Endogenous Synthesis of Arginine Plays an Important Role in Maintaining Arginine Homeostasis in Postweaning Growing Pigs[1,2]

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ABSTRACT This study was conducted to determine whether endogenous synthesis of arginine plays a role in regulating arginine homeostasis in postweaning pigs. Pigs were fed a sorghum-based diet containing 0.98% arginine and were used for studies at 75 d of age (28.4 kg body weight). Mitochondria were prepared from the jejunum and other major tissues for measuring the activities of Δ^1-pyrroline-5-carboxylate (P5C) synthase and proline oxidase (enzymes catalyzing P5C synthesis from glutamate and proline, respectively) and of ornithine aminotransferase (OAT) (the enzyme catalyzing the interconversion of P5C into ornithine). For metabolic studies, jejunal enterocytes were incubated at 37°C for 30 min in Krebs-Henseleit bicarbonate buffer containing 2 mmol/L L-glutamine, 2 mmol/L L-[U-^{14}C]proline, and 0–200 μmol/L gabaculine (an inhibitor of OAT). The activities of P5C synthase, proline oxidase and OAT were greatest in enterocytes among all of the tissues studied. Incubation of enterocytes with gabaculine resulted in decreases ($P < 0.05$) in the synthesis of ornithine and citrulline from glutamine and proline. When gabaculine was orally administered to pigs (0.83 mg/kg body weight) to inhibit intestinal synthesis of citrulline from glutamine and proline, plasma concentrations of citrulline (−26%) and arginine (−22%) decreased ($P < 0.05$), whereas those of alanine (+21%), ornithine (+17%), proline (+107%), taurine (+56%) and branched-chain amino acids (+21–40%) increased ($P < 0.05$). On the basis of dietary arginine intake and estimated arginine utilization, the endogenous synthesis of arginine in the 28-kg pig provided ≥50.2% of total daily arginine requirement. Taken together, our results suggest an important role for endogenous synthesis of arginine in regulating arginine homeostasis in postweaning growing pigs, as previously shown in neonatal pigs. J. Nutr. 127: 2342–2349, 1997.

KEY WORDS: • *arginine* • *glutamine* • *proline* • *ornithine aminotransferase* • *pigs*

Arginine is a basic amino acid and serves as an essential precursor for the synthesis of biologically important molecules such as protein, ornithine, proline, polyamines, creatinine, nitric oxide and agmatine (Barbul and Dawson 1994, Cynober et al. 1995, Li et al. 1994). Nitric oxide is an endothelium-derived relaxing factor, a neurotransmitter, a mediator of immune response and a signalling molecule (Bredt and Snyder 1994). Agmatine is a novel noncatecholamine ligand at α_2-adrenergic receptors (Li et al. 1994) and an inhibitor of nitric oxide synthase (Galea et al. 1996). Arginine also plays an important role in the detoxification of ammonia via the urea cycle and is a potent stimulator of secretion of insulin and growth hormone, important regulators of nutrient metabolism (Mulloy et al. 1982, Visek 1986). Although arginine can be synthesized by most mammals (except for cats and ferrets), it is classified as a nutritionally essential amino acid for young mammals and for adults at times of stress and illness (Visek 1986, Yu et al. 1996). Thus, regulation of arginine homeostasis is of nutritional and physiologic importance.

Factors that regulate arginine homeostasis include dietary arginine intake, endogenous synthesis and degradation of arginine, as well as intracellular protein turnover. We have recently demonstrated that endogenous synthesis of arginine plays an important role in maintaining arginine homeostasis in neonatal pigs nursed by sows (Flynn and Wu 1996). This is of nutritional and physiologic importance for neonates because of a remarkable deficiency of arginine in the milk (Davis et al. 1994, Wu and Knabe 1994). In contrast, arginine homeostasis has been suggested to be regulated mainly by dietary arginine intake and arginine oxidation rather than by endogenous arginine synthesis in adult humans (Castillo et al. 1993). In both of these studies, piglets and humans were in the fed state. The contrasting findings between piglets and adult humans may result from differences in species, age and dietary availability of arginine.

The synthesis of arginine from glutamine requires glutaminase, Δ^1-pyrroline-5-carboxylate (P5C)[4] synthase, ornithine aminotransferase (OAT), carbamoylphosphate synthase I

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[4] Abbreviations used: ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; BSA, bovine serum albumin; CPS-I, carbamoylphosphate synthase-I; γ-GK, γ-glutamyl kinase; γ-GPR, γ-glutamylphosphate reductase; OAT, ornithine aminotransferase; OCT, ornithine carbamoyltransferase; P5C, Δ^1-pyrroline-5-carboxylate.

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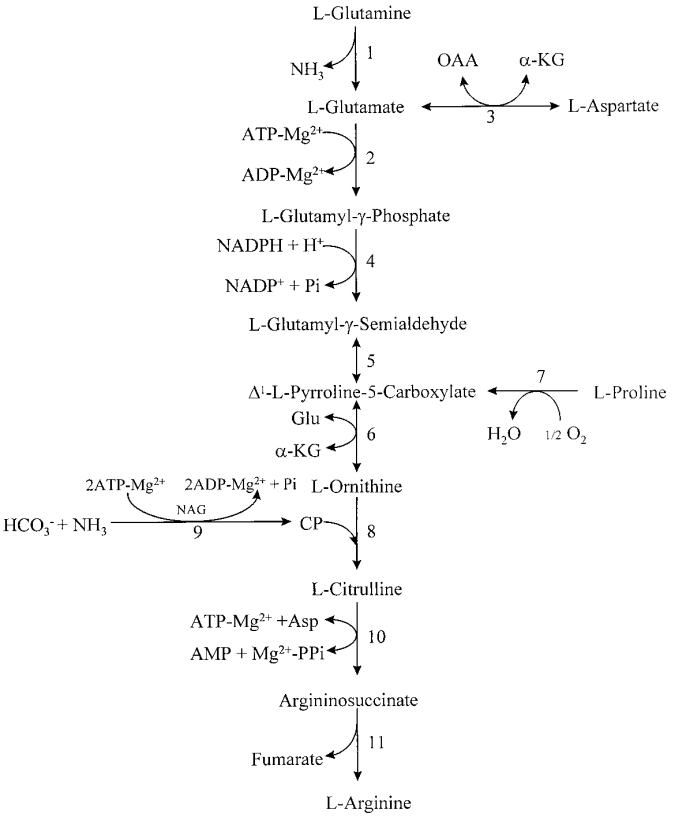

FIGURE 1 The metabolic pathway for the synthesis of arginine from glutamine and proline in enterocytes. The conversion of L-glutamate into L-arginine requires the following enzymes: *1*) phosphate-dependent glutaminase; *2*) Δ^1-L-pyrroline-5-carboxylate (P5C) synthase (a bifunctional enzyme) that exhibits γ-glutamyl kinase activity; *3*) glutamate aminotransferase; *4*) P5C synthase (a bifunctional enzyme) that exhibits γ-glutamylphosphate reductase activity; *5*) spontaneous chemical reaction; *6*) ornithine aminotransferase; *7*) proline oxidase; *8*) ornithine carbamoyltransferase; *9*) carbamoylphosphate synthase-I; *10*) argininosuccinate synthase; *11*) argininosuccinate lyase. Reactions 1–9 occur in mitochondria, and reactions 10–11 take place in the cytosol. Reaction 3 also occurs in the cytosol. Abbreviations used: Asp, L-aspartate; CP, carbamoylphosphate; Glu, L-glutamate; α-KG, α-ketoglutarate; NAG, *N*-acetyl-glutamate; OAA, oxaloacetate.

(CPS-I), ornithine carbamoyltransferase (OCT), argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) (Wu and Knabe 1995) (**Fig. 1**). Glutaminase, OAT, ASS and ASL are widely distributed in mammalian tissues, but CPS-I and OCT are located exclusively in the liver and small intestine (Curthoys and Watford 1995, Wakabayashi 1995). Because P5C synthase is almost exclusively located in enterocytes, the small intestine is the major, if not exclusive organ for synthesis of P5C from arterial glutamine and dietary glutamate/glutamine (Flynn and Wu 1996, Wakabayashi 1995). Thus, there is enormous interest in intestinal metabolism of glutamine and arginine in health and disease (Burrin and Reeds 1997). Glutamine/glutamate has generally been considered to be the only source of P5C for citrulline synthesis in the small intestine (Wakabayashi 1995). However, we have recently demonstrated that proline is an important source of P5C (via proline oxidase) and citrulline in pig enterocytes (Wu 1997). Pyrroline-5-carboxylate is the common intermediate in pathways for the synthesis of citrulline from both glutamine and proline; it is interconverted into ornithine by OAT in enterocytes (Wu 1997). An inhibition of OAT will lead to decreased synthesis of citrulline and arginine from both glutamine and proline in enterocytes, thereby resulting in arginine deficiency, as in OAT-gene knockout mice (Wang et al. 1995).

The objective of this study was to determine whether endogenous synthesis of arginine plays a role in maintaining arginine homeostasis in postweaning pigs. Intestinal synthesis of citrulline and arginine from glutamine and proline was inhibited by gabaculine, a suicide inhibitor of OAT in animal cells (Jung and Seiler 1978), including pig enterocytes (Flynn and Wu 1996).

MATERIALS AND METHODS

Chemicals. L-Glutamine, L-proline, P5C, D-glucose, bovine serum albumin (BSA; fraction V, essentially fatty-acid free), HEPES, pyridoxal phosphate, *o*-aminobenzaldehyde, dithiothreitol, phenylmethylsulfonyl fluoride, aprotinin, chymostatin, pepstatin A, phosphocreatine, creatine kinase, gabaculine (3-amino-2,3-dihydrobenzoic acid), sucrose, D-mannitol, EDTA, EGTA, Triton-X 100, Nonidet P-40, trichloroacetic acid and *o*-phthaldialdehyde were purchased from Sigma Chemical (St. Louis, MO). Dowex AG 1-X8 resin (acetate form) was obtained from Bio-Rad (Richmond, CA), and α-ketoglutarate, ATP and NADPH from Boehringer Mannheim (Indianapolis, IN). L-[U-^{14}C]Proline and L-[U-^{14}C]glutamate were purchased from American Radiolabeled Chemicals (St. Louis, MO) and Amersham (Arlington Heights, IL), respectively. HPLC-grade methanol and H_2O were obtained from Fisher Scientific (Houston, TX).

Animals. Pigs were offspring of Yorkshire × Landrance sows and Duroc × Hampshire boars, and were obtained from the Texas A&M University Swine Center. Piglets were freely nursed by sows until 28 d of age, when they were weaned to a standard sorghum-soybean meal–based diet containing 20% crude protein (Hansen et al. 1993). Beginning at 60 d of age, pigs were fed a 16% crude protein sorghum-based diet containing 0.98% arginine (**Table 1**) that met NRC nutrient requirements (NRC 1988). For analysis of amino acids (except tryptophan) in the diet, 1.0 g of the diet was hydrolyzed in 100 mL of 6 mol/L HCl at 110°C for 24 h under N_2, and amino acids in acid hydrolysates were measured by HPLC (Wu and Knabe 1994). Tryptophan in the diet was determined as described by La Rue (1985). Briefly, 0.2 g of diet was hydrolyzed in a polyallomer tube containing 10 mL of 4.2 mmol/L NaOH and 0.1 mL of thiodiglycol (an antioxidant, 25% aqueous solution) at 110°C for 20 h, and tryptophan was analyzed by HPLC (Wu and Knabe 1994). Pigs had free access to water and feed, and were used for studies at 75 d of age (28.4 ± 0.8 kg body weight; means ± SEM, n = 22). This study was approved by Texas A&M University's Institutional Animal Care and Use Committee.

Enterocyte preparation. Enterocytes were isolated from the jejunum of 75-d-old pigs, as previously described (Wu et al. 1994). Briefly, pigs were preanesthetized with an intramuscular injection of ketamine and acetylpromazine at 4.76 and 0.24 mg/kg body weight, respectively. Halothane was administered with a face mask to achieve a surgical plane of anesthesia. A mid-jejunal segment (50 cm) was removed, rinsed thoroughly with saline, filled with calcium-free oxygenated (95% O_2-5% CO_2) Krebs-Henseleit bicarbonate buffer (119 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4 and 25 mmol/L $NaHCO_3$, pH 7.4) containing 20 mmol/L HEPES (pH 7.4), 5 mmol/L EDTA (disodium), and 5 mmol/L D-glucose (95% O_2/5% CO_2), and incubated for 50 min at 37°C in a shaking water bath (70 oscillations/min). At the end of the incubation, the jejunum was gently patted with finger tips for 1 min, and the lumen was drained into polystyrene tubes. Enterocytes were obtained by centrifugation (400 × *g*, 2 min). Cells (pellet) were washed three times with oxygenated Krebs-Henseleit bicarbonate buffer (95% O_2-5% CO_2) containing 2.5 mmol/L $CaCl_2$ (no EDTA), 20 mmol/L HEPES (pH 7.4), and 5 mmol/L D-glucose. Enterocytes were viable for at least 30 min of incubation, as assessed by linear consumption of oxygen (Wu et al. 1994).

Distributions of OAT, P5C synthase and proline oxidase activities in pig tissues. Pigs (75 d old) were anesthetized as described above. Adipose tissue, brain, colon, heart, jejunum, kidney, liver, lung, pancreas, skeletal muscle (semitendinosus muscle) and spleen

TABLE 1

Composition of diet[1]

	g/kg diet
Ingredient	
Sorghum	566.6
Wheat middlings	200.0
Rice bran	71.0
Meat and bone meal	61.0
Soybean meal	48.0
Brewer's dried grains	41.5
NaCl	5.0
Limestone	2.5
Lysine-HCl, 98.8%	1.1
Medications[2]	1.0
Trace mineral premix[3]	1.0
Choline chloride premix[4]	0.8
Vitamin premix[5]	0.5
Calculated content	
Metabolizable energy, *kJ/kg*	12,870
Crude protein,[6] %	16.0
Calcium, %	0.80
Phosphorus, %	0.73

[1] Values are expressed on an as-fed basis. Dry matter content is 89.6%.

[2] Containing the following (mg/kg diet): chlortetracycline, 110.

[3] Containing the following (mg/kg diet): Cu, 15; Fe, 150; I, 0.6; Mn, 27; Zn, 150; Se, 0.03.

[4] Containing the following (mg/kg diet): choline, 48.

[5] Containing the following (mg/kg diet): retinyl palmitate, 2.42; cholecalciferol, 0.01; *all*-rac-α-tocopheryl acetate, 22; menadione sodium bisulfate complex, 6.7; riboflavin, 3.3; D-calcium pantothenate, 14.5; niacin, 27; choline 90; vitamin B-12, 0.022; D-biotin, 0.30; thiamine, 1.1; folic acid, 0.7; pyridoxine, 1.3.

[6] Containing the following amino acids (g/kg diet): Asp/Asn, 12.95; Glu/Gln, 31.65; Ser, 10.23; His, 3.80; Gly, 7.52; Thr, 6.07; Arg, 9.80; Ala, 11.93; Tyr, 6.97; Met, 3.19; al, 9.12; Phe, 8.88; Ile, 7.29; Leu, 17.98; Lys, 7.63; Trp, 1.63; Pro, 16.2; Cys, 1.70.

were dissected (Flynn and Wu 1996). The jejunum was rinsed 3 times with saline to remove luminal digesta. Mucosa was separated from the jejunal serosal smooth muscle layer with the use of a glass slide. The serosal layer was rinsed three times with saline to remove residual mucosa.

For OAT assay, tissues (0.5 g) were homogenized at 4°C in 6 mL of buffer solution (0.33 mol/L sucrose, 5 mmol/L HEPES, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.5% Triton-X 100, pH 7.4) with the use of a glass pestle. Homogenates were centrifuged at 600 × *g* and 4°C for 10 min, and the supernatant fractions were subjected to three cycles of freezing (in liquid nitrogen) and thawing (at 37°C). The lysates were used for OAT assay at 37°C for 15 min as previously described (Wu et al. 1994). Briefly, the assay mixture (2 mL) consisted of 75 mmol/L potassium phosphate buffer (pH 7.5), 20 mmol/L ornithine, 0.45 mmol/L pyridoxal phosphate, 5 mmol/L *o*-aminobenzaldehyde, 0 or 3.75 mmol/L α-ketoglutarate and cell extracts. The amount of cell extract protein in the OAT assay was 0.1, 0.5 and 5 mg for jejunal mucosa, intact jejunum and other tissues, respectively. The OAT assay was linear with time and amount of protein used.

Proline oxidase activity was measured as previously described (Herzfeld et al. 1977), except that protease inhibitors were used in tissue homogenization medium. Briefly, tissues (0.5 g) were homogenized at 4°C in 6 mL of buffer [250 mmol/L sucrose, 1 mmol/L EDTA and 2.5 mmol/L dithiothreitol in 50 mmol/L potassium phosphate buffer (pH 7.2)] containing protease inhibitors (5 mg/L phenylmethylsulfonyl fluoride, 5 mg/L aprotinin, 5 mg/L chymostatin and 5 mg/L pepstatin A). The homogenates were centrifuged at 600 × *g* and 4°C for 10 min, and the supernatant fractions were centrifuged at 12,000 × *g* for 10 min at 4°C. The pellets (mitochondria) were suspended in 0.5 mL of 50 mmol/L potassium phosphate buffer (pH 7.5) and stored at −20°C for 24 h before use for proline oxidase assay. The assay mixture (1.0 mL), which consisted of 15 mmol/L L-proline, 20 μmol/L ferricytochrome C, mitochondrial pellets and 50 mmol/L potassium phosphate buffer (pH 7.5), was incubated at 37°C for 0 or 30 min. The reaction was terminated by addition of 0.5 mL of 10% trichloroacetic acid, followed by addition of 0.1 mL of 100 mmol/L *o*-aminobenzaldehyde. After a 30-min period of standing at room temperature, the mixture was centrifuged at 600 × *g* for 5 min, and the absorbance of the supernatant fraction was measured at 440 nm. Blanks (0 min incubation) were subtracted from sample values. The mitochondrial protein content in proline oxidase assay was 0.5 mg for jejunal mucosa, 2 mg for intact jejunum, liver and kidney, and 5 mg for other tissues. The enzyme assay was linear with both time and amount of protein used for the tissues containing proline oxidase activity.

For P5C synthase assay, tissues (0.5 g) were homogenized at 4°C in 6 mL of homogenization buffer (300 mmol/L D-mannitol, 5 mmol/L HEPES, 0.2 mmol/L EDTA and 3 mmol/L dithiothreitol, pH 7.4). Homogenates were centrifuged at 600 × *g* and 4°C for 10 min, and the supernatant fraction was centrifuged at 12,000 × *g* and 4°C for 15 min. The pellet (mitochondria) was resuspended in 1.5 mL of fractionation buffer (300 mmol/L D-mannitol, 5 mmol/L HEPES, 5 mmol/L EDTA and 3 mmol/L dithiothreitol), and centrifuged at 600 × *g* and 4°C for 4 min. The supernatant fraction was centrifuged at 4000 × *g* and 4°C for 10 min. The mitochondria (pellet) were suspended in homogenization buffer and immediately used for P5C synthase assay at 23°C for 0 or 30 min (Wu and Knabe 1995). The assay mixture (1 mL) contained 0.1 mol/L HEPES (pH 7.4), 20 mmol/L $MgCl_2$, 1 mmol/L gabaculine, 1 mmol/L [U-^{14}C]glutamate (200 Bq/nmol), 3 mmol/L ATP, 0.2 mmol/L NADPH, 15 mmol/L phosphocreatine, 10 U of creatine kinase, 0.25% Nonidet P-40 and mitochondria (1 mg protein). [^{14}C]P5C was separated from [^{14}C]glutamate by anion-exchange chromatography (Dowex AG 1-X8 resin, acetate form, 200–400 mesh) (Wu et al. 1994). The recovery of P5C was >99% as determined with the use of a purified P5C standard. The P5C synthase assay was linear with time and amounts of protein used.

The name "P5C synthase" was initially chosen by Smith et al. (1980) and Wakabayashi et al. (1983) for the enzyme that catalyzes P5C synthesis from glutamate in mammalian cells. The P5C synthase assay, which was developed by Wakabayashi et al. (1983) and adapted in our studies (Wu and Knabe 1995), measured the conversion of L-glutamate into P5C. This pathway involves the following two reactions: *1*) phosphorylation of L-glutamate by γ-glutamyl kinase (γ-GK) to form L-glutamyl-γ-phosphate, and *2*) reduction of L-glutamyl-γ-phosphate by γ-glutamylphosphate reductase (γ-GPR) to form L-glutamyl-γ-semialdehyde, with the latter spontaneously cyclizing to P5C (Fig. 1). In *Escherichia coli*, γ-GK and γ-GPR are two separate enzymes encoded by two distinct genes, pro B and pro A, respectively (Deutch et al. 1984), and are considered to form an enzyme complex necessary for P5C synthesis from glutamate (Hayzer and Moses 1978a and 1978b). In both plants (Hu et al. 1992) and mammalian cells (Aral et al. 1996), the conversion of L-glutamate to P5C is catalyzed by a bifunctional enzyme (P5C synthase, a single polypeptide) that exhibits both γ-GK and γ-GPR activities.

Effect of gabaculine on synthesis of ornithine and citrulline from glutamine in enterocytes. For determining the effect of gabaculine on amino acid synthesis from glutamine, enterocytes (5 × 10^6 cells/mL) were incubated at 37°C for 0 or 30 min in the presence of 2 mL of Krebs-Henseleit bicarbonate buffer containing 20 mmol/L HEPES, 1% BSA, 5 mmol/L D-glucose, 2 mmol/L glutamine, and 0–200 μmol/L gabaculine. Incubations were terminated by addition of 200 μL of 1.5 mol/L $HClO_4$. Acidified medium was neutralized with 100 μL of 2 mol/L K_2CO_3 and used for amino acid analysis by HPLC (Wu et al. 1994). Net production of amino acids was calculated on the basis of differences in their concentrations in cell extracts between 0- and 30-min incubation periods in the presence of 2 mmol/L glutamine.

Effect of gabaculine on synthesis of ornithine and citrulline from proline in enterocytes. For the determination of amino acid synthesis from proline, enterocytes (25 × 10^6 cells/mL) were incubated at 37°C for 0 or 30 min in 2 mL of oxygenated Krebs-Henseleit bicarbonate buffer (pH 7.4, saturated with 95% O_2/5% CO_2) containing 20 mmol/L HEPES, 1% BSA, 5 mmol/L glucose, 2.0 mmol/L L-glutamine, 2 mmol/L L-[U-^{14}C]proline (150 Bq/nmol), and 0–200 μmol/L gabaculine. L-Glutamine was added to the incubation medium to provide

the following: *1*) ammonia for CPS-I, *2*) glutamate for OAT and *3*) aspartate for ASS. Incubations were initiated by addition of cells. After a 30-min incubation period, 0.5 mL of incubation medium containing cells was removed from the flask for determination of the intracellular specific activities of [^{14}C]proline, [^{14}C]ornithine and [^{14}C]citrulline as described by Wu (1997). The remaining portion of medium plus cells (1.5 mL) was acidified with 0.2 mL of 1.5 mol/L $HClO_4$ and neutralized with 0.1 mL of 2 mol/L K_2CO_3. Cell extracts were used for analysis by HPLC of amino acids and [^{14}C]ornithine, [^{14}C]citrulline and [^{14}C]arginine (Wu 1997). The intracellular specific activities of [^{14}C]proline, [^{14}C]ornithine and [^{14}C]citrulline were used to calculate production of [^{14}C]ornithine, [^{14}C]citrulline and [^{14}C]arginine from proline, respectively.

Effect of oral administration of gabaculine on plasma concentrations of free amino acids, ammonia and urea. Pigs (75 d old) were randomly assigned to one of two groups with eight animals each. One group of pigs received oral administration of gabaculine (0.83 mg/kg body weight) at 600, 1000 and 1400 h. The other group received an equivalent volume of vehicle solvent (double distilled deionized water) according to the same time schedule. Gabaculine was dissolved in 5 mL of water and then delivered orally via a 5-mL syringe. Blood samples (5 mL) were drawn from the jugular vein into heparinized tubes at 1600 h of the day before gabaculine administration (initial blood sampling) and again at 1600 h on the day of gabaculine administration (final blood sampling). Blood samples were placed in ice and centrifuged at 2000 × *g* for 15 min to obtain plasma (the supernatant fraction). Plasma (1 mL) was deproteinized with 1 ml of 1.5 mol/L $HClO_4$, and neutralized with 0.5 mL of 2 mol/L K_2CO_3. Neutralized plasma was centrifuged at 600 × *g* for 10 min, and the supernatant fraction was used for analysis of ammonia and urea by colorimetric methods (Wu and Knabe 1994) and of amino acids by HPLC (Wu et al. 1994).

Amino acid analysis by HPLC. The HPLC apparatus and precolumn derivatization of amino acids with *o*-phthaldialdehyde were as previously described (Wu 1993). Amino acids [except proline and cyst(e)ine] were separated on a Supelco 3-μm reversed-phase C18 column (4.6 × 150 mm, i.d.) guarded by a Supelco 40-μm reversed-phase C18 column (4.6 × 50 mm, i.d.; Supelco, Bellefonte, PA). The HPLC mobile phase consisted of solvent A (0.1 mol/L sodium acetate/0.5% tetrahydrofuran/9% methanol; pH 7.2) and solvent B (methanol), with a combined total flow rate of 1.1 mL/min. A gradient program with a total running time of 49 min (including the time for column regeneration) was developed for satisfactory separation of amino acids (0 min, 14% B; 15 min, 14% B; 20 min, 30% B; 24 min, 35% B; 26 min, 47% B; 34 min, 50% B; 38 min, 70% B; 40 min, 100% B; 42 min, 100% B; 42.1 min, 14% B; 48.5 min, 14% B). Proline was measured by an HPLC method involving oxidation of proline to 4-amino-1-butanol and precolumn derivatization with *o*-phthaldialdehyde (Wu 1993). Cysteine and cystine were analyzed by an HPLC method as previously described (Wu and Knabe 1994). Briefly, for cysteine analysis, a 100-μL sample was mixed with 50 μL of 50 mmol/L iodoacetic acid (an alkylating agent) for 5 min at room temperature to convert cysteine to *S*-carboxymethylcysteine. The latter then reacts with *o*-phthaldialdehyde to form a highly fluorescent derivative. For cystine analysis, a 100-μL sample was mixed with 100 μL of 28 mmol/L 2-mercaptoethanol (a reducing agent) for 5 min at room temperature to convert cystine to cysteine, and the latter was then analyzed as described above.

Protein determination. Protein in enterocytes, mitochondria and cytosolic fluid was determined by a modified Lowry procedure, with BSA as a standard (Wu et al. 1994).

Statistical analysis. Results were statistically analyzed as described by Steel and Torrie (1980). Data on enzyme activities and metabolism of glutamine and proline were analyzed by one-way ANOVA and the Student-Newman-Keuls test. Unpaired *t* test was used to analyze the differences in plasma metabolites between control and gabaculine-treated pigs. Paired *t* test was used to analyze the differences in plasma metabolites between the initial and final blood sampling periods for each group of pigs. Probabilities <0.05 were taken to indicate statistical significance.

RESULTS

Activities of OAT, proline oxidase and P5C synthase in pig tissues. Ornithine aminotransferase activity was detected in all pig tissues studied, with the greatest activity in jejunal mucosa (**Table 2**). The OAT activity in liver and kidney was ~2 and 1% of the jejunal value, respectively. Proline oxidase activity was present in all the tissues studied except for the adipose tissue, pancreas, skeletal muscle and spleen, with the greatest activity in jejunal mucosa. Proline oxidase activity in pig liver and kidney was ~14 and 25% of the jejunal value, respectively. P5C synthase activity was found only in jejunal mucosa and the intact jejunum among all of the pig tissues examined.

TABLE 2

Ornithine aminotransferase (OAT), proline oxidase and pyrroline-5-carboxylate (P5C) synthase in pig tissues[1]

Tissues	OAT	Proline oxidase	P5C synthase
	nmol P5C/(min · mg protein)		
Adipose tissue	6.5 ± 0.50^{d}	ND[2]	ND
Brain	6.7 ± 0.51^{d}	0.67 ± 0.08^{e}	ND
Colon	3.5 ± 0.28^{e}	0.61 ± 0.05^{e}	ND
Heart	6.1 ± 0.38^{d}	0.70 ± 0.06^{e}	ND
Jejunal mucosa	197.4 ± 12.5^{a}	13.4 ± 1.4^{a}	0.316 ± 0.038^{a}
Jejunum (intact)	32.1 ± 2.6^{b}	5.6 ± 0.47^{b}	0.046 ± 0.006^{b}
Jejunal serosa	10.1 ± 1.5^{c}	0.66 ± 0.08^{e}	ND
Kidney	1.8 ± 0.10^{f}	3.3 ± 0.24^{c}	ND
Liver	3.4 ± 0.30^{e}	1.9 ± 0.15^{d}	ND
Lung	3.7 ± 0.22^{e}	0.53 ± 0.07^{e}	ND
Pancreas	1.6 ± 0.11^{f}	ND	ND
Skeletal muscle	1.7 ± 0.20^{f}	ND	ND
Spleen	1.5 ± 0.17^{f}	ND	ND

[1] Data are means ± SEM, n = 6. Means with different letters in a column are different ($P < 0.05$), as analyzed by one-way ANOVA and the Student-Newman-Keuls test.

[2] ND, enzyme activities were not detected.

Inhibition by gabaculine of synthesis of ornithine and citrulline from glutamine and proline in enterocytes. Pig enterocytes synthesized ornithine and citrulline from glutamine and proline (**Tables 3** and **4**). Gabaculine (0–200 μmol/L) decreased ($P < 0.05$) the synthesis of ornithine and citrulline from glutamine and proline in a concentration-dependent manner. Gabaculine at 100 and 200 μmol/L also reduced ($P < 0.05$) alanine production from glutamine by 23 and 39%, respectively, but had no effect on production of other amino acids from glutamine. Gabaculine had no effect ($P > 0.05$) on the production of phenylalanine, an amino acid that is neither synthesized nor degraded by enterocytes (Wu et al. 1996), indicating that gabaculine had no effect on net proteolysis. No detectable amount of [^{14}C]-labeled amino acids other than [^{14}C]ornithine, [^{14}C]citrulline and [^{14}C]arginine was produced from [^{14}C]proline in enterocytes incubated in the presence of 0–200 μmol/L gabaculine.

Effects of oral administration of gabaculine on plasma concentrations of free amino acids, ammonia and urea in pigs. These data are summarized in **Table 5**. In control pigs not treated with gabaculine, initial and final plasma concentrations of amino acids, ammonia and urea did not differ ($P > 0.05$). Oral administration of gabaculine had the following effects: *1*) decreased ($P < 0.05$) plasma concentrations of arginine (−22%) and citrulline (−26%), *2*) increased ($P < 0.05$) plasma concentrations of alanine (+21%), isoleucine (+28%), leucine (+21%), ornithine (+17%), proline (+107%), taurine (+56%), threonine (+48%) and valine (+40%), and *3*) had no effect ($P > 0.05$) on other amino acids, ammonia or urea.

TABLE 3

Net production of amino acids by pig enterocytes incubated in the presence or absence of gabaculine[1,2]

Amino acid	Medium gabaculine concentration, μmol/L				
	0	10	50	100	200
	nmol/(30 min · mg protein)				
Orn	0.51 ± 0.06[a]	0.35 ± 0.04[b]	0.24 ± 0.03[c]	ND[3]	ND
Cit	5.61 ± 0.50[a]	4.29 ± 0.26[b]	1.60 ± 0.12[c]	0.52 ± 0.06[d]	ND
Glu	62.2 ± 7.0	61.4 ± 68	63.1 ± 7.5	68.2 ± 7.3	69.8 ± 8.1
Ala	57.5 ± 6.0[a]	56.4 ± 5.3[a]	51.2 ± 4.8[ab]	44.3 ± 4.0[b]	32.5 ± 2.8[c]
Asp	12.9 ± 1.2	12.0 ± 1.4	12.6 ± 1.7	13.0 ± 1.5	13.8 ± 1.2
Phe	2.37 ± 0.28	2.39 ± 0.25	2.50 ± 0.31	2.52 ± 0.26	2.33 ± 0.34

[1] Values are means ± SEM, $n = 6$. Means with different letters in a row are different ($P < 0.05$), as analyzed by one-way ANOVA and the Student-Newman-Keuls test.

[2] Pig enterocytes were incubated at 37°C for 0 or 30 min in the presence of 2 mmol/L L-glutamine plus 2 mmol/L L-proline and 0–200 μmol/L gabuculine, as described in text. Net production of amino acids was calculated on the basis of the difference in their concentrations in cell extracts between 0- and 30-min incubation periods. Basal values were 0.42 ± 0.06 ornithine (Orn), 2.30 ± 0.28 citrulline (Cit), 23.3 ± 2.6 glutamate (Glu), 13.0 ± 1.6 alanine (Ala), 3.2 ± 0.37 aspartate (Asp) and 0.31 ± 0.03 phenylalanine (Phe) nmol/mg protein (mean ± SEM, $n = 6$).

[3] ND, net production of amino acids was not detected.

Effect of oral administration of gabaculine on feed intake by pigs. Feed consumption did not differ ($P > 0.05$) between control and gabaculine-treated pigs and averaged 51.1 g/(kg body wt · d). Intake of dietary arginine was similar between control and gabaculine-treated pigs and averaged 0.50 g/(kg body wt · d).

DISCUSSION

An important role for the small intestine in synthesizing citrulline. Arginine synthesis via the urea cycle occurs in the liver and small intestine. Although arginine is formed via the urea cycle in periportal hepatocytes, an exceedingly high activity of cytosolic arginase in the liver rapidly hydrolyzes arginine to urea and ornithine and therefore precludes net synthesis of arginine by the liver under physiologic conditions (Morris 1992). Because hepatic OAT is absent from periportal hepatocytes and is restricted to perivenous hepatocytes with no CPS-I or OCT activity (Kuo et al. 1991), proline-derived P5C cannot be converted to citrulline in perivenous hepatocytes or to ornithine in periportal hepatocytes. As a result, there is no synthesis of citrulline from proline in the liver. Similarly, because the kidney contains no CPS-I or OCT activity (Edmonds et al. 1987, Morris 1992), there is no synthesis of citrulline from proline in this organ. Thus, as the only organ that contains all enzymes for synthesizing citrulline from glutamine and proline in animals (Wu 1997, Wu and Knabe 1995), the small intestine plays a major role in citrulline and arginine metabolism. In postnatal pig enterocytes, ~80–90% of utilized proline carbons were recovered in ornithine plus citrulline plus arginine (with CO_2 being a minor product) (Wu 1997). Considering the relatively large mass of the small intestine compared with the liver and kidney in growing pigs (e.g., 398, 246 and 41 g in 6-wk-old pigs, respectively) (Schoknecht and Pond 1993), we suggest that the small intestine is a major organ for metabolism of ornithine and proline and for synthesis of citrulline from glutamine and proline in postweaning pigs. The intestine-derived citrulline is utilized for arginine synthesis in the kidneys of postweaning pigs, as in adult rats (Dhanakoti et al. 1990), which have high activities of ASS and ASL (Wu and Knabe 1995).

There appear to be species differences in ornithine and proline metabolism between pigs and rats on the basis of the following findings. First, OAT was found to be present predominantly in the small intestine of neonatal and postweaning pigs (Table 2), whereas this enzyme was reported to have greatest activity in the liver and kidney of rats (Herzfeld and Knox 1968, Herzfeld et al. 1977). Second, proline oxidase activity was found to be greatest in the small intestine of neonatal and postweaning pigs (Table 2) (Samuels et al. 1989), but was reported to be negligible in or absent from the intestine of neonatal and adult rats (Herzfeld et al. 1977). Our findings

TABLE 4

Effect of gabaculine on net production of [14C]ornithine, [14C]citrulline and [14C]arginine from [14C]proline in pig enterocytes[1,2]

Amino acid	Medium gabaculine concentration (μmol/L)				
	0	10	50	100	200
	nmol/(30 min · mg protein)				
[14C]Orn	6.04 ± 0.52[a]	4.37 ± 0.05[b]	1.21 ± 0.02[c]	0.58 ± 0.07[d]	ND
[14C]Cit	8.28 ± 0.73[a]	6.50 ± 0.59[b]	1.96 ± 0.27[c]	0.66 ± 0.08[d]	ND
[14C]Arg	0.68 ± 0.08[a]	0.47 ± 0.06[b]	ND[3]	ND	ND

[1] Values are means ± SEM, $n = 6$. Means with different letters in a row are different ($P < 0.05$), as analyzed by one-way ANOVA and the Student-Newman-Keuls test.

[2] Pig enterocytes were incubated at 37°C for 30 min in the presence of 2 mmol/L L-glutamine plus 2 mmol/L L-[U-14C]proline and 0–200 μmol/L gabaculine, as described in text.

[3] ND, net production of [14C]amino acids was not detected.

TABLE 5

Plasma concentrations of free amino acids, ammonia and urea in pigs[1]

Metabolite	Control pigs		Gabaculine-treated pigs	
	Initial	Final	Initial	Final
		μmol/L		
Ala	596 ± 32	631 ± 34	582 ± 46	705 ± 32*
Arg	159 ± 16	146 ± 12	163 ± 10	127 ± 6*
Asn	62 ± 3	57 ± 2	58 ± 3	63 ± 5
Asp	13 ± 2	14 ± 1	12 ± 1	14 ± 1
Cit	64 ± 2	60 ± 3	73 ± 6	54 ± 4*
Cystine	84 ± 4	85 ± 3	82 ± 3	81 ± 2
Cysteine	4.6 ± 0.6	4.3 ± 0.5	4.4 ± 0.5	4.1 ± 0.7
Gln	513 ± 28	520 ± 37	531 ± 19	527 ± 35
Glu	172 ± 14	179 ± 15	145 ± 6	158 ± 12
Gly	664 ± 22	688 ± 29	753 ± 36	799 ± 35
His	103 ± 7	111 ± 7	100 ± 6	105 ± 7
Ile	112 ± 10	118 ± 8	102 ± 4	131 ± 5*
Leu	246 ± 15	240 ± 12	234 ± 8	283 ± 6*
Lys	88 ± 17	89 ± 11	83 ± 10	81 ± 7
Met	35 ± 2	35 ± 3	32 ± 3	36 ± 2
Orn	85 ± 6	86 ± 7	84 ± 4	98 ± 6*
Phe	87 ± 4	81 ± 3	80 ± 9	88 ± 5
Pro	395 ± 21	391 ± 28	363 ± 17	752 ± 63**‡
Ser	153 ± 16	159 ± 12	180 ± 9	175 ± 14
Taurine	56 ± 6	57 ± 7	59 ± 4	92 ± 7**‡
Thr	89 ± 16	83 ± 13	86 ± 8	127 ± 11**
Trp	47 ± 3	46 ± 2	46 ± 3	50 ± 3
Tyr	105 ± 6	93 ± 5	97 ± 7	105 ± 5
Val	280 ± 13	296 ± 13	252 ± 6	353 ± 9**
Ammonia	263 ± 7	251 ± 7	251 ± 7	238 ± 13
Urea	3517 ± 128	3614 ± 168	3246 ± 179	3628 ± 202

[1] Values are means ± SEM, $n = 8$. *$P < 0.05$ and **$P < 0.01$, different from the initial value, analyzed by paired t test; ‡ $P < 0.05$, different from the control group, as analyzed by unpaired t test.

with pigs are in contrast to the current view that proline oxidase is present primarily in the liver, kidney and brain of mammals (Phang et al. 1995).

An important role for OAT in intestinal synthesis of citrulline from glutamine and proline. Ornithine aminotransferase interconverts P5C into ornithine and therefore plays an important role in both synthesis and degradation of glutamine, proline, ornithine, citrulline and arginine (Valle and Simell 1995). An inhibition of OAT is expected to inhibit synthesis of ornithine and citrulline from both glutamine and proline in enterocytes. This suggestion is supported by our findings that gabaculine [a potent inhibitor of pig enterocyte OAT; $K_i = 19$ μmol/L (Davis 1997)] markedly decreased the synthesis of ornithine and citrulline from glutamine and proline by pig enterocytes in a concentration-dependent manner (Tables 2 and 3). Because gabaculine does not interfere directly with the urea cycle and because a short-term treatment of gabaculine in pigs (Flynn and Wu 1996) and mice (Alonso and Rubio 1989) does not result in ammonia toxicity or any other adverse effects, oral administration of gabaculine was an attractive approach to decrease synthesis of citrulline and arginine from glutamine and proline in pigs and to determine the role for endogenous arginine synthesis in regulating arginine homeostasis in vivo.

Effect of OAT inhibition on plasma concentrations of citrulline and arginine. This is the first study to determine the role for endogenous synthesis of arginine in maintaining arginine homeostasis in postweaning growing pigs. Oral administration of gabaculine resulted in decreased plasma concentrations of citrulline and arginine in postweaning 75-d-old pigs in the fed state (Table 5), as in neonatal pigs nursed by sows (Flynn and Wu 1996). Indeed, citrulline and arginine were the only two amino acids whose plasma concentrations were reduced by gabaculine treatment. Because gabaculine inhibits arginine degradation (Wu et al. 1996) and has no effect on dietary arginine intake, and because citrulline is absent from dietary and tissue proteins, we interpret the decreased plasma concentration of citrulline and arginine in gabaculine-treated pigs as likely resulting from decreased endogenous synthesis of these two amino acids. To substantiate this suggestion, studies involving stable or radioactive tracers are required to quantify rates of arginine synthesis in control and gabaculine-treated pigs.

The following calculation suggests that dietary arginine intake alone is insufficient to meet arginine needs of the 28-kg growing pig. Protein deposition in the 28-kg pig is estimated to be 128 g/d (Campbell et al. 1983). On the basis of arginine content in pig tissue protein (6.18 g arginine/100 g protein) (Wilson and Leibholz 1981), arginine requirement for net protein deposition is 7.91 g/d ($6.18 \times 128/100 = 7.91$). On the basis of the oxidation of plasma arginine to CO_2 [10.6 μmol/(kg·h)] and the conversion of plasma arginine to proline, glutamate, citrulline and ornithine [a total of 51.7 μmol/(kg·h)] in the young pig (Murch et al. 1996), the rate of catabolism of arginine to these products is 7.28 g/d. Thus a total requirement for arginine by the 28-kg pig is ≥ 15.19 g/d ($7.91 + 7.28 = 15.19$). The amount of dietary arginine entering the portal vein is estimated to be 7.56 g/d, on the basis of dietary arginine intake (14.02 g/d) and the following assumptions: *1*) digestibility of arginine in feed is 90% (Knabe

et al. 1988); and *2*) 60% of luminal free arginine is absorbed intact by the small intestine (Windmueller and Spaeth 1976). Thus endogenous synthesis of arginine in the 28-kg pig is estimated to provide ≥ 7.63 g/d of arginine (15.19 − 7.56 = 7.63), or 50.2% of total daily arginine requirement, suggesting that endogenous arginine synthesis plays an important role in regulating arginine homeostasis in postweaning growing pigs, as previously shown in neonatal pigs (Flynn and Wu 1996). This suggestion is not consistent with previous findings that arginine homeostasis is regulated mainly by dietary arginine intake and arginine oxidation rather than by endogenous arginine synthesis in adult humans (Castillo et al. 1993). It is important to determine whether endogenous synthesis of arginine from glutamine/glutamate and proline plays an important role in regulating arginine homeostasis in growing children with net protein deposition in the body.

Paradoxical effect of OAT inhibition on plasma concentrations of ornithine. Gabaculine treatment increased plasma concentrations of ornithine (17%) in postweaning, 75-d-old pigs (Table 5), but decreased plasma concentrations of ornithine by 59% in neonatal pigs (Flynn and Wu 1996). Such a paradoxical effect of OAT inhibition has been reported in mice and humans, in that a deficiency of OAT decreases plasma concentration of ornithine in neonates but resulted in hyperornithinemia in adults (Wang et al. 1995, Valle and Simell 1995). In gabaculine-treated pigs, the source of elevated plasma ornithine is likely to be arginine, because gabaculine decreased ornithine synthesis from glutamine and proline (Tables 2 and 3). Both arginase and OAT are ubiquitous in animal tissues (Jenkinson et al. 1996, Valle and Simell 1995). Arginase I is located almost exclusively in hepatocytes, whereas arginase II is widely distributed in extrahepatic tissues (Jenkinson et al. 1996). In the liver, OAT is restricted to perivenous hepatocytes (Kuo et al. 1991), which are now known to contain arginase activity (O'Sullivan et al. 1996). Thus, arginase I and arginase II play an important role in generating ornithine from arginine in hepatic and extrahepatic tissues, respectively. Because of underdeveloped arginase activity in neonatal tissues including the small intestine (Wu et al. 1996), liver (Greengard et al. 1970) and kidney (Konarska and Tomaszewski 1986), arginine may quantitatively be a more important source of ornithine in postweaning animals and adults than in neonates. Thus, an inhibition of OAT may have a greater effect on plasma ornithine accumulation in postweaning pigs than in neonatal pigs. This may contribute to increased plasma ornithine concentrations in gabaculine-treated postweaning pigs (Table 5) and in OAT-deficient mice and humans (Valle and Simell 1995, Wang et al. 1995). Because extracellular ornithine is a poor precursor of citrulline and arginine in pig enterocytes due to preferential conversion of ornithine to proline (Wu et al. 1996), an increase in plasma concentrations of ornithine would not contribute to a significant increase in the synthesis of citrulline and arginine. This is consistent with the previous findings that increasing plasma concentrations of ornithine had little effect on those of citrulline and arginine in pigs (Edmonds et al. 1987) and humans (Castillo et al. 1995). Thus, the compartmentation of ornithine metabolism at cellular and systemic levels, as well as developmental changes of tissue arginase activities may explain why plasma citrulline and arginine were decreased despite an increase in plasma concentrations of ornithine in postweaning pigs (Table 5). This may also help to explain the paradox that an OAT deficiency results in hypo-ornithinemia in neonates of mice and humans but hyperornithinemia in adults (Valle and Simell 1995, Wang 1995).

Effects of OAT inhibition on plasma concentrations of glutamine, proline and amino acids. Gabaculine is an inhibitor of OAT and other pyridoxal phosphate-dependent aminotransferases such as L-alanine transaminase, L-aspartate transaminase, and branched-chain amino acid aminotransferase (Soper and Manning 1982). These enzymes catalyze metabolism of alanine, aspartate and branched-chain amino acids (isoleucine, leucine and valine), in addition to ornithine. This may explain our in vivo observation that oral administration of gabaculine increased plasma concentrations of alanine, branched-chain amino acids and particularly proline (Table 5). This result further supports the view that OAT plays a major role in proline degradation (Valle and Simell 1995). Gabaculine treatment increased plasma concentrations of taurine in postweaning pigs (Table 5) as in neonatal pigs (Flynn and Wu 1996), suggesting that gabaculine inhibits taurine degradation whose catabolism requires pyridoxal phosphate–dependent taurine-α-ketoglutarate aminotransferase (Toyama et al. 1978). Finally, it is noteworthy that gabaculine treatment had no effect on plasma concentrations of glutamine in postweaning 75-d-old pigs (Table 5), in contrast to neonatal pigs (Flynn and Wu 1996). Because dietary glutamine does not enter the portal vein because of its extensive catabolism in the small intestine (Curthoys and Watford 1995), plasma concentrations of glutamine depend on the balance between glutamine synthesis and degradation in the body. It is likely that gabaculine decreases glutamine utilization by the small intestine to an extent similar to that by which gabaculine inhibits glutamine synthesis from branched-chain amino acids in the animal.

In conclusion, OAT and proline oxidase activities were greatest in the small intestine among all of the porcine tissues studied, and P5C synthase was exclusively located in enterocytes. The tissue distribution of these enzyme activities in postweaning 75-d-old pigs suggests that glutamine/glutamate and proline are important precursors for intestinal synthesis of citrulline and arginine. An inhibition of OAT by gabaculine decreased intestinal synthesis of citrulline and arginine, as well as plasma concentrations of both citrulline and arginine. These results, taken together with dietary arginine intake and estimated arginine utilization, suggest that endogenous synthesis of arginine from glutamine/glutamate and proline plays an important role in maintaining arginine homeostasis in postweaning growing pigs, as in neonatal pigs.

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