CK2 Phosphorylates the Angiotensin-Converting Enzyme and Regulates Its Retention in the Endothelial Cell Plasma Membrane

Karin Kohlstedt, Firouzeh Shoghi, Werner Müller-Esterl, Rudi Busse, Ingrid Fleming

Abstract—Soluble angiotensin-converting enzyme (ACE) is derived from the membrane-bound form by proteolytic cleavage of its C-terminal domain. Because intracellular events might be involved in the regulation of the cleavage process, we determined whether the cytoplasmic tail of ACE is phosphorylated and whether this process regulates secretion. Immunoprecipitation of ACE (180 kDa) from 32P-labeled endothelial cells revealed that ACE is phosphorylated. Phosphorylation was not observed in endothelial cells overexpressing a mutant form of ACE (ACEΔS, all five cytoplasmic serine residues replaced by alanine). CK2 coprecipitated with ACE from endothelial cells, and CK2 phosphorylated both ACE and a peptide corresponding to the cytoplasmic tail. Mutation of serine2270 within the CK2 consensus sequence almost abolished ACE phosphorylation. In ACE-overexpressing endothelial cells, ACE was mostly localized to the plasma membrane. However, no ACE was detected in the plasma membrane of ACEΔS-overexpressing cells, although a precursor ACE (170 kDa) was prominent in the endoplasmic reticulum and the cell supernatant contained substantial amounts of the soluble protein (175 kDa). A correlation between ACE-phosphorylation and secretion was confirmed in endothelial cells treated with the CK2-inhibitor, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, which time-dependently decreased the phosphorylation of ACE and increased its shedding. These results indicate that the CK2-mediated phosphorylation of ACE regulates its retention in the plasma membrane and may determine plasma ACE levels. (Circ Res. 2002;91:749-756.)

Key Words: angiotensin-converting enzyme ■ casein kinase 2 ■ phosphorylation ■ protein cleavage ■ ACE secretase

The angiotensin-converting enzyme (ACE; kininase II) is a membrane-bound zinc metalloendopeptidase that is involved in the extracellular conversion of angiotensin I to angiotensin II as well as the hydrolysis of bradykinin. ACE exists in two distinct isoforms, somatic and testis ACE, which are transcribed from a single gene at alternative initiation sites. The activity of somatic ACE is thought to play a crucial role in blood pressure regulation as well as in processes involved in vascular remodeling; effects best highlighted by the beneficial effects of ACE inhibitors on vascular function and cardiovascular events. ACE is expressed mainly in endothelial cells although nonendothelial cells may represent an alternative source of the enzyme in some pathological conditions.

A metalloprotease, the so-called ACE secretase, cleaves ACE between Arg1203 and Ser1204 on the extracellular side of the transmembrane domain, to generate an C-terminal truncated, soluble form of the enzyme. The cleavage/secretion of testis ACE is reported to be a regulated process that can be stimulated by phorbol 12-myristate 13-acetate (PMA), a property that ACE shares with other ectoproteins such as tumor necrosis factor-α and β-amyloid precursor protein. Apart from the fact that somatic ACE is less efficiently cleaved than testis ACE, and that ACE shedding is sensitive to metalloprotease inhibitors, little or nothing is known about the mechanisms controlling the cleavage/secretion of somatic ACE from endothelial cells.

The aim of the present study was to determine whether or not the cytoplasmic tail of ACE is involved in the regulation of ACE shedding from endothelial cells and to elucidate the signal transduction events involved in this process.

Materials and Methods

The ACE monoclonal antibody (clone 9B9) used for immunoprecipitation and immunohistochemistry recognized the N-terminal domain of the enzyme and was from Chemicon International. The monoclonal antibody used for Western blotting also recognized the N-terminal domain and was provided by Dr Peter Bünning (Aventis, Frankfurt, Germany). The mono- and polyclonal antibodies recognizing the cytoplasmic tail of ACE (ACEct) were generated in mice and rabbits immunized with a synthetic peptide corresponding to

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Amino acids 1250 to 1277 in the ACE carboxy-terminus. The monoclonal antibody against the α-subunit of protein kinase CK2 as well as the specific CK2 substrate were from Calbiochem-Novabiochem. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and the active CK2 were from Upstate Biotechnology. Biotin-NHS was from Boehringer-Mannheim and all other substances were obtained from Sigma.

Cell Culture
Human umbilical vein endothelial cells or porcine aortic endothelial cells were isolated and cultured as described.16

ACE Mutants and Endothelial Cell Transfection
The human somatic ACE cDNA used16 was provided by Dr F. Alhenc-Gelas (Hôpital Lariboisière, Paris, France), and the point mutants were generated using a PCR-based mutagenesis kit (Stratagene). The mutation of Ser1250, Ser1263, and Ser1270 to alanine (ACE1S) was verified by sequencing. Porcine aortic endothelial cells were stably transfected with wild-type ACE, S1253A, S1263A, or S1270A using calcium phosphate precipitation or electroporation as described.17 After transfection, cells were selected by culturing in the absence of Geneticin G418 (500 μg/mL). Individual clones were picked after 2 weeks and ACE protein expression and activity were assessed.

Immunoblotting and Immunoprecipitation of ACE
Cells were lysed in Nonidet lysis buffer containing Tris/HC1 (pH 8.0, 20 mmol/L), NaCl (137 mmol/mL), β-glycerophosphate (25 mmol/L), glycerol (10% v/v), Na3P04, (2 mmol/L), okadaic acid (10 mmol/L), Na2VO4, (2 mmol/L), leupeptin (2 μg/mL), pepstatin A (2 μg/mL), trypsin inhibitor (10 μg/mL), phenylmethylsulfonyl fluoride (PMSF; 44 μg/mL), and Nonidet P40 (1% v/v), left on ice for 10 minutes and centrifuged at 10 000g for 10 minutes. After preclearing with protein A/G sepharose, ACE was immunoprecipitated from the cell supernatant or from whole-cell lysates with a mixture of protein A/G sepharose or anti-mouse IgG covered Dynabeads M-450 (DYNAL). Proteins in the cell supernatant or immunoprecipitate were heated with SDS-PAGE sample buffer and separated by SDS-PAGE, as described.18 In a number of experiments, cells were incubated with an ACE antibody (9B9; 60 minutes, 4°C) before cell lysis and immunoprecipitated proteins were detected using their respective antibodies as detailed in Results, and were visualized by enhanced chemiluminescence using a commercially available kit (Amer sham, Germany).

To reprobe Western blots with alternative antibodies, the nitrocellulose membranes were incubated at 50°C for 30 minutes in a buffer containing Tris/HC1 (67.5 mmol/L, pH 6.8), β-mercaptoethanol (100 mmol/L), and SDS (2%). After extensive washing, the filters were incubated in blocking buffer containing bovine serum albumin (3%), and subsequently with the primary antibody.

Biotinylation of Cell Surface ACE
Endothelial cells overexpressing ACE or ACE1S were washed 4 times with phosphate buffered saline (PBS) containing CaCl2 (0.1 mmol/L) and MgCl2 (1 mmol/L) and incubated in biotinylation buffer (in mmol/L: tri-ethanolamine 10, pH 8.9, CaCl2 2, NaCl 125, and biotin-NHS 4) for 30 minutes at 4°C. As a negative control, some cells were treated in an identical fashion but incubated in a buffer lacking biotin. The reaction was stopped by replacing the biotinylation buffer with PBS containing NH4Cl (50 mmol/L), to bind all free biotin. After extensive washing, the cells were lysed in Nonidet lysis buffer. An aliquot (30 μL) of the whole cell lysate was removed for Western blotting before biotinylated proteins were precipitated using streptavidin-agarose beads (2 hours at 4°C, Sigma). After washing 3 times with Nonidet-lysis buffer, the agarose beads were boiled in SDS-sample buffer and subjected to SDS-PAGE. Biotinylated cell surface bound ACE was identified by Western blotting with an anti-ACE antibody.

Metabolic Labeling
Endothelial cells were extensively washed and incubated for 30 minutes in phosphate-free Tyrode’s solution (mmol/L: NaCl 132, KC1 4, CaCl2, 1.6, MgCl2 0.98, NaHCO3 11.9, and glucose 10). This was replaced by Tyrode solution containing [3H]P04 (0.125 mCi/mL), and cells were incubated for a further 12 hours as detailed in Results. ACE was immunoprecipitated from cells and cell supernatants as described and phosphorylation determined by autoradiography. The phosphorylation of ACE was quantified by scanning densitometry, and the radioactive signal was normalized with respect to the immunoprecipitated ACE protein.

Purification of Proteins Associated With the Cytoplasmic Tail of ACE Using Affinity Columns
Two peptides corresponding to the cytoplasmic tail of human wild-type ACE (RLSIRHRSLHR1SHGQPSFSEVELRHS; ACEct) and a peptide in which all of the serine residues were replaced with aspartate (Asp-ACEct) were immobilized by attachment to sepharose (activated CH-sepharose 4B, Amersh am Biosciences) via the N-terminal amino group (Eurogentec).

Endothelial cells were harvested from freshly isolated porcine aortas and lysed in Nonidet-lysis buffer. Equal amounts of protein (700 μg in 500 μL) were incubated with 50 μg of the sepharose-coupled ACEct or Asp-ACEct peptides (60 minutes, 4°C). After incubation, the incubates were packed into columns (Poly-Prep, Bio-Rad) and washed 4 times with Nonidet-lysis buffer. Proteins attached to the columns were eluted with a concentrated salt solution (100 μL Nonidet-lysis buffer containing 1 mol/L NaCl), and the volume reduced by boiling in SDS-sample buffer. Thereafter, proteins were separated by SDS-PAGE and analyzed by Western blotting with respective antibodies.

CK2 Activity Assay and In Vitro Phosphorylation of ACE
ACE-associated CK2 activity was determined using ACE immunoprecipitated from unstimulated endothelial cells. Precipitates were washed several times in CK2 assay buffer (Tris/HC1 40 mmol/L, pH 7.9: NaCl 150 mmol/mL, MgCl2 20 mmol/L, Na2VO4, 2 mmol/L, leupeptin 2 μg/mL, okadaic acid 10 mmol/L, pepstatin A 2 μg/mL, trypsin inhibitor 10 μg/mL, and PMSF 44 μg/mL) and resuspended in 25 μL CK2 assay buffer containing 10 μg of a specific CK2 substrate19 in the absence and presence of DRB (100 μmol/L in 0.1% DMSO). The kinase reaction was initiated by addition of 10 μCi of [γ-32P]ATP and 20 μmol/L ATP and proceeded at 30°C for 30 minutes. The reaction was stopped by transfer of the supernatant onto Whatman P-81 cellulose followed by extensive washing with 1% (v/v) phosphoric acid. Phosphopeptide bound to the paper strips was quantified by scintillation counting, and the results are expressed relative to the activity measured under control conditions.

In vitro phosphorylation of the immunobilized ACEct peptide, the Asp-ACEct peptide, or ACE immunoprecipitated from unstimulated endothelial cells by CK2 were assayed as described but in the presence of a constitutively active CK2 (50 ng; Upstate biotechnology, Lake Placid, NY). The reaction was initiated by the addition of 10 μCi of [γ-32P]ATP and 20 μmol/L ATP and proceeded at 30°C for 30 minutes. The reaction was stopped by addition of SDS-PAGE sample buffer. After SDS-PAGE the incorporation of 32P was assessed by scanning densitometry and normalized to the ACEct, Asp-ACEct, or ACE protein content.

Immunofluorescence
Endothelial cells were grown on glass coverslips and stimulated as described. Thereafter, cells were fixed with formaldehyde (2% in phosphate buffered saline) and washed twice with glycerin (2% in PBS) and twice in PBS. Some cells were permeabilized with Triton X-100 (0.2% v/v) before incubation with the monoclonal anti-ACE antibody (Clone 9B9; 1:1000) followed by a fluorescein-conjugated anti-mouse IgG (1:300 in PBS; Molecular Probes) for 1 hour each. Preparations were mounted with ProLong Antifade kit (Molecular Probes) and viewed using a confocal microscope.
Results

Expression and Phosphorylation of ACE in Endothelial Cells

The short cytoplasmic tail of ACE contains five serine residues, three of which are potentially phosphorylatable by either protein kinase C (PKC, Ser1253), protein kinase A (PKA, Ser1263), or CK2 (Ser1270; Figure 1). Two forms of ACE can be detected in endothelial cells, a 170-kDa immature or precursor form (preACE) mostly localized to the Golgi apparatus, and a 180-kDa mature enzyme (plasma membrane ACE, pmACE), which is inserted into the plasma membrane (Figure 2A). Inhibiting the trafficking of proteins between the endoplasmic reticulum, Golgi apparatus, and the plasma membrane using brefeldin A (5 μg/mL, 5 hours) markedly reduced the formation of the pmACE form (Figure 2A).

To determine whether or not ACE can be phosphorylated, porcine aortic endothelial cells stably transfected with human somatic ACE were labeled with 32P. Immunoprecipitation of ACE from these cells, revealed that the 180-kDa pmACE is phosphorylated (Figure 2B). The PKC inhibitor RO 31-8220 (30 nmol/L, 15 minutes to 1 hour) did not attenuate pmACE phosphorylation (data not shown), whereas the CK2 inhibitor DRB (100 μmol/L) decreased pmACE phosphorylation (Figure 2B). Identical results were obtained using primary cultures of human endothelial cells that constitutively express ACE.

CK2 Associates With and Phosphorylates the Cytoplasmic Tail of ACE

A CK2 protein doublet was coprecipitated with ACE from lysates of ACE-overexpressing porcine endothelial cells, whereas only a weak signal was observed using nontransfected cells (Figure 3A). CK2 could also be recovered from lysates of freshly isolated native porcine endothelial cells using an affinity column consisting of the sepharose-coupled ACE cytosolic tail peptide (ACEct; Figure 3B). No CK2 bound to the Asp-ACEct affinity column in which all of the serine residues were replaced with aspartate (Figure 3B). No evidence was obtained to suggest that PKC could be either coprecipitated with ACE or bound to the affinity column. The ACE-associated CK2 was active, and in an in vitro kinase assay, ACE immunoprecipitates catalyzed the phosphorylation of a specific CK2 peptide substrate. DRB significantly decreased ACE-associated CK2 activity (Figure 3C).

To demonstrate that CK2 can phosphorylate ACE, a constitutively active CK2 was incubated with [γ-32P]ATP and either pmACE, immunoprecipitated from porcine endothelial cells (Figure 4A), the ACEct peptide, or the Asp-ACEct peptide (Figure 4B). In both assay systems, CK2 phosphorylated the ACEct peptide (Figure 4B) but not the Asp-ACEct peptide (Figure 4B).
ylated ACE, and this was prevented by DRB. In the absence of CK2, ACEct was not phosphorylated, but a low level of ACE phosphorylation was observed when ACE immunoprecipitates were used. The latter observation could be attributed to the fact that the CK2 coprecipitated with the enzyme exhibited a residual activity. CK2 failed to phosphorylate the Asp-ACEct peptide (Figure 4B). When the in vitro phosphorylation studies were performed using a constitutively active PKC instead of CK2, neither the ACE immunoprecipitated from endothelial cells nor the ACEct peptide were phosphorylated.

**Figure 3.** Active CK2 associates with ACE. A, Representative Western blots (in duplicate) showing the association of CK2 with ACE. ACE immunoprecipitates from control (−ACE) and ACE overexpressing (+ACE) porcine aortic endothelial cells were subjected to SDS-PAGE and CK2 was identified using a specific antibody. Rat brain homogenate was used as a positive control (pc) for CK2. B, Representative Western blots (in duplicate) showing the association of CK2, derived from lysates of native porcine aortic endothelial cells, with the ACEct and Asp-ACEct affinity columns. Identical results were obtained in two additional experiments. C, ACE was immunoprecipitated from primary cultures of human umbilical vein endothelial cells and the immunoprecipitates were incubated with [γ-32P]ATP and a specific CK2 substrate (CK2 sub) in the absence (CTL) and presence of DRB. Results from 6 different experiments were quantified as the phosphorylation of the CK2 substrate under both conditions; ***P<0.001 vs control.

Dephosphorylated pmACE Is Not Retained in the Plasma Membrane

Because the cleavage/secrection of ACE is a regulated process, we assessed whether or not phosphorylation regulates ACE secretion. ACE overexpressing endothelial cells were labeled with 32P and, using an antibody that recognizes the N-terminal extracellular region of the protein, ACE was immunoprecipitated from the culture medium as well as from cell lysates. ACE was recovered from both samples, and although pmACE was clearly phosphorylated, solACE was not (Figure 5A). Reprobing the Western blots with two antibodies that specifically recognize the cytoplasmic tail of ACE only detected ACE in the cell lysate confirming that solACE is a C-terminal truncated form of the enzyme. To assess whether a link exists between CK2 and ACE secretion, ACE overexpressing endothelial cells were incubated with either solvent (0.1% DMSO) or DRB for up to 8 hours and the generation of solACE was analyzed. The CK2 inhibitor induced a 16- to 20-fold increase in ACE secretion by endothelial cells (Figure 5B).

A similar effect of DRB could be demonstrated using confluent primary cultures of human endothelial cells. As mentioned above, the majority of ACE in such cells is incorporated into the plasma membrane. Incubation with DRB (100 μmol/L, 8 hours) decreased the presentation of ACE at the cell surface and increased the amount of ACE that could be recovered from the cell supernatant (Figure 6). DRB did not significantly affect total cellular levels of the protein.

To determine whether the effects of DRB on the cleavage/secrection of ACE could be attributed to its effect on ACE phosphorylation, a nonphosphorylatable form of ACE (ACEΔS) was generated in which all five serine residues in the cytoplasmic tail were replaced by alanine. In confluent porcine endothelial cells overexpressing the wild-type ACE (wtACE), most of the protein was incorporated into the plasma membrane, although low amounts of preACE and solACE could be detected. However, in endothelial cells stably expressing the ACEΔS, no pmACE could be detected (Figure 7A). To confirm the lack of membrane integration, pmACE was either selectively labeled with a monoclonal antibody directed against the N-terminal domain of ACE, or by biotin, before harvesting and permeabilization. Although this procedure resulted in the recovery of pmACE from endothelial cells expressing the wild-type enzyme, no pmACE could be detected in cells expressing ACEΔS (Figure 7B).

Similar results were obtained using immunohistochemical methods and although ACE was clearly present on the extracellular surface of ACE-expressing endothelial cells, no signal could be detected in ACEΔS-expressing cells unless the cells were permeabilized with Triton X-100 (Figure 7C).

**Mutation of Ser1270 Abrogates pmACE Phosphorylation and Enhances ACE Secretion**

Given that the CK2 inhibitor reduced 32P incorporation into ACE and promoted the cleavage of pmACE, and that the ACEΔS mutant was not inserted into the plasma membrane, we determined whether or not Ser1270 (which is within the CK2 consensus sequence) plays a crucial role in regulating the membrane retention of ACE. A point mutant (Ser1270 replaced by alanine; S1270A) was therefore expressed in
porcine endothelial cells. The S1270A protein incorporated into the endothelial cell plasma membrane, albeit to a lesser extent than the wild-type enzyme, and pmACE could be immunoprecipitated from both cell types (Figure 8A). However, in contrast to wtACE, the S1270A immunoprecipitated from 32P-labeled endothelial cells was not phosphorylated. Secretion of the S1270A was significantly greater than that of wtACE under control conditions (Figure 8B). Moreover, although PMA (300 nmol/L, 8 hours) enhanced the cleavage secretion of wtACE as well as S1270A, DRB (100 μmol/L, 8 hours) accelerated the cleavage/secretion of only the wtACE and did not significantly increase the secretion of S1270A.

Discussion
The results of the present study indicate that the cytoplasmic tail of ACE expressed in endothelial cells is phosphorylated by an associated CK2 and that the cleavage/secretion of ACE is regulated by changes in CK2 activity and the phosphorylation of a specific serine residue, Ser1270.

Only three of the five serine residues within the cytoplasmic tail of ACE are located within known sequence motifs for kinases, ie, Ser1253 is a theoretical substrate for PKC, Ser1263 for PKA, and Ser1270 for CK2. However, the results of the present study indicate that the main site of ACE phosphorylation is Ser1270 and that phosphorylation can be attributed to the activity of ACE-associated CK2. Indeed, we observed that incubation of primary cultures of human umbilical vein endothelial cells as well as porcine endothelial cells stably transfected with the wild-type human somatic ACE with a CK2 inhibitor markedly decreased ACE phosphorylation. It is sometimes difficult to interpret the effects of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) on signaling processes as the inhibition of CK2 activity in endothelial cells can affect protein biosynthesis. However, we were able to show that CK2 coprecipitates with ACE from endothelial cells and that the ACE-associated CK2 is active and sensitive to DRB. Moreover, CK2 was able to phosphorylate both native ACE immunoprecipitated from endothelial cells as well as a peptide corresponding the cytoplasmic tail in vitro.

The major consequence of ACE phosphorylation by CK2 appears to be a change in the rate of the basal cleavage/secretion of the enzyme and the subsequent accumulation of ACE.
a C-terminal truncated solACE in the cell supernatant. Indeed, both CK2 inhibition with DRB as well as the mutation of Ser1270 were associated with an increase in solACE levels. The enhanced basal secretion of the S1270A mutant was the only apparent difference from the wild-type enzyme, and unlike the ACE/H9004 S mutant in which all of the serine residues were substituted by alanine, the S1270A protein did mature normally to pmACE and become inserted into the plasma membrane.

The stability of several other proteins and protein complexes is known to be modulated by CK2. For example, the phosphorylation of the lipid phosphatase, PTEN, by CK2 is reported to negatively regulate its activity, hinder its incorporation into a PTEN-associated complex, and prevent proteasomal degradation of the protein. CK2 can also phosphorylate E-cadherin and the disruption of the adherens junction is associated with a decreased phosphorylation of E-cadherin by CK2. Interestingly, the only other type I ectoprotein that is reported to be regulated by CK2 is the CD10/neutral endopeptidase which also plays a key role in the degradation of bradykinin in endothelial cells.

Although PKC has been reported to associate with testis ACE, we were unable to detect any such association in endothelial cells. Moreover, in vitro phosphorylation assays, a constitutively active PKC did not phosphorylate either the native ACE protein or the cytoplasmic tail peptide.

PKC has however been implicated in the regulation of ACE shedding as PMA stimulates the cleavage/secretion of somatic ACE, albeit to a much lesser extent than the cleavage/secretion of testis ACE. This effect is unlikely to be direct and most probably reflects the activation of the ACE secretase. Indeed, zinc metalloproteases are known to be activated by PMA, and the PMA-induced activation of the ACE secretase in the ACE 89 mouse epithelial cell line and ACE-expressing Chinese hamster ovary cells is related to the activation of PKC. Moreover, because the basal produc-
tion of solACE is insensitive to metalloprotease inhibitors such as batimastat, which effectively inhibit the shedding of ACE stimulated by PMA or by ACE antibodies, it appears that the stimulated cleavage/secretion of ACE can be attributed to the activation of such a metalloprotease while the phosphorylation of the ACE cytoplasmic tail by CK2, regulates the basal shedding of the enzyme. Such a hypothesis could account for our observation that although the basal secretion of the S1270A protein was accelerated, this process could still be stimulated by PMA.

Plasma ACE levels have recently been suggested to represent a risk factor for coronary stent restenosis, coronary artery disease, and myocardial infarction. Indeed, elevated plasma ACE activity, determined less than 4 hours after the onset of myocardial infarction in humans, has been suggested to be a significant predictor of the development of left ventricular dilation one year after infarction. Our observations suggest that the phosphorylation of ACE by CK2 enhances the stability of the enzyme in the plasma membrane. However, it remains to be determined whether or not changes in the phosphorylation of ACE Ser by CK2 modulate plasma ACE levels in vivo.

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