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Am J Physiol Gastrointest Liver Physiol 287:344-351, 2004. First published Apr 2, 2004;
doi:10.1152/ajpgi.00445.2003

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Adaptative increase of ornithine production and decrease of ammonia metabolism in rat colonocytes after hyperproteic diet ingestion

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Submitted 15 October 2003; accepted in final form 26 March 2004

Mouillé, Béatrice, Véronique Robert, and François Blachier. Adaptative increase of ornithine production and decrease of ammonia metabolism in rat colonocytes after hyperproteic diet ingestion. *Am J Physiol Gastrointest Liver Physiol* 287: G344–G351, 2004. First published April 2, 2004; 10.1152/ajpgi.00445.2003.—Chronic high-protein consumption leads to increased concentrations of $\text{NH}_4^+/\text{NH}_3$ in the colon lumen. We asked whether this increase has consequences on colonic epithelial cell metabolism. Rats were fed isocaloric diets containing 20 (P20) or 58% (P58) casein as the protein source for 7 days. $\text{NH}_4^+/\text{NH}_3$ concentration in the colonic lumen and in the colonic vein blood as well as ammonia metabolism by isolated surface colonic epithelial cells was determined. After 2 days of consumption of the P58 diet, marked increases of luminal and colonic vein blood $\text{NH}_4^+/\text{NH}_3$ concentrations were recorded when compared with the values obtained in the P20 group. Colonocytes recovered from the P58 group were characterized at that time and thereafter by an increased capacity for L-ornithine and urea production through arginase ($P < 0.05$). L-Ornithine was mostly used in the presence of NH_4Cl for the synthesis of the metabolic end product L-citrulline. After 7 days of the P58 diet consumption, however, the ammonia metabolism into L-citrulline was found lower ($P < 0.01$) when compared with the values measured in the colonocytes recovered from the P20 group despite any decrease in the related enzymatic activities (i.e., carbamoyl-phosphate synthetase I and ornithine carbamoyl transferase). This decrease was found to coincide with a return of blood $\text{NH}_4^+/\text{NH}_3$ concentration in colonic portal blood to values close to the one recorded in the P20 group. In response to increased $\text{NH}_4^+/\text{NH}_3$ concentration in the colon, the increased capacity of the colonocytes to synthesize L-ornithine is likely to correspond to an elevated L-ornithine requirement for the elimination of excessive blood ammonia in the liver urea cycle. Moreover, in the presence of NH_4Cl , colonocytes diminished their synthesis capacity of L-citrulline from L-ornithine, allowing a lower cellular utilization of this latter amino acid. These results are discussed in relationship with an adaptative process that would be related to both interorgan metabolism and to the role of the colonic epithelium as a first line of defense toward luminal $\text{NH}_4^+/\text{NH}_3$ concentrations.

colonic epithelial cells

THE DIETARY PROTEIN CONSUMPTION level is vastly different according to food availability and cultural dietary habits. In Western Europe and in the United States for example, protein consumption averages ~ 1.5 – 2 times the recommended intakes (i.e., 0.75 – $0.83 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) (13, 33) and as much as four times these values in slimming high-protein diets. Although there are many studies on the metabolic adaptations involved in response to increased protein ingestion (23, 34), the physiological consequences of high-protein diet consumption are still

poorly understood, and the maximal nondeleterious level of intake is still to be defined (17, 23).

In people consuming a Western-type diet, Chacko and Cummings (8) have reported that up to 12 g protein may escape digestion. Thus a high dietary protein level may exceed the digestive capacities and amino acid/oligopeptide absorptive capacities of the small intestine (15), resulting in an increased protein influx in the large intestine lumen. Proteins escaping digestion, including those of endogenous origin (e.g., digestive enzymes and sloughed mucosal cells), reach the large intestine, where they undergo catabolism by the colonic flora, leading to the release in the colon lumen of a number of potentially harmful compounds including phenols, indoles, amines, sulfide, and ammonia (21). $\text{NH}_4^+/\text{NH}_3$, which is the major nitrogenous end product of bacterial activities, is mainly produced through deamination of amino acids and, to a lesser extent, by urea hydrolysis in the large bowel (38, 43). The luminal $\text{NH}_4^+/\text{NH}_3$ concentration is primarily the net result of the bacterial $\text{NH}_4^+/\text{NH}_3$ production, assimilation for bacterial de novo protein synthesis and large intestine absorption. The highest concentration of $\text{NH}_4^+/\text{NH}_3$ in the body is found in the large intestine lumen. Indeed, $\text{NH}_4^+/\text{NH}_3$ concentrations in the large intestine content, which can vary greatly according to the amount of dietary proteins, average 20–70 mM in rats (19) and 3–44 mM in fecal dialysates of humans (44). An increase in the amount of dietary proteins leads to a rise in human fecal $\text{NH}_4^+/\text{NH}_3$ concentration from 15 to 30 mM (12, 14). In healthy individuals, $\text{NH}_4^+/\text{NH}_3$ is transported to the liver by the portal vein, where it is mostly removed through urea synthesis in the hepatic ornithine cycle (22) and incorporation of NH_4^+ into L-glutamate leading to L-glutamine synthesis (16). It has been demonstrated that activities of the five urea-cycle enzymes in the rat liver are increased in response to increased dietary protein intake (26, 34) as a result of pretranslational regulations (27). An increase in $\text{NH}_4^+/\text{NH}_3$ concentration in the systemic circulation is detrimental, because this compound is toxic to the central nervous system (32). Furthermore, it has been proposed that the transient depressed food intake and the consequent reduction in body weight gain during high-protein diet consumption may be partly due to a transient increase in plasma $\text{NH}_4^+/\text{NH}_3$ concentration resulting in increased entry into the central nervous system (29, 35).

We have previously demonstrated that in normal rat colonocytes, the metabolism of ammonia leads to L-citrulline production through the sequential action of two enzymes. Carbamoyl phosphate (carbamoyl-P) synthesized by the intramitochondrial

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drial carbamoyl-P synthetase I (CPSI) is channeled through ornithine carbamoyltransferase (OCT), which combines this metabolite with L-ornithine to form L-citrulline. Incorporation of ammonia into L-citrulline by the colon epithelial cells may correspond to a first line of defense against luminal $\text{NH}_4^+/\text{NH}_3$ entry into the bloodstream (25).

In this context, the purpose of this study was to determine whether ammonia detoxification metabolism by colonic epithelial cells would adapt to a hyperproteic diet that increases the $\text{NH}_4^+/\text{NH}_3$ concentration in the colonic lumen.

MATERIALS AND METHODS

Chemicals. L-[guanido- ^{14}C]arginine, L-[U- ^{14}C]arginine, L-[ureido- ^{14}C]citrulline, L-[1- ^{14}C]ornithine, L-[U- ^{14}C]ornithine, and $\text{NaH}^{14}\text{CO}_3$ were purchased from Amersham (Little Chalfont, UK) and from New England Nuclear (Boston, MA). Hyaluronidase, ornithine carbamoyltransferase, argininosuccinate lyase, inorganic pyrophosphatase, and carbamoylphosphate were from Sigma (St Louis, MO). All enzymes and coenzymes used for enzymatic assays were from Roche (Meylan, France). D,L- α -difluoromethylornithine (DFMO) was kindly provided by the Marion Merrel Dow Research Institute (Strasbourg, France). Diets were prepared by the Atelier de Preparation des Aliments Experimentaux, Institut National de la Recherche Agronomique (Jouy-en-Josas, France).

Animals. Two-month-old Fisher male rats, weighing 207 ± 3 g, were assigned to two different groups. They were housed in individual cages and fed one of the two semipurified diets containing two concentrations of casein (see the composition of the diets in Table 1). These diets were compounded so that their energy contents were similar in the two experimental diets. The diets were also equalized in minerals, vitamins, and fat. The animals had free access to the food and tap water. Individual body weights were recorded daily.

Two or four days or one week after the initiation of the experiments, the rats were intraperitoneally anesthetized between 10:00 and

11:00 AM with pentobarbital sodium (40 mg/kg body wt). All aspects of the present protocol conformed to the requirements of the laboratory's approval for animal research (1987 regulation). Fatty tissue was removed to expose the right colonic vein, and blood was collected with a heparinized syringe. The pH of the cecal and colonic contents was measured using a penetration electrode (Fisher Bioblock Scientific, Illkirch, France).

The cecum and the large bowel were then removed, and the colon was divided into three parts representing the proximal, median, and distal colon. Colonic and cecal contents of individual rats were collected by expulsion and stored at -80°C until processing. For colon epithelial cell isolation (see *Colon epithelial cell isolation and incubation*), the colon was entirely removed.

Ammonia measurement. $\text{NH}_4^+/\text{NH}_3$ concentrations in blood samples (500 μl) recovered from the right colonic vein obtained before killing were determined. Deproteinized blood, treated with ice-cold perchloric acid (PCA; final concentration: 4%), was neutralized, and the ammonium concentration was assayed, from a standard reference curve made with different concentrations of ammonium chloride, by a specific enzymatic method (1) using a Uvikon 860 recording spectrophotometer (Kontron, Les Ulis, France).

$\text{NH}_4^+/\text{NH}_3$ concentrations were measured in colonic luminal contents (500 μl). They were first deproteinized [with ice-cold PCA (final concentration: 4%)], then neutralized, and these suspensions were then used to determine ammonium concentrations using the same specific enzymatic method as described earlier.

Colon epithelial cell isolation and incubation. Colonic epithelial cells (colonocytes) were obtained from isolated colons as described (9). Briefly, the colons were removed and flushed clean with NaCl solution (9 g/l) and then with a Ca^{2+} - and Mg^{2+} -free Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 10 mM HEPES, 5 mM dithiothreitol, and 2.5 g/l bovine serum albumin and equilibrated against a mixture of O_2 and CO_2 (19:1, vol/vol). Then, the colons were perfused for 20 min at 37°C with the same buffer containing 5 mM EDTA. The luminal fluid was collected, and the cell fraction was incubated for 15 min at 37°C using the same buffer except for the absence of EDTA and the presence of hyaluronidase (4 g/l). The isolated cells were washed twice and resuspended in a Krebs-Henseleit bicarbonate-buffered medium (pH 7.4) saturated with a mixture of O_2 and CO_2 (19:1, vol/vol) containing (in mM) 120 NaCl, 4.7 KCl, 1.2 KH_2PO_4 , 1.2 Na_2SO_4 , 10 HEPES, 1.3 CaCl_2 , 2 Mg Cl_2 , and 25 NaHCO_3 and enriched with 10 g/l bovine serum albumin (incubation medium). The colonocytes were counted on a hemacytometer. Previous work (9) has determined that the isolation procedure used in this study led to the recovery of absorbing colonocytes, which represent at least 87% of the isolated cells. Colonocyte viability was checked by measuring the percentage of the cytosolic enzyme lactate dehydrogenase activity released in the extracellular medium (7). Freshly isolated colonocytes were used immediately for metabolic flux measurement, or they were frozen in liquid nitrogen and stored at -80°C for subsequent enzymatic activity measurement. Cell viability was never $<80\%$ at the onset of incubation. Isolated cells were incubated in 120 μl incubation medium containing the radiolabeled precursors. At the end of the incubation, the samples were kept at -80°C until the HPLC procedure (see *HPLC*).

Enzymatic activities. Isolated colonocytes were kept at -80°C before measurement of enzymatic activities. They were homogenized by sonication at 4°C . Arginase activity was measured at 37°C in 100 μl of a Tris-HCl buffer (100 mM, pH 7.2) containing 2 mM MnCl_2 and increasing concentrations of L-[guanido- ^{14}C]arginine. Incubation was stopped with PCA (final concentration: 2%), and urea was separated from L-arginine by HPLC (see *HPLC*). Cytosolic and particulate arginase activities were obtained from colonocytes homogenized at 4°C by mechanical disruption and sonication (5×1 s) in a Tris-HCl buffer (100 mM, pH 7.2). The homogenate was then centrifuged at 4°C for 90 min at 100,000 g. The pellet (particulate arginase activity) was then rehomogenized at 4°C by sonication ($1 \times$

Table 1. Composition of the experimental diets P20 and P58

	Control Protein Diet (P20)	High Protein Diet (P58)
Metabolizable energy, kJ/g	37.13	37.17
Dry matter, g/kg		
Casein ^a	200.0	580.0
Cornstarch ^b	440.0	160.0
Sucrose	233.5	83.5
α -Cellulose ^c	20.0	70.0
Colza oil ^d	25.0	25.0
Groundnut oil ^d	25.0	25.0
DL-Methionine ^e	1.5	1.5
Vitamin mixture ^f	10.0	10.0
Mineral mixture ^g	45.0	45.0

^aHEC01 casein, UCCS, Surgeres, France. ^bCornstarch, Cerestar, Saint-Maur, France. ^c α -Cellulose, Durieux, Marne-la-Vallée, France. ^dColza and Groundnut oil, Bailly, Aulnay-sous-Bois, France. ^eDL-methionine, Central Soya, Trappes, France. ^fVitamin mix CV102, Central Soya, Trappes, France; Vitamin A acetate (500,000 IU); Vitamin D3 (250,000 IU); Vitamin E (5,000 IU); Vitamin K (100 IU); thiamin HCl, 1.0 g/kg; riboflavin B2, 1.0 g/kg; nicotinic acid, 4.5 g/kg; D-calcium pantothenate, 3.0 g/kg; pyridoxine HCl, 1.0 g/kg; inositol, 5.0 g/kg; D-biotin, 0.02 g/kg; folic acid, 0.2 g/kg; cyanocobalamin, 0.00135 g/kg; ascorbic acid, 10.0 g/kg; para amino benzoic acid, 5.0 g/kg; choline chloride, 75.0 g/kg; choline chlorure, 150.0 g/kg. ^gMineral mix, Prolabo Merck, Darmstadt, Germany (in g/kg mineral mixture): CaHPO_4 , 2H₂O, 380.0; K_2HPO_4 , 240.0; CaCO_3 , 180.0; MgSO_4 , 7H₂O, 90.0; NaCl, 69.0; MgO, 20.0; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 8.6; ZnSO_4 , H₂O, 5.0; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 5.0; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.0; NaF, 0.8; CrK (SO_4)₂, 12H₂O, 0.5; KI, 0.04; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 4H₂O, 0.02; CoCO_3 , 0.02; Na_2SeO_3 , 0.02. P20, isocaloric diet-fed rats with 20% casein; P58, isocaloric diet-fed rats with 58% casein.

10 s) in 300 μ l of the Tris·HCl buffer. Arginase activities were finally assayed using the particulate fraction and 100,000 g cytosolic supernatant (cytosolic arginase activity) in the presence of 20 mM L-[guano- 14 C]arginine. OCT activity was measured at 37°C in 100 μ l of a Tris·HCl buffer (50 mM, pH 8.3) containing 0.5 mM carbamoylphosphate and increasing concentrations of L-[U- 14 C]ornithine. The reaction was halted at -80°C and L-citrulline was separated from L-ornithine by HPLC (see *HPLC*). CPSI activity was measured on the cells previously sonicated in the presence of 20 μ l of a cocktail of protease inhibitors [0.4 mg/ml 4-(2-aminoethyl)benzenesulfonylfluoride, 5 mg/ml EDTA- Na_2 , 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin]. The enzyme assay was then performed at 37°C for 60 min in 100 μ l of a Tris·HCl buffer (50 mM, pH 8.3) containing (in mM) 15 $\text{NaH}^{14}\text{CO}_3$, 20 ATP, 2 N-acetylglutamate, 10 MgSO_4 , 10 NH_4Cl , and 10 L-ornithine and 15 U OCT. The reaction was halted at -80°C, and L-citrulline was separated from NaHCO_3 by HPLC (see *HPLC*). Argininosuccinate synthase (ASS) was measured at 37°C in 100 μ l of a Tris·HCl buffer (10 mM, pH 7.5) in the presence of 0.1 mM L-[ureido- 14 C]citrulline, 0.5 mM L-aspartate, 20 mM KCl, 0.5 mM MgCl_2 , 1 mM ATP, and 0.5 mU inorganic pyrophosphatase and in the presence or absence of 22 mU argininosuccinate lyase (ASL). The reaction was halted with PCA (final concentration 2%), and L-arginine was separated from L-citrulline by HPLC (see *HPLC*). ASS and ASL enzymatic activities were also determined in the rat liver as a positive control. In that case, the liver was homogenized in the same buffer, centrifuged at 14,000 g for 4 min and the supernatant was used for the assay. Ornithine decarboxylase (ODC) activity was measured at 30°C in 100 μ l of a HEPES/NaOH buffer (50 mM, pH 7.2) containing 60 μ M L-[1- 14 C]ornithine, 0.5 mM dithiothreitol, 0.2 mM pyridoxal 5'-phosphate, and with or without 10 mM DFMO. The reaction was stopped with 100 μ l of 0.5 M HCl, and the $^{14}\text{CO}_2$ was trapped in methylbenzethonium hydroxide and counted by liquid scintillation. ODC activity was calculated from the difference between $^{14}\text{CO}_2$ production in the absence or presence of 10 mM DFMO, which totally inhibits ODC activity (3).

HPLC. To measure metabolic fluxes in intact cells and enzymatic activities in cellular homogenates, radioactive products were separated from precursors by HPLC and counted by liquid scintillation. L-Citrulline was separated from L-[U- 14 C]arginine or from $\text{NaH}^{14}\text{CO}_3$ using a C18 column (Kromasil, Le Mesnil le Roi, France) after O-phthalaldehyde derivatization as described (5). L-Ornithine was separated from L-[U- 14 C]arginine using a partispher C18 column (Whatman, Clifton, NJ), and the gradient was described (6). This latter method was also used for L-arginine separation from L-[ureido- 14 C]citrulline, for L-citrulline separation from L-[U- 14 C]ornithine, and for urea separation from L-[guanido- 14 C]arginine.

Presentation of results. The production of radioactive metabolites was calculated by reference to the specific activity of the precursors in the incubation medium. V_{max} values were calculated using the Lineweaver-Burk representation. The results were expressed as the means $\pm$ SE together with the number of independent experiments performed with different cell preparations (n). The statistical significance of differences between mean values was assessed by the Student's t -test.

RESULTS

Food intake and body weight. The difference in food intake between the two groups was more ample in the very beginning of the experiment than after 7 days. Indeed rats fed isocaloric diets containing 20 (P20) or 58% (P58) casein ingested, respectively, 18 and 17 g of food per day on day 7 (Fig. 1A). Probably due to a higher daily food consumption in the control diet group (P20), the animals in this group had a better daily weight gain within the first 2 days of the experiment than the animals from the high-protein diet group (P58), which initially

Fig. 1. A: daily total food intake of rats fed a 20% casein diet (P20 control diet) and 58% casein (P58) diet for 7 days. The results are expressed as means $\pm$ SE and represent 17 independent experiments. * $P < 0.05$, *** $P < 0.001$ vs. P20. B: cumulative body wt changes of rats fed the 20 or 58% casein diet. The results are expressed as means $\pm$ SE and represent 18 independent experiments. * $P < 0.05$, *** $P < 0.001$ vs. P20.

loose weight. Thereafter, the daily weight gain was similar in both groups (Fig. 1B). The mean weights of the animals at the end of the experiments (i.e., after 7 days) were not significantly different ($P = 0.1$) averaging 232.8 ± 5.6 g for the control diet vs. 223.3 ± 5.0 g for the high-protein diet ($n = 18$).

Fecal and intestinal pH and $\text{NH}_4^+/\text{NH}_3$ concentrations. Figure 2 shows the pH in the intestinal contents recovered from animals receiving P20 and P58 diets for 2 and 7 days, respectively. Two days after the consumption of the P58 diet, the pH measured in the proximal colon content (but not in the other anatomical segments) was significantly increased compared with the pH measured in the control group (P20; Fig. 2). After 7 days of consumption of the P58 regimen, however, the pH remained not significantly different than the one measured in the proximal colon content recovered from the animals in the P20 group (Fig. 2).

Figure 3 reports the $\text{NH}_4^+/\text{NH}_3$ concentrations in the colonic luminal contents and fecal samples recovered at different times following P20 and P58 diet ingestion. When compared with the $\text{NH}_4^+/\text{NH}_3$ concentrations measured in the animals receiving the P20 diet, luminal $\text{NH}_4^+/\text{NH}_3$ concentrations in the P58 group increased after 2 days in the cecum and in the proximal and median regions of the large bowel but were not modified in the distal colon and feces. Four days after the beginning of the ingestion of the experimental diets, the $\text{NH}_4^+/\text{NH}_3$ concentrations were increased in all the large intestine segments in the P58 group when compared with the values obtained in the

Fig. 2. Rats were fed 20 or 58% casein diets for 2 and 7 days, and pH was measured in the intestinal contents of the cecum, proximal, median, and distal colon. The results are expressed as means $\pm$ SE and represent 4 independent experiments. * $P < 0.05$ vs. P20.

P20 group (Fig. 3). Lastly, after 7 days of experimentation, $\text{NH}_4^+/\text{NH}_3$ concentrations were significantly higher in the P58 group in the cecum and in the proximal/median colon luminal contents when compared with the values measured in the P20 group (Fig. 3).

Colonic blood $\text{NH}_4^+/\text{NH}_3$ concentrations. When the $\text{NH}_4^+/\text{NH}_3$ concentration was measured in the blood recovered from the right colonic vein, a rise was registered in the group consuming 58% protein when compared with the group consuming 20% protein (Fig. 4). Indeed, 2 and 4 days after the beginning of the hyperproteic diet consumption, the blood $\text{NH}_4^+/\text{NH}_3$ concentration was approximately threefold higher than in the normoproteic group. However, after 7 days, the blood $\text{NH}_4^+/\text{NH}_3$ concentration was only slightly, but significantly, higher than the concentration measured in venous blood obtained from the P20 animals.

Effect of high-protein diet on L-arginine metabolism in isolated colonocytes. When measuring L-[guanido- ^{14}C]arginine conversion to radioactive urea in the isolated colonocytes, this conversion was 1.4-fold ($n = 3$, $P < 0.05$ vs. control) and 2.4-fold ($n = 7$, $P < 0.001$ vs. control) higher in the colonocytes isolated from the P58 group when compared with the conversion measured in the colonocytes isolated from the P20 group after 2 and 7 days, respectively (Table 2). When 1 mM L-[U- ^{14}C]arginine was used as a precursor, the production of radioactive L-ornithine measured in the P58 group was also significantly increased when compared with the production measured in the P20 group (Table 2). This increased L-arginine flux through arginase leading to increased urea and L-ornithine production in P58 isolated colonocytes was associated with an increase in arginase catalytic activity detected in colonocyte homogenates (Table 3). Indeed, the maximal velocity of arginase activity ($V_{\max}$) was found to be higher in the P58 group than in the P20 control group after 2 and 7 days of the experiment (Table 3).

Fig. 3. Rats were fed 20 or 58% casein diets for 2, 4, and 7 days, and ammonia concentrations were measured in the lumen of the large intestine anatomical segments and in feces. The results are expressed as means $\pm$ SE and represent 5 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. P20.

The arginase activities associated with the cytosolic and particulate fractions were found to be both increased in the P58 group both after 2 and 7 days of the experiment (Fig. 5).

Effect of NH_4Cl on the conversion of L-arginine to L-citrulline according to the experimental diets. After 2 days of the experimental diet, NH_4Cl (1 mM), when present in the incubation medium, failed to increase the basal radioactive L-citrulline production from 1 mM L-[U- ^{14}C]arginine both in colonocytes recovered from control P20 and P58 animal groups (Fig. 6). In contrast, in colonocytes isolated from the

Fig. 4. Rats were fed 20 or 58% casein diets for 2, 4, and 7 days, and ammonia concentrations were measured in the blood recovered from the right colonic vein. The results are expressed as means $\pm$ SE and represent 9 independent experiments. * $P < 0.05$, *** $P < 0.001$ vs. P20.

Table 2. Effect of the experimental diets on the L-arginine metabolism in isolated colonocytes

	pmol · 10 ⁶ cells ⁻¹ · 30 min ⁻¹	
	2 days	7 days
Production of urea from 1 mM L-[guanido- ¹⁴ C]arginine		
P20	384.6 ± 67.3	257.9 ± 56.5
P58	527.7 ± 29.3*	613.1 ± 65.6***
Production of L-ornithine from 1 mM L-[U- ¹⁴ C]arginine		
P20	419.8 ± 83.7	550.0 ± 170.5
P58	677.0 ± 111.5**	876.0 ± 261.9*

Values are means ± SE of 3–7 independent experiments. Urea and L-ornithine production were measured in colonocytes isolated after 2 or 7 days of the experimental diets. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. P20.

P20 group, 10 mM NH₄Cl increased the basal L-citrulline production in P20 fourfold as in P58 colonocytes; 20 mM NH₄Cl increased this production ninefold, and 50 mM NH₄Cl was even more active. No significant differences in L-citrulline production cellular capacity as a function of NH₄Cl concentration could be measured in P58 colonocytes after a 2 days of diet when compared with increases recorded in the P20 group. After 7 days of the experimental diet, the NH₄Cl-provoked increases in L-citrulline production by control P20 colonocytes were close to the values recorded in this group after 2 days (Fig. 6). However, the NH₄Cl-dependent L-citrulline production was found to be significantly lower in colonocytes isolated from the P58 diet (Fig. 6). The decrease in L-citrulline synthesis capacity in colonocytes isolated from the P58 animals was not associated with any measurable decrease in either the CPSI and OCT catalytic activities (Table 3). Lastly, L-citrulline was apparently not used by isolated colonocytes for L-arginine de novo production in the pathway catalyzed by ASS and ASL, representing thus a metabolic end product. Indeed, the incubation of colonocytes isolated from control P20 and P58 groups after 7 days of experimental diets, in the presence of 1 mM L-[ureido-¹⁴C]citrulline, 1 mM L-aspartate used as a cosubstrate in the reaction catalyzed by ASS, and with or without 5 mM L-valine used to inhibit arginase activity failed to lead to any detectable production of radioactive L-arginine in control P20 colonocytes and in P58 colonocytes (data not shown). Furthermore, ASS activity when measured in the presence of an excess of ASL activity (used to convert all the L-arginino-

Table 3. Effect of the experimental diets on arginase, CPSI and OCT catalytic activities

	V _{max} , nmol · mg protein ⁻¹ · 60 min ⁻¹		
	Arginase	CPSI	OCT
2 Days diet			
P20	40.9 ± 7.9		190.8 ± 48.3
P58	100.7 ± 14.9**		231.6 ± 19.2
7 Days diet			
P20	45.6 ± 12.5	0.11 ± 0.02	216.3 ± 46.9
P58	102.0 ± 16.5*	0.12 ± 0.01	242.2 ± 38.4

Values are means ± SE of 5–8 independent experiments. Enzymatic activities were measured on colonocyte homogenates obtained after two and seven days of the P20 or P58 diet ingestion. **P* < 0.05, ***P* < 0.01 vs. P20. CPSI, carbamoyl phosphate synthetase I; OCT, ornithine carbamoyltransferase.

Fig. 5. Rats were fed 20 or 58% casein diets for 2 and 7 days, and cytosolic and particulate arginase activities were measured as 20 mM L-[G-¹⁴C]arginine conversion to radioactive urea in the cytosolic and particulate fractions derived from colonocyte homogenates. The results are expressed as means ± SE and represent 3–4 independent experiments. **P* < 0.05 vs. P20.

succinate produced by ASS into L-arginine) was found to be very low in both cases, representing 33.95 ± 12.32 pmol · mg protein⁻¹ · 60 min⁻¹ (*n* = 3) in the control group and 31.69 ± 6.46 pmol · mg protein⁻¹ · 60 min⁻¹ (*n* = 3) in the hyperproteic group. This activity was found to be indeed very small when compared with the activity in the liver homogenate used as the positive control, i.e., 95.6 ± 1.4 nmol · mg protein⁻¹ · 60 min⁻¹ (*n* = 3). Conversion on day 7 of L-citrulline to L-arginine without added ASL was found to be close to the limit of detection, not exceeding 10 pmol · mg protein⁻¹ · 60 min⁻¹ in both cases.

Effect of a high-protein diet on polyamine metabolism in isolated colonocytes. L-ornithine derived from L-arginine was little used by the colonocytes for polyamine production in the ODC pathway. Indeed, ODC activity measured in colonocyte

Fig. 6. Rats were fed 20 or 58% casein diets for 2 and 7 days, and the effect of NH₄Cl on the conversion of 1 mM L-[U-¹⁴C]arginine to L-citrulline by isolated colonocytes was measured. The results are expressed as means ± SE and represent 4 independent experiments. **P* < 0.05, ***P* < 0.01 vs. P20.

homogenates recovered from the control P20 and P58 groups averaged, respectively, 2.26 ± 0.57 and 3.75 ± 0.85 pmol·mg protein⁻¹·60 min⁻¹ ($n = 4$ in both cases) after 2 days of experimental diet and 1.87 ± 0.38 and 3.23 ± 0.85 pmol·mg protein⁻¹·60 min⁻¹ ($n = 5$ in both cases) after 7 days.

DISCUSSION

Our data clearly confirm that colonic luminal $\text{NH}_4^+/\text{NH}_3$ concentrations increase as a result of enhanced protein intake and demonstrate that this exposure to an increased $\text{NH}_4^+/\text{NH}_3$ environment is associated with modifications of ammonia-related amino acid metabolic capacities in colonocytes.

As previously described (31), the rats fed the P58 diet are apparently able to adapt to the high-protein diet, because their food intake, although depressed at the beginning of the experiments, was similar to those of the P20 control group from days 3 to 7. A putative brain adaptation to increased plasma $\text{NH}_4^+/\text{NH}_3$ levels (36), together with the adaptation of the liver urea cycle enzymes to a high-protein diet (27) leading to a rapid decrease of plasma $\text{NH}_4^+/\text{NH}_3$ levels, may partly explain the recovery of the normal food intake.

The rats receiving the hyperproteic diet were found to show higher $\text{NH}_4^+/\text{NH}_3$ concentrations in the cecum and in the proximal and median colon lumen after 2 days of the experimental diet and thereafter. $\text{NH}_4^+/\text{NH}_3$ increase was more modest in the distal colon and in the feces. This may result from different bacterial $\text{NH}_4^+/\text{NH}_3$ production and/or utilization for the de novo synthesis of proteins in the different large intestine segments as well as different $\text{NH}_4^+/\text{NH}_3$ absorptive capacities in these segments. Increase of the fecal $\text{NH}_4^+/\text{NH}_3$ concentration can be measured in humans consuming a high-protein diet (12, 14). The dietary protein levels also influenced the $\text{NH}_4^+/\text{NH}_3$ concentration in the blood collected from the colonic vein, which was much higher in the P58 group within the first days of the experiment and then almost returned back to the control values after 7 days, suggesting a metabolic adaptation of colonocytes toward increased $\text{NH}_4^+/\text{NH}_3$ ammonia concentration.

We previously demonstrated (25) in colonocytes the metabolic capacity for an incomplete ornithine cycle, acting as a probable detoxification pathway against ammonia. In these cells, the direct conversion of L-arginine into L-citrulline and nitric oxide through nitric oxide synthase activity is negligible when compared with the sequential conversion of L-arginine into L-citrulline through arginase and OCT activities (25, 28). The already described adaptation of the hepatic urea cycle enzymes to an increased protein intake led us to study colonic epithelial cell metabolism in response to increased luminal ammonia concentration. Therefore, we measured the effects of the P58 diet on the metabolic pathways involved in ammonia metabolism in colonocytes. In our study, we showed that the L-arginine flux through arginase, which allows L-ornithine and urea production, was increased 2 days after the P58 diet ingestion, and this increase was found to be maintained up to the end of the experiments. The increased L-arginine flux through arginase could be explained by a concomitant increase of both cytosolic and particulate arginase activities measured in homogenates of colonocytes recovered in the hyperproteic group of animals. Studies with increased intake of dietary protein (i.e., 60% casein) indicate that the rat liver shows a

marked stimulation in its rate of L-ornithine catabolism (30). Moreover, L-ornithine availability has been shown to limit the rate of urea synthesis in isolated hepatocytes (20). Furthermore, a substantial fall of L-ornithine plasma concentration has been measured under conditions of high portal $\text{NH}_4^+/\text{NH}_3$ concentrations (24). L-Ornithine is not a constituent of proteins, and increasing protein intake thus induces an increased liver L-ornithine requirement for liver ammonia detoxification. The marked increase of arginase activity in colonocytes by a high-protein diet and the consequent increase in L-ornithine production (without any increase in the L-ornithine requirement for L-citrulline and polyamine synthesis) thus likely contribute to ensure a sufficient L-ornithine production available for its metabolism in the liver. These results suggest that in addition to small intestine enterocytes that release L-ornithine into the blood (4, 41), colonic epithelial cells are involved in a colon-liver axis for an increased L-ornithine supply to the liver in response to a high-protein diet intake. However, it is not possible to exclude in our experiments a role of decreased dietary content in carbohydrates in the isocaloric hyperproteic group of animals for the recorded increase of L-ornithine production capacity by colonocytes. Indeed, it has recently been reported (2) that a low-carbohydrate/high-fat diet resulted in a slight increase of the postabsorptive plasma L-ornithine concentration when compared with an isocaloric isoproteic high-carbohydrate/low-fat diet.

It is worth noting that increased arginase activity in the colonocytes leads to increased urea production, which may diffuse in the lumen and serve as a substrate for bacterial ureases leading to increased $\text{NH}_4^+/\text{NH}_3$ production. However, urea represents a minor source of $\text{NH}_4^+/\text{NH}_3$ in the colon (21).

A measurable amount of L-ornithine derived from L-arginine was found to be converted into L-citrulline in the presence of NH_4Cl . L-Citrulline appeared to be the end product of ammonia metabolism in colonocytes, because these cells did not convert L-citrulline back to L-arginine. This undetectable L-citrulline conversion to L-arginine in colonocytes can be explained by the very weak ASS activity in homogenates of colonocytes recovered from normoproteic and hyperproteic groups of animals, indicating that the high-protein diet did not induce ASS activity. A similar metabolic situation is found in the small intestine of weaned animals (41), where L-citrulline produced by enterocytes is recovered in the portal vein, bypasses the liver, and is finally converted into L-arginine by the kidney, allowing, through this intestine-kidney axis, the de novo synthesis of L-arginine by the body (42).

After 2 days of the experimental diet, L-citrulline production by the colonocytes in the presence of NH_4Cl was not significantly modified in the hyperproteic group when compared with the control group. In contrast, a marked significant decrease of colonocyte capacity to synthesize L-citrulline was evidenced in the hyperproteic P58 group after 7 days of the experimental diet. Surprisingly, this decreased metabolic flux did not correspond to any decrease in CPSI and OCT activities measured in homogenates of colonocytes recovered from the hyperproteic group. NH_4Cl concentrations used in the incubation medium to increase L-citrulline synthesis (i.e., up to 50 mM) were in the range of the $\text{NH}_4^+/\text{NH}_3$ concentrations found in the colon lumen, concentrations that have been shown to exert no short-term cytotoxic effect on isolated colonocytes (25). In the hyperproteic group, the decrease in the colonocyte capacity for

Fig. 7. Schematic view for the evolution of the metabolic capacities of colonic epithelial cells after 7 days of hyperproteic (P58) vs. normoproteic (P20) diet ingestion. ARG, arginine; CITR, citrulline; CP, carbamoylphosphate; CPSI, carbamoylphosphate synthetase I; OCT, ornithine carbamoyltransferase; ODC, ornithine decarboxylase; ORN, ornithine; P, putrescine.

L-citrulline production from L-arginine in the presence of NH_4Cl could thus not be explained by a decrease of the colonocyte capacity to generate L-ornithine from L-arginine, by an increased utilization of L-ornithine for polyamine synthesis, or by a decrease of the colonocyte capacity to metabolize ammonia. The most likely explanation is that the L-citrulline production capacity in colonocytes is limited by the ammonia availability for cellular metabolism.

$\text{NH}_4^+/\text{NH}_3$ generated in the luminal contents has been suggested to increase luminal pH (19). However, in the present study, significant pH differences associated with different levels of protein intake were found only in the luminal content of the proximal colon recovered after 2 days of experimental diet from the hyperproteic group of animals. Although it is obvious that luminal pH can be modified *in vivo* by numerous luminal compounds other than $\text{NH}_4^+/\text{NH}_3$, an increase in luminal pH may favor ammonia entry into the colonic cells. Indeed, because ammonia is a weak base with a pK_a equal to 9.02, the vast majority of ammonia produced by the bacterial flora exists as NH_4^+ in the colonic luminal environment (40). Thus the higher protein consumption and the associated higher luminal pH in the proximal colon after 2 days of the experimental diet will result in an enhanced ratio of nonionized NH_3 over the protonated NH_4^+ form and the absorption of NH_3 by nonionic diffusion. Indeed, NH_3 passage through cellular membranes is 400 times more rapid than for NH_4^+ (11). In other words, the *in vivo* transient increase of luminal pH may participate in the rapid increased ammonia movements through the surface epithelial colonic cells. In colonic crypt cells, a strong permeability barrier has been shown to limit NH_3 diffusion (37). However, in apical membranes from rat distal colon, the H^+/K^+ -ATPase has been shown to be able to substitute NH_4^+ for K^+ , raising the view that this enzyme may function as an NH_4^+ pump (10). In our study, the colonocyte isolation procedure used leads to the recovery of surface epithelial absorbing

colonocytes, which represent $\sim 90\%$ of the isolated cells (9), the remainder being goblet cells.

Altogether, our results (see Fig. 7) show that in response to increased $\text{NH}_4^+/\text{NH}_3$ concentration in the colonic luminal content, colonocytes are able to increase L-ornithine synthesis from L-arginine and to decrease L-ornithine utilization for L-citrulline synthesis. The resulting higher L-ornithine availability would correspond to an elevated requirement for the elimination of portal blood ammonia in the liver urea cycle. It is conceivable that in a situation of plethoric alimentary protein supply, the intestinal production of L-citrulline used for kidney L-arginine *de novo* synthesis would be lowered. From the $\text{NH}_4^+/\text{NH}_3$ concentrations measured in the large intestine lumen and venous blood at different time points following hyperproteic diet ingestion, it appears that the increased $\text{NH}_4^+/\text{NH}_3$ movement through the colonic epithelium is transient and limited. The adaptative metabolic capacities of the differentiated absorbing colonocytes toward $\text{NH}_4^+/\text{NH}_3$ likely contribute to regulate both intracellular $\text{NH}_4^+/\text{NH}_3$ concentration and ammonemia. Further work is required to get a better understanding of the metabolic events involved in the adaptation of the colonic epithelial cells to increased luminal ammonia concentrations, particularly after longer periods of hyperproteic diet consumption. This is of considerable interest, because excessive luminal $\text{NH}_4^+/\text{NH}_3$ concentrations are considered to be deleterious for colonic epithelium (18, 39) and because colonic epithelium can be viewed in an interorgan metabolic perspective as a first line of defense against luminal $\text{NH}_4^+/\text{NH}_3$ entry into the bloodstream.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Carole Attala for technical help, Wendy Brand-Williams for revising the English manuscript, and Sandrine Boguais for secretarial help.

GRANTS

The study was supported by La Ligue contre le Cancer (Comité des Yvelines).

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