Endothelin-1 mRNA expression in the rat kidney

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Cultured pig and bovine endothelial cells are capable of synthesizing endothelin-1 (ET-1). Thus the observation that the kidney contains a large number of binding sites for ET distributed in close proximity to endothelial cells suggests that ET-1 may be released from the endothelium to act locally on these receptors. In support of this hypothesis, using the technique of reverse transcription with specific amplification of cDNA, we report here that ET-1 mRNA is expressed in the rat kidney. The partial sequence of the amplified rat ET-1 cDNA confirms that the mature rat peptide is identical to that of the mouse, man and pig, but with some differences in codon usage.

INTRODUCTION

Endothelin-1 (ET-1) is a 21-amino-acid vasoconstrictor peptide which was first isolated from cultured pig aortic endothelial cell supernatants [1], and subsequently from conditioned media obtained from bovine endothelial cells [2]. Two other endothelins, ET-2 and ET-3, have now been described from analysis of human and rat genomic DNA [3,4]. A very similar peptide, vasoactive intestinal contractor (VIC or ET-4), isolated from mouse intestine, has been characterized by genomic DNA sequencing [5]. In pig and man, ET-1 is translated from a 2.3 kb mRNA [1], whereas mouse VIC is derived from a 1.4 kb transcript [5]. Transcripts for ET-3 may be 3.7 kb long in the rat [6] or 2.5 kb in length in man [7]. We have shown previously that binding sites and mRNA for ET-1 are widely distributed in human and pig tissues [8–10], and in particular, ET-1 transcription was localized to interlobular and arcuate renal arteries by macro-autoradiographical hybridization *in situ*. Using Northern analysis, McCumber and colleagues have reported that the rat kidney does not contain ET-1 mRNA, but only transcripts coding for ET-3 [6]. These results are paradoxical since: (i) a large amount of immunoreactive ET-1 peptide is present in the kidney [11]; (ii) in culture, rat mesangial cells [12] and the pig LLC-PK1 cell line [13] release ET-1 into the medium and express ET-1 mRNA; and (iii) our own *in situ* hybridization studies localized ET-1 transcripts within the rat kidney [10]. However, in the absence of positive identification of ET-1 mRNA in the intact rat kidney, these data do not prove conclusively that ET-1 is synthesized locally in this organ, since: (i) the identity of the renal immunoreactive peptide was not established unequivocally by sequencing; (ii) phenotypic alteration may have occurred during cell culture; and (iii) the oligomer probe used in the *in situ* hybridization studies may have detected closely related molecules, but not ET-1 mRNA itself. In this study we have examined whether the rat kidney contains ET-1 mRNA using the PCR technique [14] preceded by reverse transcription. Confirmation of the specificity of amplification was provided by (i) hybridization with an oligomer probe complementary to a sequence between the primers, (ii) restriction fragment analysis, and (iii) sequencing of the cDNA products.

MATERIALS AND METHODS

Kidneys from adult male Porton Wistar rats (250–300 g) were rapidly removed and stored at -70°C until used. Total RNA was extracted by a modified guanidine isothiocyanate method [15], and 50 μg was reverse-transcribed with oligo-dT₁₈ as primer, as reported previously [10]. One-twentieth of this cDNA was then amplified using the PCR [10,14] with 3 units of Taq DNA polymerase (Cambio, Cambridge, U.K.) and the supplier's buffer.

The nucleotide sequence of rat ET-1 mRNA was unknown at the time these experiments were being undertaken. Therefore, our strategy for establishing the presence of rat ET-1 cDNA and determining its sequence involved the initial use of a 36-mer forward primer (A in Fig. 1), identical in sequence to pig ET-1 cDNA coding for the first 12 *N*-terminal residues of the mature ET-1 peptide. PCR was performed with this primer and reverse primers (1 μM each), specific for pig and human ET-1 cDNA (Fig. 1, B and C respectively), designed from genomic sequences [16,17] to flank intronic DNA and thus allow cDNA and genomic DNA amplification products to be distinguished. After 40 cycles of amplification (93 $^{\circ}\text{C}$ denaturation for 30 s; 60 $^{\circ}\text{C}$ annealing for 30 s; 72 $^{\circ}\text{C}$ extension for 3 min; final extension at 72 $^{\circ}\text{C}$ for 15 min), the reaction products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The specificity of the amplification products was determined as described previously [10] by (i) Southern analysis using a 21-mer oligonucleotide D (Fig. 1) complementary to the region coding for the seven *C*-terminal residues of ET-1, which was ³²S-3'-end-labelled with terminal transferase [18], and (ii) restriction fragment analysis utilizing the restriction endonucleases *Pvu*II and *Hind*III, which distinguish pig and human ET-1 cDNA [10]. Full confirmation of specificity was obtained by direct sequencing [19] with the Sequenase dideoxy method (U.S. Biochemical Corp.) of two separate pooled samples purified after electrophoresis using glass-milk (GeneClean kit; BIO 101, La Jolla, CA, U.S.A.), as directed by the manufacturer. The sequence of this rat cDNA permitted the synthesis of a reverse anti-sense primer E (Fig. 1) specific for the rat. To prove that it was indeed ET-1 cDNA which was being amplified, a further primer was required to flank the coding sequence of the mature 21-residue peptide at the 5'

Abbreviations used: ET, endothelin; VIC, vasoactive intestinal contractor.

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Fig. 1. Positions of the primers used for PCR

Scheme of the positions of the primers used for the identification of ET-1 mRNA by cDNA amplification. Total rat kidney RNA was reverse-transcribed using oligo-dT as primer. In the first round of PCR (PCR1), forward primer A (TGCTCCTGCTCTTCC-CTGATGGATAAAGAGTGTGTC), directed at the sequence ET-1(1-12), was combined with reverse primers B (pig: GTTATGGGTCACCTT-TCTTATCTCTGTAGAGCTCGGC) or C (human: GGTCACATAACGCTCTCTGGAGGGCTT). After electrophoretic separation, the amplification products were transferred to a nylon membrane and probed with a 3'-³²S-end-labelled oligomer D, which is complementary to the sequence coding for the C-terminal seven residues of mature ET-1 (CCAGATGATGTCCAGGTGGCA) (Southern 1). After hybridization at 37 °C, the membranes were washed with increasing stringency (down to 20 mM-NaCl/0.1 % SDS/14 mM-β-mercaptoethanol). In the second round of PCR (PCR2), a primer E (TCTTTACGCCTTCTGCATGGTA), designed from the DNA sequence of the rat amplification band from PCR1, was used with a primer F (CCATGATTATCGCTCTGCTGTTTGTGGCTT) targeted against a sequence upstream of the coding region for mature ET-1. For Southern analysis of the products from PCR2, primer A was 3'-end-labelled with ³²S.

end; this forward oligodeoxynucleotide F (Fig. 1) was designed from a segment of pig and human ET-1 cDNA showing high sequence identity. The primers E and F were utilized for amplification using 40 cycles at the temperatures described above. After electrophoretic separation, the DNA band visualized by ethidium fluorescence was extracted and purified using glass-milk and sequenced by the Sequenase method.

RESULTS AND DISCUSSION

After the first round of PCR, size fractionation by agarose-gel electrophoresis revealed an amplification band of approximately the same size as that formed from pig kidney cDNA (440 bp). This product was present only when the pig, and not the human, reverse primer was utilized (results not shown), and it hybridized, after stringent washing, with the ³²S-labelled probe D complementary to a sequence occurring between the primers. Furthermore, the rat amplification product was cleaved by the endonuclease PvuII (as in the pig, but unlike in man), but not by HindIII (unlike both pig and man) (results not shown), indicating that it had not arisen by contamination from pig or human material.

A second round of PCR with primers E and F (Fig. 1) yielded an amplification band 400 bp in length, which when sequenced was found to include a region coding for a peptide identical to pig and human ET-1 (Fig. 2). Contamination of rat kidney cDNA from these other sources is excluded by the observation of differing triplet codon usage in the sequences from rat, pig and man. Within the cDNA coding for the mature ET-1 peptide, there are three, four and five base mismatches compared with the mouse [5], pig [1] and human [16,17] sequences respectively.

Our observation of the presence of ET-1 mRNA in the adult

rat kidney supports the finding by Kitamura and colleagues of immunoreactive ET in the kidney, predominantly within the inner medulla [11], and our localization of ET-1 mRNA to certain blood vessels in the pig and rat kidney [10]. Kitamura *et al.* also reported that the adult rat kidney contains the highest concentration of immunoreactive ET of all the tissues examined [11], and that chromatographically, this ET appears to be composed of approximately equimolar amounts of ET-1 and ET-3. All of this data, and the apparent lack of ET-3 mRNA transcription in cultured endothelial cells [7], would suggest that in the rat, as in pig and man, ET-1 is the endothelin synthesized by endothelial cells.

The present results provide some evidence that the transcription of the ET-1 gene observed by Sakamoto *et al.* in cultured rat mesangial cells [12] may not be due to phenotypic alteration induced by cell culture. However, our previous *in situ* hybridization study [10] did not reveal an increased prevalence of ET-1 transcripts in the glomeruli of the rat kidney where mesangial (and endothelial) cells are concentrated. Nevertheless, the ability of cells of renal origin [12,13] and of vascular smooth muscle cells [20] to synthesize ET-1 mRNA and release ET-1 into the culture medium suggests that, in some circumstances, the endothelium may not be the only source of ET-1 in the intact kidney.

In previous studies [6,7] using Northern analysis, ET-1 mRNA was not detected in adult rat and human fetal kidney samples, although ET-3 mRNA was found. These negative results for ET-1 mRNA may be due to the relative insensitivity of Northern analysis when compared with *in situ* hybridization [21] and cDNA amplification with the PCR [14]. However, it is unclear why ET-1 mRNA is difficult to detect in the kidney, despite the apparent abundance of the immunoreactive peptide [11]. One

Fig. 2. Comparison of the DNA sequence coding for rat ET-1 with those of mouse, pig and human ET-1

The sequence of the specific rat 400 bp DNA band from PCR2 (Fig. 1) contains a potential reading frame that codes for a peptide identical to mouse, pig and human ET-1. The base differences within the coding region of ET-1 confirm that the rat kidney amplification product could not have arisen by contamination with material from these other species.

possible explanation is that rapid turnover of these transcripts [22] and low steady-state levels are balanced under physiological conditions by efficient translation and persistence of the peptide. The data available, however, do not exclude the possibility that there is also extensive uptake by renal cells of circulating ET-1 produced elsewhere.

In conclusion, we have proved that ET-1 mRNA is present in the adult rat kidney, suggesting that at least part of the large amount of immunoreactive ET found there is synthesized locally, principally by the endothelium, and perhaps also, in unusual conditions, by mesangial and smooth muscle cells.

J. P. S. is an MRC Training Fellow. V.M.S.O. was a Merck Sharp and Dohm/Royal College of Physicians International Fellow. We thank the British Heart Foundation and the Wellcome Trust for support. D. J. R. N. was supported by a Squibb Cardiovascular Fellowship.

REFERENCES

- Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. & Masaki, T. (1988) *Nature* (London) **332**, 411–415
- Emori, T., Hirata, Y., Ohta, K., Shichiri, M. & Marumo, F. (1989) *Biochem. Biophys. Res. Commun.* **160**, 93–100
- Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K. & Masaki, T. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2863–2867
- Yanagisawa, M., Inoue, A., Ishikawa, T., Kasuya, Y., Kimura, S., Kumagaya, S.-I., Nakayama, K., Watanabe, T., Sakakibara, S., Goto, K. & Masaki, T. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6964–6967
- Saida, K., Mitsui, Y. & Ishida, N. (1989) *J. Biol. Chem.* **264**, 14613–14616
- MacCumber, M. W., Ross, C. A., Glaser, B. M. & Snyder, S. H. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7285–7289
- Bloch, K. D., Eddy, R. L., Shows, T. B. & Quertermous, T. (1989) *J. Biol. Chem.* **264**, 18156–18161
- Davenport, A. P., Nunez, D. J., Hall, J. A., Kaumann, A. J. & Brown, M. J. (1988) *J. Cardiovasc. Pharmacol.* **13** (Suppl. 5), S166–S170
- Davenport, A. P., Nunez, D. J. & Brown, M. J. (1989) *Clin. Sci.* **77**, 129–131
- Nunez, D. J. R., Brown, M. J., Davenport, A. P., Neylon, C. B., Schofield, J. P. S. & Wyse, R. K. (1990) *J. Clin. Invest.* **85**, 1537–1541
- Kitamura, K., Tanaka, T., Kato, J., Ogawa, T., Eto, T. & Tanaka, K. (1989) *Biochem. Biophys. Res. Commun.* **162**, 38–44
- Sakamoto, H., Sasaki, S., Hirata, Y., Imai, T., Ando, K., Ida, T., Sakurai, T., Yanagisawa, M., Masaki, T. & Marumo, F. (1990) *Biochem. Biophys. Res. Commun.* **169**, 462–468
- Ohta, K., Hirata, Y., Imai, T., Kanno, K., Emori, T., Shichiri, M. & Marumo, F. (1990) *Biochem. Biophys. Res. Commun.* **169**, 578–584
- Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239**, 487–491
- Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Itoh, Y., Yanagisawa, M., Ohkubo, S., Kimura, C., Kosaka, T., Inoue, A., Ishida, N., Mitsui, Y., Onda, H., Fujino, M. & Masaki, T. (1988) *FEBS Lett.* **231**, 440–444
- Bloch, K. D., Friedrich, S. P., Lee, M.-E., Eddy, R. L., Shows, T. B. & Quertermous, T. (1989) *J. Biol. Chem.* **264**, 10851–10857
- Emson, P. C., Nunez, D. J. & Davenport, A. P. (1990) in *Peptide Hormone Secretion: A Practical Approach* (Hutton, J. C. & Siddle, K., eds.), pp. 159–187, IRL Press/Oxford University Press
- Winship, P. R. (1989) *Nucleic Acids Res.* **17**, 1266
- Resink, T. J., Hahn, A. W. A., Scott-Burden, T., Powell, J., Weber, E. & Buhler, F. R. (1990) *Biochem. Biophys. Res. Commun.* **168**, 1303–1310
- Nunez, D. J. R., Davenport, A. P., Emson, P. C. & Brown, M. J. (1989) *Biochem. J.* **263**, 121–127
- Yanagisawa, M., Inoue, A., Takawa, Y., Mitsui, Y., Kobayashi, M. & Masaki, T. (1988) *J. Cardiovasc. Pharmacol.* **13** (Suppl. 5), S13–S17

Received 7 January 1991/26 February 1991; accepted 4 March 1991