LEUCINE IN AGE-ASSOCIATED SARCOPENIA

RÔLE DE LA LEUCINE CONTRE LE DÉVELOPPEMENT DE LA SARCOPÉNIE

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SUMMARY

A progressive loss of muscle mass has been well described in both humans and rodents during ageing. This loss of proteins results from an imbalance between protein synthesis and degradation rates. Although some authors have shown a decrease of myofibrillar protein synthesis rates in human volunteers, this imbalance is not clearly apparent when basal rates of protein turnover are measured. A decrease in muscle protein synthesis stimulation has nevertheless been detected in ageing rats during the postprandial period, suggesting that the 'meal signal' is altered during ageing. Many results now suggest that aged muscle is less sensitive to the stimulatory effect of amino acids at physiological concentrations, but is still able to respond if the increase in aminoacidaemia is sufficiently large. Indeed, amino acids play an important role in regulating muscle protein turnover both *in vitro* and *in vivo*. Of amino acids, leucine seems to play the key role in regulating the metabolic function. It inhibits proteolysis and stimulates muscle protein synthesis independently of insulin. Leucine has been shown to act as a mediator, by modulating specifically the activities of intracellular kinases linked to the translation of proteins such as phosphatidylinositol 3_ kinase and mammalian target of rapamycin – 70 kDa ribosomal protein S6 (p70S6K) kinases. We recently demonstrated in vitro that protein synthesis in ageing rat muscles becomes resistant to the stimulatory effect of leucine in its physiological concentration range. Protein synthesis was however stimulated normally when the leucine concentration was increased well above its postprandial level. We also studied the effect of meal leucine supplementation on *in vivo* protein synthesis in adult and ageing rats. Leucine supplementation had no additional effect on muscle protein synthesis in adults but totally restored its stimulation in ageing rats. Whether chronic oral leucine supplementation would be beneficial for maintaining muscle protein mass in elderly humans remains to be studied.

Keys words: ageing, leucine, amino acids, sarcopenia.

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RÉSUMÉ

Une diminution de la masse musculaire au cours du vieillissement est aujourd'hui bien décrite chez l'Homme et l'animal. Cette perte de protéines résulte d'un déséquilibre entre synthèse et dégradation des protéines musculaires. Bien que certains auteurs aient pu montrer une diminution de la synthèse des protéines myofibrillaires chez l'Homme, ce déséquilibre est difficilement apparent dans la plupart des études menées à l'état post-absorptif. Cependant, une altération de la stimulation de la synthèse des protéines a été mise en évidence chez le rat âgé au cours de la phase post-prandiale suggérant que « l'effet repas » normalement observé était altéré au cours du vieillissement. Plusieurs travaux ont montré que le muscle âgé était moins sensible à l'effet anabolique des acides aminés aux concentrations physiologiques mais qu'il était toujours en mesure de répondre si d'importantes hyper-aminoacidémies étaient générées. En effet, les acides aminés jouent un rôle majeur dans la régulation du métabolisme protéique, que ce soit in vivo *ou* in vitro*. Parmi eux, la leucine semble être celui qui présente le plus fort effet. La leucine seule est capable d'inhiber la protéolyse et de stimuler la synthèse protéique indépendamment de l'insuline. Cet acide aminé, en plus d'être un substrat, est également un véritable médiateur cellulaire en modulant spécifiquement les activités de plusieurs kinases impliquées dans la régulation de l'initiation de la synthèse des protéines i.e phosphatidylinositol 3_ kinase and mammalian target of rapamycin–70 kDa ribosomal protein S6 (p70S6K) kinases. Nous avons montré récemment* in vitro *que la synthèse protéique musculaire devenait résistante à l'effet stimulateur de la leucine chez le rat âgé dans l'intervalle de ces concentrations physiologique. Cependant, si les concentrations de leucine étaient largement supérieures aux valeurs post-prandiales, la protéosynthèse était stimulée normalement. Nous avons donc étudié l'effet d'une supplémentation en leucine du régime sur la protéosynthèse du rat adulte et âgé* in vivo*. Cette supplémentation n'a pas eu d'effet additionnel chez l'adulte mais a permis de restaurer totalement la régulation post-prandiale du métabolisme protéique musculaire chez l'âgé. L'effet bénéfique d'une telle supplémentation en nutrition entérale chronique sur le maintien de la masse musculaire au cours du vieillissement reste cependant à étudier.*

Mots-clés : âge, leucine, acide aminé, sarcopénie.

INTRODUCTION

Normal ageing is characterized by a decline in skeletal muscle mass and strength associated with increased muscle fatigability. This phenomenon, named sarcopenia, reduces physical mobility and generates a general weakness in elderly men and women (Evans *et al*. 1995). The weakness of quadriceps muscle predisposes to impaired locomotion, frequent falls and increased risk of hip fractures in the elderly. In addition, there is an increased susceptibility to illness, since skeletal muscles are the major reservoir of body proteins and consequently of amino acids, which could be used for energy production or the synthesis of acute-phase proteins by the liver. Due to the reduced muscle mass, the ability of aged individuals to fight and recover from stress is impaired and this impairment promotes the decrease of muscle mass generated by the stress itself. All these factors taken together, sarcopenia reduces the quality of life of the rapidly expanding older population in Western countries. Elucidating the mechanisms that result in muscle wasting during ageing is therefore clearly of importance. It is estimated that 20–30 billion dollars in health costs in the United States of America are spent on problems directly related to sarcopenia (Schneider & Guralnik 1990). The decrease in lean mass is associated with an increase in the total amount of lipid stores (Cohn *et al*. 1980) ***(figure 1)***. This increase in lipid stores appears during the third decade in men but is delayed in women (Forbes & Reina 1970). The accumulation of visceral and total body fat is thought to be a consequence of the reduced daily energy expenditure and represents a risk factor for the development of type II diabetes during ageing (Holloszy *et al*. 1985, 1991). In rodent models, although the whole-body protein mass is not reduced, wasting is nevertheless noticeable in white muscles, which are mainly constituted of type II (glycolytic) fibre (Holloszy *et al*. 1991). Muscle wasting results from a reduction of fibre areas, a loss of myofibrillar proteins and conversion of type II into type I (oxidative) fibre (Carlson 1992). Alterations in mechanical and biochemical properties of skeletal muscle are very similar in elderly humans and elderly rodents (Taylor *et al*. 1992). Proteins undergo a continuous process of degradation and synthesis. Thus, protein storage in skeletal muscle results directly from the overall balance between the rates of protein synthesis and breakdown. Sarcopenia observed during ageing is thus the consequence of decreased protein synthesis, increased proteolysis or a combination of the two. This review focuses on alterations in muscle protein metabolism during ageing in the postabsorptive state and examines the response of both muscle protein synthesis and proteolysis to amino acids, which are thought to be one of the major regulators of muscle protein metabolism in the postprandial state.

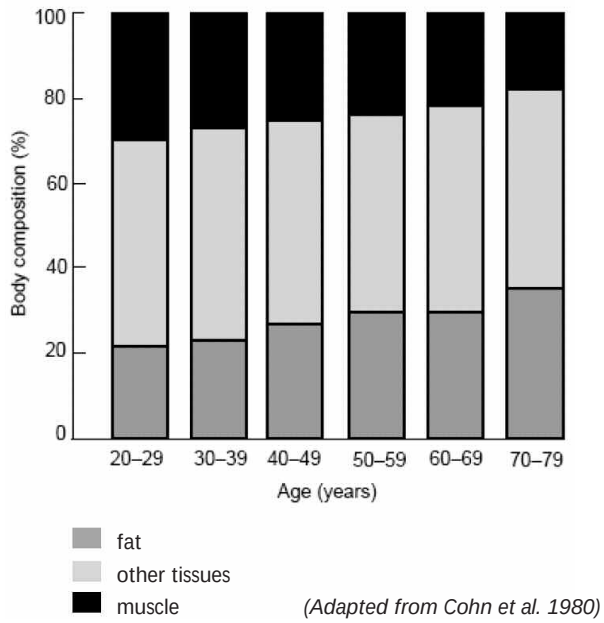

Figure 1: *Body composition in man as a function of age.*

AGEING AND POSTPRANDIAL MUSCLE PROTEIN METABOLISM

During the day, protein metabolism is modified by food intake. Whole-body proteins are stocked during postprandial periods and lost in post-absorptive periods. With a muscle protein mass that remains constant, the loss of muscle proteins is compensated by the same protein gain in the postprandial stage ***(figure 2)***. In adult volunteers, oral feeding is associated with an increase in whole-body protein synthesis and a decrease in proteolysis (Rennie *et al.* 1982; Pacy *et al.* 1994; Boirie *et al.* 1996; Volpi *et al.* 1996; Arnal *et al.* 2000). These changes are mediated by feeding-induced increases in plasma concentrations of both nutrients and hormones. Many studies suggest that amino acids and insulin play major roles in promoting postprandial protein anabolism. Feeding human subjects a protein-free diet (Volpi, 1996) or rodents (Yoshizawa *et al.* 1997a, 1998) does not induce any stimulation of protein synthesis despite a significant rise in plasma insulin, suggesting that amino acids but not insulin are essential in postprandial stimulation of protein synthesis. In accordance with this lack of an effect of insulin, refeeding causes the same effects on protein synthesis in diabetic mice and control animals despite no changes in postprandial plasma insulin concentrations (Svanberg *et al.* 1996, 1997). Likewise, provision of exogenous insulin to freely fed rodents did not increase muscle protein synthesis beyond the effect of refeeding (Garlick *et al.* 1983; Svanberg *et al.* 1996). Furthermore, the effect of feeding on protein metabolism is correlated to the amount of protein intake. Pacy *et al.* (1994) showed increased stimulation of whole-body protein synthesis increased inhibition of protein degradation when the protein content of the diet was increased from 0·36 to 2·77 g/kg per day. Studies have shown specific differences between adult and elderly subjects in the response of protein metabolism during the transition from post-absorptive

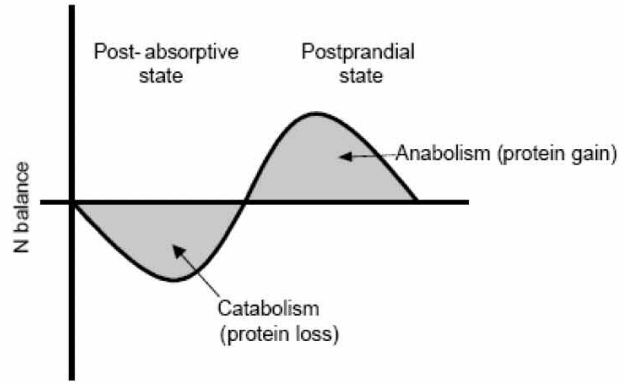

Figure 2: *Theoretical changes in nitrogen balance during the post-absorptive and postprandial states.*

to the fed state. At the whole-body level, proteolysis inhibition in the fed state was lower in ageing men or women than in adults (Boirie *et al.* 1996; Arnal *et al.* 2000). The stimulatory effect of food intake has also been investigated in skeletal muscle protein synthesis. Welle *et al.* (1994) showed no difference in whole-body incorporation of leucine into proteins in the young. However, the fractional myofibrillar protein synthesis in the vastus lateralis muscle was 28% slower in the older group. In rats, Mosoni *et al.* (1995) and Dardevet *et al.* (2002) found that protein synthesis was stimulated in adult rats but not in elderly rats. This loss of protein synthesis response to the anabolic effect of food intake could be involved in muscle protein loss that occurs during ageing, as lost protein during the post-absorptive period will not be completely recovered every day during the postprandial period in the oldest subjects.

Since amino acids play an important role in regulating protein synthesis, it has been hypothesised that during ageing, availability of amino acids could be affected. Boirie *et al.* (1997) have shown in human volunteers that the first-pass splanchnic uptake of leucine increases with age and may limit the availability of amino acids to the peripheral tissues. Volpi *et al.* (1999) confirmed this observation but showed that the delivery of amino acids to the tissues increased to the same extent in both adult and elderly individuals. In rats, no difference in dry matter intake or in essential amino acid concentrations in plasma was recorded (Dardevet *et al.* 2002), thus a defect of amino acid availability cannot be responsible per se for the defect of postprandial anabolism. Other studies have explored the direct effect of amino acids on muscle protein synthesis and have shown that protein synthesis responds normally if amino acids are infused continuously in elderly rats (Mosoni *et al.* 1993). Similarly, Volpi *et al.* (1999) observed that muscle protein synthesis was still stimulated with an increase of amino acid availability in elderly human subjects after oral amino acid administration. It is important to note that the amount of amino acids infused or administered orally in these two experiments led to sustained sizeable hyperaminoacidaemia (concentrations of most essential amino acids were more than doubled), not representative of the plasma amino acid profile observed in normal mixed meal consumption (Elia *et al.* 1989; Bergström *et al.*

1990). Recently, Arnal *et al*. (1999) demonstrated that the response of protein turnover was restored in elderly subjects if a protein-pulse feeding pattern (80% of daily proteins in one meal) was used instead of spread-protein feeding (daily proteins equally distributed). Although the plasma amino acids were not measured in this experiment, it may be assumed that amino acid availability to peripheral tissues was higher with protein-pulse feeding than protein-spread feeding. These results suggest that aged muscle is less sensitive to the stimulatory effect that amino acids have at physiological concentrations but is still able to respond if the increase in aminoacidaemia is sufficiently large.

LEUCINE AND POSTPRANDIAL ANABOLISM

Regulation of muscle protein synthesis

Several studies have indicated that branched-chain amino acids (BCAA) regulate skeletal muscle protein synthesis. *In vitro* as well as *in vivo*, addition or infusion of BCAA at 5 times fasting plasma concentrations enhanced muscle protein synthesis (Fulks *et al*. 1975; Li & Jefferson 1978). Furthermore, Garlick & Grant (1988) showed that infusion of BCAA and glucose stimulates skeletal muscle protein synthesis in post-absorptive rats as efficiently as a complete amino acid mixture and glucose. These data suggest that BCAA are responsible for the anabolic effect of amino acids on muscle protein synthesis. Buse *et al*. (1975) demonstrated *in vitro* in rat hemidiaphragms that leucine stimulated protein synthesis as effectively as a mixture of all three BCAA, suggesting that the effect of amino acids on muscle protein synthesis can be attributable to leucine alone, independently of the other BCAA. Li & Jefferson (1978) confirmed this hypothesis on hindlimb preparations, in which leucine at 10 times fasting plasma concentrations reproduced the effect of all BCAA on muscle protein synthesis. More recently, Anthony *et al*. (2000a) showed that orally administered leucine stimulated muscle protein synthesis by itself *in vivo*, independent of insulin. However, several authors have been unable to detect an effect of leucine alone on muscle protein synthesis in post-absorptive rats or ruminants (McNurlan *et al*. 1982; Funabiki *et al*. 1992; Papet *et al*. 1992). The discrepancy between *in vitro* and *in vivo* studies remains unclear. Despite differences in study design and animal species, recent work at our laboratory (Dardevet *et al*. 2000) may explain these apparent conflicting results. In our study on epitrochlearis muscle, leucine increased protein synthesis *in vitro* at physiological concentrations (100–200 µM) ***(figure 3)***. In addition, maximal stimulation was obtained where leucine concentrations ranged between the post-absorptive and postprandial levels. The stimulation of protein synthesis can be recorded *in vitro*, as muscles are incubated under the normal post-absorptive leucine concentrations (incubation medium without leucine). *In vivo* on the other hand, the presence of plasma leucine – already at the post-absorptive levels and thus close to the maximum effect of leucine on muscle protein synthesis – can prevent a further significant increase of muscle protein synthesis.

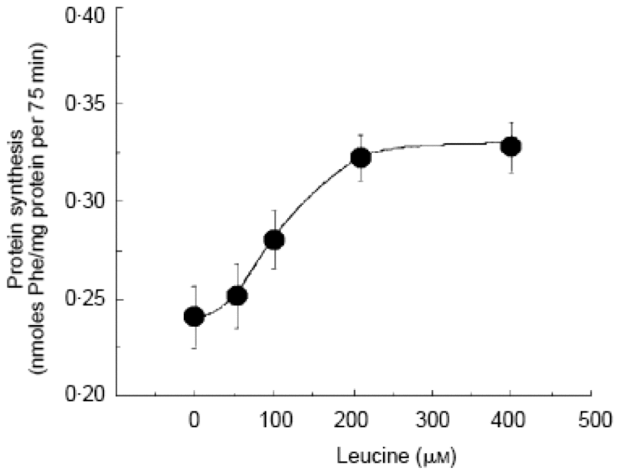

Figure 3 : *Effect of increasing leucine concentration on epitrochlearis muscle protein synthesis* in vitro *in young rats. Phe, phenylalanine.*
Mean values are shown with vertical bars representing standard errors. (Adapted from Dardevet et al. 2000)

Stimulation of muscle protein metabolism by leucine and ageing

We investigated whether a decrease in muscle protein synthesis sensitivity to leucine during ageing could explain the defect in postprandial anabolism (Dardevet *et al*. 2000). Our study clearly showed that muscle protein synthesis still responded to the leucine signal in ageing animals, but the half-maximum effect was observed at amino acid levels 2 to 3 times greater than found in young or adult rats ***(figure 4)***. This indicated that, at postprandial leucine levels, muscle protein synthesis is maximally stimulated in adult rats whereas it was still poorly increased in ageing animals. We measured the plasma amino acid concentrations in ageing and adult rats in post-absorptive and postprandial states and no difference in aminoacidaemia was found. The decreased sensitivity of muscle protein synthesis to leucine in aged rats suggests that the signalling pathway that carries the leucine signal to the protein translation machinery was less responsive to the amino acid than in adult rats. Our study (Dardevet *et al*. 2000) demonstrated that p70S6K activity was stimulated by leucine in both adults and ageing animals but, as recorded with protein synthesis, this activation occurred at higher and supraphysiological levels of leucine (half-maximum effects of 110 vs. 260 µmol/l, respectively). Thus, for full activation, postprandial leucine concentrations are sufficient in adult rats whereas up to 400 µmol/l is needed for ageing animals. Our results showed a direct correlation between sensitivity of muscle protein synthesis and sensitivity of p70S6K activation to leucine. This confirms the fact that the signalling pathway PI3_kinase–mTOR–p70S6K is involved in the stimulation of muscle protein synthesis by leucine. From this *in vitro* data, we hypothesised that the defect in postprandial stimulation of muscle protein synthesis could be overcome by increasing plasma leucine concentration *in vivo*. We studied the effect of acute meal leucine supplementation on protein synthesis in adult and ageing rats in both gastrocnemius and soleus muscles. In these *in vivo* experiments, leucine supplementation had

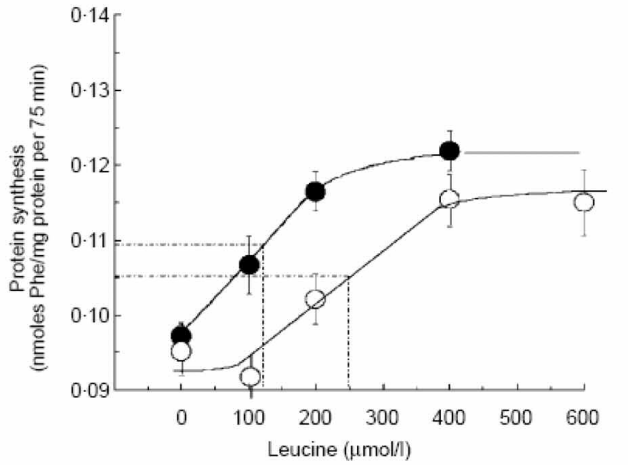

Figure 4 : *Effect of increasing leucine concentrations on epitrochlearis muscle protein synthesis measured* in vitro *in adult and old rats. The half-maximum effect for adult rats (●) was 115 µmol/l and for old rats (○) was 250 mmol/l. Mean values are shown with vertical bars representing standard errors.*
(Adapted from Dardevet et al. *2000)*

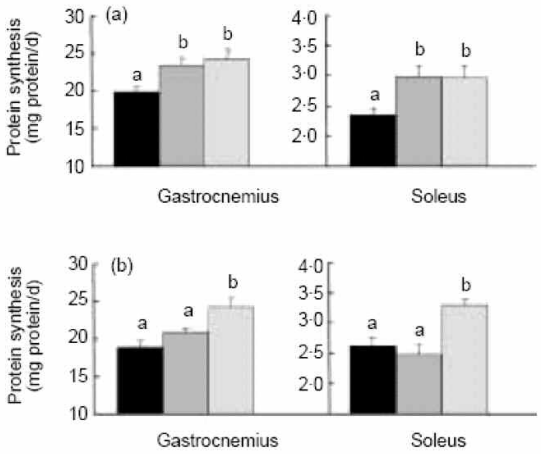

Figure 5 : *Effect of leucine supplementation on muscle protein synthesis in adult (a) and old (b) rats that were food-deprived or refed for 1 h with a control alanine diet or a control diet supplemented with leucine. Values with different letters are significantly different ($P < 0.05$).*
(Adapted from Dardevet et al. *2000)*

no additional effect on muscle protein synthesis in adults but totally restored its stimulation in ageing rats, in both muscles studied ***(figure. 5)***. We also accessed muscle proteolysis in this study. As recorded for protein synthesis, leucine supplementation restored the altered inhibition of muscle proteolysis (Combaret *et al.* 2005). Only leucine concentrations in plasma reached supraphysiological levels in both age groups (twice the control postprandial values) and confirmed *in vivo* our hypothesis that ageing rat muscles are less sensitive to the leucine signal but are still able to respond when the concentration of this amino acid is sufficiently increased. Leucine has been shown to stimulate insulin secretion, and the restoration of muscle protein synthesis in ageing rats could originate indirectly through an increase in plasma insulinaemia. This cannot explain the results in our experiment (Dardevet *et al.* 2000) since the kinetics of insulinaemia were not significantly different in the rats fed the control and the leucine-supplemented meals. Furthermore, insulin levels were not different from those of the adult groups in which stimulation of muscle protein synthesis was nevertheless recorded. However, it is important to emphasise that the presence of insulin appears to be indispensable in the postprandial stimulation of muscle protein synthesis by amino acids. Indeed, the acute decrease in postprandial insulinaemia to postabsorptive levels due to either anti-insulin serum (Millwards *et al.* 1983) or diazoxide treatment (Sinaud *et al.* 1999; Balage *et al.* 2001) greatly impaired muscle protein synthesis. Our study (Mosoni *et al.* 1993) explains why amino acid infusion, which induced a three-fold increase in plasma leucine, stimulated muscle protein synthesis to the same extent in adult and ageing rats. A similar conclusion may be deduced from the study of Volpi *et al.* (1999), who observed a similar effect of oral amino acid administration on muscle protein synthesis in adult and elderly volunteers: leucine concentrations were 2·5 to 3·5 higher than with a control meal. Recently, Arnal *et al.* (1999) demonstrated that the response of protein turnover was restored in elderly subjects if a pulse-protein feeding pattern (80% of daily protein intake in one meal) was used instead of spread-protein feeding (daily proteins equally distributed in four meals). Even if the plasma amino acids were not measured in this experiment, it could be easily assumed that amino acid availability after the high-protein meal to peripheral tissues (i.e. leucine) was higher with pulse-protein feeding than with spread-protein feeding. An increase of dietary protein intake was thus beneficial for the maintenance of muscle protein synthesis in an elderly population. However, it required 80% rather than 30% of proteins in the meal and it has been shown that high-protein diets may have deleterious effects on renal function in the elderly (Rowe 1980). In our experiments (Dardevet *et al.* 2002, Rieu *et al.* 2003), leucine alone was able to restore muscle protein synthesis, so supplementation of this amino acid represents a good alternative to high-protein diets. We recently showed that leucine supplementation was also efficient in human volunteers and this without any increase of the other amino acids during the postprandial phase (Rieu *et al.* 2006).

Leucine: an active bio-substrate

Since leucine is able to reproduce the effect of all amino acids on muscle protein synthesis, it has been hypothesised that this effect is not dependent on the amino acid concentration itself but on a specific signal initiated by leucine. It has been shown that the stimulation of skeletal muscle protein synthesis caused by feeding a complete diet is mediated by an increase in the initiation of mRNA translation (Millward *et al.* 1983; Kelly & Jefferson 1985; Preedy & Garlick 1986; Yoshizawa *et al.* 1995). Two major mechanisms have been described that contribute to the regulation of the assembly of these complexes, each of which involves reversible phosphorylation of proteins implicated in the process.

On the one hand, leucine has been shown to increase the phosphorylation of 70 kDa ribosomal protein S6 kinase (S6K1) and consequently its activation (Anthony *et al.* 2000b; Dardevet *et al.* 2000). S6K1 activation has been shown to regulate the trans-

lation of specific mRNA by modulating phosphorylation of ribosomal protein S6 ***(figure 6)***.

Previous studies *in vitro* on the other hand have reported that increased phosphorylation of eIF4E is observed in cultures of cells in response to a variety of stimuli (e.g. growth factors, hormones) and is positively correlated with changes in protein synthesis (Rhoads *et al*. 1993). *In vivo* studies do not support these results however. Indeed, the phosphorylation state of eIF4E did not change in diabetic or insulin treated diabetic rats (Kimball et al, 1996); neither did it change in skeletal muscle of overnight fasted or refed animals (Yoshizawa *et al*. 1997a). Another mechanism through which translation initiation can be regulated involves phosphorylation of the eIF4E binding protein 4EBP1. Indeed, eIF4E bound to 4E-BP1 can bind to the m7 GTP cap structure, but cannot bind to eIF4G, the active complex to stimulate translation initiation (for a review, see Anthony et al, 2001). Thus, 4E-BP1 competes with eIF4G for association to eIF4E. The ability of 4E-BP1 to bind to eIF4E is largely dependent on the phosphorylation state of 4E-BP1. Phosphorylation of 4E-BP1 releases eIF4E from the 4E-BP1–eIF4E complex, which in turn is available to bind to eIF4G ***(figure 6)***. The effect of leucine on these intracellular processes has recently been studied. Anthony *et al.* (2000a) showed that orally administered leucine alone stimulated muscle protein synthesis and that this stimulation is correlated with a hyperphosphorylation of 4E-BP1. The formation of the complex 4E-BP1–eIF4E is thus inhibited and the complex eIF4E–eIF4G is increased. One of the most tightly regulated steps in translation initiation is the binding of mRNA to the 40S subunit (Pain 1996; Rhoads 1999; Shah *et al*. 2000). This step involves the binding of eIF4E to the m7 GTP cap at the 5_ end of the mRNA and the subsequent binding of the eIF4E–mRNA complex to eIF4G, which is a critical step in the formation of the 48S pre-initiation complex (43S+60S).

The activation of p70S6K and 4E-BP1 by amino acid or leucine involves an upstream signalling pathway including the mammalian target of rapamycin (mTOR) (Patti *et al*. 1998; Kimball *et al*. 1999). *In vitro*, there is stimulation of rapamycin, a specific inhibitor of mTOR (Dardevet *et al*. 2000). *In vivo*, when intravenously injected rapamycin completely prevents leucine-dependent stimulation of muscle protein synthesis in association with a lack of hyperphosphorylation of p70S6K and 4E-BP1. Taken together, these results suggest that leucine stimulates mTOR activity in skeletal muscle for initiating protein synthesis and can be considered to be a genuine active bio-substrate. In addition to modulating activities of specific intracellular kinases and factors involved directly in the initiation of protein translation, leucine has also been shown to modulate the transcription of selected genes.

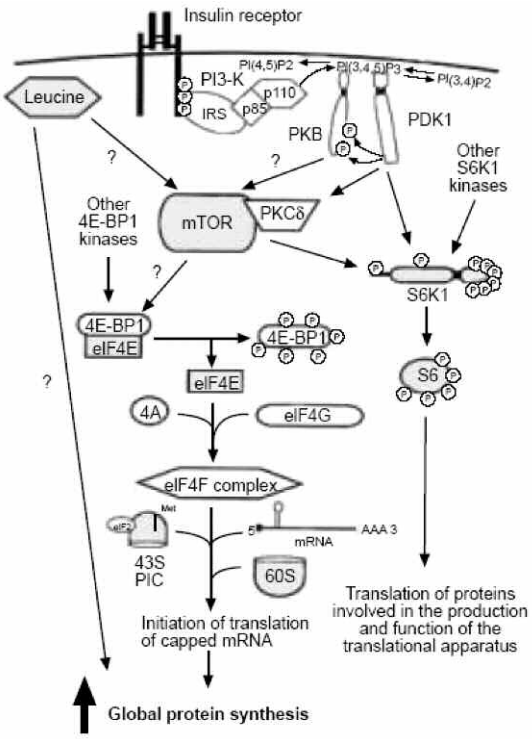

Figure 6 : *Possible mechanisms of action of leucine on muscle protein synthesis. IRS, insulin receptor substrate; PI3-K, phosphatidylinositol 3' kinase; PKB, protein kinase B; PDK, PKC, protein kinase C; S6K1, 70 kDa ribosomal protein S6 kinase; S6, ribosomal protein S6; elF 4E, eukariotic initiation factor 4E; 4E-BP1, initiation factor 4E binding protein.*
(Adapted from Anthony et al. 2001)

CONCLUSION

In conclusion, from the available data it appears that ageing skeletal muscle progressively loses its ability to respond to anabolic stimuli, particularly to dietary amino acids within the physiological range of concentrations. However, older muscle can still present an anabolic response to protein/amino acids administration if administered in large amount. For this reason, it is possible to devise nutritional strategies to maintain or slow down muscle mass loss in the elderly. Of the amino acids, leucine seems to have the greatest positive effect and thus an increase in this amino acid intake alone may represent a good and safe nutritional intervention. However, most of the data is obtained from animal models and acute experiments; so long term clinical trials are still necessary to assess the true beneficial effect of leucine supplementation in humans with respect to the reduction of muscle loss caused by ageing.

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