A Cortisol Surge Mediates the Enhanced Expression of Pig Intestinal Pyrroline-5-Carboxylate Synthase during Weaning

Guoyao Wu,*†2 Cynthia J. Meininger,† Katherine Kelly,† Malcolm Watford** and Sidney M. Morris, Jr.‡

†Faculty of Nutrition and Department of Animal Science, Texas A&M University, College Station, TX 77843; †Department of Medical Physiology, Texas A&M University System Health Science Center, College Station, TX 77843; **Department of Nutritional Sciences, Rutgers University, New Brunswick, NJ 08901; and ‡Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

ABSTRACT Citrulline synthesis from glutamine is enhanced remarkably in enterocytes of weanling pigs, but the molecular mechanism(s) involved are not known. The objective of this study was to determine whether a cortisol surge mediates the enhanced expression of intestinal citrulline-synthetic enzymes during weaning. Jejunal enterocytes were prepared from 29-d-old weanling pigs treated with or without metyrapone (an inhibitor of cortisol synthesis), or from age-matched unweaned pigs. The mRNA levels and activities of phosphate-dependent glutaminase (PDG), pyrroline-5-carboxylate synthase (P5CS), ornithine aminotransferase (OAT), carbamoyl-phosphate synthase I (CPS-I) and ornithine carbamoyltransferase (OCT) were determined. The mRNA levels for PDG, P5CS, OAT and OCT were 139, 157, 102 and 55% higher, respectively, in weanling pigs compared with suckling pigs. The activities of PDG and P5CS were 38 and 692% higher, respectively, in weanling pigs compared with unweaned pigs, but the activities of OAT, CPS-I and OCT did not differ between these two groups of pigs. The effects of metyrapone administration to weanling pigs were as follows: 1) prevention of a cortisol surge, 2) abolition of the increases in both mRNA levels and activity of P5CS, 3) no alteration in the mRNA levels and activities of PDG and CPS-I, 4) increases in the mRNA levels for OAT (216%) and OCT (39%) and in OAT activity (30%), and 5) prevention of the increase in intestinal synthesis of citrulline from glutamine. These results suggest that increased P5CS activity reflects in large part the increased levels of P5CS mRNA and is responsible for the increased synthesis of citrulline from glutamine in enterocytes of weanling pigs; these increases may be mediated by a cortisol surge during weaning that can be blocked by metyrapone administration. J. Nutr. 130: 1914–1919, 2000.

KEY WORDS: • citrulline • enterocytes • metyrapone • cortisol • weaning • pigs

The small intestine is the major source of citrulline for endogenous synthesis of arginine in mammals including pigs (Wu and Morris 1998). Arginine is the physiologic precursor for the synthesis of nitric oxide, which has been identified as the endothelium-dependent relaxing factor, a mediator of immune responses, a neurotransmitter and a signaling molecule (Bredt and Snyder 1994). In addition, arginine is an essential amino acid for young mammals (Visck 1986) and a conditionally essential amino acid for adults (Beaumier et al. 1996). Furthermore, by serving as an allosteric activator of N-acetylglutamate synthase, which synthesizes N-acetylglutamate [an activator of carbamoylphosphate synthase-I (CPS-I)],3 and as an immediate precursor of ornithine, arginine is essential for hepatic detoxification of ammonia via the urea cycle (Meijer et al. 1990). The crucial metabolic roles of arginine are emphasized by hyperammonemia, cardiovascular abnormalities, reproductive dysfunction and impaired wound healing, which are caused by an arginine deficiency (Wu et al. 2000). Thus, intestinal synthesis of citrulline is of great nutritional importance, particularly under stress conditions such as weaning and illness.

Glutamine and proline are major substrates for intestinal synthesis of citrulline in pigs (Wu et al. 1994, Wu 1997). We recently demonstrated that the synthesis of citrulline from glutamine was enhanced markedly in enterocytes (absorptive epithelial cells of the small intestine) from weanling pigs compared with suckling pigs (Dugan et al. 1995, Wu et al. 1994, Wu 1997). The pathway for converting glutamine to citrulline requires the following mitochondrial enzymes: phosphate-dependent glutaminase (PDG), pyrroline-5-carboxylase synthase (P5CS), ornithine aminotransferase (OAT), CPS-I and ornithine carbamoyltransferase (OCT) (Fig. 1). Intestinal PDG is a kidney-type isozyme (Watford 1993). An increase in P5CS activity may be responsible for the enhanced synthesis of citrulline from glutamine in enterocytes of weanling pigs.
Both mRNA levels and activities of intestinal citrulline-synthetic enzymes during weaning may reflect primarily mechanism for regulating the expression of intestinal citrulline-synthetic enzymes during weaning. This hypothesis was tested in the current study using metyrapone, an inhibitor of adrenal corticoid in pigs (Worsae and Schmidt 1980) and humans (Ganong 1991). Because glucocorticoids are potent regulators of hepatic arginine-metabolic enzymes (Morris 1992) and also urease cycle enzymes (Morris 1992, Watford 1993) and the phosphorylation of cortisol (hydrocortisone), the major circulating glucocorticoid in pigs (Worsae and Schmidt 1980) and humans (Ganong 1991). The dose of metyrapone used was based on previous studies with piglets (Sangild et al. 1995). Blood samples (3 mL) were obtained from the jugular vein of pigs immediately before and on d 19 and 20 postweanling or postmetyrapone administration for the analysis of serum cortisol using a cortisol kit (Flynn and Wu 1997b). Weanling pigs received intramuscular injections of vehicle solvent (saline) or metyrapone (5 mg/kg body) 5 min before weaning and 24 and 72 h later. This period of metyrapone administration corresponded to the cortisol surge in weaning pigs (Borbolla 1994). The dose of metyrapone used was based on previous studies with piglets (Sangild et al. 1995). Blood samples (3 mL) were obtained from the jugular vein of pigs immediately before and on d 19 and 20 postweanling or postmetyrapone administration for the analysis of plasma cortisol using a cortisol kit (Flynn and Wu 1997b). At 29 d of age, pigs were anesthetized and killed by jugular puncture for the isolation of jejunum, as previously described (Flynn and Wu 1997a). The jejunum was washed three times with saline to remove luminal content, and then used for preparing enterocytes using Ca2+-free Krebs-Henseleit bicarbonate (KHB) buffer as previously described (Wu et al. 1996, Wu 1997).

Determination of activities of citrulline-synthetic enzymes. For determining the activities of PDG, OCT, CPS-I and OCT, enterocytes (~40 mg protein) were homogenized in 5 mL of ice-cold medium (300 mmol/L sucrose, 5 mmol/L HEPES, 1 mmol/L EDTA and 3 mmol/L dithiothreitol; pH 7.4) containing protease inhibitors (5 mg/L phenylmethylsulfonyl fluoride, 5 mg/L aprotinin, 5 mg/L chymostatin and 5 mg/L pepstatin A) for preparing mitochondria, as previously described (Wu and Knabe 1995). Mitochondria were stored at −80°C for 24 h and then lysed by three cycles of freezing (liquid nitrogen) and thawing (37°C water bath). Extracts were centrifuged at 10,000 × g for 10 min at 4°C. The supernatant fluid was used for determining the activities of PDG, OCT, CPS-I and OCT at 37°C for 0, 7.5 and 15 min as previously described (Davis and Wu 1998, Wu 1993). Briefly, the PDG assay mixture (0.2 mL) consisted of 20 mmol/L L-glutamine, 150 mmol/L potassium phosphate (pH 8.2) and mitochondrial extracts (0.05 and 0.1 mg protein). The OCT assay mixture (2 mL) contained 75 mmol/L potassium phosphate buffer (pH 7.5), 20 mmol/L ornithine, 0.45 mmol/L pyridoxal phosphate, 0 or 3.75 mmol/L α-ketoglutarate, 5 mmol/L o-aminobenzaldehyde and mitochondrial extracts (0.25 and 0.5 mg protein). The CPS-I assay mixture (0.5 mL) consisted of 0.15 mol/L potassium phosphate buffer (pH 7.5), 30 mmol/L ATP, 25 mmol/L MgCl2, 5 mmol/L Na-acetylglutamate, 20 mmol/L NH4Cl, 5 mmol/L ornithine, 100 mmol/L NaHCO3, 10 μU of OCT and mitochondrial extracts (0.5 and 1 mg protein). The OCT assay mixture (0.2 mL)
to indicate statistical significance. (treatment of means (Steel and Torrie 1980). Probability ANOVA, with the Student-Newman-Keuls test for multiple compar-

concentrations of cortisol on d 2 postweaning (Table 1). Administration to weanling pigs abolished the increase in plasma

by dividing the intensity of the mRNA signal for the enzyme with

tensities were normalized on the basis of the cyclophilin mRNA

and analyzed with Multianalyst (BioRad, Hercules, CA). Band in-

blots were exposed to Biomax MS film (Kodak, Rochester, NY) for

instructions. Hybridizations were performed at 42°C for 16 h and

the Strip EZ DNA kit from Ambion according to the manufacturer’s

and mouse cyclophilin (Ambion, Austin, TX) were generated using

P5CS (Aral et al. 1996), human OAT (Mitchell et al. 1988), rat

cDNA probes for rat kidney-type PDG (Banner et al. 1988), human

OCT between unweaned and weanling pigs. There were no differences

in the mRNA levels for CPS-I between unweaned and weanling pigs.

but there were no differences (P > 0.05) in the activities of OAT, CPS-I and OCT between unweaned and weanling pigs. Metyrapone administration to weanling pigs abolished (P < 0.01) the increase in P5CS activity and had no effect (P > 0.05) on the activities of PDG, CPS-I and OCT. Intestinal OAT activity was 30% higher (P < 0.01) in metyrapone-treated weanling pigs compared with untreated weanling pigs.

Relative mRNA levels for citrulline-synthetic enzymes. Relative abundance of mRNA levels for intestinal citrulline-
synthetic enzymes (normalized on the basis of cyclophilin

mRNA) is shown in Table 3 and Figure 2. The mRNA levels for PDG, P5CS, OAT and OCT were 139, 157, 102 and 55%
higher (P < 0.01), respectively, in enterocytes of weanling pigs compared with unweaned pigs. There were no differences (P > 0.05) in the mRNA levels for CPS-I between unweaned and weanling pigs. Metyrapone administration to weanling pigs abolished the increase in P5CS mRNA levels and had no effect (P > 0.05) on PDG mRNA levels. Interestingly, the mRNA levels for OAT and OCT were 216 and 39% higher, respectively, in metyrapone-treated weanling pigs compared with untreated weanling pigs. Although some changes in

mRNA levels (e.g., OCT) were significant, they may not be of biological importance due to the semiquantitative nature of Northern blots.

**DISCUSSION**

We demonstrated recently that daily administration of cortisol to 21-d-old suckling piglets for 2 d resulted in enhanced P5CS activity and citrulline synthesis from glutamine in enterocytes (Flynn and Wu 1997a). In addition, we found that a cortisol surge was associated with increased intestinal P5CS activity during weaning, which could be abolished by admin-

**RESULTS**

**Plasma cortisol concentrations**. Plasma concentrations of cortisol were 285% greater (P < 0.01) in weanling pigs on d 2 postweaning compared with unweaned pigs, and returned to preweaning levels on d 8 postweaning (Table 1). Metyrapone administration to weanling pigs abolished the increase in plasma concentrations of cortisol on d 2 postweaning (Table 1).

### **TABLE 1**

**Plasma concentrations of cortisol in unweaned pigs and in weanling pigs that were or were not treated with metyrapone**

<table>
<thead>
<tr>
<th>Days postmetyrapone administration</th>
<th>Pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>μg/L</strong></td>
<td></td>
</tr>
<tr>
<td>Unweaned</td>
<td>22.4 ± 2.3</td>
</tr>
<tr>
<td>Wearing</td>
<td>23.0 ± 2.7b</td>
</tr>
<tr>
<td>Weanling + Metyrapone</td>
<td>22.9 ± 2.5</td>
</tr>
</tbody>
</table>

1 Data are means ± SEM, n = 8. Results were analyzed by two-way ANOVA. Means in a row with different letters are different (P < 0.01). *P < 0.01: different from the unweaned and weanling + metyrapone groups.

**Citrulline synthesis from glutamine**. Increasing medium glutamine concentrations from 1 to 5 mmol/L increased (P < 0.01) citrulline synthesis in a concentration-dependent manner in unweaned and weanling pigs (Table 2). Rates of synthesis of citrulline from glutamine were ~10-fold higher (P < 0.01) in enterocytes of weanling pigs compared with unweaned pigs. Prevention of the cortisol surge by metyrapone administration eliminated the difference in citrulline synthesis between weanling and unweaned pigs (Table 2).

### **TABLE 2**

**Citrulline synthesis from glutamine in enterocytes of unweaned pigs and of weanling pigs that were or were not treated with metyrapone**

<table>
<thead>
<tr>
<th>Medium glutamine, mmol/L</th>
<th>Unweaned pigs</th>
<th>Weanling pigs</th>
<th>Weanling pigs + metyrapone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.41 ± 0.06c</td>
<td>4.63 ± 0.51c</td>
<td>0.47 ± 0.05c</td>
</tr>
<tr>
<td>2</td>
<td>0.63 ± 0.07b</td>
<td>6.85 ± 0.60b</td>
<td>0.71 ± 0.08b</td>
</tr>
<tr>
<td>5</td>
<td>0.99 ± 0.11a</td>
<td>10.1 ± 1.2a</td>
<td>1.12 ± 0.14a</td>
</tr>
</tbody>
</table>

1 Data are means ± SEM, n = 8. Results were analyzed by two-way ANOVA. Means in a column with different letters are different (P < 0.01). *P < 0.01: different from the unweaned and weanling + metyrapone groups.
istration of RU486 (mifepristone; a glucocorticoid receptor antagonist) to weanling pigs (Flynn and Wu 1997b). Collectively these studies suggest an important role for cortisol in regulating intestinal P5CS expression and citrulline synthesis during weaning. Results of the current study provide additional evidence to indicate that increased activity of P5CS is responsible for the increased capacity of enterocytes for citrulline synthesis from glutamine in weanling piglets (Tables 2 and 3). Furthermore, the associated increases in P5CS mRNA abundance indicate that the increased P5CS activity is due primarily to regulation at the pretranslational level, probably via increased transcription of the P5CS gene. Relative increases in P5CS activity and mRNA abundance were not precisely identical (Table 3), possibly as a consequence of significant differences in half-lives and therefore in induction profiles between P5CS protein and its mRNA (Hargrove and Schmidt 1989, Hargrove 1993). Nonetheless, these considerations do not alter the principal conclusion that increased levels of P5CS mRNA are primarily responsible for the increased P5CS activity.

To substantiate our suggestion that the increased P5CS activity in enterocytes of weanling piglets largely reflects increased levels of P5CS mRNA, additional data on the P5CS protein and its half-life would be desirable. However, such information cannot be obtained at the present time because the appropriate antibody is not available. Even if the antibody were available, determination of the half-life of P5CS is not

### TABLE 3

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Relative activity or mRNA abundance</th>
<th>Unweaned pigs</th>
<th>Weanling pigs</th>
<th>Weanling pigs + metyrapone</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDG</td>
<td>Activity</td>
<td>100 ± 9.2b</td>
<td>138 ± 14a</td>
<td>136 ± 11a</td>
</tr>
<tr>
<td></td>
<td>mRNA abundance</td>
<td>100 ± 8.4b</td>
<td>239 ± 18a</td>
<td>223 ± 15a</td>
</tr>
<tr>
<td>P5CS</td>
<td>Activity</td>
<td>100 ± 11b</td>
<td>752 ± 63a</td>
<td>109 ± 13b</td>
</tr>
<tr>
<td></td>
<td>mRNA abundance</td>
<td>100 ± 9.0b</td>
<td>257 ± 15a</td>
<td>109 ± 12b</td>
</tr>
<tr>
<td>OAT</td>
<td>Activity</td>
<td>100 ± 9.7b</td>
<td>113 ± 12b</td>
<td>147 ± 16a</td>
</tr>
<tr>
<td></td>
<td>mRNA abundance</td>
<td>100 ± 13c</td>
<td>202 ± 30b</td>
<td>638 ± 75a</td>
</tr>
<tr>
<td>CPS-I</td>
<td>Activity</td>
<td>100 ± 11</td>
<td>96 ± 10</td>
<td>98 ± 9.5</td>
</tr>
<tr>
<td></td>
<td>mRNA abundance</td>
<td>100 ± 12</td>
<td>106 ± 9.5</td>
<td>97 ± 12</td>
</tr>
<tr>
<td>OCT</td>
<td>Activity</td>
<td>100 ± 9.4b</td>
<td>108 ± 12ab</td>
<td>121 ± 10b</td>
</tr>
<tr>
<td></td>
<td>mRNA abundance</td>
<td>100 ± 7.3c</td>
<td>155 ± 14b</td>
<td>216 ± 18a</td>
</tr>
</tbody>
</table>

1 Data are means ± SEM, n = 8. Results were analyzed by one-way ANOVA. Means in a row with different letters are different (P < 0.01).

Abbreviations used: CPS-I, carbamoyl-phosphate synthase-I; OAT, ornithine aminotransferase; OCT, ornithine carbamoyltransferase; PDG, phosphate-dependent glutaminase; P5CS, pyrroline-5-carboxylate synthase. Activities of PDG, P5CS, OAT, CPS-I and OCT for unweaned pigs were 84.7 ± 6.5, 0.053 ± 0.007, 248 ± 19, 6.34 ± 0.66 and 198 ± 17 nmol/(mg protein · min), respectively.

FIGURE 2 Northern blot analysis of mRNAs for citrulline-synthetic enzymes in enterocytes of unweaned pigs and of weanling pigs that were or were not treated with metyrapone. Each lane contained ~20 μg of total RNA. The panels show representative results of Northern blot analysis for each experimental condition. Each blot was probed for the indicated mRNA and also for cyclophilin mRNA. Abbreviations used: CPS-I, carbamoyl-phosphate synthase-I; CYPH, cyclophilin; OAT, ornithine aminotransferase; OCT, ornithine carbamoyltransferase; PDG, phosphate-dependent glutaminase; P5CS, pyrroline-5-carboxylate synthase.
feasible with whole piglets and also cannot be accomplished with the short-term incubation of enterocytes used here. In addition, although measurements of P5CS transcription rates would be useful, they are not essential for the conclusions of this study. In any case, we wish to note that precise methods to measure transcription rates in porcine enterocytes have not been established and also would require cloning of porcine P5CS cDNA. This is because the conditions for measurement of transcription rates are more stringent than for Northern blotting, where it is possible to use homologous (but not identical) cDNAs from related species.

Activities of the other enzymes in the citrulline biosynthetic pathway exhibited little or no change during weaning, despite the fact that, with the exception of CPS-I, there were some increases in relative abundance of the corresponding mRNAs (Table 3). As in the case of P5CS, these modest discrepancies could simply reflect differences in half-lives of the mRNAs and proteins, indicating that 8 d is an insufficient period of time for the enzyme levels to reflect increases in mRNA levels. This explanation is entirely possible because some of these enzymes have half-lives on the order of days [e.g., 4 d for rat renal OAT (Kobayashi et al. 1976) and 3–9 d for rat liver urea-cycle enzymes and OAT (Fagan et al. 1991, Morris 1992, Mueckler et al. 1983)]. The lack of CPS-I or OCT induction in pig enterocytes during weaning is in agreement with previous studies demonstrating that intestinal expression of these enzymes in rats is not inducible by glucocorticoids (Ryall et al. 1986, Wraight et al. 1985).

Metyrapone, an inhibitor of adrenal cortisol synthesis (Sangild et al. 1995), prevented a cortisol surge in weaning pigs (Table 1). Thus, metyrapone administration to weaning pigs provides a useful tool to determine whether cortisol plays a role in mediating the enhanced expression of citrulline-synthetic enzymes. A novel finding of this study is that metyrapone administration to weaning pigs completely prevented the increase in both mRNA levels and activity of intestinal P5CS (Table 3). This result suggests an essential role for a cortisol surge in mediating the enhanced expression of intestinal P5CS during weaning. In contrast, metyrapone treatment could not prevent the weaning-associated increases in mRNA levels for intestinal PDG (Table 3), suggesting that factors other than cortisol may play an important role in enhancing its mRNA levels during weaning. Interestingly, metyrapone administration to weaning pigs resulted in increased mRNA levels for intestinal OAT and OCT (Table 3). It is likely that these mRNAs are responding to increased levels of a different hormone whose action on expression of these enzymes normally is antagonized by the cortisol surge during weaning at this stage of development.

Consistent with the prevention of the induction of intestinal P5CS, metyrapone administration to weaning pigs abolished the increase in intestinal synthesis of citrulline from glutamine (Table 2). This result provides another line of evidence supporting the notion that P5CS is a key regulatory enzyme in the synthesis of citrulline from glutamine (Wu et al. 1994). Our finding also provides a molecular basis for the induction of citrulline synthesis in enterocytes of weaning pigs, which is of nutritional importance for enhancing endogenous synthesis of arginine (Wu and Morris 1998). In addition to glutamine, proline is an important substrate for citrulline synthesis in pig enterocytes (Wu 1997). We found that the synthesis of citrulline from proline increased by 26% in enterocytes of 29-d-old weaned pigs compared with age-matched unweaned pigs, probably due to increased proline oxidase activity (G. Wu, unpublished data). Because cortisol is a potent inducer of expression of intestinal P5CS, whose activity decreases markedly during the first 3 wk after birth (Wu et al. 1994, Wu and Knabe 1995), cortisol administration to 7- to 21-d-old suckling pigs may prevent the marked decline in intestinal synthesis of citrulline from glutamine (Wu et al. 1995). This hormonal intervention may help improve arginine nutrition in the sow-reared piglets, which exhibit an arginine deficiency at 7 to 21 of life (Flynn et al. 2000).

In summary, our studies indicate the following: 1) increased P5CS activity is responsible for the increased cellular capacity for intestinal synthesis of citrulline from glutamine in weaning pigs; 2) the increased P5CS activity largely reflects increased levels of P5CS mRNA, probably due to increased transcription of the P5CS gene; and 3) these increases are mediated by a cortisol surge during weaning that can be blocked by metyrapone administration.

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