Angiotensin-converting enzyme regulates bradykinin receptor gene expression

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Angiotensin-converting enzyme regulates bradykinin receptor gene expression. Am J Physiol Heart Circ Physiol 289: H1814–H1820, 2005; doi:10.1152/ajpheart.00581.2005.—The angiotensin-converting enzyme (ACE) is a membrane-bound peptidyl dipeptidase known to act on a variety of peptide substrates in the extracellular space. Its most notable functions are the formation of angiotensin II and the degradation of bradykinin. In the current experiments, we found that exogenous ACE added to vascular smooth muscle cell culture strongly induces and upregulates the genes of bradykinin receptors B1 and B2. This transcriptional regulatory property of ACE was shown to be unrelated to its known enzymatic properties. Indeed, ACE at 3.75 μg/ml added in the culture medium of vascular smooth muscle cells was found to cause marked upregulation of the mRNA expression for the B1 and B2 receptors of bradykinin by 22- and 11-fold, respectively. This phenomenon was not altered by the addition of specific angiotensin II antagonists for the AT1 or AT2 receptors. Moreover, the ACE inhibitor captopril, which inhibited ACE enzymatic activity, did not block its effect at the bradykinin receptor gene transcription level. Expression of both receptor genes was completely abolished by actinomycin D. Furthermore, transcriptional upregulation was inhibited by curcumin, suggesting involvement of different transcriptional factors in this phenomenon. Electrophoretic mobility shift assay revealed increase in NF-κB and activator protein-1 protein binding for consensus sequences, between ACE-treated cells versus untreated cells. The data indicate a novel biological function of the ACE unrelated to its well-known enzymatic function as a peptidyl dipeptidase.

Angiotensin-converting enzyme (ACE) inhibition is one of the most important advances in cardiovascular pharmacology in the last 35 years. After the first small clinical studies suggested the therapeutic potential of ACE inhibitors for the treatment of hypertension (9, 10) and heart failure (11), this class of drugs became the object of numerous large outcome trials that demonstrated their cardioprotective and nephroprotective properties and are now the most widely prescribed agents for these conditions. Yet, whereas the clinical benefits of ACE inhibition are still being investigated, the multiple functions of ACE and the mechanisms by which its inhibitors exert these benefits are not being explored.

The ACE is a membrane-bound enzyme that acts on a variety of peptide substrates. Skidgel and Erdos (32) have clarified that the peptidyl dipeptidase, which cleaves off His-Leu from angiotensin I to form angiotensin II, is identical to kininase II, which cleaves off Phe-Arg from bradykinin to form inactive residue. Therefore, its inhibition will block the generation of angiotensin II and potentiate the actions of bradykinin. A large body of literature is devoted to dissection of angiotensin-mediated and bradykinin-mediated effects of ACE inhibition, as well as to other aspects of ACE-related functions.

In a previous series of experiments studying the interactions of these two systems, we found that various experimental manipulations affect the gene expression of the bradykinin receptors B1 and B2, both in vivo and in vitro. The majority of the physiological effects of bradykinin, including its hemodynamic (vasodilation) and metabolic actions (insulin-dependent glucose transport and utilization) is mediated by the B2 receptor, whereas the B1 receptor is minimally expressed under normal conditions (26, 17, 15). In the absence of B2 receptors, such as in B2 receptor gene knockout animals, the B1 receptor becomes expressed and is capable of assuming the hemodynamic (8, 6) but not the metabolic functions of the B2 receptor (7). The B3 receptor gene is normally expressed constitutively in many tissues, whereas the B1 is inducible by various factors, notably lipopolysaccharides and tissue inflammation or damage (18). We recently found that angiotensin II in vivo and in vitro stimulates gene expression of both B1 and B2 receptors in cardiomyocytes and vascular smooth muscle cells (VSMC), whereas concurrent inhibition of the AT1 receptor with losartan can abolish this effect (14).

The current studies were designed to explore the role of ACE in the presence or absence of angiotensin II antagonists on the gene expression of the B1 and B2 receptors in VSMC from the rat aorta. We found that exogenous ACE added to cell culture medium can indeed produce significant upregulation of both receptor genes by 11- to 22-fold by a mechanism unrelated to its enzymatic properties. We also made the unexpected discovery that this enzyme, which is normally found as a type I integral membrane glycoprotein located on the surface of epithelial and endothelial cells or circulating as a free enzyme in body fluids (plasma and seminal fluid), has actually the capacity to trigger this change in gene expression level without being enzymatically active as a peptidyl dipeptidase. The signaling mechanism was shown to involve activation of two transcription factors in this process, NF-κB and activator protein (AP-1).

EXPERIMENTAL PROCEDURES

Materials. Dulbecco’s modified Eagle’s medium, penicillin-streptomycin mixture, and TRIzol were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum, Ponceau S solution, actinomycin D, and the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
were applied on Epoxy-Activated Sepharose 6B (Amersham Bio-
mM HEPES, pH 7.5, made 0.5% Triton X-100, stirred overnight, and
was centrifuged several times. The final pellet was resuspended in 5
sucrose, 50 mM HEPES, pH 7.5, buffer at
Kidney cortex was surgically removed and homogenized in 0.33 M
other contaminant in our results, we purified the ACE in our labora-
analysis of these preparations revealed impurity and contamination of
Laboratories (Lexington, KY).

ACE purification and preparation. Preliminary experiments were
done with the commercially available ACE. However, SDS-PAGE
analysis of these preparations revealed impurity and contamination of
the preparation with other proteins. To exclude involvement of any
other contaminant in our results, we purified the ACE in our labora-
tory and used it in all subsequent experiments.

Briefly, porcine kidneys were obtained from a slaughterhouse.
Kidney cortex was surgically removed and homogenized in 0.33 M
acetic acid-50 mM HEPES buffer, pH 7.5, 300 mM NaCl at pH 8.3 at 37°C. All procedures
involving protein handling, except protein concentration assay, activ-
tion of characteristic

ACE

ACE ---- 180 kDa

Fig. 1. Coomassie-stained SDS-PAGE gel showing 6 µg of purified angio-
tensin-converting enzyme (ACE, 180 kDa) after elution from lisinopril-cou-
pled chromatography column.
10 MgCl2, and 5 dithiothreitol] along with T4 polynucleotide kinase and [y-32P]ATP (3,000 Ci/mmol at 10 mCi/ml) for 10 min at 37°C. The reaction was stopped by the addition of 0.5 M EDTA and 89 μl Tris-EDTA buffer. Unincorporated label was removed with G-25 spin column. Nuclear extracts (10 μg) were preincubated for 15 min at room temperature with binding buffer [poly(dI-dC)-poly(dI-

Measurement of intracellular cAMP. Rat VSMC were exposed to 3.75 μg/ml of ACE for 20 min and 3 h and scraped in 0.1 M HCl and then centrifuged at 1,000 g for 10 min. The supernatant was collected and acetylated together with standards before the analysis. cAMP contained in each sample was determined by specific kit cAMP EIA Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

Statistical analysis. All data are expressed as means ± SE. Student’s t-test for paired and unpaired data was used as appropriate. Differences at P < 0.05 were considered significant.

RESULTS

Effects of ACE on B1 and B2 bradykinin receptor gene expression. Addition of ACE in the culture medium of rat aortic VSMC upregulated the B1 receptor gene expression after 3 h of incubation. Cells were depleted of serum for 24 h and were subsequently exposed to 3.75 μg/ml of ACE with specific activity between 90 and 100 U/mg. mRNA levels of B1 receptor were significantly upregulated compared with baseline (Fig. 2A).

With the same conditions, we analyzed levels of mRNA for the B2 receptor, which is already constitutively expressed in VSMC in basal conditions. Addition of 3.75 μg/ml ACE significantly stimulated expression of both bradykinin receptor genes: expression of the B1 receptor gene increased by 22-fold and of the B2 receptor gene by 11-fold (Fig. 2B).

A control set of experiments was performed by analyzing gene expression levels of both bradykinin B1 and B2 receptors after stimulation for 3 h with the ACE buffer. Analysis for mRNA levels showed no difference from the unstimulated control (data not shown).

To separate the enzymatic activity from other possible properties of the ACE, we repeated the above experiments after coincubation of ACE with 10–4 mol/l ACE inhibitor captopril. ACE specific activity was determined to be almost completely blocked to 0.03–0.07 U/mg. In both cases, the enzymatic blockade did not interfere with the ACE effect on B1 and B2 receptor gene expression (Fig. 2, A and B).

To investigate the possibility that the ACE effect might be mediated by generation of angiotensin II (acting via its receptors AT1 and AT2) and convincingly exclude any influence of...
enzymatic activity of the ACE on this process, we repeated the above experiments with addition of the specific AT1 receptor antagonist losartan (10^-5 mol/l) or the AT2 receptor antagonist PD-123319 (10^-5 mol/l).

In neither case was the ACE effect on B1 and B2 receptor gene expression altered by these angiotensin II antagonists (Fig. 2, A and B). In previous studies we have determined that PD-123319 and losartan given alone have no effect on B1 and B2 receptor mRNA expression (14).

The final set of experiments was designed to confirm that any changes in B1 and B2 receptor gene expression represent a transcriptionally regulated event. In cells preincubated for 10 min with the transcriptional inhibitor actinomycin D, the 3-h incubation with ACE failed to produce induction and upregulation in B1 and B2 receptor mRNA levels (Fig. 3, A and B).

Pretreatment of cells with curcumin, an inhibitor that affects several different factors that are involved in gene expression regulation, including the NF-kB and AP-1 factor, also blocked the ACE effect on both genes as shown by the decrease of B1 and B2 receptor gene expression to basal levels (Fig. 3, A and B).

**Induction of NF-kB and AP-1 transcriptional factors by ACE.** The electrophoretic mobility shift assay (EMSA) was used to study the involvement of different transcriptional factors that were shown to be responsible in the regulation of bradykinin receptor gene expression (18, 30, 25).

Both bradykinin B1 and B2 receptor genes have been shown to have a responsive element in the promoter region for AP-1 transcriptional factor (24). Collected nuclear extracts from untreated VSMC have shown fairly abundant binding for DNA consensus sequence of AP-1 factor; however, after a 3-h period of stimulation with ACE, we observed increase of signal for B1 receptor protein in the 45-kDa range. This signal was not

**Studies of the promoter region for bradykinin B1 and B2 receptor gene** suggest the presence of cAMP response element-binding protein site (25, 23). Therefore, we performed EMSA with nuclear extracts from control and ACE-treated VSMC and CREB DNA consensus sequence. As shown in Fig. 4A, there was no difference in CREB binding intensity between control and stimulated cells. As for AP-1 studies, CREB studies were confirmed by using specific unlabeled competitors and nonspecific (SP-1) unlabeled competitor.

The bradykinin B1 receptor gene is an easily inducible gene, and one of the transcriptional factors most likely to be involved is NF-kB. To explore the role of NF-kB in this phenomenon, we compared binding of nuclear protein from ACE-treated and ACE-nontreated cells for DNA consensus sequence corresponding to NF-kB binding site. ACE treatment resulted in markedly upregulated NF-kB DNA bindings compared with control (Fig. 4C). The result was confirmed by inhibiting the level of binding with different concentrations of unlabeled specific competitor and unlabeled nonspecific competitor (SP-1).

**Intracellular cAMP levels in ACE-treated VSMC.** As one of the most important second messengers in cellular signaling on activation of G protein-coupled receptors, cAMP was assayed as described in EXPERIMENTAL PROCEDURES. There was no difference in cellular levels of cAMP between cells that were not treated with ACE and cells that were treated with ACE. Additionally, we explored the possible time frame of cAMP activation in cells following the stimulation; again, there was no difference between a 20-min stimulation period versus a 3-h stimulation period (data not shown).

Bradykinin B1 and B2 receptor protein expression under ACE stimulation in VSMC. Western blot analysis of membrane protein extracts from rat VSMC with anti-B1 receptor antibodies revealed very low basal expression of bradykinin B1 receptor protein in the 45-kDa range. This signal was not
significantly changed after ACE stimulation for 4 h in serum-free media. Densitometric analysis and normalization with equal amount protein loading of the immunoreactivity signal from protein extracts from treated and untreated cells showed no statistically significant differences in the level of protein expression between the two groups (data not shown).

Bradykinin B$_2$ receptor was detected by using anti-B$_2$ receptor antibodies, displaying a strong signal in the 42- to 50-kDa range. After densitometric analysis between two different groups of protein isolate, from ACE-stimulated and unstimulated cells, we concluded that the B$_2$ receptor protein level was not altered (data not shown).

**DISCUSSION**

There are two isoforms of the ACE, both transcribed from the same gene (20). The somatic form is a large glycoprotein composed of a single polypeptide chain of about 1,300 amino acids and has been isolated from endothelial, pulmonary, and renal cells of various mammals, having an 80–90% identity between species. The germinal form, consisting of about 700 amino acids, which is almost identical to the COOH-terminal half of the somatic ACE, is only present in sperm cells of the testis (4). Although mainly bound on the surface of epithelial and endothelial cells, the ACE is also found in soluble form in many body fluids, where it is released via the proteolytic action of a membrane protein secretase (22). Whether membrane bound or in the extracellular fluid, the known role of the ACE is to hydrolyze a variety of short peptide substrates by removing a dipeptide from their COOH-terminal. Various carboxypeptidases participate in the release and metabolism of polypeptides and hormones in the circulation. Some are known to act intracellularly, either being endogenous or possibly entering the cell by receptor-mediated endocytosis, where their role seems to be processing of peptide hormones and influencing of signal transduction by acting on peptide ligands (32).

In the current experiments, we found that in vascular smooth muscle cell culture free from all plasma ingredients and their competing influences, the addition of exogenous ACE induced a highly significant upregulation of the gene expression of both the B$_1$ and B$_2$ receptors of bradykinin. This upregulation was more pronounced for the B$_1$ receptor gene. Furthermore, we explored bradykinin B$_1$ and B$_2$ receptor expression on the cell membrane after 4 h of ACE stimulation. However, we found no changes in the protein level for these receptors. There are several possible explanations for this discrepancy, all of them speculative: they include the possibility that for B$_1$ receptor, antibodies may not be sensitive and reliable enough as the B$_2$ receptor antibodies (which are specific monoclonal antibodies against this receptor), or the possibility that upregulation of protein generation requires a longer interval of stimulation with ACE, or the possibility that the ACE acts to both initiate induction of gene expression and posttranscriptionally down-regulate gene activity, hence maintaining balance in protein generation.

The fact that ACE inhibitors did not interfere with this upregulation of B$_1$ and B$_2$ receptor genes suggests that this is an additional new function of the ACE, unrelated to its enzymatic properties as a peptidyl dipeptidase. Recently, it was reported that indeed ACE does exert another function that is independent of its activity as peptidyl dipeptidase: its ability to cleave glycosylphosphatidylinositol (GPI)-anchored protein (16). This new property was shown to play a major role in the fertilization process.
Further in our experiments, we showed that the presence or absence of other humoral factors, including selective AT_1 or AT_2 receptor antagonists, did not affect ACE-induced upregulation of bradykinin receptor gene expression. Blockade of angiotensin II receptors was necessary to prove that this effect was a direct action of the ACE and not an intracrine effect of locally generated angiotensin II.

We found particularly intriguing the fact that this normally extracellular or membrane-bound enzyme could markedly affect the gene expression of the receptors of one of its main substrates and therefore tried to elucidate further the underlying mechanism. The transcriptional inhibitor actinomycin D completely prevented ACE-induced upregulation of the B_1 and B_2 receptor gene, indicating that this was a transcriptionally regulated event rather than a posttranscriptional one. Another pharmacological inhibitor, curcumin, which interacts on the level of production and activation of the transcriptional factors (notably AP-1 and NF-κB), abolished increase in the mRNA level for the bradykinin B_1 and B_2 receptor genes. Curcumin is a widely used compound in vivo and in vitro that interferes with a broad range of intracellular signaling pathways (1). It has been shown to suppress the activation of NF-κB by possibly inhibiting a kinase involved in IκBα phosphorylation. Curcumin has also been shown to block AP-1 formation and action (13). The bradykinin B_1 receptor gene was shown to have possibly two promoter regions, which have, among many others, potential binding sites for NF-κB and AP-1. Under stimulation with inflammatory stimuli and growth factors, the transcriptional factor NF-κB is usually activated and responsible for B_1 gene upregulation (29, 21). Furthermore, an AP-1 binding site was shown to play an important role in B_1 receptor gene upregulation (34). The B_2 receptor gene promoter regions have been identified to have, among others, AP-1 binding sites, NF-κB binding sites, and CREB binding sites (25). cAMP activation by different stimuli was shown to modulate B_2 receptor gene expression, possibly through CRE binding protein (23). EMSA performed with nuclear protein samples from ACE-treated VSMC showed increase of NF-κB and AP-1 nuclear accumulation compared with untreated cells, confirming indeed involvement of these two transcriptional factors in the ACE-mediated stimulation of B_1 and B_2 receptor genes expression. We also explored the possibility that CREB is increased under ACE stimulation but found no increase. VSMC were assayed for cAMP levels under ACE stimulation and presented no change, further excluding that particular pathway.

The discovery that ACE has another biological property in addition to its known enzymatic activity is intriguing. From the current experiments, we suggest that the ACE effect is exerted in the intracellular level through activation of NF-κB and AP-1 transcriptional factors, which seem to be involved in the increase of the expression of the bradykinin receptor genes. The level of transcriptional activation of these genes was very high and similar to that obtained by other inflammatory stimuli (i.e., LPS, TNF-α, and IL-1) as reported in the literature (21, 31, 24). It is tempting to speculate about the mechanisms involved. One possibility (which we are currently exploring) is the existence of a binding protein/receptor on the cell membrane surface that triggers the intracellular response of ACE stimulation. Another is that ACE acts intracellularly following endocytosis through the cell membrane perhaps by interacting with a currently unknown binding site responsible for endocytosis. In our studies, purified porcine kidney ACE was solubilized from kidney cells with Triton X-100, and hence it probably contains the COOH-terminal fragment (19) that represents a membrane anchor for this enzyme; therefore, we could assume that the enzyme itself gets reconstituted in the cell membrane under unknown conditions and as a result triggers an intracellular signaling cascade. A third possibility is that ACE might act via influence on another humoral factor unrelated to its peptidyl dipEptidase activity.

The ACE is already known to be one of the most widespread enzymes acting on a multitude of substrates in the extracellular space with new ones being discovered all the time. For example, in addition to its best known enzymatic effect (conversion of angiotensin I to angiotensin II and degradation of kinins), and its ability to cleave GPI-anchored protein (16), it has also been shown to act on a novel peptide with antifibrotic and anti-proliferative properties (27). Moreover, a novel non-ACE-mediated property of the ACE inhibitors was recently described, the direct activation of the B_1 receptor on the cell surface without an intermediate peptide ligand (12). The current data suggest that ACE has an additional property unrelated to its peptidyl dipEptidase activity. This novel biological activity involves activation of intracellular signaling cascades that produce increase in transcriptional factors NF-κB and AP-1 and result in upregulation of the bradykinin B_1 and B_2 receptor genes. The biological significance of this effect remains to be elucidated.

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DISCLOSURE

None of the authors reports conflict of interest.

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