Nutrient Metabolism

Dietary Protein or Arginine Deficiency Impairs Constitutive and Inducible Nitric Oxide Synthesis by Young Rats[1]

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ABSTRACT Effects of dietary protein or arginine deficiency on constitutive and lipopolysaccharide (LPS)-induced nitric oxide (NO) synthesis were determined in young rats by quantifying urinary nitrate excretion. In Experiment 1, 30-d-old rats (n = 16) were divided randomly into two groups (n = 8/group) and pair-fed on the basis of body weight semipurified isocaloric diets containing 20 or 5% casein. In Experiment 2, 30-d-old rats (n = 24) were divided randomly into three groups (n = 8) and pair-fed on the basis of body weight purified isonitrogenous and isocaloric diets (composed of amino acids) containing 0.0, 0.3 or 1.0% L-arginine. In both experiments, daily collection of urine was initiated 10 d after the start of pair-feeding. On d 17 after the pair-feeding was initiated, LPS (1 mg/kg body wt) was injected intraperitoneally into rats, and urine was collected daily for an additional 7 d. In Experiments 3 and 4, activities of constitutive and inducible NO synthases were measured in macrophages and various tissues from protein- or arginine-deficient rats (n = 6). Body weight was lower in rats fed the 5% casein diet or the 0.0 and 0.3% arginine diets than in those fed 20% casein or 1% arginine, respectively. Dietary protein or arginine deficiency decreased serum concentrations of arginine and urinary nitrate excretion before and after LPS treatment, indicating impaired constitutive and inducible NO synthesis. Protein malnutrition reduced constitutive and inducible NO synthase activities in brain, heart, jejunum, lung, skeletal muscle and spleen, and inducible NO synthase activity in macrophages. Because NO is a mediator of the immune response and is the endothelium-dependent relaxing factor, impaired NO synthesis may help explain immunodeficiency and cardiovascular dysfunction in protein- or arginine-deficient subjects. J. Nutr. 129: 1347–1354, 1999.

KEY WORDS: • *protein* • *arginine* • *malnutrition* • *nitric oxide* • *rats*

The recent discovery of the arginine-nitric oxide (NO)[3] pathway has united traditionally diversified research areas in biochemistry, immunology, neuroscience, nutrition, pathology and physiology (Culotta and Koshland 1992) and has stimulated renewed interest in arginine nutrition of animals and humans (Wu and Morris 1998). NO, a free radical molecule, is synthesized from L-arginine by NO synthase (NOS) in virtually all mammalian cells (Morris 1998). NO is the endothelium-derived relaxing factor, a neurotransmitter, a mediator of immune response and a signaling molecule in various physiologic processes (Ignarro 1990, Moncada and Higgs 1993). NO synthases exist in two isoforms, i.e., constitutive (cNOS) and inducible (iNOS), on the basis of Ca^{2+} requirements. cNOS is constitutively expressed and is Ca^{2+}/calmodulin–dependent, whereas iNOS is Ca^{2+}-independent and can be induced by inflammatory cytokines and bacterial endotoxin [e.g., lipopolysaccharide (LPS)] (Macmicking et al. 1997, Michel and Feron 1997). By serving as a substrate for NOS and by promoting the dimerization of NOS, L-arginine plays an important role in regulating constitutive and inducible NO synthesis (Morris 1998, Stuehr 1997).

Several studies have examined the effect of dietary protein or arginine intake on in vivo NO production by adult rats or humans. Bulgrin et al. (1993) reported that feeding an arginine-free diet reduced NO synthesis by adult rats during wound healing. However, plasma concentrations of amino acids or tissue NOS activities were not determined in that study; thus it is not known whether the reduced NO synthesis resulted from an altered availability of arginine. In healthy adult humans, feeding an arginine-free diet appeared to have no effect on in vivo constitutive NO synthesis (Castillo et al. 1995). Similarly, in adult mice with mycobacterial infection, protein malnutrition had no effect on urinary nitrate excretion (Chan et al. 1996). Despite the foregoing, little information is available on the regulation of in vivo NO synthesis by dietary protein or arginine in young mammals, for which arginine is an essential amino acid (Visek 1985).

In vitro studies have demonstrated that extracellular arginine is critical to NO synthesis by endothelial cells (Arnal et al. 1995) and activated macrophages (Norris et al. 1995). Dietary protein or arginine deficiency results in plasma arginine deficiency in animals and humans, particularly young mammals (Eisenstein and Harper 1991, Holt et al. 1963, Lunn and Austin 1983). Interestingly, arginine deficiency impairs

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[3] Abbreviations used: LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; cNOS, constitutive NOS; iNOS, inducible NOS.

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glucose metabolism and insulin secretion (Mulloy et al. 1982), which are now known to be mediated by NO (Schmidt et al. 1992, Young et al. 1997). In addition, protein malnutrition is associated with immunodeficiency (Chandra 1972 and 1991, McMurray et al. 1981 and 1986), as well as hypertension (Langley-Evans 1997) and cardiac dysfunction (Chauhan et al. 1965, Smythe et al. 1962, Wharton et al. 1967). In light of the foregoing, we hypothesized that dietary protein or arginine deficiency may impair NO synthesis in young mammals by decreasing plasma arginine concentrations and tissue NOS activities. This hypothesis was tested in young rats before and after LPS treatment by measuring urinary excretion of nitrate (the major stable oxidation product of NO) (Sakinis and Wennmalm 1998), which has been used as an indicator of in vivo NO synthesis in mice (Granger et al. 1991), rats (Wu 1995) and humans (Castillo et al. 1993 and 1995). Because extensive studies exist on the effect of dietary protein or arginine deficiency on plasma and urine concentrations of ammonia and urea in rats (e.g., Anthony and Edozien 1975, Gross et al. 1991, Lunn and Austin 1983, Milner 1985, Visek 1985), and because the effect of endotoxin on plasma and urine concentrations of urea has been reported for rats and other species (Kilpatrick-Smith et al. 1986, Komarov and Reddy 1998), these aspects of protein metabolism were not measured here.

MATERIALS AND METHODS

This study was approved by Texas A&M University's Laboratory Animal Care and Use Committee.

Chemicals and diets. Nitrate reductase, NADPH and lactate dehydrogenase were obtained from Boehringer Mannheim (Indianapolis, IN). Pyruvic acid, LPS (from *Escherichia coli*, serotype 0127:B8), L-amino acids, HEPES, calmodulin, N^G-methyl-L-arginine, FAD, FMN, EDTA, EGTA, dithiothreitol, (6R)-5,6,7,8-tetrahydro-L-biopterin, phenylmethylsulfonylfluoride, aprotinin, chymostatin, pepstatin A and Griess reagent were obtained from Sigma Chemical (St. Louis, MO). L-[U-^{14}C]Arginine was purchased from American Radiolabeled Chemicals (St. Louis, MO). Ficoll-Hypaque (d = 1.07 kg/L) was obtained from Pharmacia (Piscataway, NJ). All diets were purchased from Research Diets (New Brunswick, NJ).

Effect of dietary protein intake on in vivo basal and inducible NO synthesis (Experiment 1). Sixteen 25-d-old male Sprague-Dawley rats were obtained from Harland (Houston, Texas), divided randomly into two groups (n = 8/group) and placed in metabolism cages in a temperature- and humidity-controlled facility on a 12-h light:dark cycle. Rats had free access to deionized distilled H_2O and the semi-purified diet containing 20% casein (**Table 1**) for 5 d. After this 5-d period of adaptation, groups 1 and 2 were individually pair-fed isocaloric diets containing 20 and 5% casein, respectively (Table 1). Our preliminary studies indicated that rats fed the 20% casein diet consumed less feed than rats fed the isocaloric 5% casein diet (on the basis of 100 g body wt). Thus, pair-feeding on the basis of feed intake by rats fed the 20% casein diet was adopted to ensure equal intake of dietary energy, vitamins and minerals (on the basis of 100 g body wt). During the experiment, all rats had free access to deionized distilled water; feed consumption and body weights of the rats were measured daily. Both the diets and the drinking water contained no measurable nitrite or nitrate. Daily collection of urine was initiated 10 d after the pair-feeding. On d 17 after the pair-feeding was initiated (namely, d 7 of urine collection), LPS (1 mg/kg body wt) in 0.2 mL of saline solution was injected intraperitoneally into rats, and urine was collected each day for an additional 7 d. This dose of LPS has been reported to increase NO synthesis by rat macrophages (Wu and Brosnan 1992) and nitrate excretion by rats (Wagner et al. 1983, Wu 1995). Clean metabolism cages were used on the day of urine collection to minimize contamination by animal hair and feces (Wu 1995). Urine was collected into brown bottles containing antibiotics (60 mg Penicillin G, 100 mg Chloramphenicol, and 6 mg Amikacin) (Granger et al. 1991, Wu 1995). A strong acid solution (e.g., HCl or H_2SO_4) was not used for urine collection to prevent the potential loss of nitrite and nitrate because nitrite and nitrate can be reduced to NO gas under such conditions (Bramam and Hendrix 1989, Cox 1980).

TABLE 1

Diet composition for protein-deficient experiments (Experiments 1 and 3)

Ingredient	20% Casein diet	5% Casein diet
	g/kg diet	
Casein[1]	200	50
DL-Methionine	3	0.75
Cornstarch	150	302.25
Sucrose	500	500
Cellulose	50	50
Corn oil	50	50
Salt mix[2]	35	35
Vitamin mix[3]	10	10
Choline bitartrate	2	2
Total	1000	1000
Energy, *kJ/kg*	16,326	16,326

[1] Acid casein (88.1% protein) (New Zealand Milk Products, Santa Rosa, CA). Amino acid composition of acid casein was as follows (g amino acid/100 g protein): alanine, 2.6; arginine, 3.6; aspartate plus asparagine, 6.5; cysteine plus cystine, 0.4; glutamate plus glutamine, 20.9; glycine, 1.8; histidine, 2.6; isoleucine, 4.8; leucine, 8.8; lysine, 7.4; methionine, 2.6; phenylalanine, 5.0; proline, 11.7; serine, 5.4; threonine, 3.8; tryptophan, 1.2; tyrosine, 5.3; and valine, 5.7.

[2] Containing the following (g/kg salt mix): calcium phosphate dibasic (29.5% Ca and 22.8% P), 500; magnesium oxide (60.3% Mg), 24; manganous carbonate (47.8% Mn), 3.5; potassium citrate · 1 H_2O (36.2% K), 220; potassium sulfate (44.9% K and 18.4% S), 52; sodium chloride (39.3% Na and 60.7% Cl), 74; chromium potassium sulfate · 12 H_2O (10.4% Cr), 0.55; cupric carbonate (57.5% Cu), 0.3; potassium iodate (59.3% I), 0.01; ferric citrate (21.2% Fe), 6.0; sodium selenite (45.7% Se), 0.01; zinc carbonate (52.1% Zn), 1.6; and sucrose, 118.03.

[3] Containing the following (g/kg vitamin mix): retinyl palmitate (500,000 IU/g), 0.8; cholecalciferol (100,000 IU/g), 1.0; all-rac-α-tocopheryl acetate (500 IU/g), 10.0; menadione sodium bisulfite (62.5% menadione), 0.08; biotin (1.0%), 2.0; cyanocobalamin (0.1%), 1.0; folic acid, 0.2; nicotinic acid, 3.0; calcium pantothenate, 1.6; pyridoxine · HCl, 0.7; riboflavin, 0.6; thiamin · HCl, 0.6; sucrose, 978.42.

Effect of dietary arginine intake on in vivo basal and inducible NO synthesis (Experiment 2). This experiment was conducted as Experiment 1, except that rats were pair-fed purified diets containing 0.0, 0.3 or 1.0% L-arginine. Briefly, 25-d-old male Sprague-Dawley rats (n = 24) were divided randomly into three groups (n = 8/group) and placed in metabolism cages. Rats had free access to deionized distilled H_2O and a purified diet (composed of amino acids) containing 1.0% L-arginine (**Table 2**) for 5 d. After this 5-d period of adaptation, groups 1, 2 and 3 were individually pair-fed isonitrogenous and isocaloric diets containing 1.0, 0.3 and 0.0% L-arginine, respectively (Table 2). Dietary amino acid content was verified by HPLC analysis (Wu et al. 1997). Our preliminary studies indicated that rats fed the 1.0% arginine diet consumed less feed than those fed the isocaloric 0.0 or 0.3% arginine diets (on the basis of 100 g body wt). Thus, pair-feeding on the basis of feed intake by rats fed the 1% arginine diet was adopted to ensure equal intake of dietary energy, vitamins and minerals (on the basis of 100 g body wt). Daily collection of urine was initiated 10 d after the start of pair-feeding. On d 17 after the pair-feeding was initiated (namely, d 7 of urine collection), LPS (1 mg/kg body wt) in 0.2 mL of saline solution was injected intraperitoneally into rats, and urine was collected daily for an additional 7 d, as described in Experiment 1.

Nitrate and nitrite analysis. Urine was diluted 20–200 times with deionized distilled H_2O and analyzed for nitrate, as previously described (Wu and Brosnan 1992, Wu 1995). Briefly, 0.5 mL of diluted urine sample was incubated for 2 h at 25°C with 10 μL of

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TABLE 2

Diet composition for arginine-deficient experiments (Experiments 2 and 4)

Ingredient	0% Arg diet	0.3% Arg diet	1.0% Arg diet
		g/kg diet	
Essential amino acids			
L-Arginine (free base)	0	3	10
L-Histidine · HCl · H_2O	6	6	6
L-Isoleucine	8	8	8
L-Leucine	12	12	12
L-Lysine · HCl	14	14	14
L-Methionine	6	6	6
L-Phenylalanine	8	8	8
L-Threonine	8	8	8
L-Tryptophan	2	2	2
L-Valine	8	8	8
Nonessential amino acids			
L-Alanine	17.2	15	10
L-Asparagine · H_2O	5	5	5
L-Aspartate	17.2	15	10
L-Cystine	4	4	4
L-Glutamate	30	30	30
L-Glutamine	5	5	5
Glycine	17.2	15	10
L-Proline	5	5	5
L-Serine	5	5	5
L-Tyrosine	4	4	4
Cornstarch	538.9	542.5	550.5
Maltodextrin 10	125	125	125
Cellulose	50	50	50
Corn oil	50	50	50
Salt mix[1]	35	35	35
Sodium bicarbonate	7.5	7.5	7.5
Vitamin mix[2]	10	10	10
Choline bitrartrate	2	2	2
Total	1000	1000	1000
Energy, *kJ/kg*	14,305	14,305	14,305

[1] Same as the salt mix in Table 1.
[2] Same as the vitamin mix in Table 1.

nitrate reductase solution (5×10^3 units/L) and 75 μL of NADPH solution (0.5 g/L) to convert nitrate into nitrite. A solution (0.1 mL) consisting of 1 mmol/L pyruvate and 10 U of lactate dehydrogenase was then added to the assay mixture. After the mixture was incubated at 25°C for 1 h, the Griess reagent was added for colorimetric reaction with nitrite. The absorbance of the resulting solution was measured at 543 nm using a UV/VIS spectrophotometer (Perkin-Elmer, Oak Brook, IL). The pyruvate/lactate dehydrogenase mixture was employed to convert excess NADPH to $NADP^+$ because NADPH interferes with reaction of the Griess reagent with nitrite. Nitrite in 0.5 mL of 5- or 20-times diluted urine sample was also measured with the use of the Griess reagent but was not detectable (the detection limit was 1 μmol/L).

Serum concentrations of free amino acids. At the end of the urine collection (d 25 of the pair-feeding), blood was obtained from the tail vein using a microhematocrit tube (Wu 1995). Serum was collected by centrifugation of the blood (10,000 × *g*, 1 min), and used for amino acid analysis by a fluorimetric HPLC method as previously described (Wu et al. 1994).

Effect of dietary protein intake on NOS activities (Experiment 3). This experiment was conducted as described for Experiment 1, except that rats fed the 20 and 5% casein diets ($n = 6$/group) received intraperitoneal administration of 0 or 1 mg LPS per kg body wt on d 17 of pair-feeding and were killed 20 h later. After rats were anesthetized with CO_2, peritoneal macrophages were prepared using the Ficoll-Hypaque gradient as previously described (Wu et al. 1991), and several tissues (brain, gastrocnemius muscle, heart, jejunum, lung and spleen) were dissected. Macrophages and the tissues were used for determining activities of cNOS and iNOS by measuring conversion of L-[^{14}C]arginine into L-[^{14}C]citrulline, as described by Wu et al. (1998). Briefly, tissues (~0.5 g) were homogenized in 1 mL of 50 mmol/L HEPES buffer (pH 7.4) containing 1 mmol/L EDTA and protease inhibitors (5 mg/L phenylmethylsulfonyl-fluoride, 5 mg/L aprotinin, 5 mg/L chymostatin and 5 mg/L pepstatin). Aprotinin was recently reported to be a competitive inhibitor of cNOS and iNOS with K_i values of 50 and 78 μmol/L, respectively (Venturini et al. 1998). We found that aprotinin at the concentration (5 mg/L or 0.77 μmol/L) used in our tissue homogenization buffer had no effect on cNOS or iNOS activity. The homogenizer was rinsed with 1 mL of the buffer, and the combined homogenates were centrifuged at 600 × *g* and 4°C for 10 min. The supernatant fluid was used for NOS assays. For determining iNOS activity, the assay mixture (0.2 mL) contained 0.1 mmol/L (6R)-5,6,7,8-tetrahydro-L-biopterin, 1 mmol/L dithiothreitol, 1 mmol/L $MgCl_2$, 1 mg/L calmodulin, 0.1 mmol/L NADPH, 0.1 mmol/L FAD, 0.1 mmol/L FMN, 0.1 mmol/L L-[U-^{14}C]arginine (150 Bq/nmol), 10 mmol/L L-valine (an inhibitor of arginase), 0.1 mmol/L L-citrulline (to prevent the potential recycling of ^{14}C-citrulline into arginine), 2 mmol/L EGTA and tissue extracts (~1 mg protein). For determining total NOS activity, the assay mixture contained all of the above components, except that 2 mmol/L $CaCl_2$ replaced 2 mmol/L EGTA. Radioactivity blanks containing all of the above components plus 2 mmol/L N^G-methyl-L-arginine (an inhibitor of NOS) were included to improve assay specificity. cNOS activity was calculated by subtracting iNOS from total NOS activity.

Effect of dietary arginine intake on NOS activities (Experiment 4). This experiment was conducted as described for Experiment 2, except that rats fed the 1.0 and 0.0% arginine diets ($n = 6$/group) received intraperitoneal administration of 0 or 1 mg LPS per kg body wt on d 17 of pair-feeding and were killed 20 h later. cNOS and iNOS activities in peritoneal macrophages and tissues (brain, gastrocnemius muscle, heart, jejunum, lung and spleen) were measured as described in Experiment 3.

Statistical analysis. Results are expressed as means ± SEM. Data were analyzed by unpaired *t* test, or by 1-way or 2-way ANOVA with the Student-Newman-Keuls (SNK) multiple comparison test (Steel and Torrie 1980). Probability values < 0.05 were taken to indicate significant difference.

RESULTS

Feed intake and animal growth. During the 25-d period of pair-feeding, average daily feed intakes were 8.66 ± 0.49 and 8.74 ± 0.55 g/(100 g body wt · d), respectively, for rats fed the 20 and 5% casein diets (Experiment 1) and 9.91 ± 0.52, 9.76 ± 0.65 and 9.83 ± 0.62 g/(100 g body wt · d), respectively, for rats fed the 1.0, 0.3 and 0.0% arginine diets (Experiment 2). The greater food intake by rats fed the arginine diets (Experiment 2) compared with the casein diets (Experiment 1) was consistent with the lower energy concentrations of the arginine diets (Tables 1 and 2). In both experiments, feed intake decreased ($P < 0.05$) by 85% within 24 h of LPS administration and returned to the pre-LPS treatment value on d 3 post-LPS administration. Final body weight was lower in rats fed the 5% casein diet compared with those fed the 20% casein diet, and body weights of rats fed the 0.0 and 0.3% arginine diets were lower ($P < 0.05$) than those of rats fed the 1.0% arginine diet (**Table 3**).

Urinary excretion of nitrate by rats. Urinary excretion of nitrate was 52% lower ($P < 0.05$) in rats fed the 5% casein diet than in those consuming the 20% casein diet, before and after the LPS treatment (**Fig. 1**). Similarly, decreasing dietary arginine concentrations from 1.0 to 0.0% resulted in progressive decreases ($P < 0.05$) in urinary excretion of nitrate before and after LPS administration (**Fig. 2**). In all rats, LPS administration increased ($P < 0.05$) urinary excretion of nitrate to peak values within 24 h. Peak values of nitrate excretion were lower ($P < 0.05$) in protein- and arginine-deficient rats than

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TABLE 3

Effect of dietary protein or arginine deficiency on body weights of young rats (Experiments 1 and 2)[1]

Body weight	Exp. 1		Exp. 2		
	20% Casein	5% Casein	1.0% Arg	0.3% Arg	0.0% Arg
			g		
Initial	94.6 ± 3.2	95.1 ± 3.4	95.4 ± 3.6	94.3 ± 3.2	94.6 ± 4.1
Final	238.4 ± 6.2*	134.5 ± 4.8*,†	214.7 ± 5.6*,a	165.4 ± 4.9*,b	135.5 ± 4.3*,c

1 Initial body weights of rats at the beginning of pair-feeding (d 0) and their final body weights at the end of the experiment (d 25) were measured. Data are means ± SEM, $n = 8$, and analyzed by 2-way ANOVA and the Student-Newman-Keuls test for each experiment.

* $P < 0.05$, different from the initial value.

† $P < 0.05$, different from the 20% casein group (Exp. 1).

Means in a row with different letters (a–c) are different ($P < 0.05$) (Exp. 2).

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in those fed the 20% casein diet or the 1.0% arginine diet, respectively. By d 4 post-LPS treatment, urinary excretion of nitrate returned to basal values in all groups of rats.

Urinary nitrate excretion by LPS-treated rats represents NO synthesis by both cNOS and iNOS. Thus, inducible NO synthesis by LPS-treated rats can be calculated on the basis of urinary nitrate excretion before and after LPS administration. Peak values of inducible NO synthesis were 21% lower ($P < 0.05$) in rats fed the 5% casein diet compared with rats fed the 20% casein diet (**Table 4**). Similarly, reducing dietary arginine concentrations from 1.0 to 0.0% resulted in progressive decreases ($P < 0.05$) in the peak values of inducible NO synthesis by 17–35% (Table 4). On d 2 after LPS treatment, inducible NO synthesis remained 29 and 35% lower ($P < 0.05$), respectively, in rats fed the 0.0% arginine diet than in those fed the 1.0 and 0.3% arginine diets. In all groups of rats, there was no inducible NO synthesis on d 4 after LPS treatment, which likely resulted from the absence of iNOS in rat tissues.

Serum concentrations of free amino acids. There were no differences ($P > 0.05$) in serum concentrations of alanine, asparagine, aspartate, glutamate, glutamine and glycine between rats fed the 20 and 5% casein diets (**Table 5**). Serum concentrations of all essential amino acids, citrulline, cysteine, ornithine, taurine and tyrosine were lower ($P < 0.05$) in rats fed the 5% casein diet compared with rats consuming the 20% casein diet. Serum concentrations of most amino acids, except alanine, arginine, glutamine, glycine and ornithine, did not differ ($P > 0.05$) among rats fed the 0.0, 0.3 and 1.0% arginine diets (**Table 6**). Serum concentrations of arginine and ornithine were lower ($P < 0.05$), but those of alanine and glutamine were higher ($P < 0.05$), in rats fed the 0 and 0.3% arginine diets compared with rats fed the 1% arginine diet. Serum concentrations of glycine were lower ($P < 0.05$) in rats fed the 1.0% arginine diet than in those fed the 0.0% arginine diet.

Effects of dietary protein or arginine intake on NOS activities. Dietary protein deficiency decreased ($P < 0.05$)

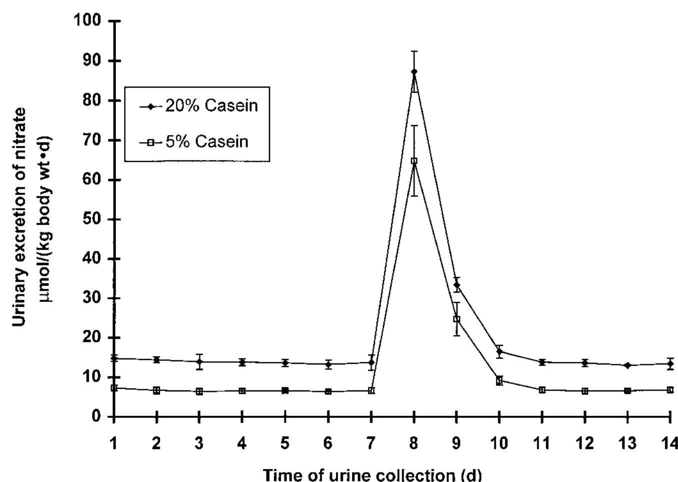

FIGURE 1 Effect of dietary protein deficiency on urinary excretion of nitrate in young rats (Experiment 1). Data are means ± SEM, $n = 8$, and analyzed by 2-way ANOVA and the Student-Newman-Keuls test. After rats were pair-fed the 20 and 5% casein diets for 10 d, daily urine collection was initiated. After the urine collection on d 7, lipopolysaccharide (LPS, 1 mg/kg body wt) was injected intraperitoneally into rats. Peak values of urinary nitrate excretion were observed within 24 h of the LPS administration. At all time points examined, urinary nitrate excretion was lower ($P < 0.05$) in rats fed the 5% casein diet compared with those fed the 20% casein diet.

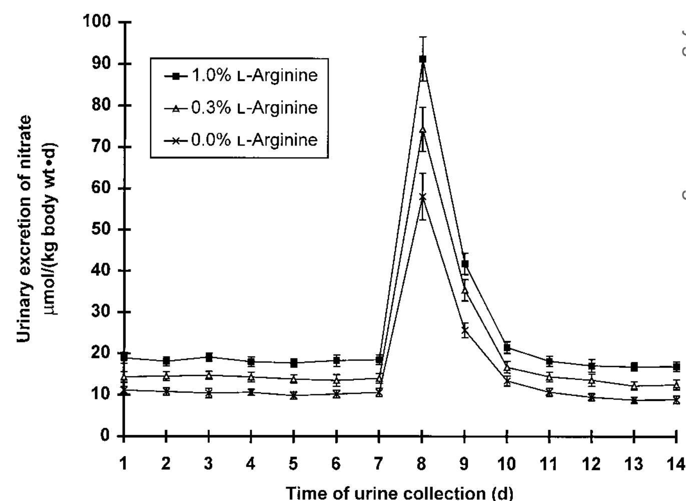

FIGURE 2 Effect of dietary arginine deficiency on urinary excretion of nitrate in young rats (Exp. 2). Data are means ± SEM, $n = 8$, and analyzed by 2-way ANOVA and the Student-Newman-Keuls test. After rats were pair-fed the 1.0, 0.3 and 0.0% arginine diets for 10 d, daily urine collection was initiated. After the urine collection on d 7, lipopolysaccharide (LPS, 1 mg/kg body wt) was injected intraperitoneally into rats. Peak values of urinary nitrate excretion were observed within 24 h of the LPS administration. At all time points examined, urinary nitrate excretion differed ($P < 0.05$) among the three groups of rats.

TABLE 4

Effect of dietary protein or arginine deficiency on inducible nitric oxide synthesis in young rats treated with lipopolysaccharide (Experiments 1 and 2)[1]

Day post-LPS treatment	Exp. 1		Exp. 2		
	20% Casein	5% Casein	1.0% Arg	0.3% Arg	0.0% Arg
			μmol/(kg body wt · d)		
1	73.6 ± 5.5	58.2 ± 5.1†	72.8 ± 6.1[a]	60.4 ± 4.9[b]	47.5 ± 4.2[c]
2	19.7 ± 2.3	18.1 ± 1.6	23.3 ± 1.7[a]	21.4 ± 2.0[a]	15.1 ± 1.3[b]
3	2.8 ± 0.34	2.6 ± 0.29	3.0 ± 0.26	2.7 ± 0.32	2.8 ± 0.29
4	ND	ND	ND	ND	ND

[1] Data are means ± SEM, $n = 8$, and analyzed by 2-way ANOVA and the Student-Newan-Keuls test for each experiment. See Figures 1 and 2 for experimental details. Inducible nitric oxide (NO) synthesis by LPS-treated rats was calculated on the basis of urinary nitrate excretion before and after lipopolysaccharide (LPS) administration. In all groups of rats, inducible NO synthesis differed ($P < 0.05$) among d 1–3 post LPS-administration.
† $P < 0.05$, different from the 20% casein group (Exp. 1). Means in a row with different letters (a–c) are different ($P < 0.05$) (Exp. 2). ND, Inducible nitric oxide synthesis was not detectable.

cNOS and iNOS activities in brain, heart, jejunum, lung, skeletal muscle and spleen by 19–25%, as well as iNOS activity in macrophages by 28% (**Table 7**). Dietary arginine deficiency had no effect on either cNOS or iNOS activity in macrophages or the tissues examined (brain, heart, jejunum, lung, skeletal muscle and spleen) (data not shown).

DISCUSSION

Nitrate is the major stable end product of NO in animals, and thus urinary excretion of nitrate has been used as an indicator of in vivo NO synthesis from L-arginine in animals and humans (e.g., Castillo et al. 1993, Sakinis and Wennmalm 1998, Wu 1995). Many cell types express cNOS, including endothelial cells, smooth muscle cells, neural cells and intestinal epithelial cells (Morris 1998). Synthesis of NO by cNOS, also refereed to as constitutive (basal) NO production, is quantitatively low but plays an important role in maintaining vascular tone (Ignarro 1990, Moncada and Higgs 1993). Thus, impaired constitutive NO synthesis results in hypertension and cardiovascular dysfunction (Huang et al. 1995). On the other hand, large amounts of NO are produced by iNOS in response to immunologic challenge or LPS treatment (Granger

TABLE 5

Serum amino acid concentrations in young rats pair-fed 20 and 5% casein diets (Experiment 1)[1]

Amino acid	20% Casein	5% Casein
	μmol/L	
Alanine	693.8 ± 90.1	721.9 ± 56.4
Arginine	207.2 ± 16.4	169.1 ± 10.3*
Asparagine	50.9 ± 4.8	53.9 ± 5.4
Aspartate	42.1 ± 2.4	42.5 ± 1.9
Citrulline	78.4 ± 4.8	63.2 ± 4.1*
Cysteine[2]	155.3 ± 10.2	60.7 ± 5.3*
Glutamate	132.5 ± 12.2	109.1 ± 16.5
Glutamine	806.8 ± 64.7	843.7 ± 71.4
Glycine	412.6 ± 31.9	476.9 ± 37.3
Histidine	73.7 ± 8.5	44.2 ± 5.0*
Isoleucine	106.6 ± 12.4	64.7 ± 3.7*
Leucine	162.8 ± 14.8	80.9 ± 10.5*
Lysine	551.1 ± 62.1	412.8 ± 50.3*
Methionine	105.4 ± 17.9	26.8 ± 2.8*
Ornithine	60.1 ± 4.2	48.4 ± 3.9*
Phenylalanine	68.6 ± 7.3	40.8 ± 2.9*
Proline	327.8 ± 15.1	113.5 ± 10.8*
Serine	357.4 ± 46.4	458.0 ± 40.8*
Taurine	548.3 ± 44.6	411.1 ± 37.9*
Threonine	547.5 ± 67.5	84.7 ± 7.6*
Tryptophan	125.6 ± 10.3	31.1 ± 3.0*
Tyrosine	157.3 ± 12.4	49.6 ± 5.1*
Valine	262.5 ± 18.9	111.9 ± 5.5*

[1] Data are means ± SEM, $n = 8$. *$P < 0.01$, significantly different from the control (20% casein) group, as analyzed by unpaired t test.
[2] Including cysteine + 1/2 cystine.

TABLE 6

Serum amino acid concentrations in young rats pair-fed 1.0, 0.3 and 0.0% L-arginine diets (Experiment 2)[1]

Amino acid	1.0% Arginine	0.3% Arginine	0.0% Arginine
		μmol/L	
Alanine	572.4 ± 16.6[c]	632.4 ± 18.2[b]	710.2 ± 21.3[a]
Arginine	223.6 ± 12.4[a]	162.0 ± 10.1[b]	108.9 ± 7.5[c]
Asparagine	53.8 ± 4.6	55.1 ± 2.9	49.5 ± 5.2
Aspartate	32.1 ± 2.8	35.2 ± 2.6	37.3 ± 3.0
Citrulline	77.4 ± 5.7	75.6 ± 4.4	72.2 ± 5.3
Cysteine[2]	168.7 ± 11.3	152.4 ± 12.6	163.9 ± 10.4
Glutamate	122.7 ± 10.1	115.8 ± 11.7	109.6 ± 13.8
Glutamine	657.3 ± 15.4[c]	761.2 ± 17.6[b]	885.7 ± 20.2[a]
Glycine	372.8 ± 28.4[b]	415.7 ± 23.7[ab]	466.3 ± 32.9[a]
Histidine	69.2 ± 4.5	66.7 ± 2.9	72.3 ± 4.4
Isoleucine	105.4 ± 7.2	108.6 ± 7.6	109.1 ± 8.2
Leucine	151.2 ± 10.1	147.4 ± 8.7	140.2 ± 9.1
Lysine	425.8 ± 18.9	421.0 ± 21.5	467.2 ± 26.4
Methionine	85.3 ± 5.7	83.7 ± 6.4	91.1 ± 7.2
Ornithine	66.4 ± 4.5[a]	50.8 ± 3.6[b]	32.6 ± 2.7[c]
Phenylalanine	60.8 ± 4.5	62.4 ± 3.8	57.1 ± 5.2
Proline	310.4 ± 15.0	318.2 ± 21.6	285.7 ± 24.4
Serine	402.3 ± 27.9	387.5 ± 20.3	409.4 ± 35.7
Taurine	487.5 ± 31.2	428.9 ± 22.3	461.3 ± 32.5
Threonine	419.2 ± 17.6	405.0 ± 20.7	412.7 ± 29.4
Tryptophan	98.3 ± 7.6	93.2 ± 5.5	91.5 ± 6.8
Tyrosine	124.2 ± 10.8	116.8 ± 9.9	110.2 ± 8.6
Valine	213.4 ± 17.7	191.8 ± 16.9	206.5 ± 15.3

[1] Data are means ± SEM, $n = 8$. Means in a row with different letters (a–c) are different ($P < 0.05$), as analyzed by one-way ANOVA and the Student-Newman-Keuls test.
[2] Including cysteine + 1/2 cystine.

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TABLE 7

Effect of dietary protein deficiency on nitric oxide synthase activities in various tissues and macrophages of young rats (Experiment 3)[1,2]

	20% Casein		5% Casein	
	− LPS	+ LPS	− LPS	+ LPS
	Constitutive NO synthase, pmol/(min · mg protein)			
Brain	87.4 ± 6.8	83.9 ± 7.5	68.1 ± 6.4†	61.5 ± 7.3†
Heart	24.6 ± 1.9	39.8 ± 3.2*	18.5 ± 1.4†	27.2 ± 2.3*,†
Jejunum	13.2 ± 1.0	17.9 ± 1.4*	8.7 ± 0.79†	12.8 ± 1.0*,†
Lung	6.6 ± 0.71	7.1 ± 0.63	5.1 ± 0.43†	5.5 ± 0.37†
Skeletal muscle	15.1 ± 1.8	17.6 ± 2.0	11.0 ± 1.2†	12.4 ± 1.4†
Spleen	10.2 ± 1.1	11.5 ± 1.4	7.1 ± 0.64†	7.9 ± 0.82†
Macrophages	ND[3]	ND	ND	ND
	Inducible NO synthase, pmol/(min · mg protein)			
Brain	ND	6.3 ± 0.55	ND	4.8 ± 0.38†
Heart	ND	27.8 ± 2.3	ND	21.6 ± 1.5†
Jejunum	ND	20.2 ± 1.7	ND	16.4 ± 1.2†
Lung	ND	72.3 ± 5.2	ND	54.6 ± 4.8†
Skeletal muscle	ND	7.3 ± 0.65	ND	5.8 ± 0.44†
Spleen	ND	128.2 ± 7.3	ND	80.4 ± 5.9†
Macrophages	ND	385.2 ± 26.1	ND	277.3 ± 24.0†

[1] Data are means ± SEM, $n = 6$, and analyzed by 2-way ANOVA and the Student-Newman-Keuls test.
[2] Rats were pair-fed the 20 and 5% casein diets for 17 d and then were or were not treated with lipopolysaccharide (LPS). Rats were killed for tissue collection 20 h after LPS treatment.
† $P < 0.05$, different from the 20% casein group.
* $P < 0.05$, different from the group receiving no LPS treatment (− LPS).
[3] ND, enzyme activity was not detectable.

et al. 1991, Wagner et al. 1983, Wu 1995). Synthesis of NO by iNOS plays an important role in killing invading pathogens by macrophages and thus in the host immunity (Macmicking et al. 1997). Studies using anti-iNOS antibody (Cook et al. 1994) and Northern blot technique (Nagasaki et al. 1996) have revealed the widespread induction of iNOS by LPS in multiple organs of rats. Major sites of NO synthesis induction in LPS-treated rats are the liver, spleen and lung as well as mononuclear cells (e.g., circulating monocytes and macrophages) (Cook et al. 1994, Nagasaki et al. 1996). Thus, LPS administration to rats provides a useful probe for studying the effect of dietary protein or arginine deficiency on in vivo inducible NO synthesis.

This study reports the effect of dietary protein deficiency on in vivo basal and inducible NO synthesis in young rats. Our results demonstrate that dietary protein deficiency decreased serum concentrations of arginine and other essential amino acids in young rats (Table 6), as reported for rats and humans (Eisenstein and Harper 1991, Holt et al. 1963, Lunn and Austin 1983). An important finding from this study is that dietary protein deficiency reduced urinary nitrate excretion before and after LPS injection in growing rats (Fig. 1). Inducible NO synthesis by LPS-treated rats was also lower in rats fed the 5% casein diet compared with rats fed the 20% casein diet (Table 4). These results suggest that dietary protein deficiency impaired constitutive NO synthesis and could not support maximal inducible NO synthesis by young rats in response to LPS treatment.

The mechanism responsible for impaired NO synthesis in protein-deficient rats is likely multifactorial. In this study, we determined effects of dietary protein intake on serum arginine availability and NOS activities in macrophages and various tissues. Serum concentrations of arginine were 20% lower in rats fed the 5% casein diet compared with rats fed the 20% casein diet (Table 5). Interestingly, constitutive NO synthesis was 52% lower in rats fed the 5% casein diet compared with rats fed the 20% casein diet (Fig. 1). Thus, decreased NO synthesis in protein-deficient rats cannot be explained solely by plasma arginine availability. It is possible that the deficiency of all essential amino acids impaired the expression of enzymes involved in the synthesis of NOS protein in rats fed the 5% casein diet. Consistent with this suggestion is our finding that dietary protein deficiency decreased cNOS activities in various rat tissues (Table 7), as recently reported for porcine placenta and endometrium during early gestation (Wu et al. 1998). Peak values of inducible NO synthesis were 21% lower in rats fed the 5% casein diet compared with those fed the 20% casein diet. This reduction of inducible NO production was associated with lower serum arginine concentrations (18%) (Table 5) and iNOS activity in various tissues (19–25%) (Table 7). These results suggest that maximal inducible NO synthesis by iNOS cannot be supported in young protein-malnourished rats.

To determine whether dietary arginine deficiency impairs NO synthesis independently of changes in cNOS or iNOS activities, we conducted an additional experiment (Experiment 2) in which young rats were fed purified diets containing 0.0, 0.3 or 1.0% L-arginine. Arginine is an essential amino acid for young mammals because endogenous arginine synthesis cannot meet arginine needs for optimal growth and health (Visek 1985). Thus, feeding the 0.0 or 0.3% arginine diet to 30-d-old rats resulted in marked growth retardation (Table 3) and decreased serum concentrations of arginine compared with feeding the 1% arginine diet (Table 6). In addition, decreasing dietary arginine concentrations from 1.0 to 0.0% caused progressive decreases in constitutive and inducible NO

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synthesis by young rats (Fig. 2, Table 4). Indeed, in rats fed the 0.0% arginine diet, serum arginine was so severely depleted that inducible NO synthesis remained depressed on d 2 post-LPS treatment. Similarly, plasma arginine deficiency was reported to be associated with reduced NO synthesis in newborn infants (Vosatka et al. 1994). In contrast to dietary protein deficiency, dietary arginine deficiency had no effect on cNOS or iNOS activities in various tissues or iNOS activity in macrophages (see Results section). These results suggest that serum concentrations of arginine (109 and 162 μmol/L) are inadequate for both basal and inducible NO generation, and demonstrate an important role for extracellular arginine in regulating in vivo NO synthesis by cNOS and iNOS in young rats. Our results are consistent with the findings from in vitro studies that increasing extracellular arginine concentrations increased NO synthesis by cNOS [e.g., in endothelial cells (Arnal et al. 1995)] and by iNOS [e.g., activated macrophages (Norris et al. 1995)] in a concentration-dependent manner. In this regard, it is noteworthy that although serum concentrations of arginine were similar in rats fed the 5% casein diet and the 0.3% arginine diet (Tables 5 and 6), urinary excretion of nitrate by rats fed the 5% casein diet was only ~45% of that by rats fed the 0.3% arginine diet (Figs. 1 and 2). These results further support the notion that in protein-deficient rats, factors other than serum arginine concentrations (e.g., cNOS activity) also contribute to the impaired constitutive NO synthesis.

Results of this study may help to explain impaired immunologic and cardiovascular functions in protein- or arginine-deficient animals and humans. Dietary protein deficiency continues to be a major nutritional problem in developing countries and also occurs in subpopulations of developed nations (Young et al. 1990). Protein malnutrition is a major contributor to the morbidity and mortality from infectious diseases, largely as a result of impaired immune response (Chandra 1972 and 1991, McMurray et al. 1981 and 1986). In addition, dietary protein deficiency results in hypertension (Langley-Evans and Jackson 1996, Langley-Evans 1997), as well as cardiac failure and cardiovascular abnormalities in humans and experimental animals (Chauhan et al. 1965, Smythe et al. 1962, Wharton et al. 1967). Dietary protein or arginine deficiency decreases plasma concentrations of arginine in animals and humans, particularly young mammals (Eisenstein and Harper 1991, Holt et al. 1963, Lunn and Austin 1983), and impairs both constitutive and inducible NO synthesis by young rats (Figs. 1 and 2). On the basis of the recent report that a 59% decrease in NO synthesis was associated with severely impaired endothelial function and abnormal vascular structure in a hypercholesterolemic rabbit model (Bode-Böger et al. 1996), a chronic reduction of NO synthesis by cNOS [e.g., 52% decrease in young rats fed the 5% casein diet compared with those fed the 20% casein diet (Fig. 1)] would have an adverse effect on the circulatory system in protein- or arginine-deficient subjects. Similarly, in light of the recent findings from iNOS-deficient mice that inducible NO synthesis was critical for host survival in *Mycobacterium tuberculosis*, *Leishmania major* or viral infection (Nathan 1997), suboptimal NO synthesis by iNOS [e.g., 20–35% decreases in protein- or arginine-deficient young rats (Table 4)] would contribute to the impaired host response to immunologic and inflammatory stimuli in patients with kwashiorkor. Thus, because NO mediates the killing of pathogenic microorganisms by activated macrophages (Macmicking et al. 1997) and is the endothelium-dependent relaxing factor (Ignarro 1990, Moncada and Higgs 1993), our findings may help to explain the immunodeficiency and increased susceptibility to infectious diseases, as well as cardiovascular dysfunction, in protein- or arginine-deficient animals and humans.

In conclusion, dietary protein or arginine deficiency impaired constitutive NO synthesis by cNOS and could not support maximal inducible NO synthesis by iNOS in young rats. Decreases in both serum arginine concentrations and NOS activities contributed to the reduced NO synthesis by protein-deficient rats. Our results may help explain the immunodeficiency and cardiovascular dysfunction in protein- or arginine-deficient subjects.

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