A comparison of the zinc contents and substrate specificities of the endothelial and testicular forms of porcine angiotensin converting enzyme and the preparation of isoenzyme-specific antisera

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Angiotensin converting enzyme (ACE; EC 3.4.15.1) was purified from porcine kidney and lung (endothelial isoenzyme) and testis (testicular isoenzyme) by affinity chromatography on lisinopril–2.8 nm–Sephacrose. Atomic-absorption spectroscopy revealed that ACE purified from kidney and lung contained 2.58 and 2.35 atoms of zinc per molecule of enzyme (M_r 147 000) respectively. In contrast, ACE purified from testis contained only 1.58 atoms of zinc per molecule of enzyme (M_r 80 000). Thus it would appear that both putative zinc-binding sites in endothelial ACE contain zinc and may therefore be catalytically active. No differences were observed in the pattern of products generated on hydrolysis of benzoyl (Bz)-Gly-His-Leu, substance P, luteinizing-hormone-releasing hormone (LH-RH) and its analogue, des-Gly¹⁰-LH-RH-ethylamide, by kidney and testicular ACE. There was also no difference in the initial rates of hydrolysis of Bz-Gly-His-Leu or substance P by the two isoenzymes, although LH-RH and its analogue were hydrolysed twice as rapidly by kidney ACE. It is therefore unlikely that the *N*-terminal catalytic site in porcine endothelial ACE is predominantly responsible for the atypical cleavage of LH-RH generating the *N*-terminal tripeptide. Two polyclonal antisera were raised to the affinity-purified forms of pig kidney and testicular ACE. Isoenzyme-specific antisera were then isolated from these by absorbing out those antibodies recognizing determinants on the other isoenzyme. Immunoelectrophoretic blot analyses and immunofluorescent staining of sections of pig kidney were used to demonstrate the specificity of the antisera. Immunofluorescent staining of sections of pig testis with the antiserum specific to testicular ACE localized testicular ACE solely to the lumen of the seminiferous tubules, whereas the antiserum specific to endothelial ACE revealed the presence of this isoenzyme only in blood vessels. The antiserum to endothelial ACE, which recognizes determinants in the unique *N*-terminal domain, was investigated as a possible specific inhibitor of the *N*-terminal catalytic site. Although this antiserum failed to inhibit testicular ACE, the effect on the activity of endothelial ACE appeared to be due to inhibition of both the *N*- and *C*-terminal catalytic sites.

INTRODUCTION

Angiotensin converting enzyme (ACE; peptidyl dipeptidase A, EC 3.4.15.1) is a zinc metallopeptidase that displays a broad distribution in mammalian tissues. The classical physiological substrates of ACE are angiotensin I, which is activated to the potent vasopressor peptide angiotensin II, and the vasodilatory peptide bradykinin, which is inactivated (Soffer, 1976). As a consequence, ACE has become a crucial enzymic target in the treatment of hypertension and congestive heart failure, leading to the development of selective inhibitors, e.g. captopril and enalaprilat, to control these conditions (Cushman & Ondetti, 1980; Abrams *et al.*, 1984; Gavras & Gavras, 1988; Mancina, 1991). ACE has been purified from a number of different mammalian tissues and appears to display some degree of heterogeneity with respect to molecular size. However, there are only two distinct isoenzymes. The larger form (M_r 140 000–180 000, depending on species) is present throughout the body on both endothelial and epithelial cell surfaces, with the highest concentrations found in lung and kidney [for reviews, see Patchett & Cordes (1985), Ehlers & Riordan (1989) and Hooper (1991)]. This form of ACE is referred to here as ‘endothelial ACE’. A distinct and smaller isoenzyme of ACE (M_r 90 000–110 000, depending on species) is found exclusively in the testes (El-Dorri *et al.*, 1982; Lanzillo *et al.*, 1985) and is referred to here as ‘testicular ACE’.

The cDNAs for both endothelial (Soubrier *et al.*, 1988; Bernstein *et al.*, 1989) and testicular (Ehlers *et al.*, 1989; Kumar *et al.*, 1989; Lattion *et al.*, 1989) ACE have now been sequenced and their corresponding amino acid sequences deduced. Endothelial ACE is composed of two highly similar domains, referred to as the *N*- and *C*-terminal domains, each bearing a putative zinc-binding motif (His-Glu-Met-Gly-His), implying the presence of two catalytic sites. In contrast, the cDNA-deduced amino acid sequence of testicular ACE, with the exception of 67 amino acids at the *N*-terminus, is identical with the *C*-terminal domain of endothelial ACE and contains only a single zinc-binding motif. For both putative catalytic sites in endothelial ACE to be active, an atom of zinc would need to be bound at each zinc-binding motif. Earlier experiments, though, predicted a single atom of zinc to be bound per molecule of endothelial ACE (Das & Soffer, 1975; Bunning *et al.*, 1983; Bunning & Riordan, 1985), thus implying that only one of the two putative catalytic sites is active. However, the construction of mutants of endothelial ACE containing only one intact zinc-binding site demonstrated that both domains were catalytically active, each able to cleave the *C*-terminal dipeptide from benzoyl (Bz)-Gly-His-Leu and angiotensin I (Wei *et al.*, 1991). One of the groups which performed some of the original zinc analyses has recently re-analysed the zinc content of ACE and obtained values of approx. 2 and 1 atoms of zinc per molecule of enzyme for the

Abbreviations used: ACE, angiotensin converting enzyme; FITC, fluorescein isothiocyanate; LH-RH, luteinizing-hormone-releasing hormone (luliberin); Bz, benzoyl.

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endothelial and testicular isoenzymes respectively (Ehlers & Riordan, 1991).

In addition to its classical specificity to act as a peptidyl dipeptidase, ACE can act *in vitro* as an endopeptidase releasing C-terminal tripeptides and or N-terminal tripeptides from certain terminally blocked peptides, e.g. substance P and luteinizing-hormone-releasing hormone (LH-RH) (Yokosawa *et al.*, 1983; Skidgel & Erdös, 1985). It is conceivable that the N- and C-terminal catalytic sites of endothelial ACE may be involved differently in these atypical cleavages. In support of this, Ehlers & Riordan (1991) have reported that, of a range of peptides examined, only LH-RH and its analogue, des-Gly¹⁰-LH-RH-ethylamide, were hydrolysed at significantly higher rates by endothelial ACE compared with testicular ACE, and that the patterns of product peaks produced were different for the two isoenzymes. These observations suggest that the two catalytic sites may have different substrate specificities.

ACE has been mapped in most tissues by immunohistochemistry employing either monoclonal or polyclonal antibodies by both optical and electron microscopy (e.g. Defendini *et al.*, 1983; Danilov *et al.*, 1987; Barnes *et al.*, 1988; Schulz *et al.*, 1988). In addition, radiolabelled inhibitors (e.g. [³H]captopril, [²⁵I]MK351A) have also been used to localize ACE by autoradiography (e.g. Mendelsohn, 1984; Strittmatter & Snyder, 1984). However, these approaches have limitations when attempting to define the distribution of testicular ACE, owing to the immunological and catalytic similarities between the two isoenzymes.

In the present study we have analysed the zinc content of porcine endothelial and testicular ACE and compared the patterns and initial rates of hydrolysis by the two isoenzymes of certain substrates which are cleaved atypically. Furthermore, we have prepared antibodies specific for each isoenzyme from polyclonal antisera generated to each isoenzyme by absorbing out the cross-reacting antibodies on a column to which the other isoenzyme had been immobilized. These isoenzyme-specific antisera have then been employed to map the two forms by immunohistochemistry. In addition, we have explored the possibility of one of these antisera acting as a selective inhibitor of the N-terminal catalytic site of endothelial ACE.

EXPERIMENTAL

Materials

Pig kidneys, lungs and testes were kindly provided by ASDA Farmstores, Lofthousegate, W. Yorkshire, U.K., and were obtained within 10 min of death. Testes were dissected from the epididymis, surrounding capsule and other associated tissues and stored at -70 °C until required. Kidney cortex was dissected out and stored similarly. Adult male New Zealand White rabbits (2-3 kg) were used to raise polyclonal antibodies and were provided and maintained by the University of Leeds Animal Service. LH-RH, des-Gly¹⁰-LH-RH-ethylamide and substance P were purchased from BACHEM (U.K.) Ltd., Saffron Walden, Essex, U.K., and were > 99% pure as determined by h.p.l.c. analysis. Bz-Gly-His-Leu and the donkey anti-rabbit IgG [fluorescein isothiocyanate (FITC)-conjugated] were from Sigma Chemical Co. Lisinopril {MK521, N-[(S)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline} and enalaprilat {MK422, N-[(S)-1-carboxy-3-phenylpropyl]-L-alanyl-L-proline} were gifts from Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A. All other materials were from sources previously noted.

Purification of ACE

ACE was purified from pig kidney, lung and testis by a modification of the method described by Hooper *et al.* (1987),

using lisinopril-2.8 nm-Sepharose as the affinity resin. The major difference was that bound enzyme was eluted with 50 mM-Na₂B₄O₇/0.1% (w/v) Triton X-100, pH 9.5 (Ehlers *et al.*, 1986) instead of with free lisinopril.

Zinc analyses

All buffers were made up with deionized water. Dialysis tubing was boiled in the presence of EDTA and then washed extensively with deionized water. All glassware was acid-washed in 6 M-HCl and rinsed thoroughly with deionized water before use. Enzyme samples (0.2 mg of protein/ml) were dialysed against three changes of 5 mM-Tris/HCl/0.1% (w/v) Triton X-100, pH 7.9 at 4 °C to remove any non-specifically bound zinc. Protein was determined by the method of Bensadoun & Weinstein (1976) and by using bicinchoninic acid in a microtitre plate assay; both methods gave results which did not differ by more than 10%. Duplicate 0.4 ml aliquots of each sample were analysed on an Instrumental Laboratories IL 157 atomic-absorption spectrometer (Thermo Electron Ltd., Birchwood, Warrington, Cheshire, U.K.). ZnSO₄ (0-6 µM) was used as standard.

Hydrolysis of peptides

Equimolar amounts (1.12 pmol) of affinity-purified pig kidney ACE (*M_r* 180000) or pig testicular ACE (*M_r* 110000) were incubated with LH-RH (0.1 mM) or des-Gly¹⁰-LH-RH-ethylamide (0.1 mM) in 50 mM-Hepes/0.3 M-NaCl, pH 7.5, for 18 h at 37 °C. With substance P (0.1 mM), equimolar amounts (0.56 pmol) of kidney or testicular ACE were incubated in 50 mM-Hepes/0.3 M-NaCl, pH 7.5, for 90 min at 37 °C. Routine assays with Bz-Gly-His-Leu (5 mM) were carried out in 0.1 M-Tris/HCl/0.3 M-NaCl/10 µM-ZnCl₂, pH 8.3. Reactions were terminated by heating at 100 °C for 4 min, and the substrate and reaction products resolved and quantified by reverse phase h.p.l.c. as described in Hooper & Turner (1987).

Production and purification of antibodies

Antibodies were raised against purified kidney and testicular ACE in New Zealand White rabbits. An IgG fraction was prepared from the serum by affinity chromatography on a column of Protein A-Sepharose. Antibodies cross-reacting with the other isoenzyme were removed from the IgG fraction by affinity chromatography on a column of the appropriate isoenzyme immobilized on CNBr-activated Sepharose as described in Matsas *et al.* (1986) for endopeptidase-24.11. RP127, which was raised to pig kidney ACE, was passed down a column of immobilized pig testicular ACE. The population of antibodies that did not bind to the immobilized testicular ACE should therefore be specific for kidney ACE and are referred to as RP127_K (see Table 1). Likewise, RP175, which was raised to pig testicular ACE, was passed down a column of immobilized pig kidney ACE. The population of antibodies that did not bind to the immobilized kidney ACE should therefore be specific for testicular ACE and are referred to as RP175_T (see Table 1).

Immunoelectrophoretic-blot analysis

SDS/PAGE was performed by the system of Laemmli (1970), with a 7-17% (w/v) polyacrylamide gradient as described previously (Relton *et al.*, 1983). Immunoelectrophoretic-blot analysis was performed as described by Hooper & Turner (1987), except that poly(vinylidene difluoride) transfer membranes were used together with a ¹²⁵I-labelled anti-rabbit IgG second antibody as described by Hooper *et al.* (1990).

Immunohistochemistry

Specimens of kidney cortex from 2-4-week-old piglets and testis from adult pigs were obtained immediately after death and

Table 1. Properties of the antibodies used in the present study

See the Experimental section for details.

Antibody	Antigen	Immobilized ligand used to purify antibody	Cross-reactivity on immunoelectrophoretic-blot analysis	
			Kidney ACE	Testicular ACE
RP127	Kidney ACE	—	Yes	Yes
RP127 _K	Kidney ACE	Testicular ACE	Yes	No
RP175	Testicular ACE	—	Yes	Yes
RP175 _T	Testicular ACE	Kidney ACE	No	Yes

blocks frozen and stored as described by Matsas *et al.* (1986). Cryostat sections (8 μ m) were cut, and immunofluorescent staining was performed by the method of Gee *et al.* (1983). Primary antibodies (approx. 2 μ g of IgG protein/ml final concentration) were applied for 2 h at 22 °C, followed by a second antibody, donkey anti-rabbit IgG (FITC-conjugated) (dilution 1 in 50). All antibodies were diluted in 0.2% (w/v) gelatin in 50 mM-Tris/HCl/0.15 M-NaCl, pH 7.4, containing a pig IgG fraction (1 mg/ml). Controls were prepared by replacing the first antibody with either 0.2% (w/v) gelatin in 50 mM-Tris/HCl/0.15 M-NaCl, pH 7.4, or a rabbit preimmune IgG. In both cases no specific immunofluorescence was observed.

Inhibitor studies using anti-ACE antibodies

Equivalent activities of affinity purified kidney or testicular ACE (8.3 nmol of Bz-Gly produced/min) were incubated in the absence and presence of RP127_K in 0.1 M-Tris/HCl/0.3 M-NaCl/10 μ M-ZnCl₂, pH 8.3, for 72 h at 4 °C. Control incubations replaced RP127_K with equivalent concentrations of a rabbit preimmune IgG. Aliquots (90 μ l) of the enzyme/antibody mixture were incubated with Bz-Gly-His-Leu (5 mM) for 45 min at 37 °C or with LH-RH (0.1 mM) for 18 h at 37 °C. After terminating the reaction by heating at 100 °C for 4 min, the substrate and reaction products were resolved and quantified by reverse-phase h.p.l.c. as described by Hooper & Turner (1987).

RESULTS AND DISCUSSION

Purification and zinc analyses of endothelial and testicular ACE

ACE from pig kidney, lung and testes was purified by affinity chromatography on lisinopril–2.8 nm–Sephacryl to apparent homogeneity as determined by SDS/PAGE (results not shown). ACE purified from kidney or lung migrated as a single polypeptide band with an apparent M_r of 180000. In contrast, ACE purified from testis migrated as a single polypeptide with an apparent M_r of 110000. The zinc content of endothelial ACE was determined by atomic-absorption spectroscopy and compared with that of the testicular enzyme (Table 2). ACE purified from pig kidney or lung had a similar zinc content of 2.58 and 2.35 atoms of zinc per molecule of enzyme respectively. In contrast, analysis of testicular ACE revealed a lower zinc content compared with the kidney or lung enzymes of 1.58 atoms of zinc per molecule of enzyme. Membrane dipeptidase, another zinc-metalloprotein, contained 0.92 atom of zinc per molecule of enzyme subunit, in agreement with previous results (Armstrong *et al.*, 1974). Thus these results indicate the presence of one atom of zinc at each of the two putative zinc-binding sites predicted

from the cDNA sequence of endothelial ACE, in agreement with the more recent results of Ehlers & Riordan (1991). The slightly higher values observed in the present study may be caused by using M_r values predicted from the human cDNA-derived sequences.

Hydrolysis of peptides by endothelial and testicular ACE

ACE acts as an endopeptidase on, among other substrates, substance P and LH-RH. Substance P is hydrolysed by ACE by one of two pathways: either the C-terminal dipeptide amide is removed, followed by the subsequent release of dipeptides from the newly formed C-terminus, or the C-terminal tripeptide amide is removed initially, with subsequent dipeptides released (Yokosawa *et al.*, 1983). In the case of LH-RH, ACE releases tripeptide fragments from both the N- and C-termini [LH-RH-(1–3)-peptide; pGlu-His-Trp and LH-RH-(8–10)-peptide; Arg-Pro-GlyNH₂] (Skidgel & Erdös, 1985). At high chloride-ion concentrations, however, release of the N-terminal tripeptide predominates producing LH-RH-(1–3)-peptide and LH-RH-(4–10)-peptide. We therefore investigated the hydrolysis of substance P, LH-RH and the LH-RH analogue des-Gly¹⁰-LH-RH-ethylamide by endothelial and testicular ACE in order to examine whether these atypical cleavages can be attributed to one or other of the catalytic sites in endothelial ACE. Equimolar amounts of affinity purified porcine kidney or testicular ACE were incubated with various peptide substrates (Table 3). Bz-Gly-His-Leu and substance P were hydrolysed at comparable initial rates by both kidney and testicular ACE. However, LH-RH and des-Gly¹⁰-LH-RH-ethylamide were hydrolysed more rapidly (2.3- and 1.9-fold respectively) by kidney ACE than by testicular ACE. The patterns of hydrolysis of substance P and des-Gly¹⁰-LH-RH-ethylamide by kidney and testicular ACE were identical (results not shown). With LH-RH the ratio of the peak areas, the N-terminal tripeptide LH-RH-(1–3)-peptide and the C-terminal heptapeptide LH-RH-(4–10)-peptide, were identical with both kidney and testicular ACE (Fig. 1). These results

Table 2. Zinc analyses of ACE purified from pig kidney, lung and testes

M_r values are based on the amino acid sequences deduced from the corresponding cDNAs for human endothelial ACE, human testicular ACE and pig membrane dipeptidase. The zinc contents are quoted as the ratio of atoms of zinc/molecule of enzyme (of M_r described above). Zinc analyses were performed and specific activities were determined using Bz-Gly-His-Leu as substrate as described in the Experimental section. Duplicate analyses were performed on three separate enzyme samples resulting from different purifications from each tissue. Results are means for three samples ($\pm$ S.E.M.), except for membrane dipeptidase, which is quoted as the mean from two separate enzyme samples which did not differ by more than 11.5%.

Enzyme	10 ⁻³ × M_r	Specific activity		Atoms of zinc: molecule of enzyme
		(μ mol of Bz-Gly/min per mg)	(μ mol of Bz-Gly/min per μ mol of protein)	
Kidney ACE	146.6*	6.20	1.05	2.58 $\pm$ 0.18:1
Lung ACE	146.6*	6.90	1.17	2.35 $\pm$ 0.23:1
Testicular ACE	80†	11.00	1.21	1.58 $\pm$ 0.10:1
Membrane dipeptidase	44.7‡	—	—	0.92:1

* Data from Soubrier *et al.* (1988).

† Data from Ehlers *et al.* (1989).

‡ Data from Rached *et al.* (1990).

Table 3. Hydrolysis of substrates by pig kidney and testicular ACE

Equimolar amounts of kidney or testicular ACE were incubated with the indicated substrate at 37 °C in 50 mM-Hepes/0.3 M-NaCl, pH 7.5, and the substrate and reaction products resolved and quantified by h.p.l.c. as described in the Experimental section. The extent of Bz-Gly-His-Leu hydrolysis was calculated by the amount of Bz-Gly produced. The extent of hydrolysis of the other peptides was calculated by comparison of the peak area of remaining substrate with the peak area of the unchanged substrate. Results are means ($\pm$ S.E.M.) for four separate determinations. The inclusion of 10 μ M-enalaprilat in control incubations completely inhibited the hydrolysis of all substrates by both kidney and testicular ACE.

Substrate	Extent of hydrolysis (%)		Ratio (kidney/ testicular)
	Kidney ACE	Testicular ACE	
Bz-Gly-His-Leu	8.6 $\pm$ 0.2	8.0 $\pm$ 0.2	1.1
Substance P	22.3 $\pm$ 0.3	18.2 $\pm$ 0.5	1.2
LH-RH	14.4 $\pm$ 1.1	6.2 $\pm$ 0.4	2.3
Des-Gly ¹⁰ -LH-RH-ethylamide	9.1 $\pm$ 0.7	4.7 $\pm$ 0.9	1.9

suggest that the *N*-terminal catalytic site in endothelial ACE may play a greater role than the *C*-terminal site in the cleavage of the Trp³-Ser⁴ bond of LH-RH, but that there are no qualitative differences in specificity between the two sites, at least in regards to the peptides examined here. This is in contrast with the recent report indicating that LH-RH and des-Gly¹⁰-LH-RH-ethylamide are hydrolysed > 300-fold faster by human endothelial ACE than by the testicular isoenzyme, and that the pattern of hydrolysis of LH-RH was different for the two isoenzymes (Ehlers & Riordan, 1991). The reasons for the apparent differences in the rate and pattern of hydrolysis of LH-RH by the two isoenzymes of ACE between these two studies are unclear, but may reflect species differences. However, from the data of Ehlers & Riordan (1991) it is not clear whether the initial rates of hydrolysis were being compared, as in the present study.

Preparation of antibodies specific to either endothelial or testicular ACE

A polyclonal antibody raised to pig kidney ACE (RP127) was used in immunoelectrophoretic blot analysis of affinity-purified kidney and testicular ACE (Fig. 2, lanes 1 and 2). RP127 recognized kidney ACE, *M_r* 180000, and the lower-*M_r* 110000 testicular isoenzyme, owing to their common determinants, in agreement with previous results (El-Dorry *et al.*, 1982). Antibodies specific to kidney ACE were prepared by absorption of the antibody RP127 on a column of testicular-ACE-Sepharose to remove cross-reacting antibodies. The population of antibodies recovered in the unbound fraction were termed RP127_k (see Table 1) and were then used in immunoelectrophoretic blot analysis of affinity-purified kidney and testicular ACE (Fig. 2, lanes 3 and 4). RP127_k recognized only kidney ACE and not testicular ACE. A polyclonal antibody (RP175) was also raised to purified testicular ACE. Affinity-purified testicular and kidney ACE were then subjected to immunoelectrophoretic blot analysis using RP175 (Fig. 2, lanes 5 and 6). RP175 recognized the *M_r*-110000 form of testicular ACE and cross-reacted to a lesser extent with the higher-*M_r* 180000 form of kidney ACE. Cross-reacting antibodies were removed from the antibody RP175 by absorption on a column of kidney-ACE-Sepharose. The population of antibodies recovered in the unbound fraction were termed RP175_t (see Table 1). Immunoelectrophoretic blot analysis of affinity-purified testicular and kidney ACE (Fig. 2, lanes 7 and 8) showed that RP175_t recognized only testicular ACE and

Fig. 1. Pattern of hydrolysis of LH-RH by pig kidney and testicular ACE

Equimolar amounts of kidney or testicular ACE were incubated with LH-RH (0.1 mM) for 18 h at 37 °C as described in the Experimental section. The substrate and reaction products were resolved by reverse-phase h.p.l.c. and monitored at 214 nm. In the presence of 10 μ M-enalaprilat, no hydrolysis by either isoenzyme was observed. (a) Kidney ACE; (b) testicular ACE. Peak 1, LH-RH-(4-10)-peptide (Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂); peak 2, LH-RH-(1-3)-peptide (pGlu-His-Trp).

not kidney ACE. Thus these results are consistent with RP175_t recognizing determinants present on the unique *N*-terminal sequence of testicular ACE and with RP127_k recognizing determinants on the unique *N*-terminal domain of endothelial ACE (see Fig. 3).

Immunofluorescent staining of sections of pig kidney

The antibody preparations were then used for immunohistochemical studies on sections of pig kidney. The localization of ACE in sections of pig kidney was viewed by immunofluorescence microscopy (Fig. 4). Both RP127 and RP127_k recognize determinants located on kidney ACE (see above). Accordingly, the pattern of immunofluorescent staining of kidney sections produced by these two antibody preparations was very similar (Figs. 4a and 4b respectively). Intense staining of glomeruli and convoluted tubules was evident, in agreement with previous

Fig. 2. Immunoelectrophoretic blot with anti-(kidney ACE) and anti-(testicular ACE) antibodies

Samples of affinity-purified pig kidney ACE (lanes 1, 3, 5 and 7; 1.0 μ g of protein) or testicular ACE (lanes 2, 4, 6 and 8; 1.0 μ g of protein) were prepared and analysed as described in the Experimental section. After electrophoretic transfer to poly(vinylidene difluoride) membranes, the tracks were blotted with either RP127 (lanes 1 and 2), RP127_k (lanes 3 and 4), RP175 (lanes 5 and 6) or RP175_T (lanes 7 and 8). Bound antiserum was detected with a 125 I-labelled second antibody, followed by autoradiography.

Fig. 3. Schematic comparison of testicular and endothelial ACE showing the putative sites of binding of the antibodies used in the present study

The structure of testicular ACE is shown at the top of the Figure and that of endothelial ACE at the bottom. T, testicular; E, endothelial. The amino acids at the junction of the domains are numbered as for the human isoenzymes (Soubrier *et al.*, 1988; Lattion *et al.*, 1989). Domains T2 and E2 have 100% identity. T1 and E1 are unique to the respective isoenzymes. The zinc-binding sites are represented by HEMGH.

reports (e.g. Defendini *et al.*, 1983). The antibody raised to pig testicular ACE (RP175) produced a pattern of fluorescent staining (Fig. 4c) that was similar to that produced by RP127 and RP127_k. This is in agreement with the immunoelectrophoretic-blot analysis (Fig. 2), which showed that RP175 cross-reacted with both testicular ACE (M_r 110000) and kidney ACE (M_r 180000). In contrast, RP175_T, which recognizes only specific determinants on testicular ACE, failed to produce fluorescent staining of sections of pig kidney (Fig. 4d), consistent with the absence of this isoenzyme in this tissue.

Immunofluorescent staining of sections of pig testis

ACE has been localized in the male reproductive tract both by immunohistochemistry using antibodies to endothelial ACE (Yotsumato *et al.*, 1984; Berg *et al.*, 1986; Brentjens *et al.*, 1986) and by autoradiography using [3 H]captopril (Strittmatter &

Fig. 4. Comparison of anti-(kidney ACE) and anti-(testicular ACE) antibodies using immunofluorescence staining of pig kidney

Sections of pig kidney cortex were prepared and stained as described in the Experimental section. Immunostaining was with (a) RP127, (b) RP127_k, (c) RP175 and (d) RP175_T. Intense fluorescence of the glomeruli (indicated by single arrows) and of the brush border of convoluted tubules (indicated by double arrows) are seen in micrographs (a), (b) and (c), whereas in (d) immunofluorescence is absent. The bar represents 50 μ m.

Snyder, 1984; Strittmatter *et al.*, 1985). The distribution of the two isoenzymes has been defined indirectly by comparing the pattern of staining either with [3 H]captopril at different stages of development and after hypophysectomy (Strittmatter *et al.*, 1985) or after preincubation of an anti-(endothelial ACE) antibody with either endothelial or testicular ACE (Berg *et al.*, 1986). These studies have demonstrated that testicular ACE is present in the seminiferous tubules of the testis but not in the interstitial Leydig cells, and that endothelial ACE is present on the luminal surface of blood vessels. In the present study we have directly compared the localization of the endothelial and testicular forms of ACE using our isoenzyme-specific antibodies. Sexually mature pigs (12 weeks old) were used for this study, since it is established that the testicular form of ACE is developmentally controlled, being expressed at the onset of puberty (Velletri, 1985). Figs. 5 and 6 show phase-contrast and fluorescence micrographs of sections of pig testis with the different antibodies. The use of RP127 as the primary antibody produced an intense fluorescence of seminiferous tubules and blood vessels (Fig. 5b). This is in marked contrast with the pattern of immunofluorescence produced using the anti-(endothelial ACE) antibody, RP127_k (Fig. 5d). In this instance the blood vessels were intensely fluorescent, whereas negligible fluorescence was observed in the seminiferous tubules (Fig. 5d). The polyclonal antibody raised to pig testicular

Fig. 5. Phase-contrast and immunofluorescence microscopy of pig testis immunostained with antibodies to kidney ACE

Sections of pig testis were prepared and stained as described in the Experimental section. (a) and (b) are phase-contrast and fluorescence micrographs respectively of the same field immunostained with RP127. (c) and (d) are phase-contrast and fluorescence micrographs respectively of the same field immunostained with RP127_K. Irrespective of the primary antibody used, the blood vessels were immunopositive (thin arrows), whereas the intense fluorescence seen towards the centre of seminiferous tubules was observed only when RP127 was applied (compare thick arrows, micrographs b and d). Leydig cells (indicated by curved arrows) were immunonegative. The bar represents 100 μ m.

ACE (RP175) produced fluorescence of seminiferous tubules and blood vessels (Fig. 6b), a pattern similar to that produced by RP127 (Fig. 5b). With the anti-(testicular ACE) antibody, RP175_T, only the seminiferous tubules were fluorescent, with the intensity of staining increasing towards the centre of the tubule (Fig. 6d), reflecting the increased expression of testicular ACE as spermatids mature (Strittmatter & Snyder, 1984; Berg *et al.*, 1986). No fluorescence was observed in the blood vessels with RP175_T. These antibody preparations specific to endothelial and testicular ACE may be useful tools for mapping the relative distribution of these two isoenzymes throughout the male genital tract during development and for confirming that it is a soluble form of testicular ACE which is present in mature spermatids (Strittmatter *et al.*, 1985).

Inhibition of kidney and testicular ACE activity by the anti-(endothelial ACE) antibody

A useful tool for elucidating the specificity and function of the *N*-terminal catalytic site of endothelial ACE would be an inhibitor selective for this site and not the *C*-terminal site. The specificity

Fig. 6. Phase-contrast and immunofluorescence microscopy of pig testis immunostained with antibodies to testicular ACE

Sections of pig testis were prepared and stained as described in the Experimental section. (a) and (b) are phase-contrast and fluorescence micrographs respectively of the same field immunostained with RP175. (c) and (d) are phase-contrast and fluorescence micrographs respectively of the same field immunostained with RP175_T. The central portion of the seminiferous tubules fluoresce brightly in both (b) and (d) (thick arrows), whereas blood vessels are only immunofluorescent with RP175 (thin arrows). Leydig cells (curved arrows) did not show bright fluorescence with either of the primary antibodies. The bar represents 100 μ m.

of RP127_K for endothelial ACE was consistent with this antibody recognizing only epitopes specific to the *N*-terminal domain (see Fig. 3). The use of RP127_K as a putative inhibitor of the catalytic site of the *N*-terminal domain was, therefore, investigated (Fig. 7). A ratio of antibody to enzyme of 15:1 and 20:1 (μ g of IgG/ μ g of protein) resulted in a marked inhibition of both Bz-Gly-His-Leu and LH-RH hydrolysis (Figs. 7a and 7b respectively) by kidney ACE. In contrast, no inhibition of testicular ACE was observed at any of the concentrations of RP127_K tested. Rabbit preimmune IgG also failed to inhibit either kidney or testicular ACE at the concentrations tested. However, it was apparent that the observed inhibition of kidney ACE was not specific for the *N*-terminal domain. The present data demonstrated that the hydrolysis of Bz-Gly-His-Leu was almost completely abolished (89% inhibition) by RP127_K, which cannot be attributed to the selective inhibition of the *N*-terminal domain. Wei *et al.* (1991), using their mutant constructs in which either the *N*- or *C*-terminal domain was deleted or inactivated, showed that, at 300 mM-chloride, the *C*-terminal domain hydrolysed Bz-Gly-His-Leu 4–5-fold faster than the *N*-terminal domain. There-

Fig. 7. Inhibition of kidney and testicular ACE by RP127_K

Antibody RP127_K was incubated with equivalent activities, in terms of Bz-Gly-His-Leu hydrolysis, of either purified kidney or testicular ACE and enzyme activity then assayed with either (a) Bz-Gly-His-Leu or (b) LH-RH as described in the Experimental section. The relative activities (%) were calculated from (a) the peak area of Bz-Gly compared with the corresponding peak from incubations performed in the absence of antibody or similarly from (b) the peak area of the *N*-terminal tripeptide (pGlu-His-Trp) of LH-RH. Each point is the mean of duplicate determinations which did not differ by more than 5%. ■, □, Kidney ACE; ●, ○, testicular ACE. Closed symbols, preincubated with RP127_K; open symbols, preincubated with rabbit preimmune IgG.

fore, approx. 20% inhibition of kidney ACE, with Bz-Gly-His-Leu as substrate, would be expected if the inhibition by RP127_K was specific for the catalytic site in the *N*-terminal domain. Although the binding of RP127_K to the *N*-terminal domain of endothelial ACE may have achieved partial, if not total, inhibition of this catalytic site, inhibition of the catalytic site in the *C*-terminal domain must also have occurred. One possibility is that binding of antibody to the *N*-terminal domain may induce a change in conformation of the enzyme which prevents access or perturbs binding of substrate to both catalytic sites. An alternative possibility may reflect the three-dimensional structure of endothelial ACE. If the enzyme is folded such that the catalytic sites are within close proximity of each other, then the binding of antibodies to epitopes located at or near the catalytic site of the *N*-terminal domain may also impede access of substrate to the *C*-terminal domain.

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