Neprilysin Inhibitors Potentiate Effects of Bradykinin on B2 Receptor

Peter A. Deddish, Branislav M. Marcic, Fulong Tan, Herbert L. Jackman, Zhenlong Chen, Ervin G. Erdős

Abstract—Some beneficial effects of angiotensin-I–converting enzyme (ACE, kininase II) inhibitor therapy are attributed to enhancing the activity of bradykinin on its B2 receptor. Independent of inhibition of bradykinin hydrolysis, ACE inhibitors enhance the action of bradykinin on its B2 receptor by inducing crosstalk between ACE and the receptor. We investigated whether inhibitors of another kininase II-type enzyme, neprilysin (neutral endopeptidase 24.11; NEP), could augment bradykinin effects unrelated to blocking its breakdown using a NEP-resistant bradykinin analog as ligand. We used transfected Chinese hamster ovary (CHO) cells stably expressing human B2 receptor and NEP (CHO/NEP-B2) or only B2 (CHO/B2) as control and human pulmonary fibroblasts (IMR90), expressing B2, but more NEP than ACE. NEP inhibitor phosphoramidon (100 nmol/L), or omapatrilat, which inhibits both NEP and ACE, did not potentiate bradykinin in CHO/B2 cells. In IMR90 cells, 10 nmol/L bradykinin elevated \([\text{Ca}^{2+}]_{i}\) and desensitized the receptor. Adding either 100 nmol/L omapatrilat or phosphoramidon resensitized the receptor to the ligand, which was abolished by receptor blocker HOE 140. Arachidonic acid release by bradykinin from CHO/NEP-B2 cells was also augmented by 100 nmol/L phosphoramidon or omapatrilat about 3-fold, and again, the inhibitors resensitized the desensitized B2 receptor. The inhibitors did not potentiate bradykinin when soluble rNEP was added to the medium of CHO/B2 cells. Similar to ACE, NEP inhibitors potentiated bradykinin independent of inhibiting inactivation. Consequently, omapatrilat could augment bradykinin effects on B2, when either ACE or NEP is expressed close to receptor on cell membrane. (Hypertension. 2002;39[part 2]:619-623.)

Key Words: G proteins calcium arachidonic acids angiotensin-converting enzyme receptors, bradykinin fibroblasts

We wished to establish whether, besides angiotensin I converting enzyme (ACE), inhibitors of another kinase II-type enzyme, neprilysin (neutral endopeptidase 24.11, CD10, NEP), can also augment the effects of bradykinin (BK) on its B2 receptor. This was to be tested on cells where expression of NEP is much higher than that of ACE. Although both enzymes cleave BK at a Pro7-Phe8 bond and are Zn metallopeptidases,1,2 they differ from each other in many ways. NEP cleaves higher molecular weight peptide substrates than does ACE. NEP hydrolizes BK with a larger turnover number, but the KN of the peptide is also higher; consequently, ACE in solution has a higher specificity constant (500 min−1 µmol/L−1) than that of NEP (40 min−1 µmol/L−1).2,3 Both human enzymes are concentrated in proximal tubular cells4–6 and seminal plasma,7 but endothelial cells are much richer in ACE, whereas fibroblasts express more NEP.8,9 ACE and NEP are ectoenzymes on plasma membranes, but ACE is type I, a transmembrane enzyme,10 whereas NEP is type II.11 The former is inserted into the membrane at its C-terminus,10,12 but, in the latter, the transmembrane anchor is at the N-terminus.11 The distribution of ACE and NEP also differs in various species; for example, in rat heart13 and urine14 NEP is a major kinase.

In the present experiments, we used a BK analog (BKan) that activates the B2 receptor, but is resistant to ACE and carboxypeptidase15,16 and, as shown below, to NEP. To study the crosstalk between the BK B2 receptor and NEP, we transfected cells to express human NEP and B2 receptor alone or together and used a human fibroblast cell line with native B2 receptor but with much more NEP than ACE. Both specific ACE and NEP inhibitors (enalaprilat and phosphoramidon) and the dual inhibitor omapatrilat were tested.

Methods

Materials

Chinese hamster ovary (CHO)-K1 cells and human fetal lung fibroblast (IMR)-90 cells were from American Type Culture Collection (Rockville, Md). Purified human recombinant neprilysin (rNEP) and the cDNA of human neprilysin were gifts from Prof Philippe Crine (Université de Montreal). The cDNA of human ACE, the
cDNA encoding the human BK B2 receptor, and the neomycin resistant gene (pHBlApr-3-neo) were from Prof P. Corvol (College de France, Paris), Syntex Co. (Palo Alto, CA), and Dr L.H. Kedes (University of Southern California, Los Angeles), respectively. Omapatrilat ([4S-4a,7a,10aβ]-octahydro-4-((2-mercaptop-1-oxo-3-phenylpropyl)amino)-5-oxo-7H-pyrido[2,1-b](1,3)-thiazepine-7-carboxylic acid) was from Bristol-Myers Squibb Research Institute. Mammalian expression vector pcDNA6/V5-His and blasticidin were from Invitrogen; lipofectin and fetal bovine serum (FBS) were from Gibco BRL. [5,6,8,9,11,12,14,15-3H(N)]-arachidonic acid (AA; 100 Ci/mmol) was from American Radiolabeled Chemicals. BK, phosphoramidon [N-(o-hexamethyloxysulfonyloxyhydroxophosphinyl)-Leu-Trp], culture media, and other chemicals were purchased from Sigma Chemicals. Enalaprilat was provided by Merck, Sharp and Dohme Research Division. [Phe8ψ-(CH2NH)Arg9]-bradykinin (Bkan) was from Novabiochem.

**Human NEP Construct**

Plasmid pRcCMV/NEP (provided by Prof Crine) was digested with EcoRI restriction enzyme to obtain the full-length NEP cDNA, which was ligated into the pcDNA6/V5-His expression vector and used for transfecting CHO/B2 cells.17

**Cell Culture**

CHO cells were grown, subcultured, and transfected as described.6,17 Following transfection, cells were subjected to selection using Ham’s F-12 medium, containing 10 μg/mL blasticidin (pcDNA6 vector contains blasticidin resistance gene). After selection, clones were harvested and propagated using cloning rings. The clone with the highest NEP activity was chosen for continued culture. IMR-90 cells were grown in minimum essential Eagle’s medium containing 10% fetal bovine serum.

**Measurement of [Ca2+]i and [3H] AA**

[Ca2+]i, was measured using a microspectro-fluorometer (PTI Delasco or Attofluor Ratiosion), and the Ca2+-sensitive fluorescent dye, fura-2/AM, on cells grown to confluence. [3H] AA release was assayed as before.17

**Potentiation of BK, Desensitization and Resensitization of the Receptor**

Monolayers of CHO/NEP-B2 cells, loaded with [3H] AA, were exposed to 10 nmol/L Bkan given alone, or together with the potentiating agent to be tested for 30 or 60 minutes. [3H] AA release was assayed into the medium was determined.

In control experiments, omapatrilat or phosphoramidon was ineffective in cells that lacked NEP or in the absence of receptor agonist. To show the involvement of the B2 receptor, its blocker HOE 140 (0.1 to 1.0 μmol/L) was used routinely. After desensitization by initial exposure of cells to kinin, sensitivity to BKan (resensitization by NEP inhibitor) was measured either by [3H] AA release or by elevation of [Ca2+]i, level.16,17

**Enzyme Assay**

Generally, the activity of NEP was determined from the percent products substrate. ACE activity was assayed with hippuryl-His-Leu at pH 8.0, 150 mmol/L NaCl, and with or without 1 μmol/L enalaprilat as inhibitor. Crude cell homogenates were prepared by harvesting cells into ice-cold Tris-maleate pH 7.4 buffer and disrupted by a sonic dismembranator (Artrek Corp); after, they were briefly centrifuged at 1,000g to remove intact cells and nuclei.

**Results**

**Resistance of Bkan to NEP Hydrolysis**

The resistance of Bkan to hydrolysis by ACE was reported.16 Whether Bkan could be cleaved by human rNEP was tested by comparing the hydrolysis of BK and Bkan under similar conditions, followed by analysis of reaction products by HPLC using standards to identify and quantitate product peaks. The Table shows that while 77±3.2% of BK was hydrolyzed by rNEP in 60 minutes, no Bkan was cleaved. Thus, besides ACE, this pseudopetide analogue of BK is also highly resistant to NEP. The interaction of Bkan with NEP was also probed by determining if Bkan could competitively inhibit the hydrolysis of BK by NEP. Bkan (25 μmol/L) did not inhibit significantly the hydrolysis of BK (50 μmol/L) by NEP after 20 minutes of preincubation with the enzyme (n=4), demonstrating a very low affinity for the active site of NEP.

**Suitability of IMR-90 Cells**

IMR-90 cells are an established, well-characterized, and widely used cell line of normal human embryonic fibroblasts. They express the BK B2 receptor.19 To determine the ratio of NEP to ACE, we assayed NEP and ACE in IMR-90 cell lysates. IMR-90 cell lysates were incubated with Glut-Ala-Ala-Phe-MNA or hippuryl-His-Leu substrates. Cleavage, or inhibition by the specific inhibitors phosphoramidon or enalaprilat (1 μmol/L), was taken as NEP and ACE activities in the cells. The actual amount of each enzyme in the lysate was calculated from the established kinetic parameters for these enzymes.20 Cell lysates contained 1.34±0.04 pmol of NEP and 97.6±9.2 fmoles (n=3) of ACE per mg protein. Thus, IMR-90 cells constitutively express bradykinin B2 receptors, NEP, and ACE with a ratio of 14:1.

**Resensitization of Bradykinin B2 Receptor by Omapatrilat**

In experiments with transfected CHO cells expressing both ACE and B2 receptor (CHO/AB), changes in [Ca2+]i were measured using BK as agonist, and either phosphoramidon, enalaprilat, or omapatrilat to resensitize the receptor. The inhibitor was added to make the cell’s B2 receptor, desensitized by the agonist, respond a second time to BK in the medium.16,17,21 Using CHO/AB cells with BK (10 or 100 nmol/L) as agonist, phosphoramidon (1 μmol/L) did not inhibit significantly the hydrolysis of BK (50 μmol/L) by NEP after 20 minutes of preincubation with the enzyme (n=4), demonstrating a very low affinity for the active site of NEP.

### Hydrolysis of Bradykinin and Bradykinin Analog by Human Recombinant Neutral Endopeptidase*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Incubation Time (min)</th>
<th>Percent Hydrolysis$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradykinin (50 μmol/L)</td>
<td>None</td>
<td>30</td>
<td>38±5.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>77±3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>0.8±0.4</td>
</tr>
<tr>
<td>Bradykin analog‡ (50 μmol/L)</td>
<td>None</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>0</td>
</tr>
</tbody>
</table>

*$Neutral endopeptidase concentration=500 pmol/L.
†PPAD=Phosphoramidon.
‡Analog=[Phe8ψ-(CH2NH)Arg9]-Bradykinin.
$Values=mean±SE; n=3.
resensitize the receptor (Figure 1A; n=3) as expected, because the cells lacked NEP. However, in these cells, the dual inhibitor omapatrilat (Figure 1B; n=3) and the ACE inhibitor enalaprilat (100 nmol/L; not shown) resensitized the B2 receptor because the cells responded to the agonist a second time, in agreement with previous findings.16,17,21 The inhibitors induced an enzyme receptor crosstalk leading to enhanced BK effect on the receptor via ACE, as these cells have no native NEP (unpublished data, 2001; results not shown).

Resensitization of B2 Receptor in IMR-90 Cells

Addition of 10 nmol/L BKan to IMR-90 cells increased [Ca2+]i level within seconds. After desensitization of the B2 receptor with the first dose of the agonist (a second dose of BKan had no effect), phosphoramidon (100 nmol/L; Figure 2A) or omapatrilat (100 nmol/L; Figure 2B) resensitized the B2 receptor because the cells responded to the agonist a second time, in agreement with previous findings.16,17,21 The inhibitors induced an enzyme receptor crosstalk leading to enhanced BK effect on the receptor via ACE, as these cells have no native NEP (unpublished data, 2001; results not shown).

Hand, increased [Ca2+]i 3.9±0.9-fold while phosphoramidon elevated it 1.8±0.1-fold when resensitizing B2 receptor to its ligand. The addition of phosphoramidon or omapatrilat to the IMR-90 cells in the absence of the peptide agonist did not change the intracellular calcium level, although cells could still respond to added ligand (not shown). Just as proven with ACE inhibitors,16,17,21 the NEP inhibitors in the absence of BK did not activate the receptor.

Figure 3 shows that the addition of HOE 140 (100 nmol/L) to the cells after stimulation with BKan (10 nmol/L), but before addition of omapatrilat (100 nmol/L; n=5), blocked
the resensitization of the receptor to the BKan present in the medium. Consequently, resensitization was due to the reactivation of the B$_2$ receptor.\textsuperscript{22,23}

### Resensitization of Receptor in CHO/NEP-B$_2$ Cells

Figure 3 shows that in transfected cells expressing both the B$_2$ receptor and NEP, the addition of phosphoramidon resensitized the B$_2$ receptor and induced a transient increase in [Ca$^{2+}$].

When CHO/NEP-B$_2$ cells were loaded with [$^3$H] AA, inhibitors of NEP also augmented the release of AA by kinin. AA release stimulated by addition of BKan (10 nmol/L) for 30 minutes was measured first in each experiment. Phosphoramidon (100 nmol/L) increased AA release by BKan 2.9±0.6-fold (n=3), and omapatrilat (100 nmol/L) increased AA release 2.6±0.4-fold (n=3). Thus, both phosphoramidon and omapatrilat increased the release of AA by BKan in CHO/NEP-B$_2$ cells.

In CHO cells expressing only B$_2$ receptor (CHO/B$_2$), but neither ACE nor NEP, addition of 100 nmol/L omapatrilat following 10 nmol/L BKan did not resensitize the B$_2$ receptor as measured by changes in [Ca$^{2+}$]. Thus, omapatrilat did not affect the B$_2$ receptor in the absence of ACE and NEP (not shown).

In other control experiments, soluble human rNEP (50 ng/mL) was added to the medium of CHO/B$_2$ cells. Under these conditions, when NEP was not expressed on the plasma membrane but instead dissolved in the medium, neither phosphoramidon nor omapatrilat resensitized the B$_2$ receptors to BKan (not shown; n=3).

### Discussion

ACE (kininase II) inhibitors have been used to treat millions of patients worldwide. Their beneficial effects,\textsuperscript{24,25} however, cannot be explained only by blocking Ang II release or BK inactivation. ACE inhibitors indirectly enhance the effect of BK on its B$_2$ receptor by inducing a protein-protein interaction (ie, a crosstalk between the enzyme and the receptor).\textsuperscript{16,17,22,23} Experiments in support of this hypothesis were done on the guinea pig ileum\textsuperscript{26} and atrial tissues,\textsuperscript{27} hog coronary arteries,\textsuperscript{28} and cultured cells that were transfected or constitutively expressed ACE and B$_2$ receptor by using BK and its partially or fully ACE-resistant analogs.\textsuperscript{16,17,22,23}

This enhancement of the B$_2$ receptor activation leads to an increased release of arachidonic acid and IP$_3$, and elevation of [Ca$^{2+}$] level, which can lead to an augmented liberation of prostaglandins and NO. Besides potentiating BK effects, ACE inhibitors resensitize the receptor after it has been desensitized by an agonist.\textsuperscript{16,17,21–23} ACE and B$_2$ receptors have to be sterically close to induce an interaction.\textsuperscript{16} The enhanced activation of BK receptor initiates a signal transduction pathway that differs from the primary effect of BK.\textsuperscript{22} As shown above, the inhibition of NEP also enhances the effect of BK and the NEP-resistant BKan on the B$_2$ receptor. This was investigated with CHO-AB cells transfected to express ACE and B$_2$ receptor\textsuperscript{17}, and CHO-NEP/B$_2$ cells transfected to express NEP and B$_2$. Specific inhibitors enalaprilat and phosphoramidon enhanced the effect of BK only in the cell line that expressed either ACE or NEP, whereas omapatrilat elevated the effect of BKan in both cell types; thus, omapatrilat acted on both ACE and NEP to potentiate BK and resensitize the receptor. In the IMR-90 cells that constitutively express NEP overwhelmingly more than ACE, enalaprilat potentiated BK much less than phosphoramidon or omapatrilat, or did not potentiate BK at all.

NEP inhibitors had no effect on the B$_2$ receptor in the absence of NEP on the cell membrane. Furthermore, instead of being expressed on the plasma membrane when soluble rNEP was added to the medium, BK was again not potentiated.

All of these results are taken as an indication that NEP inhibitors enhance the effect of BK on its receptor by a mechanism similar to that of ACE inhibitors. Depending on the cell type, activation of the B$_2$ receptor is followed by the release of important vasoactive mediators.\textsuperscript{29} The experiments showed that omapatrilat potentiated BK in cells where enalaprilat did not. The mode of action of a single inhibitor of both ACE and NEP, resulting in an indirect activation of the B$_2$ receptor, could be responsible for additional beneficial therapeutic effects of vasopeptidase inhibitors such as omapatrilat.\textsuperscript{30}
Acknowledgments
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References
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