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DETECTION OF ENDOTHELIN-1 mRNA BY RT-PCR IN ISOLATED RAT RENAL TUBULES

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SUMMARY: A combination of reverse transcription and polymerase chain reaction was utilized to detect baseline endothelin-1 (ET-1) mRNA expressed in renal tubules dissected from rats. 5' and 3' primers were constructed according to human ET-1 genomic DNA. Rat ET-1 mRNA was found to be expressed only in cortical through medullary collecting ducts in addition to glomeruli. Sites for tubular synthesis of ET-1 corroborate well with major ET-1 binding sites along the nephron, indicating autocrine/paracrine role of ET-1 in the renal tubules and supporting a prevailing concept on such function of ET-1 in many differing tissues.

A spectrum of endothelin-1 (ET-1) function in regulating renal hemodynamics and tubular transport has broadly been expanding over last few years. As far as tubular action of ET-1 is concerned, accumulating evidence suggest its dominant action along collecting ducts rather than proximal tubules or the loop of Henle. There ET-1 may regulate water and electrolytes transport perhaps by inhibiting Na-K-ATPase (1) or antagonizing vasopressin action (2, 3). The intracellular signaling of ET-1 action on the collecting ducts is probably mediated by an increase in cell Ca^{2+} concentration (4).

On the other hand a possibility of tubular synthesis of ET-1 has been raised in several reports including our demonstration of ET-1 mRNA in MDCK, a cell line of distal tubular origin (5, 6). Recently Kohan et al. reported using primary cultures of rabbit renal tubules that the amount of ET-1 released in the culture media is greatest from inner medullary collecting ducts than from medullary thick ascending limb of Henle or proximal tubules (7) and subsequently they confirmed ET-1 production in cultured inner medullary collecting ducts of the rat (8). One argument against these results may come from the fact that culture conditions does not necessary represent the basal state of the tissue in *vivo*. ET-1 synthesis may be modulated by an addition of serum or by other non-physiological conditions.

Therefore, in the present study we attempted to detect baseline ET-1 mRNA expression in isolated defined renal tubule segments. To this purpose a combination of reverse transcription and polymerase chain reaction methods was applied to detect only a minute

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amount of ET-1 mRNA in a microscopic tissue such as renal tubules. Baseline ET-1 mRNA was detected in collecting ducts from cortex through inner medulla and glomeruli as well.

MATERIALS AND METHODS

Microdissection of renal tubules: Male Wistar rats weighing 200-300 g were anesthetized with Nembutal (50 mg/kg). All experimental procedures hereafter were done in sterile conditions. The left kidney was perfused via the left renal artery with a cold 0.1% collagenase-containing modified Hanks' solution (NaCl, 137 mM; KCl, 5 mM; $MgSO_4$, 0.8 mM; Na_2HPO_4, 0.33 mM; KH_2PO_4, 0 .44 mM; $MgCl_2$, 1 mM; $CaCl_2$, 1 mM; Tris-HCl, 10 mM; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 20 mM; H_2CO_3, 0.03% (w/v); and bovine serum albumin (BSA), 0.1% ; pH 7.4). Thin pyramids were cut along the corticopapillary axis and incubated for 15 min at 37°C in the same solution as above. After rinsing with the cold Hanks' solution, tissues were transferred to the dissection solution containing 10 mM vanadyl ribonucleotide complex (VRC) (Life Technologies Inc., Gaithersburg, MD).

Microdissection of the nephron was carried out free hand under stereomicroscopic observation at 4°C and finished within 30 min. Parts of microdissected nephrons are: glomeruli (G); the early, middle, and terminal portions of the proximal tubules (S1, S2, and S3, respectively); the cortical and medullary thick ascending limbs of Henle's loop (CAL and MAL, respectively); the distal convoluted tubule (DCT); the cortical, outer medullary and inner medullary collecting ducts (CCD, OMCD, and IMCD, respectively). The lengths of these microdissected nephron segments were measured by an eyepiece micrometer. The details of microdissection of the rat nephron is described elsewhere (9).

The microdissected samples were transferred to a separate wash dish holding ice-cold Hanks' solution and rinsed vigorously to remove extraneous materials. This wash step was repeated another time. Special cares were taken to prevent contamination of other tissues. Then the respective samples of 2 µl volume were carried to a siliconized fresh microtube using a siliconized microcapillary, and kept on ice until the following reverse transcription reaction.

Reverse transcription (RT): RT was performed with oligo-dT primers using a cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany). Eight microliters of a lysing solution which yielded the final concentration of 2% Triton X-100 and 1 U/µl human placental RNase inhibitor and 5 mM dithiothreitol were added to a reaction tube to permeabilize the cells. Then, RT components were serially added according to the manufacture's protocol and the contents were mixed, briefly centrifuged. The reaction tubes were incubated at 42°C for 60 min, which gave rise to a mixture of cDNAs.

Polymerase chain reaction (PCR): PCR was performed using the GeneAmp DNA amplification reagent kit (Perkin-Elmer Cetus, Norwalk, CT). Since rat ET-1 cDNA was sequenced (10) but its gene was not revealed, we deliberately designed 5' and 3' primers by taking advantage of human ET-1 genome map (11). We first selected 20 bases of rat ET-1 cDNA sequences corresponding to the beginning of N-terminus of ET-1 peptide for 5' primer (sense DNA). The sequence of this primer was 5'-GTTGCTCCTGCTCCTCCTTG-3'. And for 3' primer (antisense DNA) 20 oligonucleotides which are located within 3' untranslated region of the cDNA was selected. The sequence of 3' primer was 5'-GCATGGAGAGCGC-AGAGTTG-3'. Both primers had 60% GC composition and resulting melting temperatures (*Tm*) were calculated at 66°C. In comparison with human ET-1 genome these two primers was expected to span three introns. Accordingly, the predicted length of PCR amplification product should be 532 base pairs (bp). An additional oligoprobe (sense DNA) positioned between 5' and 3' primers was synthesized so as to hybridize the authentic PCR products. The sequence of this 21 oligonucleotides was 5'-GAACTCCGAGCCCAAAGTACC-3'.

Before conducting PCR, RT contents (20µl) were incubated at 90°C for 10 min to denature RNA-cDNA hybrids and to inactivate the reverse transcriptase. Then 80 µl of a PCR Master Mix containing 5' and 3' primers prepared according to the manufacturer's formula was added to each tube. 2.5 units of *Taq* DNA polymerase were used per reaction. The mixtures were overlaid with 100µl water-saturated mineral oil. PCR was programmed using a temperature control system (PC-500, ASTEC, Tokyo) to perform initial melt by 94°C, 3 min and 30 or 40 cycles of the following sequential steps: 94°C, 1 min (melt); 60°C, 1 min (anneal); 72°C, 3 min (extend), and final extension by 72°C, 7 min. Samples were kept at -20°C until next Southern blot analysis.

Southern blot analysis: One hundred microliters of the reaction volume were transferred to a new microtube and subjected to ethanol-precipitation. The resulting DNA pellets were size-fractionated by 2% agarose gel electrophoresis for 2 hours together with DNA size markers ranging 79-1057 bp. DNA bands visualized by ethidium bromide staining were photographed. Then gels were blotted overnight onto Biodyne Transfer Membrane (Pall Ultrafine Filtration Corp., Glen Cove, NY) with 20x SSC (1x SSC: 0.15M NaCl, 0.015M sodium citrate; pH 7.0). The blotted membrane was baked at 80°C for 2 hours. The synthetic oligonucleotide for hybridization was end-labeled with [γ-^{32}P]ATP (>5000 Ci/mmol, New England Nuclear) to a specific activity of ~10^8 cpm/μg. Solution hybridization was carried out at 42°C overnight, and after three times wash with 1x SSC, 0.1% SDS autoradiography was performed at -80°C for 2-3 days.

RESULTS

The representative results from agarose gel electrophoresis and Southern blot hybridization of PCR products of ET-1 mRNA are shown in Figure 1. Note that predominant RT-PCR products examined on five glomeruli migrated between 495 and 550 bp as indicated by DNA size markers (lanes 1-3), compatible with the predicted length of 532 bp. By Southern blot analysis this DNA band was verified to be an authentic PCR product as the radio-labeled probe located between two distant primers specifically hybridized (lanes 4-5). On the other hand no DNA band appeared if the reverse transcriptase was omitted from the experiment (lanes 6-7), confirming that the 532 bp PCR products indeed derived from rat ET-1 mRNA but not from its genomic counterpart. It also confirmed that 5' and 3' primers we designed for RT-PCR locate on separate exons. In this experiments three different portions of proximal tubules (S1, S2 and S3) were examined along with glomeruli. However, no signals were detected in those tubule segments (lanes 8-10).

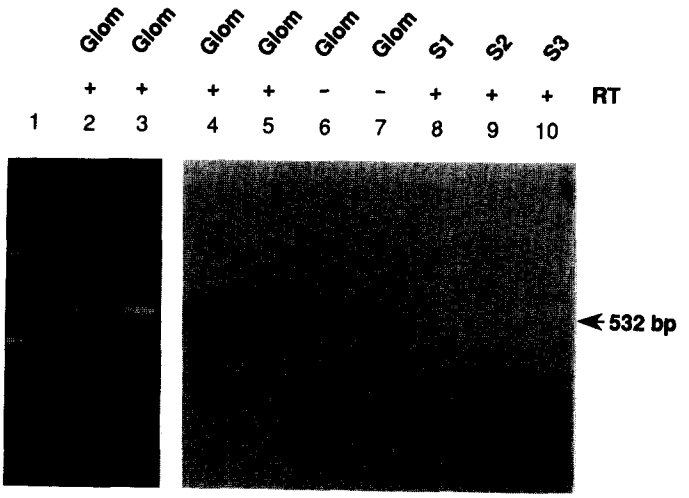

Figure 1. Analysis of RT-PCR products for rat ET-1 mRNA.
Left (lanes 1-3): ethidium bromide stained gels. DNA size markers indicate 1057, 770, 612, 495, 392, 340, and 294 bp from top to bottom. PCR products from four glomeruli appeared a single band whose molecular weight matched a predicted size of 532 bp.
Right (lanes 4-10): autoradiograms of Southern blots probed with ^{32}P-labeled oligonucleotide which locates between 5' and 3' PCR primers. Four glomeruli and 6 mm proximal tubules (S1, S2, S3) were used. Reverse transcriptase was added in RT (+) or omitted in RT (-). An amplification reaction was performed,40 cycles. An arrow indicates an expected PCR product size (532 bp). Note that no signals were detected without RT.

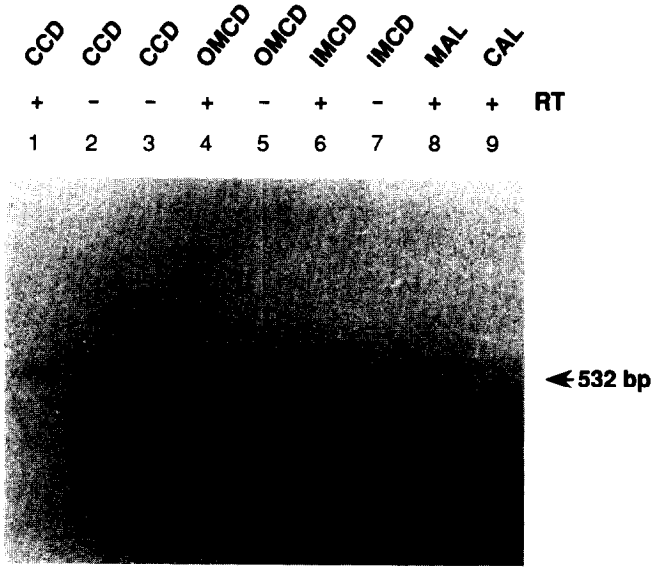

Figure 2. Southern blot analysis of RT-PCR products.
An amplification reaction was performed,30 cycles. The predicted PCR product was 532 bp as indicated by an arrow. Tubule lengths used were 2 mm for CCD and OMCD, and 1 mm for IMCD, and 4 mm for MAL and CAL. Note that no products were detected without RT.

Results of another set of experiments are shown in Figure 2. CCD, OMCD and IMCD displayed discrete signals which were again proved by Southern blot hybridization (lanes 1, 4, 6). Other tubule segments such as MAL and CAL gave no detectable signals (lanes 8-9). The signal of RT-PCR product on IMCD appear weaker than CCD or OMCD probably because a smaller amount of IMCD was used in this experiment (2 mm of CCD or OMCD vs 1 mm of IMCD). However, we did not attempt a quantitative measure for RT-PCR signals from ET-1 mRNA expression. Again removing reverse transcriptase failed to generate PCR products as shown by RT (-) in Figure 2.

DISCUSSION

Accumulating evidence suggest a potential tubular synthesis of ET-1, and the present study was designed to detect baseline ET-1 mRNA expression in microdissected renal tubule segments. Although a whole kidney has been found to express ET-1 mRNA in porcine and human (12) as well as rat (10, 13), a study to observe an exact localization of ET-1 mRNA within the kidney has been hampered by an inherent heterogeneous structure of the nephron. It seems very difficult to obtain sufficient amounts of each tubule segments for RNA blot analysis. We, therefore, utilized RT-PCR method to circumvent this problem.

RT-PCR was first applied to a detection of aldose reductase mRNA in microdissected renal tubules by Garcia-Perez and associates (14). Subsequently her laboratory demonstrated tubular localization of atrial natriuretic factor receptor mRNA using RT-PCR (15). Our method is essentially the same as theirs. An important approach of the present study was to design

specific primers for rat ET-1 mRNA. Since no genomic map for rat ET-1 gene was available when this study was initiated, we referred a human ET-1 genomic map for assuming exon/intron positioning in rat ET-1 gene. Our approach turned out to be valid because the predicted PCR products disappeared in the absence of reverse transcriptase (Fig. 1). Our data suggest that 532 bp PCR product is a reflection of an existence of ET-1 mRNA. We did not vigorously compare PCR signals among tubules because the measure for quantitation of the mRNA expression through RT-PCR was yet to be established.

Several unwanted DNAs besides predominant RT-PCR products for ET-1 mRNA were sometimes amplified (data not shown). We, however, concluded that they were not crucial to our purpose because these irrelevant DNA bands never became visible after Southern blot hybridization. Put it in another way, it is indispensable to perform Southern blot analysis to verify the identification of RT-PCR products. A combination of our RT-PCR method and an approach to deduce exon/intron mapping of the target gene in comparison with its counterpart of other species warrants a wide application for detection of the mRNA expression of a certain gene using microdissected tubule segments.

Of special note is a similarity between ET-1 mRNA expression sites revealed in the present study and ET-1 binding sites along the nephron. We recently demonstrated a profile of ET-1 binding capacity along rat nephron segments (9). Thus predominant ET-1 bindings along the tubules were seen in IMCD>OMCD=CCD with a little binding in other tubule segments. The data were compatible with our previous findings that ET-1 evokes a rise in cell Ca^{2+} only in CCD, OMCD and IMCD of mouse kidney (4). Accordingly the sites for ET-1 synthesis agree quite well with the sites for ET-1 binding and action. However, another laboratory independently reported microlocalization of ET-1 mRNA along rat nephron, showing its existence only in IMCD and glomeruli but not in CCD or OMCD (16). The discrepancy between their result and the present study may lie on the somewhat different method utilized. We believe, however, that it is more reasonable that CCD and OMCD are also able to synthesize ET-1 in the light of its autocrine and paracrine mode of action in many differing tissues (12, 17).

Our present results clearly demonstrated the evidence for ET-1 synthesis along the renal collecting ducts. Thus it is suggested that ET-1 is synthesized in and secreted from renal collecting ducts and in turn binds to neighboring collecting ducts to exert its biological effects, which illustrates an autocrine and/or paracrine role of ET-1 also in renal tubules. Regulation of ET-1 mRNA expression in the collecting ducts and physiological function of ET-1 at the same tubules need further investigation.

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