Carboxypeptidase-mediated enhancement of nitric oxide production in rat lungs and microvascular endothelial cells

Vaishali Hadkar,†* Sakonwun Sangsree,†* Stephen M. Vogel,† Viktor Brovkovych, and Randal A. Skidgel†,2

†Departments of Pharmacology and 2Anesthesiology, University of Illinois College of Medicine, Chicago, Illinois 60612

Submitted 29 September 2003; accepted in final form 12 February 2004

The precise roles of NO in sepsis and lung inflammation are still controversial, as it is beneficial in certain conditions but also can have toxic effects (22, 73). This is reflected in the conflicting reports regarding the effects of iNOS inhibition or knockout on overall survival in sepsis (22) and the variable results obtained using inhaled NO in therapy for patients with ARDS or acute lung injury (2, 67).

Induction of iNOS during sepsis can occur in many organs, but the major site of NO production that results in pulmonary edema and injury is in the lung itself (33). Of the many cell types present in the lung, alveolar macrophages and airway epithelial and endothelial cells can all be induced to express iNOS (13, 18, 30). Because iNOS is constitutively active, even at the normal low intracellular calcium concentration, its regulation is thought to be primarily at the level of transcription and once synthesized by the supply of substrate (41). For example, in the absence of extracellular Arg uptake by system y+ or depletion of extracellular arginine, iNOS activity is reduced to zero (9, 64). At wounds or other inflammatory sites, extracellular Arg can be depleted by arginase, which converts it to ornithine (1, 11). It is generally considered that normal Arg levels in plasma and cells (100–800 μM) (9, 38, 75) are sufficient to saturate iNOS, whose Km for Arg is <20 μM (29).

However, studies in a variety of different model systems have demonstrated that even in the presence of apparently saturating levels of extracellular Arg, addition of additional substrate does increase NO production (42), and this has been termed the “arginine paradox.”

Arg is a semiessential amino acid, becoming essential during growth and development or wound healing (75). However, little is known about the use of peptides and proteins as sources of Arg substrate for NO synthesis. The release of free Arg from a peptide or protein would require the action of an exopeptidase specific for cleaving basic amino acids. One class of enzyme that fits this profile is the basic or B-type metallocarboxypeptidases, which cleave a single amino acid at a time from the COOH terminus of peptides and proteins and exhibit strict specificity for COOH-terminal Arg or lysine (Lys) (57, 59). Two members of the regulatory carboxypeptidase (CP) or CPN/E subfamily of B-type metallocarboxypeptidases, CP and CPD, are widely distributed membrane-bound proteins (59). CP is a 62-kDa membrane protein anchored via glycosylphosphatidylinositol, whereas CPD is a 180-kDa single-chain glycoprotein with three homologous carboxypeptidase active site domains and a carboxyl-terminal hydrophobic transmembrane anchor (40, 59, 62). We previously showed, in a

Sepsis is a leading cause of acute respiratory distress syndrome (ARDS) and acute lung injury, which are characterized by pulmonary inflammation and increased capillary endothelial permeability caused by numerous toxic products that are released. These include superoxide, cytokines, chemokines, complement fragments, kinins, eicosanoids, and proteases (2, 67). Lipopolysaccharide (LPS) also stimulates inducible nitric oxide synthase (iNOS) mRNA expression in various organs, including the lung (35, 71). The induction of iNOS leads to generation of large amounts of NO in alveolar macrophages and lung epithelial, endothelial, and interstitial cells (25, 43).

* V. Hadkar and S. Sangsree contributed equally to this work.

Address for reprint requests and other correspondence: R. A. Skidgel, Dept. of Pharmacology (m/c 368), Univ. of Illinois College of Medicine, 835 S. Wolcott, Chicago, IL 60612 (E-mail: rskidgel@uic.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
mouse macrophage cell line stimulated with interferon-γ (IFN-γ) and LPS, that addition of a carboxypeptidase substrate stimulated NO production by sixfold, and this effect was blocked by a specific carboxypeptidase inhibitor (23). Furthermore, in cells stimulated with IFN-γ and LPS in Arg-free medium, CPD activity increased two- to threefold; immunohistochemical staining and Western analysis revealed an increase in CPD protein, and Northern analysis showed increased CPD mRNA (23). These data indicate that CPD could enhance NO production in macrophages by cleaving Arg from peptide substrates during inflammatory processes.

In the present study we investigated whether B-type carboxypeptidases can generate the Arg substrate for iNOS to increase NO production in rat lung microvascular endothelial cells (RLMVEC) and isolated perfused rat lungs under inflammatory conditions.

**MATERIALS AND METHODS**

**Materials.** Low-glucose Dulbecco’s modified Eagle’s medium (DMEM) without phenol red, custom-made arginine-free DMEM, and IFN-γ (murine recombinant) were from Gibco/Invitrogen/Life Technologies. L-Glutamine, endothelial growth factor, heparin, and DL-2-mercaptoethyl-3-guanidothiopropionic acid (MGTA) were from Calbiochem. Fetal bovine serum was from Atlanta Biologicals. Furlacyloyl-Ala-Arg (Fa-A-R) was from Bachem Biosciences. 5-Dimethylaminonaphthalene-1-sulfonyl-L-alanyl-L-arginine (dansyl-Ala-Arg) was synthesized and purified as described previously (66). RLMVEC were obtained from Clonetics (Cambrex). Gelatin and bacterial LPS from *Salmonella typhimurium* (for use with RLMVEC) or from *Escherichia coli* 0127:B8 (for injection into rats) were from Sigma. Trifluoroacetic acid (TFA) was from Pierce Chemical. Common chemicals were from Fisher Scientific.

**Cell culture.** Cells were cultured in low-glucose DMEM without phenol red containing 4 mmol/l L-glutamine, 60 g/ml endothelial cell growth factor, 90 μg/ml heparin, and 10% heat-inactivated fetal bovine serum (FBS, to destroy any endogenous serum carboxypeptidase (23). Further- more, in cells treated with IFN-γ and LPS in Arg-free medium, CPD activity increased two- to threefold; immunohistochemical staining and Western analysis revealed an increase in CPD protein, and Northern analysis showed increased CPD mRNA (23). These data indicate that CPD could enhance NO production in macrophages by cleaving Arg from peptide substrates during inflammatory processes.

**Measurement of NO production.** NO production was determined by two methods: 1) assay of nitrate accumulation in the medium and 2) measurement of NO in real time with a porphyrinic microsensor. To measure nitrate accumulation, RLMVEC treated with IFN-γ and LPS as above were washed twice with Arg-free DMEM, and then fresh medium was added containing either 1 mM Arg or 1 mM carboxypeptidase substrate Fa-A-R with or without 20 μM carboxypeptidase inhibitor MGTA. Cells were then incubated for 48 h at 37°C, and accumulated nitrite was measured in the medium with the colorimetric Griess reagent (1% sulfanilamide, 0.1% naphthalene diamine dihydrochloride, and 2% phosphoric acid) (69). Culture medium (150 μl) was mixed with 150 μl of Griess reagent, and after a 10-min incubation at room temperature the absorbance was measured at 540 nm. Nitrite concentration was calculated from a standard curve, using sodium nitrite.

For direct measurement of NO generation in real time, a porphyrinic microsensor was used as described (6, 36, 52). The microsensor is highly sensitive for NO, and its response time is rapid, providing the ability to make kinetic measurements of NO generation. The porphyrinic sensor was positioned with the help of a micromanipulator close to the endothelial cell culture surface (20 ± 1 μm). RLMVEC treated with IFN-γ and LPS as above were washed twice with Arg-free DMEM, and cells were incubated in fresh Arg-free DMEM for 3 h before the experiment. In some experiments, cells were incubated with the carboxypeptidase inhibitor MGTA for 30 min at 37°C before measurement. To initiate NO generation, 1 mM Arg or carboxypeptidase substrate Fa-A-R was added, and the responses (current vs. 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 an NO standard. The maximum concentration of NO achieved within 20 min after addition of substrate was used to quantitate the results.

**Studies using isolated perfused rat lungs.** Studies were done in accordance with institutional and National Institutes of Health (NIH) guidelines and after review and approval by the University of Illinois at Chicago Animal Care Committee. Sprague-Dawley rats (300–350 g) were pretreated with LPS (15–20 mg/kg) for 5–6 h. Rats were anesthetized with 3% halothane in 20% O₂ (balance N₂) via a vaporizer. The trachea was cannulated, and lungs were ventilated with a small-animal ventilator at 60 cycles/min, 3-ml tidal volume, and 2-ml water end-expiratory pressure with continued administration of the anesthetic gas mixture. A thoracotomy was performed, and heparin (1,000 units/ml) was injected into the right ventricle. The pulmonary artery was cannulated and perfused with modified Krebs-Henseleit solution (concentration in mM: 118 NaCl, 4.7 KCl, 1.0 CaCl₂, 1.2 MgCl₂, 5.55 d-glucose, 25 NaHCO₃, 0.33 NaH₂PO₄, and 1.0 MgTA) supplemented with 5 g/100 ml bovine serum albumin via a peristaltic pump at a rate of 0.03 ml/g body wt⁻¹ min⁻¹. A cannula was placed in the left atrium to drain venous effluent. The length of the cannula was kept constant at 4 cm in all experiments. The heart and lungs were then excised en bloc and suspended from a calibrated beam balance to continuously monitor the wet weight of the lungs. The initial portion of the venous effluent was discarded until the effluent was clear of blood cells. Subsequently 50 ml of venous effluent were recirculated and maintained at 37°C. A bubble trap and a pressure transducer (to measure pulmonary artery pressure) were part of the circuit. The same gas mixture used in ventilation was also bubbled through the perfusing liquid. The lungs were covered with Saran wrap to reduce evaporative fluid loss. The lung wet weight was nulled by use of counterweights, and the subsequent changes in the wet weight of the lung and the pulmonary artery pressures were continuously monitored. Data were digitized, logged, and displayed on the video monitor with commercially available software (Labtech Notebookpro for Windows).

The lungs underwent an equilibration perfusion for 20 min to establish an isogravimetric preparation. Then buffer was replaced with fresh buffer and for each lung; we established the control (background) rate of NO production for 50 min by measuring nitrite accumulated in the perfusate every 10 min. The perfusate buffer was then replaced with fresh buffer containing either 200 μM Arg or Fa-A-R substrate with or without either 20 μM of the carboxypeptidase inhibitor MGTA or 10 mM Lys, an inhibitor of Arg transport. Aliquots were removed at various times and assayed for nitrite with the Griess reagent as described in *Measurement of NO production* and also tested for the hydrolysis of Fa-A-R by HPLC analysis. Lung weights remained constant during the control and treatment periods.

**HPLC analysis.** Aliquots of perfusate from lungs were treated with ice-cold 10% TFA and centrifuged at 10,000 g to precipitate proteins, and the supernatant was used for HPLC analysis. We analyzed the hydrolysis of Fa-A-R with a μBondapak C18 reverse-phase column and a gradient of acetonitrile in water increasing from 5 to 30% in 20 min. Each phase contained 0.05% TFA. Peptides were detected at 214 nm, and the peaks were identified and quantitated by comparison to known amounts of authentic standards. For cell culture studies, confluent monolayers of RLMVEC in 24-well plates were incubated in Arg-free DMEM for 3 h before the experiment. Fa-A-R (1 mM) was then added and incubated for 1 h at 37°C. In some experiments, we incubated cells with the carboxypeptidase inhibitor MGTA for 30 min at 37°C before adding the substrate. Medium was collected, added to an equal volume of 10% TFA (ratio 1:1), and then centri...
Carboxypeptidase activity assays. Membrane fractions from rat lungs or RLMVEC were prepared essentially as described (45). Briefly, rat lungs perfused free of blood were homogenized with a Polytron, and RLMVEC were homogenized by sonication. Homogenates were fractionated at 4°C by sequential centrifugation at 1,000 g for 15 min, 10,000 g for 25 min, and 100,000 g for 60 min. The final membrane pellet (P3) was resuspended and assayed for CPM enzyme activity with dansyl-Ala-Arg substrate (45, 66). Samples were incubated at 37°C for 1–2 h with 200 μM dansyl-Ala-Arg in a final volume of 250 μl in 0.1 M HEPES buffer at pH 7.5 containing 0.2% Triton X-100. The assay was done at a slightly higher pH than the optimum for CPM (which is 7.0) to minimize any crossover activity of CPD in the assay. CPD is only 20–25% active at pH 7.5 (12, 40).

Western blotting. RLMVEC were lysed in 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% Na-deoxycholate, 0.02% Na azide, 1 mM PMSF, 0.5 μg/ml leupeptin, and 5 μg/ml aprotinin. Rat lung tissue was homogenized in PBS containing 1 mM PMSF, 0.5 μg/ml leupeptin, and 5 μg/ml aprotinin with a Polytron homogenizer. Cell lysates or lung homogenates were centrifuged at 14,000 g for 15 min at 4°C, the supernatant was collected, and protein was measured with a Micro BCA protein assay reagent kit (Pierce). Aliquots containing 50 μg of protein were separated on 8% SDS-polyacrylamide gels, and proteins were electrophoretically transferred to a nitrocellulose membrane. The endothelial NOS (eNOS) or iNOS bands were detected by use of mouse monoclonal anti-eNOS or anti-iNOS primary antibodies (Transduction Laboratories; 1:2,000 dilution) followed by goat anti-mouse IgG secondary antibody coupled to horseradish peroxidase (1:10,000 dilution; Santa Cruz Biotechnology) and chemiluminescence with a Pierce ECL kit. To control for protein loading, the blots were also probed for GAPDH using mouse monoclonal anti-GAPDH primary antibody (Ambion).

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed using Student’s t-test or one-way analysis of variance and the Tukey post hoc test for multiple comparisons where appropriate. Values of P < 0.05 were considered significant.

RESULTS

Carboxypeptidase substrate stimulates NO production in perfused lungs. Rats were pretreated with LPS to induce iNOS expression as previously described (34, 35, 71). Lungs were isolated and perfused with 5% Krebs solution for a 20-min stabilization period to ensure constant wet weight and arterial pressure. To show that iNOS was induced by LPS treatment, the solution was replaced with the same buffer containing 5 mM Arg, and nitrite accumulation was measured for 60 min. As shown in Fig. 1, Arg stimulated a large increase in NO production in the LPS-treated rat lung, whereas nitrite was barely detectable at 60 min in the control rat lung.

To confirm that LPS treatment upregulated iNOS expression, Western blot analysis was carried out for eNOS and iNOS in rat lung homogenates. As shown in Fig. 2, iNOS expression was clearly induced in the lungs of rats pretreated with LPS and was apparently absent in lungs from control or sham injected rats. However, faint iNOS bands were detectable in the control and sham-injected lungs when the blots were overexposed by the sensitive chemiluminescence technique (not shown). In contrast, eNOS expression was not substantially altered by LPS treatment (Fig. 2, the slightly darker band in the control lane is due to unequal protein loading as shown by the GAPDH band).

To determine the effects of carboxypeptidase substrate and inhibitor on NO production, we equilibrated rat lungs for 20 min as above and replaced the buffer with fresh 5% Krebs solution. Aliquots were removed at 10-min intervals for 50 min and assayed for nitrite to establish a control basal rate of NO production for each lung. Buffer was then replaced again with fresh buffer containing various agents, and the rate of NO production was measured for an additional 50 min. As shown in Fig. 3, there was constitutive production of NO in the absence of substrate in the perfusion buffer, indicating a basal level of Arg release from tissues or proteins in LPS-treated lungs. The basal rate of NO production for a given lung was set at 100% for comparison with the effect of added agents. When lungs from rats treated with LPS were perfused with buffer containing the specific carboxypeptidase substrate Fa-A-R, the rate of NO production increased to 220% of the control level (Figs. 3 and 4). This increase in NO production was almost completely inhibited when Fa-A-R was perfused along with the carboxypeptidase inhibitor MGTA (Fig. 4). This indicates that MGTA inhibited the increased NO production by inhibiting the carboxypeptidase-mediated release of Arg from the substrate. To confirm that the effect of MGTA was due to inhibition of carboxypeptidase activity and not to inhibition of NOS or Arg transport, we perfused MGTA along with added free Arg. Under these conditions, NO production was stimulated threefold, indicating MGTA did not block NOS or Arg transport (Fig. 4). Lys, a cationic amino acid that competitively inhibits Arg transport into cells, blocked the increased NO production seen with the carboxypeptidase substrate Fa-A-R (Fig. 4). This indicates that the substrate Fa-A-R was hydrolyzed extracellularly and the released Arg was transported into the cells and used for NO production.

Fa-A-R hydrolysis. To confirm that the substrate Fa-A-R was cleaved to release free Arg, we carried out HPLC analysis on perfusate samples. At time 0, the substrate was intact and
eluted as a single peak (Fig. 5). After 20 min of perfusion through the rat lungs, most of the substrate was hydrolyzed, and the product Fa-A appeared (Fig. 5). The smaller size of the Fa-A peak than the apparent reduction in size of Fa-A-R could be due to the lower inherent absorbance of Fa-A than Fa-A-R, which is the basis of the use of the substrate in a continuous spectrophotometric assay of carboxypeptidase activity (50). HPLC analysis of standard concentrations of Fa-A and Fa-A-R under the same conditions showed that the absorbance of Fa-A-R is 1.5-fold greater than Fa-A (not shown). This does not fully account for the smaller size of the Fa-A peak, and it indicates that there is uptake or further degradation of Fa-A by the lung. When the carboxypeptidase inhibitor MGTA was perfused along with Fa-A-R for 20 min, MGTA substantially inhibited the hydrolysis of the substrate (Fig. 5). However, when Fa-A-R was perfused along with Lys, it did not inhibit the hydrolysis of the substrate, showing that its ability to block NO production was due to inhibition of Arg transport and not carboxypeptidase activity. Quantitation of the results by integration of peak areas from the HPLC analysis showed that 69% of the Fa-A-R was hydrolyzed after 20 min, and this was reduced to 31% in the presence of the carboxypeptidase inhibitor MGTA, whereas 61% of the Fa-A-R was hydrolyzed in the presence of 10 mM Lys (average results from two different perfusions).

Rat lung carboxypeptidase activity. We previously detected high CPM activity in membrane fractions of lungs from several species, including the rat (45). To determine whether LPS treatment altered rat lung carboxypeptidase activity, we measured dansyl-Ala-Arg hydrolysis at pH 7.5 in membrane fractions of lungs from control rats or rats treated with LPS for 6 h. As shown in Fig. 6, rat lung membrane carboxypeptidase activity was high in control lungs and decreased slightly in lungs from rats treated with LPS, although the difference was not statistically significant ($P = 0.075, n = 3$).

Carboxypeptidase activity in RLMVEC. It has been well established that, because of its large surface area, the lung microvasculature is a major site for metabolism of circulating peptides by endothelial peptidases, for example angiotensin I and bradykinin hydrolysis by angiotensin-converting enzyme (58). We have also previously shown that human endothelial cells contain membrane-bound B-type CPM (45, 52, 61) and...
that CPM levels can be upregulated by inflammatory stimuli (52). To determine whether RLMVEC contain CPM-like activity and whether it is altered under inflammatory conditions, control cells and cells treated with 100 ng/ml LPS and 100 U/ml IFN-γ were lysed, and high-speed membrane fractions were prepared by differential centrifugation and assayed for CPM activity at pH 7.5 with dansyl-Ala-Arg substrate. As shown in Fig. 7, membrane fractions from control RLMVEC did contain CPM-like activity, which increased about twofold after a 16- or 24-h treatment with LPS and IFN-γ.

Expression of eNOS and iNOS in RLMVEC. To determine whether LPS and IFN-γ treatment upregulated iNOS expression or altered eNOS expression, we carried out Western blot analysis for eNOS and iNOS in RLMVEC lysates. As shown in Fig. 2, iNOS expression was clearly induced in the RLMVEC treated with LPS and IFN-γ and was absent in control cells. In contrast, eNOS expression was not altered by LPS and IFN-γ treatment (Fig. 2C).

Stimulation of NO production by carboxypeptidase substrate in RLMVEC. To determine whether carboxypeptidase substrate could stimulate NO production by RLMVEC, we first stimulated cells with 100 ng/ml LPS and 100 U/ml IFN-γ for 24 h and measured nitrite accumulation in the medium, methods similar to those previously used to measure iNOS upregulation in RLMVEC (18). Cells treated with LPS and IFN-γ as described above were washed and incubated in fresh medium containing either 1 mM Arg or the carboxypeptidase substrate Fa-A-R, and nitrite accumulation was measured over 48 h with the Griess reagent. As shown in Fig. 8, NO production increased over fourfold in cells stimulated with LPS and IFN-γ in the presence of 1 mM Arg or Fa-A-R. The NO production in the presence of Fa-A-R but not Arg was substantially blocked by the specific carboxypeptidase inhibitor MGTA (Fig. 8). Co-incubation with 10 mM Lys to block the Arg transporter blocked NO production stimulated by either Fa-A-R or Arg.

These data indicate that carboxypeptidase substrate is as effective as Arg in supporting NO production in stimulated RLMVEC. However, as the method only measures the accu-
mulation of a breakdown product of NO over a prolonged period, it is not possible to determine the kinetics of these responses. For example this technique would not distinguish between a possible slow, steady release of Arg from the carboxypeptidase substrate that reaches a maximum at the end of the incubation period and a rapid NO production stimulated by free Arg that is completed in a much shorter time. To address this point, we measured the kinetics of production of NO in real time using a sensitive porphyrinic microelectrode. Cells were incubated without (control) or with LPS and 100 U/ml IFN-γ for 8–24 h. Cells were lysed, and membrane fractions were isolated and assayed for CP activity at pH 7.5 with dansyl-Ala-Arg substrate. Results are given as specific activity (nmol/min/mg protein) and are mean values ± SE for n = 3. *Significant increases (P < 0.05) in CP activity compared with control.

Fig. 7. Upregulation of membrane-bound CP activity in RLMVEC by inflammatory stimuli. RLMVEC were treated without (Control) or with 100 ng/ml LPS and 100 U/ml IFN-γ for 8–24 h. Cells were lysed, and membrane fractions were isolated and assayed for CP activity at pH 7.5 with dansyl-Ala-Arg substrate. Results are given as specific activity (nmol/min/mg protein) and are mean values ± SE for n = 3. *Significant increases (P < 0.05) in CP activity compared with control.

To determine which NOS isoform was responsible for the Fa-A-R stimulated NO production, we used inhibitors relatively specific for either iNOS (1400W) (17) or eNOS (l-NO-nitroarginine; l-NNA) (16) when used at low concentration. The NO production stimulated by 1 mM Fa-A-R in RLMVEC was substantially blocked by 2 μM 1400W (76 ± 14% inhibition; n = 3, P < 0.01) but not significantly inhibited by the eNOS inhibitor 2 μM l-NNA (13.6 ± 10.8% inhibition; n = 3, P = 0.53). These data show that iNOS is the primary NOS isoform responsible for NO production in response to Fa-A-R in RLMVEC treated with LPS + IFN-γ.

Although NO production was similar in the presence of 1 mM Arg or Fa-A-R, based on our measurements of carboxypeptidase activity in the cells, it seemed unlikely that all 1 mM of the carboxypeptidase substrate would be cleaved by the cultured cells during the course of the NO measurement. To determine the actual amount of Arg released by the cellular carboxypeptidase activity, we measured Fa-A-R hydrolysis by HPLC analysis. In intact RLMVEC stimulated with LPS + IFN-γ and treated identically to cells used for the NO measurements, Fa-A-R was hydrolyzed at a rate of 11.0 ± 2.7 μM/h (n = 3), meaning that a total of 3.7 μM Arg would be released over the 20-min period of NO measurement. These data indicate that the initial rate of NO production in RLMVEC is stimulated more efficiently by Arg released from carboxypeptidase substrates than free bulk Arg in the medium.

Fig. 8. Stimulation of NO production in RLMVEC by CP substrate. RLMVEC treated with IFN-γ and LPS as described in MATERIALS AND METHODS were washed twice with Arg-free DMEM, and then fresh Arg-free DMEM was added containing either 1 mM Arg or 1 mM CP substrate Fa-A-R with or without 20 μM CP inhibitor MGTA or 10 mM Lys. Cells were then incubated for 48 h at 37°C, and accumulated nitrite was measured in the medium using the colorimetric Griess reagent. Results shown are means values ± SE (n = 3). † or **Significant increase (P < 0.005) in NO production compared with the corresponding control; ‡significant decrease (P < 0.005) in NO production compared with that obtained with 1 mM Fa-A-R in stimulated cells; *significant decrease (P < 0.01) in NO production compared with that obtained with 1 mM Arg in stimulated cells.
Fig. 9. Kinetic measurement of NO production in real time stimulated by CP substrate or Arg in RLMVEC. RLMVEC pretreated with IFN-γ and LPS or with medium alone (Control) were washed twice with Arg-free DMEM and then were incubated an additional 3 h in fresh Arg-free DMEM before the experiment; 1 mM Arg or CP substrate Fa-A-R was added (shown by the arrows pointing down), and NO production was measured electrochemically with a porphyrinic electrode. A: typical real-time tracings of NO production. Vertical bar shows response to standard 300 nM concentration of NO. Length of the horizontal bar indicates 20 min. B: mean values from 3 separate experiments ± SE. The maximum concentration of NO achieved within 20 min after addition of substrate was used to quantitate the results. # or ‡ Significant increases (P < 0.001) in NO production in response to Fa-A-R or Arg, respectively, compared with the corresponding control. Brackets indicate other statistical comparisons: *P < 0.05; **P < 0.01.
DISCUSSION

These data show that a carboxypeptidase substrate, after cleavage by cellular carboxypeptidases, can provide sufficient Arg to stimulate the generation of NO in perfused lungs of rats pretreated with LPS. The response is inhibited by the carboxypeptidase inhibitor MGTA as well as by the cationic amino acid Lys, which is a competitive inhibitor of Arg transport. This indicates that the substrate is hydrolyzed extracellularly by membrane carboxypeptidases in the lung. In perfused rat lungs, the most likely site for hydrolysis of the carboxypeptidase substrate would be the microvascular endothelial cells, which provide a large surface area exposed to the perfusate. Because iNOS expression can be induced in endothelial cells under inflammatory conditions as shown here and reported previously (13, 18, 30), we studied cytokine-stimulated RLMVEC and found they were capable of significant and prolonged NO generation in response to carboxypeptidase substrate as seen in the lungs. However, there are two apparent differences between the responses in the endothelial cells vs. the whole lung. First, when tested for carboxypeptidase activity, rat lung membranes were found to have high constitutive levels of activity that did not increase further after LPS stimulation, whereas endothelial cell carboxypeptidase activity was increased upon cytokine stimulation. However, a whole lung membrane fraction would include contributions from the numerous other cell types in the lung where carboxypeptidase activity might have not changed or even declined, possibly masking an increase in endothelial cells. For example, CPM is highly concentrated in type I alveolar epithelial cells in the lung (45). In addition, the perfused lungs were from rats treated with LPS for only 5–6 h, whereas the increase in carboxypeptidase activity in cultured endothelial cells did not occur at 8 h and was only apparent at the 16- and 24-h time points. Second, whereas NO production increased dramatically in perfused lungs from LPS-treated rats, maximal NO production was not apparent until 16 h after treatment of RLMVEC in culture with LPS + IFN-γ. However, even though it was not maximal, NO production was elevated after only 8 h in RLMVEC. It is also unlikely that the culture conditions can accurately mimic the in vivo effects of LPS administration, which could lead to faster iNOS induction in endothelial cells in the whole animal. Thus it is possible that most of the NO production in response to carboxypeptidase substrate in perfused lungs is contributed by the microvascular endothelial cells. However, we cannot rule out the possible involvement of other cells such as macrophages. For example, we previously showed in a mouse macrophage cell line stimulated with IFN-γ and LPS that administration of a carboxypeptidase substrate stimulated NO production by sixfold, and this effect was blocked by a specific carboxypeptidase inhibitor (23). This response was due primarily to membrane CPD in the cells that was also upregulated during cytokine stimulation (23).

Studies of the initial kinetics of NO production in RLMVEC showed that a relatively low level of Arg release from the carboxypeptidase substrate (3.7 μM over the 20-min measurement) resulted in NO production equivalent to that in the presence of 1 mM extracellular Arg. These data indicate that the initial rate of NO production is more efficiently stimulated by Arg from carboxypeptidase substrates than free bulk Arg in the medium. One explanation for this phenomenon could be a close association between the membrane carboxypeptidase and arginine transporter, such that the Arg is made available to the transporter before it can diffuse freely into the medium. Proof of this possibility will require further investigation.

For carboxypeptidase cleavage of Arg-containing peptides to affect NO production, it would be necessary for NO synthesis to be dependent on Arg levels. This might seem unlikely as the normal Arg concentration in cells and plasma is far above the $K_m$ for the NOSSs (9, 29, 38, 75). However, addition of extracellular Arg increases NO production in a variety of different model systems (27, 42, 47). Conversely, several experimental models have shown a link between Arg depletion and reduced NO synthesis (69, 74). Because iNOS is thought to be primarily regulated at the transcriptional level, extracellular Arg supply is a much more important regulator for this isoform. Indeed, in the absence of extracellular Arg uptake by system $Y^+$ or depletion of extracellular arginine, iNOS activity is abolished (9, 64). In addition, inflammatory stimuli that upregulate iNOS also upregulate cationic amino acid transporters, which enhances cellular Arg uptake (19, 26, 54). Another way in which Arg levels can be regulated is by conversion of citrulline to Arg (24). A major enzyme involved in the conversion, argininosuccinate synthetase, is upregulated by LPS or IFN-γ in murine macrophages (48) and by cytokine treatment in many other cell types (44). In contrast, arginase is a negative regulator of NO production as it competes with NOS for Arg, converting it to L-ornithine and urea (44). Arginase levels are increased in inflammatory conditions, which can lead to Arg depletion (1, 9, 11, 20, 72). Our studies reveal an additional mechanism by which the Arg supply for NO production can be maintained via the cleavage of Arg-containing peptides by cell-surface carboxypeptidases.

In our studies, we used a synthetic substrate, Fa-A-R, as a specific B-type carboxypeptidase substrate. This peptide was chosen because it cannot be cleaved by endopeptidases or endopeptidases (it lacks an "internal" peptide bond), and it is resistant to aminopeptidases as well because it does not have a free NH$_2$-terminal amino group. Although serine carboxypeptidases such as prolylcarboxypeptidase or cathepsin A/deamidase could potentially cleave it, they cleave only substrates with penultimate proline or hydrophobic residues, respectively (59), and they are localized in lysosomes, meaning they would not have access to extracellular substrates. Further evidence of the specificity of the substrate is the finding that the specific B-type metallocarboxypeptidase inhibitor MGTA (51, 55, 66) can inhibit 98–99% of the hydrolysis of a similar substrate, dansyl-Ala-Arg, in crude membrane fractions of dog, human, baboon, or guinea pig lung (7, 45).

In vivo, other enzymes could be involved in the proteolytic cleavage of peptide and protein substrates. However, the release of free Arg from peptides and proteins would require the action of an exopeptidase, i.e., an aminopeptidase or carboxypeptidase. Because of their location and specificity to cleave only COOH-terminal Arg or Lys, membrane-bound B-type carboxypeptidases (such as CPM and CPD) are candidates to be involved in this process. It is likely in our studies that the primary enzyme involved in the release of Arg from carboxypeptidase substrate was CPM, based on the following considerations. First, immunohistochemical staining of human lung microvascular endothelial cells showed a distribution for CPD and CPM similar to that found in other cell types; CPM
is primarily on the plasma membrane, and CPD is highly concentrated in the Golgi (52). Although CPD can be detected on the plasma membrane, most studies indicate this represents a maximum of ~10% of the total CPD in the cell (23, 32, 68). Second, CPD has an acidic pH optimum of 6.2 and at pH 7.4 has only ~20–25% of its activity remaining with dansyl-Ala-Ala-Arg, whereas CPM is fully active at pH 7.4. As there are no specific inhibitors that distinguish between CPM and CPD, a definitive resolution to this question will require the development of knockout animals or RNA interference approaches.

Potential in vivo substrates containing COOH-terminal Arg are numerous (56, 57) and in many cases are likely to be increased under inflammatory conditions. These include bradykinin; anaphylatoxins C3α, C4a, and C5a; enkephalin hexapeptides (i.e., Arg6- or Lys6-, Met- or Leu-enkephalin); dynorphin A1–13; dynorphin A1–11; dynorphin A1–9; fibrinopeptides 6A, 6D, A, and B; atriopeptin II; cardiodilatin 1–67; albumin propeptide; insulin C-peptide; hemoglobin; erythropoietin; and a variety of growth factors including epidermal growth factor (39). In addition to these substrates, numerous proteolytic cascades, such as the fibrinolytic system, coagulation cascade, and the complement system, consist of a series of normally inactive proenzymes that are activated after injury or during inflammation by the cleavage of an Arg-X bond, resulting in the generation of a new protein chain with a COOH-terminal Arg (3). Thus activation at each step would produce a potential carboxypeptidase substrate, which could then be cleaved to produce free Arg.

The supply of substrate Arg for NOs is important not only to maintain the beneficial effects of NO but also to inhibit the production of superoxide. Recent studies have made it clear that NOs can become important generators of superoxide under conditions where either the substrate Arg or the cofactor tetrahydrobiopterin is depleted (65), as can occur during conditions such as sepsis (28). Superoxide and its metabolites are oxidants, which can be potent stimuli of NF-κB activation (14). For example, under low substrate conditions, iNOS can produce both superoxide and NO to generate peroxynitrite (ONOO−) (65). This potent oxidant not only can cause cellular damage and dysregulation of protein function by nitrating tyrosine residues (4, 21) but also stimulates cytokine production by human monocytes (37) or IL-8 gene expression and production by human leukocytes (76).

The potential roles of NO in sepsis and lung inflammation are numerous, but whether its net effect is beneficial or deleterious is still under debate (22, 73). This is highlighted by conflicting reports on the effects of iNOS inhibition or knock-out on overall survival in sepsis (22). Assigning a precise role to NO is difficult due to its dual nature. For example, NO plays an important role in the regulation of vascular permeability and acts as a signaling molecule to mediate some of the permeability effects of agents such as substance P, bradykinin, and VEGF (8, 31). In general, low amounts of NO are protective of the endothelial barrier, whereas high amounts or coproduction of superoxide to form ONOO− can be damaging.

In addition, NO can have proinflammatory effects of its own. For example, NO can stimulate the transcription and expression of macrophage inflammatory protein-2 (MIP-2) (70), a rodent C-X-C chemokine that is involved in the pathogenesis of acute lung injury (53). In an in vivo gram-negative bacte remia model, an NOS inhibitor blocked the expression of MIP-2 and blunted lung permeability increases induced by E. coli administration to mice, which was restored by cotreatment with an NO donor (60). On the other hand, NO can blunt the inflammatory response via its ability to inhibit NF-κB activation by increasing the expression, nuclear translocation, and stabilization of its inhibitory protein IκB (49, 63). NO can also have opposing biphasic effects on NF-κB activation that depend on both the level of NO produced and the time course of the response. For example, in a mouse monocyte/macrophage cell line NO activated NF-κB early after LPS administration or at lower levels of NO and inhibited its activation at later times or at higher doses of NO (10).

Excessive production of NO by iNOS has been implicated as an important contributor to many of the manifestations of septic shock such as hypotension, myocardial depression, direct tissue injury, and organ failure (46). However, because low levels of NO have numerous beneficial effects, complete inhibition of NO production might not be an effective therapeutic strategy, as borne out in many animal studies (15, 22).

During sepsis, activation of cascades such as the coagulation, fibrinolytic, and complement systems could lead to generation of significant quantities of carboxypeptidase substrates, and under these conditions, use of a carboxypeptidase inhibitor could prove to be beneficial. This approach would target NO synthesis by iNOS because this isoform is the most dependent on extracellular Arg.

ACKNOWLEDGMENTS

Current address for Sakonwun Sangsree: Dept. of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok, Thailand, 65000.

GRANTS

These studies were supported by National Institutes of Health Grants HL-60678 and DK-41431.

REFERENCES

10. Connelly L, Palacios-Calderon M, Ameica C, Moncada S, and Hobbs AJ. Biphasic regulation of NF-kappa B activity underlies the pro-


