9. An 853 base-length of rat renin mRNA fragment was synthesized by *in vitro transcription* in the presence of T7 RNA polymerase (Boeringer Mannheim). The resulting synthetic renin mRNA fragment was used as an internal standard for measuring tissue renin mRNA contents.

Amplification of renin cDNA: Five micrograms of tissue total RNA or 20 pg of synthetic renin mRNA fragment were used for the specific first-strand synthesis of renin cDNA. EX 9 was used as the specific primer for reverse-transcription of rat renin cDNA. The reaction was carried out by incubating for 60 min at 37 °C in 20 μl of 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 50 mM KCl, 4 mM $Na_4P_2O_7$, 20 units of RNase inhibitor (Boeringer Mannheim), 0.125 mM deoxynucleotide triphosphate, 40 pmoles of EX 9 primer and 20 units of Avian myeloblastosis virus reverse transcriptase (Boeringer Mannheim). The reaction mixture was heated at 95 °C for 5 min. Kidney renin cDNA transcript was 20 times diluted with water before the amplification. Four microliters of tissue renin cDNA transcript and serial dilution of synthetic renin cDNA transcript were used for PCR amplification. Oligonucleotide primers, EX 8 and EX 9, were labeled with [γ-^{32}P] ATP (5,000 Ci/mmol; Amersham International plc., Bucks, UK) using T^4 polynucleotide kinase according to the manufacture's protocol (MEGALABEL^{TM}; Takara Shuzo Co.Ltd., Kyoto,Japan). PCR amplification was performed in a total 40 μl of 10 mM Tris-HCl buffer (pH 8.3) containing, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.125 mM deoxynucleotide triphosphate, 2 pmoles of end-labeled primers and 1 unit of Thermus aquaticus DNA polymerase (Perkin Elmer/Cetus, Norwalk, USA). The reaction was carried out by heating at 94 °C for 30 sec, followed by annealing at 50 °C for 30 sec and elongation at 72 °C for 1 min. This cycle was repeated 25 times.

Analysis of PCR products: Ten microliters of the amplified cDNAs from tissue renin mRNA and the standard renin mRNA fragment were electrophoresed on 8% polyacrylamide gels. After electrophoresis, the gels were vaccum-dried and exposed on an X-ray film (Hyper film^{TM}-MP; Amersham International plc.) for 2 hr. In addition, the gels were exposed on an imaging plate (Fuji Film Co. Ltd., Tokyo, Japan) for 5-10 min and the autoradiographic images were analyzed by Bio-Imaging Analyzer (BAS-2000; Fuji Film Co. Ltd.) for measuring the absorbance of bands of the amplified renin cDNA.

RESULTS

Figure 1 shows representative autoradiographic images of PCR products from tissue total RNA and the synthetic renin mRNA fragment. Bands of cDNA products were obtained at 189 bp for tissue renin mRNA and at 853 bp for the standard renin mRNA fragment. Figure 2 shows a standard curve for renin mRNA. Imaging plates were exposed for 5 min and absorbance units were measured by Bio-Imaging analyzer (BAS-2000). The linearity of



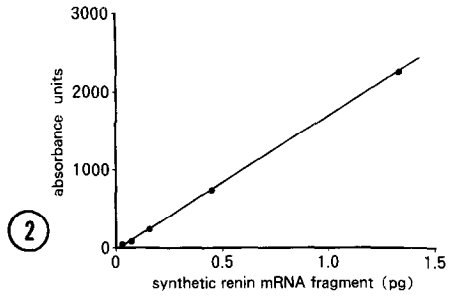


Figure 1. The autoradiographc images of the PCR products obtained from the standard mRNA fragment and tissue total RNA, which were 853 bp (A) and 189 bp (B), respectively. (A) Serial dilutions (1:3) of standard renin cDNA products (1.33-0.016 pg). (B) Tissue renin cDNA products obtained from tissue total RNA, lane 1; kidney (12.5 pg), 2; heart (250 pg), 3; aorta (250 pg), 4; adrenal gland (250 pg). The autoradiograph was exposed for 2 hr.

Figure 2. Standard curve for the synthetic renin mRNA fragment. The abscissa represents the amount of the synthetic renin mRNA fragment, and the ordinate represents the absorbance units of bands for the PCR products. The imaging plate was exposed for 5 min.

this standard curve was good using points under 2,300 absorbance units (r^2=0.999). The detection limit of this assay was about 32 fg of the full-length rat renin mRNA (1,600 bases length). Tissue renin mRNA contents were calculated from the standard curve of the synthetic renin mRNA fragment. Mean values of tissue renin mRNA contents in the kidney, heart, aorta and adrenal gland were 55.8 ± 17.8, 0.15 ± 0.05, 0.11 ± 0.03 and 0.16 ± 0.07 pg/μg of total RNA, respectively. Renin mRNA contents of these extra-renal tissues were about 300-500 folds lower than that of the kidney.

DISCUSSION

We established a simplified method for the quantitative measurement of extra-renal renin mRNA using LP-PCR. The first study of quantitative measurement of mRNA using the PCR technique was reported by Chelly et al. (12). They used an unrelated template as an internal standard for measuring dystrophin mRNA. Wang et al. (13) reported the quantification of interleukin 1α mRNA using a

synthetic mRNA fragment as an internal standard which was able to be amplified by the equivalent sequences for the target mRNA. Ekker et al. (14) reported relative renin mRNA contents in extra-renal tissues using PCR amplification. In their report, PCR products were detected by Southern blotting analysis hybridized with an internal probe of rat renin gene. Blotting assay involves transferring a target RNA or DNA to a membrane and hybridizing with a probe. In our present study, we used end-labeled primers for PCR amplification to detect a specific mRNA population, and could exclude a step of transfer to a membrane and hybridization with a probe. Therefore, LP-PCR method is a more simple and accurate method for the detection of a specific mRNA population. Recently, extra-renal renin mRNA contents have been measured by Northern blotting, solution hybridization assay or RNase protection assay (15-19). Since a synthetic renin cRNA probe was used for measuring renin mRNA contents in solution hybridization assay or RNase protection assay, the detection limits of these assays are about one tenth lower than the classical Northern blotting. However, the sensitivity of these assays is not enough to measure levels of renin mRNA in the extra-renal tissues of a single animal. We could detect low levels of renin mRNA in several tissues such as heart, aorta and adrenal gland using a single RNA sample extracted from a rat.

Suzuki et al. (19) reported that renin mRNA contents of kidney, heart and adrenal gland in WKY were 0.43, 0.076 and 0.011 pg/μg of total RNA, respectively. The value of renin mRNA content in the kidney was markedly lower than that of our result. Ganten et al. (1,2) reported that renin activity in the kidney was about 700-fold higher than that in the heart. In our study, the level of renin mRNA was about 500-fold higher in the kidney than in the heart. Paul et al. (18) reported that renin mRNA content in the mouse heart was 0.07 pg/μg of total RNA. Our result of rat heart renin mRNA content is

similar to their result. Since our detection limit of renin mRNA content was about one thousandth lower than the classical Northern blotting, we could detect the level of extra-renal renin mRNA using a single RNA sample from a rat. These results suggest that LP-PCR method may be a useful tool to investigate the extra-renal RAS.

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