

Angiotensin-Converting Enzyme (ACE) Dimerization Is the Initial Step in the ACE Inhibitor-Induced ACE Signaling Cascade in Endothelial Cells

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ABSTRACT

The binding of angiotensin-converting enzyme (ACE) inhibitors to ACE initiates a signaling cascade that involves the phosphorylation of the enzyme on Ser1270 as well as activation of the c-Jun NH₂-terminal kinase (JNK) and leads to alterations in gene expression. To clarify how ACE inhibitors activate this pathway, we determined their effect on the ability of the enzyme to dimerize and the role of ACE dimerization in the initiation of the ACE signaling cascade. In endothelial cells, ACE was detected as a monomer as well as a dimer in native gel electrophoresis and dimerization/oligomerization was confirmed using the split-ubiquitin assay in yeast. ACE inhibitors elicited a rapid, concentration-dependent increase in the dimer/monomer ratio that correlated with that of the ACE inhibitor-induced phosphorylation of ACE. Cell treatment with galactose

and glucose to prevent the putative lectin-mediated self-association of ACE or with specific antibodies shielding the N terminus of ACE failed to affect either the basal or the ACE inhibitor-induced dimerization of the enzyme. In ACE-expressing Chinese hamster ovary cells, ACE inhibitors elicited ACE dimerization and phosphorylation as well as the activation of JNK with similar kinetics to those observed in endothelial cells. However, these effects were prevented by the mutation of the essential Zn²⁺-complexing histidines in the C-terminal active site of the enzyme. Mutation of the N-terminal active site of ACE was without effect. Together, our data suggest that ACE inhibitors can initiate the ACE signaling pathway by inducing ACE dimerization, most probably via the C-terminal active site of the enzyme.

Two isoforms of the angiotensin-converting enzyme (ACE) exist in mammals: somatic or tissue ACE and testicular ACE. These enzymes are both type I transmembrane proteins consisting of an extended extracellular domain and a short cytoplasmic tail and are derived from a single gene by virtue of alternative use of transcription initiation sites (Hubert et al., 1991). The extracellular portion of testicular ACE has one active site, whereas that of somatic ACE has two active sites (the N and C domains), each of which contains the amino acid sequence HEMGH that is crucial for Zn²⁺ binding (Wei et al.,

1991). Soluble forms of ACE can also be detected in plasma and other body fluids and are generated by the enzymatic cleavage of the C-terminal extracellular portion, in the so-called juxtamembrane stalk region.

All of the ACE isoforms hydrolyze circulating peptides and catalyze the extracellular conversion of the decapeptide angiotensin I to the octapeptide angiotensin II, which is a potent vasopressor. ACE also inactivates the vasodilator peptides bradykinin and kallidin, which are derived from kininogen by the action of kallikreins (for review, see Bernstein et al., 2005). Inhibition of ACE is expected to prevent the formation of angiotensin II and to potentiate the hypotensive response to bradykinin, which would lead to the lowering of blood pressure. Somatic ACE also plays a role in vascular remodeling, effects best highlighted by the fact that the in vivo gene transfer of ACE into the uninjured rat carotid artery results in the development of vascular hypertrophy

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ABBREVIATIONS: ACE, angiotensin-converting enzyme; JNK, c-Jun NH₂-terminal kinase; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; DSS, disuccinimidyl suberate; BS₃, bis(sulfosuccinimidyl)suberate; ura, uracil; 5-FOA, fluoroorotic acid; Ub, ubiquitin; CTL, control; D, dimer; M, monomer.

independent of systemic factors and hemodynamic effects (Morishita et al., 1994). Selective overexpression of ACE in the heart also results in morphological changes in the atria, arrhythmia, and sudden death (Xiao et al., 2004). Antisense oligonucleotides against ACE, in contrast, are reported to prevent neointimal formation after balloon angioplasty (Morishita et al., 2000), and ACE inhibitors decrease vascular hypertrophy in hypertensive animals (Clozel et al., 1991). Furthermore, ACE inhibitors, such as ramiprilat, exert beneficial effects on endothelial function and vascular remodeling (Schartl et al., 1994; Mancini et al., 1996) and protect against the progression of atherosclerosis and the occurrence of cardiovascular events in humans (Heart Outcomes Prevention Evaluation Study Investigators, 2000).

Although the latter effects of ACE inhibitors are generally attributed to a decrease in the ACE-mediated generation of angiotensin II and the accumulation of bradykinin (Wiemer et al., 1991), a number of the effects of this class of compounds cannot be accounted for by inhibition of the enzyme per se. For example, it is not generally appreciated that during long-term ACE inhibition angiotensin II levels are not greatly reduced (Lee et al., 1999), and more than 80% of the angiotensin II formation in the human heart and more than 60% of that in arteries is reported to be chymase-dependent (Petrie et al., 2001). Many other reports published over the past 10 years have also shown that ACE inhibitors can amplify responses to bradykinin in situations in which the accumulation of the peptide cannot be assumed to occur. These effects have been attributed to the reactivation of the deactivated B₂ receptor and/or the cross-talk between ACE and intracellular signaling cascades and may be linked to interference with the sequestration of activated G protein-coupled receptors into caveolae (Benzing et al., 1999). We have since established that ACE functions as a signal transduction molecule and that the binding of an ACE inhibitor to ACE initiates a series of events, including the phosphorylation of ACE on Ser1270 and the activation of ACE-associated JNK, that eventually result in changes in gene expression (Kohlstedt et al., 2004, 2005). This ACE signaling cascade occurs independently of the involvement of angiotensin II and bradykinin or any of their receptors. It is however not clear how the binding of an ACE inhibitor is able to initiate the processes described. Because ACE can exist in a dimeric form in vitro (Kost et al., 2003), the aim of the present investigation was to determine whether or not ACE dimerizes in endothelial cells and whether dimerization is involved in ACE inhibitor-induced ACE signaling.

Materials and Methods

Materials. The ACE monoclonal antibodies (clone 9B9, 3A5, and 5F1) recognizing different epitopes of the N-terminal domain of the enzyme were from Chemicon International (Temecula, CA). The monoclonal antibody (C78) used for Western blotting also recognized the N-terminal domain and was provided by Dr. Peter Bünning (Sanofi-Aventis, Frankfurt, Germany). The mono- and polyclonal antibodies recognizing the cytoplasmic tail of ACE were generated in mice and rabbits immunized with a synthetic peptide corresponding to amino acids 1250 to 1277 in the ACE C terminus. The phospho-specific p-Ser1270 antibody was generated from the peptide sequence HGPQFGpSEVELR (position 1263 to 1275 in human somatic ACE protein) by Eurogentec (Seraing, Belgium). The antibodies against CD31 and JNK-1 were from Santa Cruz Biotechnology, Inc.

(Santa Cruz, CA). The anti-green fluorescent protein antibody used for immunoprecipitation of yellow green fluorescent protein-tagged ACE was from Roche Applied Science (Mannheim, Germany). All other substances were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Culture. Human umbilical vein endothelial cells were isolated and cultured as described previously (Busse and Lamontagne, 1991). Because ACE expression decreases with time in culture, all of the experiments in human endothelial cells were performed using primary cultures. Porcine aortic endothelial cells stably transfected with ACE or the nonphosphorylatable S1270A ACE mutant were generated and cultured as described previously (Kohlstedt et al., 2002). Although the porcine endothelial cells no longer endogenously expressed ACE or functional AT₁ and B₂ receptors, they expressed a number of characteristic endothelial cell proteins (von Willebrand factor, CD31, the endothelial nitric-oxide synthase, and VE-cadherin).

Histidyl and glutamyl residues in the active sites of ACE are essential for their enzymatic activity. Inactive ACE mutants were generated by either the mutation of the histidine doublet in the HEMGH sequence to lysine (H361,365K in the N domain and H959,963K in the C domain) or by mutation of the active site glutamate to aspartate (E362D in the N domain or E960D in the C domain). In addition, an ACE mutant in which both glutamate residues in the N and C domains were mutated (E362,960D) was generated and stably overexpressed in CHO cells (Wei et al., 1991). For the sake of clarity, the mutants are referred to as follows: N_{His} (H361,365K), C_{His} (H959,963K), N_{Glu} (E362D), C_{Glu} (E960D), and N+C_{Glu} (E362,960D).

Immunoblotting and Immunoprecipitation. Cells were lysed in Nonidet lysis buffer containing 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 25 mM β-glycerophosphate, 10% (v/v) glycerol, 1 mM Na₄P₂O₇, 10 nM okadaic acid, 2 mM Na₃VO₄, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 10 μg/ml trypsin inhibitor, 44 μg/ml phenylmethylsulfonyl fluoride, and 1% (v/v) Nonidet P-40 and left on ice for 10 min and centrifuged at 10,000g for 10 min. After preclearing with protein A/G-Sepharose, proteins were immunoprecipitated from the cell supernatant or from whole cell lysates with their respective primary antibodies as detailed under *Results*. Proteins in the cell supernatant or immunoprecipitates were heated with SDS-PAGE sample buffer and separated by SDS-PAGE as described previously (Kohlstedt et al., 2002). Proteins were detected using their respective antibodies and were visualized by enhanced chemiluminescence using a commercially available kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). To reprobe Western blots with alternative antibodies, the nitrocellulose membranes were incubated at 50°C for 30 min in a buffer containing 67.5 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, and 2% SDS.

For native gels, cells were lysed by the addition of H₂O supplemented with 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 10 μg/ml trypsin inhibitor, and 44 μg/ml phenylmethylsulfonyl fluoride and three cycles of freeze-thawing. The cells were then harvested by scraping, and appropriate volumes of concentrated phosphate-buffered saline were added to give a final concentration of 140 mM NaCl, 10 mM Na₂HPO₄, 2.68 mM KCl, and 1.47 mM K₂HPO₄ at pH 7.0. Lysates were then centrifuged (10 min; 4°C; 10,000g), and supernatants were used for analysis. Equal amounts of protein (20 μg) were loaded on a 6% acrylamide gel without SDS at 4°C 1) in SDS- and DTT-free sample buffer, 2) after treatment with 10 U/ml trypsin at room temperature for 1 h before addition of SDS- and DTT-free sample buffer, and 3) after boiling for 10 min in SDS-PAGE buffer. After electrophoresis (4°C; 20 mA), gels were incubated in SDS-transfer buffer for 10 min, and proteins were transferred to nitrocellulose membranes as described above and blotted with the C78 anti-ACE antibody. The molecular masses were estimated by comparison with those of the reference proteins thyroglobulin (669 kDa), ferritin (440 kDa), and catalase (232 kDa).

Chemical Cross-Linking. Cross-linking was performed using the cross-linking agents disuccinimidyl suberate (DSS) or bis(sulfo-

succinimidyl)suberate (BS₃; Pierce Chemical, Rockford, IL) according to the manufacturer's instructions. The cross-linking of ACE on the surface of confluent cells was performed at room temperature for 30 min using 1 to 2 mM DSS or BS₃. After quenching the reaction with 20 mM Tris-HCl, pH 7.4, for 15 min at room temperature, cells were lysed and immunoblotting performed as described above.

Split-Ubiquitin Assay. The split-ubiquitin (Ub) assay was carried out as described previously (Eckert and Johnsson, 2003) using the *Saccharomyces cerevisiae* strain JD53 (*Mata^{ura} 3-52, leu2-3, -112his3 Δ200, lys2-801, trpΔ63*). For the expression of a fusion protein of ACE and C-terminal portion of ubiquitin (C_{ub}), the human ACE-cDNA was cloned into the vector pMet-Ste14-C_{ub}-Rura3, replacing Ste14 (Wittke et al., 1999). Fusion constructs of ACE and the N-terminal portion of ubiquitin (N_{ub}) were generated by replacing the Ubc6 sequence by the human full-length ACE-cDNA within the pCUP314-Ubc6 plasmid. To enhance the discriminating power of the assay system, we used two N_{ub} mutants containing alanine (N_{ua}) or glycine (N_{ug}) in position 13, which have a lower affinity for C_{ub} than wild-type N_{ub} possessing isoleucine in this position (Johnsson and Varshavsky, 1994). The coding sequence for N_{ub}, N_{ua}, and N_{ug} followed by an ADH terminator sequence was cloned in-frame to the 3' end of the ACE cDNA. Transformed JD53 cells were grown at 30°C in selective medium containing 50 mg/ml uracil (ura) to an optical density₆₀₀ of 1.0, and 4 μl of 10-fold dilutions was spotted on agar plates lacking uracil or containing 1 mg/ml 5'-fluororotic acid (5-FOA) and 50 mg/ml uracil. Plates were incubated at 30°C for 2 to 4 days unless otherwise stated. The plasmids were generous gifts from Dr. Nils Johnsson (Westfälische Wilhelms-Universität Münster, Münster, Germany).

JNK Activity Assay. JNK was immunoprecipitated, and its in vitro kinase activity was measured using 2 μg of glutathione S-transferase-c-Jun as substrate as described previously (Kohlstedt et al., 2004). Reactions were stopped, and the products were resolved by 12% SDS-PAGE. The incorporation of ³²P was visualized by autoradiography and quantified by scanning densitometry.

Statistical Analysis. Data are expressed as the mean ± S.E.M., and statistical evaluation was performed using Student's *t* test for unpaired data or one-way analysis of variance followed by a Bonferroni *t* test where appropriate. Values of *p* < 0.05 were considered statistically significant.

Results

Evaluation of ACE Dimerization by Native Gel Electrophoresis and the Split-Ubiquitin Assay. To determine whether ACE exists as a homodimer in endothelial cells, porcine aortic endothelial cells stably overexpressing human somatic ACE were subjected to native gel electrophoresis. Two ACE forms were apparent: one dominant form of approximately 270 kDa, the putative monomer; and a minor form of approximately 520 kDa, the putative dimer (Fig. 1a). Because the quality of the signals obtained with ACE, which is a highly glycosylated transmembrane protein, was relatively poor, we confirmed that the ACE dimer (~520 kDa) could only be detected in SDS- and DTT-free conditions whereas under denaturing conditions (+SDS; +95°C), the lower molecular mass band (monomeric ACE) was exclusively detectable. Moreover, the treatment of the endothelial cells with trypsin to cleave ACE at the C-terminal stalk region (Woodman et al., 2000) resulted in the loss of both the 520- and the 270-kDa signals. It is noteworthy that the stimulation of endothelial cells with ramiprilat (100 μM; 7 min) enhanced the relative amount of 520-kDa ACE by 137 ± 11% (*n* = 5; *p* < 0.05) compared with the situation in unstimulated cells (Fig. 1a).

To demonstrate homodimer formation of ACE in living cells, we used the split-ubiquitin system. In brief, we used a ura auxotrophic yeast strain (JD53) lacking orotidylate decarboxylase (Ura3p) (i.e., the enzyme catalyzing the final step of UMP biosynthesis and also converting 5-FOA to the toxic metabolite 5-fluorouracil) (Wittke et al., 1999). The interaction assay is based on the reassembly of the N- and C-terminal halves, N_{ub} and C_{ub}, respectively, of Ub that are fused to the cytosolic portions of full-length human ACE (i.e., ACE-N_{ub} and ACE-C_{ub}). Uracil prototrophy is reconstituted through the fusion of a modified version of Ura3p, containing an extra N-terminal residue of arginine (RUra3p), to the

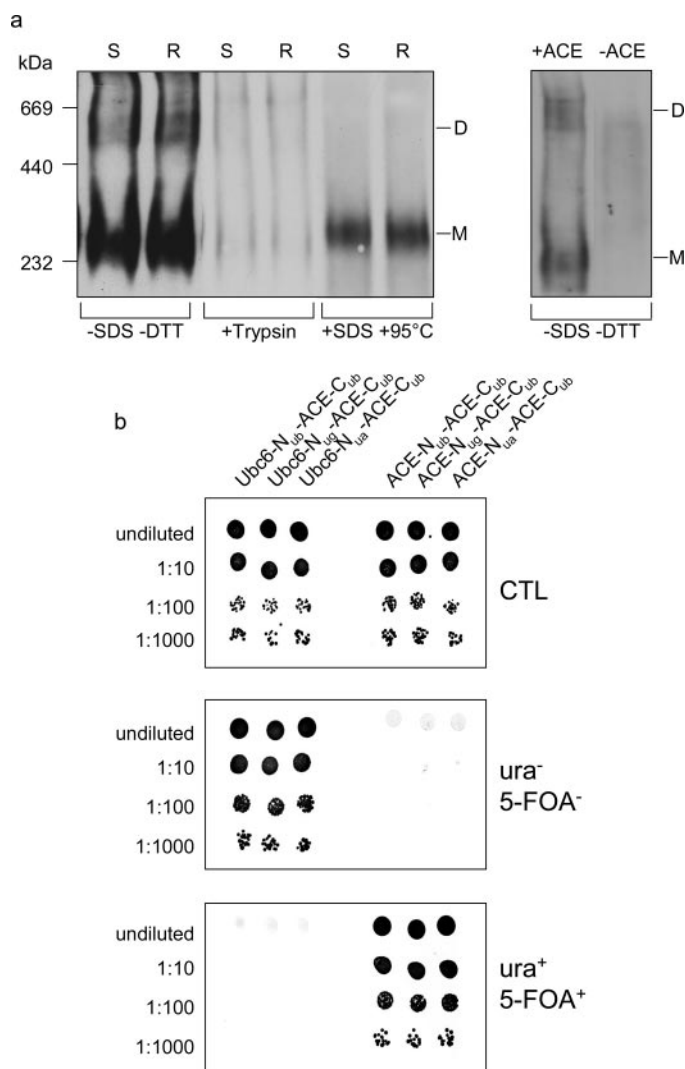


Fig. 1. Dimerization of ACE under basal conditions and in the presence of ramiprilat. a, representative Western blots showing somatic ACE monomers (M) and dimers (D) in lysates of ACE-overexpressing porcine aortic endothelial cells treated with either solvent (S) or ramiprilat (R; 100 nM; 7 min) (left side) or ACE overexpressing (+ACE) and ACE-deficient (-ACE) porcine aortic endothelial cells (right side). Experiments were performed under nonreducing conditions (-SDS-DTT). As a control, lysates were treated with trypsin (10 U/ml; 1 h) before adding nonreducing sample buffer or were heated for 10 min in Laemmli SDS sample buffer (+SDS +95°C) before application. b, serial dilutions of JD53 cells coexpressing ACE-N_{ub}, ACE-N_{ua}, or ACE-N_{ug} and ACE-C_{ub} were spotted on plates lacking Trp/His (CTL), or uracil and 5-FOA (ura⁻ 5-FAO⁻), or on plates containing uracil and 5-FOA (ura⁺ 5-FAO⁺). Interaction between ACE monomers is indicated by cell growth on ura⁺ 5-FAO⁺ plates and growth inhibition on ura⁻ 5-FAO⁻ plates and vice versa. Similar results were obtained in at least three additional experiments.

C-terminal half of ACE- C_{ub} , thereby generating ACE- C_{ub} -RUra3p. Dimerization-induced apposition of N_{ub} and C_{ub} halves in cotransformants creates a quasi-native Ub that is recognized by ubiquitin-specific proteases splitting off the C-terminally attached reporter RUra3p. Because of its extra arginine residue at the N terminus, released RUra3p is subject to rapid degradation by proteases of the N-end rule. In this way, the split-Ub system provides a direct readout of the N_{ub} - C_{ub} association state; i.e., the nonassociated Ub halves retain RUra3p activity, which allows cells to grow on uracil-deficient (ura^-) medium and inhibits growth on ura^+ plates containing 5-FOA, because of the accumulation of toxic 5-fluorouracil, whereas the associated Ub halves produced an inverse growth pattern.

Transformed JD53 cells expressed both ACE- N_{ub} and ACE- C_{ub} proteins, as determined by Western blotting (data not shown). Cells coexpressing ACE- N_{ub} and ACE- C_{ub} were unable to grow on ura^- plates but expanded on 5-FOA plates, indicating that ACE forms stable dimers (and/or higher oligomers) in intact cells (Fig. 1b). By contrast, cells expressing ACE- C_{ub} and an unrelated construct in which the ubiquitin-conjugating enzyme Ubc6 was fused to the N-terminal portion of N_{ub} grew on ura^- plates but not on ura^+ , 5-FOA⁺ plates, indicating that ACE and Ubc6 do not interact under these conditions. To enhance the discriminating power of the assay system, we also used two N_{ub} mutants containing alanine (N_{ua}) or glycine (N_{ug}) in position 13, which have a lower affinity for C_{ub} than wild-type N_{ub} . Using these modified probes, we observed a robust interaction between ACE- N_{ua} or ACE- N_{ug} and ACE- C_{ub} , confirming that ACE dimerizes/oligomerizes in living cells (Fig. 1b).

Effect of Ramiprilat on the Dimerization of ACE in Endothelial Cells. To determine the effects of the ACE inhibitor ramiprilat on the dimerization of ACE, cells were stimulated with the ACE inhibitor and then treated with the chemical cross-linkers DSS or BS₃ before ACE dimerization was assessed by SDS-PAGE and Western blotting.

Ramiprilat elicited a rapid (within 2 min) 2-fold increase in the dimerization of ACE, which was maintained for up to 60 min (Fig. 2a). In parallel experiments, the phosphorylation of ACE on Ser1270 was monitored, and, as reported previously (Kohlstedt et al., 2004), the ramiprilat-induced phosphorylation of ACE was maximal 2 min after the addition of the inhibitor but declined back to baseline over the next 10 to 15 min (Fig. 2b). The phosphorylation of ACE increases again after 24 to 48 h of ACE-inhibitor treatment (Kohlstedt et al., 2004), and this was paralleled by an increased dimerization of ACE (data not shown). A similar ramiprilat-induced dimerization of ACE was observed using primary cultures of human umbilical vein endothelial cells and was also initiated by other ACE inhibitors (i.e., enalaprilat, quinaprilat, and captopril, albeit with varying efficacies) (Fig. 2c). Primary cultures of human umbilical vein endothelial cells endogenously express ACE, and the fact that the ACE inhibitor-induced dimerization of ACE could be detected in these cells indicates that the effects observed cannot be attributed to an artifact related to the overexpression system.

The mutation of ACE Ser1270 to alanine prevents the ACE inhibitor-induced phosphorylation of ACE and the subsequent ACE signaling (Kohlstedt et al., 2004). However, the ramiprilat-induced dimerization of ACE was also observed in endothelial cells overexpressing the nonphosphorylatable

S1270A ACE mutant (Fig. 2d). The effect of ramiprilat and other ACE inhibitors on the dimerization of ACE in ACE-overexpressing porcine endothelial cells was concentration-dependent with significant effects being observed using concentrations of 10 nM or greater (Fig. 3), suggesting that the ACE inhibitor-induced dimerization of ACE is a specific effect of this class of compounds, rather than a unique property of ramiprilat.

Effect of Carbohydrates and Antibodies on ACE Dimerization and Shedding. We next assessed the role of the putative carbohydrate recognition domain (Kost et al., 2003), a lectin-like structure residing within the N-terminal portion of somatic ACE, in enzyme dimerization. Because ACE dimer formation in vitro in inverse micelles has been reported to be inhibited by galactose and other carbohydrates (Kost et al., 2000), we incubated ACE-overexpressing porcine aortic endothelial cells with 10 μ M galactose, 10 μ M glucose, or 10 μ M mannitol (as an osmotic control) for 30 min, before the addition of 100 nM ramiprilat for 2 min. The basal or ramiprilat-induced dimerization of ACE in endothelial cells was not influenced by either galactose, glucose, or mannitol (Fig. 4). Furthermore, the pretreatment of cells with the monosaccharides did not affect either the basal or the ramiprilat-induced phosphorylation of ACE on Ser1270 (data not shown). The carbohydrate recognition domain that was suggested to be important for ACE dimerization is recognized

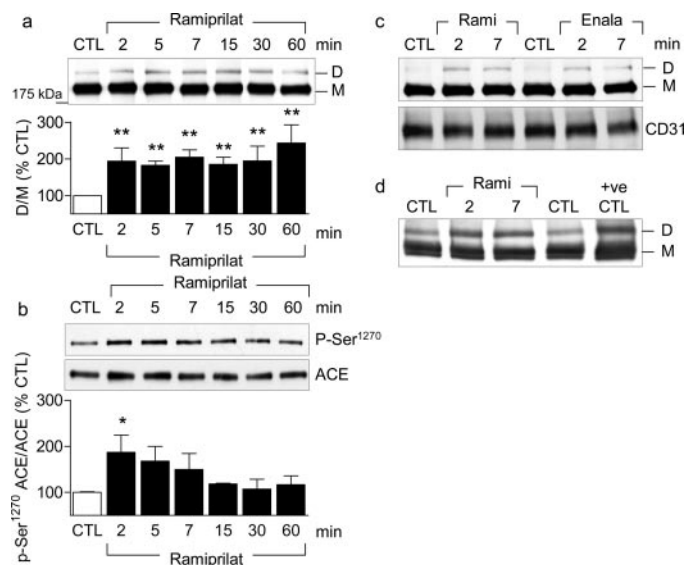


Fig. 2. Effect of inhibitors on ACE dimerization and phosphorylation in endothelial cells. Time course (2–60 min) of the ramiprilat (100 nM)-induced dimerization (assessed after chemical cross-linking) (a) and phosphorylation of ACE on Ser1270 (p-Ser1270) (b) in porcine aortic endothelial cells stably overexpressing human somatic ACE. Results were quantified by calculating the D/M ratio and comparing changes relative to the ratio detected in unstimulated endothelial cells (CTL) or by calculating the p-Ser1270ACE/ACE signal ratio by densitometry and comparing changes relative to the signal obtained in CTL cells. The bar graphs summarize the results of four to six independent experiments; *, $p < 0.05$; **, $p < 0.01$ versus CTL. c, effect of ramiprilat (Rami) and enalaprilat (Enala; each 100 nM; 2 and 7 min) on ACE dimer formation in primary cultures of human umbilical vein endothelial cells, after cross-linking with BS₃. The blots were reprobbed with CD31 to demonstrate equal loading of each lane. Identical results were obtained in four to six additional experiments. d, effect of Rami (100 nM; 2 and 7 min) on ACE dimerization in porcine aortic endothelial cells stably overexpressing the nonphosphorylatable ACE mutant S1270A. Porcine aortic endothelial cells stably overexpressing human ACE were used as a positive control (+ve CTL).

and thus shielded by the monoclonal antibody 9B9 (Kost et al., 2003). We therefore determined the effect of different monoclonal ACE antibodies (9B9, 3A5, 5F1, and C78) directed to different epitopes within the N domain (Danilov et al., 1994) on ACE dimer formation. The antibodies tested had no acute effect on ACE dimerization per se (Fig. 5a). However, the 9B9 and 3A5 antibodies (but not 5F1) significantly enhanced the amount of ACE recovered from the endothelial cell supernatant (Fig. 5b), reflecting the enhanced cleavage/secretion of the enzyme. None of the antibodies tested were able to prevent the ramiprilat-induced dimerization of ACE in ACE-overexpressing porcine aortic endothelial cells (Fig. 5c), excluding the previously speculated correlation between ACE dimerization and shedding (Kost et al., 2003).

Effect of Ramiprilat on Dimerization and Initiation of ACE Signaling in CHO Cells. Somatic ACE has two homologous N and C domains, each of which contains a catalytically active site comprising the consensus sequence HEMGH (Soubrier et al., 1988). Glu³⁶² in the N domain and Glu⁹⁶⁰ in the C domain are essential catalytic residues, and the His doublets 361/365 and 959/963 are most likely involved in the binding of Zn²⁺ (Wei et al., 1991). To determine whether the active sites are necessary for the dimer forma-

tion, we analyzed ACE dimerization in CHO cells stably transfected with either wild-type ACE or different ACE mutants; two with an inactivated N domain (N_{His} and N_{Glu}), two with an inactivated C domain (C_{His} and C_{Glu}), and one mutant with interfering mutations in both the N and C domains (N+C_{Glu}).

Mutation of the glutamyl residues within the C and/or N domains (N_{Glu}, C_{Glu}, and N+C_{Glu}) was without effect on the ramiprilat-induced dimerization of ACE or on its phosphorylation on Ser1270 (data not shown). Replacement of the two histidyl residues within the C domain of ACE (C_{His}) attenuated the basal formation of ACE dimers as well as Ser1270 phosphorylation of the ACE mutant, whereas the N_{His} mutant showed unchanged dimerization status and increased basal Ser1270 phosphorylation (Fig. 6, a and b). Moreover, although the ramiprilat-induced dimerization (Fig. 6a) and phosphorylation of ACE (Fig. 6b) were apparent in cells expressing the wild-type ACE or the N_{His} mutant, neither was

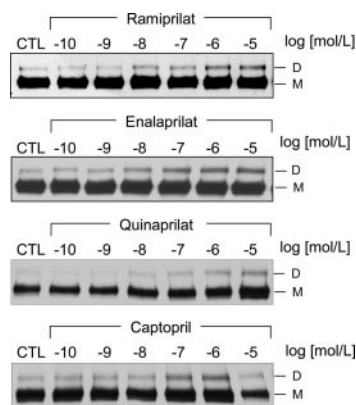


Fig. 3. Concentration-dependent effect of different ACE inhibitors on ACE dimerization. Representative Western blots showing the concentration-dependent effects of ramiprilat, quinaprilat, captopril, and enalaprilat (0.1 nM–10 μM; 2 min) on ACE dimerization, assessed after chemical cross-linking. Similar results were obtained in three additional experiments.

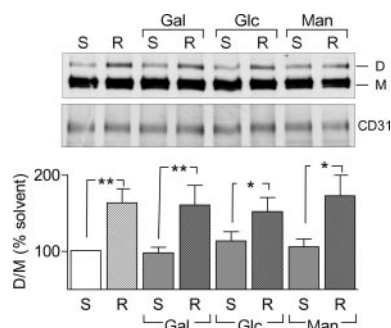


Fig. 4. Effect of carbohydrates on the dimerization of ACE in ACE-overexpressing endothelial cells. a, representative Western blots showing the effect of pretreatment with galactose (Gal), glucose (Glc), or mannitol (Man) (each 10 μM; 30 min) on ramiprilat (R; 100 nM; 2 min)-induced ACE dimerization. The results were quantified by calculating the cross-linked D/M ratio and comparing changes relative to the ratio detected in unstimulated endothelial cells (S, solvent). The bar graphs summarize results obtained in four to six different experiments; *, $p < 0.05$; **, $p < 0.01$ versus CTL.

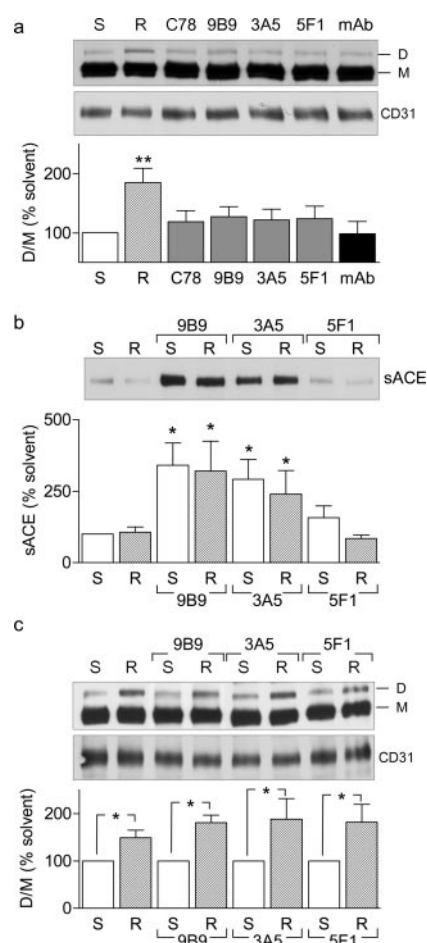


Fig. 5. Effect of antibodies on ACE dimerization and cleavage. a, effect of solvent (S), ramiprilat (R; 100 nM; 7 min), monoclonal antibodies to the ACE N domain C78, 9B9, 3A5, and 5F1 (each 10 μg/ml; 7 min) and an unrelated monoclonal antibody (mAb; anti-caveolin-1), on ACE dimerization in overexpressing porcine aortic endothelial cells. b, effect of the 9B9, 3A5, and 5F1 antibodies (each 10 μg/ml; 4 h) on the amount of soluble ACE (sACE) recovered by immunoprecipitation from the supernatant of ACE-overexpressing porcine aortic endothelial cells. c, effect of pretreatment with solvent and antibodies 9B9, 3A5, and 5F1 (each 10 μg/ml; 4 h) on the R (100 nM; 2 min)-induced dimerization of ACE, assessed after chemical cross-linking. The bar graphs summarize the results of three to six independent experiments; *, $p < 0.05$; **, $p < 0.01$ versus the respective buffer alone.

observed in CHO cells expressing the C_{His} ACE mutant. Consistent with these effects, ramiprilat was also unable to elicit the activation of JNK in C_{His} -expressing cells (Fig. 7). The effects of ramiprilat treatment on cells expressing wild-type ACE were similar to those observed in cells expressing N_{His} , or any of the Glu mutants (N_{Glu} , C_{Glu} , and $N+C_{Glu}$; Fig. 7), indicating that ramiprilat was able to elicit signaling as long as the two His residues mediating Zn^{2+} binding of the active site in the C domain were present.

Discussion

The results of the present investigation demonstrate that ACE occurs as a homodimer as well as a monomer in endothelial cells, as judged by native gel electrophoresis, and that the binding of an inhibitor to the enzyme significantly enhances ACE dimer formation. The C-terminal active site of ACE seems to be determinant for ACE dimerization and for the initiation of downstream ACE signaling. Indeed, the ACE inhibitor-induced dimerization of the enzyme as well as the ACE inhibitor-induced phosphorylation of ACE on Ser1270 and the subsequent activation of the JNK pathway was abrogated by the mutation of two critical histidyl residues that determine the binding of Zn^{2+} to the C domain. Given that dimer formation was rapid and correlated with the time course of the inhibitor-induced ACE phosphorylation on Ser1270, the dimerization of ACE may well be the initial step in the recently identified ACE signaling pathway in endothelial cells.

Mature somatic ACE exists at the endothelial cell surface and has a calculated molecular mass of 146.6 kDa; however, the enzyme is highly glycosylated, and the molecular mass of the enzyme is between 170 and 180 kDa when assessed by

denaturing polyacrylamide gel electrophoresis (Das and Soffer, 1975). Other forms of human somatic ACE exist, such as an immature form found in intracellular compartments (~170 kDa) and a soluble form (~175 kDa) found in the plasma representing a C-terminally truncated form of the enzyme that is generated by the proteolytic cleavage of its stalk region and the subsequent release from the plasma membrane (Alhenc-Gelas et al., 1989). Aggregates of ACE that might reflect enzyme dimers and/or higher oligomers have been found during purification of ACE from human lung (Nishimura et al., 1977) as well as in *in vitro* experiments by using inverse micelles and ACE-expressing COS cells (Kost et al., 2000, 2003).

Using native gel electrophoresis, we found that the ACE endogenously expressed in endothelial cells migrates not only as monomers but also as dimers. The treatment of cell lysates with SDS, to denature ACE dimers, resulted in failure to detect the larger protein, whereas the use of trypsin, which cleaves ACE at the C-terminal stalk region (Woodman et al., 2000), resulted in failure to detect the enzyme at all. The ability of ACE to homodimerize was also demonstrated using the split-ubiquitin system in yeast as well as by chemical cross-linking in primary cultures of human endothelial cells that endogenously express ACE. Moreover, the data obtained with the split ubiquitin assay indicate that ACE dimerization is greater in living cells and a more pronounced phenomenon than the *in vitro* experiments would suggest. The relatively low dimer levels detected in the cross-linking experiments can probably be attributed to methodological problems related to the detection of the dimeric form of ACE by the antibodies used. Similar problems have been described when assessing the dimer (D)/monomer (M) ratio of the endothelial nitric-oxide synthase by low-temperature SDS-PAGE (Zou et al., 2002).

Given the existence of ACE dimers, we next determined whether the binding of an ACE inhibitor to ACE altered the dimer/monomer ratio in endothelial cells. Our results showed that the treatment of endothelial cells with ramiprilat increased the dimer/monomer ratio and that the formation of ACE dimers correlated temporally with the onset of the in-

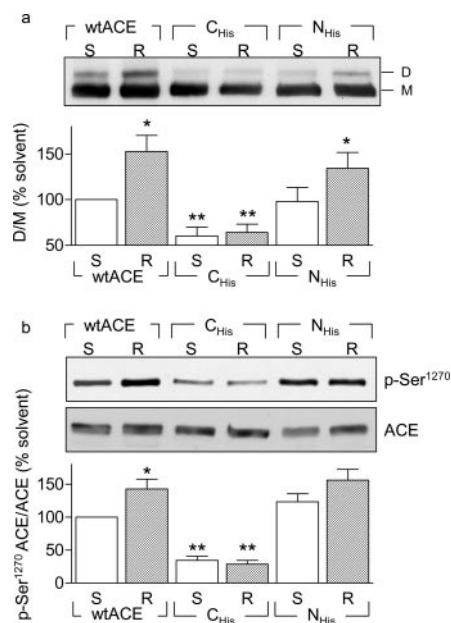


Fig. 6. Effect of ramiprilat on dimerization and phosphorylation of N_{His} and C_{His} mutants. Representative Western blots showing the effect of solvent (S) and ramiprilat (R; 100 nM; 2 min) on ACE dimerization (a) and phosphorylation (b) at Ser1270 (p-Ser1270) in CHO cells overexpressing human somatic ACE (wtACE) or N_{His} and C_{His} mutants. M and D forms of ACE are indicated. The bar graphs summarize results obtained in four to six independent experiments; *, $p < 0.05$ versus basal wild-type ACE dimerization or phosphorylation.

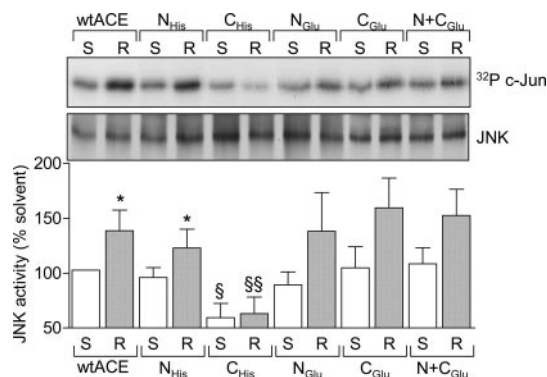


Fig. 7. Effect of ramiprilat on signaling by ACE mutants. Autoradiography of ^{32}P -labeled c-Jun and statistical analysis showing the effect of solvent (S) and ramiprilat (R; 100 nM; 7 min) on the activity of JNK immunoprecipitated from CHO cells overexpressing human somatic ACE (wtACE) or one each of the N_{His} , C_{His} , N_{Glu} , C_{Glu} , or $N+C_{Glu}$ mutants. To ensure equal immunoprecipitation of JNK, blots were reprobed with an antibody against JNK. The bar graph summarizes the results obtained in four independent experiments; *, $p < 0.05$ versus respective solvent; §, $p < 0.05$; §§, $p < 0.01$ versus basal JNK activity in cells expressing wild-type ACE.

hibitor-induced phosphorylation of the enzyme at Ser1270. The effects described do not represent a transient cellular response, because the ACE inhibitor-induced phosphorylation of ACE is biphasic and consists of a transient peak after 2 to 7 min followed by maintained elevation in phosphorylation after 6 to 48 h (Kohlstedt et al., 2004). The activation of this signaling cascade has previously been linked to changes in endothelial cell gene expression and the enhanced production of the vasoprotective autacoid prostacyclin (Kohlstedt et al., 2005). The ability to induce ACE dimer formation was not restricted to ramiprilat and was also observed after cell stimulation with enalaprilat, quinaprilat, and captopril, indicating that this is an effect of this class of compounds. Dimer formation was observed in an endothelial cell line that overexpressed human somatic ACE as well as in primary cultures of human umbilical vein endothelial cells that endogenously express ACE, indicating that the effects described cannot be simply attributed to an artifact associated with the overexpression system.

Somatic ACE contains 17 potential sites for *N*-glycosylation, mainly of the complex type (Das and Soffer, 1975). Because ACE dimerization has been attributed to interactions between its carbohydrate side chains and a carbohydrate recognition domain at its N domain that are sensitive to treatment with galactose (Kost et al., 2000), we tested the effects of galactose, glucose, or mannitol on either the basal or the ramiprilat-induced dimerization of ACE, but we were unable to find any significant interference with ACE dimerization. It has also been suggested that monoclonal antibody 9B9 directed to the putative carbohydrate recognition domain region can interfere with ACE self-association (Kost et al., 2003). We therefore assessed the effect of various antibodies directed to the N domain of ACE, on dimer formation. The monoclonal antibodies tested had no effect on the basal or ramiprilat-induced dimerization of ACE but exerted differential effects on the cleavage of the enzyme. The latter effect has been reported previously (Kost et al., 2003) and linked to the antibody-mediated inhibition of dimerization. However, although we clearly observed antibody-induced ACE shedding, we were unable to detect any link between shedding and dimerization. To determine whether the dimerization of ACE was linked to the recently described ACE signaling pathway, which involves the ACE inhibitor-induced phosphorylation of the enzyme on Ser1270 as well as the activation of the JNK pathway (Kohlstedt et al., 2002, 2004), we compared the effects of ramiprilat on the dimerization of the wild-type ACE as well as of the S1270A ACE mutant. Ramiprilat elicited dimerization of both the wild-type and the S1270A ACE, indicating that the phosphorylation status of the cytoplasmic tail of the enzyme does not influence dimer formation.

Other type I membrane glycoproteins, such as the β -secretase (β -site amyloid precursor protein cleaving enzyme) are also able to dimerize and thus regulate their intracellular and/or extracellular functions (Schmechel et al., 2004). Like ACE, β -site amyloid precursor protein cleaving enzyme can be phosphorylated on a Ser residue near its C terminus and can also be cleaved from the cell surface (Capell et al., 2000). We therefore determined whether interfering with the dimerization of ACE affected the ACE inhibitor-induced phosphorylation of the enzyme and/or the subsequent activation of JNK. Analysis of different C- or N-domain inactive

ACE mutants overexpressed in CHO cells revealed that the C domain rather than the N domain of ACE seems to be implicated in its dimerization, inasmuch as inactivation of the C domain active site via mutation of the two essential Zn^{2+} -complexing histidyl residues of the HEMGH consensus sequence rendered the enzyme unable to dimerize either under basal conditions or in response to ramiprilat. Mutation within the C domain also resulted in the loss of inhibitor-induced ACE signaling; i.e., we failed to observe a ramiprilat-induced increase in Ser1270 phosphorylation or activation of the downstream JNK pathway. Both aspects of the ACE signaling pathway remained intact after mutation of the N domain. At present, we do not know how ACE inhibitors can effect ACE dimerization via the C domain; however, it is possible that their binding induces conformational changes that eventually result in the exposure of a dimerization domain, as has been reported for the ligand-induced dimerization of the epidermal growth factor receptor (Ferguson et al., 2003).

Although the N and C domains of ACE arose as a consequence of gene duplication (Lattion et al., 1989), there is at least circumstantial evidence suggesting that there are marked differences in the function of the two sites. For example, although both the N and C domains contribute to the degradation of bradykinin, angiotensin I conversion takes place preferentially within the C domain, and selective C domain inhibition is sufficient to prevent angiotensin I-induced vasoconstriction (van Esch et al., 2005). There is also evidence suggesting that the N domain of ACE may be functionally less important, because the RXP407 peptide that specifically inhibits the ACE N domain active site has no effect on blood pressure (Junot et al., 2001).

Together, the results of the present investigation suggest that the ACE inhibitor-induced dimerization of ACE, via the C domain of the enzyme, represents the initial step in the ACE signaling pathway that involves the activation of the JNK/c-Jun pathway and leads to changes in endothelial cell gene expression.

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References

- Alhenc-Gelas F, Soubrier F, Hubert C, Allegrini J, and Corvol P (1989) The peculiar characteristics of the amino acid sequence of angiotensin I-converting enzyme, as determined by cDNA cloning of the human endothelial enzyme. *J Cardiovasc Pharmacol* 14:S6–S9.
- Benzing T, Fleming I, Blaukat A, Muller-Esterl W, and Busse R (1999) Angiotensin-converting enzyme inhibitor ramiprilat interferes with the sequestration of the B_2 kinin receptor within the plasma membrane of native endothelial cells. *Circulation* 99:2034–2040.
- Bernstein KE, Xiao HD, Frenzel K, Li P, Shen XZ, Adams JW, and Fuchs S (2005) Six truisms concerning ACE and the renin-angiotensin system deduced from the genetic analysis of mice. *Circ Res* 96:1135–1144.
- Busse R and Lamontagne D (1991) Endothelium-derived bradykinin is responsible for the increase in calcium produced by angiotensin-converting enzyme inhibitors in human endothelial cells. *Naunyn-Schmiedeberg's Arch Pharmacol* 344:126–129.
- Capell A, Steiner H, Willem M, Kaiser H, Meyer C, Walter J, Lammich S, Multhaup G, and Haass C (2000) Maturation and pro-peptide cleavage of beta-secretase. *J Biol Chem* 275:30849–30854.
- Clozel J-P, Hess P, Michael C, Schietinger K, and Baumgartner HR (1991) Inhibition of converting enzyme and neointima formation after vascular injury in rabbits and guinea pigs. *Hypertension* 18:II55–II59.
- Danilov S, Jaspard E, Churakova T, Towbin H, Savoie F, Wei L, and Alhenc-Gelas F (1994) Structure-function analysis of angiotensin I-converting enzyme using monoclonal antibodies. Selective inhibition of the amino-terminal active site. *J Biol Chem* 269:26806–26814.

- Das M and Soffer RL (1975) Pulmonary angiotensin-converting enzyme. Structural and catalytic properties. *J Biol Chem* **250**:6762–6768.
- Eckert JH and Johnsson N (2003) Pex10p links the ubiquitin conjugating enzyme Pex4p to the protein import machinery of the peroxisome. *J Cell Sci* **116**:3623–3634.
- Ferguson KM, Berger MB, Mendrola JM, Cho HS, Leahy DJ, and Lemmon MA (2003) EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. *Mol Cell* **11**:507–517.
- Heart Outcomes Prevention Evaluation Study Investigators (2000) Effects of ramipril on cardiovascular and microvascular outcomes in people with diabetes mellitus: results of the HOPE study and MICRO-HOPE substudy. Heart Outcomes Prevention Evaluation Study Investigators. *Lancet* **355**:253–259.
- Hubert C, Houot AM, Corvol P, and Soubrier F (1991) Structure of the angiotensin I-converting enzyme gene. Two alternate promoters correspond to evolutionary steps of a duplicated gene. *J Biol Chem* **266**:15377–15383.
- Johnsson N and Varshavsky A (1994) Split ubiquitin as a sensor of protein interactions in vivo. *Proc Natl Acad Sci USA* **91**:10340–10344.
- Junot C, Gonzales MF, Ezan E, Cotton J, Vazeux G, Michaud A, Azizi M, Vassiliou S, Yiotakis A, Corvol P, et al. (2001) RXP 407, a selective inhibitor of the N-domain of angiotensin I-converting enzyme, blocks in vivo the degradation of hemoregulatory peptide acetyl-Ser-Asp-Lys-Pro with no effect on angiotensin I hydrolysis. *J Pharmacol Exp Ther* **297**:606–611.
- Kohlstedt K, Brandes RP, Muller-Esterl W, Busse R, and Fleming I (2004) Angiotensin-converting enzyme is involved in outside-in signaling in endothelial cells. *Circ Res* **94**:60–67.
- Kohlstedt K, Busse R, and Fleming I (2005) Signaling via the angiotensin-converting enzyme enhances the expression of cyclooxygenase-2 in endothelial cells. *Hypertension* **45**:126–132.
- Kohlstedt K, Shoghi F, Muller-Esterl W, Busse R, and Fleming I (2002) CK2 phosphorylates the angiotensin-converting enzyme and regulates its retention in the endothelial cell plasma membrane. *Circ Res* **91**:749–756.
- Kost OA, Balyasnikova IV, Chemodanova EE, Nikolskaya II, Albrecht RF, and Danilov SM (2003) Epitope-dependent blocking of the angiotensin-converting enzyme dimerization by monoclonal antibodies to the N-terminal domain of ACE: possible link of ACE dimerization and shedding from the cell surface. *Biochemistry* **42**:6965–6976.
- Kost OA, Bovin NV, Chemodanova EE, Nasonov VV, and Orth TA (2000) New feature of angiotensin-converting enzyme: carbohydrate-recognizing domain. *J Mol Recognit* **13**:360–369.
- Lattion AL, Soubrier F, Allegrini J, Hubert C, Corvol P, and Alhenc-Gelas F (1989) The testicular transcript of the angiotensin I-converting enzyme encodes for the ancestral, non-duplicated form of the enzyme. *FEBS Lett* **252**:99–104.
- Lee AF, MacFadyen RJ, and Struthers AD (1999) Neurohormonal reactivation in heart failure patients on chronic ACE inhibitor therapy: a longitudinal study. *Eur J Heart Fail* **1**:401–406.
- Mancini GB, Henry GC, Macaya C, O'Neill BJ, Pucillo AL, Carere RG, Wargovich TJ, Mudra H, Luscher TF, Klibaner MI, et al. (1996) Angiotensin-converting enzyme inhibition with quinapril improves endothelial vasomotor dysfunction in patients with coronary artery disease. The TREND (Trial on Reversing ENdothelial Dysfunction) Study. *Circulation* **94**:258–265.
- Morishita R, Gibbons GH, Ellison KE, Lee W, Zhang L, Yu H, Kaneda Y, Ogihara T, and Dzau VJ (1994) Evidence for direct local effect of angiotensin in vascular hypertrophy. In vivo gene transfer of angiotensin converting enzyme. *J Clin Invest* **94**:978–984.
- Morishita R, Gibbons GH, Tomita N, Zhang L, Kaneda Y, Ogihara T, and Dzau VJ (2000) Antisense oligodeoxynucleotide inhibition of vascular angiotensin-converting enzyme expression attenuates neointimal formation: evidence for tissue angiotensin-converting enzyme function. *Arterioscler Thromb Vasc Biol* **20**:915–922.
- Nishimura K, Yoshida N, Hiwada K, Ueda E, and Kokubu T (1977) Purification of angiotensin I-converting enzyme from human lung. *Biochim Biophys Acta* **483**:398–408.
- Petrie MC, Padmanabhan N, McDonald JE, Hillier C, Connell JM, and McMurray JJ (2000) Antisense oligodeoxynucleotide inhibition of vascular angiotensin II generation in resistance arteries from patients with heart failure and coronary heart disease. *J Am Coll Cardiol* **37**:1056–1061.
- Scharlt M, Bocksch WG, Dreyse S, Beckmann S, Franke O, and Hunten U (1994) Remodeling of myocardium and arteries by chronic angiotensin converting enzyme inhibition in hypertensive patients. *J Hypertens Suppl* **12**:S37–S42.
- Schmechel A, Strauss M, Schlicksupp A, Pipkorn R, Haass C, Bayer TA, and Mülthaupt G (2004) Human BACE forms dimers and colocalizes with APP. *J Biol Chem* **279**:39710–39717.
- Soubrier F, Alhenc-Gelas F, Hubert C, Allegrini J, John M, Tregear G, and Corvol P (1988) Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. *Proc Natl Acad Sci USA* **85**:9386–9390.
- van Esch JHM, Tom B, Dive V, Batenburg WW, Georgiadis D, Yiotakis A, van Gool JMG, de Bruijn RJA, de Vries R, and Danser AHJ (2005) Selective angiotensin-converting enzyme C-domain inhibition is sufficient to prevent angiotensin I-induced vasoconstriction. *Hypertension* **45**:120–125.
- Wei L, Alhenc-Gelas F, Corvol P, and Clausen E (1991) The two homologous domains of human angiotensin I-converting enzyme are both catalytically active. *J Biol Chem* **266**:9002–9008.
- Wiemer G, Scholkens BA, Becker RH, and Busse R (1991) Ramiprilat enhances endothelial autacoid formation by inhibiting breakdown of endothelium-derived bradykinin. *Hypertension* **18**:558–563.
- Wittke S, Lewke N, Muller S, and Johnsson N (1999) Probing the molecular environment of membrane proteins in vivo. *Mol Biol Cell* **10**:2519–2530.
- Woodman ZL, Oppong SY, Cook S, Hooper NM, Schwager SL, Brandt WF, Ehlers MR, and Sturrock ED (2000) Shedding of somatic angiotensin-converting enzyme (ACE) is inefficient compared with testis ACE despite cleavage at identical stalk sites. *Biochem J* **347**:711–718.
- Xiao HD, Fuchs S, Campbell DJ, Lewis W, Dudley SC Jr, Kasi VS, Hoit BD, Keshelava G, Zhao H, Capecci MR, et al. (2004) Mice with cardiac-restricted angiotensin-converting enzyme (ACE) have atrial enlargement, cardiac arrhythmia and sudden death. *Am J Pathol* **165**:1019–1032.
- Zou MH, Shi C, and Cohen RA (2002) Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest* **109**:817–826.

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