NUTRIENT REGULATION OF SKELETAL MUSCLE PROTEIN METABOLISM IN ANIMALS. THE INVOLVEMENT OF HORMONES AND SUBSTRATES

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INTRODUCTION

It has been extensively demonstrated that nutrient intake plays an important role in the control of protein turnover, especially in skeletal muscle (see Reeds & Fuller, 1983; Arnal *et al.* 1987; McNurlan & Garlick, 1989; Millward & Rivers, 1989; Young *et al.* 1992; Lobley 1990, 1992, 1993 for reviews). The present review attempts to provide insights at the tissue level with respect to both protein synthesis (now more often assessed using the flooding dose method (Garlick *et al.* 1980; Attaix *et al.* 1986)) and the major proteolytic pathways (Attaix *et al.* 1994). Our particular aim is to understand the control mechanisms including hormones and substrates.

MOLECULAR MECHANISMS OF PROTEIN SYNTHESIS AND DEGRADATION

As summarized by Kimball *et al.* (1994) synthesis of new protein in eukaryotic cells is achieved *via* a complex series of discrete reactions that occur in the nucleus, cytosol and various subcellular locations. In the nucleus, transcription of specific genes by three classes of RNA polymerases results in the production of mRNA, tRNA, 5S RNA and 45S preribosomal RNA. The RNA products are processed and, in the case of mRNA and tRNA, are transported to the cytoplasm. The 45S preribosomal RNA is processed to yield the mature 18S, 5·8S and 28S RNA constituents of the ribosome. These three products along with the 5S RNA and $\sim$ 85 ribosomal proteins move to the nucleolus where they assemble into the 40S and 60S preribosomal particles which are then transported to the cytoplasm. In the cytoplasm, the ribosomal particles bind to mRNA to form polysomes, which can exist free or bound to the endoplasmic reticulum, cytoskeleton, and perhaps other subcellular structures. Protein synthesis in the cytoplasm begins with the aminoacylation of tRNA and ends with the release of a completed peptide chain from a polysome. Translation of mRNA into protein by ribosomes is usually divided into three phases: (*a*) initiation, in which the initiator methionyl-tRNA is bound to mRNA, which in turn binds first to a 40S ribosomal subunit and subsequently to a 60S subunit, thus forming a translationally competent ribosome; (*b*) elongation, during which tRNA bound amino acids are incorporated into a growing peptide chain in the order specified by the mRNA to which the ribosome is bound; and (*c*) termination, the phase when the completed peptide chain is released from the ribosome. Each of these steps requires the intervention of protein factors known collectively as eukaryotic initiation factors (eIF), elongation factors (eEF) and releasing factor (Redpath & Proud, 1994).

Like any other mammalian tissue, skeletal muscle contains multiple proteolytic systems that are presumably involved in the breakdown of specific proteins. Three major proteolytic pathways appear to be responsible for the breakdown of muscle proteins, although such a simple division is a gross oversimplification. Unfortunately, the two most studied proteolytic systems in muscle (i.e. the lysosomal and the Ca^{2+} dependent pathways) play little if any role in the breakdown of the major contractile proteins (Tawa & Goldberg, 1993; see Attaix *et al.* 1994 for a review). The third major process is ATP–ubiquitin dependent and was only recently discovered in muscle. Ubiquitin (a 76 amino acid protein highly conserved in all eukaryotes) is first activated in an ATP dependent fashion and then covalently binds to protein substrates (see Hershko & Ciechanover, 1992 for a recent review) (Fig. 1). Ubiquitination of proteins always requires ATP, the ubiquitin activating enzyme, E1, and one of the ubiquitin carrier proteins, E2, to generate the first covalent attachment of a single ubiquitin moiety to a substrate. Some conjugation reactions also

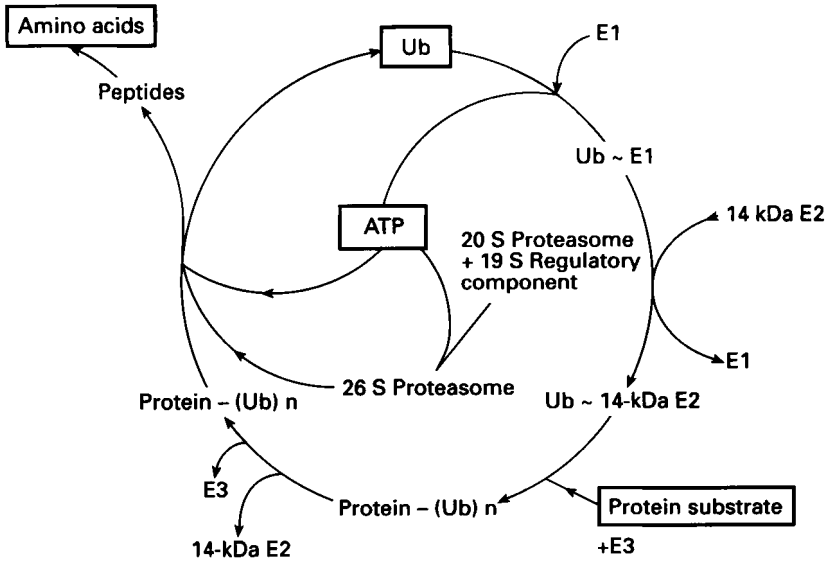

Fig. 1. ATP–ubiquitin dependent protein breakdown in skeletal muscle. Three enzymes, E1, E2 and E3 are involved in the formation of ubiquitin-protein conjugates (see the text). To date a single E2 (14-kDa E2) has been reported to be involved in muscle protein ubiquitination. ATP is required at three steps: (1) activation of ubiquitin (Ub) by E1; (2) formation of the 26 S proteasome, and (3) hydrolysis of Ub-protein conjugates. The formation of Ub-protein conjugates and their hydrolysis by the 26 S proteasome are two potential rate limiting steps in the pathway.

require a third enzyme, known as ubiquitin protein ligase, E3, for substrate recognition. Multiubiquitination of the substrate occurs by the attachment of ubiquitin chains or trees in all E3 dependent reactions. Multiubiquitinated proteins are preferentially degraded by a very large 1500 kDa (26S) proteolytic complex that requires ATP for activation and substrate hydrolysis. The 26S complex contains the 20S proteasome which has a proteolytic function, plus a 19S regulatory component. The 20S proteasome is the major neutral proteolytic system identified so far in eukaryotic cells (up to 1% of the soluble proteins), and exists in both the cytoplasm and the nucleus. The rat liver proteasome contains 14 subunits with molecular masses of 21 to 31 kDa. Proteasomes contain at least five types of endopeptidase activity (Rivett, 1993). The ATP–ubiquitin dependent proteolytic pathway is widely believed to catalyse the selective breakdown of short-lived and abnormal proteins in mammalian tissues. In skeletal muscle, it is currently presumed that this pathway is responsible for the breakdown of the long-lived myofibrillar proteins. For example, when ATP in muscle is depleted by incubation with dinitrophenol and 2-deoxyglucose to prevent oxidative phosphorylation and glycolysis, the breakdown of cell proteins, including myofibrillar components, markedly falls.

EFFECT OF HORMONES

INSULIN AND INSULIN-LIKE GROWTH FACTOR 1

It has been widely demonstrated, using continuous infusion of labelled leucine or phenylalanine, that insulin administration reduces whole body proteolysis when estimated by the endogenous amino acid appearance rate (Gelfand & Barrett, 1987; Tesseraud *et al.* 1993). This effect is very sensitive, and the strongest reduction occurs with low doses of

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Table 1. *Effect of insulin infusion and amino acid replacement on fractional rate of protein synthesis (%/d) in skeletal muscle*

Animals	Flooding amino acid	Control	Insulin
(1) Rat, 80 g BW	Phenylalanine	9·9 ± 0·6	14·4 ± 0·4*
(2) Rat, 200 g BW	Phenylalanine	6·7 ± 0·8	8·0 ± 0·5
(3) Rat, adult	Valine	7·0 ± 0·2	6·9 ± 0·2
(4) Goats, adult	Valine	4·0 ± 0·6	4·3 ± 0·4

(1) Garlick & Grant, 1988; (2) McNulty *et al.* 1993; (3) Mosoni *et al.* 1993*a*; (4) Tauveron *et al.* 1994. Insulin infusion was performed along with amino acid infusion in order to blunt the insulin-induced hypoaminoacidaemia. Plasma glucose was maintained to its basal level except in (1). Protein synthesis was always measured by the flooding dose procedure using either phenylalanine or valine. * $P < 0·05$ *v.* control.

BW, body weight.

insulin. Skeletal muscle has been recognized as a target for the antiproteolytic action of insulin. Indeed, insulin reduced 3-methylhistidine urinary excretion (a simple index of myofibrillar protein breakdown) and decreased amino acid release from the forelimb in man (Gelfand & Barrett, 1987). Supportive evidence for muscle specific actions of insulin comes from studies where infusion of the hormone reduced ubiquitin expression in skeletal muscles *in vivo* (Larbaud *et al.* 1995). Similarly, insulin also reduced the mRNA levels for the 14 kDa E2 in cultured myotubes (Wing & Banville, 1994). In contrast, Tessari *et al.* (1991) demonstrated that hyperinsulinaemia acutely decreases endogenous amino acid appearance by acting primarily at sites other than skeletal muscle; for example, in liver insulin is able to inhibit protein degradation through alterations of macroautophagy and activities of cathepsins (see Mortimore *et al.* 1989 for a review).

A stimulating effect of insulin on skeletal muscle protein synthesis has been observed in rats *in vivo*, particularly in young animals after a short fasting period (Table 1). The response was less pronounced in adult fasted rats (Garlick & Grant, 1988; Baillie & Garlick, 1992) and in this context it is noteworthy that rats become insulin resistant with age with regard to both protein synthesis and glucose metabolism (Dardevet *et al.* 1994). For example, insulin infusion under euglycaemia, eukalaemia, and amino acid replacement (to eliminate the confounding effects of insulin induced hypoglycaemia, hypokalaemia and hypoaminoacidaemia) failed to stimulate protein synthesis in skeletal muscle from both adult postabsorptive rats (Mosoni *et al.* 1993*a*) or fed goats (Tauveron *et al.* 1994). Dose response curves obtained *in vitro* in rodents (Dardevet *et al.* 1994) suggest that skeletal muscle protein synthesis is very sensitive to insulin. On the basis of the relationship between plasma insulin and protein synthesis observed *in vivo* in skeletal muscle of rats that have been refed after several days of fasting, protein synthesis appeared to be sensitive only to low insulin concentrations ($< 0·1$ nM) with no additional effect at high insulin concentration (Millward *et al.* 1988).

Insulin-like growth factor 1 (IGF-1) exerts an anabolic effect in skeletal muscle through mitogenic action and stimulation of cell differentiation (for review see Froesch *et al.* 1985). IGF-1 is also able to elicit metabolic effects in skeletal muscle. Similar to insulin, it promotes a net positive protein balance by activation of protein synthesis and/or suppression of protein degradation. These properties have been used to reverse muscle protein wasting (Bondey *et al.* 1994). We compared the effects of insulin and IGF-1 on both glucose and protein metabolism in isolated epitrochlearis muscle (Dardevet *et al.* 1994). Previous authors observed quantitatively similar effects of insulin on glucose uptake, glycolysis, and glycogen synthesis in skeletal muscle using isolated rat soleus (Dimitriadis

et al. 1992) or human muscle fibre strips. Based on dose response curves in our study, IGF-1 and insulin were also equipotent for both amino acid (system A) and glucose transport into young rat epitrochlearis muscle. IGF-1 is thus a key hormone in the regulation of muscle amino acid and glucose supply. In addition, our results showed that IGF-1 and insulin both stimulated protein synthesis in epitrochlearis muscle from young animals. However, IGF-1 was more potent than insulin since the maximum response was greater with IGF-1 than with insulin. Our results are in agreement with previous studies on isolated rat soleus muscle and ovine primary muscle cultures. *In vivo* studies also revealed that IGF-1 is a potent activator of whole body and muscle protein synthesis, and an inhibitor of proteolysis (Douglas *et al.* 1991).

Actions of insulin on nuclear events in the pathway of protein synthesis have now been clearly demonstrated, e.g. gene transcription, ribosome biogenesis plus RNA processing and transport (see Kimball *et al.* 1994 for a review). In addition, the hormone appears to regulate both the initiation and elongation phases of translation probably by modulating the state of phosphorylation of initiation (eIF-2, eIF-2β, eIF-3 and eIF-4) and elongation factors and ribosomal protein S6. The key kinases involved in this regulation have not been identified yet. Wortmannin, a specific inhibitor of PI3-kinase, decreased insulin and IGF-1 stimulated protein synthesis in epitrochlearis muscle by 59·2 and 72·7% respectively (D. Dardevet, unpublished). Wortmannin also revealed a rapid antiproteolytic action of IGF-1 *in vitro*. A similar trend was observed in insulin-treated muscles. Dardevet thus clearly demonstrated that PI3-kinase plays a critical role in the regulation of muscle protein turnover by insulin and IGF-1. Since wortmannin only partly inhibited the effect of these hormones on protein synthesis, this suggests that a part of their stimulating action was mediated by a signalling pathway independent of PI3-kinase. Surprisingly insulin and IGF-1 did not stimulate MAP-kinase activity at all incubation times tested (2–25 min) suggesting that this protein kinase was also not directly involved in the hormonal control of muscle protein metabolism.

GROWTH HORMONE

The numerous studies have been extensively reviewed (Bauman *et al.* 1982; Hart & Johnsson, 1986; MacRae & Lobley, 1991; Lobley, 1993) and it is now accepted that growth hormone (GH) is able to influence a wide range of biochemical processes. In summary, either directly or indirectly, GH stimulates anabolic processes such as cell division, skeletal growth and protein synthesis (growth promoting activity) while increasing the oxidation of fat (lipolytic activity) and inhibiting the transport of glucose into body tissues (diabetogenic activity). For example growth rate of hypophysectomized animals was restored to normal by daily injection of GH. Chronic treatment of intact farm animals with the hormone usually increases the ratio of lean meat to body fat and the efficiency of food conversion, but any effect on total body weight gain is often offset by a substantial reduction in adipose tissue. Protein accretion in GH treated animals depends on amino acid availability. For example, a poor response to GH was observed in pigs given a high energy/low protein diet whereas a major response occurred in animals fed a restricted energy high protein diet (Machlin, 1972). This also seems to be the case in lamb but to a lesser extent (MacRae & Lobley, 1991). The effect of GH was mainly due to increased muscle protein synthesis following an increased protein synthetic capacity (increased muscle RNA/protein content) without any change in protein degradation (Pell & Bates, 1987). The acute effect of GH on forearm amino acid balances and kinetics in healthy man is also consistent with this idea (Fryburg *et al.* 1991).

GLUCOCORTICOIDS

Glucocorticoid treated rats often show impaired muscle protein synthesis both *in vivo* and *in vitro* (see Kettelhut *et al.* 1988 for a review). Proteolysis does not change in fed animals or only transiently increases when large doses are administered to animals (Odedra *et al.* 1983). In contrast, glucocorticoids clearly stimulate skeletal muscle proteolysis in food deprived rats, coupled with an activation of the ATP–ubiquitin dependent proteolytic pathway (Kettelhut *et al.* 1988; Wing & Goldberg, 1993). Myofibrillar proteins represent a target for glucocorticoids with respect to their effects on both protein synthesis and degradation (Odedra *et al.* 1983).

THYROID HORMONES

Hyperthyroidism is associated with several metabolic changes including enhanced gluconeogenesis, glycogenolysis, lipid mobilization and negative nitrogen balance with muscle wasting (see Tauveron *et al.* 1992 for a review). It increased both whole body protein breakdown and synthesis in man (Tauveron *et al.* 1995). In agreement with increased proteolysis, urinary 3-methylhistidine excretion and limb efflux of amino acids were elevated (Morrison *et al.* 1988).

In fact, thyroid hormones have pleiotropic effects on cellular processes, through their actions on key target genes in different tissues. Target genes which are induced by thyroid hormones include malic enzyme and sex hormone binding globulin in the liver, myosin heavy chain and Na^{+}–Ca^{2+} ATPase in myocardium and myelin basic protein in brain. The effects of thyroid hormones on the transcription of these genes are now known to be mediated by a nuclear receptor protein which is a member of the steroid receptor superfamily. The central domain of the receptor is highly conserved amongst all members of this superfamily. It is thought to coordinate zinc to form a 'finger' that mediates binding to a specific regulatory DNA sequence or thyroid response element usually located in the promoter regions of target genes. Three types of thyroid response element have been identified. Thyroid hormones thus act as transcription factors; they may also affect mRNA maturation and degradation (see Chatterjee 1994 for a review on the mechanism of action of thyroid hormones).

Thyroid hormones increase protein synthesis in skeletal and heart muscle (Brown & Millward, 1983; Carter *et al.* 1984; Angerås & Hasselgren, 1987). The expression of specific genes encoding muscle proteins essential for muscle contraction and glucose uptake (GLUT 4), enzymes of glycolysis, pentose phosphate shunt and energy metabolism are affected. High doses of thyroid hormones increase muscle protein breakdown. The enhancement in the activity of cathepsins B (EC 3.4.22.1), D (EC 3.4.23.5) and L (EC 3.4.22.15) as well as that of leucine aminopeptidase (EC 3.4.11.1) suggests that thyroid hormones stimulate the lysosomal pathway. Whether the ATP–ubiquitin dependent system is affected by thyroid hormones in muscle remains to be investigated (Dümmler *et al.* 1994).

OTHER HORMONES

Glucagon usually stimulates liver proteolysis and amino acid oxidation. High dosage of glucagon has also been shown to decrease skeletal muscle protein synthesis both *in vitro* and *in vivo* (Preedy & Garlick, 1985). However, the physiological significance of this latter finding remains unclear since no significant specific ^{125}I-glucagon binding in muscle has been detected (M. Balage, unpublished).

Adrenalin is a potent antagonist of insulin mediated glucose metabolism *in vivo*. Its action is characterized by an increase in hepatic glucose production, due to stimulation of

both glycogenolysis and gluconeogenesis and inhibition of insulin mediated glucose uptake by peripheral tissues. Adrenalin and insulin also have opposite effects on lipolysis and ketogenesis but not on protein turnover. Indeed, short term adrenalin infusion, especially when hypoaminoacidaemia is prevented, exerts an anabolic action by decreasing whole body proteolysis (Castellino *et al.* 1990). Conflicting results have been observed with respect to the effect of adrenalin on skeletal muscle turnover (see Florini, 1987 for a review). However, β receptors, which are involved in cellular catecholamine signalling, have been found in skeletal muscle. This is in keeping with muscle anabolism since β agonists increase muscle protein deposition in various species (Reeds *et al.* 1986; MacRae & Lobley, 1991). The effect of β agonists appears to be mediated through increased synthesis (MacRae & Lobley, 1991) or reduced degradation rates (reductions in the Ca^{2+} dependent proteinases) (Parr *et al.* 1992).

ENDOCRINE INTERACTIONS

There is some evidence that insulin and IGF-1 interact. Both IGF-1 and insulin receptors are present in skeletal muscle (Dardevet *et al.* 1991, 1994). Hybrid receptors containing insulin and IGF-1 receptor subunits are also known to exist; however, their physiological importance in muscle has not been established. Insulin and IGF-1 receptors exhibit great homology in both structure and function (peptide specificity, tyrosine kinase activity, receptor autophosphorylation, and down-regulation). IGF-1 and insulin could elicit similar biological responses either by cross-reaction with the appropriate heterologous receptor or by a concurrent activation of convergent effector pathways emanating from homologous receptor activation. The receptor binding studies from Poggi *et al.* (1979) on isolated muscle are in favour of homologous receptor activation. We performed experiments which clearly demonstrated that isolated rat epitrochlearis muscle was sensitive to insulin and IGF-1 within the nanomolar range for all metabolic processes studied. Binding studies in gastrocnemius muscle showed that receptors had high affinity for their homologous hormone (1 nM and 0·5 nM for insulin and IGF-1 receptor respectively) and low affinity for the heterologous hormone (300–1000 nM and 40–60 nM for insulin and IGF-1 receptor respectively). Cross-reactivity is therefore unlikely to occur at the concentrations of ligands used in our studies, and the respective effects of insulin and IGF-1 seen were probably mediated through their own receptors. Our observation that, even at 100 nM, IGF-1 did not stimulate muscle protein synthesis or AIB uptake in adult muscle suggests that IGF-1 does not act through the insulin receptor.

It has been shown that the antiproteolytic effect of insulin in human forearm (mainly representative of skeletal muscles) was impaired by excess of glucocorticoids (Louard *et al.* 1994) or GH (Fryburg *et al.* 1992). In contrast, we recently demonstrated that this effect of insulin was improved during experimental hyperthyroidism in man. A lower insulinaemia induces a similar or enhanced reduction of whole body proteolysis (Tauveron *et al.* 1995). Thyroid hormone treatment resulted in the disappearance of plasma thyroid stimulating hormone which may in turn act on the insulin receptor tyrosine kinase because evidence is available that thyroid stimulating hormone activates cellular serine/threonine phosphatase(s) which may regulate the phosphorylation state and activity of insulin and IGF-1 receptors (Condorelli *et al.* 1992). Another explanation involves the modification of insulin action by thyroid hormones through a potentiation of the effect of catecholamines; adrenalin has been shown to control the degree of phosphorylation of the insulin receptor in muscle (Webster *et al.* 1986).

The GH–IGF-1 axis further provides a characteristic example of endocrine interactions. GH treatment usually results in elevated plasma IGF-1, mediated by a direct effect on IGF-

1 synthesis, and also on the synthesis of the components of the large 150 kDa ternary complex of IGF-1 and IGF binding protein 3. The latter is the principal carrier form of IGF (the remainder of the IGF in the circulation is bound to IGF binding protein 1, 2, or 4, each of which circulates as a complex of 30–40 kDa). The effects of GH on muscle are thought to be mediated in part *via* IGF-1. Several other hormones have also been implicated in the regulation of IGF-1 secretion and transport. For example, insulin is needed for a normal IGF-1 production but has a negative effect on the transcription of IGF binding protein 1.

Insulin has been shown to inhibit glucocorticoid stimulated proteolysis in skeletal muscle. It has also a dominant inhibitory effect over glucocorticoid stimulated phosphoenolpyruvate carboxykinase (EC 4.1.1.32) gene transcription in liver and the molecular mechanism of this interaction has now been elucidated (O'Brien & Granner, 1991).

EFFECT OF SUBSTRATES

It has been shown that the splanchnic bed is the major site for disposal of the amino acids ingested in a protein meal or administered intravenously, especially alanine and other glucogenic amino acids (Gelfand *et al.* 1986). Comparable studies in rats suggest that amino acids taken up by the liver are catabolized and replace the deficit in amino acids coming from proteolysis; indeed, liver proteolysis is inhibited (Mortimore *et al.* 1987). Tauveron *et al.* (1994) demonstrated in fed goats that dietary amino acid deposition is also the result of stimulation of liver protein synthesis. The 30-min labelling interval used in this study provides estimates of total protein synthesis, because newly synthesized secretory proteins were retained in the liver. The effect of amino acids was demonstrated under conditions in which the specific activity of the amino acid precursor pool was rigidly maintained by expansion of the amino acid pool (flooding dose of [^{3}H]valine) to minimize dilution of specific activity by amino acids released during proteolysis.

Hyperaminoacidaemia failed to stimulate skeletal muscle protein synthesis in either postabsorptive rats (Garlick & Grant, 1988; McNulty *et al.* 1993) or fed goats (Tauveron *et al.* 1994). A small effect was obtained in rats, but only by using very high or long term unphysiological hyperaminoacidaemia (Mosoni *et al.* 1993*a*). All these studies were performed using the flooding dose method (Garlick *et al.* 1980). In contrast, an increase in muscle protein synthesis by hyperaminoacidaemia was more often noted when measured by constant infusion of labelled amino acids, in man (Bennet *et al.* 1989); in pigs (Watt *et al.* 1992); or in goats (Tesseraud *et al.* 1993). These conflicting results may therefore arise from different methods of measuring protein synthesis. The crucial question concerning the accuracy of the methods based on the incorporation of labelled amino acids administered either as a continuous infusion or as a flooding dose remains unresolved (Lobley *et al.* 1992; Garlick *et al.* 1994; Rennie *et al.* 1994*a*). The flooding dose procedure yields higher synthesis rates in muscle than constant infusion, especially when plasma free amino acid is assigned to the precursor pool (Table 2). Values based on the homogenate as precursor gave comparable results. Indeed, the specific activity of the labelled amino acid is lower in muscle than plasma during constant infusion. Hyperaminoacidaemia should equalize these specific activities. The stimulation of protein synthesis by amino acids recorded during constant infusion was thus probably due to the fact that plasma labelled amino acid specific activity better reflects tracer specific activity in muscle tissue pools when plasma amino acid concentrations are higher. In other words, the effect of hyperaminoacidaemia would be to raise the rate of protein synthesis to that obtained with the flooding dose injection. One possible explanation for the absence of a consistent effect of amino acids on muscle protein

Table 2. *Comparison of estimates of fractional rates of protein synthesis (%/d) calculated from plasma or tissue pools sampled during continuous infusion of leucine or phenylalanine with values obtained from a large dose*

Precursor pool	Continuous infusion		Flooding dose
	Plasma	Tissue	Tissue
(1) Rat, 100 g BW, leucine			
Rectus abdominalis		4·7†	6·9
(2) Rat, 250 g BW, phenylalanine			
Rectus abdominalis	2·8†		6·7
Quadriceps	2·4†		6·2
(3) Lambs, 40 kg BW, phenylalanine			
Mixed muscles			
Low intake	0·8†	1·7	2·0
High intake	1·4†	2·7	2·8

(1) Pomposelli *et al.* 1985; (2) McNulty *et al.* 1993; (3) Lobley *et al.* 1992.
† $P < 0{\cdot}05$ *v.* value for flooding dose.
BW, body weight.

synthesis when using the large dose procedure is that the amino acid bolus itself might artificially elevate tissue protein synthesis (directly or through changes in endocrine or substrate status) in such a way that it is difficult to observe anabolic effects. This phenomenon, which is suggested from the changes in the incorporation of an infused amino acid when a flood is given (see discussion in Lobley *et al.* 1992; Garlick *et al.* 1994; Rennie *et al.* 1994*a*) has not been properly demonstrated so far.

Branched chain amino acids, especially leucine (or the corresponding 2-oxoisocaproic acid), have been shown to account for the beneficial effect of complete amino acid mixtures added to muscle incubated *in vitro* (Buse & Reid, 1975). We therefore determined the effect of a 4-h leucine infusion, leading to 15-fold elevated plasma leucine concentrations, on skeletal muscle and whole body protein synthesis in sucking lambs during the postprandial period (Table 3). The [^{3}H]phenylalanine flooding dose method was used to quantify the fractional rates of protein synthesis at the end of the leucine infusion. This infusion lowered other plasma amino acid concentrations but did not change protein synthesis in any muscles studied or in the whole body. In another experiment the leucine induced decreases in concentrations of other plasma amino acids were compensated by the simultaneous infusion of substantial amounts of those amino acids. Again, leucine excess did not significantly change protein synthesis (Papet *et al.* 1992). These results are consistent with those obtained using infusion of complete amino acid mixtures.

Leucine catabolites have also been shown to be involved in the regulation of protein turnover in skeletal muscle. Leucine is converted to 2-oxoisocaproate by the branched chain amino acid aminotransferase (EC 2.6.1.42). 2-oxoisocaproate can be decarboxylated to isovaleryl-CoA by the branched chain keto acid dehydrogenase (EC 1.2.4.4) present in the mitochondria. Alternatively, 2-oxoisocaproate can be oxidized by the cytosolic 2-oxoisocaproate oxygenase to produce 3-hydroxy-3-methylbutyrate, with approximately 5% of leucine oxidation occurring *via* the 3-hydroxy-3-methylbutyrate pathway in sheep and pigs (Van Koevering & Nissen, 1992). 2-oxoisocaproate occasionally stimulated protein anabolism both *in vitro* and *in vivo* (Flakoll *et al.* 1991). We examined the effect of 3-hydroxy-3-methylbutyrate in growing sheep but failed to demonstrate any effect of either acute infusion or chronic treatment on *in vivo* muscle protein synthesis (Table 3). Surprisingly, the whole body 3-methylhistidine production rate (calculated from

Table 3. *Lack of an effect of leucine and 3-hydroxy-3-methylbutyrate (HMB) on fractional rates of protein synthesis in skeletal muscles of lambs (%/d)*

Group	Control	Experimental
Acute leucine intravenous infusion, milk fed lambs (8 kg BW)		
Tensor fasciae latae	8·4	8·9
Longissimus dorsi	11·3	11·1
HMB supplementation of diet, ruminant (50 kg BW)		
Tensor fasciae latae	3·0	2·9
Longissimus dorsi	3·3	3·2

Protein synthesis was measured using a phenylalanine flood.
BW, body weight.

the plasma disappearance curve of intravenously administered [$^2H_3$]methylhistidine; Rathmacher *et al.* 1993) was increased, indicating an increase in myofibrillar protein degradation (Ostaszewski *et al.* 1994). However, this result must be considered with reserve since it has been demonstrated in sheep that only a small percentage of 3-methylhistidine, which is released from actin and myosin, is actually excreted in the urine, with the remaining majority retained as balenine in muscle. Any change in the balenine pool should have an effect on the decay curve.

A specific effect of glutamine on skeletal muscle protein turnover has also been hypothesized. Times of stress and injury result in the release of large amounts of glutamine from muscle with a corresponding marked reduction in the large intracellular glutamine pool of muscle tissue. Despite this increase in glutamine efflux, its plasma concentration is decreased, indicating that there is an increased rate of removal by other tissues and organs (e.g. gut or wounded tissues) exceeding its rate of release from muscle tissue. It has been shown that depletion of glutamine in the extracellular pool is able to stimulate glutamine efflux through activation of a special transport system (system N). This adaptive regulation appears to involve both rapid phosphorylation dependent signalling cascades and gene expression (see Rennie *et al.* 1994*b* for a review). Regulation of the system N thus appears to be particularly important in modulating the availability of glutamine to the rest of the body. The specific role of glutamine is evidenced from the fact that maintaining normal glutamine concentrations intramuscularly by administering adequate amino acid solutions or glutamine enriched amino acid solutions leads to a conservation of muscle protein (see Wolfe *et al.* 1989 for a review). In addition, glutamine itself may have some anabolic effects on muscle protein turnover (Millward & Rivers, 1989; Rennie *et al.* 1994*b*).

Substrates of energy metabolism, e.g. glucose, fatty acids, triglycerides, glycerol and ketone bodies, can also be effective in promoting nitrogen balance and retention. Their infusion elicited significant changes in whole body leucine fluxes, especially leucine oxidation (see Young *et al.* 1992 for a review) but their mechanisms of action are poorly understood.

SUBSTRATE–HORMONE INTERACTIONS

Substrates have a potential effect on protein turnover first through their action on hormone secretion. For example, it is noteworthy that the protein sparing effect of glucose is mediated by insulin. Some amino acids are also able to stimulate the secretion of insulin, glucagon, GH and IGF-1. Secondly, substrates could modify hormone action. Garlick & Grant (1988) measured protein synthesis *in vivo*, using the flooding dose method, in tissues of postabsorptive young rats that were given intravenous infusions of various combinations

of insulin and amino acids. In the absence of amino acid infusion, there was a steady rise in muscle protein synthesis with plasma insulin concentration up to 158 μunits/ml, but maximal rates were obtained at 20 μunits/ml when a complete amino acid mixture was included. The effect of the complete mixture could be reproduced by a mixture of essential amino acids or of branched chain amino acids, but not by a non-essential mixture, alanine, methionine or glutamine. It was concluded that amino acids, particularly those with branched chains, increase the sensitivity of muscle protein synthesis to insulin. In addition, other data show that amino acids can modulate the antiproteolytic actin of insulin. Hypoaminoacidaemia induced by insulin infusion in man would appear to blunt the responsiveness of the insulin-inhibited whole body leucine appearance; in contrast with the presence of near basal plasma amino acid concentrations, the effect of insulin is enhanced (Flakoll *et al.* 1989). Insulin infusion in fed goats, with appropriate glucose and amino acid replacement, revealed that the inhibition of whole body leucine appearance was more sensitive to the hormone during early lactation. This adaptation relates to the amino acid deficiency which exists during early lactation, because it did not occur under hyper-aminoacidaemia (Tesseraud *et al.* 1993). In such experiments insulin and amino acids may exert their effects on different tissues and through separate pathways but the possibility that competition for the same targets may occur cannot be excluded. Alternatively, amino acids may modulate insulin action by influencing events within the insulin action cascade. Indeed, erythroblastic leukaemic cells incubated in media containing essential amino acids, glutamine and serine subsequently bound more labelled insulin than those incubated without serine (Galbraith & Buse, 1981). Insulin receptor aggregation and auto-phosphorylation in cultured 3T3-L1 adipocytes is stimulated in the presence of cationic polyamino acids (Komori *et al.* 1989).

Substrates other than amino acids have been shown to modify insulin signalling, e.g. 3-hydroxybutyrate increases the insulin sensitivity of adipocyte glucose transport at a postreceptor level (Green *et al.* 1984), while the membrane lipid environment also has an effect on the properties of insulin receptors (Ginsberg *et al.* 1981; Liu *et al.* 1994).

SHORT TERM EFFECT OF NUTRIENTS

The short term effect of nutrients can be assessed from studies on diurnal cycling or comparisons between postprandial and postabsorptive states, i.e. short term fasting. Protein is deposited during feeding and lost in the postabsorptive state. The net balance of protein depends on the relative magnitude of the two phenomena (see Reeds & Fuller, 1983 and Arnal *et al.* 1987 for reviews).

RESPONSES OF TISSUE PROTEIN METABOLISM

In young rats, muscle protein synthesis increases sharply within 1 h of feeding (Garlick *et al.* 1983; Millward *et al.* 1983). This response is a component of the marked postprandial anabolism which leads to growth. Individual muscles respond differentially since oxidative muscles, such as soleus, are much less sensitive. Fractional or absolute synthesis rates and ribosomal efficiency were all stimulated by nutrients in responsive muscles. In adult rats, stimulation of muscle protein synthesis by feeding appears attenuated (Baillie & Garlick, 1992) and muscle mass is stable, i.e. less diurnal cycling. We have confirmed this insensitivity to feeding in old rats (Mosoni *et al.* 1995).

Evidence from starvation studies supports the observations made during the post-absorptive state. When food is withdrawn from growing rats, there is a rapid and progressive fall in both the fractional rate of protein synthesis and in ribosomal efficiency in skeletal muscle. The decrease is substantial after only 12 h of food withdrawal. A similar

magnitude of decrease occurs after 1 d of fasting in adult rats (Chérel *et al.* 1991). Short term fasting also resulted in an increase in muscular proteolysis, especially myofibrillar proteins (Kettelhut *et al.* 1988), but across the perfused hindquarters of fasted rats this does not occur through the lysosomal or the Ca^{2+} dependent pathways. Indeed, agents which block lysosomal acidification (chloroquine, NH_4Cl) or the autophagic pathway (insulin, amino acids), and the inhibitor (leupeptin) of cathepsins B, H and L and calpains, do not affect 3-methylhistidine release. In contrast, the skeletal muscle ATP–ubiquitin dependent proteolytic pathway is activated upon starvation (Kettelhut *et al.* 1988; Wing & Goldberg, 1993). For example, incubation of muscles under conditions where intracellular ATP was depleted almost totally suppressed the increased proteolysis which arises due to fasting (Wing & Goldberg, 1993). The rise in ATP dependent proteolysis observed during starvation was associated with increased ubiquitin abundance of the mRNA (Wing & Goldberg, 1993) and the 14 kDa ubiquitin conjugating enzyme (14 kDa E2) that mediates the formation of ubiquitin–protein conjugates (Wing & Banville, 1994), accumulation of ubiquitinated proteins and increased expression of subunits of the 20S proteasome (Medina *et al.* 1992). Upon refeeding, total and ATP dependent proteolysis, concentrations of ubiquitin–protein conjugates, and expression of ubiquitin, 14 kDa E2 (Wing & Banville, 1994), and proteasome subunits returned to normal. ATP–ubiquitin stimulated proteolysis was also enhanced in the soluble extracts of skeletal muscle from fasted rabbits (Medina *et al.* 1992), as expected from the increased C2 proteasome subunit mRNA abundance reported under similar conditions by Ilian & Forsberg (1992).

This diurnal cycling in protein metabolism was studied in human adults during a 48 h period of consecutive 12 h periods of feeding hourly meals and fasting, after 12 d of adaptation to diets containing 0·4–2·3 g protein/kg daily (Price *et al.* 1994). Whole body amino acid fluxes were investigated using primed constant infusions (Pacy *et al.* 1994). Based on both N and amino acid balances the authors concluded that the amplitude of the diurnal cycling increases with increasing dietary protein intake. In other words the protein requirement for N balance reflects a demand for repletion of fasting losses which increases with increasing habitual protein intake (Pacy *et al.* 1994; Price *et al.* 1994). Recent data at tissue level in growing rats revealed that decreasing dietary protein level from 20 to 10% blunted the changes in protein synthesis during the postabsorptive/postprandial transition (P. Patureau Mirand, unpublished).

MEDIATORS

Diurnal cycling of protein turnover may be mediated by substrates and hormones. The rise in plasma amino acids and insulin after feeding has been recognized as a main factor (Pacy *et al.* 1994). Although amino acids *per se* have been demonstrated to induce protein anabolism, their effect is too small to be responsible for the marked postprandial increase in muscle protein synthesis in the young rat. Based on the results of experiments using insulin infusion, the hormone alone can exert a direct effect on the postprandial decrease in proteolysis but not on the stimulation of protein synthesis (see above). The role of insulin in the changes in muscle protein synthesis which occur on feeding young animals has been evidenced from experiments which induce insulin suppression. Injection of anti-insulin antibody just before feeding in young rats successfully prevented the stimulation of *in vivo* muscle protein synthesis by food intake (Millward *et al.* 1983; McNurlan & Garlick, 1989). We recently blocked postprandial insulin secretion by diazoxide injection and a marked decrease in protein synthesis was observed in muscles taken from treated animals when compared to pair fed controls (Sinaud, S., Balage, M. & Grizard, J., unpublished, Table 4)). This suggests that insulin plays an important role in the response of muscle to feeding but

Table 4. *Effect of postprandial insulin suppression on muscle protein synthesis*

Group	Control	Experimental
Insulinaemia (μU/ml)	47 ± 10	15 ± 2*
In vivo protein synthesis (%/d)	21·3 ± 1·1	10·3 ± 0·07*

Male Wistar rats (100 g) received 6–8 g of a standard diet and were then divided into two groups. The experimental group was injected intraperitoneally with diazoxide (25 mg/100 g BW) 1 h later in order to blunt insulin secretion. The control group received the vehicle. Protein synthesis was measured in mixed muscles using a phenylalanine flood 2 h after the treatment. Each value is the mean ± SE for five individuals. * $P < 0·05$ *v.* control.

BW, body weight.

the hormone appears to be acting on protein synthesis through interactions with other postprandial components, e.g. amino acids (see substrate–hormone interactions).

Increases in circulating corticosterone occur in rats during short term fasting. There is evidence that the increase in skeletal muscle proteolysis after short term fasting is mediated by glucocorticoids because this is impaired in adrenalectomized animals. The proteolytic response to fasting of muscles from adrenalectomized animals could be partly restored by glucocorticoid treatment *in vivo* or *in vitro* (Kettelhut *et al.* 1988). Elevated glucocorticoids may also inhibit protein synthesis by inhibiting any residual insulin stimulation of protein synthesis (Millward *et al.* 1983). In accordance with this concept, the rise in plasma corticosterone in the rat during the final hours of light during the day, when food intake is low, would favour muscle protein wasting (Obled *et al.* 1977).

The plasma concentrations of GH in the rat fluctuate markedly over a 24-h period and several peaks are observed, especially during the night. GH, either directly or through IGF-1, may influence muscle protein synthesis. Circadian oscillations of the thyroid hormones culminated in the hours of darkness and that of glucagon in the light phase (Ahlersova *et al.* 1984). However, the relationship between these hormones and the diurnal cycling of protein turnover has not been clearly established. The role of the increased concentrations of plasma glucose and triglycerides after feeding coupled with the decline in free fatty acids and ketone bodies is also unknown.

EFFECT OF LONGER TERM CHANGES IN PROTEIN AND AMINO ACID INTAKE

PROTEIN DEFICIENCY

In the rat dietary protein has a powerful regulatory influence on muscle growth. Yahya & Millward (1994) and Yahya *et al.* (1994) analysed the time course response of protein turnover to protein deficiency in young rats (7, 3·5 or 0·5% protein diet *v.* 20% control). The 7·0 and 3·5% protein diets gave a graded inhibition of muscle protein synthesis due to protein deficiency, mediated by a reduction in ribosomal capacity. Transient increases in proteolysis also contributed to growth inhibition in some groups. The decrease in protein synthesis was more severe with the 0·5% protein diet where ribosomal capacity and activity both decreased; proteolysis also decreased with this diet. Tawa *et al.* (1992) apparently showed that the suppression of skeletal muscle protein breakdown observed in rats fed a protein deficient diet was accompanied by reduced lysosomal proteolysis, with levels of many lysosomal proteinases (cathepsins B, H, C, and carboxypeptidases A and C but not cathepsin D) lowered. However, following inhibition of the lysosomal proteolytic pathway, proteolysis remained lower in protein deficient animals than in control rats. In fact, the

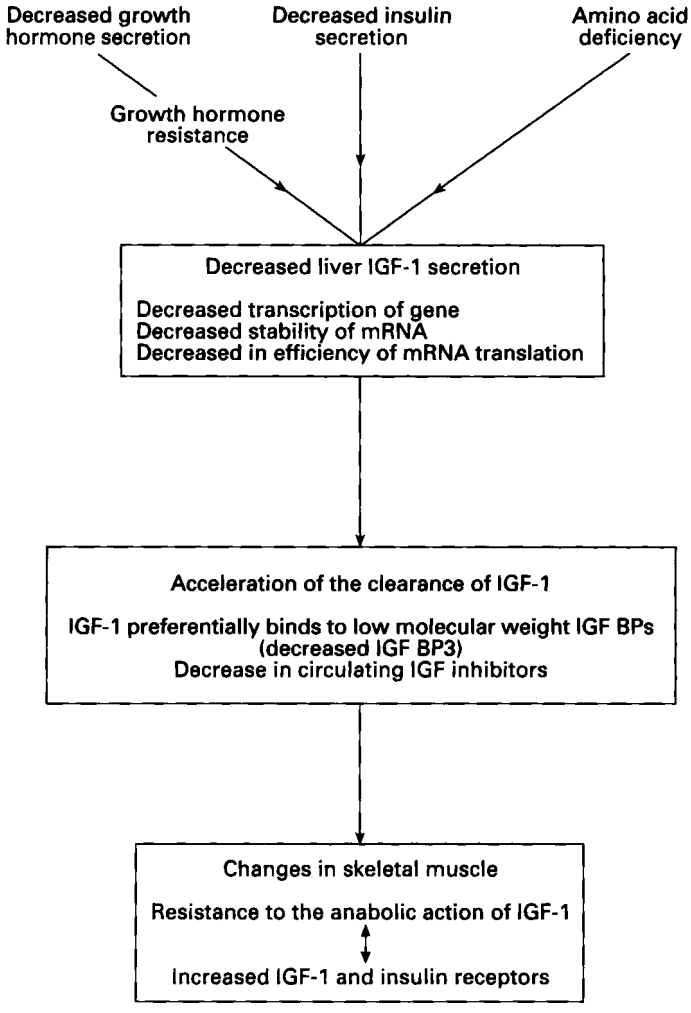

Fig. 2. Overview of the regulatory role of IGF-1 in protein restricted rats. IGF-1, insulin-like growth factor-1. BP, binding protein.

reduction in protein breakdown observed in these animals was due to a marked decrease in ATP dependent proteolysis with the muscle content of proteasome significantly less in animals fed on a protein-deficient diet (Tawa & Goldberg, 1993).

Millward and colleagues have suggested that longitudinal bone growth may be the primary target for overall somatic growth regulation since skeletal muscle growth could be dependent on a potentiating stimulus of bone growth possibly exerted by passive stretch (Yahya & Millward, 1994; Yahya *et al.* 1994). The effect of dietary protein is mediated, in part, by hormonal responses which Millward called the 'anabolic drive'. Protein deficiency clearly decreased plasma insulin, tri-iodothyronine and IGF-1 and tissue IGF-1 (see Millward & Rivers, 1989 for a review) whereas it increased plasma corticosterone (Grizard *et al.* 1977).

The decline in IGF-1 in protein restricted rats is related to a decreased transcription rate of the IGF-1 gene and stability of the 7·5 kb IGF-1 mRNA, the most abundant species (Straus & Takemoto, 1990; Fig. 2); a decrease in the efficiency of mRNA translation was

also noted (Thissen *et al.* 1991). This may originate from the amino acid deficiency *per se* and support for this hypothesis comes from experiments on primary cultures of rat hepatocyes where removal of tryptophan from the medium resulted in a dramatic decline in IGF-1 mRNA (Harp *et al.* 1991). Alternatively there may be an alteration of the hormonal control of liver IGF-1 synthesis due to both hypoinsulinaemia and resistance to the effect of GH, even though liver insulin and GH receptors were unaltered. Several observations suggest that in addition to decreasing IGF-1 production, dietary protein restriction can impair the growth promoting effects of IGF-1 (Thissen *et al.* 1994 for a review).

However, as with prolonged fasting, the biological significance of the impairment in IGF-1 action in protein-deprived rats remains unclear, due to counter-regulatory mechanisms. For example, plasma IGF-1 BP3 is reduced and IGF-1 preferentially binds to IGF BP in the small (30 kDa) complex, instead of equal binding to 150 kDa and 30 kDa proteins in control rats (Thissen *et al.* 1994). Because the small IGF–BP complex is believed to facilitate the transport of IGF-1 from serum to tissue, this may allow faster transcapillary passage and distribution to the target organs. The IGF inhibitors are absent from sera of protein restricted rats. In addition, there was an increase in both insulin and IGF-1 binding to skeletal muscle receptors of protein restricted rats (Dardevet *et al.* 1991) which may alter the sensitivity of target tissues especially towards insulin, because a high responsiveness of peripheral tissues to insulin is characteristic of protein deprived rats (at least for glucose metabolism) (Okitolonda *et al.* 1988; Crace *et al.* 1990). These adaptive mechanisms tend to conserve muscle protein when dietary protein is inadequate.

AMINO ACID BALANCE

An increase in growth performance, nitrogen retention or secreted products of animals could be achieved through improved protein quality. This is usually obtained by supplementing the diet with the limiting amino acid and will decrease the protein requirement for a given level of protein deposition. More generally, adjusting supplies of essential amino acids to an ideal protein pattern should be a way of expressing completely the growth potential of pigs or chickens, allowing them to optimize food intake (Henry, 1988; Wang & Fuller, 1989).

The effect of lysine additions, to a diet deficient in lysine but well balanced in other amino acids, was recently studied in 3-week-old chickens (Tesseraud *et al.* 1992). Weight gain varied curvilinearly with dietary lysine and was maximal at ~ 11 g lysine/kg diet. Increasing lysine supplementation in the diet resulted in increased protein deposition in the pectoralis major muscle. This occurred along with significant increases in absolute rates of both protein synthesis and degradation. Maximum protein deposition was achieved when ribosomal activity was optimal, i.e. for a dietary lysine content of ~ 9 g/kg, close to requirement. The same phenomenon was demonstrated in growing pigs with both lysine (Salter *et al.* 1990) and tryptophan (Cortamira *et al.* 1991). The possible relationships between changes in protein turnover after amino acid supplementation and hormone concentrations (e.g. insulin, IGF-1 and glucocorticoids) have not yet been established (Cortamira *et al.* 1991).

PROTEIN EXCESS

It is well known that dietary protein in excess of growth requirements, e.g. 15–30% in the growing rat, only slightly improves protein deposition in muscle. The curve depicting muscle fractional synthesis rates as a function of dietary protein also shows a plateau at high dietary protein levels (Laurent *et al.* 1984). Slight decreases in fractional synthesis rates

were occasionally observed at very high levels (30–40%), especially in food restricted rats (Taillandier, D. & Attaix, D., unpublished). However, high protein diets could have beneficial effects; for example, a 30% protein diet was able to sustain protein synthesis in soleus in unweighted rats (hindlimb suspension), whereas a 15% protein diet did not (Taillandier *et al.* 1993). High protein diets also prevented the decrease in protein synthesis after early weaning in young pigs (Sève *et al.* 1986).

Dietary protein excess results in extensive degradation of amino acids in the liver; this prevents their accumulation in the blood. The hepatic concentration of glucogenic amino acids is consistently depressed. This response was the result of a marked induction of liver amino acid uptake and catabolism, which further decreased amino acid availability to skeletal muscle, especially threonine, serine, glycine and glutamine (Moundras *et al.* 1993). For example, there was a 45-fold increase in liver threonine–serine dehydratase (EC 4.2.1.16) activity with a 60% casein diet, while alanine aminotransferase (EC 2.6.1.2) and gluconeogenic enzyme activities were also stimulated (Bois-Joyeux *et al.* 1986).

It has been suggested that corticosterone and glucagon may be involved in the regulation of nutrient partitioning between the liver and other tissues (Bois-Joyeux *et al.* 1986). In support of this concept we demonstrated that hyperglucagonaemia, caused by increasing dietary protein concentrations, is not associated with any significant modification of glucagon binding sites in rat liver (Balage *et al.* 1986). Moreover, cyclic AMP production stimulated by glucagon was enhanced (Balage *et al.* 1986). The effect of high protein feeding on IGF-1 and insulin control of metabolism has been only poorly studied, but IGF-1 receptors in muscle are not modified after feeding a 30% (compared to 15%) protein diet despite substantial increases in plasma IGF-1 concentrations (Dardevet *et al.* 1991). Insulin resistance was observed with respect to glucose metabolism (Rossetti *et al.* 1989).

EFFECT OF CHRONIC DIETARY ENERGY RESTRICTION

The specific effect of dietary energy restriction has been assessed by giving high protein diets in restricted amounts. The growth rate of young animals deteriorated in proportion to the severity of energy restriction. Body fat deposition was reduced whereas tissue protein content was only slightly modified (Grizard *et al.* 1975). It has been suggested that a delayed inhibition of bone growth by energy restriction was, in part, responsible for the relative resistance of muscle growth to energy restriction (Yahya *et al.* 1994; Yahya & Millward, 1994).

The influence of energy restriction on gastrocnemius protein turnover was examined in growing rats after 4 and 8 d of diets fed at 75, 50 and 25% of the *ad lib.* intakes, with protein intakes held constant (Yahya *et al.* 1994). The main feature of the metabolic responses to energy restriction in the first 4 d was the maintenance of relatively high fractional rates of protein synthesis in all groups except those offered the 25% diet, where there was a 40% inhibition. Only after 8 d did the fractional synthesis rate of animals fed the less severe 75 and 50% diets become significantly reduced, with a further reduction in the 25% group. These graded reductions in protein synthesis were mediated by similar decreases in ribosomal capacity and, in addition, the ribosomal efficiency was also inhibited after 4 and 8 d in both the 50 and 25% groups. Protein degradation was unchanged, except after 8 d in the 25% group, where it was elevated by 40%.

Plasma corticosterone was elevated in rats offered restricted amounts of dietary energy (Lunn *et al.* 1979) but whether this can account for the responses of muscle protein turnover to the energy deficient diets is by no means certain (see Yahya *et al.* 1994). These responses may also be mediated by insulin, which exhibited low postprandial plasma concentrations (Balage *et al.* 1990*a*). In addition, whatever method of skeletal muscle insulin

receptor preparation employed (enriched plasma membranes, crude Triton X-100 solubilized or wheat germ agglutinin purified extracts), insulin binding was similar in control and energy restricted rats (Balage *et al.* 1990*a*).

EFFECT OF PROLONGED FASTING

Adult rats show three phases of starvation as assessed from changes in daily nitrogen excretion and in the specific daily loss of body mass. In phase I (2–3 d), nitrogen excretion and daily change in mass per unit body mass decreased by ~ 60%. Both parameters stabilized in phase II (3–7 d) and they increased two-fold in phase III (7–10 d). Chérel *et al.* (1991) measured the capacity for protein synthesis and the efficiency of protein synthesis *in vivo* during these three phases of fasting. Protein sparing during phase II occurred in association with a decrease in protein degradation in the whole body, when compared to control fed rats. Fractional synthesis rates in skeletal muscles were maintained, because ribosomal efficiency was restored almost to normal from the lower values established by short term phase I fasting. Rats were in a protein wasting condition after 9 d of starvation (phase III) at which time protein degradation in the whole body had not increased and was actually ~ 80% of the value observed in fed animals. In contrast, skeletal muscle protein synthesis rates were markedly decreased, owing to the reduction in both ribosomal capacity and efficiency.

The ability of the rat and other species to conserve proteins in skeletal muscle during moderate periods of starvation depends on the continued availability of lipid fuels. In the transition from the protein sparing phase of starvation (II) to the terminal phase (III), lipid fuels become depleted and loss of protein from muscle is increased. It has been hypothesized that lipid fuels may directly modulate protein metabolism in muscle and prevent a substantial rise in circulating concentrations of catabolic hormones (Lowell & Goodman, 1987). In this respect, recent data (Le Maho, Y. & Chérel, Y., personal communication) indicate that the rise in plasma corticosterone does not seem to mediate the changes in muscle protein turnover, because blockage of glucocorticoid receptors by RU 38486 did not prevent protein wasting in phase III.

A decrease in the action of anabolic hormones probably contributes to the effect of prolonged fasting in the rat. Plasma insulin and IGF-1 decline whereas circulating IGF-1 binding proteins 1 and 2 and their liver mRNA increase; under such circumstances these latter may bind more IGF-1 and inhibit its action (see Thissen *et al.* 1994 for a review). Tissue IGF-1 mRNA abundance also decreased, along with a loss of liver GH receptors. Skeletal muscle became resistant to insulin and presumably to IGF-1. An impairment in the effect of these hormones on protein synthesis may thus occur but this is attenuated by a compensatory increase in the expression of muscle insulin and IGF-1 receptors (Lowe *et al.* 1989; Balage *et al.* 1990*b*). Interestingly, pancreatic insulin gene expression was not decreased; the decrease in proinsulin biosynthesis results only from a reduction in translation efficiency (Chen *et al.* 1989). GH and thyroid hormones also show low plasma concentrations after prolonged fasting in the rat, but their role in the regulation of protein wasting remains unclear.

ALTERATIONS DURING AGEING

During ageing, a loss of skeletal muscle mass is well described both in humans and rodents (Holloszy *et al.* 1991). This corresponds to a loss of muscle protein that must result from an imbalance between muscle protein synthesis and degradation rates. In studies measuring basal muscle protein synthesis rate *in vivo*, although a decline from the young to the mature

Table 5. *Influence of age on protein content and turnover in gastrocnemius muscle of rat*

Group	Young (1·5 months)	Adult (12 months)	Old (24 months)
Protein content (mg)	183 ± 25	557 ± 81*	397 ± 94*
Fractional synthesis rate (%/d)	13·2 ± 1·4	6·6 ± 0·7*	8·8 ± 1·3*†
Fractional breakdown rates (%/d)	11·1 ± 1·3	6·6 ± 0·8*	8·8 ± 1·3*†

Data from Mosoni *et al.* (1993*b*). Protein synthesis was measured using a flooding dose of valine. The fractional breakdown was calculated by the difference between growth rate and synthesis.
* $P < 0·05$ *v.* young. † $P < 0·05$ *v.* adult.

organism has been established, only slight differences were then obtained through old age, with no clear trend (Goldspink *et al.* 1987; Mosoni *et al.* 1993*b*). Results on basal muscle protein degradation also showed only slight differences in old age compared with adults (see Makrides, 1983 for a review; Table 5). However, muscle atrophy occurs at this period. Prospective animal studies have yielded the strongest evidence yet that diet plays a major role in longevity and the ageing process. The most consistent finding is that dietary restriction not sufficiently severe to cause malnutrition markedly extends the life span of rodents as compared with control animals fed *ad lib.* (Masoro, 1992).

We recorded an insensitivity of protein synthesis to feeding in old rats (Mosoni *et al.* 1995). This alteration in metabolic responsiveness could explain, in part, the age related loss of muscle protein because the reduced amounts of proteins synthesized during postprandial anabolism may be inadequate to compensate for protein lost during the postabsorptive phase. This would lead to a slow erosion of muscle proteins, but unfortunately such losses during the postabsorptive state are too small to be measured accurately. It is not clear whether or not this insensitivity to feeding arises from amino acids and insulin. Unphysiological hyperaminoacidaemia stimulated muscle protein synthesis *in vivo* to a similar extent in old and adult rats (Mosoni *et al.* 1993*a*). Protein synthesis exhibited the same state of insulin resistance both *in vivo* and *in vitro* (Mosoni *et al.* 1993*a*; Dardevet *et al.* 1994). In addition, studies of hormone receptor binding and gene expression did not discriminate between old and adult rats (Dardevet *et al.* 1994).

The hypothesis that GH deficiency is responsible for the decline in postprandial protein synthesis in the elderly has gained substantial support since (*a*) the amplitude of GH pulses decreases with age, and (*b*) GH increases protein synthesis in skeletal muscle of ageing rodents and muscle mass in elderly men (Costa *et al.* 1993). IGF-1 may also be involved in this regulation since the increased life span in dietary restricted rodents occurred along with a tissue enrichment in IGF-1 receptors (Costa *et al.* 1993).

We recently brought new insights into the mechanisms possibly underlying muscle wasting during ageing based on the study of the effect of dexamethazone in adult and old rats (Dardevet *et al.* 1995). Muscle wasting occurred more rapidly in old rats and the recovery of muscle mass was impaired, suggesting that old rats were more sensitive to glucocorticoid treatment. Since an increase in plasma glucocorticoids was described in old rats (Sabatino *et al.* 1991), this may create a slight but continuous imbalance between muscle protein synthesis and breakdown, generating a loss of protein over a long period. Furthermore, in states where elevated circulating glucocorticoid levels prevail (stress or pathological conditions), the inability of old rats to catch up rapidly may result in muscle atrophy.

According to measurements in incubated epitrochlearis muscles, dexamethasone induced muscle wasting mainly resulted from increased protein breakdown in the adult, but from

Table 6. *Epitrochlearis atrophy and protein turnover in adult (6–8 months) and old (22 months) male Sprague–Dawley rats following 5–6 d on dexamethazone treatment*

Group	Adult	Old
Loss of muscle weight (mg)	−29·3 ± 3·4*	−33·6 ± 3·2*
Protein synthesis	−4·4 ± 9·1	−41·9 ± 4·9*
Protein breakdown	+50·0 ± 23·2*	+20·0 ± 12·1

Dexamethazone (500 μg/kg daily) was given in the drinking water. Epitrochlearis muscles were incubated in buffer supplemented with glucose, branched chain amino acids, and [^{14}C]phenylalanine as described in Dardevet *et al.* (1994). Protein synthesis (nmol of phenylalanine incorporated into protein per mg protein per h) and breakdown (nmol tyrosine per mg protein per h) are expressed as percent differences from pair fed controls. Values are means ± SEM for 4–6 animals. * $P < 0·05$ *v.* control pair fed values.

depressed protein synthesis in the aged animals (Table 6). Increased expression of cathepsin D, m-calpain, and ubiquitin was observed in the muscles from dexamethasone treated adult and old rats. In contrast, the disappearance of the stimulatory effect of glucocorticoids on protein breakdown in ageing occurred along with a loss of ability of steroids to enhance the expression of the 14-kDa ubiquitin carrier E2, which is involved in protein substrate ubiquitinylation, and of subunits of the 20S proteasome, the proteolytic core of the 26S proteasome that degrades ubiquitin conjugates. Thus, if glucocorticoids play any role in the progressive muscle atrophy seen in ageing, this is unlikely to result from an activation of the ubiquitin–proteasome proteolytic pathway.

CONCLUSIONS

Net gain or loss of skeletal muscle protein is ultimately determined by a balance between two opposite processes, protein synthesis and degradation. Synthesis of new protein is achieved *via* a complex series of reactions that occur in the nucleus, cytosol and various subcellular locations. Most of these reaction steps and the regulatory factors involved have been identified. In contrast, mechanisms of skeletal muscle proteolysis are only poorly understood, especially with respect to the role of the three major proteolytic systems present in this tissue, i.e. the lysosomal, Ca^{2+} dependent and ATP–ubiquitin dependent proteolytic pathways.

(1) Hormones have been shown to regulate many biochemical events involved in muscular protein turnover. However, *in vivo* studies reveal only some of the targets. Insulin and IGF-1 infusions stimulate amino acid deposition in adult muscle mainly through their antiproteolytic effect; a stimulatory effect of insulin on protein synthesis has been clearly shown only in experiments which induce insulin suppression in young animals. The anabolic action of GH is related only to protein synthesis. Glucocorticoids induce muscle wasting by decreasing protein synthesis and occasionally by stimulating protein degradation, when food intake is low. Thyroid hormones stimulate protein degradation to a greater extent than protein synthesis. In addition most of these hormones interact in their effects on protein turnover.

(2) Substrates, especially amino acids, also have a potential role in the regulation of protein metabolism in muscle. Surprisingly, amino acid infusion alone results in only a slight stimulation of protein synthesis *in vivo* when assessed by the flooding dose method. Moreover neither leucine nor 3-hydroxy-3-methylbutyrate (its metabolite) have a significant effect. Experimentally it has been demonstrated that amino acids may exert their anabolic

effect *in vivo* through their interaction with insulin. For example, normal amino acid levels are needed to allow the full antiproteolytic action of insulin.

(3) Periods of absorption of nutrients result in amino acid deposition in muscle, repleting the loss of muscle protein which occurs during postabsorptive states. The magnitude of this diurnal cycling is dependent on dietary protein level. Muscle anabolism is due to both increased protein synthesis and decreased protein degradation, associated with a decrease in the expression of components of the ATP–ubiquitin dependent proteolytic pathway. This could be mainly due to a coordinated effect of insulin and amino acids. Reduced glucocorticoid action is also suspected, especially with respect to proteolysis.

(4) The role of amino acids in the control of muscle protein metabolism is clearly demonstrated from studies on the effects of longer term changes in protein and amino acid intake. Protein deficiency induced a graded inhibition of protein synthesis. Protein degradation showed contrasting results depending on the severity of the restriction. The effect of protein restriction seems to be mediated in part by a decrease in the action of IGF-1 as well as insulin. Reduction of a specific essential amino acid in the diet, e.g. lysine or tryptophan, minimizes the effect of protein deficiency. High protein diets have only a minor effect on muscle protein metabolism, except a moderate decrease in protein synthesis when given in restricted amount (an effect of energy restriction).

(5) Adult rats spare muscle protein during prolonged fasting. This is mainly a reflection of sustained protein synthesis along with a decrease in protein degradation. The terminal protein wasting occurs when protein synthesis becomes depressed (after 9 d). These changes could be mediated by both endogenous lipid fuels and decreased action of anabolic hormones (e.g. insulin and IGF-1).

(6) There is a marked loss of muscle proteins during ageing. Old animals are more sensitive to glucocorticoids. As elevated circulating glucocorticoid levels prevail in these subjects, this may create a slight but continuous imbalance between muscle protein synthesis and degradation. In addition, protein synthesis is less sensitive to feeding. The involvement of anabolic hormones in these changes has not been clearly established.

We conclude that nutrients, especially amino acids, are powerful modulators of protein metabolism in skeletal muscles. However, the mediators of the effects of nutrients are not clearly identified. The poor understanding of mechanisms arises from the complexity of interactions between various hormones and between hormones and substrates. Further developments are needed to detect specific changes in hormones and amino acid signalling.

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