

Minireview

Kinin- and angiotensin-converting enzyme (ACE) inhibitor-mediated nitric oxide production in endothelial cells

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Abstract

Carboxypeptidase cleavage of the C-terminal Arg of kinins generates specific agonists of the B₁ receptor. Activation of B₁ receptors produces nitric oxide via eNOS in bovine endothelial cells and iNOS in cytokine-stimulated human endothelial cells. Angiotensin-converting enzyme (ACE) inhibitors are direct agonists of B₁ receptors in endothelial cells, although they release NO via a different signaling pathway than peptide ligands in bovine cells. This brief review discusses carboxypeptidase M as a required processing enzyme for generating B₁ agonists, how ACE inhibitors and peptide ligands stimulate NO production and the evidence for, as well as some consequences of, the direct activation of B₁ receptors by ACE inhibitors.

Keywords: B₁ receptor; bradykinin; carboxypeptidase M; kallidin; kininase I; nitric oxide synthase.

Introduction

Kinins are active peptides containing nine (bradykinin) or ten (kallidin; Lys-bradykinin) amino acids and they are released by kallikreins from the precursor molecule, kininogen (Bhoola et al., 1992). Kinins contain C-terminal Arg residues that are essential for interaction with the constitutively expressed B₂ kinin receptors (Regoli and Barabe, 1980). One of the original pathways established for kinin metabolism was the removal of the C-terminal Arg residue by plasma carboxypeptidase N (CPN) or kininase I (Erdős and Sloane, 1962). Although this step was originally considered to inactivate kinins, more recently a different kinin receptor, named B₁, was discovered (Regoli and Barabe, 1980). Its expression is induced by tissue injury or inflammation and binds with highest affinity to kinin peptides lacking the C-terminal Arg residue (Regoli and Barabe, 1980). Later research established the presence of a different enzyme, carboxypeptidase M (CPM), on the plasma membrane of many cell types that also efficiently cleaves the C-terminal Arg (Skidgel et al.,

1984, 1989). Thus, B-type carboxypeptidases (enzymes that cleave C-terminal basic amino acids, e.g., CPM or CPN) are the required processing enzymes that generate agonists for the B₁ receptor (Figure 1), analogous to the angiotensin-converting enzyme (ACE) that converts inactive angiotensin I initially released from the angiotensinogen precursor into active angiotensin II (Skidgel and Erdős, 1993). It also follows that inhibitors of B-type carboxypeptidases should block B₁ receptor-mediated responses, much as ACE inhibitors block angiotensin II-mediated effects.

Carboxypeptidase-mediated regulation of kinin signaling and nitric oxide production

The main B-type carboxypeptidase to yield the specific ligands for kinin B₁ receptors is likely CPM (Figure 1). This is a glycosylphosphatidylinositol-anchored protein localized on the cell plasma membrane, where it has access to peptides in close proximity to the peptide receptors (Skidgel, 1988, 2004; Skidgel and Erdős, 1998). Furthermore, the recently determined crystal structure of CPM revealed an S1' binding pocket that is particularly well suited to accommodate C-terminal Arg residues (Reverter et al., 2004). Unique structural features in the C-terminal domain can orient the active site to effectively deliver the cleaved products to nearby membrane proteins such as the B₁ receptors (Reverter et al., 2004). Although the concept that a carboxypeptidase can regulate kinin signaling via the B₁ or B₂ receptors by hydrolysis of C-terminal Arg residues has been well known for many years (Skidgel, 1988; Figure 1), few pieces of evidence supported the idea that this happens in real time in a physiologically relevant way. We recently investigated this regulatory step using control and cytokine-stimulated human lung microvascular endothelial cells (HLMVECs) to simulate conditions that might be encountered in septic shock (Sangsree et al., 2003). Sepsis is a leading cause of acute lung injury, and causes pulmonary inflammation and increases capillary endothelial permeability. Nitric oxide (NO) plays an important role in regulating lung vascular permeability, and the high levels produced during inflammation, or when combined with superoxide to form peroxynitrite, can injure the endothelial barrier (Tasaka et al., 2002). CPM is highly expressed in lungs (Nagae et al., 1993) and kinins stimulate NO production via activation of the B₂ and/or B₁ receptor (Bhoola et al., 1992; Leeb-Lundberg et al., 2005). We used a porphyrinic microsensor to directly measure NO release from HLMVEC monolayers in real time as a response to kinins (Sangsree et al., 2003). Using cultured HLMVECs as controls, we added bradykinin to cause a transient

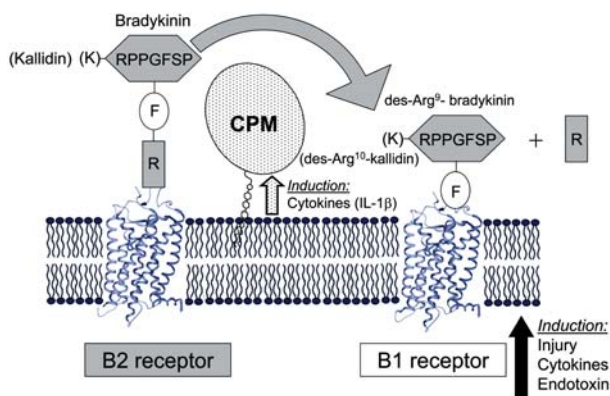


Figure 1 Schematic diagram of the putative role of carboxypeptidase M (CPM) in the regulation of kinin receptor signaling. By cleaving the C-terminal Arg of bradykinin or kallidin, CPM converts the B₂ agonists into B₁ agonists. The bradykinin (and kallidin) sequences are represented in single letter code and the C-terminal Phe (F) and Arg (R) residues are highlighted as being important determinants of B₂ vs. B₁ receptor binding. The B₂ receptor is constitutively expressed, whereas B₁ is induced by injury or inflammatory cytokines. CPM is also upregulated by inflammatory cytokines, primarily IL-1 β .

burst of NO that returned to baseline in approximately 5 min. This response was mediated by the B₂ receptor, as it was blocked by the B₂ antagonist HOE140 (Sangsree et al., 2003). In contrast, in HLMVECs pretreated with IL-1 β and IFN- γ for 16 h to upregulate B₁ receptors and inducible nitric oxide synthase (iNOS), addition of either bradykinin or kallidin resulted in a slowly increasing and prolonged high output of NO that reached a maximum in approximately 20–30 min and then slowly declined almost to baseline in ~60 min (Sangsree et al., 2003; Figure 2). To determine which receptor type was mediating the response, we used specific kinin receptor antagonists. The B₂ antagonist HOE140 inhibited only approximately 24–30% of the response, whereas a B₁ receptor antagonist, des-Arg¹⁰-Leu⁹-kallidin, inhibited ~50%; a combination of both antagonists blocked approximately 90% of the response (Figure 2). These data indicate that physiologically relevant levels of B₁ agonist were rapidly generated from bradykinin or kallidin by a cellular B-type carboxypeptidase. To prove this, a specific B-type carboxypeptidase inhibitor was used, DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid (MGTA), to block this conversion. Indeed, in the presence of this inhibitor, the output of NO was blocked to a similar extent as in the presence of a B₁ receptor antagonist (Figure 2). Although this inhibitor does not distinguish between CPM and other members of the B-type carboxypeptidase family, plasma CPN was not present in our system and the only other active membrane carboxypeptidase is carboxypeptidase D (CPD; Skidgel and Erdös, 1998). Based on our measurements of CPM and CPD activity in HLMVECs (Sangsree et al., 2003) and the known distribution of CPD (approx. 10% on the plasma membrane and 90% in the Golgi), the ratio of the two enzymes on the cell surface is approximately 5:1 CPM/CPD. In addition, CPD has an acidic pH optimum of 6.2 and only approximately 25% of its activity remains at pH

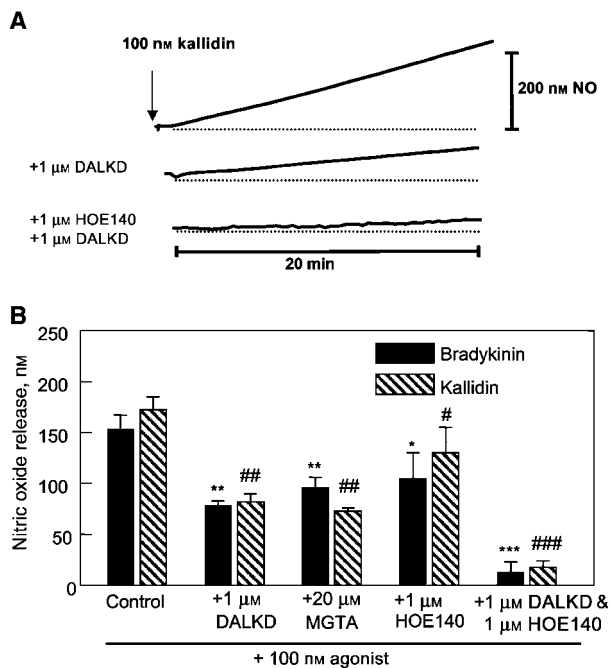


Figure 2 Carboxypeptidase-dependent enhancement of NO production by B₂ receptor agonists via conversion to B₁ receptor agonists.

HLMVECs were treated with 5 ng/ml IL-1 β and 200 U/ml IFN- γ for 16 h and then stimulated with 100 nM bradykinin or kallidin without or with preincubation with the B₁ antagonist des-Arg¹⁰-Leu⁹-kallidin (DALKD), B₂ antagonist HOE 140, or the B-type carboxypeptidase inhibitor DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid (MGTA). NO production was measured electrochemically with a porphyrinic electrode. (A) Typical real-time tracing of NO production. The vertical bar shows the response to a standard 200 nM concentration of NO. Length of the horizontal bar indicates 20 min. (B) Mean values of NO concentration at 20 min from three to eight separate experiments \pm SEM. Difference significant at $p < 0.001$ (***) or ###), $p < 0.01$ (** or ##) or $p < 0.05$ (* or #) compared with 100 nM bradykinin (*) or 100 nM kallidin (#) alone. Modified from Sangsree et al. (2003) and used with permission.

7.4, indicating a likely cell-surface activity ratio of ~20:1 CPM/CPD. To prove this point more directly, we recently used CPM-specific siRNA to knock down its expression in HLMVECs and with this technique we almost completely abolished the conversion of B₂ to B₁ agonist and thus the removal of C-terminal Arg (V. Brovkovich, S. Brovkovich and R.A. Skidgel, unpublished results). Finally, CPM activity can be upregulated approximately two-fold in HLMVECs by the same cytokine treatment (IL-1 β plus IFN- γ) that upregulates B₁ receptor expression (Sangsree et al., 2003). CPM is synthesized in its fully active form (i.e., it does not have a propeptide that renders it initially inactive) and there are no known naturally occurring endogenous inhibitors. Thus, control of its cellular activity derives either from regulation of expression or by enzymatic release from cell membranes (Li and Skidgel, 1999). Indeed, IL-1 β alone can upregulate CPM expression (both the mRNA and protein) and activity in HLMVECs (S. Sangsree and R.A. Skidgel, unpublished data). Taken together, these data show that CPM is important in inflammatory conditions because it enhanc-

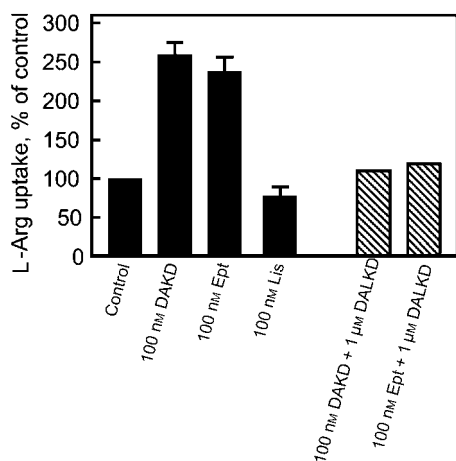


Figure 3 Stimulation of arginine uptake by des-Arg¹⁰-kallidin and enalaprilat in cytokine-stimulated endothelial cells. HLMVECs were pre-treated with 5 ng/ml IL-1 β and 200 U/ml IFN- γ for 16 h and then stimulated with 100 nM des-Arg¹⁰-kallidin (DAKD), 100 nM enalaprilat (Ept) or 100 nM lisinopril (Lis) for 1 min. The abscissa shows the uptake of radiolabeled arginine relative to control (defined as 100%). The effects of enalaprilat and des-Arg¹⁰-kallidin were blocked by the B₁ antagonist des-Arg¹⁰-Leu⁹-kallidin (DALKD). Results represent mean values \pm SEM from three to nine independent experiments carried out in triplicate (solid bars) or mean values from two independent experiments carried out in triplicate (hatched bars). Some of the data shown are taken from Ignjatovic et al. (2004).

es kinin signaling by generating a B₁ agonist, which then activates its receptor and produces high levels of NO.

Regulation of vascular tone by kinin B₁ receptors

The B₁ receptors were first identified functionally because they mediated contraction to des-Arg-kinins in vessel preparations after incubation of the tissue in physiological buffers for several hours (Regoli and Barabe, 1980). Later, it was shown that B₁ receptor expression could be upregulated in response to lipopolysaccharide, inflammatory cytokines and noxious stimuli (Regoli and Barabe, 1980; Leeb-Lundberg et al., 2005). Although vessels incubated *in vitro* for 6 h to induce B₁ receptor expression contract when stimulated by a B₁ agonist, B₁ receptor activation *in vivo* causes vasodilation (Regoli and Barabe, 1980; Leeb-Lundberg et al., 2005). *In vitro*, prolonged incubation in a muscle bath undoubtedly damages or removes the endothelial layer, resulting in contraction due to direct stimulation of the B₁ receptors on the smooth muscle cells and to an increase in intracellular calcium [Ca²⁺]_i. However, dilation *in vivo* is caused by stimulation of B₁ receptors on the endothelial cells, which generates vasodilatory mediators such as prostacyclin and NO (Leeb-Lundberg et al., 2005). We became interested in investigating pathways of B₁-mediated NO generation in pulmonary endothelial cells, especially because we found a novel mode of action of ACE inhibitors, which directly activate the bradykinin B₁ receptors without the involvement of endogenous kinins or ACE (Ignjatovic et al., 2002a).

B₁ receptor signaling pathways activated by ACE inhibitors and peptide agonists

In bovine pulmonary-artery endothelial cells that constitutively express B₁ receptors, the ACE inhibitor enalaprilat and the peptide B₁ agonist des-Arg¹⁰-kallidin (in nanomolar concentrations) stimulate NO production that is blocked by a B₁ receptor antagonist (Ignjatovic et al., 2002a). To investigate which nitric oxide synthase (NOS) isoform was activated, we used specific inhibitors to show that the endothelial NO synthase (eNOS) is primarily responsible for NO generation (Ignjatovic et al., 2004). Interestingly, des-Arg¹⁰-kallidin and the ACE inhibitor enalaprilat activated eNOS by facilitating different signaling pathways downstream of the B₁ receptors. DesArg¹⁰-kallidin enhanced inositol-phosphate generation and elevated [Ca²⁺]_i by augmenting its release from intracellular stores, followed by an influx of extracellular Ca²⁺. In contrast, enalaprilat did not cause inositol-phosphate release, but stimulated only the influx of extracellular Ca²⁺ through rare earth-sensitive channels; its effect was blocked by cholera toxin or protein kinase C inhibitors, agents which did not affect the des-Arg¹⁰-kallidin-mediated response (Ignjatovic et al., 2004).

In HLMVECs treated with cytokines (IL-1 β and IFN- γ for 16 h) to upregulate expression of B₁ receptors and iNOS, both enalaprilat and des-Arg¹⁰-kallidin caused a prolonged (>20 min) release of NO that was blocked by a B₁ but not by a B₂ receptor antagonist (Ignjatovic et al., 2004). The effect of enalaprilat was not due to inhibition of ACE (i.e., causing an increase in kinins followed by carboxypeptidase conversion to des-Arg-kinins), as the carboxypeptidase inhibitor MGTA had no effect (see below). As shown above, MGTA completely blocks the B₁ response when these cells are stimulated with a B₂ agonist kinin.

In cytokine-treated HLMVECs, NO production was primarily iNOS-dependent, in contrast to the bovine pulmonary artery endothelial cells. Consequently the intracellular calcium chelator BAPTA had no effect, as iNOS is not calcium-regulated. However, iNOS does depend on an extracellular supply of its substrate, arginine (Closs et al., 2000), so we investigated whether B₁ receptor stimulation alters arginine uptake. Indeed, both des-Arg¹⁰-kallidin and enalaprilat substantially increased Arg uptake (approx. three-fold) in cytokine-stimulated HLMVECs, which was blocked by a B₁ receptor antagonist (Figure 3). Another ACE inhibitor, ramiprilat, also stimulated Arg uptake (Ignjatovic et al., 2004), but not lisinopril, an ACE inhibitor that is inactive on B₁ receptors (Ignjatovic et al., 2002a; Figure 3). In bovine pulmonary artery endothelial cells, an ACE inhibitor or des-Arg¹⁰-kallidin did not increase Arg uptake (Ignjatovic et al., 2004). Thus, in cytokine-stimulated HLMVECs, ACE inhibitors and peptide ligands of B₁ receptors similarly increase Arg uptake and enhance NO production via iNOS, whereas in bovine pulmonary-artery endothelial cells they both stimulate eNOS activity, but through different signaling pathways (Figure 4).

In recent studies on cytokine-stimulated HLMVECs (Stanisavljevic et al., 2005) we found that B₁ receptor activation by ACE inhibitors (enalaprilat, quinaprilat) or

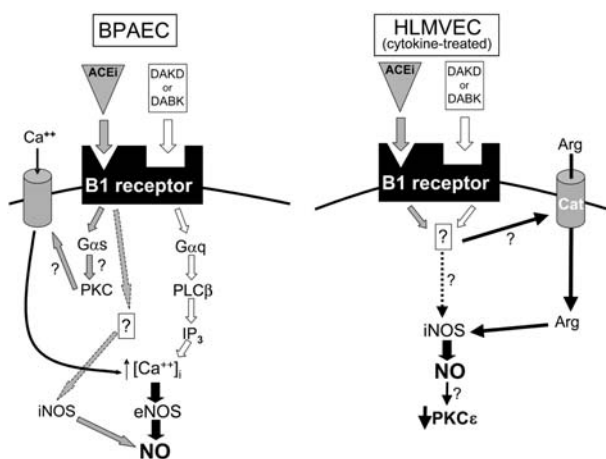


Figure 4 Schematic representation of potential B₁ receptor signaling pathways activated by peptide ligands or ACE inhibitors leading to endothelial NO production.

In bovine pulmonary artery endothelial cells (BPAEC), ACE inhibitors (ACEi) and peptide ligands (des-Arg¹⁰-kallidin, DAKD; or des-Arg⁹-bradykinin, DABK) activate different signaling pathways (represented by shaded arrows or open arrows, respectively) via different G proteins to increase intracellular calcium and stimulate NO production, primarily via eNOS. ACE inhibitors also stimulate NO production via iNOS by an undefined pathway (dashed, shaded arrow). In HLMVECs treated with cytokines IL-1 β and interferon- γ , ACE inhibitors or peptide ligands appear to activate similar signaling pathways to stimulate NO production via iNOS. Although still incompletely understood, a mechanism for this could be an increased Arg uptake, known to be an important regulator of iNOS activity. In HLMVEC, one consequence of increased NO production is the inhibition of PKC ϵ . Question marks denote pathways or mechanisms that have not yet been fully elucidated. Cat, cationic amino acid transporter; PKC, protein kinase C; PLC, phospholipase C; IP₃, inositol 1,4,5-triphosphate.

peptide ligands (des-Arg¹⁰-kallidin, des-Arg⁹-bradykinin) inhibited protein kinase C epsilon (PKC ϵ) with an IC₅₀ of 7×10^{-9} M. Despite the reported differences in binding affinity to B₁ receptors, des-Arg¹⁰-kallidin and des-Arg⁹-bradykinin were equally active, even in the presence of an aminopeptidase inhibitor that blocked the conversion of des-Arg¹⁰-kallidin to des-Arg⁹-bradykinin. NO synthase inhibitors abolished this effect and an NO donor mimicked B₁ receptor activation (Stanisavljevic et al., 2005). Thus, B₁ receptor-dependent NO production inhibits PKC ϵ (Figure 4), a novel PKC isoform upregulated in myocardium after infarction (Simonis et al., 2003).

Overall, the above data indicate that in some cell types, B₁ activation by peptide ligands or ACE inhibitors (which interact at different sites on the receptor) can result in transduction through different signaling pathways. This also raises the possibility that some cell types with only a single operative signal transduction pathway may be unresponsive to an ACE inhibitor, while responding to peptide ligands of B₁ receptors, or *vice versa*.

Evidence for the direct activation of B₁ receptors by ACE inhibitors

The ability of ACE inhibitors to activate B₁ receptors has obvious relevance to the widespread use of these drugs

to treat hypertension and a variety of cardiovascular disorders, especially following myocardial infarction. A recent publication (Fortin et al., 2003) and an eLetter response by Marceau et al. to our publication (Ignjatovic et al., 2004; see 'reply to Ignjatovic et al.' in the online version with our answer) disputed our findings that ACE inhibitors activate B₁ receptors, indicating that our results reported earlier (Ignjatovic et al., 2002a, 2004) could not be repeated. However, it should be noted that these investigators have never tried to replicate our findings using the same cells, species or model systems we used, but have used different species, cell types and transfected tagged receptors. There are many possible explanations for the differences in results, apart from one group being right and the other wrong. For example, many of the experiments of Fortin and colleagues with endogenously expressed receptors have relied on responses resulting in smooth muscle contraction (rabbit aorta, isolated mouse stomach, human umbilical vein) or ERK1/2 phosphorylation in isolated smooth muscle cells. As noted above, in vessel preparations incubated for 6 h in a Krebs solution to induce the B₁ receptor response, the endothelial layer would likely be damaged or not responsive, as evidenced by contractions obtained in response to B₁ agonist as opposed to vasodilation, which is the hallmark of the response *in vivo* (Leeb-Lundberg et al., 2005). As outlined above, our studies have focused on both human and bovine endothelial responses in which B₁ receptor stimulation produces NO that relaxes smooth muscles. Besides these differences, variations in experimental protocols could also play a role, as Fortin and colleagues routinely used prolonged serum starvation for the ERK1/2 phosphorylation studies, a pretreatment that we did not employ. In preliminary experiments, we have found that serum starvation can selectively inhibit or abolish the response to an ACE inhibitor without affecting peptide ligand-mediated responses. As different signaling pathways are undoubtedly responsible for smooth muscle contraction or ERK1/2 phosphorylation versus endothelial NO production, the most likely explanation for the discrepancy in results is that ACE inhibitors do not stimulate all the same signal transduction pathways as peptide ligands of B₁ receptors, as stated above. Indeed, this is one of the major findings in our report (Ignjatovic et al., 2004).

In experiments in which the B₁ receptor was transfected, Fortin and co-workers used yellow fluorescent protein (YFP)-tagged rabbit B₁ receptor, whereas we employed native untagged human receptors (Ignjatovic et al., 2002a, 2004). In addition, their assay systems differed, utilizing either phospholipase A₂ assays (i.e., arachidonic acid release) or ERK1/2 phosphorylation assays for the rabbit receptor instead of measuring an increase in [Ca²⁺]_i (Ignjatovic et al., 2002a, 2004). In preliminary studies, we have found that B₁ receptor stimulation in human endothelial cells or in cells transfected with the human receptor results in little or no arachidonic acid release, indicating a lack of phospholipase A₂ activation (T. Ignjatovic, S. Stanisavljevic, X. Zhang, R.A. Skidgel and E.G. Erdős, unpublished results). Under the same conditions, B₂ receptor stimulation increases arachidonic release by over five-fold. Besides the triggering of differ-

ent signal transduction pathways, it is possible that the YFP tag alters B₁ receptors so that they no longer respond to ACE inhibitors, but stay responsive to peptide ligands. We have generated a variety of tagged B₁ receptor constructs with different of markers at both the N- and C-terminus and have found that some tags can alter the signaling properties of the receptors in unpredictable ways. Thus, caution must always be used in interpreting results obtained only with tagged receptors. For example, one of the assays used by Fortin et al. to assess the stimulation of YFP-labeled B₁ receptors was based on movement into caveolae-related rafts after addition of a B₁ agonist (Sabourin et al., 2002; Fortin et al., 2003). However, another group using untagged human B₁ receptors transfected in human embryonic kidney (HEK) cells showed that B₁ agonist stimulation did not cause the movement of B₁ receptors into caveolae-related rafts, whereas under the same conditions, B₂ receptor stimulation transiently increased B₂ receptor localization in these membrane microdomains (Lamb et al., 2002). Whether this discrepancy is due to species differences or artifacts induced by tagging of the rabbit receptor has not been addressed, but raises concerns that the YFP-labeled rabbit B₁ receptor may not recapitulate all of the responses of the native human receptor.

Role of the pentameric zinc-binding motif in B₁ receptor activation by ACE inhibitors

In our initial report on the stimulation of the B₁ receptor by ACE inhibitors, we noticed a consensus Zn²⁺ binding motif (HEXXH) conserved in many metalloproteases, including ACE (Corvol et al., 2004), and also conserved in the receptor sequence of many mammalian species, a notable exception being the dog B₁ receptor, in which only one of the three critical residues is retained (Ignjatovic et al., 2002a,b). Furthermore, mutation of the first His in the human B₁ receptor motif abolished the response to ACE inhibitors, but not to peptide ligands, in transfected HEK cells. A synthetic undecapeptide containing this sequence inhibited B₁ receptor activation by an ACE inhibitor, but not by a peptide ligand, suggesting the importance of this motif in ACE inhibitor action (Ignjatovic et al., 2002a). A recently released draft of the bovine genomic sequence contains a partial sequence highly homologous with the B₁ receptor (GenBank accession no. XM_610629). This sequence contains HDWAP instead of HEAWH in the putative Zn²⁺-binding region. The Asp in the bovine sequence could fulfill the same function as the Glu in the human and other mammalian B₁ receptors; however, the change of His to Pro in the bovine sequence is not conservative. Because bovine endothelial cells expressing the B₁ receptor respond to ACE inhibitors, this raises a question as to the importance of the whole HEXXH sequence in ACE inhibitor-B₁ receptor interactions in this species. Here, caution must be used when interpreting initial reports of partial genomic sequences, which may contain pseudogenes, polymorphisms or sequencing errors; only a single base change is needed to convert the codon encoding Pro to that for His (CCC to CAC). Final confirmation requires

cloning and sequencing of the complete bovine B₁ receptor cDNA and expressing it to show whether or not it responds to ACE inhibitors. So far, data are consistent with the hypothesis that the HEXXH sequence, present in the ACE active sites and B₁ receptors from many species, is necessary for stimulation by ACE inhibitors in cells of various origins. This hypothesis is supported by the following results. (i) The human B₁ receptor response to ACE inhibitors, but not to peptide ligands, was abolished when the first His in this sequence was mutated to Ala in human cells transfected with the mutant construct. (ii) Receptor activation by an ACE inhibitor, but not a peptide ligand, was blocked by a synthetic undecapeptide containing the canonical pentameric sequence. (iii) The esterified form of enalaprilat, the pro-drug enalapril, which does not bind Zn²⁺, did not activate the B₁ receptor. Even if it is confirmed that functional bovine B₁ receptors are encoded by the sequence mentioned above, in which the second zinc-binding His is replaced by Pro, that would still not negate our results showing that mutation of the first His residue abolishes ACE inhibitor effects. This residue is also conserved in the bovine pentameric sequence and is likely crucial to bind the Zn²⁺ cofactor. Possibly, another residue in a different region of the bovine B₁ receptor (e.g., the partial sequence contains four additional His residues) can fulfill the role of a second zinc-coordinating ligand in the bovine receptor. For example, in the zinc metalloproteases, the first two zinc-binding ligands, His and Glu, are separated by only two residues, but the third zinc-binding His, over 100 residues away in the linear sequence, is very close to the other two residues in the three-dimensional structure (Skidgel, 1996; Reverter et al., 2004).

Are alternate explanations of B₁ signaling mediated by ACE inhibitors valid?

Alternative explanations for our results have been proposed (Fortin et al., 2003; see reply to Ignjatovic et al., 2004): (i) ACE inhibitors potentiate the local level of kinins endogenously generated by the cells. (ii) ACE itself acts as the signaling molecule in response to ACE inhibitors. (iii) ACE inhibitor responses are blocked by the B₁ antagonist DALKD because, as an ACE substrate, it can compete with enalaprilat for binding to an active site of ACE. However, numerous lines of evidence negate these interpretations.

Regarding the first point, the hypothetical level of kinin production that could be expected, as reported by this group (Houle et al., 2003), is quite small, orders of magnitude lower than concentrations used to stimulate the B₁ responses in our system. Second, this possibility was disproved in our first publication (Ignjatovic et al., 2002a), in which we showed that cells transfected with B₂ receptors alone did not respond to enalaprilat, but responded to added bradykinin. Had kinins been generated or their levels increased by an ACE inhibitor, the B₂ receptors should have responded. Third, cells with the mutant B₁ receptor (HEAWH to AEAWH) did not react to an ACE inhibitor, but did respond to des-Arg¹⁰-kallidin. If ACE inhibitors activated B₁ via generation of endogenous

kinins, then the mutant receptors should have responded as well. Finally, for endogenous kinins to be involved, they would first have to be converted to the des-Arg kinin B₁ agonists by a cellular kininase I-type enzyme (carboxypeptidases), as mentioned above (Figures 1 and 2). To directly address whether endogenous generation of des-Arg kinin could be involved in the ACE inhibitor response, we preincubated cytokine-stimulated HLMVECs without or with 20 μ M MGTA (the specific B-type carboxypeptidase inhibitor) for 15–20 min and then added enalaprilat (100 nM) to stimulate NO production via the B₁ receptors. Under these conditions, NO production in response to enalaprilat was the same in the control cells as in the cells preincubated with MGTA. Because blocking conversion of putative endogenous kinins does not alter the B₁-mediated response to ACE inhibitors, endogenous kinins cannot be responsible for the B₁ response.

ACE inhibitor binding to ACE was shown to activate the c-Jun N-terminal kinase (JNK) pathway that leads to increased protein expression of ACE or COX-2 (Kohlstedt et al., 2004, 2005). However, activation of this or another pathway via direct ACE signaling cannot be responsible for B₁ receptor activation, as our publications have provided compelling evidence against this possibility (Ignjatovic et al., 2002a, 2004). First, B₁-mediated responses are stimulated by ACE inhibitors in CHO and HEK cells transfected with the native human B₁ receptors, which are blocked by B₁ receptor antagonists. These cells lacked any detectable ACE expression and the untransfected cells did not respond to ACE inhibitors. Although it has been reported that ACE inhibitors can upregulate ACE expression, this effect requires new protein synthesis over a time span of several hours to days, whereas the response of B₁ receptors to ACE inhibitors is immediate. Second, cells transfected with ACE alone did not respond to ACE inhibitors. Third, lisinopril is also a highly potent ACE inhibitor, but since its structure differs from the other ACE inhibitors used, it did not stimulate the B₁ receptor in our system. Finally, although the control HLMVECs expressed ACE, they responded only negligibly to ACE inhibitor when compared to cells in which B₁ receptors were induced by cytokines. If direct ACE signaling were responsible for the response, it should have stayed the same or decreased, as cytokine treatment decreases ACE expression in human endothelial cells (Saijonmaa et al., 2001).

Another idea that can be discarded is that the B₁ antagonist blocks the effect of ACE inhibitors by interacting directly with ACE. The kinetics of ACE with the B₁ antagonist DALKD as a substrate have not been established, but they were reported for [Leu⁸]-des-Arg⁹-bradykinin, which lacks the N-terminal Lys, but is otherwise identical to DALKD, including the C-terminal region where it interacts with ACE and is cleaved (Inokuchi and Nagamatsu, 1981). In these studies, the K_m value of [Leu⁸]-des-Arg⁹-bradykinin was 250 μ M with purified lung ACE. We routinely use only a 10-fold molar excess of the B₁ antagonist peptide to block the B₁ response to ACE inhibitors (Ignjatovic et al., 2002b, 2004), so it would not be possible for a substrate with an affinity in the hundred micromolar range to effectively compete with ena-

laprilat, which has a 385 000-fold higher affinity ($K_i=6.5\times 10^{-10}$ M) for the active site of ACE (Wei et al., 1992).

Conclusions

We have considered and explored many possible alternate explanations for our results over the years, but the only conclusion consistent with our findings so far is that most ACE inhibitors directly activate the B₁ receptor and that the region containing the HEXXH sequence is important for this effect. Of the five ACE inhibitors that have been tested (enalaprilat, quinaprilat, ramiprilat, captopril and lisinopril), only lisinopril failed to activate the B₁ receptor. This is likely due to the unique structure of lisinopril, which is identical to enalaprilat except for the lysine side chain, $-(CH_2)_4-NH_2$, instead of the methyl group found in enalaprilat and most other ACE inhibitors. Thus, the presence of the large positively charged group on lisinopril could prevent access to a recessed binding site or perhaps be repelled by a positively charged residue on the receptor.

Whether ACE inhibitor-mediated B₁ receptor stimulation and NO production would be beneficial or deleterious in sepsis is difficult to predict, given the variable and sometimes contradictory results of NOS inhibitors or NOS knockout on septic shock in animal models. However, ACE inhibitor treatment improved endothelial function in septic patients (Boldt et al., 1998) and in rabbits with LPS-induced endotoxic shock (Wiel et al., 2004). In the latter case, ACE inhibitor protection was via an NO-dependent mechanism (Wiel et al., 2004).

Finally, the release of NO by ACE inhibitors after stimulating B₁ receptors may significantly contribute to the many-fold beneficial therapeutic effects of these agents (Pfeffer, 2001). One mechanism by which enhanced NO production could be beneficial in the heart is via the inhibition of PKC ϵ , as overexpression of PKC ϵ results in dilated cardiomyopathy, with failure resulting from disruption of the myofibrillar proteins and their interactions (Goldspink et al., 2004). It follows that the potential clinical application of a B₁ receptor blocker may reduce the beneficial cardiac effects of ACE inhibitor administration.

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