Do angiotensin-converting enzyme inhibitors directly stimulate the kinin B₁ receptor?

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Am J Physiol Heart Circ Physiol 285: H277–H282, 2003. First published March 20, 2003; 10.1152/ajpheart.01124.2002.—It has been recently claimed that the human B₁ receptors for kinins bind angiotensin-converting enzyme (ACE) inhibitors via a potential zinc-binding domain and are pharmacologically stimulated by these drugs. We verified whether ACE inhibitors stimulate B₁ receptors in vitro. The isolated rabbit aorta or mouse stomach responded by negligible contractions to the application of captopril, enalaprilat, or zofenoprilat. The human isolated umbilical vein also failed to respond to enalaprilat. All of these preparations were responsive to the B₁ receptor agonists des-Arg⁹-bradykinin (BK) or Lys-des-Arg⁹-BK. Furthermore, enalaprilat applied continuously had no significant interaction with the effects of Lys-des-Arg⁹-BK on the rabbit aorta. Enalaprilat failed to stimulate [³H]arachidonate release, translocate the receptors (confocal microscopy), or stimulate ERK1/2 phosphorylation (immunoblot) in HEK-293 cells stably expressing the recombinant human B₁ receptors via two types of G protein-coupled receptors (GPCRs) in mammals: the B₁ and B₂ receptors (12, 17). Angiotensin-converting enzyme (ACE) hydrolyze and inactivate kinins; thus drugs designed to inhibit ACE not only prevent the formation of the pressor hormone ANG II from ANG I but also may potentiate endogenous kinins (22). Because they are vasodilator and antihypertensive agents, endogenous kinins may produce a part of the antihypertensive effect of ACE inhibitors, depending on the experimental model (22). A novel claim about the role of B₁ receptors in the therapeutic effect of ACE inhibitors has been made lately: these drugs have been said to directly bind to a zinc atom presumably present in a consensus zinc-binding domain identified in the second extracellular loop of this receptor (8). This domain, a HEXXH motif, is conserved in all species for which the B₁ receptor is currently known, and its sequence is perfectly conserved in the rabbit and the mouse (HEAWH), relative to the human sequence (11, 16). This type of site is present in the active site of metallopeptidases such as ACE (23) but not present in the B₂ receptor sequence. Thus, according to these investigators, ACE inhibitors at nanomolar concentrations would be able to activate the kinin B₁ receptor with such consequences as calcium signaling and nitric oxide production in transfected cells expressing the recombinant human B₁ receptors or in endothelial cells (8). Several years ago, Babiuk et al. (3) studied the interaction of the ACE inhibitor captopril with the B₁ receptor agonists des-Arg⁹-bradykinin (BK) and Lys-des-Arg⁹-BK on the B₁ receptors mediating the contraction of the rabbit isolated aorta and have found essentially none. In the present experiments, we have controlled and extended these findings using the same preparation and other established smooth muscle bioassays for the B₁ receptor, the mouse isolated stomach, and human umbilical vein. ACE inhibitors such as enalaprilat (specifically claimed to activate the B₁ receptor, see Ref. 8) have been exploited in the present experiments. We have also verified the effect of enalaprilat on human or rabbit smooth muscle cells that express B₁ receptors at physiological densities or on a fusion protein composed of the rabbit B₁ receptor conjugated to the yellow fluorescent protein (B₁R-YFP). The B₁R-YFP protein is a fully functional high-affinity receptor that recently allowed to record phospholipase A₂ activation and a form of subcellular receptor translocation in response to Lys-des-Arg⁹-BK (18).

KININS ARE BLOOD-DERIVED PEPTIDES that stimulate vascular cells via two types of G protein-coupled receptors (GPCRs) in mammals: the B₁ and B₂ receptors (12, 17). Angiotensin-converting enzyme (ACE) hydrolyze and inactivate kinins; thus drugs designed to inhibit ACE not only prevent the formation of the pressor hormone ANG II from ANG I but also may potentiate endogenous kinins (22). Because they are vasodilator and antihypertensive agents, endogenous kinins may produce a part of the antihypertensive effect of ACE inhibitors, depending on the experimental model (22). A novel claim about the role of B₁ receptors in the therapeutic effect of ACE inhibitors has been made lately: these drugs have been said to directly bind to a zinc atom presumably present in a consensus zinc-binding domain identified in the second extracellular loop of this receptor (8). This domain, a HEXXH motif, is conserved in all species for which the B₁ receptor is currently known, and its sequence is perfectly conserved in the rabbit and the mouse (HEAWH), relative to the human sequence (11, 16). This type of site is present in the active site of metallopeptidases such as ACE (23) but not present in the B₂ receptor sequence. Thus, according to these investigators, ACE inhibitors at nanomolar concentrations would be able to activate the kinin B₁ receptor with such consequences as calcium signaling and nitric oxide production in transfected cells expressing the recombinant human B₁ receptors or in endothelial cells (8). Several years ago, Babiuk et al. (3) studied the interaction of the ACE inhibitor captopril with the B₁ receptor agonists des-Arg⁹-bradykinin (BK) and Lys-des-Arg⁹-BK on the B₁ receptors mediating the contraction of the rabbit isolated aorta and have found essentially none. In the present experiments, we have controlled and extended these findings using the same preparation and other established smooth muscle bioassays for the B₁ receptor, the mouse isolated stomach, and human umbilical vein. ACE inhibitors such as enalaprilat (specifically claimed to activate the B₁ receptor, see Ref. 8) have been exploited in the present experiments. We have also verified the effect of enalaprilat on human or rabbit smooth muscle cells that express B₁ receptors at physiological densities or on a fusion protein composed of the rabbit B₁ receptor conjugated to the yellow fluorescent protein (B₁R-YFP). The B₁R-YFP protein is a fully functional high-affinity receptor that recently allowed to record phospholipase A₂ activation and a form of subcellular receptor translocation in response to Lys-des-Arg⁹-BK (18).

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Materials and Methods

Drugs. Enalaprilat (Vasotec IV) was manufactured by Merck. Zofenoprilat and B-9858 were gifts from Menarini Ricerche (Florence, Italy) and Laboratoires Fournier (Daux, France), respectively. Des-Arg9-BK was purchased from Bachem Bioscience (King of Prussia, PA), human recombinant interleukin-1β (IL-1β) was from R&D Systems (Minneapolis, MN), and the remaining drugs were from Sigma (St. Louis, MO).

Contractility studies. Exploratory studies were conducted to compare direct contractile effects of the B1 receptor agonist des-Arg9-BK to that of selected ACE inhibitors in two smooth muscle bioassays: the isolated rabbit aorta and mouse stomach. Murine tissues were obtained either from C57BL/6 mice or from a described mouse strain in which the B2 receptor gene had been inactivated (4); they were prepared as described (1). Rabbit aortic rings (New Zealand White, 1.5–2 kg, Charles River, St. Constant, Canada) were suspended under a tension of 2 g in 5-ml tissue baths containing oxygenated (95% O2,5% CO2) and warmed (37°C) Krebs solution as described (9).

Kinins contract the isolated human umbilical vein via preformed B2 receptors responsive to BK and via inducible B1 receptors responsive to both des-Arg9-BK and Lys-des-Arg9-BK (6, 13, 20). Further contractility studies in the rabbit aorta and human umbilical vein preparations (conducted as described, see Ref. 13) were based on the construction of cumulative concentration-response curves for the high-affinity B1 receptor agonist Lys-des-Arg9-BK. Two cumulative concentration-response curves were constructed at 3 and 6 h from the beginning of the incubation of the preparations with ample washings between the interventions. This protocol accounts for the fact that the maximal response to this kinin increases as a function of the postisolation incubation time in these preparations, a behavior specific for agonists of B1 receptors and attributed to the postisolation formation of these receptors (9, 19, 20). The stimulant drug was always Lys-des-Arg9-BK for the first curve and this kinin or enalaprilat for the second one. Some rabbit tissues stimulated with the kinin twice were continuously treated with either ZnCl2 (100 nM) or enalaprilat (1 μM) to monitor any effect on the sensitivity, maximal effect, or sensitization process. Some rabbit tissues acutely stimulated with enalapril at 6 h had been also treated with ZnCl2. Contractility results were expressed in grams of tension.

It was verified in separate preparations of isolated rabbit aortas with intact endothelium that the ACE inhibitor enalaprilat could reduce the apparent contractile potency of ANG I, largely attributed to the in situ conversion of this peptide into ANG II (21). To do so, cumulative concentration-effect curves for either ANG I or ANG II were established twice at 1 and 5 h postmounting; enalaprilat (1 μM) was applied 30 min before the construction of the second curve.

Cellular systems. The derivation of a HEK-293 cell line stably expressing B1-R-YFP and its properties have been described elsewhere (18). These cells were used in a phospholipase A2 assay (24-well plates) and confocal microscopy (35-mm petri dishes), precisely as described (18), and also in a ERK1/2 phosphorylation assay (see ERK1/2 phosphorylation assay). Primary cultures of vascular smooth muscle cells from the rabbit aorta and human umbilical artery were obtained as described (2, 19).

ERK1/2 phosphorylation assay. To extend the investigation of cellular responses mediated by B1 receptors, we tested the presence of phospho-ERK1/2 in resting or drug-treated cells (untransfected HEK-293 cells, HEK-293 cells stably expressing B1-R-YFP or primary cultures of smooth muscle cells from the rabbit aorta or human umbilical artery). These kinases are known to be activated by the peptide agonist of the B1 receptors des-Arg9-BK in cellular systems (15). Confluent 75-cm2 HEK-293 cell flasks were cultured overnight with medium containing a reduced fetal bovine serum concentration (0.5%) to minimize the background phosphorylation of the tested kinases. Rabbit aortic smooth muscle cells express kinin B1 receptors in a regulated manner and in higher abundance following treatment with IL-1β (19). Human umbilical artery smooth muscle cells were also shown to express the messenger RNA coding for the kinin B1 receptor in a regulated manner (stimulation by IL-1β treatment) (2). Accordingly, the human cells bind the selective B1 receptor radioligand [3H]Lys-des-Arg9-BK in a more intense manner if pretreated with IL-1β (T. Sabourin and F. Marceau, unpublished observations). Smooth muscle cells of either origin (75 cm2 flasks) were starved in fetal bovine serum (0.5%) 36 h before kinase assay and treated with recombinant IL-1β (5 ng/ml) 4 h before use to maximize the receptor abundance. The cells were treated with the stimulants agonist Lys-des-Arg9-BK (10 nM) or enalaprilat (1 μM; both applied 30 min before extraction in HEK-293 cells or 10 min in muscle cells), the B1 receptor antagonists Lys-[Leu8]des-Arg9-BK (1 μM) or B-9858 (Lys-Lys-[Hyp4,Igl5,Ngl7]-Oic)-des-Arg9-BK; see Ref. 10; 10 nM; antagonists applied 40 min before extraction in HEK-293 cells or 10 min in muscle cells), or combinations of these drugs. Total cell extracts applicable to immunoblots were then prepared as outlined elsewhere (7). Transferred proteins were revealed using two types of antibodies for each sample: phospho-ERK1/2 (monoclonal, dilution 1/1,000, New England Biolabs) and total ERK1/2 (to show comparable loading; polyclonal, dilution 1/1,000, New England Biolabs). Staining was revealed using the appropriate peroxidase-conjugated secondary antibodies (1/16,000 for each).

RESULTS

Contractility studies. The isolated aorta from the rabbit or isolated stomach from the mouse, either from control or B2 receptor gene knockout animals, responded by negligible contractions to the application of captopril (10−7–10−5 M), enalaprilat (10−6 M), or zofenoprilat (10−5 M) (Table 1). All these preparations were responsive to the B1 receptor agonist des-Arg9-BK.

Table 1. Effect of ACE inhibition on B1 receptors rabbits and mice

<table>
<thead>
<tr>
<th></th>
<th>Rabbit Aorta</th>
<th>Mouse Stomach</th>
<th>Mouse Stomach (B2 Receptor Knockout)</th>
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<tbody>
<tr>
<td>des-Arg9-BK</td>
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<tr>
<td>10−7 M</td>
<td>0.750 ± 0.098</td>
<td>0.543 ± 0.012</td>
<td>0.470 ± 0.014</td>
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<tr>
<td>Captopril</td>
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<tr>
<td>10−5 M</td>
<td>0</td>
<td>0</td>
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<tr>
<td>10−6 M</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Zofenoprilat</td>
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<tr>
<td>10−5 M</td>
<td>0.003 ± 0.001</td>
<td>0</td>
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<tr>
<td>Enalaprilat</td>
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<tr>
<td>10−5 M</td>
<td>0.005 ± 0.003</td>
<td>0</td>
<td>0.067 ± 0.002</td>
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</table>

Values are expressed in grams of isometric tension and are means ± SE of 4–8 determinations. All assays were performed in the presence of 10−6 M ZnCl2. ACE, angiotensin-converting enzyme.

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In another series of experiments, no direct activation of B1 receptors by enalaprilat (1 nM-1 μM, applied at 6 h) was recorded in tissues previously proven to respond to the alternate B1 receptor agonist Lys-des-Arg^9^-BK (Fig. 1). Furthermore, enalaprilat applied continuously had no significant interaction with the effects of Lys-des-Arg^9^-BK (Fig. 1). Because hypothetical loading of B1 receptors with ZnCl2 has been suggested to favor the stimulant action of ACE inhibitors, we have continuously treated some tissues with this salt (100 nM). Zinc preloading is not permissive for a direct action of enalaprilat nor does it modify the contractile effect of Lys-des-Arg^9^-BK (Fig. 1).

Other sets of isolated rabbit aortas have been stimulated with ANG I or ANG II twice, the second time in the presence of enalaprilat (1 μM; Fig. 2). The drug produced a 15.8-fold shift to the right of the concentration-effect curve (loss of apparent potency) for ANG I but exerted negligible effects on the response to ANG II.

As observed with the isolated rabbit aorta, the isolated umbilical vein incubated for 6 h failed to respond by significant contractions to enalaprilat (1 nM-1 μM) (Fig. 3). The human preparation responded to Lys-des-Arg^9^-BK, but the change of contractile response to this B1 receptor agonist as a function of time (from 3 to 6 h postmounting) was less important than for the rabbit aorta (compare Fig. 3 with Fig. 1).

Cellular system expressing B1 receptors. Enalaprilat (1–1,000 nM) failed to stimulate [3H]arachidonate release in a phospholipase A2 assay based on HEK-293 cells stably expressing B1R-YFP (Fig. 4). In the same series of experiments, Lys-des-Arg^9^-BK was highly active in this respect (Fig. 4). Untransfected HEK-293 cells are unresponsive to this peptide in this assay (18).

Confocal fluorescence microscopy reveals that B1R-YFP is expressed essentially as an evenly distributed membrane protein (Fig. 5, see Ref. 18). Enalaprilat (1 μM, 30 min) failed to modify the cellular distribution of B1R-YFP, whereas Lys-des-Arg^9^-BK (10 nM, 30 min) concentrated fluorescent receptors in structures that remained close to the membrane surface (previously proposed to be cholesterol-rich caveolae-related rafts) (18).

ERK1/2 was phosphorylated in HEK-293 cells stimulated with Lys-des-Arg^9^-BK (10 nM) if they expressed B1R-YFP but not in untransfected cells (Fig. 6, top). The response was further shown to be dependent on B1 receptor signaling as it was abrogated by the antagonist B-9858. Enalaprilat (1 μM) failed to stimulate ERK1/2 phosphorylation in either type of HEK-293 cells (Fig. 6). Similarly, ERK1/2 was phosphorylated in response to Lys-des-Arg^9^-BK in smooth muscle cells derived from either the rabbit aorta or human umbilical artery (Fig. 6, bottom).
This response was abrogated by the antagonist Lys-[Leu⁸]-des-Arg⁹-BK. The phospho-ERK1/2 content of enalaprilat-treated smooth muscle cells was not different from that of control cells.

**DISCUSSION**

We failed to reproduce the claim of Ignjatovic et al. (8): it cannot be generally supported that ACE inhibitors directly activate B₁ receptor signaling in tissue-based or cellular bioassays in the present experiments, including in systems based on the human form of the receptor. Especially the ACE inhibitors failed to contract the smooth muscle preparations responsive to the conventional peptide agonists (Table 1, Figs. 1 and 3). The B₁ receptor seems to be constitutively expressed in the mouse stomach preparation (1); neither ACE inhibitors nor des-Arg⁹-BK were more active in the stomach isolated from B₂ receptor knockout mice, in which a certain compensatory upregulation of B₁ receptors has been proposed to apply to the cardiovascular and renal systems (5). Enalaprilat also failed to influence the potency, maximal effect, or sensitization behavior of the response to Lys-des-Arg⁹-BK in the rabbit aorta (Fig. 1). The isolated human umbilical vein is an established bioassay for the human form of the B₁ receptor, and it did not respond to enalaprilat (Fig. 3). Moreover, preloading the rabbit or mouse tissues with zinc, a procedure inspired by Ignjatovic et al. (8), also failed to reveal an effect of enalaprilat, captopril, or zofenoprilat (Table 1, Fig. 1). As reported with the early ACE inhibitor teprotide (21), enalaprilat reduced the apparent potency of ANG I without affecting that of ANG II in the rabbit aorta contractility assay, providing a positive control for the presence of ACE in the preparation and for the activity of the drug.

One of the cellular systems that we exploit here, HEK-293 cells expressing recombinant B₁R-YFP, allowed the testing of ACE inhibitors effects on three additional functional endpoints different from contractility (Figs. 4–6). Enalaprilat failed to stimulate phospholipase A₂ activity, ERK1/2 phosphorylation, or receptor translocation, whereas the peptide agonist Lys-des-Arg⁹-BK was active in all cases. Arterial smooth muscle cells of rabbit or human origin express wild-type B₁ receptors at a more physiological density; these cellular systems respond to Lys-des-Arg⁹-BK, but not to enalaprilat, as judged by the phosphorylation of ERK1/2 (Fig. 6). Different transduction mechanisms, largely unexplored for the B₁ receptor, may link GPCRs to these noncontractile cell responses.

Despite of our discrepant results, we give credit to Ignjatovic et al. (8) for pointing out a plausible and well-conserved metal binding site in a B₁ receptor extracellular domain that may assume other roles, such as the formation of heterodimers with other molecular partners that remain to be identified, an enzymatic function, or the direct binding of other peptides.
kallikrein-kinin system components. It is not clear whether B₁ receptor-mediated signaling has been observed in the cellular systems previously described (8). One possibility is that a low endogenous level of kinins is produced in the systems, either because the cells synthesize components of the kallikrein-kinin system or have taken up some from the culture media. This type of artifact is probably involved in the pharmacological actions of human tissue kallikrein in systems that express the rabbit BK B₂ receptors (7).

Whether B₁ receptors are automatically involved in the therapeutic effects of ACE inhibitors is not supported by the present experiments. The des-Arg⁹-kinins that are optimal B₁ receptor agonists are low-affinity substrates for ACE; when this enzyme is blocked, there is clinical evidence that the individual efficacy of alternate degradation pathways predicts the probability of angioedema, a severe ACE inhibitor-associated side effect (14). Therefore, B₁ receptors may be associated with the inflammatory side effects of ACE inhibitors.

In summary, ACE inhibitors do not stimulate cell or tissue responses that are stimulated with the conventional peptide agonists at the natural or recombinant B₁ receptors.
ACE INHIBITORS AND KININ B1 RECEPTORS

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