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Effect of Leucine-Rich Dietary Protein on *in Vitro* Protein Synthesis in Porcine Muscle (42214)

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Abstract. Experiments were conducted to determine the effect of feeding diets containing leucine-rich proteins on *in vitro* protein synthesis in porcine muscle. Swine (10 kg initial weight) were fed for 4 weeks diets composed mainly of corn gluten meal, corn and soybean meal, and containing a total of 2.00, 2.33, 2.92, 3.12, 3.53, and 4.01% leucine. At the end of the growing period, six swine fed each diet were killed and samples of biceps femoris, longissimus dorsi, and triceps brachii were excised. Incorporation of [^{14}C]phenylalanine into newly synthesized protein was measured using a cell-free *in vitro* system following recombination of purified soluble protein and ribosomal fractions. The feeding of diets containing increasing amounts of leucine-rich protein increased the free leucine concentration in plasma and skeletal muscle. There was no significant effect of diet on incorporation of [^{14}C]phenylalanine into muscle protein following simple recombination of soluble protein and ribosomal fractions from the same tissues. Combination of muscle soluble protein from animals fed 2.00% leucine with ribosomal fractions of animals fed increasing quantities of leucine-rich protein, however, indicated increased protein synthetic activity of the ribosomal fraction in all muscles tested. Protein synthetic activity of the soluble protein fraction was not affected by diet. It was concluded that the feeding of leucine-rich dietary proteins beyond requirements for maximal rate of growth can increase the protein synthetic potential of porcine muscle cells although whole body growth is depressed.

Studies in our laboratory (1) have indicated that small dietary excesses of leucine can increase incorporation of valine into protein of various tissues in the chick. Similarly, there is considerable support for the concept that branched-chain amino acids (2) and leucine in particular (3, 4) can promote protein synthesis in rat diaphragm. Leucine has also been shown to promote protein synthesis and reduce protein degradation in perfused rat skeletal muscle (5) and heart (6). Such observations have prompted investigation of the effect of branched-chain amino acids on *in vitro* protein synthesis in rat muscle following burn injury (7). It has also been reported that increased concentrations of branched-chain amino acids in parenterally administered infusion solutions can improve nitrogen retention in normal (8) and postoperative rats (9). Freund *et al.* (10) have recently reviewed clinical studies of the nitrogen sparing effects of branched-chain amino acids. It was advocated on the basis of substantial clinical experience that a balanced amino acid solution supplemented with 45% branched-chain amino acids be used in treating patients in the postinjury state.

The biochemical mechanism by which leucine regulates muscle protein turnover is not known although altered muscle concentrations of leucyl-tRNA are not thought to play a role (11). Buse *et al.*, however, reported increased polyribosomal aggregation in muscle of rats following single ip injections of leucine (12).

The technique of Von der Decken and Omstedt (13) for determining rates of muscle protein synthesis *in vitro* permits separate evaluation of the protein synthetic activity of both the ribosomal and soluble protein fractions of the cell. With combinations of ribosomes and soluble protein fractions from animals fed different diets it is possible to obtain information on the biochemical mechanism by which nutrients may regulate protein turnover in individual tissues.

Experiments were therefore conducted to determine the biochemical mechanism by which leucine regulates protein turnover and to determine if leucine-rich dietary proteins can promote protein synthesis in edible muscles of growing swine.

Materials and Methods. *Experimental design.* A total of 168 Yorkshire barrow swine (10 kg initial weight, 28 pigs per diet) were fed

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diets containing 2.00, 2.33, 2.92, 3.12, 3.53, and 4.01% leucine. Leucine in excess of the dietary requirement for maximal growth was supplied by the substitution of corn gluten meal and crystalline leucine for soybean meal (Table I). Animals were housed in stainless-steel floor pens, 4 swine per pen. Food consumption per pen and individual weight gain were determined for 4 weeks. At the end of the growth period 6 swine from each diet group were killed and samples were taken of blood, biceps femoris, longissimus dorsi, and triceps brachii.

Amino acid analysis. Amino acid composition of diets and free amino acids in plasma and muscles were determined as described by Smith and Austic (16).

In vitro assay of protein synthesis. The procedure used for *in vitro* assay of muscle protein synthesis was a modification of those of Von der Decken and Omstedt (13) and Alexis *et al.* (17).

Muscles from six swine fed each diet were assayed and net protein synthesis was determined by incubating recombined ribosomes and soluble protein from each muscle sample. Estimates of the effect of diet on ribosomal activity were made by combining ribosomal fractions from swine fed each diet with a pooled soluble protein fraction from swine fed the control diet containing 2.00% leucine. Activity of the soluble protein fraction was similarly quantified by combining pooled ribosomes from those animals fed the control diet with soluble protein fractions from animals fed diets containing higher concentrations of leucine.

Tissue (6 g) was homogenized (Ultra-Turrax, Tekmar Co., Cincinnati, Ohio) on ice for 30 sec in the presence of 12 ml medium A (0.25 *M* sucrose, 100 m*M* Tris–HCl (pH 7.8), 0.185 *M* KCl, 9.0 m*M* $MgCl_2 \cdot 6H_2O$). Two milliliters 10% Triton X-100 was added and the resulting mixture was again homogenized for 30 sec. Samples were then centrifuged at 27,000*g* at 4°C for 20 min. Supernatant (3.5 ml) was carefully layered over 4.0 ml medium B (0.35 *M* sucrose, 35 m*M* Tris–HCl (pH 7.8), 0.185 *M* KCl, 9.0 m*M* $MgCl_2 \cdot 6H_2O$) and centrifuged at 105,000*g*, 4°C for 2 hr (Model L8-55, 50Ti rotor, Beckman Instruments Inc., Palo Alto, Calif.).

The resulting supernatant containing soluble protein as removed and stored on ice. The ribosomal pellet was washed twice with medium C (0.25 *M* sucrose, 35 m*M* Tris-HCl (pH 7.8), 0.1 *M* KCl, 9.0 m*M* $MgCl_2 \cdot H_2O$) and the washings were discarded. The ribosomal pellet was then resuspended in 1 ml medium C and the RNA concentration of the suspension was determined by the method of Fleck and Munro (18).

A 5.0 ml aliquot of the soluble protein fraction was purified by applying it to a 2.3 × 30 cm column of Sephadex G-25 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and

TABLE I. COMPOSITION OF DIETS

Ingredient (%)	Concentration of dietary leucine (%)					
	2.00	2.33	2.92	3.12	3.53	4.01
Soybean meal	33.0	25.7	17.4	9.1	1.2	0.6
Corn gluten meal	—	6.0	12.0	18.0	24.0	24.0
Corn	63.8	65.1	67.4	69.5	71.1	71.4
L-leucine[a]	—	—	—	—	—	0.3
L-lysine HCl[a]	—	—	—	0.17	0.35	0.37
L-tryptophan[a]	—	—	—	—	0.13	0.13
Iodized salt	0.5	0.5	0.5	0.5	0.5	0.5
Limestone	0.9	0.9	0.9	0.9	0.9	0.9
$CaHPO_4$	1.2	1.2	1.2	1.2	1.2	1.2
Vitamin premix[b]	0.5	0.5	0.5	0.5	0.5	0.5
Mineral premix[c]	0.1	0.1	0.1	0.1	0.1	0.1

[a] United States Biochemical Corporation, Cleveland, Ohio.
[b] Described by James and Smith (14).
[c] Described by Smith (15).

eluting with medium C. The eluant was collected in 3-ml fractions and the concentration of protein was determined in each (19).

The reaction mixture included 25 μg of ribosomal RNA in suspension, 0.5 mg of soluble protein in solution, 31.2 μl cofactor solution, 62.5 μl amino acid solution (20), 5 μl [U-^{14}C]phenylalanine (100 μCi/ml in ethanol: water (2:98), New England Nuclear, Ltd., Lachine, Quebec), and medium C to give a total volume of 250 μl. The cofactor solution (pH 7.8) included 10 m*M* phosphoenol pyruvate, 1 m*M* ATP, 0.1 m*M* GTP, pyruvate kinase (40 μg/ml), 7 m*M* $MgCl_2 \cdot 6H_2O$, 80 m*M* KCl, 0.25 *M* sucrose and 35 m*M* Tris–HCl (all reagents supplied by Sigma Chemical Co., St. Louis, Mo.).

The reaction was initiated by the addition of ribosomes and soluble protein to the remaining components of the mixture which had been incubated at 37°C for 2 min. All samples were then incubated at 37°C for 60 min after which the reaction was stopped by the addition of 250 μl 10% trichloroacetic acid (TCA). All soluble proteins were precipitated by heating the acidified solution at 90°C for 25 min. The precipitated protein was transferred to a test tube with 10 ml 5% TCA. All samples were filtered under vacuum and filters were washed 12 times with 5% TCA followed by 12 times with 95% ethanol and once with water (5 ml/washing). Filters were transferred to liquid scintillation counting vials and 0.5 ml of protein solubilizer (Protosol, New England Nuclear) was added. Samples were incubated for 30 min at 60°C, neutralized with 0.2 ml glacial acetic acid, and mixed with liquid scintillation counting fluid (Biofluor, New England Nuclear). Radioactivity was determined by liquid scintillation spectrophotometry.

Statistical analyses. Data were statistically analyzed by analysis of variance appropriate for completely randomized design (21) with individual comparisons made as orthogonal contrasts where appropriate (22).

Results. The feeding of diets containing increasing amounts of leucine caused reduced growth of swine ($P < 0.01$) although the reduction in food consumption was not statistically significant (Table II). Increasing dietary leucine also successfully increased the concentration of free leucine in plasma and longissimus dorsi ($P < 0.05$) while concentrations of isoleucine and valine were not affected.

Dietary leucine concentrations had no overall effect on protein synthesis following simple recombination of ribosomes and soluble protein from each muscle sample (Table III). Synthesis was highest in triceps brachii tissues. The same result was observed when ribosomes from animals fed the 2.00% leucine control diet were combined with soluble protein from animals fed diets containing higher concentrations of leucine. In all three muscles assayed, however, there was significantly increased protein synthesis when soluble protein from animals fed the control 2.00% leucine

TABLE II. WEIGHT GAIN, FOOD CONSUMPTION AND TISSUE FREE LEUCINE CONCENTRATION OF SWINE FED LEUCINE-RICH PROTEIN

Dietary leucine (%)	Weight gain	Food cons	Plasma leu	LD leu	TB leu	BF leu
2.00	16.2	27.3	26.58	0.23	0.24	0.29
2.33	15.5	27.0	32.80	0.26	0.27	0.37
2.92	13.8[a]	25.9	44.60[a]	0.29	0.30	0.48
3.12	12.1[a]	24.8	50.55[a]	0.31[a]	0.34	0.39
3.53	12.4[a]	24.5	43.60[a]	0.37[a]	0.34	0.33
4.01	11.7[a]	22.6	36.00	0.36[a]	0.39	0.44
n	28	7	5	5	5	5
SD	∓4.0	∓6.3	∓19.37	∓0.07	∓0.09	∓0.27

Note. Abbreviations: leu = leucine, cons = consumption, LD = longissimus dorsi, TB = triceps brachii, BF = biceps femoris. Units: weight gain and food cons = kg; plasma leu = μmole/dl; LD, TB, and BF = μmole/g. All values are means.

[a] Significantly different from control (2.00% leucine) values at $P < 0.05$.

TABLE III. EFFECT OF LEUCINE-RICH DIETARY PROTEIN ON *in Vitro* INCORPORATION OF [^{14}C]PHENYLALANINE INTO PROTEIN OF PORCINE MUSCLE (dpm/60 min/0.5 mg Protein)

Dietary leucine (%)	Simple recombination	Recombination with varying ribosomes	Recombination with varying protein
		Longissimus dorsi	
2.00	8,058	7,537	9,042
2.33	10,525	9,653[a]	10,273
2.92	8,470	9,471[b]	9,463
3.12	9,059	9,599[b]	9,638
3.53	10,099	10,455[a]	10,949
4.01	8,512	10,748[a]	9,325
		Triceps brachii	
2.00	17,416	15,991	18,509
2.33	15,152	16,685	17,636
2.92	14,668	17,454	16,265
3.12	15,453	16,693	19,934
3.53	13,874	18,045[b]	17,419
4.10	14,251	18,742[a]	16,925
		Biceps femoris	
2.00	8,917	8,812	8,588
2.33	7,521	9,234	7,827
2.92	8,712	9,471	9,114
3.12	8,658	10,109[b]	8,404
3.53	9,640	10,727[a]	10,336
4.01	7,676	11,816[a]	8,893

Note. Simple recombination is determination of ^{14}C incorporated by ribosomes and soluble protein obtained from the same tissue. Varying ribosomes refers to ^{14}C incorporated by a combination of soluble protein from animals fed the control diet (2.00% leucine) and ribosomes from animals fed varying concentrations of leucine. Varying protein refers to ^{14}C incorporated by a combination of ribosomes from animals fed the control diet and soluble protein from animals fed varying concentrations of leucine. Values are means (n = 6); SD = ∓941, 6808, and 2838 for longissimus dorsi, triceps brachii, and biceps femoris, respectively.

[a,b] Significantly different from control values at $P < 0.01$ and $P < 0.05$, respectively.

diet was combined with ribosomes from animals fed diets containing increasing concentrations of leucine ($P < 0.05$).

Discussion. The growth depression seen when surplus dietary leucine was fed was similar to that observed by other investigators (23, 24). Since the leucine content of the diet was adjusted mainly by the feeding of leucine-rich protein instead of crystalline leucine, it was not possible to maintain a constant dietary concentration of all other essential amino acids. Amino acid analysis of the diets indicated, however, that only the concentration of leucine increased with the addition of corn gluten protein while only the concentrations of tryptophan and lysine decreased. It was necessary to add supplemental tryptophan and lysine at the highest levels of corn gluten meal in order to meet minimal requirements for growth (25). Only excesses of the branched-chain amino acids have been shown to influence protein synthesis in muscle (2–5), however, so the feeding of these intact proteins was considered a valid test of the effect of leucine on protein synthetic capacity of muscle in the present study.

The triceps brachii, longissimus dorsi, and biceps femoris were chosen to test the effects of leucine-rich dietary proteins on protein synthesis because these tissues are high nutritional quality animal protein food products. It was considered important to test these tissues individually since small but significant changes in protein synthesis in specific muscles could be masked by changes in other tissues when effects of leucine on whole body protein synthesis are measured. This is likely the reason for the lack of effect of leucine on whole body protein synthesis as reported by McNurlan *et al.* (26). The significant increase in

free leucine concentration in longissimus dorsi indicated that the effect of feeding leucine-rich protein on plasma concentrations of leucine was reflected at the tissue level and any leucine-induced changes in muscle protein synthesis should, therefore, be detectable. Any increases in concentration of leucine in triceps brachii and biceps femoris were not statistically significant because of increased variability between individuals for these tissues.

Substantial differences were seen when comparing the rate of protein synthesis in various muscles following simple recombination of soluble protein and ribosomes from the same tissues. Synthetic activity was particularly great in the triceps brachii. This may reflect the different rates of growth for different types of muscles in immature swine (27, 28).

The consistent effect of increasing dietary leucine on increased synthetic activity of the ribosomal fraction confirms that such diets can alter muscle protein metabolism. The significance of this observation is increased by the fact that it was seen in all three of the muscle types examined. This finding supports the observations of Buse *et al.* (12) and indicates that any protein anabolic effects of leucine in muscle are at least partly mediated through increased ribosomal activity and not through changes in the activity of soluble proteins.

It is not clear why the consistently increasing protein synthetic activity of muscle ribosomal fractions was not reflected in increased protein synthesis following simple recombination of ribosomal and soluble protein fractions. Unknown factor(s) in the soluble protein fraction may be limiting total protein synthesis despite the increased ribosomal activity. This did not result in decreasing protein synthesis, however, when the activity of the soluble protein fractions from animals fed increasing amounts of leucine-rich protein were combined with control ribosomes.

The design of the current experiments did not permit a separate measurement of the effect of dietary leucine on protein catabolism but leucine has been reported to reduce *in vitro* intracellular proteolysis in rat muscle (11). The contribution of decreased proteolysis to net protein synthesis was shown to increase with increasing leucine concentration.

It can be concluded that the feeding of leucine-rich proteins can increase the protein synthetic potential of muscle. The reduced growth of animals fed such diets *ad libitum,* however, indicates that such an effect would most likely be of benefit when leucine intake can be controlled such as in clinical situations involving total parenteral nutrition.

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1. Smith TK. Effect of dietary leucine on incorporation of valine into chick tissue protein. Nutr Rep Int **22:** 545–553, 1980.
2. Fulks RM, Li JB, Goldberg AL. Effect of insulin, glucose and amino acids on protein turnover in rat diaphragm. J Biol Chem **250:**290–298, 1975.
3. Buse MG, Reid SS. Leucine. A possible regulator of protein turnover in muscle. J Clin Invest **56:**1250–1261, 1975.
4. Buse MG, Weigand DA. Studies concerning the specificity of the effect of leucine on the turnover of proteins in muscles of control and diabetic rats. Biochim Biophys Acta **475:**81–89, 1977.
5. Li JB, Jefferson LS. Influence of amino acid availability on protein turnover in perfused skeletal muscle. Biochim Biophys Acta **544:**351–359, 1978.
6. Chua B, Siehl DL, Morgan HE. Effect of leucine and metabolites of branched-chain amino acids on protein turnover in heart. J Biol Chem **254:**8358–8362, 1979.
7. Odessey R, Parr B. Effect of insulin and leucine on protein turnover in rat soleus muscle after burn injury. Metabolism **31:**82–87, 1982.
8. Kishi T, Iwasawa T, Itoh H, Chibata I. Evaluation of an infusion solution enriched with branched-chain amino acid in parenteral nutrition of rats. J Nutr **110:** 710–719, 1980.
9. Kishi T, Iwasawa Y, Itoh H, Chibata I. Nutritional effect of a branched chain amino acid-rich solution parenterally administered into postoperative rats. Nutr Rep Int **25:**809–817, 1982.
10. Freund HR, Gimmon Z, Fischer JE. Nitrogen sparing effects and mechanisms of branched chain amino acids. In: Kleinberger G, Deutsch E, eds. New Aspects of Clinical Nutrition. Basel, Karger, pp346–360, 1983.
11. Tischler ME, Desautels M, Goldberg AL. Does leucine, leucyl-tRNA, or some metabolite of leucine regulate protein synthesis and degradation in skeletal and cardiac muscle? J Biol Chem **257:**1613–1621, 1982.
12. Buse MG, Atwell R, Mancusi, V. In vitro effect of branched chain amino acids on the ribosomal cycle in muscles of fasted rats. Horm Metab Res **11:**289–292, 1979.
13. Von der Decken A, Omstedt PT. Dietary protein

quality measured by in vitro protein synthesis in rat skeletal muscle ribosomes. J Nutr **102**:1555–1562, 1972.
14. James LJ, Smith TK. Effect of alfalfa on zearalenone toxicity and metabolism in rats and swine. J Anim Sci **55**:110–118, 1982.
15. Smith TK. Spent canola oil bleaching clays: Potential for treatment of T-2 toxicosis in rats and short-term inclusion in diets for immature swine. Canad J Anim Sci **64**:725–732, 1984.
16. Smith TK, Austic RE. The branched-chain amino acid antagonism in chicks. J Nutr **108**:1180–1191, 1978.
17. Alexis SD, Vilaire G, and Young VR. Cell-free studies of protein synthesis with skeletal muscle from normal and potassium-depleted rats. J Nutr **101**:273–286, 1971.
18. Fleck A, Munro HN. The precision of ultraviolet absorption measurements in the Schmidt–Thannhausser procedure for nucleic acid estimation. Biochim Biophys Acta **55**:571–583, 1962.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurements with the Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
20. Roodyn DB, Reis PJ, Work TS. Protein synthesis in mitochondria. Requirements for the incorporation of radioactive amino acids into mitochondrial protein. Biochem J **80**:9–21, 1961.
21. Snedecor GW, Cochran WG. Statistical Methods, 6th Ed. Ames, Iowa, Iowa State Univ Press, p135, 1967.
22. Ostel R, Mensing RW. Statistics in Research, 3rd Ed. Ames, Iowa, Iowa State Univ Press, p276, 1975.
23. Oestemer GA, Hanson LE, Meade RJ. Leucine-isoleucine interrelationship in the young pig. J Anim Sci **36**:674–678, 1973.
24. Henry Y, Duee PH, Rerat A. Isoleucine requirement of the growing pig and leucine-isoleucine interrelationship. J Anim Sci **42**:357–364, 1976.
25. Subcommittee on Swine Nutrition. Nutrient Requirements of Swine, 8th Ed. Washington DC, Natl Acad Sci, 1979.
26. McNurlan MA, Fern EB, Garlick PJ. Failure of leucine to stimulate protein synthesis in vivo. Biochem J **204**: 831–838, 1982.
27. Richmond RJ, Berg RT. Relative growth patterns of individual muscles in the pig. Canad J Anim Sci **62**: 575–586, 1982.
28. Richmond RJ, Berg RT. Effects of sex, genotype and nutrition on the relative growth of muscles in the pig. Canad J Anim Sci **62**:587–596, 1982.

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