Supplement: Protein Metabolism in Response to Ingestion Pattern and Composition of Proteins

Regulation of Muscle Protein by Amino Acids[1,2]

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ABSTRACT Amino acid availability is a potent regulator of muscle protein synthesis (MPS). We have performed a series of studies using stable isotope methodology and the arteriovenous balance approach to quantify many aspects of the response of MPS, breakdown, and the balance between synthesis and breakdown to changes in the availability of amino acids. A constant intake of amino acids stimulates MPS in a dose-dependent manner until concentrations are approximately doubled, after which further increases in concentration are ineffective. MPS rises more rapidly after bolus ingestion to a peak rate of MPS higher than during constant intake, but the response is transient. A reduction in amino acid availability below basal levels inhibits MPS. Ingestion of nonessential amino acids is not needed to stimulate MPS. When carbohydrate alone is ingested there is minimal effect on MPS, but there is an interactive effect with amino acid ingestion, meaning the response to amino acids plus glucose is more than the sum of their individual effects. Finally, acute anabolic responses in net MPS correspond quantitatively to differences in 24-h net muscle balances. J. Nutr. 132: 3219S–3224S, 2002.

KEY WORDS: • *amino acids* • *skeletal muscle* • *anabolism* • *net balance* • *stable isotope*

Determining the "protein requirement" for normal man, as well as the "requirements" for individual amino acids, has been the topic of research for >50 y, yet arguments persist. One of the central problems is identifying a definable end point. Skeletal muscle comprises the majority of protein in the body, and the amount of muscle can vary severalfold between individuals who are functional in terms of acts of daily living. Thus, there is likely no simple value for "requirement." For example, one can not reasonably determine requirements on the basis of simply enabling daily function if the individual is a competitive weightlifter who needs enough intake to maintain much more muscle mass than is necessary for minimal activity. Furthermore, as will be evident from the data presented in this paper, the effectiveness of protein or amino acid intake depends not only on the form in which the substrate is ingested but also on the pattern of ingestion and the interaction with other factors, such as exercise and nonprotein caloric intake. Thus, it is probably more reasonable to consider the way in which ingested protein/amino acid and energy intake affect the rate of muscle protein synthesis (MPS) in different circumstances than to try to identify a particular numerical value for requirement.

MATERIALS AND METHODS

The results presented in this paper have been generally obtained using a model of leg amino acid/protein kinetics based on the arteriovenous (A-V) difference method (1). This method uses stable isotope tracer methodology. When a tracer of an essential amino acid (EAA) such as phenylalanine (Phe) is used that is neither synthesized nor oxidized in muscle, uptake and appearance of the tracer can be equated to rates of MPS and breakdown. The difference between synthesis and breakdown, i.e., the net balance, is the pertinent parameter relevant to the net gain (anabolism) or loss (catabolism) of muscle. Therefore, the primary end point discussed in this paper will be net Phe balance across muscle. Net balance is the result of the balance between MPS and breakdown, which are each regulated, potentially independently. Therefore, in addition to net balance, regulation of both MPS and breakdown will be discussed. In that regard, some data will be expressed as fractional synthetic rate, which is determined by the rate of direct incorporation of tracer into the product protein (2).

RESULTS AND DISCUSSION

Response to amino acids at rest

There is a close relationship in normal volunteers between the availability of amino acids, i.e., the rate at which amino acids enter the free intracellular pool from inward transport and from breakdown, and the rate of MPS. Furthermore, when amino acids are infused intravenously the rate of inward transport is stimulated and the rate of MPS is stimulated corre-

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[4] Abbreviations used: A-V, arteriovenous; EAA, essential amino acid; eIF, eukaryotic initiation factor; Met-tRNA, MPS, muscle protein synthesis; NEAA, nonessential amino acid; Phe, phenylalanine; tRNA, transfer RNA.

spondingly (**Fig. 1**) (3). The simplest interpretation of these observations is that amino acid availability drives MPS via availability. However, this explanation is not consistent with all available data. Thus, we have shown that even when plasma amino acids double and MPS increases correspondingly, the intracellular concentrations of EAA is either not increased or even decreases slightly (Bohe, J., Low, A., Wolfe, R. R. and Rennie, M. J., unpublished results). Furthermore, basal concentrations of amino acids are apparently sufficient to maintain sufficient charging of transfer RNA (tRNA) to support a significant increase in the rate of MPS (4). Thus, it is more likely that changes that occur in extracellular amino acid concentrations serve as signals to activate the synthetic process, and that once synthesis is activated this results in an increased rate of inward transport. Thus, regulation of the intracellular concentrations serves to maintain availability of amino acids as they are used at an accelerated rate for incorporation into protein (5).

The concept of extracellular changes in amino acid concentrations signaling changes in synthesis also applies to the circumstance of a reduction below normal basal values. We used hemodialysis in anesthetized pigs to reduce blood amino acid concentrations to ~50% below the normal basal level (Kobayashi, H., Borsheim, E., Traber, D. L., Badalamenti, J., Anthony, T. G., Kimball, S. R., Jefferson, L. S. and Wolfe, R. R., unpublished results). After 2 h, amino acids were replaced in half of the animals. When concentrations were reduced there was a corresponding reduction in MPS. Inward transport was also reduced, so intracellular concentrations remained constant. Synthesis returned to the basal rate when amino acids were replaced, whereas synthesis remained suppressed for the entire 4 h of hemodialysis in which amino acids were not replaced. Thus, return of the signal to increase or decrease synthesis was similar in both circumstances; i.e., changes in extracellular concentrations signal corresponding changes in synthesis. However, at a molecular level the mechanisms are apparently different in the two circumstances.

The initiation of mRNA translation is a key regulatory process that responds to changes in amino acid concentrations. Translation initiation is a complex multi-step process requiring more than a dozen eukaryotic initiation factors (eIF) (6,7). At least two steps in the initiation pathway are subject to regulation in vivo: *1*) binding of initiator methionyl-tRNA to the 40 S ribosomal subunit, and *2*) binding of mRNA to the 43 S preinitiation complex. The activity of eIF2B is involved in the first step. Adequate amino acid availability, i.e., charging of tRNA, is necessary for a decrease in eIF2a phosphorylation and, in turn, the increase in eIF2B activity necessary to initiate the synthetic process. In the 1970s several investigators expressed the perspective that amino acids acted via the extent of charging of tRNA (8–10). However, it has been shown that the K_m values for the tRNA charging enzymes are generally low, and thus there should be sufficient amino acids for adequate charging in muscle in the basal state (11). Furthermore, although direct measurements of tRNA charging have been limited, it appears that in liver the tissue content of charged tRNA is relatively stable in a number of circumstances in which protein synthesis changes, so it is unlikely that tRNA charging is a direct regulator of synthesis (12). Furthermore, we have shown that during the infusion of amino acids at rates sufficient to increase plasma concentrations in the normal physiological range, MPS is stimulated whereas the intracellular concentrations remain either unchanged or slightly depressed (Bohe, J., Low, A., Wolfe, R. R. and Rennie, M. J., unpublished results). Because the amino acids involved in charging of muscle tRNA apparently come from the intracellular pool (13), our results are consistent with the notion that a stimulation of MPS above the normal basal rate is not mediated by an increase in tRNA charging. Thus, it appears under physiological circumstances that factors such as amino acids that stimulate MPS above the basal rate do not act via changes in eIF2B activity. In contrast, we found that when plasma amino acid concentrations were decreased by hemodialysis the guanine nucleotide exchange activity of eIF2B dropped promptly in association with the reduction in protein synthesis (Kobayashi, H., Borsheim, E., Traber, D. L., Badalamenti, J., Anthony, T. G., Kimball, S. R., Jefferson, L. S. and Wolfe, R. R., unpublished results). When the amino acids were replaced after 2 h of dialysis, the decreased eIF2B activity returned to normal and MPS increased. It thus appears that, when amino acid availability is insufficient, initiation of protein synthesis is limited by insufficient eIF2B activity and therefore other mechanisms that might normally stimulate MPS via the binding of mRNA to the 43 S preinitiation complex (e.g., insulin) would be expected to be ineffective. It could therefore appear that eIF2B activity functions in a permissive role at the physiological level. Rather, stimulation of an increase in MPS above the basal rate is apparently regulated by increased binding of mRNA to the 43 S preinitiation complex, which is caused by increased eIF4G association with eIF4E (4).

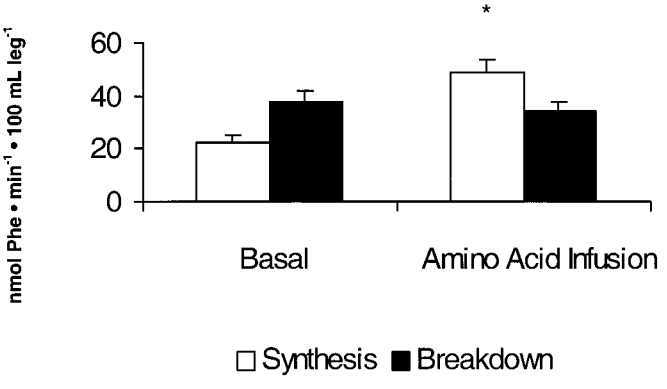

FIGURE 1 Effect of balanced amino acid infusion (164 mg/h · kg) on MPS and breakdown (from Ref. 3).

Amino acids and muscle protein breakdown

In vitro experiments indicate a role of amino acids in regulating muscle protein breakdown (14). However, in normal subjects infusion or ingestion of amino acids had no effect on breakdown (3). Similarly, reduction of amino acid concentrations in normal pigs had no effect on breakdown (Kobayashi, H., Borsheim, E., Traber, D. L., Badalamenti, J., Anthony, T. G., Kimball, S. R., Jefferson, L. S. and Wolfe, R. R., unpublished results). However, the response is different when breakdown is accelerated in a catabolic state. In severely burned patients muscle protein catabolism results from an accelerated rate of breakdown that exceeds the extent of increase in synthesis (15). In these patients infusion of amino acids suppressed breakdown, whereas synthesis was not affected. Thus, an elevation in amino acid concentrations in vivo only suppresses accelerated muscle protein breakdown.

Composition of amino acid mixture and MPS

Amino acids that can be synthesized in the body at rates adequate to meet basal requirements are considered to be

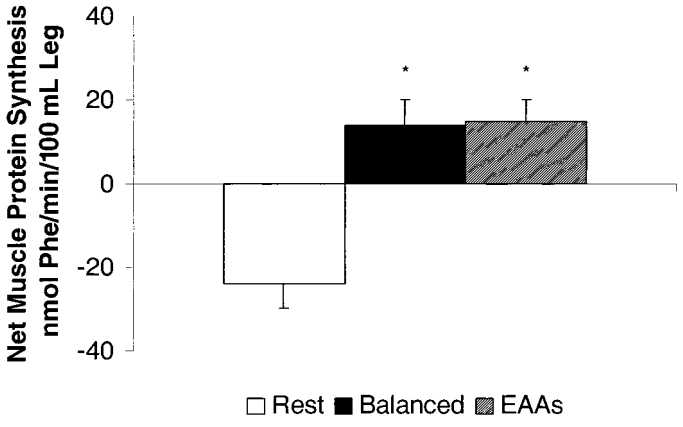

FIGURE 2 Comparison of net muscle protein synthesis (MPS) in response to infusion of a balanced solution of only the essential amino acids (EAA) (164 mg/h · kg) versus infusion of only the EAA within the mixture (~50% of the balanced mixture).

nonessential amino acids (NEAA). However, it is not clear whether ingestion of one or more of these amino acids is necessary to support accelerated MPS resulting from supplement ingestion. We had previously shown that MPS was stimulated to a similar extent when 40 g of either a balanced mixture of EAA and NEAA or the same amount of EAA only was ingested in small doses over 3 h (16). These results indicated that ingestion of NEAA was not normally needed for MPS to be stimulated when EAA were ingested. In contrast, because substitution of ~50% of the EAA with NEAA did not reduce the magnitude of stimulation it could be that the NEAA can be used as effectively as the EAA. Alternatively, because we have shown that there is a limit to the extent to which MPS can be stimulated during constant intake (Bohe, J., Low, A., Wolfe, R. R. and Rennie, M. J., unpublished results), it is possible that the 20 g of EAA given in the balanced mixture already exceeded the maximal dose. In this case, amino acids given in excess of 20 g whether or not the amino acids were "essential" would not be used for synthesis. To more directly test whether the ingestion of NEAA is necessary for the stimulation of MPS, individuals were given 30 g of a balanced mixture of amino acids (EAA + NEAA) continuously over 3 h. The study was then repeated, but only the EAA in the mixture were given (~14 g). The results are summarized in **Figure 2**. The responses of MPS and net muscle balance (shown in Fig. 2) were almost identical, although only half the total amino acids were given in the EAA group as in the group given EAA + NEAA. These results showed that NEAA are not needed for the stimulation of MPS by exogenous EAA.

Bolus versus constant infusion

The response of MPS to a bolus of amino acids is quite different from the response to a constant infusion. There is a rapid increase in MPS in conjunction with the rise in EAA concentration, and an equally rapid fall as the EAA concentrations begin to decrease (**Fig. 3**). However, the rate of MPS returned to the basal value well before the concentration fell to the basal level. In fact, in the example shown in Figure 3, after ingestion of 15 g of EAA, net balance (as well as the rate of MPS) had returned to the basal rate when the plasma concentration was still more than double the basal value. In contrast, when plasma concentrations were increased to a steady-state concentration for 3 h at a value twice above the basal value, the maximal stimulation of MPS was achieved (17). Furthermore, whereas the response to a bolus is transient, the peak response may be 6- to 10-fold the basal value. Nonetheless, because of the transient nature of the response, the total synthetic response (area under curve of synthesis vs.

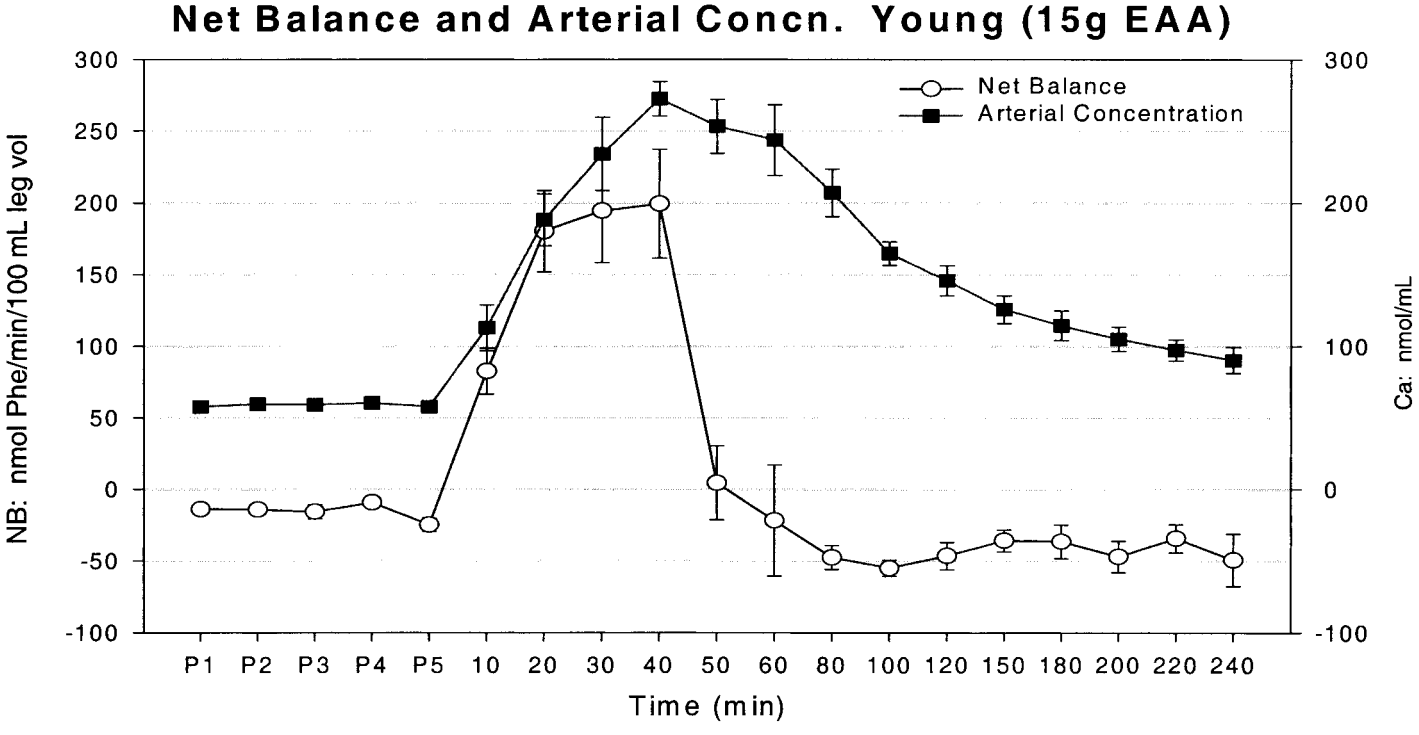

FIGURE 3 Plasma phenylalanine (Phe) concentration and net balance in response to 15 g of essential amino acids (EAA) taken as bolus.

time) was less than when similar doses of amino acids were taken in the constant intake mode as opposed to the bolus ingestion (**Fig. 4**). Thus, different mechanisms appear to be operative in response to the mode of ingestion. Clarification of these mechanisms could lead to amplifying the response to a given amount of intake.

Interaction of amino acids and carbohydrate intake

To test the independent and potentially interactive effects of amino acids and carbohydrate intake normal volunteers were given drinks containing 30 g of a balanced amino acid mixture alone, 30 g of glucose alone or a combination of 30 g each of amino acids and glucose. The drinks were consumed in small amounts over 3 h, resulting in essentially steady state elevations in concentrations of amino acids, glucose and insulin in the plasma. The stimulation of net MPS by amino acids alone is shown in Figure 2. Glucose alone had no significant effect on MPS. However, when the glucose was added to the amino acids there was an interactive effect, meaning that the stimulation of net MPS was more than the sum of the individual responses (17). This interactive effect is understandable when considered in light of the response of MPS to insulin and the discussion above of the permissive nature of eIF2B activity.

Whereas insulin has long been recognized as an important anabolic hormone, its specific role in regulation of MPS in human subjects has been the subject of debate. Whereas in vitro experiments have clearly demonstrated a stimulatory effect of insulin on MPS, studies in human subjects have yielded inconsistent results (see Ref. 18 for review). However, when the role of amino acid concentrations in the regulation of MPS is considered, the apparently contradictory in vivo results can be largely reconciled. When glucose/insulin are given alone, such as during the euglycemic/hyperinsulinemic clamp procedure, plasma amino acid levels fall. The effect of a reduction in amino acid levels was discussed above. Thus, although insulin itself has a stimulatory effect on synthesis, an adequate eIF2B activity is required for that potential effect to translate into an increased rate of synthesis, and this is not the case when amino acid levels fall. Consequently, studies in which plasma amino acid levels were allowed to drop during insulin infusion have generally failed to show a stimulatory effect of insulin on MPS. In contrast, when the effect of insulin has been assessed in the context of maintained (e.g., Ref. 19) or elevated (e.g., Ref. 20), concentrations of amino acids have been found to stimulate synthesis. Thus, the interactive response of MPS to carbohydrate plus amino acids described above can be predicted, although carbohydrate intake alone has minimal effect.

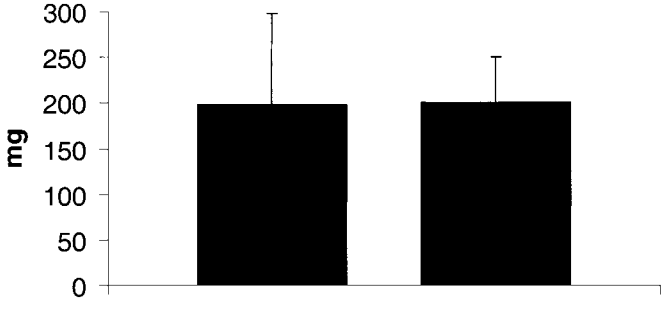

FIGURE 4 Comparison of area under the curve (i.e., total response) over 3 h after ingestion of 15 g of essential amino acid (EAA) as bolus or constantly throughout the 3 h.

Resistance exercise and amino acid intake

The response of muscle protein metabolism varies in magnitude according to the exact nature of intensity of exercise and the method used to quantify the response. Studies determining the fractional synthetic rate have consistently shown a stimulation after exercise (e.g., Ref. 21), and although results from the A-V balance method have been less consistent, a stimulation of synthesis has been observed with this method as well when the exercise involved several muscle groups of the leg (22). However, breakdown is also elevated, so that the balance between synthesis and breakdown does not become positive (22). Thus, nutritional intake is required for resistance exercise to be anabolic.

The response to amino acid intake after exercise is dependent on the composition and amount, as well as the pattern and timing of ingestion in relation to the performance of exercise. We assessed the effect of composition and amount of amino acids by comparing the response to two doses, given 1 h apart, of 6 g each of a mixture of EAA only versus 6 g of a balanced mixture (EAA + NEAA) (Borsheim, E., Tipton, K. D., Wolf, S. E. and Wolfe, R. R., unpublished results). The balanced mixture contained ~3 g of EAA per dose. The total response to the two doses of 6 g of EAA was double the response to the balanced mixture. In addition, in each case the response to the second dose was the same as to the first. This was true even though when the second dose was given the response of net MPS to the fist dose had returned to the basal rate, despite the persistent elevation in concentration after the first dose. Thus, it appears that an increasing concentration activates the synthetic process and the decline in concentration decreases synthesis, irrespective of the absolute value of the concentration. We also assessed the effect of the addition of 35 g of glucose to either the balanced or EAA mixtures (Borsheim, E., Tipton, K. D., Wolf, S. E. and Wolfe, R. R., unpublished results; Miller, S. L., Tipton, K. D., Chinkes, D. L., Wolf, S. E. and Wolfe, R. R., unpublished results). In contrast to the situation at rest, no interactive or even additive glucose effect was observed. This absence of anabolic effect of carbohydrate is consistent with the absence of action of local hyperinsulinemia on muscle protein metabolism after exercise (23). In contrast, local hyperinsulinemia stimulates MPS in resting individuals (20).

Timing of intake in relation to exercise

In the experiments described above the amino acids ± glucose were given after exercise. No difference in response was observed when EAA + glucose were given at 1 versus 3 h after exercise (24). Similarly, when the same mixture was given immediately after exercise the response of MPS was the same as when given 1 h after exercise (25). However, when the EAA/glucose mixture was given immediately before exercise the response was greatly amplified. Not only was the increase during exercise approximately fourfold above the basal value, the response in the first hour after exercise was just as large as the response over the same time interval when the supplement was given immediately after exercise (**Fig. 5**). When total area under the curve was calculated, the response was approximately threefold greater when the drink was given after exercise than at rest, and approximately double the response as when ingested after exercise (25).

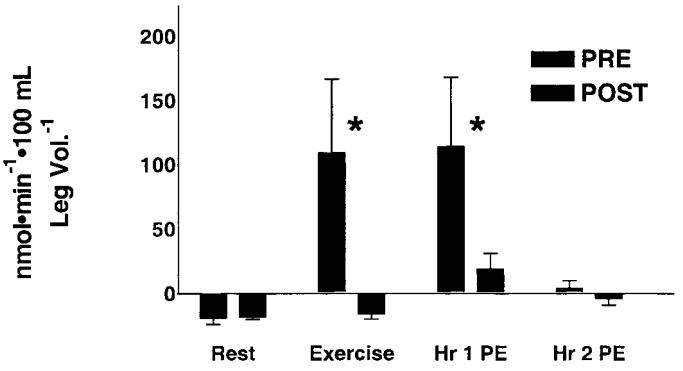

FIGURE 5 Effect of timing of ingestion of mixture of 6 g of essential amino acid (EAA) + 35 g of glucose on net muscle phenylalanine (Phe) balance in relation to performance of resistance exercise. PRE, Ingestion immediately before performance of exercise; POST, ingestion 1 h after exercise (from Ref. 26).

Diurnal changes in muscle protein balance

In a general sense protein synthesis is stimulated after food is ingested and it is decreased when the individual is in the postabsorptive state. Conventionally this corresponds to a diurnal cycle, with synthesis being elevated in waking hours and depressed during sleep. It has been proposed that, once maintenance requirements are achieved, additional protein intake has no effect on 24-h whole-body protein balance, because the elevated peak during the day is balanced by a greater nadir in the evening (26). However, this proposed response is at odds with considerable experimental data indicating that, as protein intake increases, total amino acid retention increases, even when intake is above that required for amino acid balance (27). We therefore tested whether acute acceleration of MPS, as we have demonstrated above in response to exercise plus amino acids, translates to a comparable increase in net balance over 24 h, or rather if an acute increase is counterbalanced by a corresponding nadir in the night. On 1 day normal volunteers were given two doses of amino acids plus glucose, one before and one after a bout of resistance exercise, to provide a potent acute stimulation of MPS. On a second occasion they rested during the corresponding time. The subjects were given the same meals and treated identically over 24 h, other than the exercise/amino acid supplementation. A-V samples were taken throughout the entire 24 h. The results showed that the stimulated net balance over 3 h in the exercise/amino acid supplementation group translated to almost the identical gain over 24 h. Net balance throughout the night was almost identical in the two groups. Fractional synthetic rate values over the 24 h confirmed the conclusions from the 24-h net balance data. Thus, acute stimulation of muscle protein metabolism does not affect rates of synthesis at later times when there is no food intake.

Relation of findings to protein requirements

The results presented here establish a direct role of amino acids in regulating net muscle protein balance. Furthermore, the response is directly affected by the exact composition and amount of mixture of amino acids ingested, the timing of ingestion in relation to exercise and the amount and nature of nonprotein energy ingested with amino acids. When these findings are considered in the context of the estimation of protein or amino acid "requirements," it is clear that multiple results could be achieved for the same AMINO ACID intake, depending on the interaction of the various factors discussed above. Thus, attempts to identify a unique AMINO ACID "requirement" are likely to be unsuccessful. Furthermore, our results, along with virtually all data in the literature on the topic, suggest that increasing AMINO ACID intake will increase muscle mass, with all other variables remaining constant. Whereas this concept runs counter to popular perception, real-life examples abound in obese individuals who have significantly elevated muscle mass despite living sedentary lives (e.g., Ref. 28). Thus, it is likely that increasing AMINO ACID intake in accord with the principles discussed above to optimize effectiveness will promote muscle anabolism, whether in depleted individuals such as the elderly or in active athletes trying to increase muscle mass. The exact nutritional approach will determine the extent of anabolic response.

SUMMARY

Plasma concentrations of EAA regulate muscle protein metabolism in human subjects. The magnitude and duration of response is dependent on the magnitude of the change in concentrations, the timing of increases in concentration in relation to exercise and the concurrent insulin response resulting from any carbohydrate ingested with EAA.

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