Transport and Utilization of Arginine and Arginine-Containing Peptides by Rat Alveolar Macrophages

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Purpose. To demonstrate that rat alveolar macrophages (AM) exhibited the PepT1-like transporter for the uptake of arginine (Arg)-containing small peptides and utilized these peptides as direct substrates for nitric oxide (NO) production. NO is an important mediator that, on one hand, protects the lung from bacteria infection and, on the other hand, augments inflammatory lung injury.

Method. The uptake of small peptides by rat AM was evaluated using fluorescein isothiocyanate (FITC)-labeled (*) peptides (Arg-Lys*, Gly-Sar-Lys*, and β-Ala-Lys*), high-performance liquid chromatography (HPLC) analysis of potential peptide degradation, and known inhibitors of Arg and PepT1 transport. NO production by AM through Arg and Arg-containing peptides was studied with and without inhibition by transport inhibitors. The presence of PepT1-like transporter on AM was evaluated using anti-PepT1 antisera and Western blot analysis. The substrate specificity of Arg-Gly and Arg-Gly-Asp was determined using purified inducible NO synthase (iNOS). The availability of Arg-containing peptides in the lung was determined by HPLC analysis of bronchoalveolar lavage (BAL) fluid.

Results. The FITC-labeled peptides were internalized by AM without degradation. The uptake of Arg-Lys*, β-Ala-Lys*, and Gly-Sar-Lys* was blocked (~50%) by cephradine (an inhibitor of PepT1 for peptide transport) but not by Lys (an inhibitor on cationic amino acid transporter 2B for Arg transport). The NO production by AM through Arg-containing peptides was significantly blocked only by PepT1 inhibitors and by an anti-PepT1 antibody in a dose-dependent manner. These inhibitors had no effect on the AM production of NO using Arg as a substrate. Arg-Gly and Arg-Gly-Asp were found to be direct substrates for iNOS with similar K_m and V_{max} values to those of Arg. But, the production of NO by AM using these peptides as substrates was 2-fold higher than using Arg as a substrate. Both Arg-Gly and Arg-Gly-Asp were found in the BAL fluid. The presence of a PepT1-like transporter on AM was confirmed by Western blotting.

Conclusion. This study shows that AM exhibit PepT1-like transporter for small peptide uptake. Arginine-containing peptides, through the PepT1 transporter system, can serve as direct substrates of iNOS for the production of NO by AM.

KEY WORDS: alveolar macrophages; PepT1 transporter; arginine-containing peptides; substrates for iNOS; NO production

INTRODUCTION

Peptide transporters located on various epithelial cells play a pivotal role in the efficient absorption of protein digestion products including amino acids, dipeptides, and tripeptides. In fact, up to 78% of the amino acid content in plasma has been found to be in the form of dipeptides and tripeptides in experimental animals (1). The removal of such a large amount of small peptides from plasma across an organ could be the result of dipeptide hydrolysis in the plasma or of direct absorption by the organs. Lochs *et al.* (2) have shown, however, that the hydrolysis in plasma was not a major mechanism for the disappearance of dipeptides from circulation. This suggests that the direct utilization of these small peptides could occur in various tissues. Indeed, several studies have shown that direct utilization of small peptides occurs in various organ and tissues including the liver, kidney, brain, and placenta (3–5). The functions of small peptides have received increasing attention in recent studies. For example, Wang *et al.* (6) reported that either dimethionine- or trimethionine-containing peptides was more efficient than free methionine in the synthesis of secreted proteins in mammary tissue of lactating mice.

In addition to its vasodilatory effect, nitric oxide (NO) is an important signal transduction mediator in a variety of physiologic systems. In pulmonary host defense, NO is produced by alveolar macrophages (AMs) through inducible NO synthase (iNOS) activity to provide cytotoxic effects against invading bacteria (7) or to regulate cellular cytokine secretion and cyclooxygenase activity (8) in response to inflammatory stimulation. The induction of NO production from AMs, such as that by lipopolysaccharide (LPS), depends not only on the activity of the iNOS, but also on the availability of the substrate arginine (Arg). Recent studies have shown that Arg is taken up by AMs through the cationic amino acid transporter 2B (CAT-2B) (9). LPS, which stimulates the production of iNOS, also facilitates the AM uptake of Arg (10). However, the substrate for NO synthesis may be not restricted to Arg. Thiemermann *et al.* (11) have shown that Arg-containing dipeptides were, in fact, better fitted for the active site of NO synthase than Arg in endothelial cells. Furthermore, the presence of a peptide transport protein in pulmonary type II cells has been reported (12). Most recently, Groneberg *et al.* (13) further illustrated that peptide transporter(s) was indeed located on alveolar type II pneumocytes, the bronchial epithelium, and the small-vessel endothelium of mammalian lungs.

We hypothesize that AMs, the major cell type for pulmonary host defense, exhibit peptide transporters that may play a key role in lung peptide homeostasis. NO formed by iNOS has been implicated in a variety of inflammatory and autoimmune diseases. The mechanism(s) by which NO is produced is thus of great biomedical interest. To provide more insight into the small peptide transport and iNOS activity in the lung, the present study investigated the potential role of a pepT1-like transporter for the uptake of Arg-containing dipeptides and tripeptides by AMs and the cellular utilization of these small peptides as a direct substrate for NO synthesis. For comparison, the transport and utilization of Arg by AM in NO synthesis were concurrently studied.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (Hla: (SD)CVF) weighing 200–250 g (Hilltop Lab Animals, Scottsdale, Pennsylvania) were used as a source for AMs throughout this study. *Esch-*

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erichia coli LPS, Arg, Arg-Sar, Arg-Gly, Arg-Lys, Arg-Gly-Asp, cephradine, and cephalexin were obtained from Sigma Co (St. Louis, Missouri). Fluorescein isothiocyanate (FITC)-labeled lysine (Lys*) and lysine-containing peptides (i.e., β-Ala-Lys*, Arg-Lys*, and Gly-Sar-Lys*) were purchased from Genemed Synthesis, Inc. (San Francisco, California). To probe the presence of pepT1-like transporter on AMs, two peptide segments (peptide I and peptide II) corresponding to P457-471 (PGHRHTLLVWGPNLY) and P480-494 (QKPEKGENGIRFVST) of the extracellular domain of rat PepT1 (14) were synthesized, and their antirabbit antisera were developed commercially by Genemed Synthesis, Inc. Peptides I and II represent two sections of the extracellular domain that show the highest degree of amino acid sequence homology among rat, rabbit, and human PepT1. Peptide II exhibits relatively higher hydrophilicity than peptide I due to the presence of more charged amino acid residues (K, lysine; R, Arg; E, glutamate). Purified iNOS was obtained from CalbioChem (San Francisco, California). Rat PepT1 protein was kindly received from Dr. You-jun Fei (Department of Biochemistry, University of Georgia) as a gift. All other reagents were purchased from Sigma Co.

Isolation of AMs

Rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally; Butler, Columbus, Ohio) and were euthanized by exsanguination of the abdominal aorta. The trachea was cannulated, and the lungs were lavaged with a Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) medium (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, and 5.5 mM glucose; pH 7.4), at a volume of 6 ml for the first lavage and 8 ml for the subsequent lavages. The total lavage volume was 80 ml. The bronchoalveolar lavage (BAL) fluid was centrifuged at 500 g for 10 min at 4°C. The supernatant from the first lavage was saved separately for the determination of protein content, lactate dehydrogenase (LDH) activity, using an automated Cobas Fara II analyzer (Roche Diagnostic Systems, Montclair, New Jersey) with standard diagnostic reagents and following the manufacturer's procedures. Cell pellets from all samples of BAL fluid were combined and suspended in 1 ml of PBS to determine the total cell and differential cell counts using an electronic cell counter (Coulter Electronics, Hialeah, Florida) that was equipped with a cell-sizing unit (15). AMs were determined by their unique cell diameter and were used in primary cell culture experiments.

Analysis of Arg and Arg-Containing Peptides in Plasma and BAL Fluid

In certain experiments, a blood sample (~7 ml) was obtained from the abdominal aorta of a sacrificed rat and was kept in heparinized ice-cold tubes. Arg-Sar (1 mM) was added to the blood samples as an internal standard for subsequent high-performance liquid chromatography (HPLC) analysis, was centrifuged at 1500 g at 4°C for 10 min, and the plasma samples were collected. An aliquot of 2.5 ml of plasma for each sample was taken and mixed with an equal volume of 6% sulfosalicylic acid for deproteinization. Following centrifugation at 2000 g at 4°C for 30 min, the supernatant was loaded into a C18 cartridge (Vac 20, Waters Corporation, Milford, Massachusetts) and was eluted in succession with 30 ml of 0.1% trifluoroacetic acid in methanol/water solution (95:1; pH 2.4), 30 ml of 30% methanol (pH 2.4), and 10 ml of deionized water (pH 8.0). The third fraction was collected and lyophilized. The freeze-dried fraction was redissolved in 250 μl of water. Aliquots of 50-μl samples were stored at –80°C until time for analysis.

For the analysis of Arg and Arg-containing peptides in BAL fluid, rats were anesthetized and lavaged with 2 ml of the PBS, as described above. The recovered BAL fluid (~1 ml) for each rat was centrifuged at 500 g for 5 min. The supernatant for each sample was mixed with an equal volume of 6% sulfosalicylic acid. The treated fluid was centrifuged at 2000 g at 4°C for 20 min and was loaded into a C18 cartridge (Vac 6, Waters Corporation). The column was eluted in succession with 15 ml of water-saturated ethyl acetate (pH 2.4), 15 ml of 30% methanol in water (pH 2.4), and 3 ml of deionized water (pH 8.0). The last fraction was collected and lyophilized. The freeze-dried fraction was redissolved in 100 μl of water. Aliquots of 20-μl samples were stored at –80°C until time for analysis.

The concentrations of Arg and Arg-containing peptides in plasma and BAL fluid were determined using a high-performance liquid chromatographic (HLPC) system equipped with a 600E system controller, a WISP 701B autosampler, a 486 tunable absorbance detector, and a 746 data module (Waters Corporation). Chromatographic separation of Arg and the small peptides was achieved using a C18 reversed-phase column (150 × 4.6 mm, 3 μm; Thermo Electron Corp., Waltham, Massachusetts) and a mobile phase of 10% of CH_3CN in 0.1% trifluoroacetic acid in water (pH 2.4), delivered at a flow rate of 1 ml/min. The Arg compounds were detected by ultraviolet at 215 nm. The retention times for L-Arg, Arg-Gly, Arg-Gly-Asp, and Arg-Sar (internal standard) were 5.8, 7.5, 12.6, and 16.8 min, respectively.

The accuracy of the above-described method was validated by repeated intraday and interday measurements of samples containing known concentrations of Arg, Arg-Gly, and Arg-Gly-Asp. The results showed consistent recovery of these compounds following the sample purification procedure at greater than 80% from the blood and over 90% from the BAL fluid.

Cell Culture Studies

Determination of Cellular Uptake

The potential cytotoxic effect of various testing compounds was assessed by measuring the extracellular LDH activity in various AM incubation mixtures. LDH was monitored spectrophotometrically at 340 nm using an automated Cobas FARAII analyzer for the reduction of pyruvate coupled with the oxidation of nicotinamide adenine dinucleotide phosphate. Only viable cell preparations were used in subsequent experiments. To determine the cellular uptake of amino acids and peptides, AMs were seeded at 10^6 cells/well in 24-well culture plates in Earle's balanced salt medium. Following an adherence period, the nonadherent cells were removed by rinsing the cellular monolayers three times with the same medium. The adhered cell cultures were incubated with 5 μM FITC, Lys*, β-Ala-Lys*, Arg-Lys*, or Gly-Sar-Lys* with and without 100 μM lysine or cephradine, with a final

volume of 1 ml, in Arg-free Earle's salt solution for 2 h. Lysine and cephradine were used as inhibitors for CAT-2B and PepT1 transport, respectively. At the end of incubation, the uptake experiment was terminated by replacing the supernatant with ice-cold Earle's salt solution. The cells were washed four times with ice-cold Earle's salt solution and sonicated (MSE sonicator, Fisher, Pittsburgh, Pennsylvania) for 15 min. After centrifugation for 5 min, the supernatant for each sample was collected and measured for relative fluorescence intensity at an excitation of 494 nm and an emission of 519 nm.

Determination of NO Production

AMs were seeded at 1×10^6 cells/ml/well in 24-well culture plates containing Arg-free Earle's salt solution with 2 mM glutamine and 5% fetal bovine serum with/without 1 μg/ml LPS. Two hundred micromolar Arg or Arg-containing peptides (i.e., Arg-Gly and Arg-Gly-Asp) were then added and incubated at 37°C for 24 h. The AM-conditioned supernatants were collected and measured for NO production according to the Griess assay (16). The inhibitory effects of various inhibitors including lysine, cephradine, and the rabbit antirat PepT1 antisera on NO production also were determined by adding varying concentrations of an inhibitor to an LPS-stimulated AM culture using the same procedure.

Western Blot Analysis of Peptide Transporter on AM

Sample Preparation

AMs were seeded and cultured with or without LPS (1 μg/ml) in Earle's salt culture medium for 24 h. Cells were collected and washed with ice-cold PBS buffer. Following centrifugation, AMs were suspended in a homogenizing buffer (20% glycerol, 0.1 M Tris·HCl, and 10 mM EDTA, pH 7.4) containing various protease inhibitors (1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 100 μg/ml aprotinin). The cell suspension was homogenized with a glass tissue grinder and was sonicated for 10 s on ice. The homogenates were centrifuged at 5000 g for 10 min, and the pellet (the nuclear fraction) was discarded. The supernatant was centrifuged at 50,000 g for 2 h, and the resulting pellet (cell membrane fraction) was resuspended in the homogenizing buffer. The protein concentration of the membrane fraction was determined, and 50-μl aliquots of the membrane preparations were stored at –80°C.

Western Blot

The cell membrane protein (80 μg) was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a Novex precast gradient gel (4–12%; Invitrogen, Carlsbad, CA). The proteins were transferred electrophoretically to a nitrocellulose membrane. Immunoblotting was conducted with a 1-h incubation with polyclonal rabbit antibodies that were specific to rat PepT1. After washing, the blot was incubated with secondary antibody labeled with horseradish peroxidase-conjugated goat antirabbit immunoglobulin G (Pierce, Rockford, Illinois) and was detected by enhanced chemiluminescence. The signal densities of the protein bands were measured using a Fluochem 8000 densitometer (Alpha Innotech Corp., Alexandria, Virginia).

Enzyme Kinetics Studies

The substrate specificity of purified iNOS for Arg and Arg-containing peptides was tested using a modified method of Stuehr *et al.* (17). In brief, 0.1 mg (2 units) of iNOS was incubated with 200 μM Arg or Arg-containing peptides (i.e., Arg-Gly and Arg-Gly-Asp), 4 μM (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride, 4 μM flavin adenine dinucleotide (FAD), 3 mM dithiothreitol, 2 mM nicotinamide adenine dinucleotide phosphate, and 40 mM Tris HCl buffer (pH 7.9) for various time periods. The reaction mixtures were measured for NO production using the Griess assay (16). Incubation of Arg-containing peptides with the reaction mixture in the absence of iNOS also was carried out. The solutions were analyzed for potential hydrolysis of the peptides by HPLC.

For the kinetics studies, Arg, Arg-Gly, and Arg-Gly-Asp were incubated with iNOS (0.1 mg) at varying substrate concentrations (15, 20, 30, 60, 120, 180, or 240 μM). The values of K_m and V_{max} were determined. The inhibition of iNOS activity was determined using N^G-monomethyl-L-Arg (L-NMMA) as a specific inhibitor at concentrations of 1, 4, 8, 12, 16, 20, 24, and 28 μM. The 50% inhibitory concentration (IC_{50}) was calculated using the Winnonlin Nonlinear Estimation Program (version 03.0A, Pharsight Corporation, Mountain View, California).

Statistical Analysis

All data are expressed as the mean ± SD of at least six separate experiments, with measurements in duplicate or triplicate in each experiment. Statistical analysis was conducted using a one-way analysis of variance with a Tukey multiple comparison procedure. The significance level is set at $P < 0.05$.

RESULTS AND DISCUSSION

Uptake of Fluorescence-Labeled Small Peptides by AM

To illustrate that small peptides may be directly utilized by the lung, the uptake of lysine and small peptides by AMs was studied using FITC-labeled compounds. Figure 1 shows that FITC itself is not internalized by AMs, but that both Lys* and the FITC-labeled peptides (β-Ala-Lys*, Arg-Lys*, and Gly-Sar-Lys*) are readily taken up by AMs. The internalization of the FITC-labeled compounds also was observed through fluorescence light microscopy, which showed evenly distributed fluorescence within the cells (data not shown). In comparison, AMs treated with FITC itself did not show fluo-

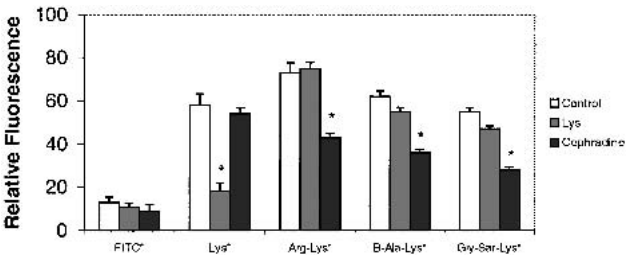

Fig. 1. Transporter-mediated uptake of 5 μM FITC-labeled lysine (Lys*) and small peptides (Arg-Lys*, β-Ala-Lys*, and Gly-Sar-Lys*) by AMs (10^6 cells). Lys (1 mM) and cephradine (1 mM) were used as inhibitors for CAT-2B and PepT1 transporters, respectively. *, significant difference from control at $P < 0.05$ (n = 6).

rescence. Gly-Sar-Lys*, which contains the sarcosine residue, is not subjected to enzymatic hydrolysis to yield the amino acids. In addition, we have analyzed the peptides in the AM culture media by HPLC and found no peptide degradation (data not shown), suggesting that the small peptides were internalized as intact molecules. The uptake of lysine* was significantly block by nonlabeled lysine, a known competitive inhibitor for CAT-2B transporter, but not by cephradine, which has been shown to block PepT1-mediated peptide transport (5). On the other hand, the uptake of Arg-Lys*, β-Ala-Lys*, and Gly-Sar-Lys* was not affected by the addition of nonlabeled lysine but was significantly inhibited by the presence of cephradine. These results indicate that lysine and the small peptides were internalized through distinct transport processes (i.e., small peptides were transported through a membrane transporter that is different from the transporter responsible for lysine uptake).

Utilization of Arg-Containing Peptides by AM for NO Production

The production of NO by nonstimulated and LPS-stimulated AMs in the presence of 200 μM Arg or Arg-containing dipeptides and tripeptide is shown in Table 1. Although both Arg and the small peptides were utilized by AMs to produce NO, the production of nitrite through the peptides in LPS-primed AMs was consistently higher (~2-fold) than that of the Arg system.

Figure 2 shows the effect of various inhibitors of membrane transport on NO production in LPS-stimulated AM. Lysophosphatidylcholine (LPC) has been identified as a potent inhibitor of CAT-2B-mediated amino acid transport. Our results showed that LPC at 5 μM significantly inhibited NO production through Arg but had little or no effect on the AM production of NO through Arg-containing peptides. In addition, lysine (1 mM) markedly inhibited NO generation through the Arg system but failed to block NO production that had been generated through Arg-Gly or Arg-Gly-Asp. Interestingly, lysine, which did not block the uptake of Arg-Lys by AMs, inhibited AM production of NO through the use of Arg-Lys as a substrate. This was not expected. It is possible that because both the Arg and lysine moieties are cationic, this small peptide can be transported by both the amino acid and peptide transporters, or that the inhibition of NO production by lysine occurs at the enzyme level after the uptake process. Figure 2 further shows that the PepT1 inhibitors cephalexin and cephradine selectively inhibited NO production through the Arg-containing peptides but had no significant effect on the Arg system.

The above results clearly show that LPS-induced iNOS activity in AMs for NO synthesis is largely dependent upon the transport or uptake of the substrate(s). Although Arg is transported through CAT-2B, the Arg-containing peptides are internalized through a peptide transporter. This finding is different from that of an earlier study on chicken macrophages, which suggested that Arg-containing dipeptides were hydrolyzed to produce Arg before cellular uptake (18). The fact that these peptides resulted in increased AM production of NO (2-fold more than that produced through Arg) suggests that these small peptides are more efficiently taken up by the cells and/or they may serve as direct and better substrates for iNOS.

Table I. Production of NO by Nonstimulated and LPS-Stimulated AMs in the Presence of Arg and Arg-Containing Peptides[a]

Substrate (200 μM)	Nitrite (μM)	
	Without LPS stimulation	With LPS stimulation
AM only (10^6 cells)	1.4 ± 0.2	2.5 ± 0.3
Arg	2.1 ± 0.4	21.5 ± 3.5
Arg-Lys	7.2 ± 1.3	39.2 ± 5.1[b]
Arg-Gly	5.1 ± 0.3	41.9 ± 3.6[b]
Arg-Gly-Asp	3.7 ± 1.0	38.4 ± 2.1[b]

[a] Values given as mean ± SD of six separate experiments.
[b] Indicates significant difference from using arginine as the substrate.

Identification of PepT1-Like Transporter on AMs

A number of transporters, including PepT1 and PepT2, have been identified for dipeptide and tripeptide transport (5,19). It is reasonable to suggest that in various organ systems in which the transport or absorption of small peptides is necessary there are similar peptide transporters. In the lung, it has already been reported that a peptide transporter (or transporters) is present in type II cells, the bronchial epithelium, and the small vessel endothelium (13). We hypothesize that a PepT1-like transporter is responsible for the uptake of Arg-containing dipeptides and tripeptides in AMs. For this reason, two peptide segments of the extracellular domain of rat PepT1 were chosen for antibody production. These peptide segments represent regions of the extracellular domain that have the highest degree of amino acid sequence homology among rat, rabbit, and human PepT1 proteins, which makes it possible that these regions may be involved in the binding of small peptides. Figure 3 shows the effects of anti-PepT1 antiserum I and anti-PepT1 antiserum II on NO production by LPS-stimulated AMs using Arg or Arg-containing peptides as a substrate. These antisera were commercially developed, and both react with the PepT1 protein. Antiserum I had no effect on NO synthesis in any of the systems. But the production of NO using the Arg-containing peptides as a substrate was significantly blocked by antiserum II. Neither antibody affected the NO production using Arg as the substrate. The inhibitory effect of anti-PepT1 antiserum II on AM uti-

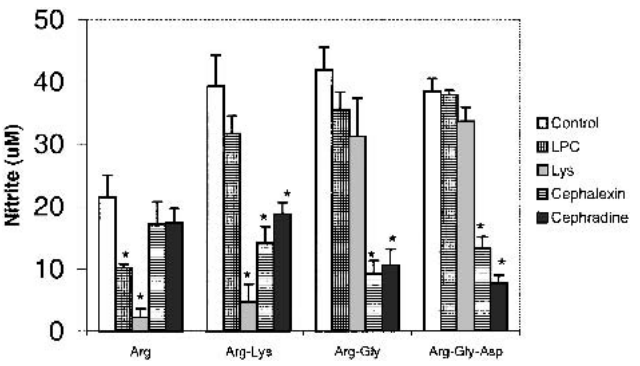

Fig. 2. Effects of transporter inhibitors on the production of NO by LPS-stimulated AM using Arg or Arg-containing peptides as the substrate. Concentration of inhibitors: Lys, 1 mM; cephradine or cephalexin, 1 mM; LPC, 5 μM. *, significant difference from control at $P < 0.05$ (n = 6).

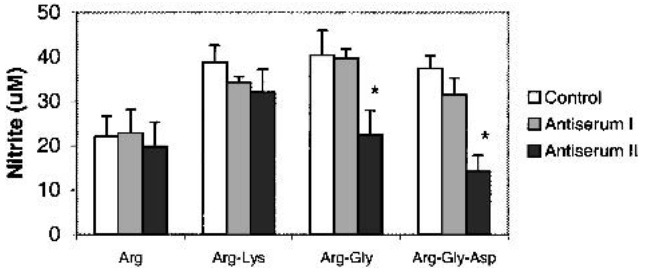

Fig. 3. Effects of anti-PepT1 antiserum I (1:10) and anti-PepT1 antiserum II (1:10) on AM production of NO using Arg or Arg-containing peptides as substrates. *, significant difference from the control at $P < 0.05$ (n = 6).

lization of Arg-containing peptides for NO production was dose-dependent (Fig. 4). In comparison, antiserum I in the same concentration range did not inhibit the production of NO by AMs (data not shown). It is interesting to point out that the inhibitory effect of antiserum II on Arg-lys is considerably weaker than its inhibition on other peptides. This again suggests a possibility that the uptake of Arg-Lys by AMs may involve other transporters such as the cationic amino acid transporters.

Antiserum II is derived from the peptide segment of PepT1 that contains more charged amino acid residues than peptide I, the corresponding peptide segment for antiserum I. This makes peptide II a more likely segment to be involved in the proton-coupled small peptide transport process. The fact that antiserum II inhibits AM utilization of the Arg-containing peptides indicates that AMs indeed exhibit a PepT1-like transporter and that antiserum II was able to bind with and inactivate the extracellular binding site of the transporter that is crucial to the peptide transport process.

Figure 5 shows the Western blot analysis of the presence of a PepT1-like transporter protein in the cell membrane of AMs. A 120-kD band was detected in the membrane fraction of both LPS-activated and nonactivated AMs using antiserum II, which corresponds to the PepT1 standard protein. Although the antiserum I is reactive to rat PepT1, the 120-kD protein band was not observed in antiserum I-treated membrane fractions, suggesting that the corresponding peptide I segment is probably not present in, or specific to, the peptide transporter in AMs. Interestingly, when the amino acid sequences of peptide I (P457-471) and peptide II (P480-494) from rat PepT1 are compared to those of rat PepT2, there is

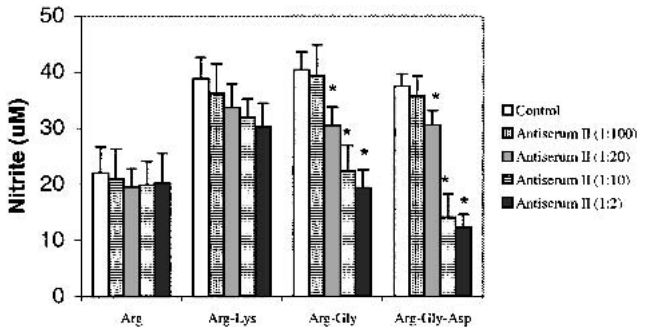

Fig. 4. The dose-dependent effects of anti-PepT1 antiserum II on NO production by LPS-stimulated AMs using Arg or Arg-containing peptides as the substrate. The antiserum dilutions were 1:100, 1:20, 1:10, and 1:2.

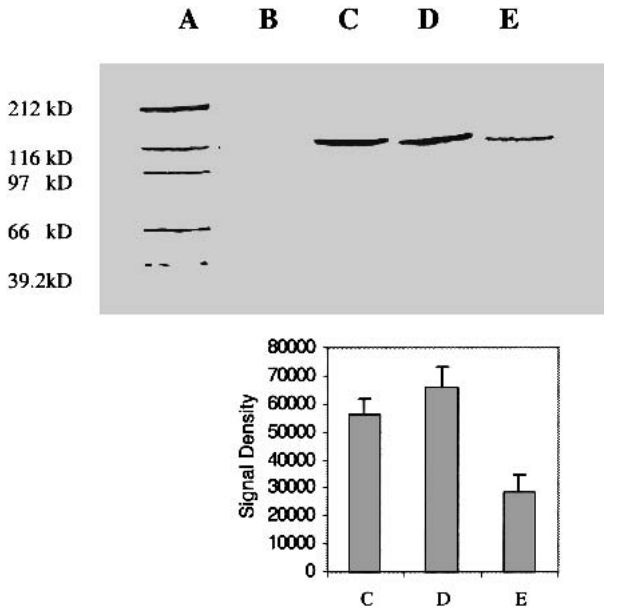

Fig. 5. Western blot analysis of AM membrane proteins using anti-PepT1 antiserum II. Keys: A, molecular weight marker; B, preimmune serum; C, PepT1 standard protein; D, LPS-stimulated AMs; and E, nonstimulated AMs. The signal densities of corresponding bands were measured and compared (n = 3).

only 13% homology for peptide I, but 50% homology for peptide II. This supports our results showing that peptide II is involved in the process of small peptide transport. The density of the protein band from LPS-activated AMs was significantly higher (by 130%) than that of the nonactivated AMs, suggesting that LPS, which is known to induce iNOS, also enhances the uptake of small peptides by AMs through the PepT1-like transporter. These results further confirm the presence of a PepT1-like transporter in AMs and suggest a role for AMs in regulating lung peptide homeostasis.

Substrate Specificity Studies

Arg, Arg-Gly, and Arg-Gly-Asp showed dose- and time-dependent generation of NO when incubated with purified iNOS. The NO production through these compounds was blocked by L-NMMA through competitive inhibition in a dose-dependent manner. HPLC analysis of the reaction mixture in the absence of iNOS showed no degradation of the Arg-containing peptides (data not shown). These results indicate that Arg-Gly and Arg-Gly-Asp are direct substrates for iNOS and share a common binding site with Arg and L-NMMA. Figure 6 shows the Lineweaver-Burk plots for Arg, Arg-Gly, and Arg-Gly-Asp, and the corresponding K_m and V_{max} values are given in Table 2. The measured K_m for Arg is 59.3 μM, which is slightly higher than the value (16 μM) reported in the literature (20). In comparison to Arg, the Arg-containing peptides shared similar values of K_m and V_{max}. Furthermore, the enzyme-substrate reaction for all compounds was inhibited by L-NMMA with a similar IC_{50} value of about 14 μM. These results show that Arg and the Arg-containing dipeptides and tripeptides are equally effective as the substrate for iNOS. It should be mentioned that Arg-Sar, which contains the N-monomethylglycine moiety, was not a substrate for iNOS.

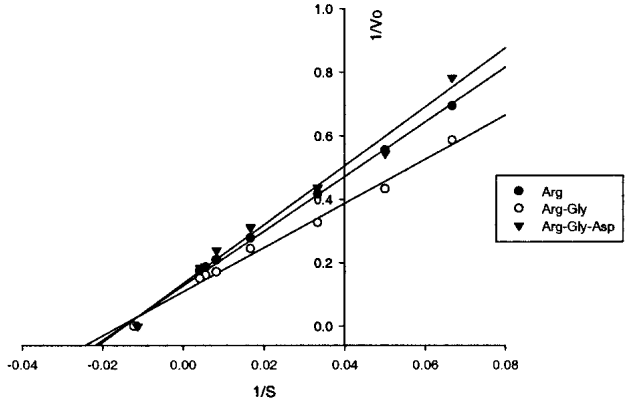

Fig. 6. The Lineweaver-Burk plots for iNOs activity using Arg, Arg-Gly, or Arg-Gly-Asp as the substrate (n = 5). The enzymatic reactions were carried out by incubating 0.1 mg iNOs with Arg or Arg-containing peptides at concentrations of 15, 20, 30, 60, 120, 180, and 240 μM for 4 min. The initial rates of NO synthesis were measured spectrophotometrically using the Griess assay.

Previously, we have shown that Arg-containing peptides give a 2-fold increase in NO production by LPS-stimulated AMs when compared to Arg. The fact that these compounds are equal in substrate specificity for iNOS indicates that the membrane transport system for the cellular uptake of the substrates plays an important role in the AM production of NO. This study suggests that the uptake of small peptides by AMs is in fact more efficient than their uptake of Arg. One possibility for this occurrence is that the CAT-2B transporter, which transports cationic amino acids, may be blocked by biologic compounds such as other amino acids or LPC, whereas the PepT1-like transporter is more selective in transporting peptide molecules.

Measurement of Arg and Arg-Containing Peptides in Plasma and Lungs

To provide more insight into the possibility that Arg-containing peptides play a role in the AM production of NO, we have measured the concentrations of Arg, Arg-Gly, and Arg-Gly-Asp in plasma and BAL fluid using HPLC. The results are given in Table 3. In rat plasma, the concentration of Arg was found to be 81.5 μM. A value of 124 μM has been reported in the literature (21). In the rat lung, the recovered BAL fluid contains 82.6 μM Arg, a value close to that of the plasma. The concentrations of small Arg-containing peptides in rat plasma or in rat lungs have not been reported. Our studies show that these peptides are in fact present in significant levels in plasma and BAL fluid. The concentration ratio of Arg to the combined total of the peptides is about 3:1 in the plasma and 4.3:1 in the BAL fluid. These values are significant, particularly in light of the fact that other small Arg-containing peptides also may be present, and that these Arg-containing peptides result in higher production of NO than Arg at an equal molar concentration. It is likely that a significant portion of the NO production by AMs in response to pulmonary inflammatory stimulation results from the enhanced uptake of Arg-containing peptides through the PepT1-like transporter.

Table II. K_m, V_{max}, and IC_{50} Values for the Enzyme Kinectics of iNOs Using Arg, Arg-Gly, and Arg-Gly-Asp as Substrates and L-NMMA as a Competitive NO Inhibitor[a]

Substrate	K_m(μM)	V_{max}(μmol/min/mg protein)	IC_{50} (μM)
Arg	59.3 ± 2.1	6.6 ± 0.7	14.2 ± 1.1
Arg-Gly	56.0 ± 1.8	8.4 ± 1.2	12.8 ± 0.8
Arg-Gly-Asp	60.1 ± 2.2	6.8 ± 1.3	14.0 ± 0.6

[a] Value given as mean ± SD of six separate experiments.

Table III. Concentrations of Arg and Arg-Containing Peptides In Rat Plasma and BAL Fluid[a]

	Concentration (μM)	
Substrate	Blood	BAL fluid
Arg	81.5 ± 3.28	82.61 ± 4.16
Arg-Gly	19.7 ± 2.41	11.37 ± 2.46
Arg-Gly-Asp	8.8 ± 1.03	7.62 ± 1.61

[a] Values given as mean ± SD (n = 6).

CONCLUSION

The present study demonstrates that Arg-containing dipeptides and tripeptides are direct substrates to the iNOS. Arg-containing peptides such as Arg-Gly and Arg-Gly-Asp, which are present in significant concentrations in plasma and the lungs, can be readily internalized through a peptide transport process and utilized for NO synthesis by a variety of cell types. This notion is in agreement with several reports that many dipeptides and tripeptides in plasma are directly absorbed by various organs without hydrolytic degradation (2,5). In the pulmonary system, where, on one hand, NO production protects the lung from bacteria infection (7,22) and, on the other hand, exacerbates inflammatory lung injury, AMs are shown to exhibit PepT1-like transporters and to produce high levels of NO by directly using Arg-containing peptides as substrates. This study suggests a plausible mechanism through which a significant portion of the NO production by AMs may be generated.

The transport and utilization of Arg-containing peptides by AMs may have a broader implication for the regulatory role of peptide transporters in various biologic systems. Studies have shown that renal-impaired patients may develop hypertension due to reduced production of NO by endothelial cells. The reason for the reduction of NO synthesis has been attributed to a blockage of Arg transport through the CAT-2B transporter by compounds such as LPC, a natural CAT-2B inhibitor (9,23). Our studies showed that LPC indeed blocked AM production of NO through Arg uptake but had no effect on the cellular uptake and utilization of the Arg-containing peptides. This suggests that Arg-containing peptides, which are internalized through a peptide transporter, may be considered as an alternative source of substrates for NO synthesis.

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