Oral *N*-Carbamylglutamate Supplementation Increases Protein Synthesis in Skeletal Muscle of Piglets[1]

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Abstract

This study investigated the potential mechanisms by which oral supplementation of *N*-carbamylglutamate (NCG), an analogue of endogenous *N*-acetylglutamate (an activator of arginine synthesis) increases growth rate in sow-reared piglets. Two piglets of equal body weight (BW) and of the same gender from each lactating sow were allotted to receive oral administration of 0 (control) or 50 mg of NCG/kg BW every 12 h for 7 d. Piglets ($n = 32$; BW = 3 kg) were studied in the food-deprived or fed state following the 7 d of treatment. Overnight food-deprived piglets were given NCG or water (control) at time 0 and 60 min. Piglets studied in the fed state were gavage-fed sow's milk with their respective NCG treatment at 0 and 60 min. At 60 min, the piglets were administered a flooding dose of [^{3}H]phenylalanine and killed at 90 min to measure tissue protein synthesis. Piglets treated with NCG gained 28% more weight than control pigs ($P < 0.001$) over the 7-d period. Fed pigs had greater rates of protein synthesis in longissimus dorsi and gastrocnemius muscles and duodenum compared with food-deprived pigs ($P < 0.001$). Absolute protein synthesis rates in longissimus dorsi ($P = 0.050$) and gastrocnemius ($P = 0.068$) muscles were 30 and 21% greater, respectively, in NCG-treated compared with control pigs. Piglets supplemented with NCG also had greater plasma concentrations of arginine and somatotropin than control pigs ($P < 0.001$). The results suggest that oral NCG supplementation increases plasma arginine and somatotropin levels, leading to an increase in growth rate and muscle protein synthesis in nursing piglets. J. Nutr. 137: 315–319, 2007.

Introduction

Preweaning growth rate is a major determinant of neonatal survival and postweaning growth performance of pigs (1). Unfortunately, the nursing piglet may not reach its maximum growth rate largely due to milk production limitations of the lactating sow (2,3). When piglets are provided with ad libitum intake of a milk replacer, weight gain is increased compared with nursing piglets. However, the use of milk replacer systems in commercial pork production can be cost-prohibitive currently. Although the metabolic basis for the reduced performance of sow-reared piglets is not completely known, it may be due to an inadequate supply of an essential amino acid(s) and/or energy (2,4).

The sub-maximal growth of nursing piglets occurs after ~8 d of age (3,5). Interestingly, plasma arginine concentrations in nursing piglets begin to decrease at 7 d of age and are reduced dramatically by 14 d of age compared with the other essential amino acids (6). The decrease in plasma arginine is due to the relatively low concentration of arginine in sow's milk (6,7) and the decreasing activity of mitochondrial *N*-acetylglutamate synthase (NAGS)[5] in enterocytes, a required enzyme for intestinal arginine synthesis (8). Endogenous synthesis of arginine in neonates decreases by ~70% in 7-d-old pigs and declines further in 14- to 21-d-old piglets (9,10). Although piglets can conserve dietary arginine during a dietary deficiency by decreasing hydrolysis and increasing recycling (11), this metabolic adaptation does not ameliorate the reduced growth. Both metabolic and growth data indicate that arginine deficiency is a major factor that limits the maximal growth of milk-fed piglets (2,8).

N-Carbamylglutamate (NCG) is a metabolically stable analogue of *N*-acetylglutamate (NAG), which activates carbamylphosphate synthase-1 (CPS-1), a key enzyme in arginine synthesis in enterocytes (8,12). Oral supplementation of piglets with NCG increases endogenous synthesis of arginine, resulting in an increase in plasma arginine concentration (8). We hypothesize that arginine availability limits piglet growth and that supplementing NCG to piglets will increase endogenous provision

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[5] Abbreviations used: ASR, absolute protein synthesis rate; BW, body weight; CPS-1, carbamylphosphate synthase-1; DTM, dissected tissue mass; FSR, fractional protein synthesis rate; NAG, *N*-acetylglutamate; NAGS, *N*-acetylglutamate synthase; NCG, *N*-carbamylglutamate; TPC, tissue protein concentration; TPM, tissue protein mass.

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of arginine to support maximal muscle protein synthesis and growth rate. The objective of this study was to test this hypothesis using nursing piglets.

Materials and Methods

Two experiments were conducted to determine the mechanism by which oral NCG administration increases growth rate in nursing piglets. In Expt. 1, piglets were evaluated in the food-deprived state. In Expt. 2, piglets were studied in the fed state. This project was completed in accordance with the guidelines of the United States Research Council for the care and use of animals and was approved by the Texas A&M University and Baylor College of Medicine Institutional Animal Care and Use Committees.

Animals. The piglets ($n = 32$) used in this study were the offspring of Yorkshire × Landrace sows and Duroc × Hampshire boars and were maintained at the Texas A&M University Swine Center. To eliminate a potential effect of sow's milk production and litter size on neonatal growth, litter size was standardized to 10 pigs per sow and 2 piglets of equal body weight (BW) and of the same gender from each lactating sow were allotted to each treatment. Piglets received 0 (control) or 50 mg of NCG (Sigma-Aldrich) per kg BW every 12 h for 7 d. NCG was mixed with water and slowly administered with a 5-mL syringe into the mouth of the piglet. Control animals received an equal volume of water. The lactating sows had free access to water and feed; dietary metabolizable energy, protein, lysine, calcium, and phosphorus were 13.2 MJ/kg, 14.1, 0.61, 0.80, and 0.60%, respectively (6,7). At d 4 after initiation of NCG treatment, a jugular vein catheter was surgically inserted into piglets, as previously described (13). At d 7 after initiation of the NCG treatment, tissue protein synthesis was measured in piglets in either the food-deprived or fed state.

Experiment 1: Food-deprived state. Following 7 d of treatment, control and NCG-supplemented 14-d-old piglets were removed from the sows to prevent suckling for 12 h before being placed awake in a sling restraint system and administered water or NCG at time 0 and 60 min. Blood samples were collected every 30 min from time 0 to 90 min. At 60 min, pigs were injected via the catheter with a flooding dose of [^{3}H]phenylalanine to determine rates of tissue protein synthesis. At 90 min, the pigs were killed and tissue samples were collected.

Experiment 2: Fed state. Following 7 d of treatment, control and NCG-supplemented 16-d-old piglets were removed from the sows in treatment pairs and immediately placed awake in a sling restraint system and intragastrically gavage-fed sow's milk at a rate of 7.5 mL/kg BW, with water or NCG at time 0 and 60 min. The volume of the intragastric feeding of sow's milk was equivalent to the normal intake of these piglets based on previous work (14). Blood samples were collected every 30 min from time 0 to 90 min. At 60 min, pigs were injected via the jugular vein catheter with a flooding dose of [^{3}H]phenylalanine to determine rates of tissue protein synthesis. After the piglets were killed at 90 min, the right longissimus dorsi muscle spanning the last 5 ribs, the gastrocnemius, liver, and small intestine were removed and weighed. The weights of these tissues represent the dissected tissue mass (DTM) and were used to calculate absolute protein synthesis rates (ASR) for both experiments.

Tissue protein synthesis in vivo. Tissue protein synthesis was measured in vivo using a modification of the flooding dose technique (15). Pigs were injected via the jugular vein catheter with 10 mL/kg BW of a flooding dose of phenylalanine (Amersham), which provided 1.5 mmol phenylalanine/kg BW and 37 MBq of L-[4-^{3}H]phenylalanine/kg BW. Samples of whole blood were taken at 5, 15, and 30 min after the injection of [^{3}H]phenylalanine for measurement of the specific radioactivity of the extracellular free pool of phenylalanine. Immediately after the 30-min blood sample was taken, pigs were given a lethal injection of sodium pentobarbital (50 mg/kg BW). Fractional rates of protein synthesis (FSR; percentage of protein mass synthesized in a day) for each tissue were calculated as

$$\text{FSR}\,(\%/\text{d}) = [(S_b/S_a) \cdot (\text{T}/\text{t})] \cdot 100,$$

where S_b is the specific radioactivity of the protein-bound phenylalanine, S_a is the specific radioactivity of the tissue-free phenylalanine at the time of tissue collection and the linear regression of the blood specific radioactivity of the animal at 5, 15, and 30 min against time, the constant T = 1,440 min/d, and t is the time of labeling in minutes of the specific tissue. We have previously demonstrated that the specific radioactivity of the tissue-free phenylalanine after a flooding dose of phenylalanine is in equilibrium with the aminoacyl-tRNA specific radioactivity; hence, the tissue-free phenylalanine reflects the specific radioactivity of the tissue precursor pool (16).

The ASR was calculated as the FSR times the tissue protein mass (TPM) as previously described (17)

$$\text{ASR}\,(\text{mg protein/d}) = \text{FSR} \cdot \text{TPM}.$$

TPM was calculated as the tissue protein concentration (TPC) times the DTM:

$$\text{TPM} = \text{TPC} \cdot \text{DTM}.$$

The dissected TPM obtained in Expt. 2 were used for the calculation of the ASR of the tissues for pigs in both experiments.

Chemical analysis. Plasma amino acids were analyzed by HPLC methods involving precolumn derivatization with *o*-phthaldialdehyde, as previously described (18), except that quantification was performed using Millennium-32 software (Waters). A sample of tissue homogenate was used to determine total TPC as described by the manufacturer (Protein Assay kit 23235, Pierce). Ammonia in plasma was analyzed using glutamate dehydrogenase, with ammonium chloride as a standard. Urea in plasma was analyzed by an enzymatic method involving urease and glutamate dehydrogenase as previously described (19). The concentration of plasma glucose was analyzed using a YSI 2300 STAT Plus (Yellow Springs Instruments). Plasma insulin and somatotropin (growth hormone) were determined using RIA kits (Linco) for porcine insulin and growth hormone, respectively (2).

Statistical analysis. The data from these 2 studies were combined and analyzed using the general linear modeling procedure of SAS (SAS Institute). The statistical model included the main effects and interaction of feeding state (food deprived vs. fed) and treatment (control vs. NCG). Initial BW was used as a covariate for the growth performance and protein synthesis data. Plasma hormone and metabolite concentrations were analyzed as repeated-measures using the general linear modeling procedure of SAS. $P < 0.05$ was considered significant.

Results

Following 7 d of treatment, NCG-supplemented pigs were heavier than controls and gained 28% more BW ($P < 0.001$; **Table 1**). In addition, pigs in the fed state had greater final BW and weight gain compared with food-deprived pigs ($P = 0.033$). There were no feeding state × treatment interactions ($P > 0.31$). Fractional protein synthesis rate in the longissimus dorsi, gastrocnemius, and duodenum were greater in fed pigs compared with food-deprived pigs ($P < 0.001$). Although pigs orally administered NCG had higher FSR in skeletal muscle compared with control pigs, these affects were not significant ($P > 0.24$). NCG treatment increased the absolute rates of protein synthesis in the longissimus dorsi muscle by 30% ($P = 0.050$) and in the gastrocnemius muscle by 21% ($P = 0.068$). In addition, there was a slight increase in the absolute protein synthesis rate in the duodenum ($P = 0.103$) with NCG treatment. However, the ASR in the liver ($P = 0.804$) were not increased in pigs treated with NCG. The absolute rate of protein synthesis in the longissimus dorsi ($P < 0.001$), gastrocnemius ($P < 0.001$), and duodenum ($P < 0.001$) were greater in fed pigs compared with food-deprived

TABLE 1 Growth performance and protein synthesis rates of piglets in the food-deprived or fed state that were orally supplemented with NCG

	Food deprived		Fed			*P*-value[1]	
	Control	NCG	Control	NCG	SEM	State	Treatment
Initial BW, *g*	2710	2780	3160	3209	65	0.001	0.588
Final BW, *g*	3946	4409	4713	5143	106	0.033	0.001
Gain, *g*	1236	1629	1553	1934	59	0.033	0.001
FSR[2], *%/d*							
Longissimus dorsi	5.9	6.9	17.1	18.5	1.1	0.001	0.241
Gastrocnemius	8.3	9.1	14.9	15.4	0.7	0.001	0.498
Liver	80.0	78.8	81.4	79.1	1.4	0.749	0.573
Duodenum	41.3	40.7	61.5	64.8	2.3	0.001	0.441
ASR[3], *mg/d*							
Longissimus dorsi	147	205	435	552	36	0.001	0.050
Gastrocnemius	223	284	397	469	24	0.005	0.068
Liver	11232	11623	11179	11451	324	0.212	0.804
Duodenum	4838	5124	6910	7912	294	0.001	0.103

[1] There were no significant state × treatment interactions; therefore, this term was removed from the statistical model. Initial BW was used as a covariate for the statistical analysis. Values are the means and pooled SEM; $n = 7$–9 per treatment group.
[2] Fractional protein synthesis rate.
[3] Absolute protein synthesis rate.

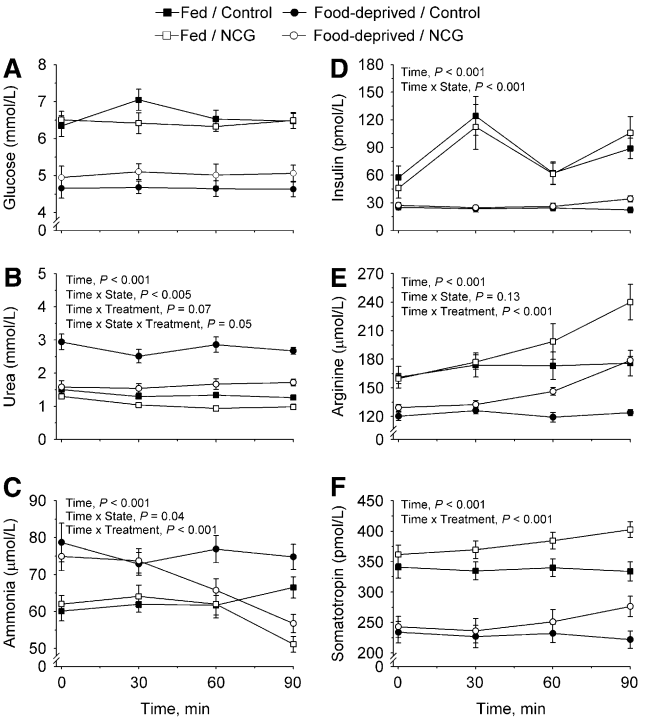

Figure 1 Plasma glucose (*A*), urea (*B*), ammonia (*C*), insulin (*D*), arginine (*E*), and somatotropin (*F*) in piglets after 7 d of treatment with NCG or water twice daily. Piglets studied in the fed state were gavage-fed sow's milk at time 0 and 60 min, and piglets studied in the food-deprived state were removed from the sow 12 h prior to time 0 min. In both the fed and food-deprived states, the piglets were orally administered 50 mg/kg BW of NCG or water (control) at time 0 and 60 min on the day of measurement of tissue protein synthesis. Values are means and pooled SEM; $n = 7$–9 per treatment group.

pigs; however, hepatic protein synthesis did not differ ($P = 0.212$) between the 2 groups.

Plasma concentrations of metabolites and hormones are presented in **Figure 1**. Although plasma concentrations of glucose were lower in food-deprived pigs compared with fed pigs ($P < 0.001$), the concentrations did not change over time ($P = 0.28$; Fig. 1*A*). Subsequently, there were no significant time × state, time × treatment, or time × state × treatment interactions. Concentrations of all other metabolites and hormones that were measured in this experiment changed over time ($P < 0.001$). Urea concentrations were affected by feeding state and NCG supplementation (Fig. 1*B*). Control pigs that were food deprived had the highest concentrations of urea over the 90-min sampling period (time × state × treatment, $P = 0.05$). Plasma ammonia concentrations in piglets that were supplemented with NCG decreased over time compared with control pigs (time × treatment, $P < 0.001$; Fig. 1C). In addition, plasma ammonia concentrations in food-deprived pigs decreased to a greater extent over time compared with fed pigs (time × state, $P = 0.04$). Supplementing piglets with NCG resulted in a net decrease in both urea and ammonia concentrations.

Plasma insulin concentrations in fed piglets increased over time compared with the food-deprived piglets (time × state, $P < 0.001$; Fig. 1*D*). Although plasma arginine concentrations were numerically greater in the fed state compared with the food-deprived state, these differences were not significant ($P = 0.13$; Fig. 1*E*). Plasma concentrations of arginine and somatotropin (Fig. 1*F*) increased over time in NCG-supplemented pigs, whereas the concentrations in control pigs did not change (time × treatment, $P < 0.001$).

Amino acid concentrations at 90 min are presented in **Table 2**. Concentrations of the essential amino acids at 90 min after NCG administration were not affected ($P > 0.50$, Table 2). Only the plasma concentrations of citrulline ($P < 0.001$) and ornithine ($P < 0.025$) (intermediates in the pathways of arginine synthesis from glutamine and proline) were greater in NCG-supplemented pigs compared with control pigs.

Discussion

This study investigated the mechanisms by which NCG supplementation increases growth rate in sow-reared piglets. We confirmed previous findings that supplementing piglets with NCG increases growth rate (8) and further demonstrated that the elevation in growth rate was accompanied by increased absolute rates of protein synthesis in skeletal muscle, as well as higher plasma concentrations of arginine and somatotropin. The results suggest that by increasing the endogenous synthesis of arginine, and thus its concentration in the plasma, we have indirectly supplied the limiting amino acid needed for maximum growth rate and muscle protein synthesis in the nursing piglet. Alternatively, the increases in growth rate and skeletal muscle protein synthesis may be due to the increased concentrations of somatotropin in these piglets, as arginine is a known secretagogue of somatotropin (20).

Arginine has a versatile and important role in nutrition and metabolism. In addition to serving as an essential building block for tissue proteins, arginine is a necessary precursor for the synthesis of creatine, polyamines, and nitric oxide (21). Arginine plays a crucial role in ammonia detoxification via the hepatic urea cycle by acting as an allosteric activator of NAGS to synthesize NAG, which in turn activates CPS-1 (22). Thus, plasma concentrations of arginine and ammonia are sensitive indicators of arginine status, where a hallmark of arginine deficiency is an elevated concentration of plasma ammonia (23). Supplementing the piglets with NCG in this study clearly increased plasma concentrations of arginine in both the food-deprived and fed states. This increase in plasma arginine coincided with a decrease in

TABLE 2 Concentrations of amino acids in plasma of food-deprived and fed piglets at 90 min after oral NCG administration

	Food deprived		Fed			*P*-value[1]	
	Control	NCG	Control	NCG	SEM	State	Treatment
Essential amino acids			*μmol/L*				
Histidine	98	99	97	95	2.2	0.601	0.868
Isoleucine	96	93	127	123	3.9	0.001	0.575
Leucine	152	144	197	194	5.8	0.001	0.515
Lysine	211	203	213	210	4.5	0.598	0.525
Methionine	64	67	79	77	1.9	0.001	0.864
Phenylalaine	86	84	91	93	2.5	0.153	0.990
Proline	434	456	569	550	13.0	0.001	0.946
Threonine	265	270	260	266	5.6	0.718	0.616
Tryptophan	67	68	65	63	1.8	0.285	0.834
Valine	240	243	291	289	6.1	0.001	0.927
Other amino acids							
Alanine	411	409	508	520	12.6	0.001	0.769
Asparagine	86	82	98	96	2.6	0.011	0.531
Aspartic acid	14	13	15	14	0.5	0.198	0.577
Citrulline	71	90	88	120	3.8	0.001	0.001
Cysteine	148	145	174	167	3.6	0.001	0.436
Glutamine	442	402	514	519	11.3	0.001	0.256
Glutamic acid	87	82	143	134	6.1	0.001	0.393
Glycine	1009	1013	960	943	15.3	0.053	0.831
Ornithine	75	83	90	98	2.3	0.001	0.025
Serine	225	216	239	243	7.1	0.149	0.857
Taurine	131	133	128	122	3.9	0.437	0.799
Tyrosine	174	181	178	174	4.1	0.832	0.855

[1] There were no significant state × treatment interactions; therefore, this term was removed from the statistical model. Values are the means and pooled SEM; $n = 7$–9 per treatment group.

plasma ammonia concentrations, with the most pronounced decrease in the food-deprived pigs. The concentrations of plasma arginine and ammonia in control piglets are consistent with previously published values (6,24). Based on BW gain and the absolute rates of protein synthesis in skeletal muscle, arginine may be the first limiting amino acid needed for maximum growth in sow-reared piglets. The plasma concentrations of urea would corroborate this finding, as higher concentrations would indicate lower rates of net protein synthesis and a higher rate of amino acid catabolism (24) due to the imbalance of essential amino acids relative to ideal amino acid ratios for protein accretion in piglets (2,25).

Plasma concentrations of arginine begin to exhibit the most pronounced decline in nursing piglets at 7 d of age among essential amino acids (6). Providing milk replacer with higher levels of arginine than that found in sow's milk increases piglet growth rate (2). However, at present, utilizing a milk replacer system in commercial pork production can be cost-prohibitive and requires additional maintenance by the producer. Although oral supplementation with high doses of arginine or citrulline twice daily can increase plasma arginine concentrations, an undesirable result is a reduction in plasma concentrations of other essential amino acids (e.g. lysine and tryptophan) in plasma, due to sharing the same transport systems (e.g. arginine and lysine; citrulline and tryptophan) in pig enterocytes (8).

A viable alternative to supplementing arginine or citrulline is the use of NCG. This compound has been used to treat patients with hyperammonemia due to a NAGS deficiency (26). As a metabolically stable activator of CPS-1, NCG stimulates the synthesis of citrulline and arginine from glutamine and proline in pig enterocytes (8). Importantly, oral administration of NCG to 4-d-old sow-reared piglets increased plasma arginine concentrations by 68% and weight gain by 1.3 kg during a 10-d period, without altering body composition (8). Interestingly, the higher absolute rates of skeletal muscle protein synthesis we observed in this study did not entirely account for the increases in BW. We hypothesize that arginine may not only increase muscle protein synthesis but may also inhibit muscle protein degradation in piglets.

Alternatively, adipose deposition may have contributed to the increases in weight gain observed in this study. However, Wu et al. (8) previously measured fat content in NCG-supplemented vs. control piglets and did not find a difference. Further, Fu et al. (27) reported that supplementing arginine to Zucker diabetic rats selectively decreased abdominal and epididymal adipose mass. These reductions in adipose mass were accompanied by increases in lipolysis and the activation of genes responsible for fatty acid oxidation (27). Thus, we do not expect an increase in arginine availability to stimulate a disproportional accretion of fat in piglets.

Our previous studies showed that feeding stimulates protein synthesis in pigs and the magnitude of this response decreased rapidly with development (27). The feeding-induced increase in protein synthesis can be reproduced with the infusion of insulin and amino acids (28). Skeletal muscle protein synthesis is stimulated by insulin and amino acids, whereas liver and visceral tissue protein synthesis is stimulated only by amino acid infusion. In this study, protein synthesis was again greater in skeletal muscle and duodenum of fed compared with food-deprived piglets; however, there was no effect of feeding on liver protein synthesis (Table 1).

Arginine is a potent stimulator of insulin and growth hormone secretion (29). Although plasma insulin levels were not affected by NCG treatment in the current study, somatotropin levels increased in NCG-treated pigs along with plasma arginine concentrations. Accordingly, growing pigs given exogenous somatotropin had higher rates of gain (30) and muscle protein synthesis (31) than control pigs. The effect of arginine on somatotropin secretion may result from an inhibition of somatostatin secretion (20).

In conclusion, the supplementation of NCG to nursing piglets increases growth rate and BW, likely by increasing plasma arginine concentration, a limiting amino acid for maximal neonatal pig growth. The elevated levels of somatotropin also may have contributed to the increased growth rate of these piglets. The results suggest that an increase in protein synthesis in skeletal muscle is a primary mechanism responsible for the enhanced growth of NCG-treated piglets.

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