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TUNICAMYCIN PREVENTS STIMULATION OF PROTEIN SYNTHESIS BY BRANCHED CHAIN AMINO ACIDS IN ISOLATED RAT MUSCLES

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Quarter rat diaphragms were incubated for 150 min. with or without stimulators of protein synthesis and/or inhibitors of protein glycosylation. Tyrosine incorporation into protein was measured during the last 120 min. Branched chain amino acids stimulated protein synthesis by 40-60%. Tunicamycin or 2-deoxy-D-glucose did not affect baseline protein synthesis, but inhibited or abolished stimulation of protein synthesis by branched chain amino acids. Tunicamycin did not prevent stimulation of protein synthesis by insulin or affect amino acid transport under these conditions. The data suggest that a) glycoprotein(s) may be involved in the post-transcriptional stimulation of protein synthesis by leucine, b) leucine and insulin may stimulate peptide chain initiation in muscle through different mechanisms.

The special role of branched chain amino acids, particularly leucine, in stimulating muscle protein synthesis, has been demonstrated in incubated isolated muscles (1-5), perfused hindquarters (6,7) and isolated hearts (5,7,8) of the rat. The effect is best demonstrated in muscles of fasted animals (2), in young rather than mature rats (6). In vivo, the effect may be limited to the catabolic state (9,10), and there appears to be differential susceptibility between muscle types (11). The mechanism by which the three branched chain amino acids together or leucine alone stimulate protein synthesis is poorly understood; it appears to entail stimulation of translation at the level of peptide chain initiation (6,12), and does not require mRNA synthesis (2,6). The rate of protein synthesis correlates with the intracellular concentration of leucine, and does not reflect the saturation of leucyl-tRNA, the km of which is very low (5,7). The branched chain amino acids are metabolized by skeletal and heart muscle (reviewed in 13). Leucine participates in the regulation of numerous processes, it inhibits protein degradation in

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muscle (1,2,5-7), heart (5,7,8) and liver (14), stimulates the production of glutamate and glutamine by these tissues (13,15,16), inhibits hepatic urea genesis (16), and inhibits the oxidation of pyruvate and glucose (17). β-Hydroxy-β-methylglutaryl-CoA (HMG-CoA) is a product of leucine catabolism and a precursor in the pathway of cholesterol and dolichol synthesis (18). Leucine is a substrate for cholesterol synthesis in skeletal muscle (19). Dolichols function in their phosphorylated state as carbohydrate carriers and donors in the biosynthesis of cellular glycoproteins. The concentration of dolicholphosphate may be limiting protein glycosylation (20,21).

Glycoproteins participate in numerous metbolic processes (21-28), although there is no report of their involvement in peptide chain initiation. We postulated that if stimulation of muscle protein synthesis by branched chain amino acids is linked in some way to glycoprotein synthesis, inhibitors of protein glycosylation may block their effect. Two inhibitors were tested. Tunicamycin is an antibiotic which inhibits the transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetylglucosamine to dolichol monophosphate and thereby blocks the formation of N-linked glycoproteins (22-28). 2-deoxy-D-glucose[2] (2DG) is a glucose analogue, which is phosphorylated by cells to 2-DG-6P but not further metabolized. It is converted however into both the UDP and GDP derivative and competes with UDP-N-acetylglucosamine for binding to dolichol-phosphate. Like tunicamycin, 2-DG inhibits core glycosylation of proteins, but unlike tunicamycin, 2-DG also inhibits elongation of protein linked carbohydrate chains (25,29,30).

MATERIAL AND METHODS

Material. Two lots of tunicamycin were tested, one was purchased from Calbiochem-Behring, the other was a gift of the Natural Products Branch of NIH. The racemic b (i.e. ± amino-endo) isomer of 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH), a non-utilizable analogue of leucine, was a gift of Dr. Halvor N. Christensen, University of Michigan. Carboxyl labelled [^{14}C]BCH as well as L-[^{14}C-1]α-aminoisobutyric acid (AIB), D-[^{3}H]mannitol and L-[ring-2-6-^{3}H]tyrosine were from New England Nuclear. 2-DG, unlabelled amino acids and crystalline sodium pyruvate were from Sigma; all reagents used were of the

[2] Abbreviations used: 2-DG = 2-deoxy-D-glucose; BCH = DL-b-2-aminobicyclo[2-2-1]heptane-2-carboxylic acid; AIB = α-aminoisobutyric acid; PCA = perchloric acid.

highest purity obtainable. Monocomponent, crystalline porcine insulin (26.5 U/mg) was a gift from Dr. Ronald Chance, Eli Lilly Company.

Muscle preparation. Male, Wistar rats (90-110 g) were purchased from Charles River Breeding Laboratories and fasted 18 hrs before experiments. Rats were decapitated with a guillotine, the two hemidiaphragms were rapidly removed, bissected and weighed; quarter diaphragms were incubated with shaking at 37°C in 2 ml of Gey and Gey's balanced salt solution (bicarbonate buffer), pH 7.4, gas phase 95% O_2 + 5% CO_2 as described (3,31). Glucose was added to the media as an energy source in all studies, except in experiments using 2-DG were pyruvate was substituted to avoid competition of glucose with 2-DG for transport.

Protein synthesis. Quarter diaphragms were preincubated in media supplemented with 11 mM glucose or 11 mM pyruvate and 0.35 mM L-tyrosine with or without additions e.g. branched chain amino acids (1 mM each), insulin (1 mU/ml), tunicamycin (1 μg/ml) or 2-DG (3 mg/ml). One quarter diaphragm of each rat was placed into control medium and the others distributed between media containing a stimulator of protein synthesis, an inhibitor of protein glycosylation and a combination of inhibitor and stimulator. Following 30 min preincubation, the tissues were transferred to identical media supplemented with 0.5 μCi/ml L[^{3}H]tyrosine and incubated for 2 hrs. They were then homogenized in 2 ml ice-cold 6% perchloric acid (PCA) with a Polytron tissue homogenizer. Following centrifugation the pellet was solubilized in 1 N NaOH and prepared for scintillation counting as described previously (3,31). Aliquots of deproteinized media and of the PCA soluble fraction of muscle homogenates were used for fluorometric analysis of tyrosine (32) and for liquid scintillation counting. The spec. act. of intracellular tyrosine in muscle was calculated as described (3,31). Incorporation of tyrosine into protein (nmole/mg tissue/2 hrs) was determined by dividing protein spec. act. (dpm/mg tissue) by the intracellular spec. act. of free tyrosine (dpm/nmole) (1). Protein synthesis proceeds linearly with time under these conditions for 2 hrs (1,31).

Amino acid transport. Weighed, quarter rat diaphragms were preincubated for 2 hrs in media containing 5.5 mM glucose, 0.1 mM mannitol and a non-utilizable amino acid, AIB (0.1 mM) or BCH (2 mM) with or without tunicamycin (1 μg/ml). They were then transferred to identical media supplemented with [^{3}H]mannitol (1.5 μCi/ml, as an extracellular marker) and 0.2 μCi/ml of either [^{14}C]AIB or [^{14}C]BCH and incubated for 10-30 min. After incubation, the muscles were solubilized in boiling 1 N NaOH. Aliquots of media and muscle extracts were counted in a Beckman LS-7000 β-scintillation counter with a double isotope program. In calculations of the intracellular concentration of AIB or BCH it was assumed that the medium was at equilibrium with the extracellular space in which mannitol and extracellular AIB or BCH were distributed identically.

Statistical analyses. Means ± SEM are shown. Differences between means were analyzed by paired, two tailed Student's t-test.

RESULTS

Protein synthesis (Table 1). Addition of the 3 branched chain amino acids to the incubation medium increased protein synthesis 40-60% over baseline (Studies 1, 2 and 4). Tunicamycin (1 μg/ml) did not affect the rate of protein synthesis in unsupplemented media (Studies 1-3), but decreased (Study 1) or abolished (Study 2) the stimulatory effect of branched chain amino acids on protein synthesis. Studies 1 and 2 differ in that different preparations of tunicamycin were tested. When protein synthesis was

Table 1. Tunicamycin or 2-deoxyglucose-D-glucose prevent stimulation of protein synthesis by branched chain amino acids in muscles

Additions to medium	Protein synthesis (% of no addition control)	P
Study 1		
BCAA	160.3 ± 12.0 (28)	<0.001 vs control
Tunicamycin[a]	99.4 ± 6.4 (26)	
BCAA + Tunicamycin[a]	136.2 ± 11.2 (28)	<0.02 vs. BCAA
Study 2		
BCAA	140.7 ± 16.3 (9)	<0.001 vs control
Tunicamycin[b]	97.9 ± 11.2 (9)	
BCAA + Tunicamycin[b]	106.5 ± 7.4 (9)	<0.05 vs. BCAA
Study 3		
Insulin	127 ± 8.1 (11)	<0.01 vs. control
Tunicamycin[b]	102.7 ± 10.7 (11)	
Insulin + Tunicamycin[b]	145.7 ± 13.2 (11)	<0.01 vs. control NS vs. insulin
Study 4		
BCAA	138.7 ± 14.6 (6)	<0.05 vs. control
2-deoxy-D-glucose	93.8 ± 9.9 (6)	
BCAA + 2DG	92.8 ± 9.1 (5)	<0.02 vs. BCAA

Quarter rat diaphragms were preincubated for 30 min. and incubated for 2 hrs. as described in Methods in media containing 11 mM glucose (Studies 1-3) or 11 mM pyruvate (Study 4), 350 μM tyrosine and the additions indicated. L-[ring-2,6-^{3}H]tyrosine (0.5 μCi/ml) was included during incubation. Protein synthesis was measured as tyrosine incorporation into proteins (nmol/g tissue/2 hrs), which in no addition controls was 242 ± 13 (n = 48, Study 1-3) and 130 ± 6 (n = 6, Study 4). All data are normalized to % of no addition control muscle from the same rat; means ± SEM are shown, with the number of observations in parentheses. BCAA = branched chain amino acids, leucine, isoleucine, valine, 1 mM each. Tunicamycin[a] was from Calbiochem-Boehring, Tunicamycin[b] from Natural Products Branch, NIH, 1 μg/ml; 2-deoxy-D-glucose was 3 mg/ml; crystalline pork insulin 1 mU/ml. P values were calculated by Student's paired t test.

stimulated by the addition of insulin, without branched chain amino acids, tunicamycin did not affect the rate of either baseline or insulin stimulated protein synthesis (Study 3).

In media supplemented with pyruvate (Study 4), baseline protein synthesis was slower than in glucose containing media, but, as in glucose containing media, branched chain amino acids stimulated the rate of protein synthesis, confirming earlier observations (31). 2DG had the same effect as tunicamycin, baseline protein synthesis was unaffected, but stimulation by branched chain amino acids was abolished (Study 4).

Amino acid transport. In chick embryo fibroblasts, tunicamycin treatment for 24 hrs decreases amino acid transport, suggesting that glycosylation of the carrier proteins is required for their effective function, or for their

insertion into the plasma membrane, or for protection from intracellular degradation (25). Stimulation of protein synthesis by branched chain amino acids may have been prevented by tunicamycin if it inhibited the intracellular accumulation of the branched chain amino acids and/or labelling of the intracellular tyrosine pool. To test this hypothesis, we incubated quarter diaphragms with or without tunicamycin at the same dose and for the same length of time as in the experiments shown in Table 1; transport of non-utilizable amino acids was measured during the last 10-30 min. AIB is preferentially transported on the "A"-system, and BCH on the "L"-system, the latter transports the branched chain and aromatic amino acids (33). Neither system appeared to be affected by tunicamycin (Fig. 1a and 1b). In additional experiments (carried out as in Table 1), the intracellular spec. act. of tyrosine was constant between 30 and 120 min (31), and was not affected by either tunicamycin or branched chain amino acids, separately or together (Fig. 1c).

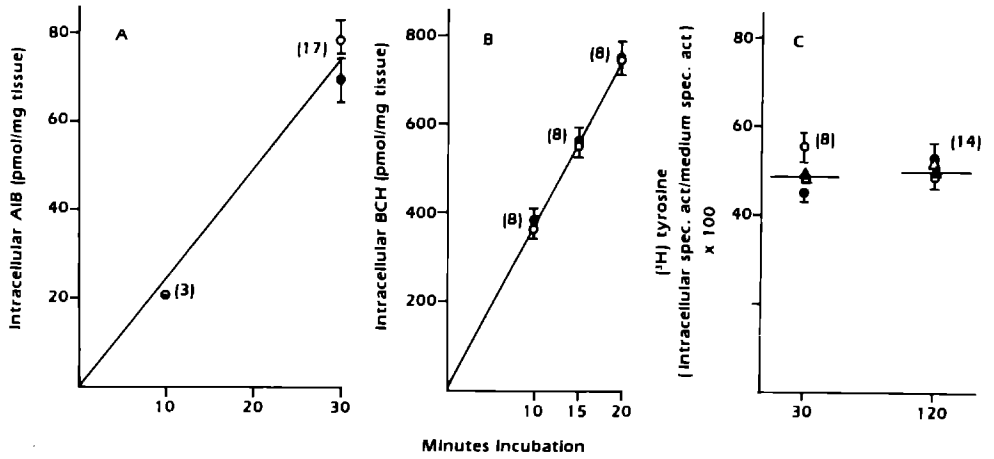

Fig. 1. Two-hour incubation with tunicamycin does not affect amino acid transport in isolated muscles. In Figs. A and B quarter diaphragms were preincubated for 2 hrs in media containing 5.5 mM glucose, 0.1 mM mannitol and 0.1 mM AIB, (Fig. 1A) or 2 mM BCH (Fig. 1B) with ●—● or without ○—○ 1 μg/ml tunicamycin. Muscles were then transferred to identical media supplemented with 1.5 μCi/ml [^{3}H]mannitol (extracellular marker) and 0.2 μCi/ml [^{14}C]AIB (Fig. 1A) or 0.2 μCi/ml [^{14}C]BCH (Fig. 1B) and incubated for the times indicated. The penetration of labelled, non-utilizable amino acids into the intracellular water was measured as described in Methods. In Fig. 1C quarter diaphragms were preincubated for 30 min. and incubated for 30 or 120 min. as described in Table 1; the spec. act. of tyrosine in the medium and in the intracellular water was measured as described in Methods. 0 = No addition control, Δ = BCAA, ● = tunicamycin, ▲ = BCAA + tunicamycin. Means ± SEM are shown, except where the SEM are too small for illustration. The number of observations/per point are indicated in parentheses. The horizontal lines in Fig. 1C indicate group means.

DISCUSSION

The data suggest that protein glycosylation is required for branched chain amino acid induced stimulation of protein synthesis in muscle, the effect could be permissive or the branched chain amino acids may stimulate overall protein synthesis (4) by increasing synthesis or glycosylation of a critical glycoprotein. In either case, this would implicate glycoprotein(s) in the post-transcriptional regulation of peptide chain initiation. It is possible however that both tunicamycin and 2-DG inhibited protein synthesis by unspecified mechanisms, unrelated to protein glycosylation. The dose of tunicamycin used here (1 μg/ml) inhibits protein glycosylation by 95% during relatively short incubations in organ culture or in cell culture, causing only mild inhibition of protein synthesis (22-24); 10-20X lower concentrations are effective inhibitors of protein glycosylation in cell-culture systems, where prolonged exposure e.g. 24 hrs, is feasible (25-28). Since baseline protein synthesis was unaffected in our experiments by tunicamycin or by 2-DG, and tunicamycin did not inhibit stimulation of protein synthesis by insulin, the effects observed may be specific.

The insulin receptor (24) and amino acid carrier proteins (25) are glycoproteins; the lack of impairment of either amino acid transport or of the insulin response after relatively short exposure to tunicamycin (120-150 min) suggests that the turnover of these structures in muscle is relatively slow. The half-life of the insulin receptor in 3T3-L1 mouse adipocytes is 7.5 hrs (24), - it has not been determined in skeletal muscle; in incubated diaphragms puromycin induced inhibition of AIB transport requires 3-4 hrs exposure to the drug (34).

The branched chain amino acid responsible for stimulation of protein synthesis and inhibition of protein degradation in muscle, in vitro, appears to be leucine (2,3,5,7,8). Our data is consistent with hypotheses that leucine may stimulate the synthesis or the glycosylation of a relatively short-lived glycoprotein, which participates in the post-transcriptional regulation of protein synthesis, or glycosylation may protect such a protein from intra-

cellular proteolysis (26-28) or inactivation, or prevent the activation of a translational inhibitor (2). Glycosylation has been shown to protect proteins from intracellular degradation (26-28). The data also suggest that while both insulin (35) and leucine (6,12) are thought to stimulate peptide chain initiation the molecular mechanisms may be different.

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