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Signaling via the Angiotensin-Converting Enzyme Results in the Phosphorylation of the Nonmuscle Myosin Heavy Chain IIA

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ABSTRACT

The phosphorylation of the short C-terminal cytoplasmic domain of the somatic angiotensin-converting enzyme (ACE) is involved in the regulation of enzyme shedding. We determined whether the phosphorylation of the cytoplasmic domain of ACE (ACEct) on Ser1270 regulates the cleavage/secretion of the enzyme by affecting its association with other proteins. ACE was associated with β-actin and the nonmuscle myosin heavy chain IIA (MYH9) in endothelial cells, as determined by coimmunoprecipitation experiments as well as an ACEct affinity column. The ACE-associated MYH9 immunoprecipitated from ^{32}P-labeled endothelial cells was basally phosphorylated and cell stimulation with ACE inhibitors, or with bradykinin, increased the phosphorylation of MYH9. Casein kinase 2 (CK2) but not protein kinase C phosphorylated MYH9 in vitro, CK2 coprecipitated with MYH9 from endothelial cells and the phosphorylation of MYH9 in intact cells paralleled the phosphorylation of ACE on Ser1270 by CK2. The CK2 inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole attenuated the phosphorylation of ACE and MYH9, disrupted their association, and enhanced the cleavage/secretion of ACE from the plasma membrane. Cytochalasin D decreased the interaction between ACE and MYH9 and stimulated ACE shedding. Although MYH9 was still able to associate with residual amounts of a nonphosphorylatable S1270A ACE mutant, no ACE inhibitor-induced increase in MYH9 phosphorylation could be detected in S1270A-expressing cells. These data indicate that the interaction of ACE with MYH9 determines ACE shedding and is modulated by phosphorylation processes. Furthermore, because ACE inhibitors affect the phosphorylation of MYH9, the phosphorylation of this class II myosin might contribute to the phenomenon of ACE signaling in endothelial cells.

The angiotensin-converting enzyme (ACE) is a type I transmembrane protein composed of a large N-terminal extracellular domain and a short cytoplasmic tail. Although only one gene encodes ACE, it contains two transcription initiation sites (Kumar et al., 1991), and thus two isoforms of the protein exist: a somatic isoform that possesses two catalytic sites and a germinal or testes isoform that contains only one (Kumar et al., 1991). Both ACE isoforms can be found as soluble enzymes in plasma/seminal fluid and are generated by enzymatic cleavage of the C-terminal tail (Ramchandran et al., 1994).

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The somatic isoform of ACE is mainly expressed in the vascular endothelium and because it catalyzes the formation of the potent vasoconstrictor angiotensin II as well as the degradation of the vasodilator bradykinin (Jaspard et al., 1992), it is assumed to play an important role in the regulation of blood pressure. Indeed, a variety of animal studies and clinical trials have shown that the inhibition of somatic ACE may prevent the decrease in the bioavailability of nitric oxide associated with "endothelial dysfunction" and/or restore endothelial function (Dzau et al., 2002). The physiological/pathophysiological relevance of soluble ACE is unclear (Xiao et al., 2004) and although a point mutation in the juxtamembrane stalk of ACE causes a dramatic increase in serum ACE levels but no clinical abnormalities (Kramers et al., 2001), elevated plasma ACE levels have been reported to represent a risk factor for cardiovascular diseases (Cambien et al., 1994; Oosterga et al., 1997).

The molecular mechanisms that regulate the cleavage of

ABBREVIATIONS: ACE, angiotensin-converting enzyme; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; RO 31-8220, 3-[1-[3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl) maleimide (bisindolylmaleimide IX); PMA, phorbol 12-myristate 13-acetate; HUVEC, human umbilical vein endothelial cell(s); PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; MYH9, nonmuscle myosin heavy chain IIA; ACEct, cytoplasmic tail of human somatic ACE; PBS, phosphate-buffered saline; JNK, c-Jun N-terminal kinase; CK2, casein kinase 2; CaMKII, calmodulin-dependent kinase II; PKC, protein kinase C.

ACE are unknown, as is the identity of the ACE secretase, which shares some properties with other membrane protein cleaving secretases in that it can be activated by phorbol esters and is sensitive to metalloprotease inhibitors (Ramchandran et al., 1994). We reported recently that the serine phosphorylation of the cytoplasmic tail of ACE by the kinase CK2 can also regulate the shedding process via mechanism independent of the secretase (Kohlstedt et al., 2002). Indeed, the replacement of all five of the serine residues in the cytoplasmic tail of human somatic ACE by alanine was sufficient to exclude it completely from the plasma membrane (Kohlstedt et al., 2002). Preventing the phosphorylation of ACE on Ser1270 however accelerated the cleavage secretion of the enzyme to an extent similar to that reported for a deletion mutant in which the entire cytoplasmic domain of testis ACE was deleted (Chubb et al., 2004).

The aim of this investigation was to determine whether the phosphorylation of the cytoplasmic domain of ACE regulates the cleavage/secretion of the enzyme by determining its association with other proteins. Experiments were performed in primary cultures of human endothelial cells that express ACE, as well as in three porcine aortic endothelial cell lines. These endothelial cells were ACE-deficient or they overexpressed either human somatic ACE or a nonphosphorylatable ACE mutant in which Ser1270 was replaced by alanine (S1270A).

Materials and Methods

Materials. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was from Upstate Biotechnology (Lake Placid, NY), whereas RO 31-8220 and phorbol 12-myristate 13-acetate (PMA) were from Calbiochem-Novabiochem (Bad Soden, Germany). All other substances were obtained from Sigma (Munich, Germany).

Cell Culture. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described previously (Busse and Lamontagne, 1991). As ACE expression decreases with time in culture, all of the experiments in human endothelial cells were performed using primary cultures. The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki (World Medical Association, 1997). Porcine aortic endothelial cells stably transfected with ACE or the S1253A, S1263A, or S1270A ACE mutants were generated and cultured as described previously (Kohlstedt et al., 2002). Although the porcine endothelial cells no longer endogenously expressed ACE or functional angiotensin II and bradykinin receptors, they expressed a number of characteristic endothelial cell proteins (von Willebrand factor, CD31, the endothelial nitric-oxide synthase, and vascular/endothelial cadherin).

Immunoblotting and Immunoprecipitation of ACE and MYH9. Cells were lysed in Nonidet lysis buffer containing 20 mM Tris/HCl, pH 8.0, 137 mM NaCl, 25 mM β-glycerophosphate, 10% glycerol (v/v), 2 mM $Na_4P_2O_7$, 10 nM okadaic acid, 2 mM Na_3VO_4, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 10 μg/ml trypsin inhibitor, 44 μg/ml phenylmethylsulfonyl fluoride, and 1% Nonidet P-40 (v/v), left on ice for 10 min and centrifuged at 10,000*g* 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. Proteins in the cell supernatant or immunoprecipitates were heated with SDS-PAGE sample buffer and separated by SDS-PAGE as described previously (Fleming et al., 1998). Proteins were detected using their respective antibodies, and visualized by enhanced chemiluminescence using a commercially available kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). In some experiments, either gels or PVDF membranes were stained with silver or Coomassie Brilliant Blue, and the excised bands were identified by sequencing.

The ACE monoclonal antibody used for immunoprecipitation and immunohistochemistry was from Chemicon International (Temecula, CA). The monoclonal antibody used for Western blotting was provided by Dr. Peter Bünning (Sanofi-Aventis, Frankfurt, Germany), and the antibody against nonmuscle myosin heavy chain (MYH9) was from DPC Biermann (Bad Nauheim, Germany).

To analyze the association between ACE and MYH9 in vivo in male mice (C57 black 6, 6 weeks old; Charles River Laboratories, Wilmington, MA), mice were anesthetized (isofuran 1.5%) and euthanized by a transverse cut through the large abdominal vessel. The lungs were perfused rapidly with ice-cold phosphate-buffered saline and snap-frozen, homogenized, and lysed in Nonidet lysis buffer, before ACE was immunoprecipitated as described above.

Purification of Proteins Associated with the Cytoplasmic Tail of ACE Using Affinity Columns. A peptide corresponding to the cytoplasmic tail of human somatic ACE (ACEct) was immobilized by attachment to Sepharose. HUVEC were lysed in Nonidet P-40 lysis buffer, and equal amounts of protein (700 μg in 500 μl) were incubated with 50 μg of the Sepharose-coupled ACEct peptide as described previously (Kohlstedt et al., 2002). Thereafter, the ACEct-Sepharose was recovered and boiled in SDS sample buffer, and ACEct-associated proteins were separated by SDS-PAGE and analyzed by Western blotting.

Protein Identification. Bands were excised from Coomassie Blue-stained PVDF blots, blocked with polyvinylpyrrolidone (2 mg/ml in methanol for 2 h at room temperature) and extensively washed with water. Enzymatic digestion was performed in NH_4HCO_3 (50 mM; pH 8.0) adding 0.5 μg trypsin (Promega) for 16 h at 37°C. An aliquot (1 μl) of the supernatant was spotted onto a sample plate with 1 μl of matrix [α-cyano-4-hydroxycinnamic acid, 8 mg/ml in 50% (v/v) acetonitrile, and 1% (v/v) trifluoroacetic acid]. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry acquisition was performed on a Voyager STR mass spectrometer (Applied Biosystems, Foster City, CA) set to reflectron mode. Known trypsin auto-cleavage peptide masses (842.51 and 2211.10 Da) were used for a two-point internal calibration for each spectrum. Monoisotopic peptide masses were searched against the theoretical peptide masses of all human proteins in the Swiss-Prot and TrEMBL protein databases using the Mascot search program (Matrix Science Ltd., London, UK). Another aliquot of 10-μl supernatant was separated by reversed-phase high-performance liquid chromatography, and fractions were analyzed by N-terminal sequencing using an Edman Sequenator Procise 434 (Applied Biosystems).

Immunofluorescence. HUVEC were grown to confluence and fixed with formaldehyde (2% in phosphate-buffered saline, PBS), washed twice with glycine (2% in PBS) and then twice in PBS. Cells were permeabilized with 0.2% Triton X-100 (v/v) before incubation with the anti-ACE or anti-MYH9 antibodies followed by fluorescein- or Texas Red-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) for 1 h each. Preparations were mounted with ProLong Antifade kit (Invitrogen) and viewed using a confocal microscope.

Metabolic Labeling. Endothelial cells were labeled with ^{32}P as described previously (Kohlstedt et al., 2002) for 12 h. ACE was immunoprecipitated, and its phosphorylation was determined by autoradiography. The phosphorylation of ACE and ACE-associated MYH9 were quantified by scanning densitometry and the radioactive signal was normalized with respect to the immunoprecipitated ACE protein.

In Vitro Phosphorylation of MYH9. MYH9 was immunoprecipitated from unstimulated HUVEC. Precipitates were washed several times in the respective kinase assay buffer, to assess the activity of c-Jun N-terminal kinase (JNK) (Kohlstedt et al., 2004), CK2 (Kohlstedt et al., 2002), calmodulin-dependent kinase II (CaMKII; 20 mM MOPS, pH 7.2, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 1 mM DTT, and 6.7 μM calmodulin) or protein kinase C (PKC; 0 mM Tris/HCl, pH 7.5, 5

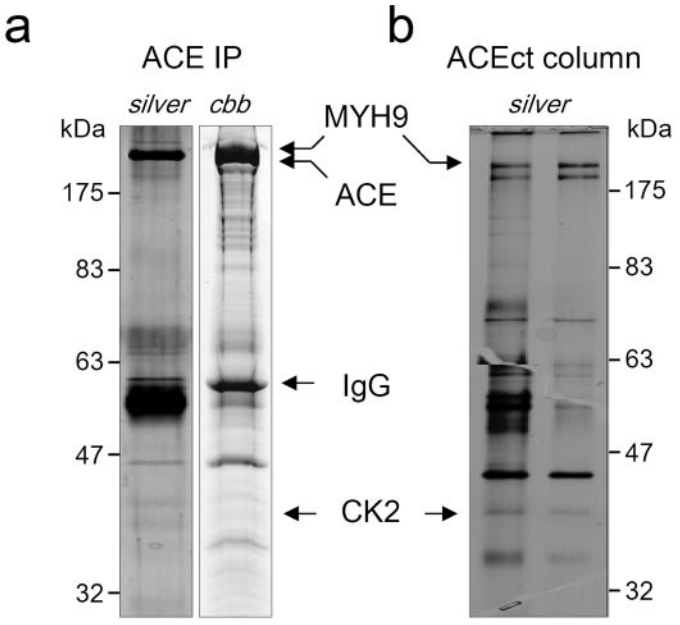
mM $CaCl_2$, and 100 mM $MgCl_2$). Immunoprecipitates were resuspended in 25 μl of assay buffer, and the relevant active kinases were added [i.e., active JNK (immunoprecipitated from endothelial cells stimulated with 1 μM anisomycin for 1 min), active CaMKII (immunoprecipitated from endothelial cells stimulated with 1 μM ionomycin for 1 min), 50 ng of constitutively active PKC (Calbiochem), or 50 ng of CK2 (Upstate Biotechnology, Lake Placid, NY)]. Before use, the activity of the kinases used was assessed by testing their ability to phosphorylate known substrates [i.e., acetylated myelin basic protein for PKC, glutathione *S*-transferase-c-Jun for JNK, and CaMKII substrate (Calbiochem-Novabiochem) for CaMKII (data not shown)]. The kinase reactions were initiated by addition of 10 μCi of [γ-^{32}P]ATP and 20 μM ATP and proceeded at 30°C for 30 min. The reaction was stopped by the addition of SDS-PAGE sample buffer. After SDS-PAGE, the incorporation of ^{32}P was assessed by scanning densitometry and normalized to the protein content.

CK2 Activity Assay. ACE-associated CK2 activity was determined using ACE immunoprecipitated from endothelial cells as described previously (Kohlstedt et al., 2002).

Statistical Analysis. Data are expressed as 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

Identification of ACE-Associated Cytoskeletal Proteins in Endothelial Cells. ACE was immunoprecipitated from ACE-overexpressing porcine aortic endothelial cells, and the coprecipitated proteins were separated by SDS-PAGE and visualized either by Coomassie Brilliant Blue



c

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SDEEVDGKADGAEAKPAE

Fig. 1. Identification of nonmuscle myosin heavy chain IIA (MYH9) as an ACE-associated protein. Representative silver staining (silver) and Coomassie Brilliant Blue staining (cbb) of ACE immunoprecipitates (ACE IP) from porcine aortic endothelial cells overexpressing human somatic ACE (a) or proteins extracted from an ACEct affinity column loaded with HUVEC lysates (b). c, the peptide mass fingerprint analysis of the 225-kDa band identified MYH9 as ACE- or ACEct-associated protein. Peptides detected are highlighted (bold, underlined) in the protein sequence. The tryptic peptide KFDQLLAEEK (molecular mass of 1219.64 Da; larger font and italics) was isolated and N-terminally sequenced for verification.

staining or silver staining (Fig. 1a). In a parallel approach, HUVEC lysates were loaded onto an ACEct-affinity column, and the bound proteins were separated by SDS-PAGE and visualized by silver staining (Fig. 1b). In addition to CK2 [previously identified by Western blot analysis (Kohlstedt et al., 2002)], several proteins of similar molecular masses were recovered using both protocols. A prominent protein band of approximately 225 kDa was detected in ACE immunoprecipitates as well as associated with the ACEct column.

To identify the 225-kDa protein, ACE was immunoprecipitated from HUVEC, and the coprecipitated 225-kDa protein was recovered. Peptide mass fingerprinting was used to identify the 225-kDa ACE-associated protein as nonmuscle myosin heavy chain 9 (MYH9; gi:12667788). The tryptic peptide KFDQLLAEEK (1219.64 Da) was isolated from these samples and N-terminally sequenced for verification (Fig. 1c).

Association and Colocalization of ACE with MYH9 in Endothelial Cells. We next analyzed the intracellular localization of ACE and MYH9 by immunofluorescence using specific antibodies. Fluorescent labeling of the proteins revealed that ACE and MYH9 are colocalized in the plasma membrane of HUVEC (Fig. 2a). ACE and β-actin coprecipitated with MYH9 from human endothelial cells (Fig. 2b). β-Actin may account for the approximately 42-kDa protein detected in ACE immunoprecipitates as well as in the ACEct column eluates (see Fig. 1). In the reverse experiment, MYH9 coprecipitated with ACE from ACE-overexpressing porcine aortic endothelial cells but not from ACE-deficient cells (Fig. 2c). Given that identical results were obtained using HUVEC and the porcine aortic endothelial cell line, the association of ACE with β-actin and MYH9 was not simply an artifact related to the overexpression system. Moreover, MYH9 also associates with ACE in vivo as demonstrated by coprecipitation of MYH9 with ACE from murine lungs (Fig. 2d).

Effect of Ramiprilat on the Phosphorylation of ACE-Associated MYH9 in Endothelial Cells. ACE inhibitors elicit the time-dependent activation of ACE-associated CK2 and the rapid increase in the phosphorylation on ACE Ser1270 (Kohlstedt et al., 2004). Because the binding of MYH9 to the ACEct column suggested that ACE and MYH9 are linked intracellularly, and MYH9 can be regulated by phosphorylation (Brzeska and Korn, 1996), we determined the effects of ACE inhibitors on the phosphorylation of ACE-associated MYH9.

^{32}P-labeled HUVEC were stimulated with ramiprilat for 2 to 7 min, and ACE was immunoprecipitated (Fig. 3a). Ramiprilat not only transiently increased ACE phosphorylation but also stimulated the phosphorylation of the coprecipitated MYH9 (Fig. 3a). The example provided is overexposed to highlight the effects of ramiprilat on MYH9 phosphorylation but, as reported previously (Kohlstedt et al., 2004), the phosphorylation of ACE peaked 2 min after application of the ACE inhibitor. The time course of MYH9 phosphorylation was slightly different from that of ACE and peaked after 5 to 7 min, indicating that the phosphorylation of ACE preceded that of MYH9. Comparable effects were elicited by another ACE inhibitor, perindoprilat (data not shown).

Three of the five serine residues in the cytoplasmic tail of ACE (Ser1253, Ser1263, and Ser1270) are potentially phosphorylatable. In porcine aortic endothelial cells overexpressing either the wild-type ACE or S1253A or S1263A ACE mutants, ramiprilat enhanced the phosphorylation of ACE as well as that of the ACE-associated MYH9. However, in cells overexpressing the nonphosphorylatable S1270A ACE mutant, ramiprilat failed to elicit the phosphorylation of the ACE-associated MYH9 (Fig. 3b). The association of MYH9 with S1270A ACE was decreased in comparison with its association with the wild-type or S1253A and S1263A ACE mutants. The association of MYH9 with S1253A, S1263A, and S1270A ACE relative to that of MYH9 with wild-type ACE was 0.9 ± 0.2, 1.1 ± 0.2, and 0.3 ± 0.1, respectively ($p < 0.01$, $n = 4–6$). The decreased association of MYH9 with S1270A ACE might reflect a destabilization of ACE in the plasma membrane and therefore is consistent with our previous observation that the cleavage/secretion of this nonphosphorylatable ACE mutant is enhanced (Kohlstedt et al., 2002).

Effect of ACE Substrates on Phosphorylation of ACE-Associated MYH9. To determine whether ACE-associated MYH9 can also be phosphorylated after cell stimulation with ACE substrates, ^{32}P-labeled HUVEC were stimulated with bradykinin or angiotensin I. As reported previously, the bradykinin-induced phosphorylation of ACE peaked after 2 min (Kohlstedt et al., 2004), whereas the phosphorylation of ACE-

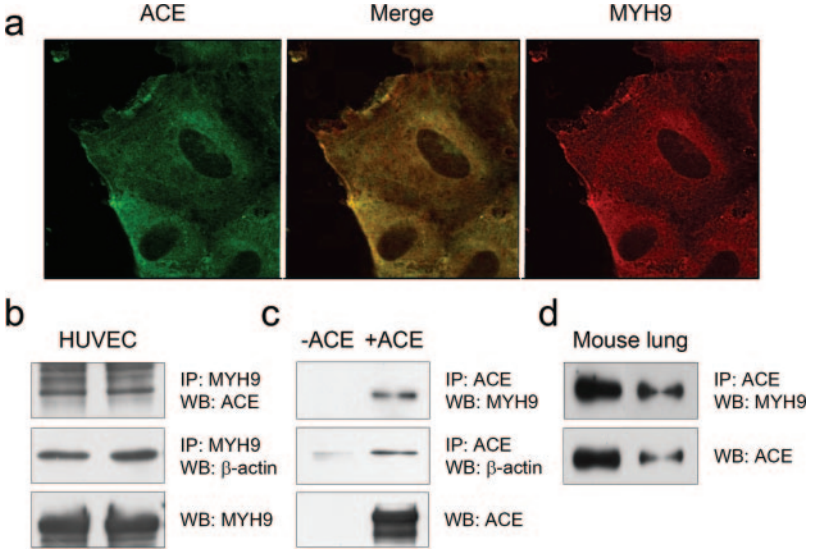

Fig. 2. Colocalization and precipitation of MYH9 and ACE from endothelial cells. a, immunohistochemical staining of ACE (green) and MYH9 (red) in HUVEC. b, representative Western blots showing the coprecipitation of ACE and β-actin with MYH9 from HUVEC (duplicates). c, coprecipitation of MYH9 and β-actin with ACE from ACE-deficient (−ACE) porcine aortic endothelial cells and cells overexpressing human somatic ACE (+ACE). Identical results were obtained in three additional experiments. d, coprecipitation of MYH9 with ACE from mouse lung (duplicates). Identical results were obtained using material from six mice.

associated MYH9 was slightly delayed and peaked after approximately 5 min (Fig. 4a). Again, the autoradiographs shown are overexposed to reveal the phosphorylation of MYH9, whereas nonsaturated examples were used for the densitometric analysis. Angiotensin I did not affect the phosphorylation of ACE-associated MYH9 (Fig. 4b).

Phosphorylation of ACE-Associated MYH9 Is Mediated via CK2. The protein sequence of MYH9 contains potentially phosphorylatable serine and threonine residues, mostly within recognition sequences for PKC or CK2 (Murakami et al., 1998), although phosphorylation by other kinases has been proposed. We therefore analyzed the effect of four kinases (PKC, CK2, JNK, and CaMKII) on the phosphorylation of MYH9 in vitro. Only CK2 was able to phosphorylate the MYH9 immunoprecipitated from porcine aortic endothelial cells (Fig. 5a). Stimulation of intact endothelial cells with the PKC activator PMA also failed to enhance the phosphorylation of ACE-associated MYH9 (Fig. 5b). However, PMA stimulated the cleavage/secretion of ACE, which resulted in its attenuated recovery. The PKC inhibitor RO 31-8220, on the other hand, enhanced the recovery of ACE from endothelial cells and slightly increased the phosphorylation of ACE-associated MYH9.

Because an interaction between PKC and CK2 has been described previously (Bren et al., 2000), we determined whether RO 31-8220 affects the activity of ACE-associated CK2. The CK2 that coprecipitated with ACE from porcine endothelial cells was active, and its activity was attenuated by DRB. Pretreatment of the endothelial cells with RO 31-8220 significantly increased the activity of CK2 (Fig. 5c), indicating that PKC may intrinsically attenuate the activity of ACE-associated CK2 and that CK2 may phosphorylate MYH9 in intact endothelial cells. Indeed, CK2 coprecipitated with MYH9 from porcine aortic endothelial cells (Fig. 5d) and DRB time-dependently attenuated both the basal phosphorylation of ACE and that of ACE-associated MYH9 (Fig. 5e). At the same time, the CK2 inhibitor increased the cleavage/secretion of ACE and thus reduced the recovery of ACE-associated MYH9. Similar results were obtained with a second CK2 inhibitor apigenin (data not shown).

Because not all PKC isoforms are sensitive to PMA or RO 31-8220, it is necessary to note that we were unable to detect the association of ACE with different PKC isoforms (α, β, γ, δ, ϵ, ζ, and η) by coimmunoprecipitation or using the ACEct affinity columns in either the ACE-overexpressing porcine endothelial cells or in HUVEC.

Effect of Colchicine and Cytochalasin D on Association between ACE and MYH9. To determine whether the interaction between MYH9, β-actin, and ACE is sensitive to disruption of the actin cytoskeleton or microtubule system, ACE-overexpressing porcine aortic endothelial cells were stimulated with colchicine or cytochalasin D before MYH9 was immunoprecipitated. Although colchicine failed to influence the interaction of the two proteins, disruption of the actin cytoskeleton with cytochalasin D attenuated the association of ACE with MYH9 (Fig. 6a). To determine whether this loss of physical interaction affects ACE cleavage/secretion, the cell supernatant was collected and soluble ACE was recovered by immunoprecipitation. Soluble ACE was detected in all samples, but significantly more ACE was recovered from the supernatant of cytochalasin D-treated cells than from the supernatant of cells treated with either solvent or colchicine (Fig. 6b). Reprobing the Western blots with an antibody raised against the ACEct peptide confirmed that the secreted protein lacked the C-terminal cytoplasmic sequence.

In the supernatant of cells expressing S1270A ACE, more soluble ACE (2- to 4-fold more) was detected than in the supernatant from wild-type cells, and neither colchicine nor cytochalasin D altered the cleavage/secretion of ACE (Fig. 6c) or the association of the residual membrane-bound nonphosphorylatable S1270A ACE mutant with MYH9 (Fig. 6d).

Discussion

The results of the present study demonstrate that the ectodomain shedding of somatic ACE is functionally regulated via the interaction of the ACE cytoplasmic tail with the actin cytoskeleton, more specifically with β-actin and MYH9.

A role for the actin cytoskeleton in anchoring ACE in the plasma membrane was inferred recently on the basis of the observation that cytochalasin D markedly increased the

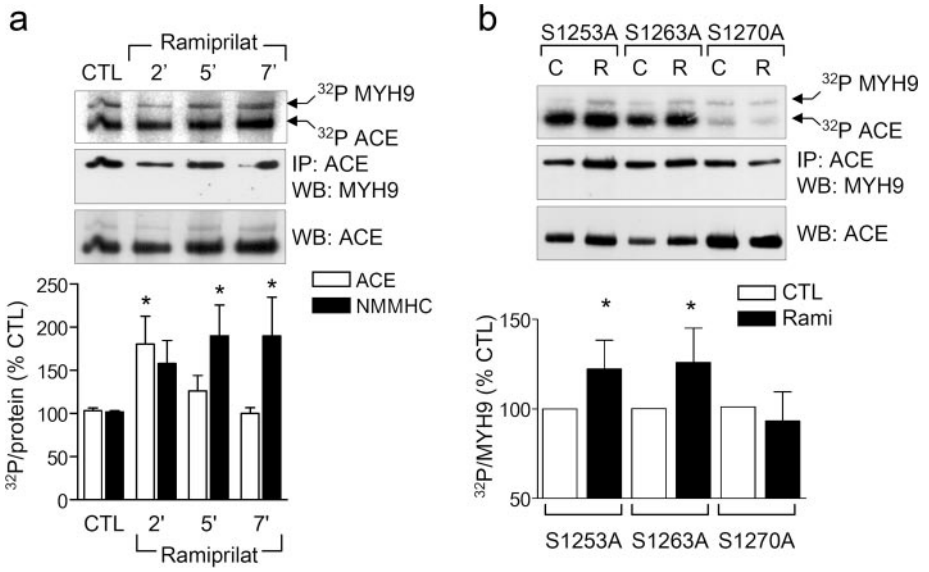

Fig. 3. Effect of ramiprilat on phosphorylation of ACE associated MYH9. a, representative autoradiography (^{32}P) and Western blots (WB) showing the time-dependent effect of ramiprilat (100 nM, 2 to 7 min) on phosphorylation of MYH9 and ACE, immunoprecipitated from HUVEC. The bar graph summarizes data from three independent experiments. b, autoradiography (^{32}P) and Western blots (WB) showing the effect of ramiprilat (R, 100 nM, 2 min) on the phosphorylation of ACE and MYH9 in ACE immunoprecipitates from porcine aortic endothelial cells overexpressing the ACE point mutants S1253A, S1263A, or S1270A. The bar graph summarizes data obtained in three independent experiments. *, $p < 0.05$ versus the respective control (CTL).

shedding of testis ACE (Chubb et al., 2004). Although the cleavage/secretion of ACE has been attributed to an "ACE sheddase" that is distinct from ADAM10 (a disintegrin and metalloproteinase domain 10) and the tumor necrosis factor-α–converting enzyme (Allinson et al., 2004), it seems that the cytoplasmic tail of ACE is also able to modulate its shedding. We reported previously that the phosphorylation of the cytoplasmic tail of ACE on Ser1270 regulates its retention in the plasma membrane (Kohlstedt et al., 2002) and in the supernatant of cells expressing ACE containing a point mutation of Ser1270 to alanine (S1270A), more soluble ACE was detected than in the supernatant from wild-type cells. In the present study, we observed that whereas cytochalasin D disrupted the association of ACE with MYH9 and elicited shedding of the wild-type ACE, it was unable to increase the shedding of the S1270A ACE mutant or to attenuate the association of the residual membrane-bound nonphosphorylatable S1270A ACE mutant with MYH9. These results indicate that phosphorylation processes functionally connect ACE with MYH9 and might regulate the strength of their interaction and thus the stability of ACE in the plasma membrane, although we cannot exclude an indirect effect on the activity of the ACE sheddase.

Endothelial cell stimulation with the ACE inhibitors ramiprilat and perindoprilat enhanced the phosphorylation of ACE-associated MYH9, a response that lagged slightly behind that of ACE on Ser1270. It is currently not known whether the phosphorylation of ACE-associated MYH9 is a transient phenomenon or phosphorylation also occurs in parallel with the maintained phase of ACE phosphorylation, which can be detected with a phosphospecific antibody over 24 to 48 h (Kohlstedt et al., 2004). Bradykinin has also been

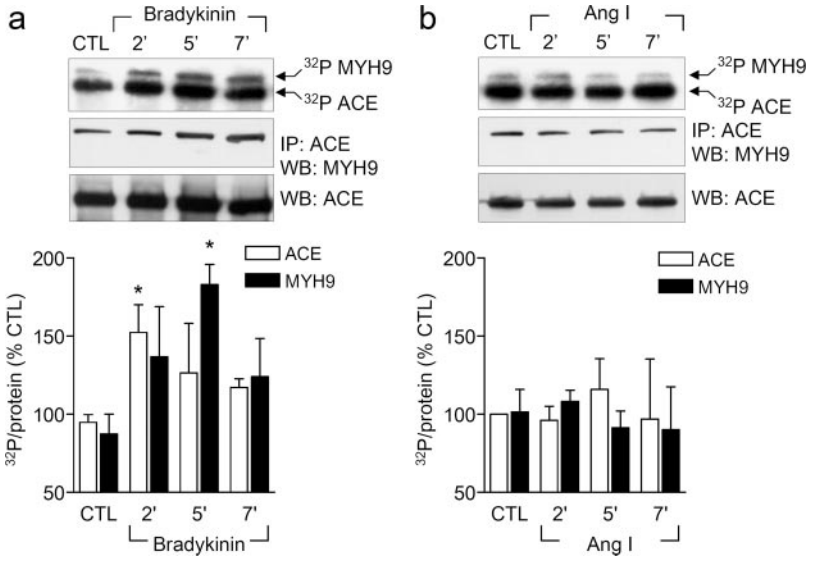

Fig. 4. Effect of bradykinin and angiotensin I, on the phosphorylation of ACE-associated MYH9. Autoradiograph (^{32}P) and Western blots (WB) showing the time-dependent effect of bradykinin (100 nM, 2–7 min) (a) and angiotensin I (Ang I, 100 nM, 2–7 min) (b) on the phosphorylation of ACE and MYH9 immunoprecipitated from ACE-overexpressing porcine aortic endothelial cells. The bar graphs summarize the time-dependent changes in ACE and MYH9 phosphorylation from three to seven independent experiments, *, $p < 0.05$ versus the respective control (CTL).

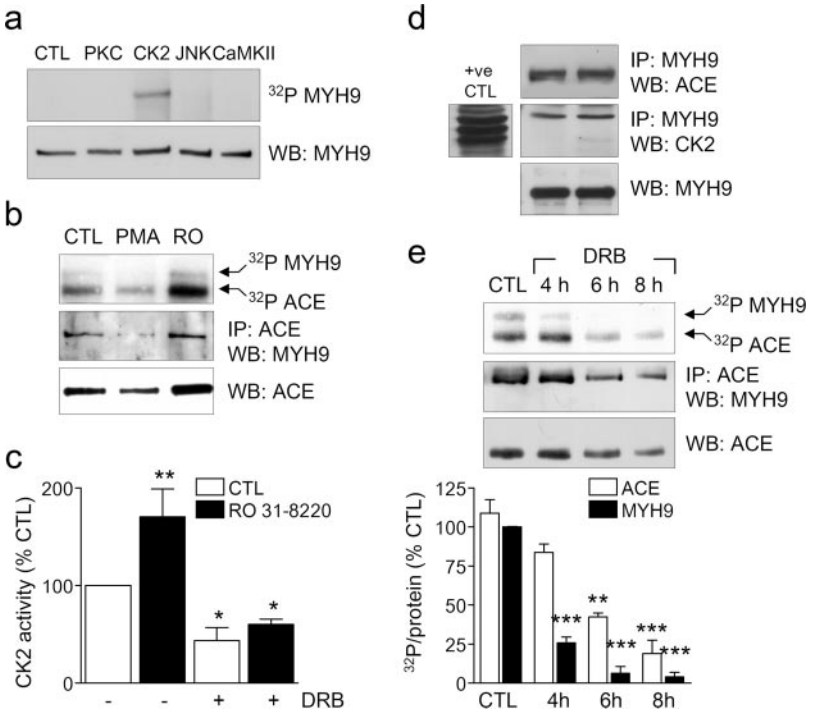

Fig. 5. CK2 mediates the phosphorylation of MYH9. a, effect of solvent (CTL), PKC, CK2, JNK, and CaMKII on phosphorylation of MYH9 in vitro. The autoradiograph shown is representative of the results obtained in three additional experiments. b, effect of PMA (1 μM, 15 min) and RO 31-8220 (30 nM, 15 min) on the phosphorylation (^{32}P) of ACE and MYH9, coprecipitated from ^{32}P-labeled HUVEC. c, bar graph showing the effect of pretreating ACE-overexpressing endothelial cells with RO 31-8220 (30 nM, 15 min) on the activity of ACE-associated CK2 in the absence and presence of DRB (100 μM, $n = 5$). d, Western blots showing the coprecipitation of CK2 with MYH9 from porcine aortic endothelial cells. Rat brain homogenate was used as a positive control (+veCTL) for CK2. e, effect of DRB (100 μM, 4–10 h) on the phosphorylation of ACE and MYH9 in ACE immunoprecipitates from ^{32}P-labeled HUVEC. The bar graph summarizes the results of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus the respective control (CTL).

reported to transiently enhance the phosphorylation of MYH9 in PC-12 cells and N1E-115 neuroblastoma cells; although the MYH9 kinase involved is unknown, the signaling cascade involved is dependent on intracellular Ca^{2+} and the activation of Rac (van Leeuwen et al., 1999). Such an effect of bradykinin on the phosphorylation of ACE-associated MYH9 reported here can be excluded, however, because the porcine aortic endothelial cells analyzed did not express functional B_2 kinin receptors. Angiotensin I, although it is an ACE substrate, does not elicit the phosphorylation of ACE on Ser1270 (Kohlstedt et al., 2004) and was also unable to elicit the phosphorylation of MYH9. Because selective inhibition of the C-domain active center of ACE is sufficient to inhibit the conversion of angiotensin I, whereas bradykinin is a substrate for both the N- and C-terminal active centers (van Esch et al., 2005), it is tempting to suggest that angiotensin I does not interact with the appropriate site on ACE to elicit ACE signaling.

A number of kinases have been reported to phosphorylate MYH9, including CK2 (Murakami et al., 1990, 1998), PKC (Conti et al., 1991; Murakami et al., 1998), and CaMKII (Rieker et al., 1987). However, in an in vitro kinase assay, we observed that although a constitutively active CK2 phosphorylated MYH9, no phosphorylation was detected with either a constitutively active PKC or activated CaMKII precipitated from ionomycin-treated endothelial cells. Inhibition of CK2 attenuated not only the phosphorylation of ACE and MYH9 in endothelial cells but also the association of the two proteins and increased ACE cleavage/secretion. Taken together, our findings suggest that 1) CK2 associates with both MYH9 and ACE in endothelial cells, 2) ramiprilat, which enhances the activity of ACE-associated CK2, enhanced MYH9 phosphorylation, and 3) a CK2 inhibitor attenuates the phosphorylation of MYH9, all of which strongly suggest that the kinase responsible for the phosphorylation of the ACE-associated MYH9 is CK2.

Although PKC has been reported to associate with the cytoplasmic tail of ACE in a mouse epithelial cell line transfected with a rabbit testis ACE expression vector (Santhamma and Sen, 2000), we were unable to detect any association between PKC and the cytoplasmic tail of the human somatic enzyme in primary cultures of human endothelial cells (K. Kohlstedt, R. Kellner, R. Busse, I. Fleming, unpublished observation). However, there are some differences in the cytoplasmic domains of the two proteins; for example, the cleavage/secretion of rabbit testicular ACE has been reported to be regulated by the tyrosine phosphorylation of its cytoplasmic tail (Santhamma et al., 2004), whereas the cytoplasmic domain of human somatic ACE does not contain a tyrosine residue. We were also unable to detect a direct effect of PKC on the phosphorylation of ACE-associated MYH9. Indeed, the PKC activator PMA stimulated the cleavage/secretion of ACE and thus disrupted the association of the two proteins, an effect than can be accounted for by the activation of the ACE secretase (Ramchandran et al., 1994). Our data suggest that PKC, rather than having a direct effect on either ACE or MYH9, exerts a regulatory influence on the activity of CK2, inasmuch as the pretreatment of endothelial cells with the PKC inhibitor RO 31-8220 enhanced the activity of ACE-associated CK2 as well as the phosphorylation of both ACE and MYH9. One property of CK2 is that it can form heterocomplexes with other kinases, which regulates its function and substrate specificity (Hathaway and Traugh, 1982). There is at least circumstantial evidence of such an interaction between PKC-ζ and CK2 in a monoblastic cell line (U937 cells) and the PKC-ζ/CK2 complex influences the basal turnover of IκBα (Bren et al., 2000).

The ACE-associated MYH9 is one of the three conventional class II myosin isoforms found in humans (Simons et al., 1991; Berg et al., 2001). The physiological relevance of this myosin-II heavy chain phosphorylation in vertebrate cells is largely unknown, but it is reported to be involved in regula-

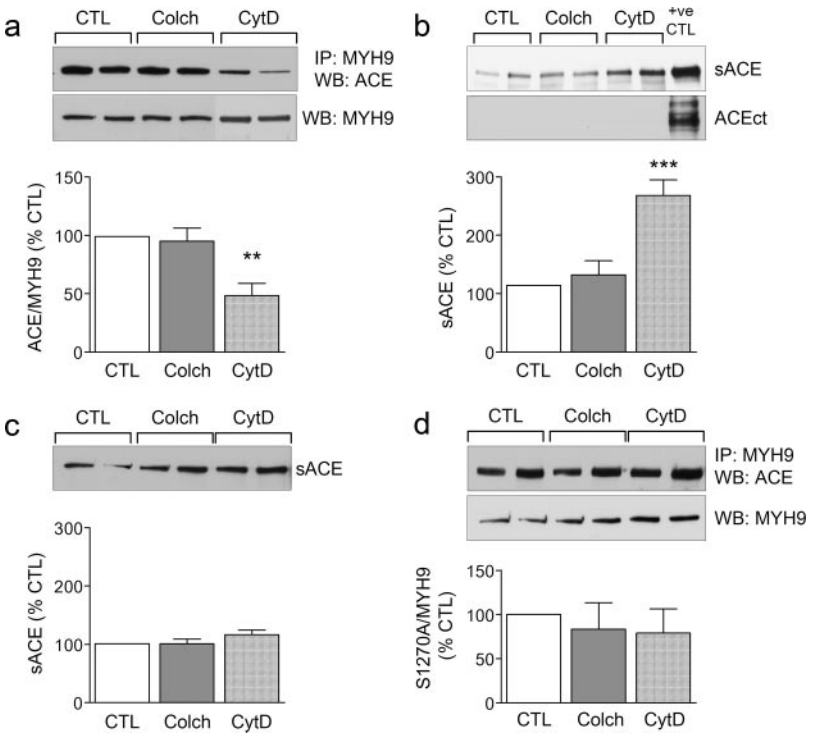

Fig. 6. Effect of cytoskeletal disruption on ACE cleavage/secretion and the association of ACE with MYH9. Representative Western blots and bar graphs showing the effects of solvent (CTL), colchicine (Colch, 20 ng/ml, 30 min), or cytochalasin D (CytD, 2 μM, 4 h) on the association of ACE (a) and S1270A ACE with MYH9 (d) in MYH9 immunoprecipitates from ACE- and S1270A-overexpressing porcine aortic endothelial cells (duplicates) and on the release of soluble ACE (sACE) into the supernatant of ACE- (b) and S1270A- (c) overexpressing endothelial cells (duplicates). To verify ACE cleavage, membranes were reprobed with an antibody directed against the cytoplasmic tail of ACE (ACEct). Human somatic ACE was used as a positive control (+veCTL). The bar graphs summarize data obtained in four to five independent experiments. **, $p < 0.01$; ***, $p < 0.001$ versus control (CTL).

tion of filament (dis)assembly and myosin activity (Brzeska and Korn, 1996; Murakami et al., 1998) as well as in the regulation of vesicle secretion (Wilson et al., 1998) and cell locomotion (Brzeska and Korn, 1996). The phosphorylation and degradation of MYH9 has been detected in endothelial cells undergoing apoptosis (Suarez-Huerta et al., 2000), but very little else is known about the role of MYH9 in endothelial cells or the consequences of its phosphorylation on activity and/or intracellular localization (Kolega, 1998). In smooth muscle cells, MYH9 has been reported to associate $\alpha v\beta 3$ integrins and the focal adhesion kinase after cell stimulation with thrombospondin-1 and may therefore play a role in intracellular signaling (Sajid et al., 2000). Given our previous report that the CK2-mediated phosphorylation of ACE on Ser1270 is one of the initial steps in a specific signaling cascade activated by the binding of an ACE inhibitor to the enzyme (Kohlstedt et al., 2004), it is tempting to speculate that the ACE inhibitor-induced phosphorylation of MYH9 represents a further branch in the "ACE signaling" cascade.

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