Potentiation of des-Arg⁹-Kallidin-Induced Vasoconstrictor Responses by Metallopeptidase Inhibition in Isolated Human Umbilical Artery

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ABSTRACT

Several metallopeptidases have been reported to be involved in bradykinin (BK) B₂ receptor agonist metabolism. Our goal was to evaluate in vitro roles of metallopeptidases [e.g., neutral endopeptidase (NEP), aminopeptidase M (APM), and angiotensin-converting enzyme (ACE)] as functional inactivators of the selective BK₂ receptor agonist Lys-des-Arg⁹-BK (DAKD) in isolated human umbilical artery (HUA) rings. Concentration-response curves (CRCs) to DAKD were performed after a 5-h incubation period. Treatment with 10⁻⁹ M phosphoramidon (NEP inhibitor) or 10⁻⁹ M amastatin (APM inhibitor) potentiated DAKD-elicited responses, whereas 1⁻³⁰ M captopril (ACE inhibitor) had no significant effects. However, when the three enzymes were simultaneously inhibited, a significant potentiation over responses obtained under concurrent NEP and aminopeptidase M inhibition was observed. In contrast, responses induced by the peptidase resistant BK₂ receptor agonist Sar⁹-Phe⁸-des-Arg⁹-BK were not modified by triple peptidase inhibition. In addition, endothelial denudation failed to alter DAKD-induced responses in HUA. Finally, in the presence of NEP, ACE, and APM inhibition, Lys-des-Arg⁹-[Leu⁸]-BK, the potent BK₂ receptor antagonist, produced a parallel, concentration-dependent, rightward shift of DAKD CRCs. The obtained pKᵦ (8.57) and the Schild slope not different from unity are in agreement with an interaction at a single homogeneous BK₂ receptor population. In summary, this work constitutes the first pharmacological evidence that metallopeptidases NEP, APM, and ACE represent a relevant inactivation mechanism of the endogenous BK₂ receptor agonist DAKD in isolated HUA.

Functional studies have originally indicated the existence of two bradykinin (BK) B₂ receptors in mammalian tissues (Regoli et al., 1977). Such studies were later confirmed by molecular and genomic approaches (McEachern et al., 1991; Menke et al., 1994; for BK₂ and BK₁ receptor subtypes, respectively). BK and the decapeptide Lys-BK (kallidin) are endogenous agonists at BK₁ receptors (B₂ receptors). The cleavage of these peptides by the serine peptidases carboxypeptidase N and carboxypeptidase M yields the naturally occurring BK₂ receptor agonists des-Arg⁹-BK and Lys-des-Arg⁹-BK (DAKD). Several metallopeptidases, including neutral endopeptidase (NEP; EC 3.4.24.11), aminopeptidase M (APM; EC 3.4.11.2), and angiotensin-converting enzyme or kininase II (ACE; EC 3.4.15.1), have been reported to be involved in BK₁ receptor agonist inactivation (Marceau et al., 1998). BK₂ receptors are constitutively expressed in a variety of tissues and mediate most of the in vivo effects of kinins (Bathon and Proud, 1991). On the other hand, BK₁ receptors are not present in any significant amount in normal tissues, and their expression is often inducible rather than constitutive (Regoli et al., 1978; Sardi et al., 2000). Synthesis of BK₁ receptors can be induced during tissue isolation trauma and incubation or under certain pathophysiological conditions conveying tissue injury or inflammation (Marceau et al., 1998). Among them, a relevant role has been attributed to BK₁ receptor function in different processes, such as endotoxic shock (Pesquero et al., 2000; deBlois and Horlick, 2001), bronchial hyper-responsiveness (Huang et al., 1999), ischemia (Tschop et al., 2004), ischemia reperfusion (Lagneux et al., 2003), and postischemic neovascularization (Emanuelli et al., 2002). Taking this into account, the aim of the present work was to evaluate, through functional studies, the possible roles NEP, ACE, and APM might play in the biological inactivation of DAKD in isolated HUA and to val-

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REFERENCES: BK, bradykinin; DAKD, Lys-des-Arg⁹-BK; NEP, neutral endopeptidase; APM, aminopeptidase M; ACE, angiotensin-converting enzyme; HUA, human umbilical artery; CRC, concentration-response curves; 5-HT, serotonin; KD, kallidin.
idate this vessel as yet another BKβ receptor model prepa-

Materials and Methods

Tissue Preparation. Human umbilical cords were obtained from normal full-term deliveries and excised midway between the child and the placenta. Immediately, cords were placed in modified Krebs’ solution at 4°C of the following composition: 119 mM NaCl, 4.7 mM KCl, 25 mM NaHCO3, 1.2 mM KH2PO4, 2.5 mM CaCl2, 1.0 mM MgSO4, 0.004 mM EDTA, and 11 mM d-glucose. Written informed consent was obtained from each parturient.

Usually within 3 h after delivery, the samples were placed onto dissecting dishes containing Krebs’ solution, and arteries (internal diameter approximately 1 mm) were carefully dissected free from Wharton’s jelly using micro-dissecting instruments and cut into rings of approximately 3-mm width. Whenever necessary, endothelium was removed by gently rubbing the inner side of HUA rings with a roughened steel rod.

Functional Studies. Immediately after dissection, rings were suspended in 5-ml organ baths and stretched with an optimal resting tension of 2 to 4 g (Tufan et al., 2003). Changes in tension were measured with Grass isometric force transducers (FT-03C; Grass Instruments Co., Quincy, MA) and displayed on Grass model 7D polygraphs. During the incubation period, Krebs’ solution was maintained at 37°C and at pH 7.4 by constant bubbling with 95% O2/5% CO2. Bath solution was replaced every 15 min with fresh bubbled buffer. Rings were incubated for 5 h, and then CRGs to DAKD or Sar2-d-Phe8-des-Arg9-BK were obtained by the cumulative addition of agonists in 0.25-log10 increments. Since the majority of isolated smooth muscle preparations become responsive to BKβ receptor agonists as a function of time, HUA preparations were incubated for 5 h prior to obtaining the CRGs to DAKD or Sar2-d-Phe8-des-Arg9-BK. Antagonist and peptidase inhibitors were applied 30 min before and during the addition of either agonist. Neither antagonist nor peptidase inhibitors produced any significant increase in basal tone of HUA rings when applied.

At the end of each CRC, 10 μM serotonin (5-HT) was applied to determine the tissue maximal contractile response (Altura et al., 1972), which was 7.96 ± 0.24 g (n = 140). All experiments were performed in parallel with rings from the same umbilical cord. Only one agonist CRC was performed in each ring.

Drugs. The following compounds were used for functional studies: 5-hydroxytryptamine creatine sulfate complex from Sigma/RBI (Natick, MA); des-Arg9-KD and Lys-des-Arg9-[Leu8]-BK (des-Arg9-5-hydroxytryptamine creatine sulfate complex from Sigma/RBI performed in parallel with rings from the same umbilical cord. Only 10 responses were expressed as the percentage of tissue maximum response elicited by Sar2-d-Phe8-des-Arg9-BK from Phoenix Pharmaceuticals Inc. (Belmont, CA); amastatin hydrochloride [(S)-2,3]-3-amino-2-hydroxy-5-methylhexanoyl-Val-Val-Asp hydrochloride) and captopril ([N-(S)-3-mercapto-2-methylpropionyl]-t-proline) from Sigma-Aldrich (St. Louis, MO); phosphoramidon, a selective NEP inhibitor, significantly augmented maximum DAKD-elicited contractions in HUA (control: EC50 7.42 ± 0.09, n = 26; treated: EC50 8.24 ± 0.17, n = 8, P < 0.05; Fig. 2B; Table 1). Maximum responses produced by DAKD were not significantly modified by treatment of rings

where Y is the response, X is the arithmetic dose, and a is the response when X = 0. EC50 was transformed into pEC50 (−log EC50).

When using antagonists, statistical differences in nH were determined by constraining nH of treated tissues equal to nH of control values and assessing for significance by using F statistics, where F values lower than 0.05 were taken to indicate significant differences (DeLean et al., 1978). When criteria for competitive antagonism were satisfied, that is, the antagonist produced a parallel rightward shift of the agonist curve without attenuation in maximum response, antagonist pA2 values and slopes of Schild regressions were calculated by graphical extrapolation as described by Arunlakshana and Schild (1959). Where the slope of the Schild plot was not significantly different from unity, the regression was recalculated with Schild slope constrained to unity, and the affinity value obtained was then referred to as pKp. The measured slope of Schild plot is presented to illustrate whether agonist-antagonist interactions are consistent with an involvement of one or more receptor subtypes in mediating response of the agonist (Kenakin, 1992). Statistical analysis was performed by means of unpaired Student’s t test or one-way analysis of variance followed by Tukey’s post-test, when appropriate.

Results

Contractile Effects of DAKD on Isolated HUA. Cumulative addition of DAKD to HUA rings, after a 5-h incubation period, produced a concentration-dependent contraction. The estimated pEC50 was 7.82 ± 0.28 (n = 8), and the maximum response (Emax) was 48.7 ± 7.5% of maximal contraction obtained with 10 μM 5-HT (Fig. 1). Endothelium removal did not modify DAKD-elicited responses in HUA rings (pEC50 8.00 ± 0.15, n = 8, P > 0.05; Emax 56.8 ± 10.0%, P > 0.05).

Potentiation of DAKD-Elicited Responses in HUA by NEP and APM Inhibition. Treatment with 10 μM phosphoramidon, a selective NEP inhibitor, significantly augmented maximum DAKD-elicited contractions in HUA (control: Emax 49.3 ± 7.1%, n = 26; treated: Emax 87.4 ± 1.1%, n = 6, P < 0.05; Fig. 2A; Table 1).

In addition, a significant leftward shift of DAKD-induced responses was observed by treatment of HUA rings with 10 μM amastatin, a selective APM inhibitor (control: pEC50 7.42 ± 0.09, n = 26; treated: pEC50 8.24 ± 0.17, n = 8, P < 0.05; Fig. 2B; Table 1). Maximum responses produced by DAKD were not significantly modified by treatment of rings
of triple peptidase inhibition was significantly increased over CRCs obtained in the presence of 10 μM phosphoramidon and 10 μM amastatin (pEC_{50} 8.13 ± 0.11, n = 8, P < 0.05; Fig. 3; Table 1).

**Effects of Triple Peptidase Inhibition on Sar^{D-Phe\text{d}}-Phe^{\text{a}}-des-Arg^{9}-BK-Induced Responses in HUA.** Sar^{D-Phe\text{d}}-Phe^{\text{a}}-des-Arg^{9}-BK produced a concentration-dependent contractile response of HUA rings (pEC_{50} 6.84 ± 0.13, E\text{max} 70.3 ± 11.8%, n = 9; Fig. 4). Exposure to 1 μM captopril, 10 μM phosphoramidon, and 10 μM amastatin did not produce modifications of Sar^{D-Phe\text{d}}-Phe^{\text{a}}-des-Arg^{9}-BK-induced responses (pEC_{50} 6.83 ± 0.11, E\text{max} 65.3 ± 11.6%, n = 9, P > 0.05; Fig. 4).

**Antagonism of Lys-des-Arg^{9}(\text{Leu})^{b}-BK on DAKD-Induced Responses in HUA.** In rings pretreated with 1 μM captopril, 10 μM phosphoramidon, and 10 μM amastatin, exposure to increasing concentrations of the selective BKB_{2} receptor antagonist des-Arg^{9}-Leu^{a}-KD (10, 30, and 100 nM) produced a competitive rightward shift of DAKD-induced responses (Fig. 5A; Table 2). Schild plot yielded a pK_{b} \text{ value} of 8.34 ± 0.14 and a slope of 1.29 ± 0.30. Since the slope was not significantly different from unity, regression was recalculated with the slope value constrained to unity, and the pK_{b} was obtained (pK_{b} 8.57 ± 0.12; Fig. 5B).

**Discussion**

In HUA, inhibition of NEP's enzymatic activity caused a significant potentiation of vasoconstrictor responses induced by DAKD. NEP, a membrane-bound M13 zinc metalloproteinase, was first detected in the brush border of animal kidney by Kerr and Kenny (1974). Since then, NEP presence has been described in many vascular tissues, such as rat aorta smooth muscle, adventitial and endothelial cells (González et al., 1998), human endothelial cells (Llorens-Cortes et al., 1992), and cardiomyocytes (Kokkonen et al., 1999). Gafford et al. (1983) have shown that the selective BKB_{2} receptor agonist BK is hydrolyzed by NEP. The primary site of hydrolysis in BK was reported to be at the Pro^{7}-Phe^{8} bond. However, a secondary site for cleavage, Gly^{4}-Phe^{5}, was detected in that study. It has been proposed that the primary site for cleavage of DAKD by NEP might be at the Pro^{7}-Phe^{8} bond (Marceau et al., 1998). Additionally, in functional studies, NEP's ability to inactivate different vasoactive peptides in isolated smooth muscle preparations has been evaluated. For instance, BK-induced relaxation of vascular as well as bronchial smooth muscle has been shown to be potentiated by inhibition of NEP (Frossard et al., 1990; Miyamoto et al., 2002). However, to our knowledge, no reports have been made regarding any functional relevance of NEP on the BKB_{2} receptor agonists' elicited responses. In summary, our results constitute the first functional evidence that NEP might be involved in the biological inactivation of the naturally occurring BKB_{2} receptor agonist DAKD in HUA biophase.

APM is a transmembrane M1 zinc metalloproteinase. Proud et al. (1987) have reported that APM is able to cleave the amino terminal Lys- of DAKD and Lys-BK into des-Arg^{9}-BK and BK, respectively. Palmieri et al. (1989) characterized APM activity in cultured porcine aorta endothelium and smooth muscle cells and suggested that vascular APM may modulate vasoactive peptide levels in vivo, particularly within the microenvironment of endothelial and smooth muscle cell surface receptors. Correspondingly, it has been dem-
onstrated that APM inhibition can potentiate in vitro responses produced by angiotensin III in different isolated vessels (Robertson et al., 1992; Li et al., 1997). In addition, in lipopolysaccharide-pretreated rabbits, inhibition of APM by previous treatment with amastatin increased the duration of the hypotension induced by DAKD (Drapeau et al., 1991). The authors suggested that APM represents an inactivation pathway for the potent natural BKB1 receptor agonist since its metabolite, des-Arg9-BK, is a much less potent BKB1 receptor agonist. The present study, in agreement with these hypotheses, demonstrates that in HUA, inhibition of APM significantly potentiates DAKD-induced contractile responses, suggesting that this enzyme might be another im-

### TABLE 1

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<th>Treatment</th>
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<td>C</td>
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P, phosphoramidon (10 μM); A, amastatin (10 μM); C, captopril (1 μM).

* Significant differences vs. control ($P < 0.05$).
† Significant differences vs. single inhibition ($P < 0.05$).
‡ Significant differences vs. dual inhibition ($P < 0.05$).

![Fig. 3](image1) Further potentiation of DAKD-induced responses in the presence of 10 μM phosphoramidon and 10 μM amastatin ($\bullet$; $n = 8$) by the addition of 1 μM captopril ($\square$; $n = 6$) on HUA rings. Vertical lines show S.E.M. Abscissa scale, $-\log_{10}$ of molar concentration. Asterisk expresses significant differences between $pEC_{max}$.

![Fig. 4](image2) Lack of potentiation of Sar$^a$-D-Phe$^b$-des-Arg$^a$-BK-induced responses in control ($\bullet$; $n = 9$) HUA rings when exposed to 10 μM phosphoramidon, 10 μM amastatin, and 1 μM captopril ($\square$; $n = 9$). Vertical lines show S.E.M. Abscissa scale, $-\log_{10}$ of molar concentration.

Fig. 3. Additional potentiation of DAKD-induced responses in the presence of 10 μM phosphoramidon and 10 μM amastatin ($\bullet$; $n = 8$) by the addition of 1 μM captopril ($\square$; $n = 6$) on HUA rings. Vertical lines show S.E.M. Abscissa scale, $-\log_{10}$ of molar concentration. Asterisk expresses significant differences between $pEC_{max}$.

Fig. 4. Lack of potentiation of Sar$^a$-D-Phe$^b$-des-Arg$^a$-BK-induced responses in control ($\bullet$; $n = 9$) HUA rings when exposed to 10 μM phosphoramidon, 10 μM amastatin, and 1 μM captopril ($\square$; $n = 9$). Vertical lines show S.E.M. Abscissa scale, $-\log_{10}$ of molar concentration.

Fig. 5. A, concentration-response curves for DAKD on control ($\bullet$; $n = 10$) HUA rings and on tissues exposed to 10 μM phosphoramidon, 10 μM amastatin, and 1 μM captopril for 30 min. Vertical lines show S.E.M. Abscissa scale, $-\log_{10}$ of molar concentration. Asterisk expresses significant differences between $pEC_{max}$. B, Schild plot of concentration-response curves for DAKD on tissues exposed to Lys-des-Arg$^a$-[Leu$^b$]-BK (10, 30, or 100 nM).

important pathway of kinin inactivation in this tissue. According to available bibliography, there is no previous evidence of potentiation of in vitro contractile responses by DAKD in human isolated vascular tissue induced by APM inhibition.

ACE is a single-chain transmembrane M2 zinc metallopeptidase that converts angiotensin I into angiotensin II by removing the C-terminal dipeptide from the former (Skeggs et al., 1956). This enzyme has been described in endothelial cells of many organs (Erdos, 1990), in human heart (Kokkonen et al., 1999), and in rat aorta smooth muscle (Arnal et al., 1994). ACE cleaves the C-terminal tripeptide from BKB1 receptor agonists, yielding BK1-5, but at a slower rate and with less affinity than the removal of the C-terminal dipeptide from BK (Marceau et al., 1998). It has been shown...
that ACE inhibition potentiates in vitro effects of BK in vascular tissues (Miyamoto et al., 2002). However, in our model, ACE inhibition by captopril did not modify DAKD-induced contractile responses. Similarly, Babiuk et al. (1982) failed to observe any potentiation of vasoconstrictor responses elicited by des-Arg^9^-BK or DAKD in isolated rabbit aorta by ACE inhibition. Nevertheless, in the present study, when ACE, NEP, and APM were simultaneously inhibited, DAKD-induced responses in HUA were potentiated when compared with those obtained in conditions of concurrent APM and NEP inhibition.

Together, the above-mentioned results support our hypothesis that the potentiation of DAKD-induced responses in HUA was due to impairment of its biotransformation in biophase. In addition, the absence of potentiation of vasoconstrictor responses elicited by des-Arg^9^-BK or DAKD by systemic treatment with a BKB1 receptor antagonist (Drapeau et al., 1993), by triple enzymatic inhibition substantiates our hypothesis. On the other hand, a direct agonist effect of ACE inhibitors has been proposed by Ignjatovic et al. (2002), who observed in several cell culture models that enalaprilat produced an agonist-like action, which they considered belonged to stimulation of BKB1 receptors. However, Fortin et al. (2003) have failed to observe such effects when evaluating responses to captopril, enalaprilat, and zofenoprilat on isolated rabbit aorta and mouse stomach, two BKB1 receptor model preparations. In addition, in the same study enalaprilat produced negligible vasomotor effects on human umbilical vein, yet another extensively characterized BKB1 receptor model. The absence of vasoconstrictor responses observed with captopril in HUA suggests a lack of activity of ACE inhibitors on BKB1 receptors in this tissue.

NEP, APM, and ACE have been characterized in endothelial cells of several species, including human (Erdos, 1990; Llorens-Cortes et al., 1992; Walsh et al., 1993). However, when we evaluated DAKD-elicited contractile responses in HUA, no differences were observed between responses obtained in intact and de-endothelialized rings. The lack of potentiation of responses induced by DAKD in HUA rings without endothelium suggests that, in this tissue, the enzymatic activity of NEP, APM, and ACE responsible for biological inactivation of this agonist is not endothelial.

The only work to date that proposed the presence of BKB1 receptors mediating contraction in HUA was carried out in presence of indomethacin, with low oxygen tension in Krebs' media and with unusually low obtained maximum responses (Abbas et al., 1998). In our study, CRCs to DAKD obtained in the presence of triple enzymatic inhibition yielded a maximum effect approximately 2-fold and a potency approximately 30-fold higher when compared with those obtained in control HUA rings. Taking into account this marked potentiation, we considered that the enzymatic inhibition of BKB1 receptor agonist metabolism was the best condition for a more reliable pharmacological characterization of the receptor population involved in DAKD-elicited responses in HUA. In this condition, a highly selective BKB1 receptor antagonist, Lys-des-Arg^9^-[Leu^8^-]-BK, competitively antagonized DAKD-induced responses, yielding a pK_B value of 8.57. This finding is in agreement with previously reported affinities of Lys-des-Arg^9^-[Leu^8^-]-BK for the human BKB1 receptor (K_i value of 1.3 nM; Menke et al., 1994). In addition, the obtained Schild slope was not different from unity, consistent with involvement of a single homogeneous receptor population in DAKD-elicited responses (Kenakin, 1992).

Although evidence from different models suggests that the BKB2 receptor subtype mediates most kinin actions under physiological conditions, the rapid and long-lasting up-regulation of BKB1 receptors after inflammatory insult implies that this receptor may become the dominant subtype in mediating chronic inflammation (Dry and Perkins, 1993). The relevance of the BKB1 receptor subtype in different pathophysiological conditions has been reported. BKB1 gene deletion prevents endotoxic shock by lipopolysaccharide (LPS) in mice (Pesquero et al., 2000) and, in accordance with this report, deBlois and Horlick (2001) have suggested that hemodynamic and inflammatory phenomena observed after LPS treatment in green monkeys, a nonhuman primate model, are mainly mediated by BKB1 receptor activation. Additionally, associated with other inflammatory stimuli, it has been described that BKB1 receptor mRNA expression is increased in an allergen-induced bronchial hyper-responsive- ness model in rats (Huang et al., 1999). Moreover, allergic lung inflammation in ovalbumin-sensitized mice is diminished by systemic treatment with a BKB1 receptor antagonist. The role of BKB1 receptors in ischemic processes has also been studied. Lagneux et al. (2003) have shown that des-Arg^9^-BK infusion in an isolated rat heart low-flow ischemia-reperfusion model significantly reduced infarct size. Additionally, Tschope et al. (2004) have reported that the cardioprotective effects of angiotensin receptor 1 blockade after experimental myocardial infarction, by coronary artery ligation, are partly mediated by secondary activation of the BKB1 receptor pathway. Moreover, Emanueli et al. (2002) have established a functional role for the BKB1 receptor in posts ischemic neovascularization by means of a murine model of limb ischemia. According to the authors, BKB1 receptor signaling plays an essential role in reparative angiogenesis by modulating endothelial cell proliferation and survival. In addition, they suggest that potentiation of this mechanism may exert a therapeutic effect by accelerating spontaneous tissue healing. Together, all these data underline the potential relevance of BKB1 receptor-mediated actions in different

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<td>100 nM</td>
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<td>85.4 ± 7.4</td>
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* Significant differences vs. control (P < 0.05).

Table 2: Effects of Lys-des-Arg^9^-[Leu^8^-]-BK on DAKD-induced responses in HUA

E_{max} is expressed as a percentage of maximum responses obtained with 10 μM 5-HT. Values are expressed as mean ± S.E.M.
pathological conditions. Taking into account the present results, the possible potentiation of BKβ receptor agonist-induced responses by NEP, APE, and/or ACE inhibition in the above-mentioned pathophysiological models could be tested. Data obtained from these future studies, and from the present one, could potentially contribute to the development of new therapeutic strategies for these pathologies.

In summary, this work constitutes the first pharmacological evidence that metallopeptidases NEP, APE, and ACE represent a relevant inactivating mechanism of the endogenous BKβ receptor agonist DAKD in isolated HUA.

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