Lactate inhibits citrulline and arginine synthesis from proline in pig enterocytes

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Lactate inhibits citrulline and arginine synthesis from proline in pig enterocytes. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G1079–G1086, 1999.—Hypocitrullinemia and hypoargininemia but hyperprolinemia are associated with elevated plasma concentration of lactate in infants. Because the small intestine may be a major organ for initiating proline catabolism via proline oxidase in the body and is the major source of circulating citrulline and arginine in neonates, we hypothesized that lactate is an inhibitor of intestinal synthesis of citrulline and arginine from proline. To test this hypothesis, jejunum was obtained from 14-day-old suckling pigs for preparation of enterocyte mitochondria and metabolic studies. Mitochondria were used for measuring proline oxidase activity in the presence of 0–10 mM L-lactate. For metabolic studies, enterocytes were incubated at 37°C for 30 min in Krebs bicarbonate buffer (pH 7.4) containing 5 mM D-glucose, 2 mM L-glutamine, 2 mM L-[U-14C]proline, and 0, 1, 5, or 10 mM L-lactate. Kinetics analysis revealed noncompetitive inhibition of intestinal proline oxidase by lactate (decreased maximal velocity and unaltered Michaelis constant). Lactate had no effect on either activities of other enzymes for arginine synthesis from proline or proline uptake by enterocytes but decreased the synthesis of ornithine, citrulline, and arginine from proline in a concentration-dependent manner. These results demonstrate that lactate decreased intestinal synthesis of citrulline and arginine from proline via an inhibition of proline oxidase and provide a biochemical basis for explaining hyperprolinemia, hypocitrullinemia, and hypoargininemia in infants with hyperlactacidemia.

proline oxidase; amino acids; intestine; pigs

A SEVERE DEFICIENCY of citrulline and arginine (plasma concentrations below detection limit) has been reported in the infant with elevated plasma concentration of lactate (hyperlactacidemia; up to 14 mM) due to an inherited deficiency of pyruvate dehydrogenase activity (4). Life-threatening hyperammonemia occurs in the patient as a result of arginine deficiency and is effectively prevented by exogenous arginine administration (4), suggesting the presence of intact urea cycle enzymes. A deficiency of arginine has also been reported in adult patients with elevated lactate concentrations (16). It is well documented that hyperprolinemia is associated with hyperlactacidemia in humans (4, 8, 13, 16). Interestingly, Kowaloff et al. (15) observed that lactate markedly inhibited the activity of rat liver proline oxidase and suggested that such a regulatory effect of lactate could explain in part the in vivo correlation between hyperprolinemia and elevated plasma concentrations of lactate. However, the mechanism for hypocitrullinemia and hypoargininemia in humans with hyperlactacidemia has not been elucidated.

Proline oxidase (a mitochondrial enzyme) oxidizes proline to form pyrroline-5-carboxylate (P5C) and is the first key regulatory enzyme involved in proline degradation in mammals (1). This enzyme has been known to be present in the liver, kidney, and brain, and it was traditionally thought to be absent from the small intestine of postnatal animals (14). However, we and others have demonstrated the presence of proline oxidase activity in the pig small intestine (19, 24). Indeed, the activity of proline oxidase (expressed on the basis of tissue weight) was 10- and 6-fold greater in the small intestine than in the liver and kidney of the piglet, respectively (19). Furthermore, we identified the presence of proline oxidase primarily in enterocytes of the small intestine and demonstrated the synthesis of citrulline and arginine from proline in these cells (24, 25) (Fig. 1). On the basis of tissue distribution of proline oxidase activity in the pig, we suggested that the small intestine is the major organ for initiating proline catabolism in the body (24–26). Both metabolic and enzymological evidence indicates that the small intestine is the major source of circulating citrulline for endogenous synthesis of arginine in neonates and adults (32).

In light of the foregoing, we hypothesized that lactate inhibits enterocyte proline oxidase, thereby suppressing proline catabolism and synthesis of citrulline and arginine from proline in the small intestine. Such a regulatory effect of lactate may offer a biochemical basis for explaining hypocitrullinemia and hypoargininemia as well as hyperprolinemia in the infant with elevated plasma concentrations of lactate (4). This hypothesis was tested with use of the suckling pig, an excellent animal model for studying infant nutrition and metabolism (24, 29). Our results demonstrated that lactate markedly inhibited intestinal proline oxidase and decreased the synthesis of citrulline and arginine from proline in enterocytes.

MATERIALS AND METHODS

Chemicals. L-Amino acids, L-lactic acid, D-glucose, o-aminobenzaldehyde, ferrocytochrome c (from horse heart), BSA (fraction V, essentially fatty acid free), HEPES, EDTA, di-thiothreitol (DTT), phenylmethylsulfonyl fluoride, apro- tinin, chymostatin, pepstatin A, inulin, and sucrose were ob-

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L-[U-14C]glutamine, and [3H]inulin were purchased from Sigma Chemical (St. Louis, MO). L-[U-14C]proline and pig enterocytes. Enzymes that catalyze the indicated reactions are 1) ornithine oxidase, 2) ornithine aminotransferase, 3) ornithine carbamoyltransferase, 4) argininosuccinate lyase, and 6) carbamoyl phosphate synthase I. Reaction 6 requires N-acetylglutamate (synthesized from glutamate and acetyl-CoA) as an essential activator for carbamoyl phosphate synthase I. Reaction 6 requires N-acetylglutamate (synthesized from glutamate and acetyl-CoA) as an essential activator for carbamoyl phosphate synthase I. In pig enterocytes, glutamine metabolism provides glutamate, ammonia, aspartate, and ATP for conversion of proline into citrulline and arginine (30).

Fig. 1. Synthesis of ornithine, citrulline, and arginine from proline in pig enterocytes. Enzymes that catalyze the indicated reactions are 1) proline oxidase, 2) ornithine aminotransferase, 3) ornithine carbamoyl transferase, 4) argininosuccinate synthase, 5) argininosuccinate lyase, and 6) carbamoyl phosphate synthase I. Reaction 6 requires N-acetylglutamate (synthesized from glutamate and acetyl-CoA) as an essential activator for carbamoyl phosphate synthase I. In pig enterocytes, glutamine metabolism provides glutamate, ammonia, aspartate, and ATP for conversion of proline into citrulline and arginine (30).

Enterocytes or liver was homogenized in 4 ml of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.2, containing Complete (Roche Applied Science, Indianapolis, IN) protease inhibitor cocktail, 0.5 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, and 5 µg/ml PMSF. The homogenates were centrifuged at 1,000 x g for 10 min at 4°C. The supernatant was used for enzyme assays.

Proline oxidase activity was determined as previously described (24). Briefly, the enzyme assay mixture (1.0 ml), which consisted of 50 mM potassium phosphate buffer (pH 7.5), was incubated at 37°C for 0, 1, 2, 5, 7.5, 10, 15, or 20 mM proline. To determine the effect of pyruvate, ornithine, citrulline, or arginine on proline oxidase activity, these metabolites were added individually to the enzyme assay mixture at 0.05, 0.5, 1.0, 5.0, 7.5, 10, 15, or 20 mM proline.

Determination of proline oxidase activity. Enterocytes (−50 mg protein) or liver (−0.5 g) were washed three times in 10 ml buffer (in mM, 250 sucrose, 1 EDTA, 50 potassium phosphate buffer, pH 7.2) by centrifugation at 600 g and 4°C for 2 min. Enterocytes or liver was homogenized in 4 ml homogenization buffer (in mM, 250 sucrose, 1 EDTA, 2.5 DTT, 50 potassium phosphate buffer, pH 7.2). Protease inhibitors (5 µg/ml phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml chymostatin, 5 µg/ml pepstatin A) were added to the homogenization buffer to prevent enzyme degradation. The homogenates were centrifuged at 600 g, 4°C, for 10 min. The supernatant was centrifuged at 12,000 g, 4°C, for 10 min. The resultant mitochondrial pellets were suspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.5), stored at −80°C, and used for enzyme assay within 3 days. Proline oxidase activity was determined as previously described (24). Briefly, the enzyme assay mixture (1.0 ml), which consisted of 50 mM potassium phosphate buffer (pH 7.5), was incubated at 37°C for 0, 1, 2, 5, 7.5, 10, 15, or 20 mM proline. To determine the effect of pyruvate, ornithine, citrulline, or arginine on proline oxidase activity, these metabolites were added individually to the enzyme assay mixture at 0, 1, 5, or 10 mM proline.

Proline metabolism in enterocytes. Incubations were performed at 37°C for 0 or 30 min in triplicate in 25 ml polypropylene flasks placed in a shaking water bath. Incubation medium (2 ml KHB buffer) contained 1% BSA, 5 mM CaCl₂, and 20 mM HEPES (pH 7.4), by centrifugation at 600 g for 2 min and then suspended in this KHB buffer.

Enterocytes ( −50 mg protein) or liver (−0.5 g) were washed three times in 10 ml buffer (in mM, 250 sucrose, EDTA, 50 potassium phosphate buffer, pH 7.2) by centrifugation at 600 g and 4°C for 2 min. Enterocytes or liver was homogenized in 4 ml homogenization buffer (in mM, 250 sucrose, EDTA, 2.5 DTT, 50 potassium phosphate buffer, pH 7.2). Protease inhibitors (5 µg/ml phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml chymostatin, 5 µg/ml pepstatin A) were added to the homogenization buffer to prevent enzyme degradation. The homogenates were centrifuged at 600 g, 4°C, for 10 min. The supernatant was centrifuged at 12,000 g, 4°C, for 10 min. The resultant mitochondrial pellets were suspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.5), stored at −80°C, and used for enzyme assay within 3 days. Proline oxidase activity was determined as previously described (24). Briefly, the enzyme assay mixture (1.0 ml), which consisted of 50 mM potassium phosphate buffer (pH 7.5), was incubated at 37°C for 0, 1, 2, 5, 7.5, 10, 15, or 20 mM proline. To determine the effect of pyruvate, ornithine, citrulline, or arginine on proline oxidase activity, these metabolites were added individually to the enzyme assay mixture at 0, 1, 5, or 10 mM proline.

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LACTATE AND PROLINE METABOLISM

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d-glucose, 2 mM L-glutamine, 2 mM L-[U-14C]proline, or 0, 5,
or 10 mM L-lactate. These concentrations of lactate were
chosen for enteroocyte incubations to mimic plasma lactate
concentrations (up to 14 mM) in the infant with hypoglyce-
minaemia and hypoparaglinenima (4). Lactate stock solution
was adjusted to pH 7.4 with 10 mM NaOH before addition to the
incubation medium. Glutamine was added to the incubation
medium for provision of ammonia, glutamate, aspartate, and
ATP, which are all required for conversion of [14C]proline into
measurement of P5C in cells plus medium at the end of a
30-min incubation period, 0.5 ml of 10% TCA was added to the
incubation medium to terminate the reaction, followed by
addition of 0.1 ml of 100 mM o-aminobenzaldehyde. The
absorbance of the supernatant at 440 nm was measured, and
after subtraction from the blank value (0 min incubation) it
was used to calculate net P5C accumulation by enterocytes.
To determine amino acids and [14C]-labeled amino acids in cells
plus medium at the end of a 30-min incubation period, 0.2 ml
of 1.5 M HClO4 was added to the incubation medium to
terminate the reaction, followed by addition of 0.1 ml of 2 M
K2CO3. Neutralized extracts were used for amino acid analy-
sis by HPLC and for quantification of [14C]ornithine, [14C]ci-
trulline, and [14C]arginine, as previously described (24).
[14C]P5C was separated by anion-exchange chromatography,
and its radioactivity was measured using a Packard liquid
scintillation counter as previously described (29). Net synthe-
thesis of unlabeled ornithine and citrulline (amino acids not
found in proteins) from proline and glutamine was calculated
on the basis of concentration differences in medium plus cell
extracts between the presence and absence of substrates (29).
Because arginine can be formed from net proteolysis and catabolized by incubated enterocytes, net
synthesis of unlabeled arginine from proline and glutamine
was calculated on the basis of concentration differences in
medium plus cell extracts between the presence and absence
of substrates after 30-min incubation (29).

Uptake of glutamine and proline by enterocytes. Uptake of
glutamine was measured as described by Bradford and
McGivan (3). Briefly, 1 ml of KHB medium (pH 7.4), which
contained enterocytes (2 mg protein), 5 mM glucose, 2 mM
NH4Cl, 5 mM ornithine, 100 mM NaHCO3, 10 units of
added OCT (from Streptococcus faecalis, Sigma Chemical),
and mitochondrial extracts (0.5 mg protein). The ASS assay
mixture (40 µl) contained (in mM) 75 potassium phosphate
buffer (pH 7.5), 10 ornithine, 0.45 pyridoxal phosphate, 0 or
3.75 α-ketoglutarate, and 5 o-aminobenzaldehyde, and mito-
chondrial pellet (0.02 mg protein). The incubation medium for OCT (2.0 ml) contained 0.1 M potassium phosphate buffer (pH 7.5), 15 mM ornithine, 40 mM carbamoyl phosphate, and mitochondrial extracts (0.04 mg protein). 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 MgCl2, 5 mM N-acetylglutamate, 20 mM NH4Cl, 5 mM ornithine, 100 mM NaHCO3, 10 units of added OCT (from Streptococcus faecalis, Sigma Chemical), and mitochondrial extracts (0.5 mg protein). The ASS assay mixture (0.2 ml) consisted of (in mM) 75 potassium phosphate buffer (pH 7.5), 10 citrulline, 5 aspartate, 5 ATP, and 5 MgSO4, and cytosolic extracts (0.4 mg protein). The ASL assay mixture (40 µl) contained (in mM) 129 sodium phosphate buffer (pH 7.0), 10 argininosuccinate, and 65 EDTA, and cytosolic extracts (0.1 mg protein).

Protein determination. Protein in enterocytes and mitochon-
drial extracts was determined by a modified Lowry procedure
using BSA as a standard (30).

Statistical analysis. Results are expressed as means ± SE.
Data were analyzed by one-way ANOVA and Student-
Neuman-Keuls multiple comparison test or by paired t-test
(21). Probability values < 0.05 were used to indicate statisti-
cal significance.

Table 1. Inhibition of proline oxidase activity by L-lactate in pig enterocytes and liver

<table>
<thead>
<tr>
<th>L-Lactate Concentration, mM</th>
<th>Pig Enterocytes</th>
<th>Pig Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mM Proline</td>
<td>20 mM Proline</td>
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<tr>
<td>-----------------------------</td>
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<td>-------------</td>
</tr>
<tr>
<td>0</td>
<td>14.8 ± 0.31a</td>
<td>22.9 ± 0.90a</td>
</tr>
<tr>
<td>1</td>
<td>13.4 ± 0.24a</td>
<td>20.8 ± 1.1b</td>
</tr>
<tr>
<td>5</td>
<td>7.2 ± 0.36b</td>
<td>12.9 ± 0.42b</td>
</tr>
<tr>
<td>10</td>
<td>5.6 ± 0.37b</td>
<td>8.8 ± 0.53b</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol·min⁻¹·mg protein⁻¹·mL⁻¹; n = 6. Means with different letters in columns are significantly different (P < 0.05) as analyzed by 1-way ANOVA and Student-Newman-Keuls (SNK) test. *P < 0.01 vs. 5 mM proline group as analyzed by paired t-test.
RESULTS

Proline oxidase activity. Large amounts of proline oxidase activity were found in enterocyte mitochondria (Table 1). As previously reported (19, 26), proline oxidase activity in the pig liver was much less ($P < 0.01$) than in enterocytes (Table 1). Proline oxidase activity in pig enterocytes and liver was inhibited ($P < 0.05$) by lactate in a concentration-dependent manner (Table 1). From the double reciprocal plot of $1/S$ vs. $1/V$ (Fig. 2), apparent $K_m$ and $V_{max}$ values of proline oxidase in pig enterocytes were determined to be $3.39 \pm 0.25\text{mM}$ and $49.4 \pm 9.4\text{nmol/min-mg protein^{-1}}$, respectively, in the absence of lactate. Similarly, apparent $K_m$ and $V_{max}$ values of pig liver proline oxidase were determined to be $2.58 \pm 0.21\text{mM}$ and $2.16 \pm 0.14\text{nmol/min-mg protein^{-1}}$, respectively, in the absence of lactate (Fig. 3). Results of enzyme kinetics indicated that lactate decreased the maximum velocity of enzyme activity ($V_{max}$ value) but did not affect its affinity for proline (unaltered $K_m$ value) in pig enterocytes (Fig. 2) and liver (Fig. 3). In contrast, pyruvate or products of intestinal proline metabolism (ornithine, citrulline, and...
arginine) had no effect (P > 0.05) on enterocyte proline oxidase activity (Table 2).

Table 2. Effect of pyruvate, ornithine, citrulline, and arginine on proline oxidase activity in pig enterocytes

<table>
<thead>
<tr>
<th>Addition of Amino Acid</th>
<th>5 mM Proline</th>
<th>20 mM Proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12.1 ± 1.5</td>
<td>21.6 ± 0.79*</td>
</tr>
<tr>
<td>1 mM Pyruvate</td>
<td>13.6 ± 1.2</td>
<td>20.9 ± 2.2*</td>
</tr>
<tr>
<td>5 mM Pyruvate</td>
<td>14.1 ± 1.2</td>
<td>21.7 ± 2.4*</td>
</tr>
<tr>
<td>1 mM L-Ornithine</td>
<td>13.3 ± 1.6</td>
<td>22.6 ± 1.9*</td>
</tr>
<tr>
<td>5 mM L-Ornithine</td>
<td>13.2 ± 0.83</td>
<td>23.8 ± 0.82</td>
</tr>
<tr>
<td>1 mM L-Citrulline</td>
<td>13.7 ± 1.8</td>
<td>22.0 ± 0.94*</td>
</tr>
<tr>
<td>5 mM L-Citrulline</td>
<td>13.0 ± 1.7</td>
<td>21.3 ± 2.0*</td>
</tr>
<tr>
<td>1 mM L-Arginine</td>
<td>11.4 ± 0.87</td>
<td>21.8 ± 1.0*</td>
</tr>
<tr>
<td>5 mM L-Arginine</td>
<td>12.0 ± 1.1</td>
<td>20.2 ± 0.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol·min⁻¹·mg protein⁻¹; n = 4. Addition of each amino acid had no significant effect (P > 0.05) on proline oxidase activity as analyzed by 1-way ANOVA. *P < 0.01 vs. 5 mM proline group as analyzed by paired t-test.

Synthesis of ornithine, citrulline, and arginine from proline. Radiochemical analysis of [¹⁴C]proline products showed that large amounts of [¹⁴C]ornithine and [¹⁴C]citrulline and to a lesser extent [¹⁴C]arginine were formed from 2 mM [U-¹⁴C]proline in pig enterocytes (Table 3). Consistent with the inhibition of proline oxidase activity, increasing extracellular lactate concentrations from 0 to 5 or 10 mM decreased (P < 0.05) the synthesis of [¹⁴C]ornithine, [¹⁴C]citrulline, [¹⁴C]arginine, and total [¹⁴C]P5C by enterocytes in a concentration-dependent manner (Table 3). HPLC analysis of amino acids also revealed that lactate markedly decreased (P < 0.05) the formation of ornithine, citrulline, and arginine by enterocytes incubated with proline and glutamine (Table 4). Net accumulation of P5C was also reduced (P < 0.05) by lactate in a concentration-dependent manner (Table 4). Proline utilization, measured on the basis of proline disappearance from the incubation medium, was 16.0 ± 1.4, 15.3 ± 1.6, 12.7 ± 1.1, and 10.4 ± 0.86 nmol·30 min⁻¹·mg protein⁻¹ (means ± SE, n = 6), respectively, in the presence of 0, 1, 5, and 10 mM lactate. Lactate at 5 and 10 mM decreased (P < 0.05) proline utilization by 21 and 35%, respectively.

It should be noted that, in enterocytes from 14-day-old pigs, some of the P5C derived primarily from proline was converted to ornithine, citrulline, and arginine in pig enterocytes (Fig. 1), and thus the P5C measured at the end of 30-min incubation represented its net accumulation but not its total synthesis (Table 4). Total synthesis of P5C should include net P5C accumulation and formation of ornithine, citrulline, and arginine, plus arginine. Pig enterocytes contain both proline oxidase (a mitochondrial enzyme) (24) and P5C reductase (a cytosolic enzyme converting P5C to proline) (31), which constitute a potential intracellular proline-P5C cycle. Thus, to quantify P5C at the end of the 30-min incubation, we prefer not to use the term “net synthesis of P5C” from proline because it may imply a net result of the potential proline-P5C cycle. On the other hand, because ornithine, citrulline, and arginine were further metabolized in enterocytes (32), the measurements of these three amino acids represented their net synthesis but not their total synthesis.


<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.05 ± 0.19a</td>
<td>4.18 ± 0.33a</td>
<td>14.7 ± 1.0a</td>
</tr>
<tr>
<td>1</td>
<td>4.89 ± 0.26a</td>
<td>4.03 ± 0.29a</td>
<td>14.3 ± 1.2*</td>
</tr>
<tr>
<td>5</td>
<td>3.77 ± 0.18a</td>
<td>3.21 ± 0.27b</td>
<td>10.9 ± 0.92*</td>
</tr>
<tr>
<td>10</td>
<td>2.86 ± 0.15b</td>
<td>2.54 ± 0.22c</td>
<td>8.5 ± 0.80*</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol·30 min⁻¹·mg protein⁻¹; n = 6. Enterocytes were incubated for 30 min in presence of 2 mM L-glutamine, 2 mM L-proline, and 0, 1, 5 or 10 mM L-lactate. Means with different letters in column are significantly different (P < 0.05) as analyzed by 1-way ANOVA and SNK test.

Table 4. Effects of lactate on the net accumulation of pyrroline-5-carboxylate (P5C) and net synthesis of ornithine, citrulline, and arginine in pig enterocytes incubated in presence of 2 mM proline and 2 mM glutamine

<table>
<thead>
<tr>
<th>Medium L-Lactate, mM</th>
<th>Net Accumulation of P5C</th>
<th>Ornithine</th>
<th>Citrulline</th>
<th>Arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.42 ± 0.37a</td>
<td>4.72 ± 0.35a</td>
<td>1.34 ± 0.10a</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.28 ± 0.26a</td>
<td>4.61 ± 0.28a</td>
<td>1.19 ± 0.14a</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3.45 ± 0.26a</td>
<td>3.83 ± 0.29a</td>
<td>0.72 ± 0.06a</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2.73 ± 0.25a</td>
<td>2.91 ± 0.23b</td>
<td>0.53 ± 0.04c</td>
<td></td>
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</tbody>
</table>

Values are means ± SE in nmol·30 min⁻¹·mg protein⁻¹; n = 6. Enterocytes were incubated for 30 min in presence of 2 mM L-glutamine, 2 mM L-proline, and 0, 1, 5, or 10 mM L-lactate. Means with different letters in column are significantly different (P < 0.05) as analyzed by 1-way ANOVA and SNK test.

DISCUSSION

Endogenous synthesis of arginine has recently attracted considerable interest (32) since the discovery in...
Table 5. Effect of L-lactate on glutamine and proline uptake by pig enterocytes

<table>
<thead>
<tr>
<th>Lactate Concentration, mM</th>
<th>Glutamine Uptake</th>
<th>Proline Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.9 ± 2.3</td>
<td>4.42 ± 0.50</td>
</tr>
<tr>
<td>1</td>
<td>12.3 ± 2.6</td>
<td>3.46 ± 0.47</td>
</tr>
<tr>
<td>5</td>
<td>13.4 ± 2.0</td>
<td>4.27 ± 0.53</td>
</tr>
<tr>
<td>10</td>
<td>12.0 ± 2.5</td>
<td>4.29 ± 0.46</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol·min⁻¹·mg protein⁻¹; n = 6. Enterocytes were incubated for 2 min in presence of 2 mM L-[U-¹⁴C]glutamine or [U-¹⁴C]proline. Incubation medium also contained 0, 1, 5, or 10 mM L-lactate. Intracellular ¹⁴C radioactivity was measured as an indicator of glutamine or proline uptake. L-Lactate had no significant effect (P > 0.05) on glutamine or proline uptake by enterocytes as analyzed by 1-way ANOVA. *P < 0.01 vs. glutamine uptake as analyzed by paired t-test.

1988 of arginine as the physiological precursor of nitric oxide (NO), a free radical with enormous physiological, immunological, and pathological importance (17). In addition to glutamine as a substrate for intestinal citrulline synthesis (30), we have recently demonstrated a novel pathway for the synthesis of citrulline and arginine from proline via proline oxidase in enterocytes (24), the major source of circulating citrulline for endogenous arginine synthesis in neonates and adults (32). Because intestinal synthesis of citrulline and arginine from glutamine decreases progressively in neonatal pigs during the suckling period (29, 30), proline is the major substrate for citrulline and arginine synthesis in enterocytes during this period (24–26). The endogenous synthesis of arginine from proline as well as glutamine plays an important role in maintaining arginine homeostasis in neonates (9), because arginine is remarkably deficient in the milk of most mammals, including humans, pigs, and mice (7), and both proline and glutamine are abundant amino acids in the milk (28). As a result, hypoargininemia in the patient with hyperlactacidemia remains unknown. Because there is no net production of citrulline or arginine by the liver due to a very high arginase activity and tight channeling of hepatic urea cycle enzymes (32), inhibition of proline catabolism by lactate in the liver is not likely to contribute to citrulline or arginine deficiency in animals or humans with hyperlactacidemia. Therefore, we determined a possible role of lactate in regulating intestinal proline oxidase and other enzymes that synthesize arginine from proline.

An important finding of this study is that lactate at concentrations found in patients with hyperlactacidemia (4, 8, 13, 16) markedly inhibited mitochondrial proline oxidase in pig enterocytes (Table 1, Fig. 2). Among all enzymes that synthesize arginine from proline, proline oxidase was the only enzyme whose activity was inhibited by lactate (Table 6). Neither pyruvate (the immediate product of lactate) nor proline metabolites (ornithine, citrulline, and arginine) affected intestinal proline oxidase activity (Table 2). Because lactate is virtually not metabolized by mitochondria, an inhibition of proline oxidase activity in lysed mitochondria (Table 1) suggests that lactate directly inactivates the enzyme. Kinetics analysis indicated noncompetitive inhibition of intestinal proline oxidase by lactate (decreased Vₘₐₓ and unaltered Kₘ) (Fig. 2), as described by Segel (20). In this class of enzyme inhibition, an inhibitor (e.g., lactate) bears no structural resemblance to the substrate (e.g., proline) but binds to either free enzyme (e.g., proline oxidase) or to enzyme-substrate complex, thus reducing enzyme activity (20). Lactate was also a noncompetitive inhibitor of pig liver proline oxidase (Fig. 3). In contrast, lactate appeared to inhibit rat liver proline oxidase by decreasing the affinity of the enzyme for proline (increased Kₘ value) without altering Vₘₐₓ (competitive inhibition) (15). In competitive inhibition, an inhibitor of the enzyme is structurally similar to its substrate (20). Lactate does not resemble proline in structure, and thus it is not clear how lactate could be a competitive inhibitor of rat liver proline oxidase in the previous study (15).
To demonstrate physiological or pathophysiological relevance of inhibition of proline oxidase by lactate, metabolic studies were conducted with enterocytes incubated in the presence of 1 mM lactate (physiological plasma concentrations of lactate) and elevated lactate concentrations (5 and 10 mM) found in plasma of humans with hyperlactacidemia, hyperammonemia, and hyperprolinemia (4). Lactate is readily transported across plasma membrane and mitochondrial membrane (11, 12). Thus increasing extracellular lactate concentrations results in an increase in intracellular and intramitochondrial lactate concentrations (11). Because lactate had no effect on uptake of glutamine or proline by enterocytes (Table 5), proline uptake by mitochondria (15), or enzymes converting P5C to arginine (Table 6), inhibition of intestinal proline catabolism by lactate likely occurs at the level of proline oxidase.

Consistent with inhibition of proline oxidase, lactate decreased the synthesis of [14C]ornithine, [14C]citrulline, [14C]arginine, and total [14C]P5C from [U-14C]proline by pig enterocytes in a concentration-dependent manner (Table 3). HPLC analysis of amino acids also indicated a decrease in the formation of ornithine, citrulline, and arginine by enterocytes incubated in the presence of 5 and 10 mM lactate (Table 4). Physiological plasma concentrations of lactate (1 mM) had no effect on the synthesis of citrulline and arginine from proline in pig enterocytes (Tables 3 and 4). Because there is limited synthesis of P5C, ornithine, citrulline, and arginine from glutamine in enterocytes of 14-day-old pigs (29, 30), the net accumulation of large amounts of these metabolites in cells incubated in the presence of both glutamine and proline was derived mainly from proline. Thus both radiochemical and HPLC analyses demonstrated a concentration-dependent inhibition by lactate of the synthesis of ornithine, citrulline, arginine, and P5C from proline in pig enterocytes. Similar percentage of proline-derived P5C converted into ornithine (28–30%), citrulline (30–32%), and arginine (5–6%) suggests that lactate did not affect conversion of P5C to these amino acids in enterocytes. This is consistent with our results that lactate had no effect on activities of OAT, OCT, CPS I, ASS, or ASL in enterocytes (Table 6). Because the small intestine may be the major organ for initiating proline catabolism in the body on the basis of tissue distribution of proline oxidase activity in the pig and it is almost the exclusive source of circulating citrulline for endogenous arginine synthesis (24–26), our finding of inhibition of intestinal proline catabolism and synthesis of citrulline and arginine from proline provides a hitherto unrecognized metabolic basis for explaining hypocitrullinemia, hypocitrullinemia, and hyperprolinemia in humans with hyperlactacidemia (4, 16).

Results of this study may also have implications to understanding impaired intestinal function and pathophysiology in ischemia and sepsis that are associated with elevated plasma lactate concentrations. When the small intestine is subject to endotoxin or ischemia, local production of lactate by enterocytes or infiltrating immunocytes and plasma concentrations of lactate (up to 10 mM) is markedly increased due to enhanced glycolysis (22). In light of our present findings, an increase in lactate concentrations would result in an inhibition of proline oxidase and a decrease in the synthesis of citrulline and arginine from proline by enterocytes. This would lead to a local deficiency of arginine in intestinal mucosa and consequently to decreased NO synthesis. Inasmuch as NO plays an important role in regulating intestinal barrier function (2), decreased intestinal proline metabolism may contribute to impaired intestinal integrity and injury. In this regard, it is noteworthy that provision of exogenous arginine prevents intestinal damage associated with gut ischemia or sepsis (10, 18). In addition, an inhibition of intestinal proline catabolism may help to explain hyperlactacidemia (23) and hyperprolinemia (5) associated with elevated plasma concentrations of lactate in humans with sepsis.

In summary, results of both enzymological and metabolic studies demonstrate that lactate inhibited intestinal synthesis of citrulline and arginine from proline via an inhibition of proline oxidase. This study of intestinal proline catabolism and the previous study of hepatic proline oxidase (15) together help explain the in vivo correlation between hyperprolinemia and elevated plasma concentrations of lactate in animals and humans. Our findings also provide a novel biochemical basis for explaining hypocitrullinemia, hypocitrullinemia, and hyperprolinemia in infants with hyperlactacidemia.

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