

## Regulation of Cardiovascular Signaling by Kinins and Products of Similar Converting Enzyme Systems

### Kininase I-type carboxypeptidases enhance nitric oxide production in endothelial cells by generating bradykinin B<sub>1</sub> receptor agonists

Sakonwun Sangsree,<sup>1</sup> Viktor Brovkovich,<sup>1</sup> Richard D. Minshall,<sup>1,2</sup> and Randal A. Skidgel<sup>1,2</sup>

Departments of <sup>1</sup>Pharmacology and <sup>2</sup>Anesthesiology, University of Illinois College of Medicine, Chicago, Illinois 60612

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**Sangsree, Sakonwun, Viktor Brovkovich, Richard D. Minshall, and Randal A. Skidgel.** Kininase I-type carboxypeptidases enhance nitric oxide production in endothelial cells by generating bradykinin B<sub>1</sub> receptor agonists. *Am J Physiol Heart Circ Physiol* 284: H1959–H1968, 2003. First published March 6, 2003; 10.1152/ajpheart.00036.2003. Kininase I-type carboxypeptidases convert native kinin agonists for B<sub>2</sub> receptors into B<sub>1</sub> receptor agonists by specifically removing the COOH-terminal Arg residue. The membrane localization of carboxypeptidase M (CPM) and carboxypeptidase D (CPD) make them ideally situated to regulate kinin activity. Nitric oxide (NO) release from human lung microvascular endothelial cells (HLMVEC) was measured directly in real time with a porphyrinic microsensor. Bradykinin (1–100 nM) elicited a transient (5 min) peak of generation of NO that was blocked by the B<sub>2</sub> antagonist HOE 140, whereas B<sub>1</sub> agonist des-Arg<sup>10</sup>-kallidin caused a small linear increase in NO over 20 min. Treatment of HLMVEC with 5 ng/ml interleukin-1 $\beta$  and 200 U/ml interferon- $\gamma$  for 16 h upregulated B<sub>1</sub> receptors as shown by an approximately fourfold increase in prolonged (>20 min) output of NO in response to des-Arg<sup>10</sup>-kallidin, which was blocked by the B<sub>1</sub> antagonist des-Arg<sup>10</sup>-Leu<sup>9</sup>-kallidin. B<sub>2</sub> receptor agonists bradykinin or kallidin also generated prolonged NO production in treated HLMVEC, which was significantly reduced by either a B<sub>1</sub> antagonist or carboxypeptidase inhibitor, and completely abolished with a combination of B<sub>1</sub> and B<sub>2</sub> receptor antagonists. Furthermore, CPM and CPD activities were increased about twofold in membrane fractions of HLMVEC treated with interleukin-1 $\beta$  and interferon- $\gamma$  compared with control cells. Immunostaining localized CPD primarily in a perinuclear/Golgi region, whereas CPM was on the cell membrane. These data show that cellular kininase I-type carboxypeptidases can enhance kinin signaling and NO production by converting B<sub>2</sub> agonists to B<sub>1</sub> agonists, especially in inflammatory conditions.

carboxypeptidase M; carboxypeptidase D; bradykinin B<sub>2</sub> receptor; interleukin 1- $\beta$ ; interferon- $\gamma$

METALLOCARBOXYPEPTIDASES specifically catalyze the hydrolysis of the COOH-terminal peptide bond of peptides and proteins (44). Although the release of a single COOH-terminal amino acid could appear to be of limited physiological importance, it can have profound effects on the biological activity of peptides and proteins (42, 44, 46). This was first established by the pioneering work of Erdös and Sloane (12, 16), who discovered a B-type carboxypeptidase (which cleave only COOH-terminal Arg or Lys) in plasma that inactivates bradykinin. It was initially named kininase I to distinguish it from another bradykinin inactivator named kininase II, which was later shown to be identical with angiotensin-converting enzyme (ACE) (12, 13). Although kininase I was later renamed carboxypeptidase N (CPN), the kininase I and II designation is still used to categorize enzymes that cleave either one (Arg<sup>9</sup>) or two (Phe<sup>8</sup>-Arg<sup>9</sup>) amino acids from the COOH-terminus of bradykinin (13, 47). Later discoveries of the first membrane-bound carboxypeptidase (CPM) (45, 48) and a secretory vesicle carboxypeptidase (CPE or CPH) involved in peptide hormone processing (17, 18) provided evidence for the existence of a subfamily of carboxypeptidases that have important functions in regulating peptide hormone activity (42). Cloning and sequencing confirmed this distinction and they came to be known as the “regulatory carboxypeptidases” (or more recently the CPN/E subfamily) as a way to distinguish them from the pancreatic carboxypeptidases (or CPA/B subfamily) (40, 44, 55). Interestingly, all catalytically active members of the regulatory or CPN/E subfamily are B-type carboxypeptidases, which cleave only COOH-terminal Arg or Lys residues. The regulatory carboxypeptidases play important roles in the maintenance of physiological homeostasis and the organism’s response to pathological perturbations (40, 44, 46).

Address for reprint requests and other correspondence: R. A. Skidgel, Dept. of Pharmacology (m/c 868), Univ. of Illinois College of Medicine, 835 S. Wolcott, Chicago, IL 60612 (E-mail: rskidgel@uic.edu).

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There are only two true membrane-bound regulatory carboxypeptidases: CPM and CPD. CPM is a widely distributed ectoenzyme anchored to plasma membranes via glycosylphosphatidylinositol (9, 46, 49). CPM cleaves only basic COOH-terminal residues, and arginine is cleaved faster than lysine in a variety of synthetic peptide substrates (45). The naturally occurring peptide substrates of CPM include bradykinin, Arg<sup>6</sup>- and Lys<sup>6</sup>-enkephalins, dynorphin A<sub>1-13</sub>, epidermal growth factor (EGF), and hemoglobin (33, 35, 45). CPD was discovered as a unique B-type carboxypeptidase in rat and bovine tissues (51) as well as in human and mouse cells (34), with an acidic pH optimum of about 6.2. Subsequent cloning and sequencing of the human and rat cDNAs (54, 60) showed CPD to be the mammalian homolog of duck gp180, a protein originally identified as a duck hepatitis B-virus binding protein (26). CPD is a widely distributed 180-kDa glycoprotein containing three tandem homologous carboxypeptidase domains, of which the first two are active and the third domain is inactive, due to mutation of several critical active site residues (40, 46). CPD is a type I membrane protein with a single transmembrane sequence near the COOH-terminus and is primarily found in the trans-Golgi network in the cell, where it participates in protein processing in the constitutive secretory pathway (40, 46, 57). However, some CPD can be found on the plasma membrane where it can also function as a cell surface protein, for example, in binding hepatitis B-virus on duck liver cells (25, 26, 52) or by cleaving extracellular substrates and generating Arg to enhance nitric oxide (NO) production in macrophages (19).

Kinins are important peptide hormones that stimulate a variety of signal transduction pathways via two known G protein-coupled receptors named B<sub>1</sub> and B<sub>2</sub> (3). Activation of either the B<sub>1</sub> or B<sub>2</sub> receptor leads to stimulation of similar signal transduction pathways, causing increases in intracellular calcium and the release of mediators such as prostaglandins and NO (3, 31). However, there are several major differences between the two receptor types. B<sub>1</sub> receptor stimulation results in a more prolonged response because the receptor is resistant to desensitization and internalization in contrast to the B<sub>2</sub> receptor, which is rapidly sequestered and desensitized (2, 10, 27, 31). Another distinguishing feature is that the B<sub>2</sub> receptor is constitutively expressed, whereas the B<sub>1</sub> receptor is not normally expressed in most tissues, but injury or inflammatory mediators upregulate its transcription (3, 31). In human cells, interleukin-1 $\beta$  (IL-1 $\beta$ ) is especially effective in upregulating expression (31). Finally, bradykinin and Lys-bradykinin (kallidin) are specific ligands for the B<sub>2</sub> receptor, whereas the carboxypeptidase-derived products des-Arg<sup>9</sup>-bradykinin and des-Arg<sup>10</sup>-kallidin are ligands for the B<sub>1</sub> receptor (3, 39). Thus B-type carboxypeptidases not only inactivate B<sub>2</sub> agonists but act as required processing enzymes for the generation of B<sub>1</sub> agonists. In vitro, bradykinin is one of the best biological substrates for CPM, which cleaves only the COOH-terminal Arg<sup>9</sup> to form des-Arg<sup>9</sup>-brady-

kinin (33, 45). The membrane localization of CPM and CPD makes them ideally situated to regulate bradykinin activity near cell surface receptors. However, their roles in generating B<sub>1</sub> agonists have not been directly addressed experimentally at the cellular level. The goal of this study was to investigate the ability of endothelial cell carboxypeptidases to affect NO production in stimulated endothelial cells through the generation of B<sub>1</sub> agonists.

## MATERIALS AND METHODS

**Materials.** Microvascular Endothelial Cell Growth Medium-2 Kit (EGM-2 BulletKit) was from Clonetics (San Diego, CA). Phenol red-free Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DME/F-12) was from Sigma (St. Louis, MO). dl-2-Mercaptomethyl-3-guanidinoethylthio-propanoic acid (MGTA) and IL-1 $\beta$  were from Calbiochem (La Jolla, CA). FBS was from Atlanta Biologicals (Norcross, GA). Human recombinant interferon- $\gamma$  (IFN- $\gamma$ ) was from Life Technologies (Gaithersburg, MD). 5-Dimethylaminonaphthalene-1-sulfonyl-L-alanyl-L-arginine (dansyl-Ala-Arg) was synthesized and purified as described previously (53). Gelatin, PBS, N<sup>G</sup>-monomethyl-L-arginine (L-NMMA), bradykinin, kallidin, des-Arg<sup>9</sup>-bradykinin, des-Arg<sup>10</sup>-kallidin, and des-Arg<sup>10</sup>-Leu<sup>9</sup>-kallidin were from Sigma. HOE 140 was a gift from Dr. B. A. Schölkens (Hoechst; Frankfurt, Germany). Common chemicals were from Fisher Scientific (Pittsburgh, PA).

**Cell culture.** Human lung microvascular endothelial cells (HLMVEC) were obtained from Clonetics, BioWhittaker (San Diego, CA). Cells were cultured in EGM-2 containing 10 ng/ml human epidermal growth factor, 5 ng/ml vascular endothelial growth factor, 2 ng/ml human fibroblast growth factor, 2 ng/ml insulin-like growth factor, 0.2  $\mu$ g/ml ascorbic acid, 50 ng/ml gentamicin-amphotericin-B, and 10% FBS that was heat inactivated at 56°C for 30 min (to destroy any endogenous serum carboxypeptidase). For NO measurements, cells (from passages 4–8) were seeded at a density of  $1 \times 10^6$  cells/well on gelatin-coated 12-well plates. To upregulate B<sub>1</sub> receptor expression, 24 h after reaching confluence, HLMVEC were washed once with PBS and then incubated in fresh fully supplemented EGM-2 (as above except with 0.5% FBS) containing 5 ng/ml IL-1 $\beta$  and 200 U/ml IFN- $\gamma$  at 37°C for an additional 16 h. Control cells were treated identically except the medium lacked IL-1 $\beta$  and IFN- $\gamma$  during the 16-h incubation.

**Measurement of NO production.** NO production was determined by measurement of NO in real time with a porphyrinic microsensor (5, 30). The microsensor consisted of 5–7 carbon fibers (diameter 0.2  $\mu$ m) that were electroplated with highly conductive polymeric porphyrin, which facilitates the electron transfer from NO to the sensor. This microsensor is supercoated with a negatively charged polymer (Nafion); negatively charged species such as nitrite or nitrate cannot gain access to the porphyrinic surface and therefore do not interfere with NO measurements. This microsensor is highly sensitive for NO ( $10^{-9}$  mol/l detection limit), and its response time is rapid (on the order of  $10^{-4}$  s), providing the ability to make kinetic measurements of NO concentration. The porphyrinic sensor was positioned with the help of a micromanipulator close to the endothelial cell culture surface ( $20 \pm 1$   $\mu$ m). A three-electrode system, which includes the porphyrinic NO-sensitive electrode, counter platinum electrode, and calomel reference electrode, was used with a constant potential of 700 mV. Before the experiment was started, the medium was changed to phenol red-free and serum-free DME/

F-12 medium. In some experiments, cells were incubated with either the specific B-type carboxypeptidase inhibitor MGTA, B<sub>2</sub> receptor antagonist HOE 140, or the B<sub>1</sub> receptor antagonist des-Arg<sup>10</sup>-Leu<sup>9</sup>-kallidin for 10 min at 37°C before the addition of the agonist. To initiate NO generation, bradykinin, kallidin, or des-Arg<sup>10</sup>-kallidin was added and the responses (current versus time) were recorded continuously. Current generated was proportional to the NO released, and a computer-based Camry VP600 potentiostat was used to monitor NO concentration over time. Each electrode was calibrated with a NO standard.

**Cell harvesting and membrane fractionation.** HLMVEC grown in culture dishes were washed two times with cold PBS, scraped off into a small volume of PBS, and pelleted at 1,000 *g* for 10 min. Cell pellets were resuspended in 1 ml of 50 mM HEPES and 0.25 M sucrose, pH 7.4. The cell suspension was put on ice, and cells were then lysed by sonication three times for 15 s each. The high-speed membrane fraction was obtained by sequential differential centrifugation as described (37). Briefly, lysates were centrifuged at 1,000 *g* for 10 min, and the supernatant was centrifuged at 10,000 *g* for 25 min. The resulting supernatant was then centrifuged at 100,000 *g* for 1 h to obtain the final membrane (P<sub>3</sub>) fraction, which was resuspended in the original fractionation buffer.

**Carboxypeptidase activity assays.** CPM and CPD enzyme activity was measured using dansyl-Ala-Arg substrate (34, 41, 53). Samples were incubated at 37°C for 2 h with 200 μM dansyl-Ala-Arg in a final volume of 250 μl in either 0.1 M HEPES with 0.2% Triton X-100 buffer at pH 7.5 for CPM or 0.2 M sodium acetate with 0.2% Triton X-100 at pH 5.5 for CPD. The assay was done at a slightly higher pH than the optimum for CPM (which is 7.0) and a slightly lower pH than the optimum for CPD (which is 6.2) to minimize any cross-over activity of CPM in the CPD assay and vice versa. The reaction was stopped with 150 μl of 1 M citric acid, and the fluorescent product was extracted into chloroform and measured at 340-nm excitation/495-nm emission. Specific activity was calculated based on the protein concentration as determined by the method of Bradford.

**Immunostaining.** Confluent monolayers of HLMVEC on gelatin-coated glass coverslips were incubated in the absence or presence of 5 ng/ml IL-1β and 200 U/ml IFN-γ for 16 h at 37°C. Medium was changed to phenol red-free DME/F-12 for 1 h, and cells were then fixed in 4% paraformaldehyde for 20 min. Cells were washed with 100 mM glycine three times for 10 min to quench free aldehydes and followed by another three washes with Hank's balanced salt solution (HBSS). The cells were preincubated with blocking buffer (5% normal goat serum, 0.2% BSA, 0.1% Triton X-100 in HBSS, and 0.01% Na<sub>3</sub>N) for 1 h at room temperature. The primary antibodies mouse monoclonal anti-CPM IgG (Novocastra Laboratories, diluted 1:50) or 1:500 anti-CPD antiserum (19, 34) were incubated at 4°C overnight. Normal mouse IgG or normal rabbit antiserum (for CPM or CPD, respectively) was used as controls. After three washes with HBSS, cell were preincubated for 1 h with blocking buffer and then incubated with secondary antibodies (goat anti-mouse Alexa fluor 488 or goat anti-rabbit Alexa fluor 546, diluted 1:750) for 2 h. Cells were washed and then mounted on glass slides using Pro-Long Antifade mounting medium. Confocal microscopy was performed on a Zeiss LSM 510 using a ×63 1.3 numerical aperture objective in optical sections <1 μm in thickness (pinhole set to achieve 1 Airy unit).

**Data analysis.** Data are presented as means ± SE. Statistical analysis was done with ANOVA using the Tukey-Kramer multiple-comparison test. Values of *P* < 0.05 were considered significant.

## RESULTS

A porphyrinic microsensor was used to measure NO production in HLMVEC. This technique has the advantage of measuring nanomolar concentrations of authentic NO in real time (5, 30) as opposed to end-point measurements of accumulated breakdown products as in most other methods. When HLMVEC were exposed to increasing concentrations of bradykinin, a dose-dependent increase in NO production was seen that was transient and returned to baseline in about 5 min (Fig. 1A). The NO production was due to stimulation of the B<sub>2</sub> receptor as the B<sub>2</sub> receptor antagonist HOE 140 blocked the response (Fig. 1A). In the presence of medium alone, there was no apparent production of NO (Fig. 1B). Stimulation of control cells with the B<sub>1</sub>-specific agonist 100 nM des-Arg<sup>10</sup>-kallidin (kallidin = Lys-bradykinin) also stimulated the production of NO but in a completely different pattern with a slow linear rise that was blocked by the B<sub>1</sub> receptor antagonist des-Arg<sup>10</sup>-Leu<sup>9</sup>-kallidin (Fig. 1B). These data indicate that HLMVEC constitutively express both B<sub>1</sub> and B<sub>2</sub> receptors.

To induce upregulation of the B<sub>1</sub> receptor, HLMVEC were stimulated with 5 ng/ml IL-1β and 200 U/ml IFN-γ for 16 h. In the presence of medium alone, there was no generation of NO in the stimulated HLMVEC (Fig. 2). The B<sub>1</sub> agonist des-Arg<sup>10</sup>-kallidin caused a dose-dependent progressively increasing and prolonged generation of NO that was almost linear and still increasing after 20 min (Fig. 2). The response to 100 nM des-Arg<sup>10</sup>-kallidin was about fourfold greater in the stimulated cells compared with that generated by the control cells (Figs. 1B and 2). Extension of the measurement time revealed a plateau in the increase in NO production between 20 and 30 min and then a slow decline, although NO concentration was still elevated over baseline at 60 min (not shown). Because the electrode directly measures NO, which is unstable and continually breaking down, a plateau in the response indicates achievement of a steady-state level of NO production that is balanced by NO degradation. The response to des-Arg<sup>10</sup>-kallidin in stimulated HLMVEC was blocked by 20 μM L-NMMA, the NO synthase inhibitor, and by 1 μM des-Arg<sup>10</sup>-Leu<sup>9</sup>-kallidin, the specific B<sub>1</sub> receptor antagonist, but not by the B<sub>2</sub> antagonist HOE-140 (Fig. 3).

Interestingly, addition of the B<sub>2</sub> agonists bradykinin or kallidin to HLMVEC that had been pretreated with IL-1β and IFN-γ also increased NO production but in a pattern similar to that seen with des-Arg<sup>10</sup>-kallidin (Fig. 4). The response was significantly inhibited by 20 μM MGTA, the specific kininase I-type carboxypeptidase inhibitor, indicating that bradykinin and kallidin were converted to the corresponding des-Arg B<sub>1</sub> agonists by endothelial carboxypeptidase activity. In control experiments, MGTA did not inhibit the generation of NO in response to bradykinin in control cells (Fig. 1) or to des-Arg<sup>10</sup>-kallidin in HLMVEC stimulated with IL-1β and IFN-γ (Fig. 3). Further proof of the generation of the B<sub>1</sub> agonists was obtained by use of the B<sub>1</sub>

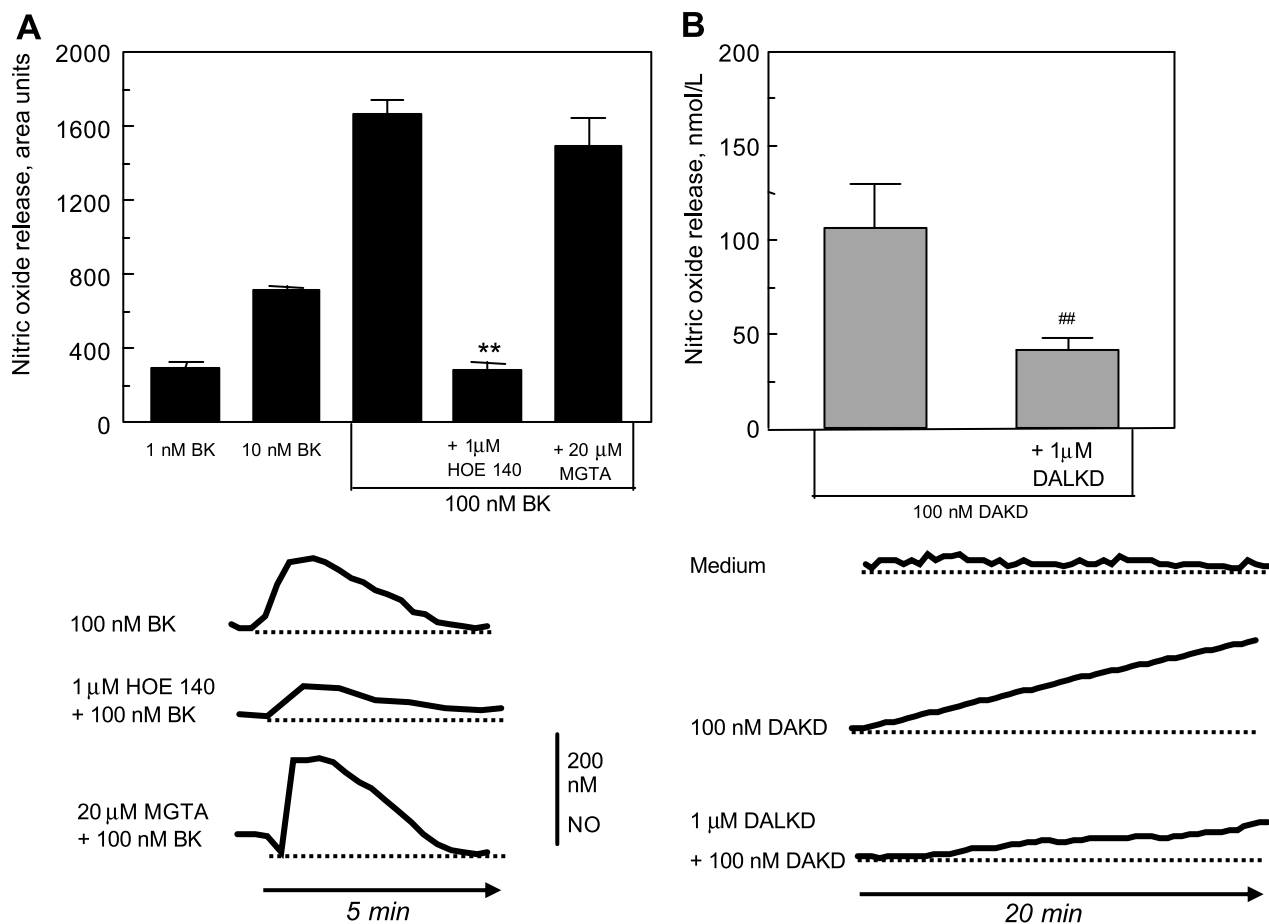


Fig. 1. Stimulation of nitric oxide (NO) production by bradykinin (BK) in control human lung microvascular endothelial cells (HLMVEC). Cells were stimulated with BK (A) at concentrations of 1 nM ( $n = 3$ ), 10 nM ( $n = 3$ ), or 100 nM ( $n = 6$ ) or 100 nM des-Arg<sup>10</sup>-kallidin (DAKD; B,  $n = 3$ ), and NO production for 10 min was measured electrochemically with a porphyrinic electrode. In some cases, HLMVEC were preincubated for 10 min with the B<sub>2</sub> antagonist HOE 140 ( $n = 5$ ) or the carboxypeptidase inhibitor *dl*-2-mercaptomethyl-3-guanidinoethylthiopropionic acid (MGTA,  $n = 4$ ) before addition of 100 nM BK or with 1 μM des-Arg<sup>10</sup>-Leu<sup>9</sup>-kallidin (DALKD;  $n = 3$ ) before addition of 100 nM des-Arg<sup>10</sup>-kallidin (DAKD). *Bottom*: typical real-time tracings of NO production. Vertical bar shows response to standard 200 nM concentration of NO. Length of horizontal arrow indicates 5 min (*left*) or 20 min (*right*). *Top*: mean values  $\pm$  SE. NO release was measured as area under the response curve (A) or as NO concentration reached at 20 min (B). \*\* or ## Difference significant at  $P < 0.01$  compared with 100 nM agonist alone.

antagonist des-Arg<sup>10</sup>-Leu<sup>9</sup>-kallidin, which inhibited the response to bradykinin or kallidin to a similar extent as MGTA (Fig. 4). The B<sub>2</sub> receptor antagonist HOE 140 elicited a smaller inhibition that was nevertheless statistically significant, indicating that some of the response could be due to direct stimulation of the B<sub>2</sub> receptor and is consistent with the lack of complete inhibition by the B<sub>1</sub> antagonist (Fig. 4). Indeed, in the presence of both B<sub>1</sub> and B<sub>2</sub> receptor antagonists, the generation of NO in response to either bradykinin or kallidin was almost completely inhibited in stimulated HLMVEC. Because we had noticed a small B<sub>1</sub> receptor response in control HLMVEC (Fig. 1), we carried out additional experiments in control cells and continued the measurement of NO production in response to bradykinin beyond the 5-min peak due to B<sub>2</sub> receptor stimulation, and we observed a very small linear increase in NO production consistent with the B<sub>1</sub> pattern of generation we found in stimulated cells (not shown).

The B<sub>1</sub> antagonist des-Arg<sup>10</sup>-Leu<sup>9</sup>-kallidin did not inhibit the initial response (peak at 5 min) to bradykinin but did block the delayed slow rise, indicating it is due to B<sub>1</sub> receptor stimulation.

To determine whether HLMVEC contained membrane-bound kininase I (or B-type) carboxypeptidase activity, we isolated a high-speed membrane fraction from the cells and assayed with the specific B-type carboxypeptidase substrate dansyl-Ala-Arg (41, 53). To differentiate between CPM, which has a neutral pH optimum, and CPD, which has an acidic pH optimum, assays were carried out at both pH 5.5 (for CPD) and pH 7.5 (for CPM). These conditions minimize overlap between the two enzymes because CPM is only 31% active at pH 5.5 and CPD is only 20–25% active at pH 7.5 (8, 34). There was significant carboxypeptidase activity at both pH values:  $36.8 \pm 5.2$  nmol·h<sup>-1</sup>·mg protein<sup>-1</sup> ( $n = 4$ ) at pH 5.5 and  $18.7 \pm 0.5$  nmol·h<sup>-1</sup>·mg protein<sup>-1</sup> ( $n = 4$ ) at pH 7.5, indicating

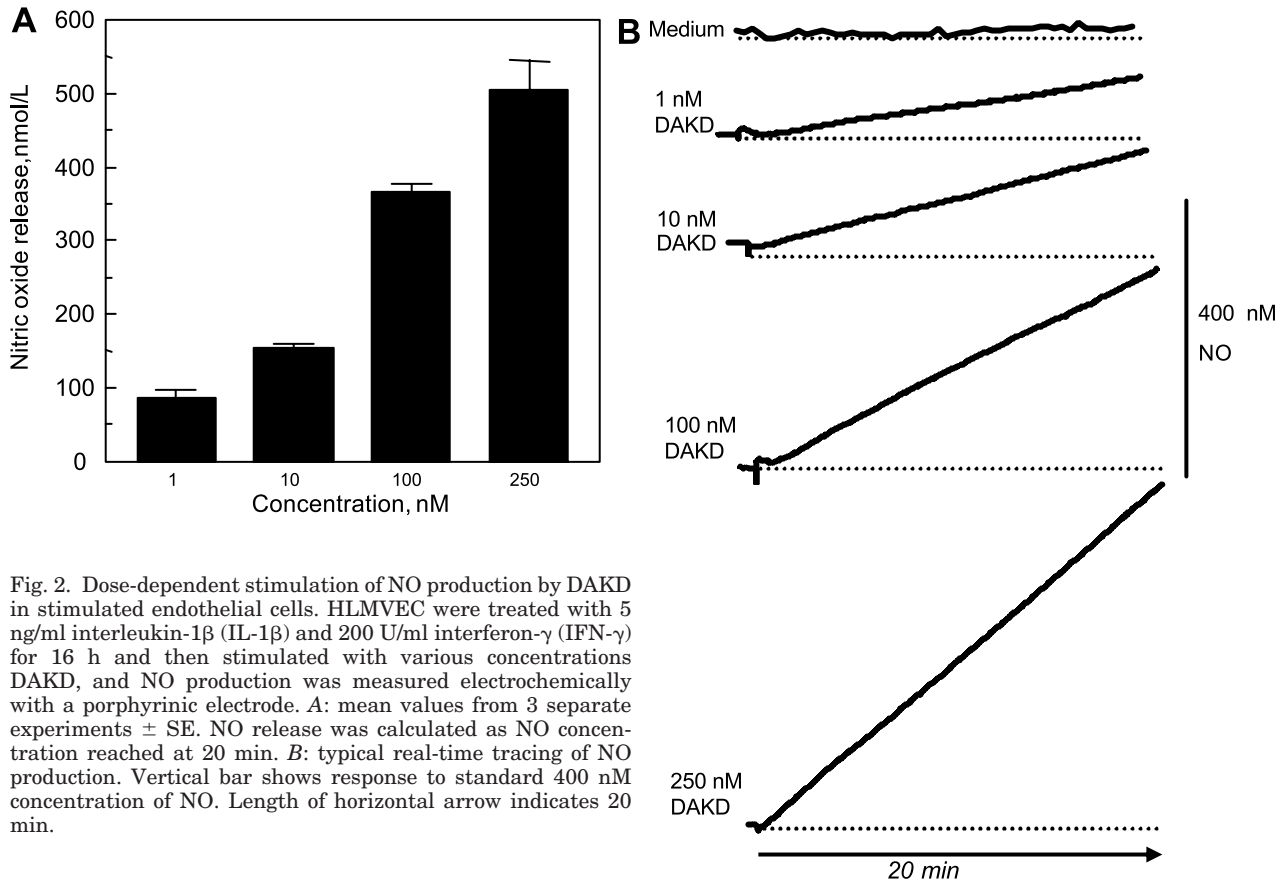


Fig. 2. Dose-dependent stimulation of NO production by DAKD in stimulated endothelial cells. HLMVEC were treated with 5 ng/ml interleukin-1 $\beta$  (IL-1 $\beta$ ) and 200 U/ml interferon- $\gamma$  (IFN- $\gamma$ ) for 16 h and then stimulated with various concentrations DAKD, and NO production was measured electrochemically with a porphyrinic electrode. *A*: mean values from 3 separate experiments  $\pm$  SE. NO release was calculated as NO concentration reached at 20 min. *B*: typical real-time tracing of NO production. Vertical bar shows response to standard 400 nM concentration of NO. Length of horizontal arrow indicates 20 min.

that the cells contain both CPM and CPD. The specific B-type carboxypeptidase inhibitor MGTA (10  $\mu$ M) inhibited  $97 \pm 3.5\%$  of the CPM activity and  $96.5 \pm 4.7\%$  of the CPD activity measured with dansyl-Ala-Arg ( $n = 4$ ). To determine whether inflammatory cytokines that upregulate the B<sub>1</sub> receptor might also upregulate carboxypeptidase activity, HLMVEC that were treated with 5 ng/ml IL-1 $\beta$  and 200 U/ml IFN- $\gamma$  for 16 h were fractionated and assayed for CPD and CPM activity as described above. After stimulation, there was about a twofold increase in both CPD and CPM activity (Fig. 5), indicating an upregulation of the enzymes that generate B<sub>1</sub> agonists under conditions that also upregulate B<sub>1</sub> receptors.

To investigate the subcellular localization of CPM and CPD in endothelial cells, immunocytochemical labeling and laser scanning confocal microscopy were performed on control HLMVEC and cells stimulated with 5 ng/ml IL-1 $\beta$  and 200 U/ml IFN- $\gamma$  for 16 h (Fig. 6). CPD was found primarily in a perinuclear region consistent with its localization in the Golgi, whereas CPM staining was primarily on the plasma membrane in a punctuate pattern that is commonly found with glycosylphosphatidylinositol-anchored proteins (Fig. 6). The intensity of CPM and CPD staining increased after cytokine treatment, which is consistent with the increases in membrane carboxypeptidase activity measured above.

**DISCUSSION**

These studies show that kinin-dependent NO production in endothelial cells can be enhanced by upregulation of the B<sub>1</sub> receptor, whose activation leads to a prolonged response. This phenomenon likely does not require exposure of cells to inflammatory cytokines. For example, bovine pulmonary artery endothelial cells, which constitutively express B<sub>1</sub>, also generate a greater and more prolonged output of NO in response to direct stimulation of B<sub>1</sub> by either ACE inhibitors or peptide ligand compared with B<sub>2</sub> stimulation (21). However, B<sub>1</sub> agonists can only be generated physiologically by carboxypeptidase conversion of B<sub>2</sub> agonists to their des-Arg forms, which highlights the importance of membrane kininase-I type enzymes in regulating this response, as shown here. In this way, kininase I degradation of kinins is actually a processing step, analogous to ACE, which cleaves angiotensin I to generate angiotensin II, the agonist for the AT1 receptor.

We found significant CPD and CPM activity in membrane fractions of HLMVEC. However, subcellular fractionation does not separate plasma membranes from intracellular membranes; therefore, these assays cannot determine how much of the activity was on the plasma membrane. Immunohistochemical staining of HLMVEC revealed the same distribution for CPD and CPM found in other cell types; CPM is primarily on the plasma membrane and CPD is highly concentrated in

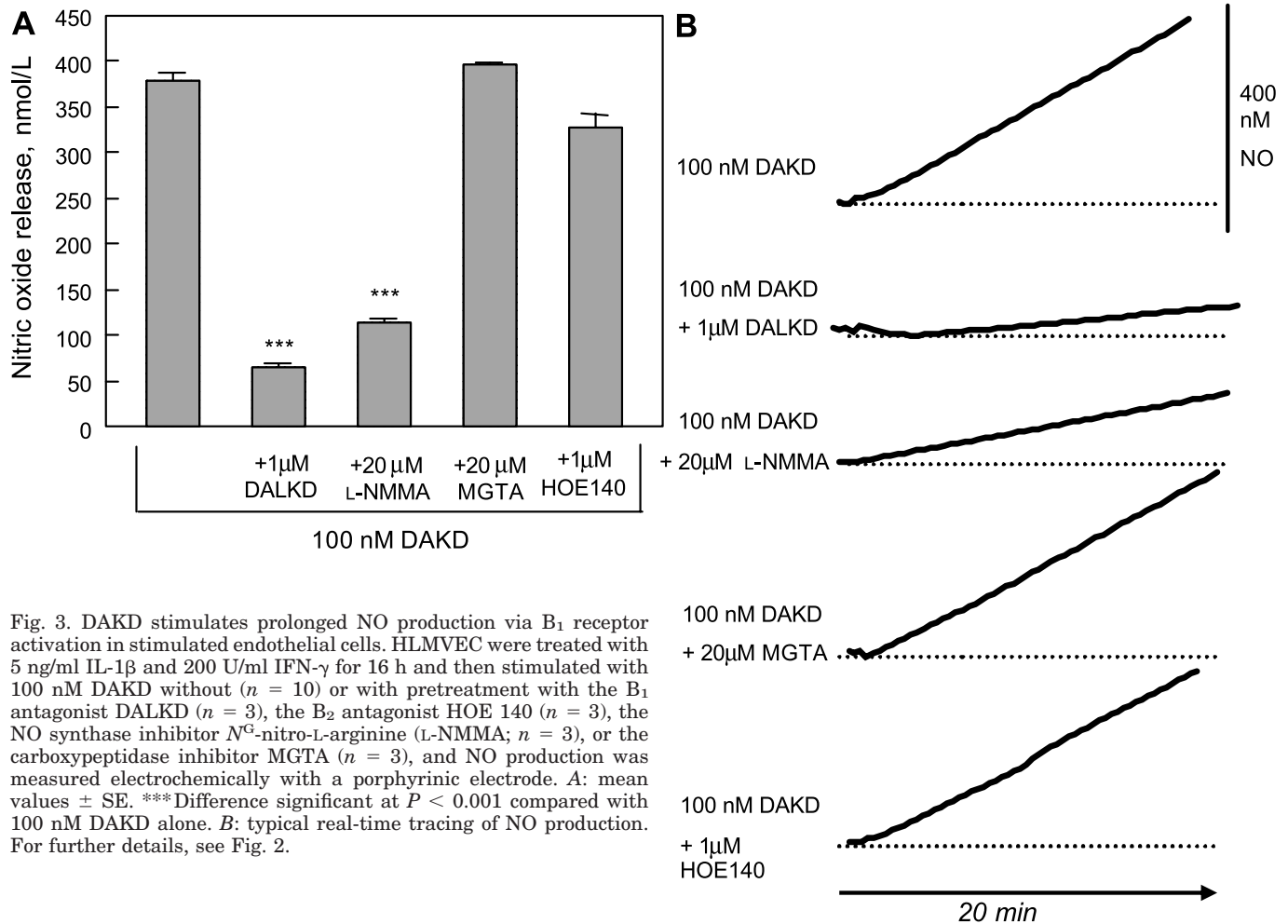


Fig. 3. DAKD stimulates prolonged NO production via B<sub>1</sub> receptor activation in stimulated endothelial cells. HLMVEC were treated with 5 ng/ml IL-1 $\beta$  and 200 U/ml IFN- $\gamma$  for 16 h and then stimulated with 100 nM DAKD without ( $n = 10$ ) or with pretreatment with the B<sub>1</sub> antagonist DALKD ( $n = 3$ ), the B<sub>2</sub> antagonist HOE 140 ( $n = 3$ ), the NO synthase inhibitor N<sup>G</sup>-nitro-L-arginine (L-NMMA;  $n = 3$ ), or the carboxypeptidase inhibitor MGTA ( $n = 3$ ), and NO production was measured electrochemically with a porphyrinic electrode. **A**: mean values  $\pm$  SE. \*\*\*Difference significant at  $P < 0.001$  compared with 100 nM DAKD alone. **B**: typical real-time tracing of NO production. For further details, see Fig. 2.

the Golgi. Although CPD can be detected on the plasma membrane, the maximum reported is about 10% of the total CPD in the cell (19, 25, 57). It is likely in our studies that the primary enzyme involved in the conversion of kinins to des-Arg kinins was CPM based on the following considerations. First, although the total membrane CPD activity was about two times higher than CPM activity, assuming that 10% of the CPD is on the plasma membrane, the ratio would be about 5:1 CPM:CPD. Second, CPD has an acidic pH optimum of 6.2 and at pH 7.4 CPD has only about 20–25% of its activity remaining with dansyl-Ala-Arg, whereas CPM is fully active at pH 7.4. There are no specific inhibitors that will distinguish between CPM and CPD, therefore, a definitive resolution to this question will require the development of knockout animals or RNA interference approaches.

Another way in which membrane carboxypeptidases can enhance NO production is by cleaving substrates to generate free Arg to be utilized by NO synthase as was shown in a macrophage cell line (19). However, this is unlikely to play a significant role in the present experiments, which were carried out in the presence of medium containing 0.7 mM free Arg. The maximum amount of Arg that would be generated by carboxypep-

tidase cleavage of all the added kallidin or bradykinin would be only 100 nM, which is 7,000-fold lower than the Arg in the medium.

An important physiological indicator of B<sub>1</sub> receptor stimulation is NO (31). The ability to measure NO production in real time revealed a striking difference in the initial time course of NO production after addition of the agonist in control HLMVEC. Thus, the B<sub>2</sub> receptor-mediated response was relatively transient and short lived, whereas the B<sub>1</sub> receptor response resulted in a slowly rising, almost linear increase in the generation of NO. The presence of a B<sub>1</sub> response in control cells was unexpected because the receptor is usually absent in normal tissues or cells and requires injury or inflammatory mediators to upregulate its transcription (3, 31). Although it is possible that our culture conditions caused upregulation of B<sub>1</sub> receptors, we took great care to eliminate potential sources of endotoxin contamination in medium, buffers, etc. It is also possible that these cells constitutively express B<sub>1</sub> receptors at a low level as found, for example, in bovine pulmonary artery endothelial cells (21, 50). The difference in pattern of NO production in response to B<sub>1</sub> or B<sub>2</sub> agonists is consistent with the marked difference in the ability of the B<sub>2</sub> and B<sub>1</sub> receptors to be desensitized

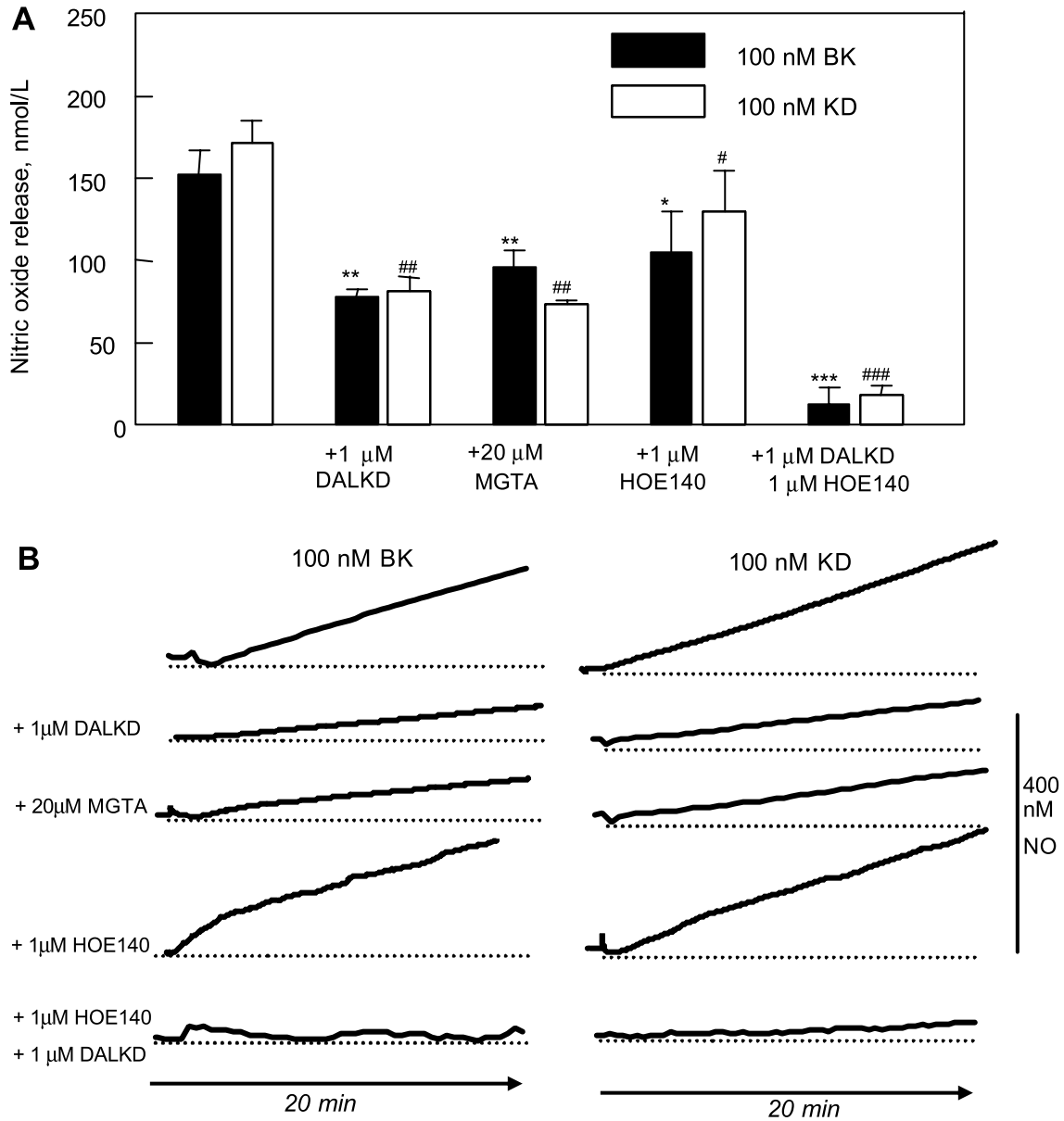


Fig. 4. B<sub>2</sub> receptor agonists stimulate prolonged NO production via B<sub>1</sub> and B<sub>2</sub> receptor activation in stimulated endothelial cells. HLMVEC were treated with 5 ng/ml IL-1 $\beta$  and 200 U/ml IFN- $\gamma$  for 16 h and then stimulated with 100 nM BK or kallidin (KD) without or with preincubation with the B<sub>1</sub> antagonist DALKD, B<sub>2</sub> antagonist HOE 140, the B<sub>1</sub> and B<sub>2</sub> antagonists combined, NO synthase inhibitor L-NMMA, or the carboxypeptidase inhibitor MGTA, and NO production was measured electrochemically with a porphyrinic electrode. A: mean values from 3 to 8 separate experiments  $\pm$  SE. Difference significant at  $P < 0.001$  (\*\*\*) or (###),  $P < 0.01$  (\*\* or ##), or  $P < 0.05$  (\* or #) compared with 100 nM BK (\*) or 100 nM KD (#) alone. B: typical real-time tracing of NO production in response to BK (left) or KD (right). For further details, see Fig. 2.

after stimulation; the B<sub>2</sub> receptor is rapidly sequestered and the response terminated, whereas the B<sub>1</sub> receptor is not, resulting in a more prolonged response (2, 10, 27, 31). However, in HLMVEC stimulated with IL-1 $\beta$  and IFN- $\gamma$ , it is apparent that B<sub>2</sub> receptor stimulation can also result in prolonged generation of NO in a pattern similar to that found with B<sub>1</sub> stimulation. Thus, in the presence of a B<sub>1</sub> antagonist in stimulated cells, a B<sub>2</sub> agonist stimulated a slow linear increase in NO production that was inhibited by a B<sub>2</sub> antagonist (Fig. 4). The reason for the prolonged B<sub>2</sub> response in

stimulated cells is unclear, but it is possible that sequestration/desensitization of B<sub>2</sub> is altered under these conditions. Alternatively, activation of NO synthase in stimulated cells might differ from that in control cells in the absence of a change in receptor desensitization. For example, B<sub>2</sub>-stimulated phosphorylation of endothelial NO synthase might predominate in stimulated cells [which would generate a slower but more prolonged response (43)] compared with only calcium activation in control cells. Another possibility is that either B<sub>1</sub> or B<sub>2</sub> receptor activation in stimulated

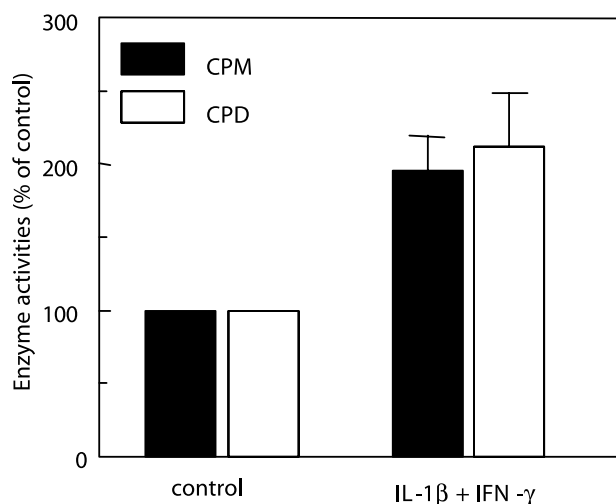
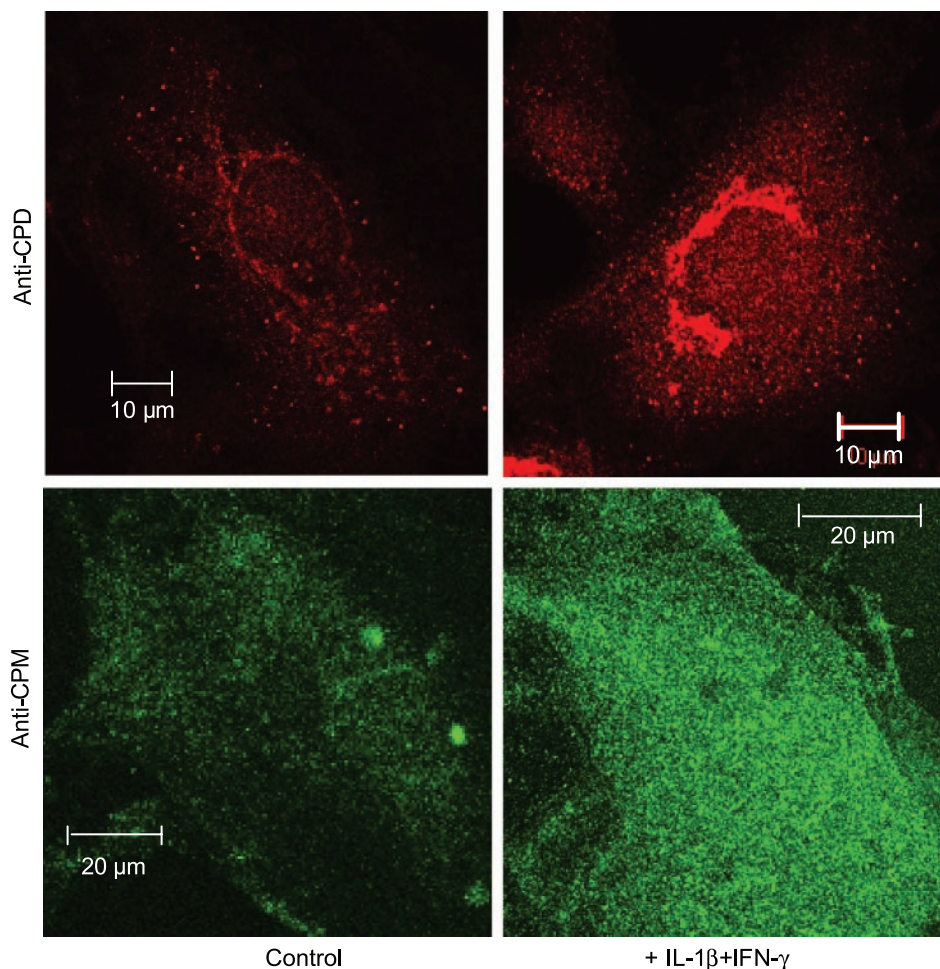


Fig. 5. Upregulation of carboxypeptidase M (CPM) and carboxypeptidase D (CPD) activity in endothelial cells by inflammatory cytokines. HLMVEC were treated without (control) or with 5 ng/ml IL-1 $\beta$  and 200 U/ml IFN- $\gamma$  for 16 h. Cells were lysed and membrane fractions were isolated and assayed for CPM or CPD activity with dansyl-Ala-Arg. Results are calculated as %control (untreated = 100%) and are means  $\pm$  SE for  $n = 4$ .

HLMVEC activates inducible NO synthase instead of endothelial NO synthase, which leads to a different pattern of NO production. These will be interesting areas to explore in future investigations.

The ability of endothelial cells to generate higher levels of NO under inflammatory conditions could have important physiological and pathological relevance. NO is a reactive radical gas that regulates cellular functions in both physiological and pathological conditions (20, 23, 29, 38). NO has a variety of activities in the cardiovascular system, including regulation of vascular tone and endothelial barrier function (24, 36). In physiological states, NO can serve a protective function, but, under conditions of high output, NO may contribute to tissue damage by reacting with superoxide to form peroxynitrite, a strong oxidant (22). NO not only participates in the inflammatory response via its physiological effects, but also by its ability to regulate the expression of inflammatory proteins (56, 59) via its regulation of the transcription factor, nuclear factor-kB (7). Thus it has been suggested that NO may exert both deleterious and protective effects in inflammatory conditions such as sepsis, depending on species, timing, the cell type, inflammatory stimulus, the NO concentration, and NO-related metabolites generated (58).

Fig. 6. Effect of cytokines on CPM and CPD expression and localization in endothelial cells. HLMVEC were exposed to vehicle or 5 ng/ml IL-1 $\beta$  and 200 U/ml IFN- $\gamma$  for 16 h, fixed, and immunostained as described in MATERIALS AND METHODS. Both CPD (top, red) and CPM (bottom, green) immunostaining increased after cytokine treatment. CPM expression was mainly observed on the cell membrane, whereas CPD was localized primarily to the perinuclear/Golgi region. No membrane or Golgi staining was seen in cells stained with control normal mouse IgG or normal rabbit serum (not shown). Results are representative of 4 separate experiments.



Kinins cause numerous biological effects, including regulation of smooth muscle tone or salt and water excretion in the kidney, and are important mediators of the inflammatory response (3, 31). Kinins also have beneficial effects in the heart and are now thought to be major mediators by which inhibitors of ACE have beneficial cardiovascular effects that go beyond lowering blood pressure (14, 28). The B<sub>1</sub> receptor may have protective roles in cardiovascular diseases (1, 4, 6), and ACE inhibitors were recently found to directly bind and activate the B<sub>1</sub> bradykinin receptor through a unique zinc-binding motif (21). ACE inhibitors also induce expression of the B<sub>1</sub> receptor in rodent kidney, heart, and vasculature, which was shown to play a role in the hypotensive effect of ACE inhibitors (32). Because ACE is a predominant peptidase for the degradation of bradykinin and kallidin in vivo (11, 12, 15), blockade with ACE inhibitors would increase the level of kinin substrates for conversion by cellular carboxypeptidases. Indeed, the generation of des-Arg kinins has been reported to increase in several studies in the presence of ACE inhibitors (31). Thus kininase I-type carboxypeptidases could potentially play a role in some of the therapeutic effects of ACE inhibitors through enhancement of signaling through the B<sub>1</sub> receptor.

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REFERENCES

1. **Agata J, Miao RQ, Yayama K, Chao L, and Chao J.** Bradykinin B(1) receptor mediates inhibition of neointima formation in rat artery after balloon angioplasty. *Hypertension* 36: 364–370, 2000.
2. **Austin CE, Faussner A, Robinson HE, Chakravarty S, Kyle DJ, Bathon JM, and Proud D.** Stable expression of the human kinin B1 receptor in Chinese hamster ovary cells. Characterization of ligand binding and effector pathways. *J Biol Chem* 272: 11420–11425, 1997.
3. **Bhoola KD, Figueroa CD, and Worthy K.** Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol Rev* 44: 1–80, 1992.
4. **Bouchard JF, Chouinard J, and Lamontagne D.** Role of kinins in the endothelial protective effect of ischaemic preconditioning. *Br J Pharmacol* 123: 413–420, 1998.
5. **Brovkovych V, Stolarczyk E, Oman J, Tomboulian P, and Malinski T.** Direct electrochemical measurement of nitric oxide in vascular endothelium. *J Pharm Biomed Anal* 19: 135–143, 1999.
6. **Chahine R, Adam A, Yamaguchi N, Gaspo R, Regoli D, and Nadeau R.** Protective effects of bradykinin on the ischaemic heart: implication of the B1 receptor. *Br J Pharmacol* 108: 318–322, 1993.
7. **Connelly L, Palacios-Callender M, Ameixa C, Moncada S, and Hobbs AJ.** Biphasic regulation of NF-kappa B activity underlies the pro- and anti-inflammatory actions of nitric oxide. *J Immunol* 166: 3873–3881, 2001.
8. **Deddish PA, Skidgel RA, and Erdös EG.** Enhanced Co<sup>2+</sup> activation and inhibitor binding of carboxypeptidase M at low pH. Similarity to carboxypeptidase H (enkephalin convertase). *Biochem J* 261: 289–291, 1989.
9. **Deddish PA, Skidgel RA, Kriho VB, Li XY, Becker RP, and Erdös EG.** Carboxypeptidase M in Madin-Darby canine kidney cells. Evidence that carboxypeptidase M has a phosphatidylinositol glycan anchor. *J Biol Chem* 265: 15083–15089, 1990.

10. **De Weerd WFC and Leeb-Lundberg LMF.** Bradykinin sequestrers B<sub>2</sub> bradykinin receptors and the receptor-coupled Ga subunits Ga<sub>q</sub> and Ga<sub>i</sub> in caveolae in DDT<sub>1</sub> MF-2 smooth muscle cells. *J Biol Chem* 272: 17858–17866, 1997.
11. **Erdös EG.** Angiotensin I converting enzyme and the changes in our concepts through the years. Lewis K Dahl memorial lecture. *Hypertension* 16: 363–370, 1990.
12. **Erdös EG (Editor).** Kininases. In: *Handbook of Experimental Pharmacology*. Heidelberg: Springer-Verlag, 1979, p. 427–487.
13. **Erdös EG.** Kinins, the long march—a personal view. *Cardiovasc Res* 54: 485–491, 2002.
14. **Erdös EG, Deddish PA, and Marcic BM.** Potentiation of bradykinin actions by ACE inhibitors. *Trends Endocrinol Metab* 10: 223–229, 1999.
15. **Erdös EG and Marcic BM.** Kinins, receptors, kininases and inhibitors—where did they lead us? *Biol Chem* 382: 43–47, 2001.
16. **Erdös EG and Sloane EM.** An enzyme in human blood plasma that inactivates bradykinin and kallidins. *Biochem Pharmacol* 11: 582–592, 1962.
17. **Fricke LD.** Carboxypeptidase E. *Annu Rev Physiol* 50: 309–321, 1988.
18. **Fricke LD and Snyder SH.** Purification and characterization of enkephalin convertase, an enkephalin-synthesizing carboxypeptidase. *J Biol Chem* 258: 10950–10955, 1983.
19. **Hadkar V and Skidgel RA.** Carboxypeptidase D is upregulated in RAW 264.7 macrophages and stimulates nitric oxide synthesis by cells in arginine-free medium. *Mol Pharmacol* 59: 1324–1332, 2001.
20. **Hobbs AJ, Higgs A, and Moncada S.** Inhibition of nitric oxide synthase as a potential therapeutic target. *Annu Rev Pharmacol Toxicol* 39: 191–220, 1999.
21. **Ignjatovic T, Tan F, Brovkovych V, Skidgel RA, and Erdos EG.** Novel mode of action of angiotensin I converting enzyme inhibitors: direct activation of bradykinin B1 receptor. *J Biol Chem* 277: 16847–16852, 2002.
22. **Ischiropoulos H, Zhu L, and Beckman JS.** Peroxynitrite formation from macrophage-derived nitric oxide. *Arch Biochem Biophys* 298: 446–451, 1992.
23. **Kirkeboen KA and Strand OA.** The role of nitric oxide in sepsis—an overview. *Acta Anaesthesiol Scand* 43: 275–288, 1999.
24. **Kubes P.** Nitric oxide affects microvascular permeability in the intact and inflamed vasculature. *Microcirculation* 2: 235–244, 1995.
25. **Kuroki K, Cheung R, Marion PL, and Ganem D.** A cell surface protein that binds avian hepatitis B virus particles. *J Virol* 68: 2091–2096, 1994.
26. **Kuroki K, Eng F, Ishikawa T, Turck C, Harada F, and Ganem D.** gp180, a host cell glycoprotein that binds duck hepatitis B virus particles, is encoded by a member of the carboxypeptidase gene family. *J Biol Chem* 270: 15022–15028, 1995.
27. **Lamb ME, De Weerd WF, and Leeb-Lundberg LM.** Agonist-promoted trafficking of human bradykinin receptors: arrestin- and dynamin-independent sequestration of the B2 receptor and bradykinin in HEK293 cells. *Biochem J* 355: 741–750, 2001.
28. **Linz W, Wiemer G, Gohlke P, Unger T, and Schölkens BA.** Contribution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors. *Pharmacol Rev* 47: 25–49, 1995.
29. **Loscalzo J and Welch G.** Nitric oxide and its role in the cardiovascular system. *Prog Cardiovasc Dis* 38: 87–104, 1995.
30. **Malinski T and Taha Z.** Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor. *Nature* 358: 676–678, 1992.
31. **Marceau F, Hess JF, and Bachvarov DR.** The B<sub>1</sub> receptors for kinins. *Pharmacol Rev* 50: 357–386, 1998.
32. **Marin-Castano ME, Schanstra JP, Neau E, Praddaude F, Pecher C, Ader JL, Girolami JP, and Bascands JL.** Induction of functional bradykinin B<sub>1</sub>-receptors in normotensive rats and mice under chronic Angiotensin-converting enzyme inhibitor treatment. *Circulation* 105: 627–632, 2002.
33. **McGwire GB and Skidgel RA.** Extracellular conversion of epidermal growth factor (EGF) to des-Arg53-EGF by carboxypeptidase M. *J Biol Chem* 270: 17154–17158, 1995.

34. **McGwire GB, Tan F, Michel B, Rehli M, and Skidgel RA.** Identification of a membrane-bound carboxypeptidase as the mammalian homolog of duck gp180, a hepatitis B virus-binding protein. *Life Sci* 60: 715–724, 1997.
35. **Michel B, Igc R, Leray V, Deddish PA, and Erdös EG.** Removal of Arg<sup>141</sup> from the a chain of human hemoglobin by carboxypeptidase N and M. *Circ Res* 78: 635–642, 1996.
36. **Moncada S, Palmer RM, and Higgs EA.** Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43: 109–142, 1991.
37. **Nagae A, Deddish PA, Becker RP, Anderson CH, Abe M, Tan F, Skidgel RA, and Erdös EG.** Carboxypeptidase M in brain and peripheral nerves. *J Neurochem* 59: 2201–2212, 1992.
38. **Palmer RM, Ashton DS, and Moncada S.** Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333: 664–666, 1988.
39. **Regoli D and Barabe J.** Pharmacology of bradykinin and related kinins. *Pharmacol Rev* 32: 1–46, 1980.
40. **Reznik SE and Fricker LD.** Carboxypeptidases from A to Z: implications in embryonic development and Wnt binding. *Cell Mol Life Sci* 58: 1790–1804, 2001.
41. **Skidgel RA.** Assays for arginine/lysine carboxypeptidases: Carboxypeptidase H (E; enkephalin convertase), M and N. In: *Methods in Neurosciences: Peptide Technology*, edited by Conn PM. Orlando: Academic, 1991, p. 373–385.
42. **Skidgel RA.** Basic carboxypeptidases: regulators of peptide hormone activity. *Trends Pharmacol Sci* 9: 299–304, 1988.
43. **Skidgel RA.** Proliferation of regulatory mechanisms for eNOS: an emerging role for the cytoskeleton. *Am J Physiol Lung Cell Mol Physiol* 282: L1179–L1182, 2002.
44. **Skidgel RA.** Structure and function of mammalian zinc carboxypeptidases. In: *Zinc Metalloproteases in Health and Disease*, edited by Hooper NM. London: Taylor and Francis, 1996, p. 241–283.
45. **Skidgel RA, Davis RM, and Tan F.** Human carboxypeptidase M. Purification and characterization of a membrane-bound carboxypeptidase that cleaves peptide hormones. *J Biol Chem* 264: 2236–2241, 1989.
46. **Skidgel RA and Erdös EG.** Cellular carboxypeptidases. *Immunol Rev* 161: 129–141, 1998.
47. **Skidgel RA and Erdös EG.** Enzymatic breakdown of bradykinin. In: *Pro-inflammatory and Anti-inflammatory Peptides*, edited by Said SI. New York: Marcel-Dekker, 1998, p. 459–476.
48. **Skidgel RA, Johnson AR, and Erdös EG.** Hydrolysis of opioid hexapeptides by carboxypeptidase N. Presence of carboxypeptidase in cell membranes. *Biochem Pharmacol* 33: 3471–3478, 1984.
49. **Skidgel RA, Tan F, Deddish PA, and Li XY.** Structure, function and membrane anchoring of carboxypeptidase M. *Biomed Biochim Acta* 50: 815–820, 1991.
50. **Smith JAM, Webb C, Holford J, and Burgess GM.** Signal transduction pathways for B<sub>1</sub> and B<sub>2</sub> bradykinin receptors in bovine pulmonary artery endothelial cells. *Mol Pharmacol* 47: 525–534, 1995.
51. **Song L and Fricker LD.** Purification and characterization of carboxypeptidase D, a novel carboxypeptidase E-like enzyme, from bovine pituitary. *J Biol Chem* 270: 25007–25013, 1995.
52. **Spangenberg HC, Lee HB, Li J, Tan F, Skidgel R, Wands JR, and Tong S.** A short sequence within domain C of duck carboxypeptidase D is critical for duck hepatitis B virus binding and determines host specificity. *J Virol* 75: 10630–10642, 2001.
53. **Tan F, Deddish PA, and Skidgel RA.** Human carboxypeptidase M. *Methods Enzymol* 248: 663–675, 1995.
54. **Tan F, Rehli M, Krause SW, and Skidgel RA.** Sequence of human carboxypeptidase D reveals it to be a member of the regulatory carboxypeptidase family with three tandem active site domains. *Biochem J* 327: 81–87, 1997.
55. **Timblin B, Rehli M, and Skidgel RA.** Structural characterization of the human carboxypeptidase D gene and its promoter. *Int Immunopharmacol* 2: 1907–1917, 2002.
56. **Tsao PS, Wang B, Buitrago R, Shyy JY, and Cooke JP.** Nitric oxide regulates monocyte chemotactic protein-1. *Circulation* 96: 934–940, 1997.
57. **Varlamov O and Fricker LD.** Intracellular trafficking of metalloproteinase D in AtT-20 cells: localization to the trans-Golgi network and recycling from the cell surface. *J Cell Sci* 111: 877–885, 1998.
58. **Wolkow PP.** Involvement and dual effects of nitric oxide in septic shock. *Inflamm Res* 47: 152–166, 1998.
59. **Xie QW, Kashiwabara Y, and Nathan C.** Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem* 269: 4705–4708, 1994.
60. **Xin X, Varlamov O, Day R, Dong W, Bridgett MM, Leiter EH, and Fricker LD.** Cloning and sequence analysis of cDNA encoding rat carboxypeptidase D. *DNA Cell Biol* 16: 897–909, 1997.