The Expression of α_{2B} -Adrenoceptor mRNA in Sprague-Dawley Rat Kidney

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Based on radio-ligand binding assays, most of the α_{2B} -adrenoceptors in rat kidney are found in the proximal tubules and are of the α_{2B} -adrenoceptors subtype. To determine more accurately the distribution of the α_{2B} -adrenoceptors in the rat kidney, we studied the expression of α_{2B} -adrenoceptors mRNA in rat kidney slices and microdissected nephron fragments using reverse transcription-polymerase chain reaction (RT-PCR) technique. Sprague-Dawley rat kidney was dissected into cortex, outer medulla and inner medulla. Total RNA was prepared from the kidney slices. Microdissected single nephron segments about 1 mm in length were transferred to tubes containing lysis buffer. RT-PCR was performed using a GeneAmp RNA PCR kit from Perkin-Elmer Corporation. The PCR primers were designed based on the sequences of the third cytoplasmic loop of the rat α_{2B} -gene. After the PCR, the products were analyzed by agarose gel electrophoresis. The specificity of PCR amplified products was examined by restriction endonuclease digestion and internal oligonucleotide hybridization. Our results indicate that α_{2B} -adrenoceptors mRNA is expressed in rat kidney cortex, outer medulla and inner medulla. Microdissection and RT-PCR showed that α_{2B} -adrenoceptor mRNA was expressed in the proximal straight tubules (S₃), cortical and medullary thick ascending limbs of Henle (CTALH, MTALH) and cortical and outer medullary collecting tubules (CCT, OMCT). (*Hypertens Res* 1993; 16: 259-263)

Key Words: α_{2B} -adrenoceptor mRNA, RT-PCR, kidney

Alpha₂-adrenoceptors are present in many organs of the body. The diverse cellular consequences of α_2 -adrenoceptor activation include inhibition of adenylate cyclase, alterations in Ca²⁺ translocation, and acceleration of Na⁺/H⁺ exchange.

Alpha₁- and α_2 -adrenoceptors have been identified in the rat kidney (1, 2). A role of renal α_2 -adrenoceptors in the pathogenesis of genetic hypertension is suggested by the unusually high α_2 -adrenoceptor density in the kidneys of genetically hypertensive rats and the observation that a high salt diet increases renal α_2 -adrenoceptor density and increases blood pressure (3). Based on radioligand binding assays, most renal α -adrenoceptors are of the α_2 -adrenoceptor subtype (1-4) with the majority being found in proximal tubules (4-7). Stanko and his colleagues (8) did competition experiments in rat renal proximal tubules, cortex and whole kidney membranes. Their results suggested that α_2 -adrenoceptors in the rat kidney are likely of the α_{2B} -adrenoceptor subtype. However, because of the limitation of the sensitivity of the radioligand assay, it is impossible to study the subtype specific α_2 -adrenoceptors in microdissected single nephron fragment.

The emergence of molecular cloning techniques

in the study of receptor structure and function has allowed the unequivocal demonstration of multiple α_2 -adrenoceptors. To date, three closely related rat α_2 -adrenoceptor genes have been cloned (9, 10). Using subtype specific hybridization probes in Northern blot analysis, Lynch and Lanier showed that α_{2A} - and α_{2B} -adrenoceptors are expressed in rat kidney. However, Northern blot is not sensitive enough to measure mRNA expressed in a very low level or in a very small quantity of tissue.

The recently developed reverse transcription-polymerase chain reaction (RT-PCR) is a very sensitive technique to study the expression and regulation of a gene. Since no information has been published on the regional distribution of α_{2B} -adrenoceptor subtype in rat kidney, we designed this experiment to locate α_{2B} -adrenoceptor mRNA expression in rat kidney slices and microdissected nephron fragments using RT-PCR technique.

Methods

The Sprague-Dawley rat kidney was very carefully dissected into cortex, inner and outer medulla. Due to the small amount of tissue obtained from the inner medulla, tissues from two rats were pooled.

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Total cellular RNA from cortex, inner and outer medulla was prepared based on the protocol in Molecular Cloning: A Laboratory Manual (11). After dissection, the kidney tissue was immediately suspended in 5 ml of 4 M guanidine-isothiocyanide solution and transferred to a dounce homogenizer. The suspension was homogenized gently to ensure both complete disruption of the cell and a uniform mixture. Solid cesium chloride was added to a final concentration of 0.5g/ml. The samples were then layered over a 5 ml cushion of 5.7 M cesium chloride and 100 mM Na₂ EDTA, pH 7.6, completely filling the 11 ml centrifuge tubes. The samples were centrifuged for 12-16 h at 55,000 rpm in a Sorvall RC 70 ultracentrifuge (T-1270 rotor) at 20°C. The supernatant was discarded. The clear RNA pellet in the bottom of the tube was resuspended in 200 µl of 10 mM Tris-HCl and 1 mM EDTA, pH 7.5, and extracted once with phenol, and once with an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1). The RNA was precipitated with ethanol. The RNA pellet was then resuspended in 200 µl of TE buffer and quantified by UV absorbance at 260 nm.

Tubule dissection was performed in dissection solution held on an ice block using dissecting forceps (sharpened Dumont No. 5), a dissection microscope (8-64X magnification), and dark-field illumination. Tubule segments were identified based on previously described criteria (12) and as specified by Krapf and Solioz (13). Dissected segments were transferred by pipet to clean dissection buffer and measured with an ocular micrometer to be 1 mm in length. Each millimeter was captured with a small glass bead (diameter, 500 µm) and transferred with dissection forceps to a 0.5 ml Eppendorf tube with 10 µl of lysis buffer containing 2% Triton X-100, 2.0 units/µl of RNase inhibitor and 5 mM dithiothreitol. The reaction tubes were placed on ice until the reverse transcription was initiated as described below.

RT-PCR was performed by using a GeneAmp RNA PCR kit from Perkin-Elmer Corporation (Norwalk, CT). The final concentrations of contents in 20 µl reverse transcription mixture were: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM of each dATP, dGTP, dCTP and dTTP, 1 unit/µl of RNase inhibitor, 2.5 µM of random primers and 50 units of Moloney Murine Leukemia virus reverse transcriptase and 0.5 µg each of RNA from cortex, inner and outer medulla or microdissected single nephron fragment. Reaction tubes were centrifuged for a few seconds and 50 µl of mineral oil was overlaid to reduce evaporation. The reaction tubes were incubated at room temperature for 15 min, then transferred to a 42°C water bath and incubated for 15 min. The reaction tubes were next transferred to a 99°C heat block for 5 min to denature the reverse transcriptase, followed by 5 min incubation at 5°C. Negative control reactions containing all the reagents except the reverse transcriptase were performed in parallel.

After reverse transcription, 80 µl of PCR reagent mixture was added. The final concentration of each component of PCR reaction (100 µl) was: 10 mM

Tris-HCl, 50 mM KCl, 2 mM MgCl₂, 2.5 units *Taq* polymerase and 0.2 µM of each sense and antisense primers. For the rat α_{2B} -adrenoceptor gene, the sense primer is defined by bases 947-970 (5' ATC CTC GTC TAC CTG CGA ATC TAC) and antisense primer encompass bases 1439-1463 (5' CTG TGT CCG TCT GCG CCA CCA CTG). The amplified product is predicted to be 517 bp. PCR was performed in an automated thermal cycler (Eri-comp, San Diego, CA) which was programmed as follows: 95°C for 3 min (initial melt), followed by 40 cycles of 95°C for 1 minute (denature), 58°C for 1 minute (anneal) and 72°C for 1 minute (extend). PCR was completed by a final extension at 72°C for 5 min.

For the analysis of the PCR products, 15 µl of the reaction mixture was loaded on a 2% NuSieve GTG/1% agarose gel and electrophoresed.

Restriction enzyme digestion of PCR amplified products and Southern blot analysis were performed to examine the specificity of the amplified products. After electrophoresis, the gel was denatured and transferred to a nylon membrane according to the procedure described by Southern (14) as modified by Smith (15). The filter membrane was baked for 1 h at 80°C. Prehybridization was performed in a solution containing 5 × SSC, 5 × Denhardt's solution, 0.1% SDS and 100 µg/ml salmon sperm DNA and 20% formamide at 42°C. After 2-3 h, the prehybridization solution was changed to the hybridization solution which contained 5 × SSC, 1 × Denhardt's solution, 0.1% SDS, 100 µg/ml salmon sperm DNA, 20% formamide and [γ -³²P] ATP labeled internal oligonucleotide probe. Twenty base pairs of rat α_{2B} -adrenoceptor internal oligonucleotides (# 1128-1147 bp, 5' GAC ACC CCA AGC CTC CAA GA) were labeled with [γ -³²P]ATP at the 5' end using T₄ polynucleotide kinase. In brief, 300-600 pmole of purified oligonucleotides were incubated at 37°C for 30 min in the presence of T₄ polynucleotide kinase buffer, 10 units of T₄ polynucleotide kinase and 5 µl of fresh [γ -³²P] ATP in a total volume of 20 µl. The labeled oligonucleotide probes were purified to remove free [γ -³²P]ATP. After hybridization for 12-16 h, the filters were washed with 3 × SSC, 0.1% SDS at 42°C for 20 min followed by a wash with 0.1 × SSC, 0.1% SDS at 42°C for another 20 min. The filter was then exposed to Kodak X-ray film.

Results

Figure 1 shows the 2% NuSieve GTG /1% agarose gel of the amplified rat kidney α_{2B} -adrenoceptor mRNA products. One-hundred base pair ladder from BRL is used as DNA size marker. Lanes 1 and 2 are RT-PCR products from rat kidney cortex. Lanes 3 and 4 are from the inner medulla and lanes 5 and 6 are from the outer medulla. Clear single band of 517 bp for rat α_{2B} -adrenoceptor was seen for lanes 1, 3 and 5, while lanes 2, 4 and 6 are the controls without reverse transcriptase. This result indicates that rat α_{2B} -adrenoceptor mRNA is expressed in renal cortex, inner medulla and outer

Fig. 1. Analysis of RT-PCR products for the rat α_{2B} -adrenoceptor mRNA from the dissected rat kidney in 2% NuSieve GTG/1% agarose gel stained with ethidium bromide. The leftmost lane is a 100 bp ladder DNA size marker, from the bottom to top: 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, etc. Lanes 1 and 2 are RT-PCR products from the kidney cortex, lanes 3 and 4 from the inner medulla, and lanes 5 and 6 from outer medulla. Lanes 2, 4 and 6 are the negative controls without reverse transcriptase.

medulla. The negative results of lanes 2, 4 and 6 indicate that the amplified products are from RNA, but not from genomic DNA, contamination.

Figure 2 shows the restriction endonuclease digestion of RT-PCR products. After digestion with Bsu36 I, the amplified α_{2B} -adrenoceptor produced two bands of 283 bp and 234 bp. The patterns of restriction endonuclease digestion of the amplified products are the same as predicted from the rat α_{2B} -adrenoceptor gene sequences, thus confirming that the amplified products are from the rat α_{2B} -adrenoceptor mRNA.

The specificity of PCR amplified products was further examined by the internal oligonucleotide hybridization. Fig. 3 shows the Southern blot of

Fig. 3. Autoradiogram of Southern blot and internal oligonucleotide hybridization of RT-PCR amplified α_{2B} -adrenoceptor mRNA products.

STD uncut Bsu36I

Fig. 2. Restriction endonuclease digestion of RT-PCR amplified products for rat α_{2B} -adrenoceptor mRNA.

RT-PCR products hybridized with α_{2B} -adrenoceptor specific oligonucleotide probes. The specific binding of the α_{2B} -adrenoceptor internal oligonucleotides to the PCR products confirmed their identity.

Microdissection and RT-PCR showed that α_{2B} -adrenoceptor mRNA was expressed in the proximal straight tubules (S₁), cortical and medullary thick ascending limbs of Henle (CTALH, MTALH) and cortical and outer medullary collecting tubules (CCT, OMCT) (Fig.4).

Discussion

Based on the radioligand binding studies, Bylund suggests the existence of several α_2 -adrenoceptor subtypes (16). α_2 -antagonists, [3 H]yohimbine and [3 H]rauwolscine have been used, popularly to measure the α_2 -adrenoceptor density, and may interact with more than one subtype. Another disadvantage intrinsic to radio-ligand binding assay is that this method is not sensitive enough to measure the α_2 -adrenoceptor density in very small quantities of tissue or in tissue with very low quantities of α_2 -adrenoceptors.

Methods commonly used for the detection and

Fig. 4. The expression of α_{2B} -adrenoceptor mRNA in microdissected nephron fragments. Microdissection and RT-PCR showed that α_{2B} -adrenoceptor mRNA was expressed in the proximal straight tubules (S_3), cortical and medullary thick ascending limbs of Henle (CTALH, MTALH) and cortical and outer medullary collecting tubules (CCT, OMCT).

analysis of RNA molecules include *in situ* hybridization, Northern blot and ribonuclease protection assay. The most sensitive of these methods is *in situ* hybridization, in which 10 to 100 molecules can be detected in a single cell. However, the *in situ* hybridization method can be technically very difficult and does not lend itself well to the processing of large numbers of samples. However, RT-PCR has provided a breakthrough in this area. Usually, less than 1 μ g of cytoplasmic RNA is sufficient for amplification of rare mRNA sequences. In this study, we have used the RT-PCR technique to locate the expression of α_2 -adrenoceptor subtype specific mRNA in the rat kidney.

The α_{2B} -adrenoceptor subtype in the neonatal rat lung was identified by showing that rauwolscine was 3 times more potent than yohimbine (17). Stanke *et al.*, using competitive binding techniques, suggested that α_2 -adrenoceptors in renal proximal tubules, cortex and whole kidney membranes were the α_{2B} -adrenoceptor subtype (8). Using the RT-PCR technique, we have, for the first time, demonstrated that α_{2B} -adrenoceptor mRNA was expressed not only in the rat kidney cortex, but also in the rat kidney inner and outer medulla. Using RT-PCR and microdissecting techniques, we further locate the expression of α_{2B} -adrenoceptor mRNA in the following nephron fragments: proximal straight tubules (S_3), cortical and medullary thick ascending limbs of Henle (CTALH, MTALH) and cortical and outer

medullary collecting tubules (CCT, OMCT).

In summary, our results show that α_{2B} -adrenoceptor mRNA is expressed in rat kidney. Restriction endonuclease digestion and Southern blot analysis confirmed that the RT-PCR amplified products are from α_{2B} -adrenoceptor mRNA. Our knowledge of renal α_2 -adrenoceptor distribution will help us to evaluate these receptors in normotensive rats and their changes in genetically hypertensive rats. It will also assist the study of the pathophysiological role of the α_2 -adrenoceptors in hypertension through activating or blocking the expression of α_2 -adrenoceptor in a specific nephron fragment.

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