Ingrid Fleming

Abstract—Inhibition of the angiotensin-converting enzyme (ACE) protects against the progression of several cardiovascular diseases. Because of its dual role in regulating angiotensin II and bradykinin levels, the positive clinical effects of ACE inhibitors were thought to be the consequence of concomitant reductions in the production of angiotensin II and the degradation of bradykinin. Recent evidence suggests that some of the beneficial effects of ACE inhibitors on cardiovascular function and homeostasis can be attributed to novel mechanisms. These include the accumulation of the ACE substrate N-acetyl-seryl-aspartyl-lysyl-proline, which blocks collagen deposition in the injured heart, as well as the activation of an ACE signaling cascade that involves the activation of the kinase CK2 and the c-Jun N-terminal kinase in endothelial cells and leads to changes in gene expression. Moreover, at least one other ACE homologue (ACE2) is proposed to counteract the detrimental effects associated with the activation of the classical renin-angiotensin system. These data reveal hitherto unexpected levels of internal regulation of the renin-angiotensin system. (Circ Res. 2006;98:887-896.)

Key Words: ACE ■ angiotensin II ■ basic science ■ c-Jun NH2-terminal kinase ■ macrophages ■ signal transduction

Circulating angiotensin II (Ang II) is the main effector of the renin-angiotensin system (RAS) and is involved in the global regulation of sympathetic activity and blood pressure as well as fluid and electrolyte balance. The angiotensin-converting enzyme (ACE) is an ectoenzyme that plays a role in the generation of Ang II by catalyzing the extracellular conversion of the decapeptide Ang I. In the classical scheme of things, two enzymes regulate the RAS: ACE and renin, the latter being released from the juxtaglomerular cells of the kidney into the circulation, where it converts angiotensinogen to Ang I. In addition to Ang I, ACE hydrolyzes a number of other substrates (see below) but probably the most important, at least for the regulation of vascular tone, is the potent vasodilator bradykinin. However, we clearly do not know all there is to know about RAS, and a number of reports over the last few years have indicated that several other ACE substrates have marked effects on cardiovascular homeostasis and that ACE homologues exist that modulate the activity of the classical RAS.

Angiotensin-Converting Enzyme

Two distinct forms of ACE (dipeptidyl-carboxypeptidase I/kininase II) are expressed in humans, a somatic form that is particularly abundant on the endothelial surface of lung vessels (but that is also expressed in all other endothelial cells types as well as in some smooth muscle cells, monocytes, T lymphocytes, and adipocytes), and a smaller germinal form found exclusively in testis. Both forms of ACE exist at the cell surface as ectoenzymes, where they hydrolyze circulating peptides. A soluble form of ACE (soluble or plasma ACE),
which is derived from the membrane-bound form through the action of the ACE secretase, is also present in serum and other body fluids.1

Testicular ACE is the ancestral form of the molecule with a single active site, and somewhat surprisingly, its crystal structure was published only recently.2 Somatic ACE arose as a consequence of gene duplication3 and contains two active sites (termed the N and C domains). The structure of the N-terminal domain of somatic ACE is still unknown, but the C-terminal domain is expected to be identical to that of testis ACE. There appear to be differences in the function of the two sites, and Ang I conversion is reported to take place preferentially within the C domain. Indeed, selective C domain inhibition is sufficient to prevent Ang I–induced vasoconstriction, at least in small porcine coronary arteries.4 On the other hand, both the N and C domains contribute to the degradation of bradykinin,5 whereas Ang 1-7 is cleaved by the N-terminal active site of ACE and inhibits the enzymatic activity of the C-terminal site.5 There is also evidence suggesting that the N domain of ACE may be functionally less relevant because the RXP407 peptide that specifically inhibits the ACE N domain active site has no effect on blood pressure.6

More recently, testis ACE was reported to possess a glycosylphosphatidylinositol (GPI) hydrolase activity. This activity was not significantly inhibited by ACE inhibitors and not affected by substitution of core amino acid residues essential for peptidase activity, suggesting that the active site for GPI hydrolase (GPIlase) activity is distinct from that of the dipeptidyl carboxypeptidase. This novel function of ACE was implicated in the cleavage of GPI-anchored proteins such as TESP5 and PH-20, two proteins involved in capacitation, from the sperm membrane, and the loss of this activity was proposed to account for the infertility of male ACE−/− mice.7 However, location and access were determinant in bringing enzyme and substrate together, at least in F9 cells, because lipid rafts needed to be disrupted with filipin in order for ACE to access GPI-anchored proteins.7 Although an attractive hypothesis, two groups have recently performed similar experiments and come to the conclusion that there is no overwhelming evidence to indicate that ACE possesses GPIlase activity, and the infertility of male ACE−/− mice can be attributed entirely to the loss of its dipeptidyl carboxypeptidase activity.8,9

Soluble ACE
A metalloprotease, the so-called ACE secretase, cleaves ACE between Arg1203 and Ser1204 on the extracellular side of the transmembrane domain10 to generate a C-terminal truncated, soluble, or plasma form of the enzyme.11 Soluble ACE in healthy subjects arises essentially from the endothelium, but in disease states, it can be found in other biological fluids including cerebrospinal and bronchoalveolar fluids. Plasma ACE levels have recently been suggested to represent a risk factor for coronary stent restenosis,12 coronary artery disease,13 and myocardial infarction.14 Indeed, elevated plasma ACE activity, determined less than four 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.15

For a short while, it was tempting to speculate that one of the major roles of soluble ACE would be to cleave GPI-anchored proteins from the plasma membrane of blood and vascular cells because this enzyme is not bound by the rules governing the localization of the membrane-bound enzyme. However, there is no hard evidence that soluble ACE possesses GPIlase activity.8,9 Moreover, given that even small amounts of serum inhibit the GPIlase activity of ACE,16 it is highly unlikely that soluble ACE plays an important role in the release of GPI-anchored proteins in vivo.

The Importance of the Local RAS
Cells and tissues not implicated in the classical RAS are now known to possess all the molecular machinery necessary to generate Ang II. Tissues classed as expressing a functional local RAS express angiotensinogen, renin, renin-binding protein, ACE, chymase, as well as Ang II receptor 1 (AT1) or AT2 and secrete Ang II.

The Heart and the Systemic Vasculature
All the components of the RAS have been found in the cardiomyocyes as well as in endothelial cells and vascular smooth muscle cells. These cell types are able to generate Ang II, and proinflammatory/proatherosclerotic stimuli such as high cholesterol and insulin are able to activate this local RAS to increase oxygen-derived free radical production and induce oxidative stress.

There is increasing evidence that the local RAS may be involved in the maintenance of cardiovascular structure and repair. ACE levels are increased in many forms of vascular and cardiac hypertrophy, and the administration of ACE inhibitors has led to regression of hypertrophy. This effect of ACE on vascular remodeling is highlighted by the report that the in vivo gene transfer of ACE into the uninjured rat carotid artery results in the development of vascular hypertrophy independent of systemic factors and hemodynamic effects.17 Selective overexpression of ACE in the heart also results in morphological changes in the atria, arrhythmia, and sudden death.18

Macrophages
In atherosclerotic human coronary arteries, ACE immunoreactivity is associated with macrophages as well as with smooth muscle cells and T lymphocytes.19,20 Given that Ang II activates monocytes and stimulates the expression of tissue factor as well as the release of proinflammatory cytokines, the induction of ACE in monocytes most likely exacerbates inflammatory responses. Indeed, ACE expression is reported to be higher in ruptured plaques than in fibrosclerotic plaques, and in the former, ACE is highly expressed in macrophages accumulated around the attenuated fibrous cap. Such findings indicate that the presence of ACE within lesions, atheromatous plaques, and ruptured plaques contributes greatly to the further progression of atherosclerosis.20

Adipose Tissue
Human preadipocytes possess a complete functional RAS, and undifferentiated preadipocytes as well as immature adi-
pocytes secrete Ang II. The expression of the RAS components seems to relate to body weight because obese women are reported to have higher circulating angiotensinogen, renin, aldosterone, and ACE levels than lean women and lower angiotensinogen gene expression in adipose tissue. On the other hand, weight reduction (≈5%) reduced angiotensinogen, renin, and aldosterone levels and decreased ACE expression. Although ACE−/− and AT1−/− mice have no obvious changes in fat deposition, an ACE inhibitor and an AT1 receptor antagonist were found to reduce adipocyte size and to increase insulin sensitivity in Sprague-Dawley rats fed a fructose-rich diet. The increase in insulin sensitivity also fits with a recent report that in subjects with essential hypertension and insulin resistance, RAS blockade with either an ACE inhibitor or an AT1 antagonist increased the secretion of the insulin-sensitizing adipokine adiponectin.

The importance of local RAS in monocytes as well as within adipose tissue and the vascular wall may well lie in the modulation of cell activation or differentiation and the subsequent release of cytokines and adipokines, which also affect the progression of cardiovascular disease. Inhibition of local tissue-specific RAS may also account for the observation that ACE inhibitors with high tissue affinity confer a greater degree of vascular RAS suppression than those with low tissue affinity despite similar suppression of the circulating RAS.

**Chymase**

Although circulating Ang II levels decrease in response to acute ACE inhibitor treatment, circulating Ang II levels tend to increase in patients taking ACE inhibitors over long periods. This phenomenon highlights the fact that ACE is not the only enzyme implicated in the generation of Ang II and the ACE inhibitor–induced elevation in Ang I facilitates its hydrolysis by other peptidases.

Chymases are serine proteases belonging to the chymotrypsin family and are found in mast cells in multiple tissues and species. Human α-chymase is able to convert a number of substrates including Ang II from Ang I, interleukin-1β from its precursor, and endothelin-1 from big endothelin as well as to activate matrix metalloproteases. Chymase is thought to be responsible for >80% of tissue Ang II formation in the human heart and >60% of that in arteries. Therefore, it is not surprising that chymase has been implicated in the pathogenesis of cardiovascular diseases, particularly in cardiac hypertrophy, heart failure, atherosclerosis, and restenosis. In mast cell–deficient (Kit<sup>−/−</sup>/Kit<sup>−/−</sup>) mice, chymase cannot be detected in the vasculature, and there is no additional effect of AT1 receptor blockade on the blood pressure of animals receiving an ACE inhibitor. Moreover, genetic deletion of ACE results in marked differences in circulating plasma Ang II and Ang I, but tissue (heart, kidney, and lung) concentrations of Ang II and the Ang II/Ang I ratio are not different in mice expressing different amounts of ACE (ie, ACE<sup>−/−</sup>, ACE<sup>+/−</sup>, and ACE<sup>+/+</sup> mice). Because the latter observations were correlated with an increase in chymase activity in the kidneys and hearts of ACE<sup>−/−</sup> mice, it is tempting to speculate that chymase is important in maintaining steady-state Ang II levels in tissue, even though the contribution of chymase to total Ang II production (including that in plasma) is estimated as being <2%. In humans, the chymase-specific substrate [Pro<sup>1</sup>/Phe-Ala<sup>2</sup>] Ang II induces a vasoconstriction that is unaffected by ACE inhibition, and non–ACE-dependent Ang II generation occurs in resistance arteries from patients with coronary artery disease. However, differences in the contribution of chymase to the generation of Ang II in different tissues and between species makes it difficult to estimate the importance of this enzyme.

Although selective chymase inhibitors have been developed and promising results have been obtained in animal models of myocardial infarction, cardiomyopathy, and tachycardia-induced heart failure, these substances have yet to be tested in humans.

**ACE Substrates**

Both of the ACE isoforms hydrolyze a spectrum of circulating peptides and catalyze the hydrolysis of substance P, Ang 1-9, N-acetyl-seraryl-aspartyl-lysyl-proline (AcSDKP), cholecystokinin, hemopressin, and amyloid β-protein in addition to Ang I and the vasodilator peptides bradykinin and kallidin.

**Bradykinin**

ACE is identical to kininase II, the enzyme that metabolizes bradykinin to inactive fragments. In fact, ACE more readily hydrolyzes bradykinin than Ang I. Therefore, one consequence of ACE inhibition is that the metabolism of bradykinin is attenuated and the local concentration of this potent vasodilator in the vicinity of the endothelium is enhanced. It became clear relatively early on just how many of the actions of ACE inhibitors can be attributed to effects on the metabolism of both Ang I and bradykinin. For example, coadministration of the B<sub>2</sub> receptor antagonist icatibant attenuated the mean arterial blood pressure response to perindopril as well as the in vivo flow-dependent vasodilatation of human resistance and epicardial coronary arteries and the radial artery. The vasodilator effects of bradykinin are thought to be particularly potent because it is one of the rare stimuli that elicits the activation of the three most important endothelium-derived vasodilator autacoids (ie, NO, prostacyclin, and the endothelium-derived hyperpolarizing factor). Assessing the role of bradykinin is rather difficult in rodents, particularly in mice, in which the B<sub>2</sub> kinin receptor is expressed only in some vascular beds, and Ang II seems to be the stronger arm of the RAS in these animals. However, in a mouse model of chronic heart failure induced by myocardial infarction, ACE inhibition was associated with improved cardiac function and remodeling, and the effects of the inhibitors were attenuated in mice lacking the B<sub>2</sub> receptor. Moreover, in addition to their effects on vascular tone, ACE inhibitors improve neovascularization in the diabetic ischemic leg via a mechanism that is no longer apparent in B<sub>2</sub> receptor knockout mice. These compounds also partially prevent the development of left ventricular hypertrophy via an effect that is sensitive to icatibant and can most probably be attributed to the enhanced expression and activity of the endothelial NO synthase.
Angiotensin 1-7

Ang 1-7 possesses just one amino acid less than Ang II (Figure 1), and although it is cleaved by the N-terminal active site of ACE at half the rate of bradykinin, it actually inhibits the enzymatic activity of the C-terminal site. Although initially thought to be without effect, Ang 1-7 is reported to potentiate the effects of bradykinin as well as those of ACE-resistant bradykinin analogues. Somewhat intriguingly, the potentiation of bradykinin-induced vasodilation in spontaneously hypertensive rats treated short-term or long-term with ACE inhibitors was reverted by an Ang 1-7 antagonist, thus unmasking a key role for an Ang 1-7-related mechanism in mediating the effects of this class of compounds.

Current interest in Ang 1-7 as a biological mediator has been stimulated by reports that it can be generated from Ang II by ACE2, a monocarboxypeptidase that shares ~42% identity with the catalytic domain of somatic ACE. Ang 1-7 is a vasodilator, and the fact that the expression of ACE2 is increased in disease and after treatment with ACE inhibitors, the ACE2/Ang 1-7 axis has been suggested to act as a natural damping mechanism for the activation of the classical RAS.

The actions of Ang 1-7 often functionally antagonize those of Ang II, for example, although Ang II increases blood pressure, Ang 1-7 is a vasodilator; it decreases blood pressure in hypertensive animals and reduces vascular cell growth. Ang 1-7 also participates in the regulation of cardiac function and preserves cardiac function, coronary perfusion, and aortic endothelial function in a rat model for heart failure. Ang 1-7 can bind to AT1 and AT2 receptors at high concentrations, as well as to its own receptor: Mas, an orphan receptor. Deletion of Mas abolishes the binding of Ang 1-7 to mouse kidneys as well as the Ang 1-7-induced relaxation of isolated aorta. There is also evidence that Mas receptor activation modulates some of the effects of AT1 and AT2 receptors by physically associating with them to generate hetero-oligomers and functionally antagonizing the actions of Ang II.

**N-Acetyl-Seryl-Aspartyl-Lysyl-Proline**

N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP or goralatide) is a potent natural inhibitor of hematopoietic stem cell proliferation that is degraded mainly by ACE. In vitro, Ac-SDKP is a potent angiogenic factor and inhibits collagen production by cardiac fibroblasts, whereas in vivo, it blocks collagen deposition in the left ventricle of rats with hypertension or myocardial infarction. ACE inhibitor treatment results in a significant increase in plasma Ac-SDKP levels in humans, and in rats, the exogenous application of Ac-SDKP exerts many effects (including inhibition of Ang II–induced cell proliferation, left ventricular macrophage/mast cell infiltration, and collagen deposition) that are generally associated with ACE inhibitor therapy. Thus, it is tempting to suggest that some of the beneficial effects of ACE inhibitors are not directly related to their effects on the RAS but rather are a consequence of increased circulating Ac-SDKP levels. Certainly, in rats made hypertensive by Ang II infusion, a monoclonal antibody to Ac-SDKP prevented the ACE inhibitor–induced decrease in left ventricular collagen deposition as well as monocyte infiltration, cell proliferation, and transforming growth factor-β expression. Prevention of the degradation of Ac-SDKP has been celebrated as a novel mechanism of action of ACE inhibitors and clearly represents one of the most important observations in this field over the last five years. However, we still have a lot to learn about the cells targeted by Ac-SDKP and the molecular mechanisms involved in mediating its effects.

**Amyloid β-Protein**

An early and pathogenically important feature of Alzheimer disease is the progressive accumulation and deposition of the amyloid β-protein in brain regions serving memory and cognition. Biochemical, cell biological, animal modeling, genetic, and emerging clinical data all suggest that amyloid β-protein is an upstream initiator of the disease process and its associated neuropathology. One way to undermine the accumulation of amyloid β protein and potentially delay the development of the disease could be to enhance its degradation by proteases expressed in the brain. The enzymes in question are the neutral endopeptidase, the endothelin-converting enzymes, and ACE.

There is circumstantial (not to mention controversial) evidence supporting a link between ACE and amyloid β-protein levels in the brain. Elevated levels of ACE have been reported in the temporal cortex of brains from Alzheimer disease patients, whereas no apparent link could be detected in other clinical studies. Enhanced cortical ACE activity was also associated with a prominent perivascular ACE and Ang II immunoreactivity surrounding some cortical vessels pointing to an underlying microvascular pathology in the process of neurodegeneration. Interestingly, the ACE degradation product, a truncated 33-residue peptide, exhibited decreased aggregation and cytotoxic potential than the full-length amyloid β-protein. Thus, reduced ACE activity, such as that realized after prolonged pharmacological inhibition, could be expected to accelerate amyloid β-protein accumulation and accelerate disease development; just such an effect has been demonstrated in vitro.

**ACE to B2 Kinin Receptor Cross-Talk**

Over the last decade, there have been a number of reports showing that ACE inhibitors can amplify responses to bradykinin, although accumulation of the peptide cannot be assumed to occur, such as in continuously perfused systems. However, whether or not a cross-talk between these two

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**Figure 1.** Peptides hydrolyzed by the ACEs.
The Case for . . .

One example of the type of experiment that led to the speculation that a cross-talk exists is the ACE-inhibitor induced Ca\textsuperscript{2+} signal observed in bradykinin-desensitized endothelial cells (Figure 2). In these cells, superfusion with a high concentration of bradykinin elicits a biphasic Ca\textsuperscript{2+} response but desensitizes the B\textsubscript{2} kinin receptor so that a 10-fold higher concentration of bradykinin is unable to elicit a second response. However, under the same experimental conditions, the addition of an ACE inhibitor, in this case ramiprilat, elicits a Ca\textsuperscript{2+} signal that is similar to the initial response. The ACE inhibitor–induced Ca\textsuperscript{2+} signal is related to the activation of the B\textsubscript{2} receptor because it is sensitive to the B\textsubscript{2} kinin receptor antagonist icatibant. However, it is highly unlikely that ramiprilat is able to immediately increase the local concentration of bradykinin to such an extent that the desensitized receptor can be reactivated. Because ACE inhibitors do not directly bind to the B\textsubscript{2} receptor and the expression of both ACE and the B\textsubscript{2} receptor are required for the activation of the B\textsubscript{2} receptor because it is sensitive to the B\textsubscript{2} kinin receptor antagonist icatibant. However, the analogues used in these initial experiments are now known to be not as resistant as originally thought to degradation by ACE, and markedly different results have been obtained with a new generation of bradykinin analogues (see below).

For ACE to B\textsubscript{2} receptor cross-talk to take place, the two proteins need to communicate with each other either directly or via closely associated signaling molecules. There has been a report of a direct physical interaction between the two proteins in Chinese hamster ovary (CHO) cells overexpressing the B\textsubscript{2} kinin receptor and ACE. This interaction was assumed to occur between extracellular structures because cross-talk could still be demonstrated between the B\textsubscript{2} receptor and an ACE mutant in which the terminal 18-aa residues were deleted from the cytosolic tail. Moreover, the localization of ACE to lipid rafts by replacing the transmembrane and cytosolic portions of the molecule with a GPI anchor resulted in a loss of the ACE inhibitor–induced resensitization of the B\textsubscript{2} receptor. However, treatment of the latter cells with filipin to disperse lipid rafts restored the cross-talk. When interpreting the results of such experiments, it is important to realize that the activated B\textsubscript{2} receptor is sequestered into caveolae whereas ACE is generally excluded from this cell compartment as well as from lipid rafts. This means that if the two proteins do physically interact, they must do so in another cell compartment. The events involved in the de novo activation of B\textsubscript{2} receptors may well differ from those involved in the reactivation of desensitized receptors because the latter but not the former are reportedly sensitive to pharmacological inhibitors of protein kinase C (PKC) and serine/threonine phosphatases.

Angiotensin peptides, specifically Ang 1-9 and Ang 1-7, have been suggested to mediate the cross-talk between ACE and the B\textsubscript{2} receptor in the CHO overexpression system. The Ang 1-7 and Ang 1-9–induced reactivation of the B\textsubscript{2} kinin receptor in CHO cells (assessed by determining arachidonic acid release) was sensitive to a series of pharmacological inhibitors of PKC and phosphatase as well as to the tyrosine kinase inhibitor genistein. A more detailed analysis of the molecular mechanisms underlying this phenomenon was not performed, but the effect was attributed to a potential conformational change in the ACE–B\textsubscript{2} receptor heterodimer. No evidence has been provided to indicate that a similar interaction occurs under physiological conditions, but the time course of the events in question needs to be taken into account because the ACE inhibitor–induced resensitization of the B\textsubscript{2} receptor is immediate. It is difficult to envisage that Ang 1-7 or Ang 1-9 levels (or bradykinin for that matter) can increase rapidly enough to account for many of the experimental findings reported to date in endothelial cells or in the isolated perfused heart.

Little detailed biochemical work has been performed in cells that constitutively express ACE and the B\textsubscript{2} kinin receptor. The only investigation using “native” endothelial cells (ie, directly scraped off isolated porcine aortae) reported that although bradykinin initiated the sequestration of the B\textsubscript{2} receptor to caveolin-rich membranes, pretreatment of these cells with an ACE inhibitor significantly attenuated the recovery of B\textsubscript{2} kinin receptors from caveolae while increasing that from membranes lacking caveolin. This effect could not be attributed to the inhibition of bradykinin degradation because no effect was seen in the presence of an inhibitory concentration of the synthetic ACE substrate hippuryl-L-histidyl-L-leucine. Ramiprilat also decreased [\textsuperscript{3}H]bradykinin binding to caveolar membranes when applied either before or after bradykinin stimulation. These data led to the suggestion that ACE inhibitors interfere with the targeting of the B\textsubscript{2} receptor to caveolae, implying that effects other than the inhibition of ACE activity per se may account for the effects of this class of compounds.

The Case Against . . .

The most damming evidence against the ACE–B\textsubscript{2} cross-talk hypothesis is that although it is evident after the addition of

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**Figure 2.** ACE inhibitors initiate a cross-talk between ACE and the B\textsubscript{2} kinin receptor. Human umbilical vein endothelial cells were loaded with fura-2 and mounted in a perfusion chamber on a fluorescent microscope. After stabilization of the Ca\textsuperscript{2+} signal, the perfusion buffer was replaced with one containing bradykinin (Bk; 100 nmol/L; BK1); after ∼10 minutes, the buffer was again replaced with one containing either a higher concentration of Bk (1 μmol/L; BK2) or the combination of Bk (100 nmol/L) and ramiprilat (100 nmol/L) and the signal monitored for an additional 10 minutes. Adapted from Benzing et al.

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The most damming evidence against the ACE–B\textsubscript{2} cross-talk hypothesis is that although it is evident after the addition of
ACE inhibitors to bradykinin-desensitized cells, it is generally not observed in cells or in blood vessels in which the B2 receptor is desensitized with the new generation of ACE-resistant B2 receptor agonists.\textsuperscript{92–95} Such data suggest that the metabolism of bradykinin by ACE is indeed so fast in the microenvironment of the B2 receptor that concentrations of the peptide increase to micromolar levels within milliseconds of the addition of ACE inhibitors. However, little information is available regarding the effects of these ACE-resistant agonists on receptor occupancy, efficacy, or speed of inactivation and sequestration.

The Verdict

Unfortunately, in the case of ACE–B2 receptor cross-talk, we seem to have a “hung jury” with the cases for and against currently prohibiting a real conclusion. In an attempt to investigate the phenomenon of ACE–B2 receptor cross-talk from a completely different aspect, we reasoned that the binding of an ACE inhibitor to ACE should be able to elicit an intracellular event and that ACE should be capable of outside-in signaling. Moreover, the cytoplasmic tail of ACE should be able to bind soluble intracellular signal molecules or adaptor proteins that initiate a chain of events ultimately linking to effects such as the reactivation of the B2 kinin receptor. Although the so-called ACE signaling pathway (outlined in the next section) was identified as the result of studies to address this hypothesis, it has not yet been possible to find a direct link between the binding of an ACE inhibitor to ACE and the reactivation of the B2 kinin receptor (our unpublished observation, 2005).

Signal Transduction by ACE

In all of the species studied to date, the short cytoplasmic tail of somatic ACE contains between three and five serine residues, one of which (Ser\textsuperscript{1270} human sequence) is located in a highly conserved 13-aa sequence at the extreme C-terminal end of the protein. In endothelial cells, Ser\textsuperscript{1270} is phosphorylated by the kinase CK2, which also physically interacts with the protein. The basal phosphorylation of ACE by CK2 stabilizes its localization in the plasma membrane because the mutation of this site and the inhibition of CK2 both enhance the cleavage/secretion of the enzyme.\textsuperscript{96} In contrast, the cytoplasmic tail of rabbit testis ACE is reported to be tyrosine phosphorylated rather than serine phosphorylated.\textsuperscript{97} However, the latter modification is not relevant to that of the human somatic enzyme, which does not contain a tyrosine residue.\textsuperscript{98}

What Are the Components of the ACE Signaling Pathway?

CK2 is not the only protein that associates with the cytoplasmic tail of ACE in endothelial cells. Using ACE immunoprecipitated from ACE overexpressing cells as well as an affinity column composed of a peptide corresponding to the cytoplasmic tail of ACE, mitogen-activated protein kinase kinase 7 and c-Jun N-terminal kinase (JNK) were also found to associate with the intracellular domain of the human enzyme. ACE phosphorylation is an essential step in the ACE signaling cascade, and the CK2-dependent phosphorylation of Ser\textsuperscript{1270} is required for the activation of ACE-associated JNK. The increase in JNK activity results in the translocation of phosphorylated c-Jun to the nucleus, an enhanced binding of the activator protein-1 transcription factor to DNA followed by the increased expression of genes such as ACE\textsuperscript{99} and cyclooxygenase-2 (COX-2; Figure 3). Although at first glance, it seems unlikely that an ACE inhibitor–induced increase in the expression of ACE or COX-2 could be associated with improved endothelial cell function, increased ACE levels have been demonstrated in lung tissue and plasma from ACE inhibitor–treated rats\textsuperscript{101} and in the serum from patients who distinctly benefit from ACE inhibitor therapy.\textsuperscript{102} Moreover, prostacyclin production is significantly increased in subjects treated with ACE inhibitors,\textsuperscript{103} and selective COX-2 inhibition diminishes the positive effects of ACE inhibitors on blood pressure.\textsuperscript{104,105}

A number of other proteins are reported to associate with the cytoplasmic domain of somatic ACE. For example, \(\beta\)-actin and the nonmuscle myosin heavy chain II A (MYH9) associate with ACE in endothelial cells and seem to play a role in the cleavage/secretion of the enzyme.\textsuperscript{106} Moreover, ACE inhibitors elicit the phosphorylation of MYH9 by CK2, which is dependent on the initial phosphorylation of ACE itself. Although the cellular consequences of these events are not entirely clear, it seems that the phosphorylation of MYH9 stabilizes its association with ACE and anchors it more firmly in the cell membrane, thus decreasing soluble ACE levels.\textsuperscript{106}

Rabbit testis ACE associates with a number of other proteins including ribophorin and the chaperone immunoglobulin-binding protein.\textsuperscript{107} The latter interaction is not likely to affect signaling but very likely to affect protein maturation because the overexpression of immunoglobulin-binding protein inhibited ACE secretion, an effect that could be attributed to the retention of the enzyme in the endoplas-
CK2 is that it can form heterocomplexes with other kinases, reported on the phosphorylation of ACE on Ser1270, no other because although a weak effect of bradykinin has been identified agonist, ACE acts as a signal transduction molecule.

ACE generates Ang II from Ang I, degrades bradykinin to inactive peptides, and after the binding of an ACE substrate was able to activate ACE-associated CK2 or PKC. On the basis of the observations outlined above, it is tempting to suggest that ACE has three functions: it can form heterocomplexes with other kinases, phosphorylate both ACE and MYH9. One property of CK2 is that it can form heterocomplexes with other kinases, which regulates its function and substrate specificity. There is at least circumstantial evidence of such an interaction between PKC-ζ and CK2 in a monolastic cell line (U937 cells) and the PKC-ζ/CK2 complex influences the basal turnover of IκBα. Calmodulin binds to the cytoplasmic domain of both rabbit testis ACE and human somatic ACE (our unpublished observation, 2004).

The ACE signaling pathway (Figure 3) has been mainly addressed in ACE-overexpressing endothelial cell lines as well as in primary cultures of endothelial cells. The evidence that such a pathway exists in vivo in humans and in mice is mainly circumstantial, and a detailed analysis of the long-term effects of the signaling pathway outlined above must await the conclusion of studies using mice carrying a mutation in Ser820. An additional intriguing point relates to the existence of an endogenous ligand for the signaling pathway because although a weak effect of bradykinin has been reported on the phosphorylation of ACE on Ser820, no other ACE substrate was able to activate ACE-associated CK2 or JNK. On the basis of the observations outlined above, it is tempting to suggest that ACE has three functions: (1) it generates Ang II from Ang I, (2) it degrades bradykinin to inactive peptides, and (3) after the binding of an ACE inhibitor that might mimic the function of an as yet unidentified agonist, ACE acts as a signal transduction molecule.

**Conclusion**

A few years ago, we thought we knew all about the RAS and a lot about its regulation. The identification of a novel ACE (ACE2) as well as an ACE-dependent signaling pathway and intracellular Ang II receptors implies that the RAS has still unexpected facets with clinical implications. One of the most intriguing aspects that remain to be elucidated is probably the light of the clinical effectiveness of ACE inhibitors in delaying the onset of diabetes.

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