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

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Dissertation submitted to the
School of Pharmacy at West Virginia University
in partial fulfillment of the requirements for the degree
of

Doctor of Philosophy
in
Pharmaceutical Science

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ABSTRACT

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

Xiaodong Yang

Purpose. To demonstrate that alveolar macrophages (AM) from rats exhibit pepT1-like transporter for the uptake of small arginine-containing peptides (ACPs) and utilized these peptides as direct substrates for nitric oxide (NO) production.

Method. A HPLC assay was developed for quantitative measurement of Arg and ACPs in rat plasma and bronchoalveolar lavage (BAL) fluid. The uptake of small peptides by rat AM was evaluated using fluorescein isothiocynate (FITC)-labeled (*) peptides (Arg-Lys*, β-Ala-Lys*, and Gly-Sar-Lys*), HPLC analysis of potential peptide degradation, and known inhibitors on arginine (Arg) and PepT1 transport. NO production by AM through Arg and ACPs was studied with and without inhibition by transport inhibitors. The presence of PepT1-like transporter on AM was evaluated using antipepT1 antisera and Western blot analysis. The substrate specificity of Arg-Gly and Arg-Gly-Asp was determined using purified inducible nitric oxide synthase (iNOS). The availability of ACPs in the lung was determined by the HPLC analysis of plasma and (BAL) fluid.

Results. The FITC-labeled peptides were internalized by AM without degradation. Uptake of Arg-Lys*, β-Ala-Lys*, and Gly-Sar* was blocked (~50%) by cephradine, but not by Lys (an inhibitor on CAT-2B for arginine transport). The NO production by AM through ACPs was significantly blocked by PepT1 inhibitors and by an antiPepT1 antibody in a dose-dependent manner. These inhibitors had no effect on 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 Km and Vmax values to those of Arg. But the production of NO by AM using ACPs as substrate was 2-fold higher than using Arg as a substrate. Both Arg-Gly and Arg-Gly-Asp were found in rat plasma and BAL fluid. The presence of a PepT1-like transporter on AM was confirmed by Western blot.

Conclusion. This study shows that AM exhibits PepT1-like transporter for small peptide uptake. ACPs, through PepT1-like transporter, can serve as direct substrates for AM production of NO, an important mediator on both protection the lung from bacteria infection and augments inflammation lung injury.

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Chapter I

Statement of Question, Hypothesis, and Specific Aims of This Study

Peptide transporters play a pivotal role in efficient absorption of protein digestion products (mainly di- or tri-peptides) through plasma membranes in the small intestine. As a result of the transport of these small peptides, up to 78% of the amino acids in the plasma are in the form of di- or tri-peptide in experimental animals (Seal and Parker 1991). Removal of such large amounts of small peptides from the plasma into an organ could be the result of dipeptide hydrolysis in plasma or of actual utilization of peptides by the organ. Lochs et al. (1988) showed that the hydrolysis in plasma was not a major mechanism for the disappearance of dipeptides from circulation. This suggests that direct utilization of these peptides could occur in various tissues.

Indeed, direct utilization of small peptides by different tissues has been reported (Krzysik and Adibi, 1977; McCormick and Webb, 1982). Fei et al. (1994) reported that small peptides might be directly used by the liver, kidney, brain and placenta. More recently, Wang et al (1996), for example, reported that di- or tri-methionine-containing peptides were more efficiently utilized than free methionine in the synthesis of mammary tissue proteins secreted from lactating mice. The

utilization of small peptides in anabolic process in other tissues, including the lung remains unclear.

In pulmonary host defense, nitric oxide (NO) is produced in particularly large amounts by alveolar macrophages (AM) to provide cytostatic/cytotoxic effects against invading bacteria. Induction of NO production by AM and agents such as lipopolysaccharide (LPS), depends not only on the activity of the inducible nitric oxide synthase (iNOS), but also on the availability of the substrate arginine. Recent studies have shown that arginine is taken up by AM through the cationic amino acid transporter 2B (CAT-2B) (Caivano, 1998). LPS, which stimulates the production of iNOS, also facilitates AM uptake of arginine (Kakuda et al., 1999).

The substrate for NO synthesis may not be necessarily restricted to arginine. Thiemermann et al. (1991) reported that in endothelial cells, arginine-containing dipeptides fit the active site of NO synthase than arginine in endothelial cells. In addition, Meredith and Boyd (1995) reported the presence of a peptide transport protein in the pulmonary type II cells and suggested that this transporter may play a role in lung peptide homeostasis. Recently Groneberg et al. (2001) demonstrated the presence of a peptide transporter in alveolar type II pneumocytes, bronchial epithelium, and endothelium of small vessels of mammalian lungs. Due to the availability of small peptides in plasma and the presence of peptide transporter in the

alveolar epithelium and endothelium, direct utilization of these small peptides is therefore feasible.

The hypothesis of this study was that alveolar macrophages can take up arginine-containing peptides and directly utilize them as substrates for NO production, and that this process is regulated by a peptide transporter.

A number of transporters including PepT1 and PepT2 located in the intestinal and kidney epithelial cells, respectively, have been identified for di- or tri-peptide transport (Fei et al., 1994, Saito et al. 1996). It is reasonable to suggest that in various organ systems, where transport or absorption of small peptide is necessary, there are similar peptide transporters.

In order to characterize the potential presence of a peptide transporter on AM, this research used the structure of PepT1 (Figure 1) as a model to test the underlying hypothesis. In this approach, two peptide segments, peptide I and peptide II (figure 1-3), while exhibit high homology in amino acid sequence among rat, rabbit, and human PepT1, were chosen to produce the anti-PepT1 antisera, and used as proteins for the detection of a peptide transporter on AM.

The specific aims of this study were:
(1) To develop a reliable method for the analysis of arginine and arginine-containing peptides in the biological system,

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(2)   To establish that appreciable amount of arginine-
      containing small peptides are present in the lungs

(3)   To demonstrate the presence of a peptide transporter
      in alveolar macrophages (AM) and the uptake of
      arginine-containing peptides through this transporter,
      and

(4)   To determine the arginine-containing peptides are
      direct substrates for the inducible nitric oxide
      synthase (iNOS) in AM for NO production.

      The outcome of this research should provide a
plausible mechanism for the role of arginine-containing
peptides in vivo NO production.

Chapter II

Review of Literature

1. Peptide Transporters

The existence of a peptide transporter in human small intestine was hypothesized more than 25 years ago (Matthews and Adibi, 1976). Due to the technical difficulty, this transporter protein was not identified until recent years. The first peptide transporter, called (PepT1) was cloned by Fei *et al*. (1994) from rabbit small intestine. The second one, called PepT2, was cloned from absorptive cells of the renal proximal tubule (Saito et al., 1996). The discovery of peptide transporters not only supported the hypothesis but also provided a useful tool for studying the utilization of peptides by various tissues.

1.1. Physiological Importance

1.1.1. Peptide Transporter in the Small Intestine

The physiological importance of a small peptide transporter became apparent with the observation of small peptides in the gut lumen, then their disappearance from small intestine, and reappearance in portal vein after a protein meal. Adibi et al. (1971 and 1981) investigated whether intact absorption played a role in the small peptide disappearance. A series of small peptides were infused directly into the upper small intestine of human

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volunteers and their fates were determined. The results of these studies suggested the absorption of large amount dipeptides and tripeptides in an intact form.

This observation challenged the traditional idea that dietary proteins must be broken down to amino acids in the gut lumen before absorption could occur. The importance of peptide transporter was further confirmed by the fact of patients with genetic impairments of amino acid absorption (Cystinuria and Hartnup diseases). These impaired individuals do not experience protein malnutrition.

Cystinuria patients lack the basic amino acid transporter and can not absorb arginine from their intestine. However, the dipeptide Arg-Leu is well absorbed in these patients. Hartnup disease is a hereditary condition in which the active transport of several neutral amino aids is deficient from both renal tubules and the small intestine. It exhibits a pellagra-like syndrome, but is benign, and patients fare quite well nutritionally by absorbing small peptides. This would not have been expected if the amino acid transporters, instead of the peptide transporters, were mainly responsible for absorption of protein digestion products.

1.1.2. Peptide Transporter in Kidney

In addition to the PepT1 in small intestine, a peptide transporter (PepT2), is also present in absorptive cells of the renal proximal tubule (Saito et al., 1996). This

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transporter via the active transport process plays a significant role in conserving peptide bound amino nitrogen which might otherwise be lost in the urine.

Such a physiologically significant role, however, was not readily accepted because it was generally assumed that the concentrations of small peptides in the circulation were very low. However, recent studies (Gardner, 1994, Matthews 1994, Schlagheck and Webb, 1984, Seal and Parker, 1991) have provided clear evidence that up to 70% of the plasma amino acid pool is in the peptide-bound form.

1.2. Regulation of Peptide Transporter

The knowledge on the regulation of peptide transporters is limited. The ability of the intestine to absorb intact peptides varies with age. In several animal species including man, the peptide transport system is established in the small intestine prior to birth (Guandalini and Rubino, 1982, Himukai et al., 1980, Sagawa et al., 1979). The peptide absorptive capacity is maximal at birth and then decreases with age to reach adult levels.

The intestinal peptide transport system is also regulated by diet. A high-protein diet enhances the ability of the intestine to absorb peptides. A switch from a low- to a high-protein diet resulted in a 1.5-2 fold increase in the pepT1 mRNA level in rat intestine (Ferraris et al., 1988). Vazquez et al. (1985) showed that metabolic perturbations, such as starvation, alter peptide transport

in the jejunum of human volunteers. Short-term restriction of diet, for example, increases the intestinal peptide transport activity. However, the mechanism that might regulate this transporter was not studied.

Our present knowledge of the regulation of the intestinal peptide transport system by hormones is limited. Little is known about the regulation of the peptide transport in the kidney. The principal peptide transporter expressed in the kidney through the action of hormones and/or second messengers has not been studied.

1.3. Therapeutic Application

1.3.1. Pharmacological Importance

The peptide transport systems present in the small intestine and the kidney also have pharmacological relevance. Many orally active peptide-like drugs possess structural features similar to those of the physiologic substrates of the peptide transport system. The intestinal peptide transport system recognizes these peptide-like drugs (e.g. β-lactam antibiotics) as substrates and acts as a vehicle for their effective absorption (Okano et al., 1986).

The peptide transport system in the kidney is responsible for active reabsorption of these antibiotics from the glomerular filtrate and, hence, increases their half-life in the circulation. Thus, the intestinal and

renal peptide transport systems play an important pharmacologic role in determining the efficiency of these antibiotics.

It is obvious that the peptide antibiotics are not the only pharmacologically relevant compounds that use the peptide transport system as a vehicle for cellular uptake. The transport system also participates in the transport of many other therapeutically and biologically active peptides, such as angiotensin-converting enzyme inhibitors, renin inhibitors, and anticancer drugs (Inui et al., 1992).

1.3.2. Clinical Importance

The peptide transport system in the small intestine and the kidney has received increasing attention in recent years. In current clinical practice, short-chain peptides are being seriously considered as viable substitutes for free amino acids in enteral and parenteral solutions. The reason for that is that since the transport of peptides in the small intestine is the primary mode of nitrogen absorption, it is logical to employ all peptides instead of free amino acids as the source of nitrogen in enteral solutions for patients.

Available evidence in laboratory animals and in man strongly suggests that enteral solutions containing small peptides may provide an absorptive advantage to patients with severely reduced intestinal absorptive area and to patients who are acutely cachectic (trauma, sepsis, and

burns). Moreover, synthetic di- and tri-peptides offer an effective alternative means of amino acid delivery using enteral solutions for those amino acids that are unstable or sparingly soluble in free form (e.g. tyrosine, cystine, glutamine).

Expanded knowledge about extraintestinal peptide assimilation in animals, especially the extraordinary ability of the mammalian kidney to extract small peptides, is supporting the view that small peptides can be substitute for free amino acids, not only in enteral solutions, but also in parenteral solutions. Recent studies have shown that it is possible to supply daily nitrogen requirements, intravenously, in the form of small peptides to animals and man (Grimble et al., 1988, Steinhardt et al., 1984).

The low osmolality of peptide-based parenteral solutions is another advantage, especially in patients with severe fluid restriction. Furthermore, some free amino acids, e.g. glutamine, are relatively unstable in solution and during heat sterilization. However, when included in the form of a dipeptide, alanyl-glutamine (Ala-Gln), for intravenous solutions, unlike free glutamine, is stable. Human studies have shown that utilization of alanyl-glutamine is highly efficient and that the supplementation of the parenteral solutions with this peptide significantly reduces postoperative nitrogen losses and prevents the post-operative reduction in muscle glutamine content (Stehle et al., 1989).

Thus, the peptide-based parenteral solutions may offer a variety of advantages in a clinical setting, and the basis for the clinical efficacy of these solutions is the ability of extraintestinal tissues, primarily the kidneys, to utilize peptides via the peptide transport.

In addition, the water solubility of some amino acids, particularly tyrosine, is limited. Water solubility of tyrosine can be greatly increased by attachment of this amino acid to a water-soluble amino acid in dipeptide form. Therefore, mixtures of small peptides are used widely as the nitrogen source for nutrition.

1.4. Tissue Distribution, Amino Acid Sequence, Antibody to Peptide Transporters

PepT1 is expressed predominantly in epithelial cells of the small intestine. Rat PepT1 is a 710 amino acid protein (rat) and is a highly conserved between species, 77% and 83%, with that of rabbit and human, respectively (Miyamoto et al., 1996). mRNA of PepT1 has been found in other tissues including kidney, liver, brain, and pancreas (Fei et al., 1994; Liang et al., 1995).

PepT2 is expressed predominantly in the kidney, and to a small extent in the central nerve system and spleen. It is a 729 amino acid protein and showed 60-80%, and 83% amino acid sequence identity with that of rabbit and human, respectively.

Both PepT1 and PepT2 share three common structural features: (1) twelve α-helical transmembrane domains; (2) one large extracellular loop that is positioned between transmembrane domain 9 and 10; (3) intracellular location of both N- and C- terminus amino acid. Overall amino acid identity is 48% between PepT1 and PepT2 in the rat. Giacomini (1999) suggested that the extracellular loop might play an important role in the interaction between substrates and transporter.

Anti-peptide antibodies to transporters have been used to identify and localize transporter protein (Saito et al., 1995; Sai et al., 1996). These anti-PepT1 antibodies against synthetic peptides corresponding to the C-terminal 13-15 amino acids of the transporter were used in immunoblotting and immunohistochemistry studies.

1.5. Peptide Transporter in Lung

The evidences for the presence of a peptide transporter in lungs have increased. Morimoto et al. (1993) reported that dipeptides could be transported across the alveolar epithelial cell monolayers. Transport of tripeptides in the lung has been investigated in detail (Helliwell et al., 1994). Meredith and Boyd (1995) suggested that a proton-coupled peptide transport protein is present in the apical surface of the pulmonary type II cells, and that this transporter may play a role in lung peptide homeostasis.

Recently, a peptide transporter was reported to be present in mammal lung cells, including alveolar type II pneumocytes, bronchial epithelium, and endothelium of small vessels (Groneberg et al., 2001). The presence of the peptide transporter in various cell types suggested that direct utilization of peptides could occur also in the lung.

2. Arginine and Arginine-Containing Peptides in Plasma and Bronchoalveolar Lavage (BAL) Fluid

In our classical understanding of protein absorption, we believe that dietary proteins are completely hydrolyzed to free amino acids in the gut and that only free amino acids can be transported by intestinal mucosa into the circulation. There is now substantial evidence that this concept is not valid. In fact, after a protein meal, most amino acid constituents of proteins are not absorbed as free amino acids but as dipeptides and tripeptides (Adibi and Kim, 1981).

Webb (1986) reported that when comparing the appearance of amino acids in portal plasma after a meal, more than 70% were associated with the peptide amino acid in experimental calves. If these peptide amino acids are of dietary origin, then this large contribution will be significant. Even if these peptide amino acids are not of dietary protein origin, they present a large quantity of amino acids with which other tissues in the animal must

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deal. There is excellent agreement between Seal and Parker (1991) and Gardner (1982, 1983) who reported that about 50% peptide amino acids in rat and 65-78% in steer and sheep.

The distribution of the free amino acids arginine in rat plasma and tissues has been reported (Barbul, 1990). The concentration of arginine is 79-124 μM in plasma and 0.03-0.28 μmol/g in different tissues. However, no information is available about the distribution of arginine-containing di- or tri-peptides in plasma and BAL fluid.

3. Nitric Oxide, Arginine, and Arginine-Containing Peptides

Nitric oxide (NO) is an important signal transduction mediator in a variety of physiological systems (reviewed in Schmidt and Walter, 1994). In pulmonary host defense, NO is produced by alveolar macrophages (AM) in response to inflammatory stimulation to provide cytotoxic effects against invading bacteria (Beckerman et al. 1993), or to regulate cellular cytokine secretion and cyclooxygenase activity (Raso et al. 2001). The induction of NO production from AM, such as by lipopolysaccharide endotoxin (LPS), depends not only on the activity of the inducible nitric oxide synthase (iNOS), but also on the availability of the substrate arginine.

NO synthesis from arginine is a reaction, which involves two separate mono-oxygenation steps (Stuehr et al., 1991). N^{ω}-Hydroxyarginine is an intermediate species

formed by a reaction requiring one O_2 and one NADPH and the presence of tetrahydrobiopterin (BH_4). This reaction appears to be similar to those carried out by the aromatic amino acid hydroxylases, which also require BH_4. The second step in the NO synthase reaction results in the oxidation of N^{ω}-Hydroxyarginine to form citrulline and NO. Briefly the production of NO can be described by following formulae:

$$Arg + NADPH + H^+ + O_2 \rightarrow HydroxyArg + NADP^+ + H_2O$$

$$HydroxyArg + 1/2 \ (NADPH + H^2) + O_2 \rightarrow Cit + NO + H_2O$$
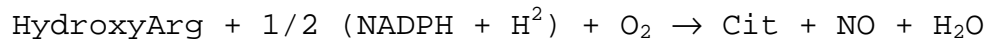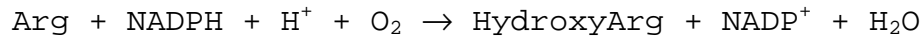
Arginine as substrate for NO production has been studied in detail (Barbul, 1990). However, little is known whether or not arginine is the only substrate. Thiemermann et al. (1991) showed that Arg-Phe fits the active site of endothelial cell NOS than arginine and that this peptide is not degraded to free amino acid during the reaction. This suggested that the substrate specificity of the NOS in endothelial cells is not necessarily restricted to L-Arg.

4. Uptake of Arginine, Arginine-Containing Peptides by Alveolar Macrophages for NO Production

System y^+ is widely believed to be the major carrier of cationic amino acids in adult tissues (Malandro and killberg, 1996). Arginine is transported across cell membranes by system y+ which is Na^+ independent and pH insensitive (White et al., 1982).

Three cationic amino acid transporters have been identified termed CAT-1, CAT-2, and CAT-2B. CAT-1 and CAT-2B are present in macrophages and monocytes. In RAW264 macrophages, CAT-1 is accounted for the basal rate of arginine uptake, while CAT-2B, induced by the lipopolysaccharide (LPS), a component of the bacterial cell wall, is responsible for the increased rate of arginine uptake. The uptake of arginine can be competitively blocked by cationic amino acid e.g. lysine (Bogle et al. 1992).

The importance of CAT transporters and arginine uptake for the production of NO in AM is well documented, however, whether arginine-containing peptides can be taken up as direct substrates for NO production remains to be determined.

5. Nitric Oxide Synthases (NOS)

Nitric oxide is produced from arginine by nitric oxide synthase (NOS). NOS was first described in 1989 (Knowles et al., 1989), first purified in 1990 (Bredt and Snyder, 1990), and first cloned in 1991 (Bredt et al., 1991). There are three NOS isoforms have been identified in mammalian cells to date. Type I NOS (nNOS, originally identified as a constitutive protein in neuronal tissue) and type III NOS (eNOS, originally identified as constitutive in vascular endothelial cells) are calcium-dependent enzymes that are expressed in a cell-specific

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manner and their activation produces the NO that mediates most of the cGMP messenger functions of this molecule. In contrast, type II NOS (iNOS, originally identified as being inducible by cytokines in macrophages and hepatocytes) has an ubiquitous tissue distribution and is only expressed during cell-mediated immune responses. For this reason it is usually referred to as inducible NOS (iNOS). The iNOS in macrophages is only found to be expressed after induction with LPS or cytokines. The half-saturating concentration (K_m) of the substrate L-arginine measured in vitro for iNOS is about 30 μM (Closs et al., 2000).

6. Measurement of NO

In general, two techniques have been commonly used as the basis for the measurement of NO, the oxidation of hemoglobin and the formation of $NO_2^- + NO_3^-$.

NO rapidly reacts with oxyhemoglobin to form NO_3^- and met-hemoglobin. The resulting spectral changes have been used as the basis of a spectrophotometric assay of NO synthase (Knowles et al., 1990). With dual wavelength measurement at 401 and 421 nm this method has a sensitivity of less than 20nM.

Formation of $NO_2^- + NO_3^-$, the oxygenation products of NO can be measured by a variety of techniques. One of them uses reduction of NO_3^- to NO_2^- by nitrate reductase or metallic catalysts followed by the colorimetric Griess reaction (Stuehr et al., 1989).

7. Inhibition of NO Synthase

The study of NO synthesis in mammalian systems has been greatly facilitated by the identification of competitive inhibitors of NO synthase. N-Monomethyl-L-arginine (L-NMMA) is one of the most commonly used. L-NMMA has been shown to be a competitive inhibitor (competitive with L-arginine) of all the form of NOS so far examined, with an IC_{50} of ~7.4 μM for iNOS (Stuehr et al. 1989). A range of arginine analogs has been found to inhibit NO synthases. Like L-NMMA, these compounds are competitive inhibitors.

Another way of inhibiting NO synthase in biological systems is to limit the supply of one of its substrates or cofactors. This type of indirect inhibition has been demonstrated using inhibitors (N-acetyl-5-hydroxytryptamine) of tetrahydrobiopterin (BH_4) synthesis. Because of the turnover of BH_4 in intact cells and tissues, inhibition of its synthesis eventually results in deletion of BH_4 to concentrations that limit NO synthesis.

NOS has been demonstrated to be subject to feedback inhibition by NO: two forms of NOS, brain constitutive nNOS and macrophage inducible iNOS were shown to be inhibited by NO either produced by the enzyme itself or generated from chemical NO donors (Stuehr and Griffith, 1992). It is likely that this inhibition results form interaction with the heme of NO synthase.

8. Remaining Questions about the Peptide Transporters

A number of issues regarding the peptide transporters still remain to be investigated.

(1) Are there additional H^+/peptide transporters other than PepT1 and PepT2? What is the functional relationship, if any, between these transporters?

(2) Is the H^+/peptide cotransporter system is expressed in the plasma membrane of mammalian tissues other than the intestine and kidney?

Northern blot analysis reveals the presence of mRNA transcripts that hybridize to the PepT1 cDNA probe in liver, brain, and pancreas. However, no information is available on H^+-couple peptide transport in these organs, whether the mRNA transcripts code for peptide transporters or for different transporters with homology to the peptide transporters remains to been investigated.

Chapter III

Materials and Methods

1. Materials

Male Sprague-Dawley rats weighing 200-250 g (Hilltop Labs. Scottsdale, PA) were used as a source for alveolar macrophages (AM) throughout this studies. E.Coli lipopolysaccharide (LPS), arginine (Arg), Arg-Sar, Arg-Gly, Arg-Lys, Arg-Gly-Asp, cephradine, and cephalexin were obtained from Sigma Co (St. Louis, MO). Fluorescein isothiocynate (FITC)-labeled lysine (Lys*) and lysine-containing peptides (β-Ala-Lys*, Arg-Lys*, and Gly-Sar-Lys*) were purchased from Genemed Synthesis, Inc (San Francisco, CA). In order to probe the presence of pepT1-like transporter on AM, two peptide segments (Peptide I and Peptide II) corresponding to P457-471 (PGHRHTLLVWGPNLY) and P480-494 (QKPEKGENGIRFVST) of the extracellular domain of rat pepT1 (Miyamoto, 1996) were synthesized and their anti-rabbit anti-sera [anti-p457-471 antiserum (antiserum I) and anti-p480-494 antiserum (antiserum II)] 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 homologous 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, arginine; E glutamate). Purified iNOs was obtained from CalbioChem (San Francisco, CA). Rat pepT1 protein was

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kindly received from Dr. You-jun Fei (Department of Biochemistry, University of Georgia) as a gift. All other reagents were purchased from Sigma Co. (St. Louis, MO).

2. Methods

2.1. Plasma Sample Preparation

Male Sprague-Dawley rats (~ 250 g) were anesthetized with sodium pentobarbital (0.2 g/kg body weight). seven ml of blood was collected from the abdominal aorta and kept in the heparinized ice-cold tubes. The blood samples were spiked with 1 mM Arg-Sar as an internal standard for subsequent HPLC analysis, centrifuged at 1500 x g at 4 ^{0}C for 10 min, and the plasma samples were collected. Two and half ml of plasma were taken and boiled for 15 min (deproteinization). The treated plasma was centrifuged at 2000 x g at 4^{0}C for 30 min.

The supernatant was loaded on to a C18 cartridge (Vac 20, Waters) and the column was eluted with 30ml of 0.1% TFA in methanol/water (95:1, pH2.4), then 30 ml of water containing 30% methanol (pH 2.4), and 10 ml of deionized water (pH 8). The third fraction was collected and lyophilized. The freeze-dried fraction was re-dissolved in 250 ul water. 50 ul of aliquots were stored at –80^{0}C until it was analyzed for ACPs measurement.

2.2. Bronchoalveolar Lavage Fluid Sample Preparation

The bronchoalveolar lavage fluids were obtained by pulmonary lavage. Briefly, rats were lavaged with 2 ml of the phosphate buffered medium as described above. The recovered BAL fluid (~ 1 ml) for each rat was centrifuged at 500 x g for 5 minutes. The supernatant for each sample was mixed with an equal volume of 6% sulfosalicylic acid. The treated fluid was centrifuged at 2000 x g at 4 ^{0}C for 20 min, and loaded into a C18 cartridge (Vac 6, Waters). 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 re-dissolved in 100 μl water. Aliqots of 20 μl samples were stored at –80^{0}C until time for analysis.

2.3. Separation of Arginine and Arginine-Containing Peptides by HPLC

The separation of arginine and arginine-containing peptides was achieved by using high performance liquid chromatography (HLPC). The HPLC system consisted a Waters 600E system controller, Waters WISP 701B autosampler, Waters 486 tunable absorbance detector, and Waters 746 data module (Waters Corp., Milford, MA). The HPLC separation method was developed by using a C18 reversed-phase column (Keystone Scientific, 150 x 4.6 mm, 3 um) and a mobile

phase consisting of 0.1% trifluoroacetic acid (TFA, pH 2.4) in water as solvent A and 0.1% TFA in CH_3CN as solvent B. The flow rate was 1 ml/min with 90% of solvent A and 10% solvent B. Elutes were detected by UV detection at 215 nm. Commercially available L-arginine, Arg-Gly, and Arg-Gly-Asp were chosen as standards and Arg-Sar as an internal standard.

2.4. Quantitative Determination of Arginine and Arginine-Containing Peptides

Standard samples for each compound of analysis was prepared by spiking known concentrations of the compound in the appropriate fluid, followed by the extraction procedures described above. Following HPLC analysis, a standard curve was generated by plotting the peak area ratio of compound/internal standard against the concentration ratio of the compound /internal standard. Linear regression analysis was made, and the standard curve was used to determine the concentration of the compound.

2.5. Accuracy and Precision

Plasma samples were repeatedly measured by HPLC according to the method described above at different times within a day, or different days. The samples were kept in ice (within a day) or frozen at $-80\ ^0C$ (different days). Precision values of the within-day (interassay) and between-day (intraassay) were determined in six replicates at each concentration of 0.2, 1.0, and 2.0 ug/ul for Arg;

0.05, 0.1, and 1.0 ug/ul for Arg-Gly; 0.01, 0.1, and 0.2 ug/ul for Arg-Gly-Asp in plasma and bronchoalveolar lavage fluid. The mean concentrations and the coefficients of variation were calculated. The accuracy of the assay was determined by comparing the nominal concentrations with the corresponding concentrations via linear regression.

2.6. Sample Recovery Experiment

A standard curve for the internal standard, Arg-Sar, was first generated by the plot of a series of Arg-Sar standard solutions (0.2, 0.5, 1.0, 1.5, and 2.0 mM) against the corresponded peak area. Arg-Sar was added into 10 ml blood sample or 1 ml lavage fluid (20 uM final concentration). The sample was then processed as described in 2.1. or 2.2. The amount of Arg-Sar recovered from the plasma or lavage fluid was determined according to the method described in 2.4. The recovery rate was calculated using following formula:

(amount measured / amount added) X 100%

20 uM of arginine and arginine-containing peptides were added into 10 ml blood or 1 ml lavage fluid, respectively. 10 ml blood and 1 ml lavage fluid without arginine and arginine-containing peptides addition were used as controls. After following the same procedure as Arg-Sar described above, the recover ratios for Arg, Arg-Gly, Arg-Gly-Asp were determined by using a similar method except for the subtraction of the corresponding amounts of Arg, Arg-Gly, Arg-Gly-Asp in the blood.

[(amount measured – amount in control) / amount added] X
100%

2.7. Arginine-Containing Peptides Uptake and Utilization by Alveolar Macrophages

2.7.1. Isolation of Alveolar Macrophages

Male Sprague-Dawley rats (~ 250 g) were anesthetized
with sodium pentobarbital (0.2 g/kg body weight) and
exsanguinated by cutting the renal artery. Alveolar
macrophages were obtained by pulmonary lavage with a Ca^{2+},
Mg^{2+}-free phosphate-buffered medium (145 mM NaCl, 5mM KCl,
1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, and 5.5 mM glucose, pH 7.4).
Lavaged cells were centrifuged at a 500 x g for 5 minutes,
washed, and resuspended in the same phosphate-buffered
medium. Cell counts and purity was measured using an
electronic cell counter equipped with a cell sizing
attachment (Coulter Electronics, Hialeah, FL).

2.7.2. Intracellular Fluorescence Uptake by Alveolar Macrophages

Isolated AM were dispersed into 24 well culture plates
at 10^6 cells/well and was incubated with 5 uM of FITC, Lys*,
β-Ala-Lys*, Arg-Lys*, or Gly-Sar-Lys* with/without 100 uM
inhibitors (lysine and cephradine) in Earle's salt solution
for 2 hours at final volume of 1 ml. The uptake was
terminated by discarding the supernatant and adding ice-
cold Earle's balanced salt solution. The cells, were

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washed 4 times with ice-cold Earle's salt solution and were sonicated (MSE sonicator, Fisher, Pittsburgh, PA) for 15 minutes. After a 5 minutes centrifugation, the supernatant was collected and measured for relative fluorescence intensity at λ_{ex} = 494 nm, λ_{em} = 519 nm.

2.7.3. Utilization of Arginine-Containing Peptides by Alveolar Macrophages for Nitric Oxide (NO) Production

Isolated AM were cultured at 1×10^{6} AM/ml in arginine-free Earle's salt solution containing 2mM of glutamine and 5% of fetal bovine serum with/without LPS (1ug/ml)(Thomas et al. 1993). 200uM of arginine or arginine-containing peptides (Arg-Gly, Arg-Gly-Asp) were added and incubated at 37°C for 24 hours. The AM-conditioned media were collected. NO production was determined by measuring the accumulation of nitrite using Greiss assay (Smith, et al. 1975).

2.7.4. Inhibitory Effects of Anti-Rat PepT1 Antiserums on NO Production in AM

The cells were co-incubated with different concentrations of two rabbit anti-rat PepT1 antiserums (I,II) and arginine-containing peptides. The inhibitory effects of inhibitors or antiserums on NO production by LPS-activated alveolar macrophages were determined according to the procedure described above.

2.7.5. Lactate Dehydrogenase (LDH) Determination

Cell viability was determined by measuring the lactate dehydrogenase (LDH) from release of AM into the extracellular medium in various AM incubation mixtures using an automated Cobas FARA II (Rosh, San Francisco, CA). LDH activity was monitored spectrophotometrically at 340 nm as it reductated pyruvate and oxidated coupled with the oxidation of NADH.

2.8. Presence of Peptide Transporter in Alveolar Macrophages

2.8.1. Sample Preparation

Isolated AM were seeded and cultured with or without LPS (1 ug/ml) in Earle's salt culture medium for 24 hours. Cells were collected, washed with ice-cold PBS buffer, and centrifuged. AM were suspended in 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 sonicated for 10 second on ice. Homogenates were then centrifuged at 5,000 x g for 10 min. and the pellet (the nuclear fraction) was discarded. The supernatant fluid was centrifuged at 50,000 x g for 2 hours and resulting pellet (cell membrane fraction) was resuspended in homogenizing buffer. Protein concentration of the membrane fraction was determined by Lowry's assay.

50 ml aliquots of the membrane preparations were stored at
–80 ^{0}C.

2.8.2. Western Blot

A membrane fraction containing 80 ug protein was
loaded on 7% of SDS-PAGE and then transferred to a
nitrocellulose membrane. The membrane was incubated for 1
hour with polyclonal rabbit antibodies specific to rat
PepT1. After washing, the blot was incubated sequentially
with secondary antibody labeled with horseradish
peroxidase-conjugated goat anti-rabbit IgG (Pierce,
Rockford, IL) and detected by enhanced chemiluminescence.
The signal densities of the protein bands were measured
using a Fluochem 8000 densitometer (Alpha Innotech Corp.,
Alexandria, VA).

2.9. Substrate Specificity Studies

2.9.1. Utilization of Arginine-Containing Peptides by iNOS in vitro for NO Production

Arginine-containing peptides were incubated with iNOS
and NO production measured using a modified method of
Stuehr *et al.* (1992). Briefly, 0.1mg of iNOS was incubated
with 200 uM of arginine, arginine-containing peptides (Arg-
Gly, Arg-Gly-Asp), 4 uM of H$_4$biopterin, 4 uM of FAD, 3mM of
dithiothreitol (DTT), 2mM of NADPH, and 40 mM of Tris HCl
buffer (pH 7.9 total volume of 1 ml). NO production was
determined by Greiss assay. The production of NO by

arginine-containing peptides was compared with that by arginine.

2.9.2. Enzyme Kinetic Studies of Arginine-Containing Peptides for NO Production

Arginine, and arginine-containing peptides including Arg-Gly and Arg-Gly-Asp at the concentration of 15, 20, 30, 60, 120,180, 240uM were incubated with iNOS, and Km and Vmax were determined using the Michaelis-Menten equation. For inhibition studies, N^G-monomethyl-L-arginine (L-NMMA) was added to the reaction mixtures at concentration of 0, 1, 4, 8, 12, 16, 20, 24, and 28 uM. The IC_{50} was calculated by using the Winnonlin Nonlinear Estimation Program (V03.0A).

3. Statistical Analysis

Data are presented as mean ± standard deviations of at lease six measurements from different animals in all experiments. Statistical analysis was conducted using a one-way analysis of variance (ANOVA) with a Tukey multiple comparison procedure with significance set at $p < 0.05$.

Chapter IV

Results and Discussion

1. Separation of arginine and arginine-containing peptides by HPLC

Arg-Gly and Arg-Gly-Asp are chosen as representative of the arginine-containing di-and tri-peptides. Arg-Sar, which contains the N-monomethylglycine residue, is not found in biological samples. Due to its structural similarity to ACPs, stable and resistant to peptidase degradation, Arg-Sar was chosen as internal standard for the HPLC quantitative assay.

Chromatographs of base line separation of arginine (Arg, R), arginine-containing peptides (Arg-Gly, RG; Arg-Gly-Asp, RGD), and internal standard (Arg-Sar, RS) were shown in Figure 4. The retention time was 5.8 min (Arg), 7.5 min (Arg-Gly), 12.6 min (Arg-Gly-Asp), and 16.8 min (Arg-Sar) in rat plasma, respectively. The similar results were in rat BAL fluid (Figure 5).

2. Standard Curves

Standard curves were generated for arginine or arginine-containing peptides by plotting the peak area ratio of compound/internal standard against the concentration ratio of the compound /internal standard. The standard curves were obtained in the concentration range of 0.2 – 2.0 μg/μl for Arg, 0.05 – 1.0 μg/μl for Arg-

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Gly, and 0.01 – 0.2 µg/µl. The limit of quantitation (LOQ) was set at 0.2 µg/µl for Arg, 0.05 µg/µl for Arg-Gly, and 0.01 µg/µl for Arg-Gly-Asp in both plasma and BAL fluid.

3. Sample Recovery Experiment

six samples of plasma and lavage fluid, with or without the addition of standard arginine and arginine-containing peptides, were processed and analyzed as described in the method section. Recovery rates of Arg, and arginine-containing peptides from rat plasma and BAL fluid were calculated and shown in table I.

Table I shows the % recovery of arginine and the peptides in plasma and in BAL fluid samples for the developed HPLC method. The sample preparation procedure resulted in a ~15 % loss of added concentration in plasma and less then 10 % loss of concentrations in BAL fluid. These results are within acceptable ranges for biological samples. In addition, the recovery rate for all testing compounds is about the same, suggesting that this method should be very consistent for the analysis of the individual and relative quantities of arginine and the arginine-containing peptides.

4. Accuracy and Precision

The accuracy and precision for quantitative assay of Arg and ACPs from plasma and BAL fluid were processed as

described in method section and the results were shown in Table II-IV.

The within-day precision expressed as the coefficients of variation (% C.V.). As shown in these tables, in plasam, the values for Arg ranged from 7.9 to 11.8%. For Arg-Gly, the value ranged from 7.9 to 13. For Arg-Gly-Asp, the value ranged from 4.3 to 14.1.

The accuracy of the assay in plasma varied in the range from 99.4 to 105.2% for Arg; from 102.5 to 104 for Arg-Gly; and from 101.3 to 102.2% for Arg-Gly-Asp.

The similar CV % and accuracy results were obtained in BAL fluids. CV% value for Arg ranged from 8.3 to 11.4%; for Arg-Gly from 5.2 to 11.3%; for Arg-Gly-Asp from 5.7 to 11.6 (table not shown). For accuracy, Arg from 99.1 to 100.8%; Arg-Gly from 98.9 to 100.4%; and Arg-Gly-Asp from 100.1 to 101.3%.

According to FDA "Specific Recommendation for Method Validation", the acceptance criteria are not more than 15% CV for precision and not more than 15% deviation for the nominal value for accuracy. The method for quantitative assay for Arg and ACPs reached the FDA requirement, suggesting the HPLC method for quantitative assay for Arg and ACPs are reliable.

5. Determination of Arginine and Arginine-Containing Peptides in Plasma and Bronchoalveolar Lavage Fluid.

The concentrations of arginine and arginine-containing peptides in plasma and bronchoalveolar lavage fluid are shown in table V. The concentration of arginine in rat plasma was 81 uM, which is similar to that previously reported by Barbul (1990). The plasma concentration of Arg-Gly, and Arg-Gly-Asp was 19.7 uM and 8.8 um, respectively. These results demonstrate for the first time the presence of arginine-containing peptides in rat plasma.

Considering the possible different combinations of arginine and other amino acids, the total concentration of arginine-containing peptides in plasma is high. This suggests that such as a large quantity of arginine-containing peptides may be utilized by different tissues. Notably, the similarly high concentration of arginine-containing peptides in bronchoalveolar lavage fluid suggests that these small peptides may also be directly utilized by the lung tissues.

6. Intracellular Fluorescence Uptake by AM

To test the whether the small peptides can be directly utilized by the lung, the uptake of fluorescently conjugated peptides by AM was compared to that of fluorescently conjugated amino acid and fluorescent probe (FITC) only. As shown in Fig 3, the uptake of FITC by AM was minimal. In contrast FITC-labeled Lys (Lys*) and small

peptides (β-Ala-Lys*, Arg-Lys*, and Gly-Sar-Lys*) showed enhanced intracellular accumulation, suggesting active transport-mediated uptake of the compounds. The uptake of Lys* by AM was completely blocked by non-labeled lysine but was not inhibited by cephradine, a reported substrate for PepT1 transporter. The uptake of the small peptides was consistently inhibited by cephradine but not by lysine. These results suggested that the small peptides were transported through a membrane transporter that is different from the lysine transporter, and were internalized by AM without prior degradation.

7. Utilization of Arginine-Containing Peptides by Alveolar Macrophages for Nitric Oxide (NO) Production

To determine if AM can directly utilize the small peptides for NO production, NO production of these peptides in LPS-activated AM was measured and compared with that by arginine. Table VI shows the production of NO by non-stimulated and LPS-stimulated AM in the presence of 200 μM arginine or arginine-containing di- and tri-peptides. While both arginine and the small peptides were utilized by AM to produce NO, the production of nitrite through the peptides was consistently higher (~ 2-fold) than that of the arginine system, in LPS-primed cells.

Figure 7 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 arginine but had little or no effect on AM production of NO through arginine-containing peptides. In addition, lysine (1 mM) markedly inhibited NO generation through the arginine system, but failed to block NO production generated through Arg-Gly or Arg-Gly-Asp. Interestingly, lysine also blocked AM production of NO through the use of Arg-Lys as a substrate. This was not expected, but presumably due to the fact that both the arginine and lysine moieties are cationic, thus allowing this small peptide to be transported by both the amino acid and peptide transporters. Figure 2 further shows that the PepT1 inhibitors, cephalexin and cephradine, selectively inhibited NO production through the arginine-containing peptides, but had little effect on the arginine system.

The above results clearly show that LPS-induced iNOS activity in AM for NO synthesis is largely dependent upon the transport or uptake of the substrate(s). While arginine is transported through CAT-2B, the arginine-containing peptides are internalized through a peptide transporter. This finding is different from that of an earlier study on chicken macrophages, which suggested that arginine-containing dipeptides were hydrolyzed to produce arginine before cellular uptake (Su and Austic, 1998). The fact that these peptides resulted in increased production of NO, 2-fold to that produced through arginine, suggests that these small peptides are more efficiently taken up by the cells or they may serve as direct and better substrates for iNOS.

8. Inhibitory Effects of Anti-Rat PepT1 Antiserums on Utilization of Arginine-Containing Peptides for NO Production in AM

A number of transporters including PepT1 and PepT2 have been identified for di- and tri-peptide transport (Fei et al., 1994; Saito et al., 1996). It is reasonable to suggest that in various organ systems, where transport or absorption of small peptides is necessary, there are similar peptide transporters. In the lung, it is already reported that peptide transporter(s) is present in Type II cells, the broncho epithelium, and the small vessel endothelium (Groneberg et al., 2001). We hypothesize that a PepT1-like transporter is responsible for the uptake of arginine-containing di- and tri-peptides in AM. 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 homologous among rat, rabbit, and human PepT1 proteins, which makes it possible that these regions may be involved in the binding of small peptides. Figure 8 shows the effects of anti-pepT1 anti-serum I and anti-pepT1 anti-serum II on NO production by LPS-stimulated AM using arginine or arginine-containing peptides as a substrate. Anti-serum I had no effect on NO synthesis in any of the systems. But the production of NO was significantly blocked by anti-serum II. Neither antibody affected the NO production using arginine as the substrate. The inhibitory

effect of antipepT1 antiserum II on AM utilization of arginine-containing peptides for NO production was dose-dependent (Figure 9). In comparison, antiserum I in the same concentration range did not inhibit the production of NO by AM (Figure 10). It is interesting to point out that the inhibitory effect of anti-serum II on Arg-lys is considerably weaker than its inhibition on other peptides. This again suggests that Arg-lys may be transported through both the peptide and 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 arginine-containing peptides indicate that AM indeed exhibit a pepT1-like transporter, and that antiserum II was able to bind and inactivate the extracellular binding site of the transporter that is crucial to the peptide transport process.

9. Lactate Dehydrogenase (LDH) Determination

The LDH level was determined in control groups (without LPS stimulation or any compounds added) and treatment groups (stimulated with LPS or compounds added). No statistically significant difference between the two groups was found, suggesting that no significant cytotoxic effects occurred during experiments.

10. Detection of PepT1-like Transporter Protein in Cell Membrane of AM

Figure 11 shows the Western blotting analysis of the presence of a PepT1-like transporter protein in the cell membrane of AM. In compared to the purified rat PepT1 protein, a 120 kD band, was detected in the membrane fraction of both LPS-activated and non-activated AM, using antiserum II. This protein band was not observed in antiserum I-treated membrane fractions, suggesting that the corresponding peptide I segment is probably not present in the peptide transporter in alveolar macrophages.

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 only 13 % homology for peptide I, but 50 % for peptide II. This supports our data that peptide II is involved in the process of small peptide transport. The density of the protein band from LPS-activated AM was significantly higher (by 2.3-folds) than that of the non-activated AM, suggesting that LPS, which is known to induce iNOS, also enhance AM uptake of small peptides through the pepT1-like transporter. These results further confirm the presence of PepT1-like transporter in AM and suggest a role for AM in regulating lung peptide homeostasis.

11. Substrate Specificity Studies

Arginine-containing peptides were incubated with iNOS in vitro to investigate whether the small peptides can be used as direct substrates for iNOS in NO production. The results show that these compounds can be used as direct substrates for NO production and that these peptides or amino acid produced a similar amount of NO at certain time (Fig 9, 10, 11). Kinetic studies showed that Arginine and arginine-containing peptides shared similar Km and Vmax (Fig 12; Table IV), suggesting that the efficiency for NO production is similar among these compounds. The NO production can be blocked by N^G-monomethyl-L-arginine (L-NMMA) with a similar IC50 value (Table VII). This finding suggested that these compounds might share a similar active site of iNOS.

This result appeared to be contradictory to the observation that arginine-containing peptides resulted in more NO production by AM than using arginine as a substrate. One possible explanation is that the CAT-2B transporter, which transports cationic amino acids, may be blocked by biological compounds such as other amino acids, LPC, etc, whereas the PepT1-like transporter in the membrane of AM is more selective in transporting peptide molecules.

P457-471   P480-494

NH$_2$

COOH

+ + +
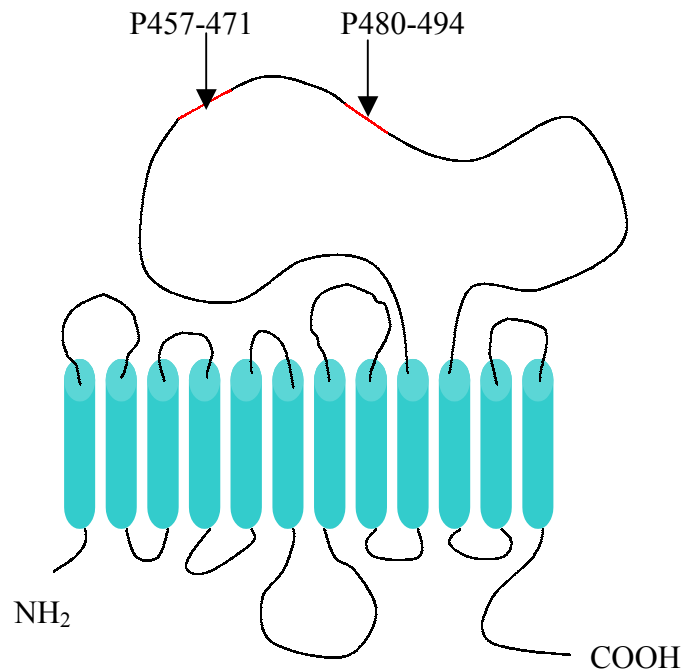
Peptide I (P457-471): **PGHRHTLLVWGPNLY**

+ -+ -   +

Peptide II (P480-494): **QKPEKGENGIRFVST**

Figure 1. Structure of PepT1 (Fei et al., 1994) and location of two chosen peptide segments for anti-PepT1 antibody development.

PepT1 protein has 12 transcellular domains. Both N-terminal and C-terminal locate intracellularly. It has a big extracellular loop which is believed as a important part for its transport functioning. Two peptide segments (peptide I and peptide II) were chosen from the loop for the development of anti-PepT1 antibody development.

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MGMSKSLSCFGYPLSIFFIVVNEFCERFSYYGMRALLILYFRNFIGWDDNL
1
STVIYHTFVALCYLTPILGALIADAWLGKFKTIVWLSIVYTIGQAVTSLSSV
2   3
NELTDNNHDGTPDSLPVHVAVCMIGLLLIALGTGGIKPCVSAFGGDQFEEG
4
QEKQRNRFFSIFYLAINAGSLLSTIITPMVRVQQCGIHVKQACYPLAFGIPAI
5
LMAVSLIVFIIGSGMYKKFKPQGNILSKVVKCICFAIKNRFRHRSKQFPKRA
6
HWLDWAKEKYDERLIAQIKMVTRVLFLYIPLPMFWALFDQQGSRWTLQA
7
TTMSGRIGILEIQPDQMQTVNTILIIILVPIMDAVVYPLIAKCGLNFTSLKKM
8
TIGMFLSAMAFVAAAILQVEIDKTLPVFPKANEVQIKVLNVGSENMIISLPG
9
QTVTLNQMSQTNEFMTFNEDTLTSINITSGSQVTMITPSLE**PGHRHTLLV**

WGPNLYRVVNDGLT**QKPEKGENGIRFVST**YSQPINVTMSGKVYEHIASY

NASEYQFFTSGVKGFTVSSAGISEQCDFESPYLEFGSAYTYLITSQATGCPQ

VTEFEDIPPNTMNMAWQIPQYFLITSGEVVFSITGLEFSYSQAPSNMKSVL
10
QAGWLLTVAVGNIIVLIVAGAGQINKQWAEYILFAALLLVVCVIFAIMARF
11   12
YTYVNPAEIEAQFEEDEKKKNPEKNDLYPSLAPVSQTQ

Figure 2. Amino acid sequence of PepT1 in rat intestinal epithelial cell (Miyamoto et al., 1996).

The numbers of 1 - 12 represent 12 transcellular domains. The extracellular loop is shown as black color and the peptide I and II is shown as red color.

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                Peptide I segment

   Rat:         PGHRHTLLVWGPNLY
   Rabbit:      A**Q***A*****N*
   Human:       Q**Q***A*****H*


                Peptide II segment

   Rat:         QKPEKGENGIRFVST
   Rabbit:      **SD*********N*
   Human:       *************N*
```

Figure 3. Comparison of amino acid sequence identity of peptide I and peptide II segments chosen as anti-pepT1 antibody development in different species.

Peptide I segment shares 73% of amino acid identities among rat, rabbit, and human, respectively. In comparison to peptide I, peptide II not only shares higher identity (80%) between rat and rabbit but also shares the highest identity between rat and human (93%).
* represents the identical amino acid.
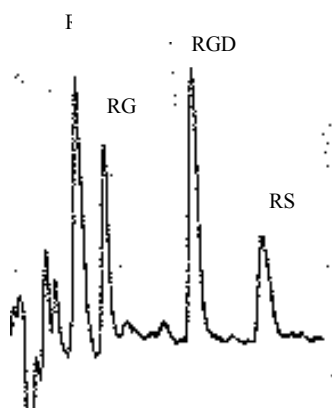
Figure 4. Chromatogram of HPLC Separation of Arginine and Arginine-Containing Peptides in Plasma Sample. The elutes were Arg (R), Arg-Gly (RG), Arg-Gly-Asp (RGD), and Arg-Sar (RS).
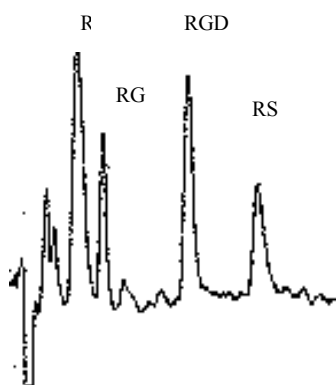
Figure 5. Chromatogram of HPLC Separation of Arginine and Arginine-Containing Peptides in Bronchoalveolar lavage fluid Sample. The elutes were Arg (R), Arg-Gly (RG), Arg-Gly-Asp (RGD), and Arg-Sar (RS).

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Table I. Recovery Rate of Arginine and Arginine-
Containing Peptides from Blood and Bronchoalveolar
Lavage Fluid

	Recovery rate (%)	
	Blood	Lavage Fluid
Arg	83.74 ± 1.24	90.32 ± 2.34
Arg-Gly	86.65 ± 2.34	91.46 ± 1.86
Arg-Gly-Asp	87.32 ± 2.06	90.47 ± 2.41

N=5, data are expressed as Mean ± standard deviation
(SD).

Table II. Evaluation of accuracy and precision for the assay of arginine in plasma sample

	Arginine (µg/µl)		
	0.2	1.0	2.0
Within-day			
Calculated	0.20	1.08	2.13
	0.22	0.99	2.00
	0.185	1.16	1.89
	0.171	1.10	1.96
	0.20	0.96	2.21
	0.21	0.94	1.78
Average ± S.D.	0.20 ± 0.02	1.04 ± 0.09	2.00 ± 0.16
C.V. (%)	11.8	8.4	7.9
Accuracy	101.1	105.2	99.4
Between-day			
Day 1	0.23	0.87	2.16
Day 2	0.197	1.03	2.04
Day 3	0.188	1.12	1.97
Day 4	0.25	0.99	1.85
Day 5	0.21	0.97	2.00
Day 6	0.22	1.05	2.07
Average ± S.D.	0.22 ± 0.02	1.00 ± 0.08	2.01 ± 0.10
C.V. (%)	10.5	8.4	5.2

Table III. Evaluation of accuracy and precision for the assay of Arg-Gly in plasma sample

	Arg-Gly (µg/µl)		
	0.05	0.1	1.0
Within-day			
Calculated	0.05	0.13	0.98
	0.046	0.12	1.07
	0.060	0.09	1.03
	0.061	0.11	0.89
	0.063	0.12	1.12
	0.05	0.118	1.06
Average ± S.D.	0.055 ± 0.01	0.11 ± 0.01	1.02 ± 0.08
C.V. (%)	13.0	11.9	7.9
Accuracy	103	102.5	104
Between-day			
Day 1	0.047	0.086	0.98
Day 2	0.05	0.09	1.05
Day 3	0.064	0.12	1.11
Day 4	0.046	0.11	1.00
Day 5	0.052	0.10	0.99
Day 6	0.061	0.97	1.07
Average ± S.D.	0.05 ± 0.01	0.10 ± 0.01	1.03 ± 0.05
C.V. (%)	14.0	12.6	5.0

Table IV. Evaluation of accuracy and precision for the assay of Arg-Gly-Asp in plasma sample

	Arg-Gly-Asp ($\mu g/\mu l$)		
	0.01	0.1	0.2
Within-day			
Calculated	0.01	0.09	0.21
	0.012	0.12	0.20
	0.013	0.113	0.196
	0.01	0.09	0.206
	0.009	0.12	0.189
	0.01	0.118	0.19
Average ± S.D.	0.01 ± 0.01	0.11 ± 0.01	0.20 ± 0.01
C.V. (%)	14.1	13.4	4.3
Accuracy	101.3	102.2	101.4
Between-day			
Day 1	0.047	0.11	0.19
Day 2	0.05	0.12	0.23
Day 3	0.064	0.09	0.18
Day 4	0.046	0.08	0.20
Day 5	0.052	0.10	0.19
Day 6	0.061	0.09	0.18
Average ± S.D.	0.05 ± 0.01	0.10 ± 0.02	0.20 ± 0.02
C.V. (%)	14.0	15.0	9.6

Table V. Concentrations of Arginine and Arginine-Containing Peptides in rat Plasma and Bronchoalveolar Lavage Fluid.

	Concentration (uM)	
	Blood	BAL Fluid
Arg	81.45 ± 3.28	82.61 ± 4.16
Arg-Gly	19.65 ± 2.41	11.37 ± 2.46
Arg-Gly-Asp	8.77 ± 1.03	7.62 ± 1.61

N=6, data are expressed as Mean ± standard deviation (SD).
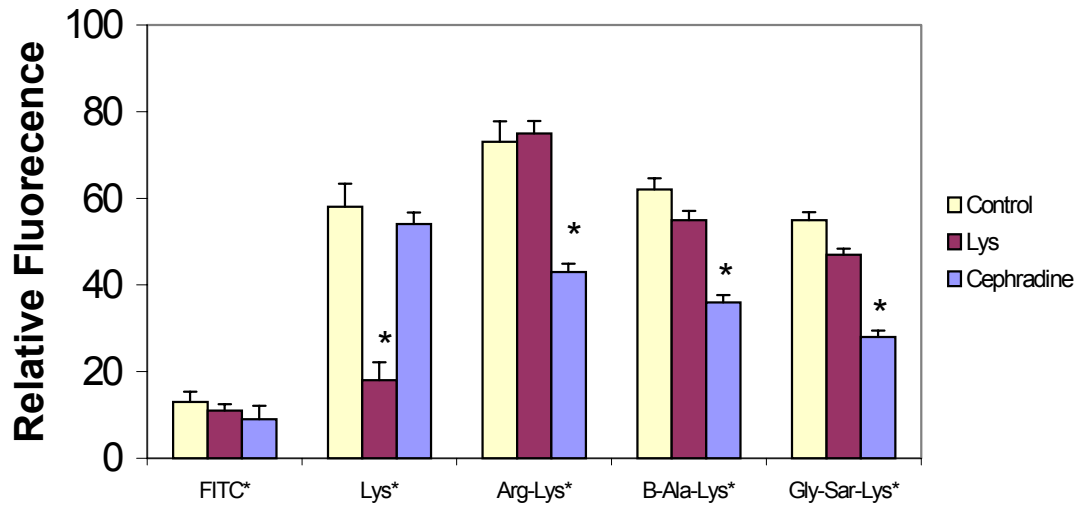
Figure 6. Transporter-mediated uptake of 5 μM FITC-labeled lysine (Lys*) and small peptides (Arg-Lys*, β-Ala-Lys*, Gly-Sar-Lys*) by alveolar macrophages (10^6 Cells). Lys (1 mM) and cephradine (1mM) was used as inhibitors for CAT-2B and PepT1 transporters, respectively. * Significant difference from control at $P < 0.05$, n = 6.

Table VI. Production of NO by non-stimulated and LPS-stimulated AM in the presence of arginine and arginine-containing peptides

Substrate	Nitrite (μM)	
	-LPS	+LPS
Cells only	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*
Arg-Gly	5.1 ± 0.3	41.9 ± 3.6*
Arg-Gly-Asp	3.7 ± 1.0	38.4 ± 2.1*

At equal molar concentration (200uM) of Arg and ACPs, NO production from 10^6 AM with/without LPS stimulation was measured following 24-hour incubation and expressed as nitrite (uM). Each value represents the mean ± SD of six separation experiments.
* indicates significant difference from AM + Arg, $p < 0.05$
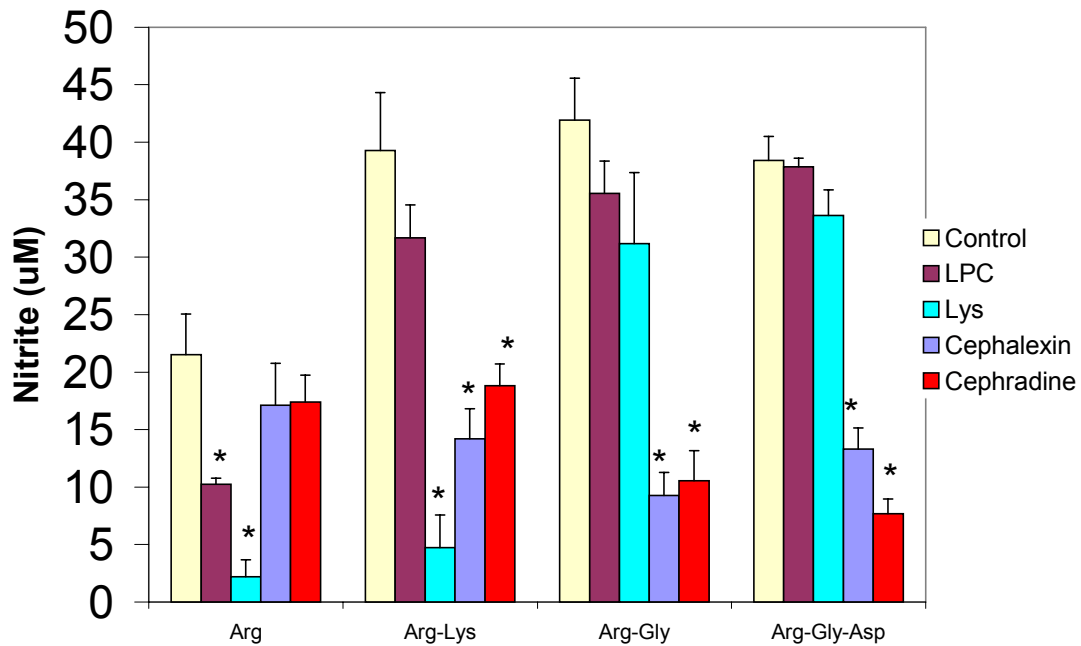
Figure 7. Inhibitory effects of CAT and PepT1 transporter inhibitors on NO production by LPS-stimulated AM. 1mM of Lys, cephradine, and cephalexin; 5uM of LPC were used as inhibitors. * indicates significant difference from control at P < 0.05; n = 6.
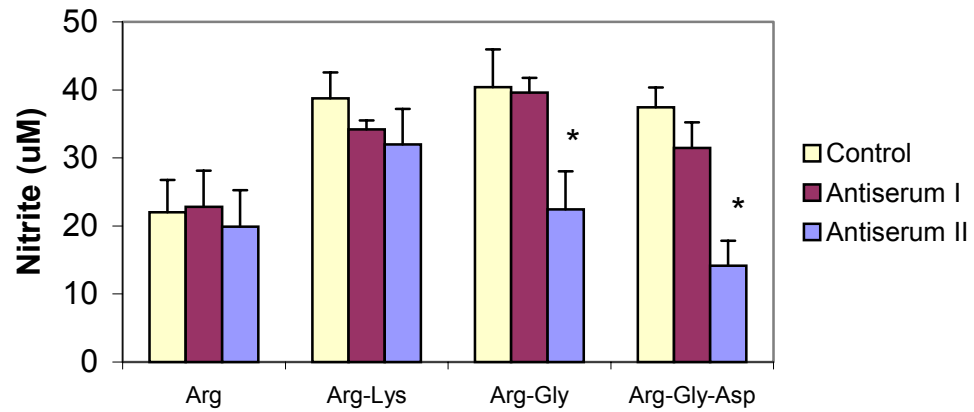
Figure 8. Effects of anti-PepT1 antiserum I (1:10) and anti-PepT1 antiserum II (1:10) on AM production of NO using arginine and arginine-containing peptides as substrates.

* indicates significant difference from the control (Arg). $P < 0.05$, n = 6.

Figure 9. The dose-dependent effects of anti-PepT1
antiserum II on NO production by LPS-stimulated AM using
arginine or arginine-containing peptides as the substrates.

The antiserum dilutions were 1:100, 1:20, 1:10, 1:2.
Antiserum II significantly block the NO production by Arg-
Lys, Arg-Gly-Asp in a dose dependent manner. N=6, $P < 0.05$.
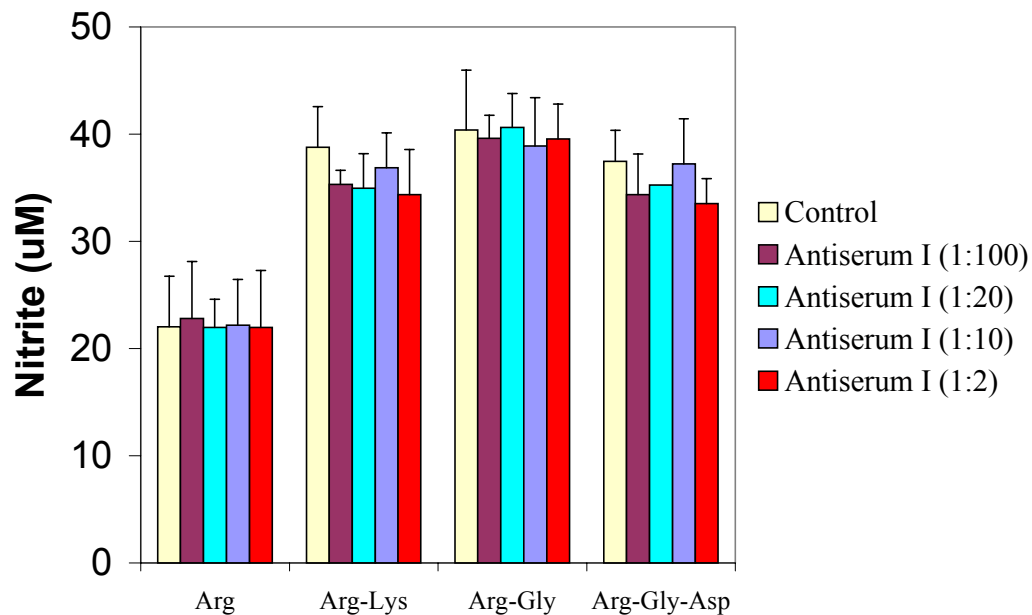
Figure 10. The dose-dependent effects of anti-PepT1 antiserum I on NO production by LPS-stimulated AM using arginine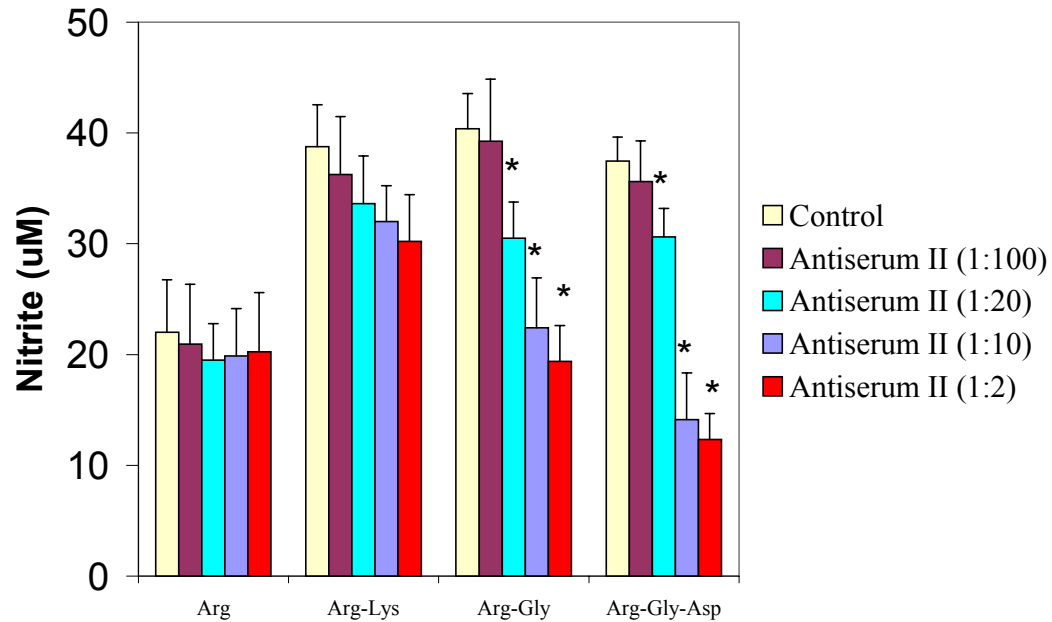 or ACPs as the substrates. The antiserum dilutions were 1:100, 1:20, 1:10, 1:2. N=6, P < 0.05. Unlike to the antiserum II, no dose-dependent effects of the antiserum I on NO production by LPS-stimulated AM were observed.

55
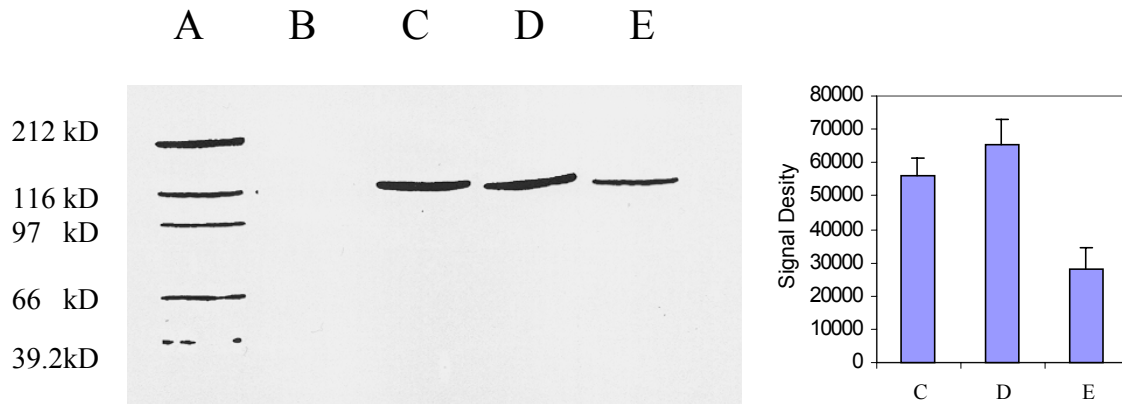
Figure 11. Western Blot of PepT1-like Transporter on the membrane fraction of AM.

A 120 kD bands were detected in membrane fraction of both LPS-stimulated and non-stimulated AM using anti-PepT1 antiserum II. Western Blot of AM membrane proteins using anti-p480-494 antiserum (antiserum II). The signal densities of corresponding bands were measured.

A: Molecular weight marker; B: Preimmune serum; C: PepT1 standard protein; D: LPS-stimulated AM; E: Non-stimulated AM.

Figure 12. Michaelis-Menten Saturation Curve of Arginine.
n = 5.   The Kinetics was carried out by incubation of 0.1
mg iNOS with arginine as substrates at concentration of 15,
20, 30, 60, 120, 180, and 240 μM for 4 min.   The initial
rates of NO synthesis were measured spectrophotometrically
using the Greiss assay.
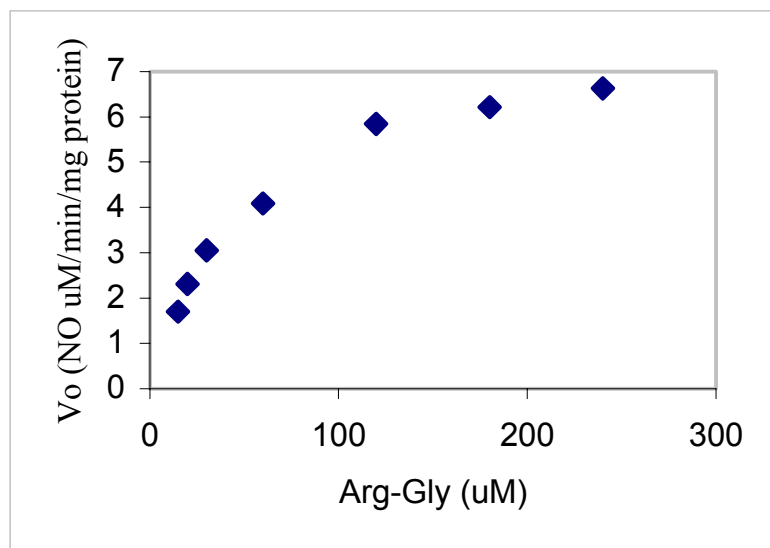
Figure 13. Michaelis-Menten Saturation Curve of Arg-Gly. n = 6. The Kinetics was carried out by incubation of 0.1 mg iNOS with Arg-Gly as substrates at concentration of 15, 20, 30, 60, 120, 180, and 240 μM for 4 min. The initial rates of NO synthesis were measured spectrophotometrically using the Greiss assay.
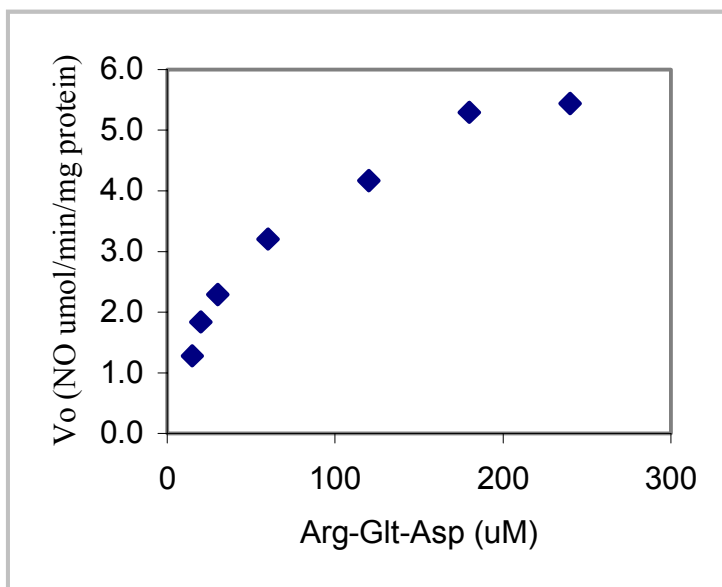
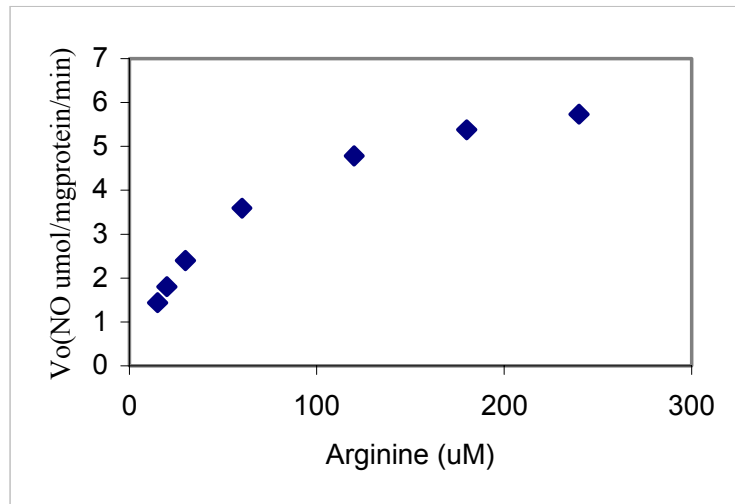**Figure 14.** Michaelis-Menten Saturation Curve of Arg-Gly-Asp.
n = 5. The Kinetics was carried out by incubation of 0.1 mg iNOS with Arg-Gly-Asp as substrates at concentration of 15, 20, 30, 60, 120, 180, and 240 μM for 4 min. The initial rates of NO synthesis were measured spectrophotometrically using the Greiss assay.
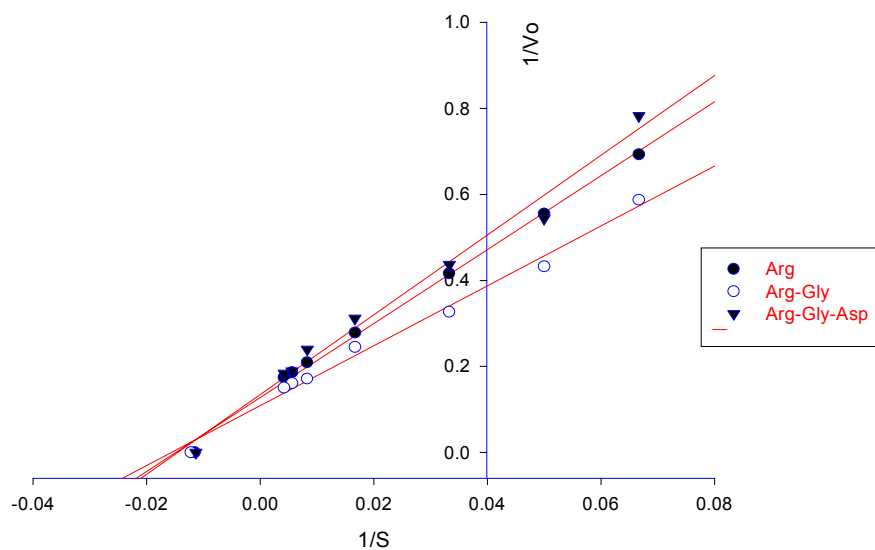
Figure 15. Lineweaver-Burk plots for iNOS activity using Arg, Arg-Gly, and Arg-Gly-Asp as substrate (n=5).

      The enzyme reactions were carried out by incubation 0.1 mg iNOS with arginine or arginine-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 Greiss assay.

Table VII. K_m, V_{max}, and IC_{50} values for the enzyme kinetics of iNOS using Arg, Arg-Gly, and Arg-Gly-Asp as substrates and L-NMMA as a competitive NO inhibitor.

Substrate	K_m (μM)	V_{max} (μmol/min/mg protein)	IC_{50} (L-NMMA) (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

Each value represents the mean ± SD of six separation experiments.

Chapter V

Summary and Conclusion

A HPLC method to analyze arginine and arginine-containing peptides from blood and bronchoalveolar lavage fluid has been established. The accuracy and precision of assay is within 15% deviation and 15% C.V., respectively, which reach the FDA requirement for analytical method validation guide. The limit of quantitation (LOQ) was in nano-range without derivatization, 0.2 µg/µl for Arginine, 0.05 µg/µl for Arg-Gly, and 0.01 µg/µl for Arg-Gly-Asp in both plasma and BAL fluid. So the method is simple, accuracy and reliable.

This project also demonstrates that arginine-containing di- and tri-peptides are direct substrates to the inducible nitric oxide synthase. Arginine-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 di- and tri-peptides in plasma are directly absorbed by various organs without hydrolytic degradation (Lochs et al., 1988; Fei et al., 1994). In the pulmonary system, where NO production on one hand protects the lung from bacteria infection (Boockvar et al., 1994) and on the other hand exacerbates inflammatory lung injury, AM are shown to exhibit PepT1-like transporter and produce high levels of NO by directly using arginine-containing peptides as

substrates. This study suggests a plausible mechanism through which a significant portion of the NO production by AM may be generated.

The transport and utilization of arginine-containing peptides by AM may have a broader implication on the regulatory role of peptide transporters in various biological 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 arginine transport through the CAT-2B transporter by compounds such as LPC, a natural CAT-2B inhibitor (Kikuta et al., 1998; Caivano, 1998). Our studies showed that LPC indeed blocked AM production of NO through arginine uptake but had no effect on the cellular uptake and utilization of the arginine-containing peptides. This suggests that arginine-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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