Urea synthesis in enterocytes of developing pigs

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Urea synthesis from ammonia, glutamine and arginine was determined in enterocytes from newborn (0-day-old), 2–21-day-old suckling, and 29–58-day-old post-weaning pigs. Pigs were weaned at 21 days of age. Cells were incubated for 30 min at 37 °C in Krebs–Henseleit bicarbonate buffer (pH 7.4) containing (i) 0.5–2 mM NH_4Cl plus 0.05–2 mM ornithine and 2 mM aspartate, (ii) 1–5 mM glutamine, or (iii) 0.5–2 mM arginine. In enterocytes from newborn and suckling pigs, there was no measurable synthesis of urea from ammonia, glutamine or arginine, and analysis of amino acids by a sensitive fluorimetric HPLC method revealed the formation of negligible amounts of ornithine from arginine. In contrast, in cells from post-weaning pigs, relatively large amounts of urea and ornithine were produced from ammonia, glutamine and arginine in a dose-dependent manner. To elucidate the mechanism of the developmental change of urea synthesis in pig enterocytes, the activities of urea-cycle enzymes were determined. The activities of enterocyte carbamoyl phosphate synthase I and ornithine carbamoyltransferase were lower in post-weaning pigs than in suckling ones, whereas there was no difference in argininosuccinate lyase. The activities of argininosuccinate synthase and arginase were increased by 4-fold and 50–100-fold, respectively, in enterocytes from post-weaning pigs compared with suckling pigs. The induction of arginase appears to be sufficient to account for the formation of urea from ammonia, glutamine and arginine in post-weaning pig enterocytes. These results demonstrate for the first time the presence of synthesis of urea from extracellular or intramitochondrially generated ammonia in enterocytes of post-weaning pigs. This hitherto unrecognized urea synthesis in these cells may be a first line of defence against the potential toxicity of ammonia produced by the extensive intestinal degradation of glutamine (a major fuel for enterocytes) and derived from diet and luminal micro-organisms.

INTRODUCTION

It is generally accepted that the liver is the only organ in which urea is formed from ammonia via the urea cycle in mammalian species [1,2]. The synthesis of urea from ammonia involves carbamoyl phosphate synthase I (CPS I) (EC 6.3.4.16), ornithine carbamoyltransferase (OCT) (EC 2.1.3.3), argininosuccinate synthase (ASS) (EC 6.3.4.5), argininosuccinate lyase (ASL) (EC 4.3.2.1), and arginase (EC 3.5.3.1). The major function of the hepatic urea cycle is to convert ammonia into water-soluble and non-toxic urea, although this cycle has also been proposed to play an important role in the regulation of acid–base balance in mammalian species [3]. The physiological importance of the urea cycle is epitomized by the previous observations that a deficiency in one of the enzymes involved results in ammonia toxicity, retarded growth, and even death in humans and other mammals [4,5].

The small intestine is not only the major site of nutrient absorption but also an important organ for the metabolism of amino acids, particularly glutamine, citrulline and arginine [6,7]. It is often assumed that the ammonia produced from intestinal degradation of amino acids is released into the portal vein and taken up by the liver for ureagenesis [8,9]. However, little information is available on ammonia metabolism in intestinal epithelial cells or the small intestine. Interestingly, a number of studies have demonstrated the presence of CPS I, OCT and arginase in pig enterocytes [10,11], CPS I, ASL and arginase in mouse intestinal mucosa [12], and CPS I, OCT, ASS and arginase in the rat small intestine [13–18]. On the basis of the presence of some urea-cycle enzymes in the small intestine, several investigators have previously hypothesized that a metabolically significant urea cycle may be functioning in this organ [12,15]. This hypothesis, however, has not been tested in intestinal epithelial cells or the small intestine.

The objective of this study was to determine whether urea is synthesized from extracellular and intracellularly-generated ammonia in enterocytes of the pig, a widely used animal model for studying mammalian intestinal development [19,20]. The results demonstrate urea synthesis from ammonia, glutamine and arginine in enterocytes from post-weaning, but not pre-weaning, pigs.

MATERIALS AND METHODS

Chemicals

L-Glutamine, L-arginine, L-ornithine, L-aspartate, L-argininosuccinate, D-glucose, ammonium chloride, sodium azide, BSA (fraction V, essentially fatty-acids free), Hepes, ornithine carbamoyltransferase and N^G-nitro-L-arginine were purchased from Sigma Chemical Co. (St. Louis, MO). Glutamate dehydrogenase, α-ketoglutaric acid, β-NADH and ADP were obtained from Boehringer Mannheim (Indianapolis, IN). HPLC-grade methanol and water were obtained from Fisher Scientific (Houston, TX). [^{14}C]Urea was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). RPMI-1640 medium and fetal calf serum (FCS) were obtained from GIBCO (Grand Island, NY). Percoll was obtained from Pharmacia (Alameda, CA).

Antibodies

Monoclonal antibodies recognizing porcine macrophages/monocytes (anti-M), T-cells (anti-CD5) and B-cells (anti-IgM) were obtained from Veterinary Medical Research and Development, Inc. (Pullman, WA). Fluorescein-conjugated goat affinity-purified $F(AB')_2$-fragment to mouse immunoglobulins

Abbreviations used: ASL: argininosuccinate lyase; ASS: argininosuccinate synthase; CP, carbamoyl phosphate; CPS I, carbamoyl phosphate synthase I; FCS: fetal calf serum; KHB, Krebs–Henseleit bicarbonate; IEL: intraepithelial lymphocytes; OCT, ornithine carbamoyltransferase.

(IgG, IgA, IgM) was purchased from Organon Teknika Corp. (West Chester, PA), and used as the secondary antibody.

Animals and the isolation of jejunum

Pigs were offspring of Yorkshire × Landrace sows and Duroc × Hampshire boars, and were obtained from the Swine Center of Texas A&M University. Newborn (0-day-old) pigs were used within 1 h of farrowing and did not receive colostrum or water. Suckling pigs were nursed by their mothers until 21 days of age, when they were removed from the sows. Post-weaning pigs were fed a corn/soybean-meal-based diet (20% protein) that met the NRC requirements of all nutrients [11,21]. Jejunum was dissected from individual anaesthetized pigs, as previously described [11]. The experiments were performed in accordance with the guidelines of the National Research Council for the care and use of animals, and were approved by Texas A&M University's Institutional Animal Care Committee.

Preparation and incubation of enterocytes

Enterocytes were prepared from the pig jejunum, using oxygenated (O_2/CO_2, 19:1) Ca^{2+}-free Krebs–Henseleit bicarbonate (KHB) buffer supplemented with 5 mM EDTA, as described by Wu et al. [11,21]. The consumption of O_2 by the prepared enterocytes in the presence of 1 mM glutamine plus 5 mM glucose was linear for at least 30 min, as measured with a Clark-type polarographic oxygen probe [11], indicating the viability of these cells. Incubations were performed at 37 °C in 25 ml polypropylene conical flasks placed in a shaking water bath (70 rev./min). Cells (4–5 mg protein per ml) were incubated for 30 min in the presence of 2 ml KHB of buffer (pH 7.4; saturated with O_2/CO_2, 19:1) containing 20 mM HEPES, 1% BSA and 5 mM glucose. The incubation medium also contained (i) 0.5–2 mM NH_4Cl plus 0.05–2 mM ornithine and 2 mM aspartate, (ii) 1–5 mM glutamine, or (iii) 0.5–2 mM arginine with or without 2 mM N^G-nitro-L-arginine. The control incubation medium contained none of these added substrates. The gas phase in the flask was O_2/CO_2 (19:1). Incubations were initiated by addition of cells and terminated by addition of 0.2 ml of 1.5 M $HClO_4$. Neutralized cell extracts were used for measurements of urea and amino acids as described below. It was established in preliminary experiments that the rates of urea synthesis from glutamine, arginine and ammonia in post-weaning pig enterocytes were linear during the 30 min incubation period.

Measurements of metabolites

Amino acids including arginine, citrulline, ornithine and proline in neutralized cell extracts were quantified by sensitive fluorimetric HPLC methods involving pre-column derivatization with *o*-phthaldialdehyde [11]. For analysis of urea, 1 ml of neutralized sample was loaded into Dowex AG 50W-X8 resin (H^+ form, 200–400 mesh) (0.6 cm × 4 cm). After washing the column with 10 ml water, urea was eluted with 10 ml water. The water used was deionized and glass-distilled, and had a pH of 6.02. The solution was freeze-dried and the residue was suspended in 0.5 ml water for analysis of urea by a sensitive fluorimetric method involving urease and glutamate dehydrogenase [22]. The collected 10 ml fraction contained 100% of added urea but no ammonia, as determined with urea and NH_4Cl standards.

Determination of carbamoyl phosphate content in enterocytes

Cells were incubated for 30 min at 37 °C in the presence of (i) 5 mM glutamine, or (ii) NH_4Cl plus ornithine and aspartate (2 mM each). At the end of the incubation period, enterocytes were obtained by centrifugation (10000 *g*, 1 min) and were lysed by three cycles of freezing and thawing at 37 °C. The cell extracts were used for determination of carbamoyl phosphate with ornithine and ornithine carbamoyltransferase [11].

Measurements of the activities of urea-cycle enzymes in enterocytes

Enterocytes were suspended in KHB buffer containing 1 mM dithiothreitol and 0.5% Triton X-100, and cell extracts were prepared by three cycles of freezing in liquid nitrogen and thawing at 37 °C. The supernatant fluids obtained after centrifugation (13000 *g*, 1 min) were used for the assays of CPS I, OCT, ASS, ASL and arginase. The activities of CPS I and OCT were determined as described by Wu et al. [11]. Briefly, the assay mixture for CPS I (0.5 ml) consisted of 0.15 M potassium phosphate buffer (pH 7.5), 25 mM ATP, 25 mM $MgCl_2$, 5 mM *N*-acetylglutamate, 10 mM NH_4Cl, 5 mM ornithine, 5 mM $NaHCO_3$, and 10 units of ornithine carbamoyltransferase. The assay medium for OCT (0.2 ml) contained 0.1 M potassium phosphate buffer (pH 7.5), 20 mM ornithine, 10 mM carbamoyl phosphate, and cell extracts (0.4 mg protein).

The activities of ASS, ASL and arginase were measured as described by Dhanakoti et al. [23], except that argininosuccinate (AS), arginine and ornithine were measured by a sensitive fluorimetric HPLC method [11]. The ASS assay mixture (200 μl) consisted of 75 mM potassium phosphate buffer (pH 7.5), 10 mM citrulline, 10 mM aspartate, 5 mM ATP, and cell lysates (0.4 mg protein), and was incubated at 37 °C for 0 or 20 min. The incubation was terminated by addition of 50 μl of 1.5 M $HClO_4$. Neutralized extracts were used for analysis of AS and arginine by HPLC using *o*-phthaldialdehyde pre-column derivatization [11]. The sum of AS and arginine was used to indicate ASS activity, because some AS (less than 10%) was converted to arginine by ASL in cell extracts. The ASL assay mixture (40 μl) contained 129 mM sodium phosphate buffer (pH 7.0), 20 mM AS and 65 mM EDTA, and was incubated at 37 °C for 0 or 15 min. The incubation was terminated by addition of 20 μl of 1.5 M $HClO_4$, and neutralized extracts were used for arginine analysis by HPLC [11] to indicate ASL activity. For arginase assay, a mixture of 50 μl of 100 mM $MnCl_2$ and 50 μl of cell lysate (0.5 mg protein) was incubated at 55 °C for 5 min. After cooling to room temperature, the mixture was incubated with 50 μl of 30 mM arginine (in 150 mM potassium phosphate buffer, pH 7.5) at 37 °C for 0 or 10 min. The incubation was terminated by addition of 50 μl of 1.5 M $HClO_4$, and neutralized extracts were used for analysis of ornithine and urea as described above. Because the amount of urea produced by arginase in extracts of enterocytes from 0–21-day-old pigs was below or close to the detection limit of the fluorimetric enzymic method used, the measurement of ornithine by HPLC was used to indicate arginase activity.

It was established in preliminary experiments that all enzyme assays were linear with the times and the amounts of cell protein used. Protein was determined by a modified Lowry method, with BSA as standard [11].

Flow cytometric analysis of mononuclear cells in enterocyte preparations

This was performed as described for rat lymphocytes and macrophages [24], with some modifications. Briefly, enterocyte suspensions (5×10^5 cells) in 50 μl of RPMI-1640 medium containing 10% (w/v) FCS and 0.5% sodium azide (FACS

medium) were incubated with 50 μl of FACS medium, anti-M (15 μg/ml), anti-IgM (15 μg/ml) or anti-CD5 (15 μg/ml) at 4 °C for 30 min. The cell pellets were washed three times with 200 μl of FACS medium, and then incubated with the fluorescein-conjugated secondary antibody at 4 °C for 30 min. The pellets were again washed three times with 200 μl of FACS medium and mixed with 300 μl of phosphate-buffered saline (pH 7.2) containing 1% (w/v) sodium azide and 1% (w/v) paraformaldehyde. Viable cells (20000) were analysed in each sample for indirect fluorescence on a FACScan flow cytometer [25].

Measurements of arginase activity and formation of urea from arginine, glutamine or ammonia in intraepithelial lymphocytes (IEL)

IEL were prepared from jejunum of 21-day-old pigs and 29–58-day-old pigs weaned at 21 days of age, as previously described [37]. The proportions of T-cells, B-cells and macrophages in the IEL preparation were 92±6%, 5±0.4%, and 0% (means±S.E.M., $n = 6$), respectively, as assessed by the above flow cytometric analysis. Arginase activity in IEL extracts was measured at 37 °C for 0 or 10 min as described above. For metabolic studies, IEL were incubated (4–5 mg protein/ml) at 37 °C for 0 or 1 h in 2 ml of KHB buffer (pH 7.4, saturated with O_2/CO_2, 19:1) containing 1% BSA and 5 mM glucose. The incubation medium also contained 0 or 2 mM arginine, 2 mM glutamine, or NH_4Cl plus ornithine and aspartate (2 mM each). Incubation was terminated by addition of 0.2 ml of 1.5 M $HClO_4$. The acidified medium was neutralized with 0.1 ml of 2 M K_2CO_3, and extracts were used for analysis of urea and amino acids, as described above.

Statistical analysis

Data were analysed by paired t-test, or by one-way ANOVA with the Student–Newman–Keuls test, as indicated in tables [26]. Probabilities less than 0.05 were taken to indicate statistical significance.

RESULTS

Composition of cell population

The cells isolated by the method used were predominantly (more than 85%) enterocytes of columnar shape [21]. The proportion of macrophages, T-cells or B-cells was less than 1% of total cell populations in enterocytes from newborn and 2–7-day-old suckling pigs. Similar data were obtained for macrophages and B-cells in enterocytes from 14–58-day-old pigs (Table 1). The proportion of T-cells in enterocyte preparations was increased ($P < 0.05$) in post-weaning pigs compared with pre-weaning pigs (Table 1).

Table 1 Proportions of macrophages, T-cells and B-cells in pig enterocyte preparations

Flow cytometric analysis of mononuclear cells was performed with monoclonal antibodies recognizing porcine macrophages, T-cells and B-cells, as described in the text. Values are means±S.E.M., $n = 6$. Within a column, means sharing different letters (*a,b*) are different ($P < 0.05$), as analysed by one-way ANOVA.

Age of pigs (days)	Macrophages (%)	T-cells (%)	B-cells (%)
14	< 1	1.5±0.4 *a*	< 1
21	< 1	2.0±0.5 *a*	< 1
29	< 1	7.5±0.6 *b*	1.4±0.3
58	< 1	7.8±0.4 *b*	1.6±0.4

Urea synthesis from ammonia and glutamine in enterocytes

Table 2 summarizes data on the synthesis of urea from ammonia and glutamine in enterocytes of developing pigs. The higher basal (0 min) concentrations of urea in enterocytes from 2–21-day-old suckling pigs than in cells from 0-day-old and post-weaning pigs were likely to be due to the uptake by enterocytes of urea-rich sow's colostrum and milk (5–7 mM) before the dissection of jejunum [27]. There was no measurable production of urea in 0–21-day-old pig enterocytes incubated in the presence of either 1–5 mM glutamine or 0.5–2 mM NH_4Cl. In contrast, urea was formed from both substrates in enterocytes of post-weaning pigs, in a dose-dependent manner. There was no measurable hydrolysis of urea in enterocytes from all ages of the pigs used, as determined with the addition of 0.5 mM [^{14}C]urea to the incubation medium and the subsequent collection of $^{14}CO_2$ in Hyamine hydroxide after a 30 min incubation period. In the presence of 2 mM NH_4Cl plus 0.05 mM ornithine (plasma concentration in pigs) [27] and 2 mM aspartate, the rates of urea synthesis were 15.6±1.8 and 16.3±2.0 nmol per mg protein per 30 min in enterocytes from 29- and 58-day-old pigs, respectively (means±S.E.M., $n = 6$).

Formation of urea, ornithine and citrulline from arginine in enterocytes

Results are shown in Table 3. There was no measurable production of urea from 0.5–2 mM arginine in enterocytes from 0–14-day-old pigs. Analysis of amino acids by HPLC revealed the formation of small amounts of ornithine from arginine (0.5–2 mM) in enterocytes of newborn and 2–21-day-old suckling pigs. In contrast, relatively large amounts of urea, ornithine and proline were produced from arginine (0.5–2 mM) in enterocytes from post-weaning pigs, in a dose-dependent manner. There was no measurable synthesis of glutamate, alanine, aspartate or asparagine in 0–58-day-old pig enterocytes incubated in the presence of 0.5–2.0 mM arginine, compared with the absence of arginine (results not shown). The rates of production of citrulline from 2 mM arginine were 1.03±0.12, 0.37±0.03, 0.43±0.05, 0.06±0.01, 0.14±0.02, 1.64±0.24, and 1.30±0.16 nmol per mg protein per 30 min in enterocytes from pigs 0, 2, 7, 14, 21, 29 and 58 days old, respectively, which were inhibited by 90–95% ($P < 0.01$) in the presence of 2 mM N^G-nitro-L-arginine.

There was no measurable utilization of arginine by enterocytes from newborn and 2–21-day-old suckling pigs. In cells from 29–58-day-old post-weaning pigs, the rates of arginine utilization (calculated on the basis of arginine disappearance from the incubation medium) were 12.5±1.76 and 14.3±1.59, and 31.1±2.25 and 33.7±3.18 nmol per mg protein per 30 min in the presence of 0.5 and 2 mM arginine, respectively (means±S.E.M., $n = 8$).

Carbamoyl phosphate (CP) concentrations in enterocytes

There was no significant difference ($P > 0.05$) in CP concentrations in enterocytes among 0-58 day-old pigs, in the presence or absence of glutamine or ammonia. The concentrations of CP in enterocytes incubated for 30 min in the absence of added substrates and the presence of 5 mM glutamine, or NH_4Cl plus ornithine and aspartate (2 mM each), were 29.6±2.4, 44.2±3.5 and 46.0±3.2 nmol per mg protein, respectively. The presence of

Table 2 Synthesis of urea from glutamine and ammonia in pig enterocytes

Enterocytes were incubated at 37 °C for 0 or 30 min in KHB buffer (pH 7.4) containing the substrates as indicated. Rates of synthesis of urea were calculated by subtraction of the 0 min (basal) urea concentrations in cell extracts, which were 4.73 ± 0.46, 15.4 ± 1.16, 13.2 ± 2.01, 12.4 ± 1.68, 11.5 ± 1.48, 4.31 ± 0.45 and 3.73 ± 0.28 nmol per mg protein for enterocytes from pigs 0, 2, 7, 14, 21, 29 and 58 days old, respectively. Values are means ± S.E.M., $n = 8$. Within a row, means sharing different letters (*a–c*) are different ($P < 0.05$), as analysed by one-way ANOVA. n.d., Urea synthesis was not measurable.

	Urea synthesis (nmol/30 min per mg of protein)				
Age of pigs (days)	No substrates	1 mM Gln	5 mM Gln	0.5 mM NH_4Cl + 2 mM Orn + 2 mM Asp	2 mM NH_4Cl + 2 mM Orn + 2 mM Asp
0–21	n.d.	n.d.	n.d.	n.d.	n.d.
29	n.d.	6.27 ± 0.74 *c*	15.2 ± 1.28 *b*	13.4 ± 1.56 *b*	21.6 ± 2.07 *a*
58	n.d.	7.91 ± 0.82 *c*	16.5 ± 1.43 *b*	14.6 ± 1.28 *b*	23.4 ± 3.25 *a*

Table 3 Production of urea, ornithine and proline from arginine in pig enterocytes

Enterocytes were incubated at 37 °C for 0 or 30 min in KHB buffer (pH 7.4) containing 0, 0.5 or 2 mM arginine. Rates of synthesis of urea, ornithine and proline were calculated by subtraction of their 0 min (basal) concentrations in cell extracts. See Table 1 for basal concentrations of urea in cell extracts. The basal concentrations of ornithine were 0.31 ± 0.02, 0.32 ± 0.02, 0.28 ± 0.02, 0.22 ± 0.03, 0.24 ± 0.02, 0.37 ± 0.04 and 0.38 ± 0.05 nmol per mg protein for enterocytes from pigs 0, 2, 7, 14, 21, 29 and 58 days old, respectively. The basal concentrations of proline were 1.46 ± 0.14, 1.38 ± 0.17, 1.31 ± 0.16, 1.44 ± 0.23, 1.26 ± 0.16, 1.17 ± 0.26 and 1.28 ± 0.21 nmol per mg protein, respectively, for enterocytes from pigs of the same age range. n.d., Synthesis of the metabolite was not measurable. Results are given in nmol per mg protein per 30 min. Within a row for each metabolite, means sharing different letters (*a–c*) are different ($P < 0.05$), as analysed by one-way ANOVA.

	0 mM Arg			0.5 mM Arg			2 mM Arg		
Age of pigs (days)	Urea	Orn	Pro	Urea	Orn	Pro	Urea	Orn	Pro
0	n.d.	0.11 ± 0.02 *a*	0.51 ± 0.07	n.d.	0.18 ± 0.02 *b*	0.64 ± 0.10	n.d.	0.32 ± 0.04 *c*	0.82 ± 0.17
2	n.d.	0.09 ± 0.01 *a*	0.47 ± 0.06	n.d.	0.15 ± 0.02 *b*	0.63 ± 0.09	n.d.	0.29 ± 0.03 *c*	0.52 ± 0.10
7	n.d.	0.08 ± 0.01 *a*	0.44 ± 0.07	n.d.	0.17 ± 0.01 *b*	0.38 ± 0.07	n.d.	0.28 ± 0.02 *c*	0.47 ± 0.11
14	n.d.	0.04 ± 0.01 *a*	0.58 ± 0.08	n.d.	0.12 ± 0.02 *b*	0.53 ± 0.07	n.d.	0.26 ± 0.03 *c*	0.45 ± 0.09
21	n.d.	0.07 ± 0.01 *a*	0.71 ± 0.10	n.d.	0.16 ± 0.03 *b*	0.90 ± 0.15	n.d.	0.65 ± 0.07 *c*	0.83 ± 0.12
29	n.d.	0.16 ± 0.02 *a*	0.64 ± 0.12 *a*	17.2 ± 1.5 *a*	4.13 ± 0.53 *b*	9.06 ± 0.82 *b*	26.5 ± 3.1 *b*	11.5 ± 1.3 *c*	16.6 ± 1.5 *c*
58	n.d.	0.20 ± 0.03 *a*	0.68 ± 0.09 *a*	16.1 ± 1.8 *a*	4.08 ± 0.37 *b*	9.15 ± 1.03 *b*	28.4 ± 3.6 *b*	11.8 ± 1.7 *c*	19.5 ± 2.0 *c*

Table 4 Activities of urea-cycle enzymes in pig enterocytes

Enzyme activities were measured as described in the text. Values (nmol/min per mg protein) are means ± S.E.M., $n = 8$. Within each column, means sharing different letters (*a–d*) are different ($P < 0.05$), as analysed by one-way ANOVA.

Age of pigs (days)	CPS I	OCT	ASS	ASL	Arginase
0	3.41 ± 0.23 *a*	142.5 ± 8.7 *a*	1.61 ± 0.13 *a*	19.3 ± 1.13 *a*	0.019 ± 0.003 *c*
2	2.25 ± 0.16 *b*	129.7 ± 9.5 *a*	1.52 ± 0.12 *a*	3.14 ± 0.34 *b*	0.021 ± 0.002 *c*
7	2.30 ± 0.25 *b*	135.3 ± 9.2 *a*	1.58 ± 0.23 *a*	3.28 ± 0.37 *b*	0.020 ± 0.003 *c*
14	2.47 ± 0.33 *b*	133.9 ± 8.0 *a*	0.38 ± 0.05 *b*	3.02 ± 0.26 *b*	0.023 ± 0.004 *c*
21	2.36 ± 0.21 *b*	88.5 ± 7.9 *b*	0.43 ± 0.06 *b*	2.86 ± 0.39 *b*	0.047 ± 0.006 *b*
29	1.43 ± 0.16 *c*	66.1 ± 6.5 *c*	1.81 ± 0.22 *a*	2.91 ± 0.22 *b*	2.51 ± 0.32 *a*
58	1.51 ± 0.18 *c*	30.6 ± 3.1 *d*	1.72 ± 0.25 *a*	3.08 ± 0.33 *b*	2.71 ± 0.36 *a*

glutamine or ammonia increased ($P < 0.05$) CP concentrations in cells from all ages of the pigs used.

Activities of the urea-cycle enzymes in enterocytes

The activities of CPS I, OCT, ASS, ASL and arginase in pig enterocytes are presented in Table 4. The activity of CPS I decreased ($P < 0.05$) by 37% and 60% in cells from suckling and post-weaning pigs, respectively, compared with newborn ones. The activity of OCT was similar in enterocytes among 0–14-day-old pigs, and progressively decreased ($P < 0.05$) in cells from 21–58-day-old pigs. The activity of ASS in enterocytes was similar among 0–7-day-old pigs, but decreased ($P < 0.05$) by 78% in 14–21-day-old pigs compared with 0–7-day-old ones. Interestingly, ASS activity was enhanced 4-fold in cells from post-weaning pigs compared with 14–21-day-old pigs, to a value similar to that found in 0–7-day-old pigs. Note that the activity of ASL in enterocytes was similar among 2–58-day-old pigs and

Table 5 Arginase activity and formation of ornithine from arginine in intraepithelial lymphocytes from jejunum of pre- and post-weaning pigs

Arginase activity was measured as described in the text. For metabolic studies, intraepithelial lymphocytes were incubated at 37 °C for 0 or 60 min in KHB buffer (pH 7.4) containing 2 mM arginine. Rates of formation of ornithine were calculated by subtraction of 0 min (basal) ornithine concentrations in cell extracts, which were 0.36 ± 0.05, 0.40 ± 0.05 and 0.38 ± 0.04 in lymphocytes from pigs 21, 29 and 58 days old, respectively. Pigs were weaned at 21 days of age. Values are means ± S.E.M., $n = 6$. * $P < 0.01$: different from the value for the 0 mM Arg group, as analysed by paired *t*-test.

Age of pigs (days)	Arginase activity (nmol/min per mg protein)	Formation of Orn from Arg (nmol per 30 min per mg protein)	
		0 mM Arg	2 mM Arg
21	0.069 ± 0.008	0.046 ± 0.004	0.63 ± 0.05*
29	0.072 ± 0.006	0.052 ± 0.004	0.71 ± 0.06*
58	0.075 ± 0.008	0.048 ± 0.006	0.66 ± 0.08*

was about 80% lower than that in newborn pigs. Arginase activity in enterocytes was negligible or low in newborn and suckling pigs, but increased ($P < 0.05$) 50–100 fold in post-weaning pigs. It is noteworthy that OCT had the highest activity among all the urea-cycle enzymes in 0–58-day-old pig enterocytes.

Arginase activity and formation of urea from arginine, glutamine or ammonia in IEL

Arginase activity and rate of ornithine formation from arginine in IEL (Table 5) were only 3% and 6%, respectively, of those in the enterocyte preparations from post-weaning pigs. In IEL from pre- and post-weaning pigs, there was no detectable formation of urea from arginine (2 mM), glutamine (2 mM), or ammonia plus ornithine and aspartate (2 mM each) (results not shown). Likewise, no amino acids other than ornithine were formed from arginine in IEL (results not shown).

DISCUSSION

Urea synthesis in pig enterocytes

The *in vitro* small-intestine preparations from post-weaning rats [28] and hamsters [29] have been reported to metabolize arginine to urea because of the presence of arginase activity in this organ. Perfusion studies *in vivo* have also shown that 33% of absorbed arginine was hydrolysed to urea plus ornithine in adult-rat jejunum [30], indicating a substantial intestinal hydrolysis of arginine. Despite these previous studies, little is known of ammonia metabolism in the small intestine. The present study demonstrates that urea was formed from ammonia, glutamine and arginine in enterocytes from post-weaning pigs, but not newborn or suckling pigs (Tables 2 and 3). Analysis of amino acids by the sensitive fluorimetric HPLC method revealed the formation of negligible amounts of ornithine from arginine in enterocytes from newborn and suckling pigs, indicating negligible hydrolysis of arginine by arginase. To the best of the author's knowledge, this study is the first to document the synthesis of urea from extracellular or intramitochondrially generated ammonia in enterocytes and thus provides direct evidence to support the previous suggestion that a metabolically significant urea cycle may be functioning in the small intestine [12,15]. Interestingly, CP concentrations in pig enterocytes were higher than those reported for livers of rats [15] and post-weaning pigs (9.2 ± 0.65 nmol per mg protein; G. Wu, unpublished work). As in rat hepatocytes [31], arginine-derived ornithine is further converted to proline in pig enterocytes (Table 3). However, in contrast to hepatocytes, which cannot form ornithine from glutamine/glutamate owing to the lack of pyrroline-5-carboxylate synthase [31], enterocytes from post-weaning pigs readily convert glutamine/glutamate into ornithine [11], which is required for urea synthesis from ammonia. The present findings suggest that the hepatocyte is not the only cell type capable of synthesizing urea from ammonia in mammals and may help to interpret the previous data on the complex kinetics of urea recycling in animals including humans [32].

Glutamine is extensively degraded in enterocytes of rats and pigs, with the production of large amounts of ammonia [11,33]. The oxidation of glutamine in enterocytes also generates ATP, HCO_3^-, ornithine and aspartate [11], which are all required for converting ammonia into urea. Thus it is expected that some of the glutamine-derived ammonia may be converted into urea in enterocytes, as shown in Table 1. In pig enterocytes, mitochondrial phosphate-dependent glutaminase hydrolyses glutamine into ammonia and glutamate [11]. This intramitochondrially generated ammonia can be efficiently coupled with urea synthesis via substrate channelling [34], as CPS I for converting NH_3 plus HCO_3^- into carbamoyl phosphate and ornithine-synthesizing enzymes are also located in mitochondria [12–18]. However, because of the lower activity of CPS I, ASS, ASL and arginase in enterocytes (Table 4) than in hepatocytes [1], only about 5% of glutamine-derived ammonia [11] is converted to urea in incubated enterocytes from 29–58-day-old post-weaning pigs, indicating the low capacity for synthesis of urea from glutamine or ammonia in these cells.

It is worth mentioning that the pigs used in this study were nursed by sows or fed under normal production conditions, and therefore were not germ-free. The micro-organisms as present in the intestinal lumen are known to contain all urea-cycle enzymes for converting ammonia to urea [35]. However, it is unlikely that the formation of urea from ammonia, glutamine and arginine in pig enterocytes (Tables 2 and 3) resulted from possible microbial contaminations on the basis of the following observations. First, there was no hydrolysis of [^{14}C]urea to $^{14}CO_2$ plus ammonia in the pig enterocytes used, indicating the absence of urease, an abundant enzyme in micro-organisms [35]. Secondly, there was no measurable activity of arginine decarboxylase, another microbial enzyme [36], in the pig enterocyte preparations used. By using monoclonal antibodies recognizing porcine macrophages, T-cells and B-cells, it was found that macrophages and B-cells were either absent or negligible in enterocytes preparations from pre-and post-weaning pigs and that T-cells accounted for about 8% of total cell population in enterocyte preparations from post-weaning pigs (Table 1). To determine whether resting IEL (predominantly T-cells) might be capable of synthesizing urea from glutamine, ammonia or arginine, IEL were prepared from jejunum of pre- and post-weaning pigs. Arginase activity and rate of ornithine formation from arginine in IEL were only 3% and 6% of those in the enterocyte preparations from post-weaning pigs, respectively (Table 5). Because there was no detectable formation of urea from arginine, glutamine or ammonia in IEL, urea synthesis in the enterocyte preparations from post-weaning pigs (Tables 2 and 3) was unlikely to be due to a 'joint venture' of mononuclear cells.

Urea-cycle enzymes in pig enterocytes

To elucidate the mechanism of the developmental change of urea synthesis in pig enterocytes, the activities of the urea-cycle

enzymes were determined in these cells. All of the urea-cycle enzymes were found to be present in pig enterocytes (Table 4). The activities of CPS I and OCT decreased but that of ASS increased in post-weaning pigs compared with suckling ones. Of particular interest is the result that the activity of arginase was increased 50–100-fold in enterocytes from post-weaning pigs compared with newborn and suckling ones. An induction of arginase has also been reported in the small intestine of post-weaning rats [38] and mice [12]. The negligible activity of arginase in enterocytes of newborn and suckling pigs minimized the hydrolysis of arginine into urea plus ornithine, whereas the induction of this enzyme in enterocytes of post-weaning pigs resulted in the production of relatively large amounts of urea, ornithine and proline from arginine (Table 3). Thus arginase activity, but not CPS I or carbamoyl phosphate concentration, appears to be a limiting factor for urea synthesis in enterocytes. This is in contrast with hepatocytes in which, owing to the exceedingly high activity of arginase, CPS I and ASS are generally considered to be major regulatory enzymes in urea synthesis from ammonia [1].

Arginase activity and the formation of urea from arginine (1 mM) were recently demonstrated in enterocytes of 0–8-day-old and 2-month-old post-weaning pigs [10,39]. However, arginase activity was found to be 40–60-fold lower than the rate of urea production from arginine in these cells [10]. This previously reported arginase activity was too low to account for the rate of urea formation from arginine in pig enterocytes. It is not known whether arginase activity was underestimated or the production of urea was overestimated in these previous studies with pig enterocytes [10,39]. The arginase activity obtained from the present study is about 50–100 times that reported by Blachier and co-workers [10,39] and seems to be sufficient to account for the rate of urea production from arginine, ammonia and glutamine in post-weaning pig enterocytes (Tables 2 and 3).

The mechanism for the induction of arginase in post-weaning pig enterocytes has not been elucidated in the present study. It can be ruled out that the induction of arginase was due to the increasing age of the pigs, because recent studies from this laboratory have shown that there was no increase in enterocyte arginase activity between unweaned 21- and 29-day-old pigs (G. Wu, unpublished work). It is known that weaning is associated with increased plasma concentrations of cortisol in the pig, which is the most abundant circulating steroid hormone and the major glucocorticoid secreted by the adrenal cortex in this species [40]. Interestingly, the administration of cortisol to 5–8-day-old rats has been reported to result in a marked increase in hepatic arginase activity to adult levels in 24 h [38]. Furthermore, cortisol is now known to increase both the mRNA level and the activity of arginase in cultured rat hepatocytes [2]. Thus it is tempting to suggest that cortisol mediates the induction of arginase and urea synthesis in enterocytes of post-weaning pigs. Further studies are required to test this suggestion.

The induction of enterocyte arginase in post-weaning pigs plays a dual metabolic role: converting ammonia into urea and synthesizing proline from arginine in the small intestine. The lack of proline synthesis from arginine in enterocytes from newborn and suckling pigs (Table 3) can be satisfactorily explained by the near absence of arginase in these cells (Table 4). Because there was no measurable production from arginine of amino acids other than ornithine, citrulline and proline in pig enterocytes, it is likely that arginine is catabolized mainly via arginase, ornithine aminotransferase and pyrroline-5-carboxylate reductase. Consistent with this suggestion are the relatively low NO synthase activity [39], the low rate of synthesis of citrulline (a co-product of NO synthase) from arginine (this study), the absence of arginine decarboxylase (this study), and negligible oxidation of arginine to CO_2 [10,41] in enterocytes from newborn, suckling and post-weaning pigs.

Possible physiological significance of arginase induction and urea synthesis in enterocytes

The induction of arginase in enterocytes of post-weaning pigs resulted in the formation of urea from extracellular or intramitochondrially generated ammonia in a dose-dependent manner. Thus the small intestine can be regarded as an organ with a metabolically significant urea cycle, as previously suggested [12,15]. It is worth pointing out that the rate of urea synthesis in incubated enterocytes is low compared with hepatocytes [1] and that the liver is undoubtedly the major organ for ureagenesis in mammals [1,42]. However, as the small intestine is the barrier separating the internal from the external environment, urea synthesis in enterocytes may be a first line of defence against the potential toxicity of ammonia produced by the extensive intestinal degradation of glutamine (a major fuel for enterocytes) or derived from diet and luminal micro-organisms. Alternatively, the induction of arginase in post-weaning pig enterocytes may play an important role in promoting intestinal maturation by providing ornithine for the synthesis of polyamines, which are essential for gastrointestinal growth and development [43]. The previously reported negligible conversion of arginine to polyamines in enterocytes of neonatal pigs [44] is consistent with the near absence of arginase activity for ornithine synthesis in these cells (Table 4). Because post-weaning pigs do not receive sow's milk, which is relatively rich in polyamines [45,46], the intestinal synthesis of polyamines from arginine or glutamine-derived ornithine may be of physiological and nutritional importance.

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