Initiation of Protein Biosynthesis in Skeletal Muscles at Feeding

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2010

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Scepticism is the beginning of Faith

Lord Henry in The Picture of Dorian Gray, ch 17,

Oscar Wilde 1890

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Abstract:

Background and aim. Artificial nutrition by intravenous feeding has for decades indicated less than optimal support of whole-body protein metabolism and balance during some treatment conditions. Therefore, the present project was aimed to evaluate the role and effects by amino acid provision to skeletal muscle cells in the light of other known important factors as amino acid infusion kinetics, IGF-I and insulin in support of myofibrillar protein synthesis.

Methods. Murine L6 and human rhabdomyosarcoma cells were cultured at standardized conditions in the presence of various amino acid concentrations. Commercially available amino acid formulations were infused by constant rates to patients scheduled for elective surgery and to ICU patients. Transgenic female mice with selective knockout of the IGF-I gene in hepatocytes were used in refeeding experiments to evaluate the role of circulating IGF-I for muscle protein synthesis, which was estimated by the flooding dose technique ($[^{14}C]$-phenylalanine). Protein factors for translational control of protein synthesis and cell signaling (4E-BPI, eIF4E, p70s6k, mTOR) were estimated in Western blots. Transcripts of muscle IGF-I, IGF-IR, PI3-kinase, AKT, mTOR, acta 1 (α-actin), mhc2A (myosin) and slc38a2/Snat 2 (amino acid transporter) were quantified by qPCR. Plasma amino acids were measured by HPLC.

Results. Incorporation rate of amino acids into muscle proteins gave incorrect results in a variety of experimental conditions. Methods independent of labeled amino acids (ribosome profiles, initiation factor analyses) indicated that essential amino acids activate initiation of protein translation, while non-essential amino acids had no such effects. Insulin at physiologic concentration (100 μM/ml) did not stimulate global muscle protein synthesis, but did so at supraphysiologic concentrations (3mU/ml). Circulating IGF-I was not critical for activation of muscle protein translation, while tissue produced IGF-I and IGF-IR controlled feeding induced protein synthesis, which involved mTOR signaling in skeletal muscles. In general, provision of exogenous amino acids was related to plasma concentrations and probably to steady state levels of amino acids in peripheral tissues of patients. Amino acids caused activation of translation initiation of muscle proteins as demonstrated for myosin heavy chain and α-actin. These effects by amino acids were in part supported by increased transcription and utilization of amino acid transporters as Snat 2 mRNA in muscles. Microarray analysis indicated up-regulation of genes in the mevalonate-pathway following amino acid exposure, important for steroidogenesis and lipid metabolism, which may imply new and additional mechanisms behind anabolic reactions in muscle cells related to nutrition.

Conclusion. Our results re-emphasize that labeled amino acids should be used with great caution for quantification of muscle protein synthesis. Measurements of regulating factors in the control of initiation of muscle protein synthesis represent alternative and convenient applications in estimation of directional changes in protein synthesis during non steady state conditions as demonstrated by overnight preoperative provision of standard TPN to patients. Our results confirm that muscle cells are sensitive to alterations in extracellular concentrations of amino acids, which signal to activate translation of transcripts for myofibrillar proteins. Such dynamics are highly dependent on the presence of membrane transporters of amino acids.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their roman numerals in the text.

I   Iresjö BM, Svanberg E, Lundholm K.

Reevaluation of amino acid stimulation of protein synthesis in murine- and human-derived skeletal muscle cells assessed by independent techniques.

Am J Physiol Endocrinol Metab. 2005 May;288(5):E1028-37.

II   Iresjö BM, Körner U, Larsson B, Henriksson BÅ, Lundholm K.

Appearance of individual amino acid concentrations in arterial blood during steady-state infusions of different amino acid formulations to ICU patients in support of whole-body protein metabolism.

JPEN J Parenter Enteral Nutr. 2006 Jul-Aug;30(4):277-85.

III   Iresjö BM, Körner U, Hyltander A, Ljungman D, Lundholm K.

Initiation factors for translation of proteins in the rectus abdominis muscle from patients on overnight standard parenteral nutrition before surgery.

Clin Sci (Lond). 2008 May;114(9):603-10.

IV   Iresjö BM, Svensson J, Ohlsson C, Lundholm K.

Liver derived circulating IGF-I is not critical for activation of skeletal muscle protein synthesis following oral feeding.

Submitted 2010.

V   Iresjö BM, Lundholm K.

Induction of myosin heavy chain 2A and α-actin synthesis by amino acids in skeletal muscle.

Manuscript.

LIST OF CONTENTS

ABBREVIATIONS

AA	Amino acids
AKT	Proteinkinase B-alpha
BCAA	Branched chain amino acids
DHEA	Dihydroepiandosterone
DNA/cdna	Deoxyribonucleic acid, complementary DNA
eIF4A	Eukaryotic initiation factor 4A
eIF4B	Eukaryotic initiation factor 4B
eIF4E	Eukaryotic initiation factor 4E
eIF4F	Eukaryotic initiation factor complex F (eIF4E,4G,4A)
eIF4G	Eukaryotic initiation factor complex 4G
FFA	Free fatty acids
FCS	Foetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GH	Growth hormone
IGF-BP	Insulin growth factor binding protein
IGF-I	Insulin like growth factor 1
IGF-IR	Insulin like growth factor 1 receptor
IRS-I	Insulin receptor substrate 1
JNK	Jun terminal kinase
MHC/mhc	Myosin heavy chain
mTOR	Mammalian target of Rapamycin
p70s6k	Ribosomal protein S6 Kinase, 70 kDa;
PATI	Proton-coupled amino acid transporter 1
PCR	Polymerase chain reaction
PiPc	Polyinosinic- polycytidylic acid
PI3K	Phosphatidylinositol 3-kinase
PVDF	Polyvinylidene difluoride
RNA/mRNA/tRNA	Ribonucleic acid, messenger RNA, transfer RNA
rps6	Ribosomal protein S6
Shc	Src homology and collagen kinase
Snat 2/slc28a2	Sodium-coupled neutral amino acid transporter 2
SREBP	Sterol regulatory element binding protein
TPN	Total parenteral nutrition
18S	18S ribosomal RNA

INTRODUCTION

Complications are significantly increased postoperatively in hospitalized and malnourished patients (1). It is therefore obvious and confirmed that systematic nutritional support reduces morbidity and mortality during treatment of cancer disease (2). However, years of clinical experience underlines difficulties to improve protein status in catabolic patients on artificial nutrition (3). For years, this fact made our research group to focus on mechanisms behind induction of protein anabolism in peripheral tissues both in healthy volunteers and in weight losing patients subsequent to various disease conditions (4). The earlier results demonstrate that conventional artificial nutrition attenuates further weight loss and protects body composition to some extent, but it remains difficult to support a clear-cut re-synthesis of body proteins, particularly in skeletal muscles (5). Thus, it remains a challenge to understand important factors behind stimulation of protein synthesis during normal and artificial feeding in patients.

Chronically malnourished patients have been evaluated in previous investigations during both enteral and parenteral nutrition. The results demonstrated that fat and carbohydrate metabolism were reasonably adequate (4), while insufficient induction of protein synthesis seemed to be related to the kinetics of infused amino acids (6). We therefore evaluated potential effects by insulin, recombinant IGF-I and ghrelin for anabolism in provision to malnourished patients. These studies demonstrated that effects by insulin were not significant in contrast to traditional opinions regarding muscle protein synthesis (7), while IGF-I was more potent although it did not improve nitrogen balance in postoperative surgical patients (8). However, ghrelin had clear cut effects to improve fat synthesis, and also implied a trend in effects to improve muscle anabolism (9). Therefore, continuing research is now focused on factors that may stimulate protein synthesis in skeletal muscles during both normal feeding and artificial nutrition. Such a stimulation appears not to be a matter of neural innervation, since denervated skeletal muscles showed normal activation of protein synthesis during re-feeding (10) , which was also observed in mice with type I and type II diabetes (11).

Our investigations in man have preferentially been conducted by measurements of amino acid balances across resting and exercising arms and legs (6, 12). Methods for measurement of protein synthesis also included applications of stable isotopes (12, 13). In addition, methods to

measure the release of 3-methylhistidine (3MH) from skeletal muscles were developed (14). The combination of stable isotopes and release of 3MH allowed determination of protein breakdown of sarcoplasmic and myofibrillar proteins in muscle tissue (15). Usually, these techniques do however not give complete and detailed information about the control of protein synthesis in vivo, which is usually used as a unanimous concept in the scientific literature, although there are a large number of proteins with specific regulation, functions and different turnover. Therefore, techniques that made it possible to distinguish alterations among different proteins were applied. This approach demonstrated that proteins with basic isoelectric points showed decreased synthesis in conditions of partial starvation, while "neutral proteins" appeared moderately decreased and "acid proteins" were surprisingly unaffected (10, 16). Thus, a traditional conceptual view with an overall decreased protein synthesis in skeletal muscles during partial starvation represents a too simplistic conclusion. We have therefore tried to combine methods to evaluate transcription and measurements of phosphorylation/de-phosphorylation and subsequent association of initiating protein complexes for the translational process (17). Experiments in animals as well as in cultured murine L6- and human rhabdomyosarcoma cells were evaluated for expression of mRNA for IGF-I, IGF-IR and GH-R at starvation-refeeding and following stimulation by hormones (insulin, IGF-I, GH) and growth factors. These studies confirmed that our methods are sensitive enough to measure physiological alterations related to preoperative intravenous feeding in humans (18).

In the present studies we have focused on physiological experiments to evaluate the role of locally produced IGF-I and IGF-IR in muscle cells to promote effects by amino acids to induce initiation of protein translation in muscle. These experiments involved measurements of rate controlling steps for initiation of translation as binding eIF4E to m_7GTP at the 5′ part of mRNA and subsequent binding of eIF4E mRNA complex to eIF4G and eIF4G binding to eIF4A and eIF3. The phosphorylation state of these proteins was altered by refeeding in normal mice, cultured cells and muscle tissue biopsies from patients (17, 18). Transduction signals behind the stimulatory networks have not yet been entirely clarified, but may be related to the transport carrier of amino acids across the cell membrane, which is now a focus of our ongoing research program, since tissue and cell expression of amino acid transporters are reported to be altered by provision of amino acids in various applications as presented in my thesis. Future research will be focused on the signal pathway(s) behind amino acid stimulation of myofibrillar proteins synthesis (myosin, actin) in skeletal muscles. An ultimate

goal of the present thesis was therefore to create a methodological basis for future translational and clinical studies that may facilitate and optimize nutritional treatment and supportive care to hospitalized and malnourished patients in combination with additional medical treatments.

AIMS OF THE PRESENT STUDY

It is likely that nutritional treatment is still less than optimal in several clinical conditions connected with severe disease, particularly concerning re-synthesis of skeletal muscle proteins. This phenomenon is in part associated with insufficient ability of artificial nutrition to stimulate protein synthesis to the extent as observed after oral refeeding, Therefore, an increased knowledge is required to understand how amino acids and hormones interact to control initiation of protein synthesis in cells.

Specific aims were:

to re-evaluate effects of amino acids for activation of protein synthesis in muscle cells by comparing results from different applications and methods (I and V).

to apply both new and well-recognized scientific methods for monitoring translation initiation of protein synthesis in human skeletal muscle tissue in order to optimize intravenous feeding in conjunction with surgical treatments (II and III).

to investigate the role liver derived IGF-I may have to stimulate feeding induced protein synthesis in skeletal muscle tissue in vivo (IV).

METHODOLOGICAL CONSIDERATIONS

A combination of methods and study models were used to investigate different aspects related to activation of protein synthesis after different kinds of feeding in our present studies. Animal models and cell culture experiments were mainly used with subsequent evaluations in clinical applications on patients. Applied methodology is described in brief and discussed from a general perspective, since all methods are described in detail in paper I-V.

Experimental and clinical models

Animal models

A starvation-refeeding model of mice was used to evaluate the effect of feeding on activation of protein synthesis and intracellular signaling in skeletal muscles. Adult, female C57Bl/6 mice of 20-22 g body weight were used (Paper I and V). All animals were housed in ordinary plastic cages with bedding and had free access to water at all conditions. Mice were transferred to new cages upon removal of food at 8 pm. They were either starved overnight for 12 hours before killing; starved overnight and thereafter refed for 3 hours with standard rodent chow before killing; or were freely fed with access to food continuously. A 3 hour refeeding period was used as we have previously demonstrated that fractional protein synthesis rate in skeletal muscles was close to maximum at this time post-feeding. Mice were preferred over rats as experimental animals, since mice are weight stable, non-growing animals as adults.

Transgenic mice with selective and inducible knockout of the IGF-I gene in liver cells were used to evaluate the role of liver-derived IGF-I for activation of protein synthesis after feeding and these mice were starved and refed as described above (Paper IV). Knockout mice were created by others using the Cre/LoxP conditional knockout system (19). Injections with polyinosinic-polycytidylic acid (PIPC) results in a specific and complete inactivation of the IGF-I gene in hepatocytes, which will cause IGF-I in the circulation to decrease by 70-80%. The mRNA expression of IGF-I in skeletal muscle tissue is however unaffected in this model upon silencing of liver IGF-I expression, which makes it possible to separate effects of local IGF-I produced in the muscle cells from liver-derived IGF-I appearing in the blood circulation. PIPC treated littermates, homozygos for lox P but lacking Mx-Cre, served as

controls. Plasma IGF-I concentrations were measured in all mice groups 1 week after PIPC treatment to confirm decreased plasma IGF-I levels in knock out animals before animals were used in refeeding experiments.

Cell culture experiments

Standard cell culture media as used for growth and maintenance of cells contain high concentrations of amino acids. Therefore, a model with initial amino acid starvation of cells was developed in order to create conditions for studies of activation of cellular protein synthesis by amino acid exposure. Cells were always cultured to confluence before a period of amino acid starvation started, in order to create conditions to measure protein synthesis related to "feeding effects" of amino acids. This approach may minimize the influence of increased protein synthesis related to stimulation of cell proliferation upon such triggers. Then, when cells became confluent they were first exposed to a medium with reduced amino acid concentrations for 24 hours, and thereafter amino acids were provided in either low (starved cells) or normal (refed cells) concentrations for 18 hours of further culture. Rat L6 myoblasts originating from rat thigh skeletal muscle and a human rhabdomyosarcoma cell line (RD) were used. L6 cells were used in the majority of our experiments. These cells fuse in culture to form multinucleated myotubes and exhibit many properties of mature muscle cells recognized in skeletal muscle tissue (20). RD cells were essentially used to confirm results from L6 cells in a human cell line (Paper I).

Cell culture experiments were used as a complement to in vivo studies as it makes it possible to study cellular events under relatively stable and standardized conditions and makes it possible to study effects by amino acids separated from other stimuli such as hormones. Foetal calf serum (FCS) was therefore removed from the culture medium at the beginning of a starvation period. This was well tolerated by both cell lines and did not affect cell viability according to visual inspection. It must however be emphasized that cells in culture are not equivalent to muscle tissue in vivo with respect to cell differentiation or cell content of contractile myofibrillar proteins (21). Contents of cell surface receptors and intracellular signaling pathways may also differ between cultured cells and muscle cells in vivo (22).

Clinical studies

Two kinds of patient groups were recruited in studies on patients who would benefit from nutritional support. One investigation was aimed to evaluate to what extent different amino

acid compositions of amino acids solutions for clinical nutrition may affect the appearance of steady state concentrations of arterial amino acids in plasma (Paper II). These patients were treated at the ICU department at Sahlgrenska University hospital due to various severe conditions. They were included for investigations when deemed to need parenteral nutrition for clinical reasons for at least 1 week. If so, they received three different, commercially available amino acid solutions with different amino acid compositions (Vamin 18, Vamin-Glucos, Glavamin). All patients received subsequently all three solutions and each solution was infused during 24 hours in random order to minimize carry-over effects. Arterial blood samples for analyses of plasma amino acids and substrates were drawn at the end of each infusion period. Amino acids were infused together with fat and carbohydrates. Amino nitrogen corresponded to 0.2gN/Kg body weight·day^{-1} and the energy intake of glucose and lipids was 20 kcal/kg/day with a caloric glucose to lipid ratio of 60:40 (23). The applied infusion rate was based on our earlier demonstration that amino acid flux switched from net outflow to net inflow across peripheral tissues in healthy individuals as well as in patients (12). Amino acid concentrations from a group of male healthy volunteers eating a standardized meal served as a reference group in comparison to amino acid profiles in peripheral blood of our patients.

Twelve patients who underwent surgery in the upper gastrointestinal tract were included in another study (Paper III), where the purpose was to evaluate if a standard TPN regimen could activate translation initiation factors in skeletal muscles. These patients were randomized to receive overnight infusions of either saline or total parenteral nutrition, which was supplied as all in one bag (Kabiven Perifer). Amino nitrogen corresponded to 0.16 gN/Kg body weight·day^{-1} and energy intake was glucose and lipids at 30 kcal/Kg·day^{-1} with a glucose ratio of about 45 %. The infusion rate was chosen to represent a standard TPN regimen used in most clinical settings. Infusions lasted for at least 12 hours before operation and continued until biopsies for measurements of translation initiation factors were taken from the rectus abdominal muscle directly after anesthesia induction. Muscle biopsies were immediately placed in RNA later solution and stored at + 4 °C for 24 hours before storage at -20 °C. This handling preserved initiation factor complexes better than immediate snap freezing in liquid N$_2$ and storage at -70°C, and made it possible to collect samples from patients appearing over a period of time which would have been a major problem otherwise because of the labile nature of such protein complexes.

Measurements of protein synthesis

Isotope based methods

The flooding dose technique was used to measure protein synthesis rate in vivo in animal experiments (24, 25). This method is based on administration of radioactive amino acid (tracer) together with a large amount of the same unlabelled amino acid (tracee). A flooding dose of phenylalanine leads to a plasma concentration of phenylalanine 5-10 times higher than normal, when provided at 150 µmol phe/100g and 0.4 µCi L-[U- ^{14}C]-phenylalanine. In this way it is assumed that the large amount of the tracee amino acid rapidly equilibrates to extinguish differences in the specific radioactivity of phenylalanine between extra- and intracellular pools of phenylalanine. This would also minimize effects of different tracer/tracee ratios among different groups of animals in experiments. Variations that might exist due to differences in nutritional status or stress factors among animals. The flooding dose approach for measurement of protein synthesis in vivo is not suitable for any choice of amino acid. Theoretically, it should thus be an amino acid with comparatively low physiologic concentration difference between plasma to extracellular to intracellular concentrations. Besides, the amino acid should be comparatively slowly metabolized in any organ tissue and it should be easily dissolved in water. Thus, phenylalanine is one of the most appropriate amino acids based on such circumstances.

In cell culture experiments, pulse labeling was used where the tracer (35-S-methonine) was diluted in the cell culture medium at the beginning of a refeeding period. In a monolayer cell culture it is assumed that steady state equilibrium of amino acids occur, which creates an even distribution of the tracer between extra- and intra-cellular pools for incorporation of the labeled amino acid into proteins. The protein synthesis rate can then be calculated according to the formula: R= [dpm/µg protein (cells)]/[dpm/nmol];

where dpm/µg is the specific radioactivity of the labeled amino acid in proteins at the end of incorporation and dpm/nmol is the constant specific radioactivity of the labeled amino acid in the immediate precursor pool (incubation medium) throughout the incubation period.

Immuno precipitation and Western Blotting

Measurements of changes in translation initiation factor complexes may be one possibility to evaluate protein synthesis activation with a tracer independent method without pool assumptions. A rate limiting step in the initiation phase of protein synthesis is binding of

mRNA to the cap binding protein eIF4E and assembly of the eIF4F complex (eIF4E, eIF4G, eIF4A). Complex formation is inhibited when the eIF4E protein is blocked by 4E-binding protein 1 (4E-BP1) (26). Total cell proteins are first immunoprecipitated, in isolation of initiation complex formations, with an anti eIF4E antibody to collect the mRNA cap binding protein eIF4E together with attached proteins followed by electrophoretic separation and transfer of the proteins to a binding surface membrane. Such membranes are thereafter treated with antibodies against eIF4G or 4E-BP1. Estimations of protein synthesis activation are made by comparing semi-quantitative increases or decreases of inhibitory (eIF4E·4E-BP1) or activating (eIF4E·eIF4G) complexes. Attachment of 4E-BP1 to eIF4E is regulated by phosphorylation. Unphosphorylated protein forms are bound to eIF4E, while phosphorylated 4E-BP1 is unbound (27). Phoshorylation status of 4E-BP1 is then determined by electrophoretic separation of all phoshorylated forms. By these applications, activation of protein synthesis can be indicative. However, it may be uncertain to what extent initiated proteins are completely terminated during translation, even though small changes in phoshorylation status and complex formation between samples can be detected.

Ribosome profiles

Activation of protein synthesis was also evaluated by an additional tracer independent method in some of our experiments. Ribosomes in cells were separated by density centrifugation in a non-linear sucrose gradient (28, 29). In this way ribosomes can be separated according to size. After centrifugation, the sucrose gradients were pumped through an UV-detector, the absorbance at $\lambda 260$ nm was measured and the area under the curve was registered. Multiple ribosomes in the process of translating mRNAs into proteins can then be separated from single ribosomes that are not involved in the translation process at the time of measurement. This method, which indicates the proportion of ribosomes in polysomes versus free- and subunits of ribosomes, gives a good estimate of increased translation but is not sensitive enough to reflect small changes in translational activity.

RNA metabolism

RNA extraction

An important factor in studies of mRNA expression is the quality of extracted tissue RNA. The RNA should be free from DNA and protein contamination and should not be degraded. All RNA preparations in our studies were isolated with RNeasy mini kit or RNeasy fibrous tissue mini kit (Qiagen) with a DNAse step included to remove contaminating DNA. RNeasy Kits are simple to use and produces RNA of high purity. RNA quality and concentration were always confirmed using a bioanalyzer (Agilent 2100, Agilent technologies) and nanodrop respectively (Nanodrop, Saveen &Werner).

Real time PCR

Transcripts of intracellular signaling pathways were measured by quantification of mRNA levels by real time PCR, which is a sensitive method for quantification of specific mRNAs (30-32). RNA is reverse transcribed into cDNA before PCR reactions. A primer pair is designed for the gene of interest preferably spanning an exon-exon boundary to allow amplification of cDNA but not of genomic DNA. The target sequence is then amplified in a PCR reaction. In the first step, the double-stranded DNA is heat denatured (95°C) into two single strands to allow binding of the primers, which are short, synthetic sequences of single stranded DNA. After annealing of the primers (usually at a temperature around 55-65°C) the temperature is raised to 72°C and a Taq DNA polymerase begins to synthesize new double stranded DNA at the 3' end of the primer. There are two new DNA strands identical to the original target at the end of each cycle. The amount of synthesized DNA can be quantified by measurement of the fluorescence emission of SybrGreen I dye after each cycle. The dye has barely no fluorescence when it is free in solution, but the emission of fluorescence is greatly enhanced when it binds to double stranded DNA. Thus, the SybrGreen I signal correlates with the amount of product amplified during PCR. One problem of using SybrGreen I dye for detection is that the dye can bind to any double-stranded DNA and thus detect contaminating DNA in reverse transcribed RNA samples. One way to overcome this problem is to use RNA specific primers spanning over an exon-exon, which does not allow amplification of genomic DNA. All our samples were also DNAse treated. Another problem is that the dye may bind unspecifically at inappropriate annealing temperature. A melting curve analysis was therefore performed for all analyses to check that only one transcript was present. It was also verified that all PCR products had the expected base pair size. Quantitative results were produced with

the relative standard curve method (33). GAPDH were used as housekeeping gene in human and animal analyses with 18S rRNA in cell culture analyses, since GAPDH were not expressed at constant levels in our cultured cells.

Expression (DNA) microarrays

Microarray technology is a powerful tool to analyze expression levels (transcripts) of thousands of genes at the same time (34). Therefore, this technique was used to search for changes in transcript levels after amino acid refeeding in L6 cells (Paper V). Microarrays are produced by several manufacturers and the technology differs slightly between producers, but the core principle behind microarrays is hybridization between two DNA strands. Agilent Whole Rat Genome 4 x 44K expression arrays in combination with a 2 colour detection system was used in our present studies. In the Agilent arrays used, 60-mer oligonucleotide sequences (probes) are in situ synthesized on a glass slide. The 60-mer long probes are more specific to the target than shorter probes used in other systems like the 25-mer probes used by Affymetrix.

RNA must first be transcribed into complimentary DNA (cDNA) by reverse transcription in order to detect RNA expression. In the Agilent system, samples are labeled with the fluorescent dyes in the cDNA synthesis step and RNA from starved and refed cells was labelled with either Cy3-dCTP or Cy5-dCTP respectively. These samples were then hybridized in competition on Whole Rat Genome 4 x 44K expression arrays, which generates a ratio of the difference between the two samples for each probe. A critical step in microarray studies is the data processing. After the fluorescence intensities were quantified in the Agilent G 2565 AA scanner, data were extracted and preprocessed with Feature Extraction software according to the manufacturer's recommendations to obtain dye normalized, outlier- and background subtracted values to be used for further analysis. RNA from 4+4 independent samples were analyzed. The preprocessed values were imported into Genespring GX 10 software (Agilent technologies) where probes with low signal to background ratio were excluded before further analysis.

Comments on protein synthesis methodology

An ideal method for estimating the true rate of protein synthesis should minimally rely on assumptions, but such a method may not exist and will probably not be available because of the complexity involved in the building of cellular proteins. Methods based on incorporation of stable or radioactive isotopes are always based on assumptions on the incorporations from the tRNA pool, which theoretically should represent the immediate precursor pool for protein synthesis (35-45). Using estimations of protein synthesis from non-tracer based methods always leaves you with the question; is a new and complete protein finally created; and if so, to what extent? Tracer methods are good in the sense they provide a quantitative measure of protein synthesis, which is not obtained by other methods. Evaluations of activation of protein synthesis from phosphorylation changes in translation initiation factors provide information that the process has started but not that it was finally and correctly completed. It may have been interrupted during elongation or termination phases before completion. Ribosome profiles, on the other hand, provide you insights into the translation process and ribosome distribution. It probably gives a rather reliable indication of increased protein synthesis in one condition compared to another. It is however a tedious and time consuming method to perform, demanding comparatively large amount of tissue and cell material, and it can never provide information on small changes of protein synthesis activity. So, what is the method of choice? This is a question without a final answer. One must also keep in mind that cells contain thousands of proteins to add one more layer of complexity to this issue; and protein synthesis is usually referred to and conceptually regarded as an over-all protein synthesis phenomenon in cells. However, the most important proteins to synthesize in skeletal muscles may be myofibrillar proteins of the contractile muscle unit in weight-losing patients who would benefit from nutritional treatment. It is the protein composition and the presence of these proteins that determine the power and force generation in muscles (46). With loss of contractile proteins the muscle will be weak and its performance deteriorated. Studies also indicate that cytoplasmic and myofibrillar proteins are synthesized at different rates (10, 47). Therefore, analyses of mRNA transcrips of myofibrillar proteins as actin and myosin heavy chains may be used to reflect eventual changes of myofibrillar transcription and subsequent protein synthesis. Cellular concentrations of mRNA transcripts are however only a static measure of the amount of mRNA at a specific time-point. By comparing transcript concentrations between conditions you also make assumptions about increased and decreased gene transcription in one condition compared to another. mRNA concentrations can however

be affected by several factors and mRNA seems to display complicated cycles from synthesis in the nucleus until targeted for destruction at the end of its life span (48). mRNA concentrations are also a reflection of the balance between transcription and translation. Finally, is the half-life of a specific mRNA always the same in different tissues (49)? Or may its life span in tissues be shortened by increased translation, i.e. consumed by use? Therefore, estimation of gene transcription, pre- mRNA analyses by qPCR or nuclear run on assays, are additional possibilities for estimations of transcription and translation analyses of protein metabolism.

RESULTS

Cell culture experiments

Amino acids and incorporation of labeled amino acids into protein

Essential amino acids stimulated incorporation rate of phenylalanine and tyrosine into cellular proteins to the same extent, while non-essential amino acids lacked stimulation (Fig. 1A, I). Increased incorporation to cellular protein was also seen when medium concentrations of either tyrosine (6-405 μM) or phenylalanine (6-405 μM) was increased in combination with constant specific radioactivity of L-[^{14}C] tyrosine or L-[^{14}C] phenylalanine respectively. However, increased concentration of tyrosine did not stimulate the incorporation rate of phenylalanine and vice versa. Also, a lack of stimulation of either tyrosine or phenylalanine incorporation rate by essential amino acids was evident when tyrosine or phenylalanine was present at high medium concentration (405 μM) in flooding dose experiment (Fig. 1 B, I). Similar results were seen when groups of amino acids (aromatics, BCAA, sulphur containing AA, Arg, Gln plus others) were used to evaluate potential stimulation of tyrosine and arginine incorporation (Fig. 2A,B, I). Thus, stimulation of tyrosine, phenylalanine and arginine incorporation rates was demonstrated by groups of amino acids that contained the tracer at increasing tracee concentration. Thus, the aromatics stimulated the incorporation of tyrosine but not of arginine and vice versa. Similar results were obtained when amino acid incorporation was evaluated among different proteins separated by gel electrophoresis.

Autoradiograms of labeled cell proteins after separation in SDS gel gradient electrophoresis indicated that a number of proteins were stimulated up to five fold by normal amino acid concentrations (Fig. 3, I). However, when the same experiment was performed and both radioactivity of [^{35}S]-methionine and the tracee concentration was kept constant, it was observed that only some protein fractions indicated truly increased incorporation; i.e. the tracer was incorporated more extensively in the presence of high tracee concentrations compared to low tracee concentrations in the medium (Fig. 4, I). These results indicate that incorporation of amino acids to mixed proteins was not from one homogenous precursor pool, or that amino acids initiated various mRNAs differently (Fig. 1A, B).
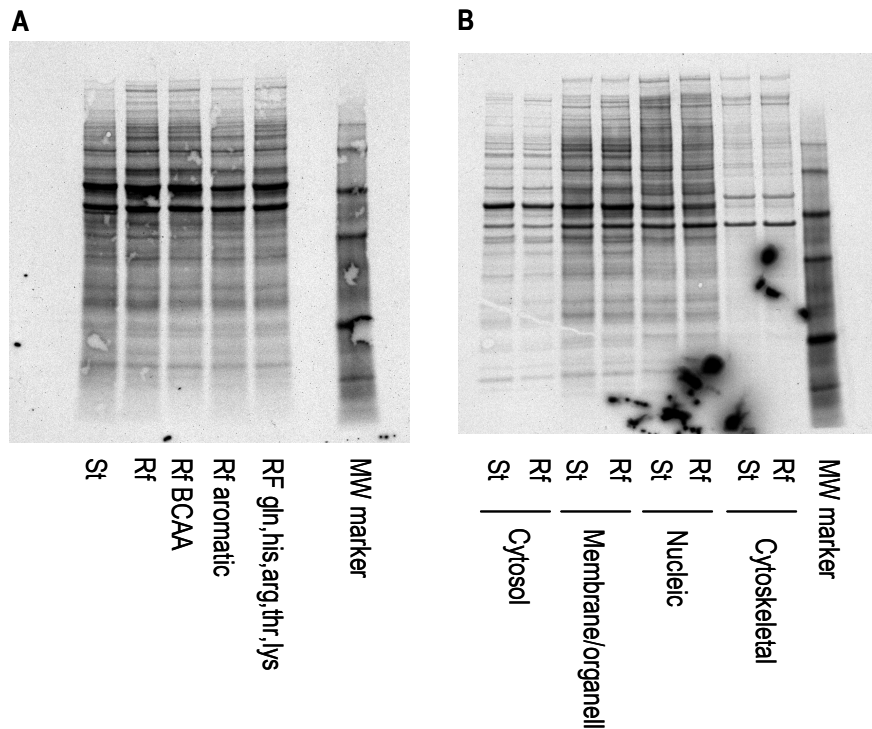
Fig. 1A. Autoradiogram of [^{35}S]-methionine labeled proteins from starved (St) and refed (Rf) L6 cells incubated with low (0.28 mM) or normal (9 mM) concentrations of all amino acids or groups of amino acids. 1B. Autoradiogram of [^{35}S]-methionine labeled proteins from either starved or refed L6 cells solubilized and extracted into four sub-cellular fractions (cytosol, membrane/organelles, nucleus, cytoskeletal, using Calbiochems sub-cellular proteome extraction kit). Proteins were separated in a 4-12% Nupage minigel with MES buffer system in the presence of SDS. Equal amounts of protein from starved and refed samples were applied on the gel. The specific radioactivity of [^{35}S]-methionine and tracee concentration of methionine were kept constant among all samples.

Ribosome profiles

Amino acids at normal concentrations stimulated protein synthesis indicated by significantly more polysomes in such cells. This effect was related to essential amino acids, while non-essential amino acids did not promote formation of polyribosomes ($p<0.01$) (Fig.6, I).

Effects of amino acids on initiation of translation

4E-BP1 was found in complex with eIF4E in cells treated with low amino acid concentrations (0.28 mM), while cells exposed to normal amino acid concentration (9 mM) had almost no 4E-BP1 bound to eIF4E. Cells provided with normal amounts of amino acids (9 mM) had almost all 4E-BP1 in the γ form, while cells incubated in the presence of low amino acid concentration had no 4E-BP1 present in the highest phosphorylated γ form. Increased amount of eIF4G・eIF4E in complex was seen in cells provided normal amino acid concentrations.

This confirms that global protein synthesis was more active in the presence of 9 mM amino acids in the medium compared to 0.28 mM (Fig. 7, I).

It was confirmed that essential amino acids activated initiation of translation, while non-essential amino acids had no such effect (Fig. 8, I). Branched chain amino acids (leu, ile, val) stimulated initiation of translation while the aromatic (trp, phe, tyr) and sulphatic amino acids (met, cys) did not (Fig. 9, I). A group of amino acids with glutamine, histidine, threonine, arginine and lysine showed increased initiation of translation. The presence of all three branched amino acids in combination were more potent than any of the individual branched chain amino acids. Also, glutamine in combination with histidine, arginine, threonine and lysine were more potent than glutamine alone (Fig. 10, I).

Effects of insulin on protein synthesis

The addition of insulin at physiologic concentration (100 μU/ml) had no significant effect on amino acid incorporation at either low or normal concentrations of amino acids, while pharmacological insulin concentrations (3 mU/ml) stimulated phenylalanine incorporation at low amino acid concentration, but significantly more at normal amino acid concentration (9 mM) (Fig. 11, I). Similar results were obtained by Western blot analyses on initiation factors (Fig. 12, I).

Transcription and amino acids

L6 cells expressed both actin and Myosin heavy chain proteins (Fig. 1, V), but 2A transcripts were below detection levels when analyzed by real-time PCR. Acta 1 transcripts were higher in starved cells compared to cells refed by amino acids (p<0.05).

Snat 2 (slc38a2) transcripts were decreased in refed cells compared to amino acid starved cells (p<0.05) as well as in cells refed by various combinations of amino acids. Cells refed by branched chain amino acids (leu, ile, val) or by glutamine in combination with other amino acids (arg, thr, his, lys) showed decreased levels of snat 2 transcripts. Refeeding by aromatic (phe, tyr, trp) or sulphatic amino acids (met, cys) did not alter snat 2 mRNA levels (Fig. 4, V).

A Gene ontology (GO) search to find categories with significant enrichment of changed genes, between amino acid starved and refed cells, was performed on results obtained from hybridization of 41 090 probes on the Agilent 4 x 44K whole genome rat array. Nine GO

categories with enrichment of entities were found; all related to lipid, cholesterol and steroid metabolism (Table 1, V). Additionally, mRNAs for several amino acid transporters and contractile proteins demonstrated alterations in transcript levels in response to provision of amino acids to cell cultures (Table 3, V).

Animal experiments

Plasma concentrations

Plasma levels of IGF-I were significantly lower in liver IGF-I knockout mice (Li-IGF-I$^{-/-}$) compared to wild type mice at all nutritional conditions (<0.001), while glucose and insulin levels were comparable. Overnight starvation caused a significant decrease in plasma glucose compared to freely fed knockouts and wt mice (p<0.05) (Table 1, IV). Branched chain and essential amino acids were significantly altered during starvation followed by refeeding in wild type mice, but not so in liver IGF-I knockouts. These alterations were also reflected in concentrations of all amino acids (Table 2, IV).

Protein metabolism in skeletal muscles

Basal fractional synthesis rate in liver and muscle tissue was comparable in freely fed wild type and liver IGF-I knockout mice. Refeeding increased liver (p<0.10) and muscle protein synthesis (p<0.01) compared to starvation in both wild type and liver IGF-I knockout animals (Fig. 1A,B, IV).

mRNA transcript levels were comparable in freely fed wild type and knockout mice (IGF-I, IGF-IR, PI3-kinase, AKT and mTOR). However, IGF-I transcripts decreased significantly in wild type mice during starvation but not so in knockouts. IGF-IR increased during starvation in both wild type and knockout mice and remained increased during refeeding (Fig. 2A-B, IV). Significant changes in transcript levels of PI3K and AKT were not observed. By contrast, mTOR levels were increased in starved knockout animals compared to refed mice without any similar change in wild type mice (Fig. 2C, IV).

The 4E-BP1・eIF4E complex, 4E-BP1 phosphorylation state, p70s6k phosphorylation and mTOR phosphorylation were comparable among wild type and liver IGF-I knockout mice. Starvation increased muscle content of 4E-BP1・eIF4E complexes with corresponding

normalization in refed animals (Fig. 3A, IV). Similarly, the 4E-BP1 phosphorylation state was decreased in starved mice compared to freely fed mice with increase in refed wild type and liver IGF-I knockout mice. p70s6k and mTOR[2448] was less phosphorylated in starved wild type and IGF-I liver knockout mice with complete reversal in both groups during refeeding (Fig 3C,D,E, IV).

Analyses of eIF4G· eIF4E complex, 4E-BP1· eIF4E complex, 4E-BP1 phosphorylation state and p70s6k confirmed that translation initiation of protein synthesis was increased in refed mice compared to muscles from starved mice (Fig. 7, I). Starvation-refeeding of normal mice (C57 BL/6) reduced and then stimulated initiation of global muscle protein synthesis (p< 0.01). Mhc2A and acta 1 transcripts were numerically decreased in skeletal muscles from refed normal mice compared to starved mice, but the difference did not reach statistical significance (Acta 1 p<0.10, MCH p<0.18, n=16) (Fig. 6, V).

Clinical investigations

Plasma concentrations at infusions of different amino acid formulations

Plasma insulin increased on Vamin 18 and Glavamin infusions compared to basal levels. Plasma glucose and serum urea did not change during steady state infusions of different amino acid solutions compared to preinfusion levels. Vamin-Glucos caused lower serum urea compared to Vamin 18 and Glavamin infusions. Plasma lactate increased during Vamin 18 infusions compared to both basal state and to the infusions of the other amino acid solutions (Table 2, II).

The sum of all amino acids in arterial plasma increased during steady state infusions of the three different amino acid solutions versus basal state in ICU patients (Table 3, II), as well as of the sum of all essential amino acids. Of non-essential amino acids, only glutamine, taurine, and tyrosine did not increase versus basal state (Table 3, II). Alanine, arginine, citrulline, glycine, histidine, serine and ornitine showed different concentrations among the amino acid solutions. Seen together, Vamin 18 displayed the most clear and highest increase in arterial concentrations of amino acids (Table 4, II).

Healthy subjects had significantly higher overall concentrations of amino acids in fasted state compared to ICU patients on TPN ($p<0.01$). The sum of all amino acids, as well as the sum of all essential and non-essential amino acids increased significantly in venous plasma around 60 min following oral intake and remained significantly increased for at least 5 hours. ICU patients remained with significantly lower ($p<0.01$) overall concentrations of amino acids during steady-state infusions of the amino acid formulations (Fig. 3, II). Unexpectedly, the sum of all amino acid in ICU patients on amino acid infusions only reached overall fasting levels in healthy volunteers (Fig. 3, II).

Plasma concentrations at infusion of complete TPN formulation
Plasma glucose and serum insulin, glycerol, triglycerides and S-FFA did not change by provision of preoperative TPN compared to saline infusions (Table 3, III). However, total plasma amino acids increased during TPN compared to saline infusions. Methionine, phenylalanine and threonine increased significantly in the group of essential amino acids, while tryptophan and lysine did not change. Only isoleucine increased among the branch chain amino acids (Table 2, III). Of non-essential amino acids alanine, arginine, aspartic acid, glycine and histidine increased significantly.

Protein metabolism in skeletal muscles during TPN
Provision of overnight TPN increased formation of active eIF4G·eIF4E complex ($p<0.05$). The inhibitory complex 4E-BP1·eIF4E decreased by TPN ($p<0.06$) (Fig. 1B, IV). Overnight nutrition increased the amount of 4E-BP1 in the most phosphorylated form to 72% compared to 60% in patients with only saline infusions ($p<0.05$)(Fig. 1C, III). TPN over night increased the total amount of p70s6k kinase as well as phosphorylation of the protein. p70s6k increased by 30% compared to saline treated patients by TPN ($p<0.05$). Skeletal muscle tissue from patients on TPN displayed decreased MHC2A transcript levels compared to muscle tissue from patients who received saline only ($p<0.05$) (Fig. 5, V).

DISCUSSION

Around 45 % of the body-weight in adult humans is skeletal muscles, an important nitrogen reserve in different stress conditions such as trauma, infection and starvation. A balanced regulation of muscle protein synthesis and degradation is needed to maintain muscle mass at appropriate functional levels (50). Feeding stimulates synthesis while acute and chronic starvation will increase and decrease degradation respectively (51). Mechanisms behind controlled protein balance in skeletal muscles over time have been extensively described in humans and animals based on studies in a variety of models from subcellular, cellular and tissues to organ levels (50). Yet, integrated signals behind protein balance in skeletal muscles are not fully understood, although several studies suggest amino acids in combination with hormones as insulin, IGF-I and GH as key factors (52), communicated by intracellular phosphoproteins (53-55). This thesis project has focused on one side of this balance, the synthesis although factors affecting protein degradation are always interconnected to the synthesis level by feedback mechanisms. Our focus has also been effects of oral and parenteral feeding, since improvements in artificial nutrition could be of great benefit to large groups of in- and out-hospital patients.

Muscle tissue

In healthy humans there is a continuous control of protein synthesis and degradation which maintains the muscle mass at an appropriate functional status close to constant levels despite variations in food availability. In stressed conditions this balance is however disturbed and muscle breakdown accelerates the loss of contractile proteins at the expense of functional capacity, while protein synthesis may be depressed due to hormonal and substrate alterations in order to deviate substrates to more immediate needs as synthesis of acute phase proteins in the liver among other purposes (56).

Muscle tissue is a postmitotic tissue unlike most other tissues in the body. The myofibers become permanently differentiated soon after birth and will not undergo mitotic division to increase myonuclear numbers. All skeletal muscles consist of numerous fibers and each of these fibers is made up of successively smaller units. Each such muscle fiber contains several hundred to thousands of myofibrils, which has side by side about 1500 myosin filaments and

3000 actin filaments that execute muscle contractions. Thus, it is the cross-wise organization of actin and myosin that gives skeletal muscle its striated appearance. The myofibrils appear inside the muscle fiber in a matrix called sarcoplasm, which is composed of well-recognized intracellular components. The myosin filament is made up by 200 or more individual myosin molecules, which are composed of two heavy chain molecules twisted around each other and 4 light chains attached to the myosin heads. The actin molecules are also twisted around each other in a helix similarly as the myosin molecule with tropomyosin and troponin attached to the actin molecule. (57) (Fig. 2). The size of the contractile unit (actin, myosin, troponin and tropomyosin) has to be unchanged or increase in order to maintain or increase the strength of muscles. Requirements for additional nuclei to support muscle hypertrophy appear to be met by cell proliferation, differentiation and finally fusion of satellite cells, which may be a type of muscle stem sells located between the basal lamina and the sarcolemma of muscle fibers close to existing myofibers (58).This process seems to be largely dependent on IGF-I exposure (59).

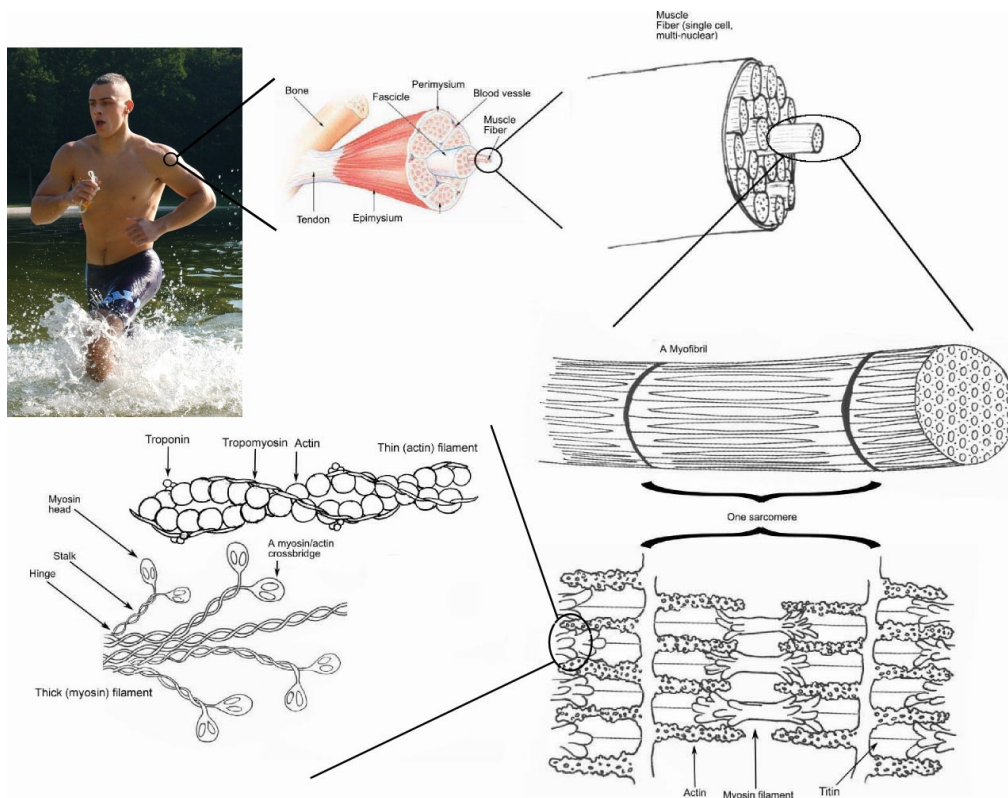

Fig 2. Structure of skeletal muscle tissue from the level of bundles down to myofibrils, sarcomeres and myofilamentory proteins as described in text.

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Several forms of Myosin heavy chain proteins exist and adult human skeletal muscle tissue may express three different isoforms of myosin as heavy chain MHC-I, MHC-IIa and MHC-IIx. In rodents one additional form MHC-IIb is described (46). The myosin gene family is located in a cluster on chromosome 17 in humans and on chromosome 11 in mice (60). Several studies have confirmed that mRNA abundance for the different isoforms correlate with the relative contents of the different MHC protein isoforms expressed in skeletal muscle tissue (61, 62).

Muscle strength is mainly determined by the cross sectional area of the muscle bundles and fibers and is reflected by the number of sarcomeres working in parallel. The speed of movement of a muscle is primarily regulated by the MHC isoforms within various fibers. Changes in MHC expression are common after exercise or muscle inactivity, but MHC isoform shifts seem to appear in a certain pattern only; Type 1-2A-2X-2B (60, 63). Disuse atrophy is related to upregulation of fast MHC isoforms while muscle activity usually up-regulates the appearance of slower forms of MHC, although it varies with type of muscle exercise (64). Fiber type changes in muscles have also been observed in mice suffering from cancer cachexia (65).

Recently, naturally occurring antisense RNA of MHC mRNA have been found to be involved in the shift of MHC isoforms in skeletal muscles as illustrated by decreased MHC2A mRNA and increased MHC2X mRNA at the same time after atrophy induction (66, 67). In our studies we found that MHC 2A mRNA as well α-actin mRNA decreased after provision of amino acids to cultured muscle cells and in skeletal muscle tissue from patients after provision of parenteral nutrition. It is not likely that appearance of antisense transcripts would be involved in feeding induced changes of MHC 2A levels or in changes of mRNA levels for α-actin protein, which is a single isoform without fiber-type specificity. Also, it has not been recognized that nutrition would be associated with fiber type changes. Lately it has been reported that introns of myosin genes encode several microRNAs (68-70), suggested to be involved in myogenesis and myosin heavy chain production (71, 72). microRNAs are small RNA fragments that can repress translation of mRNAs. Interestingly, the expression of several microRNAs were altered in skeletal muscles tissues following oral ingestion of essential amino acids and thus may be involved in control of muscle protein balance (73).

Protein synthesis

The formation and synthesis of proteins is a complex process initially depending upon the transcription of DNA into mRNA followed by translation of spliced mRNA into proteins. Translational control of existing mRNAs allows for rapid changes in cellular concentrations of proteins compared to transcriptional regulation. The process of protein translation, which is the synthesis of proteins, can be divided into 3 distinct phases; initiation, whereby mRNA bind to 40S and 60S subunits to form a ribosome complex capable of translation; elongation, by which tRNA-bound amino acids are incorporated into the growing polypeptide chains according to the mRNA template; and finally termination, where completed proteins are released from the ribosomes. Most of the control of protein synthesis is probably exerted at the initiation phase (26). This process requires several steps and involves more than a dozen of eukaryotic initiation factors (eIFs), although it is not fully understood how this process is executed. It is assumed that two steps are particularly important in control of protein synthesis rates; the binding of initiator methionyl-tRNA to the 40 S ribosomal subunit, mediated by eIF2; and the assembly of the eIF4F-complex. In the latter step, eIF4E binds to the cap structure present at the 5´end of eukaryotic mRNAs and then, the eIF4E-mRNA complex binds to factor eIF4G and eIF4A to form an eIF4F-complex competent for protein synthesis (Fig. 3). The availability of eIF4E to bind mRNA is regulated by a small protein termed 4E-binding protein 1 (4E-BP1). The 4E-BP1 competes with eIF4G for the same binding site on eIF4E and thus prevents assembly of the active eIF4F complex. The 4E-BP1 is regulated by the extent of phosphorylation. When 4E-BP1 becomes phosphorylated at specific sites it results in release of eIF4E from the inactive 4E-BP · eIF4E complex, which allows eIF4E to bind to eIF4G to form active eIF4F complex (27).

In our studies we used the above described steps for estimation of translation and activation of protein synthesis. We also measured phosphorylation of other key regulatory proteins involved in cell signaling such as mTOR and p70 s6kinase. mTOR is a downstream target in the PI3K/akt signaling pathway (among others), and integrates signaling from several stimuli related to energy status, hormones and amino acid availability. mTOR is either directly or indirectly responsible for phosphorylation of several substrates in the protein synthesis machinery including eIF4G, p70s6k and 4E-BP1. p70S6kinase can also phosphorylate several proteins including rpS6, eIF4B, S6K1 Aly/REF-like target (SKAR) as well as eukaryotic elongation factor 2 kinase. It therefore affects both the initiation and elongation of mRNA
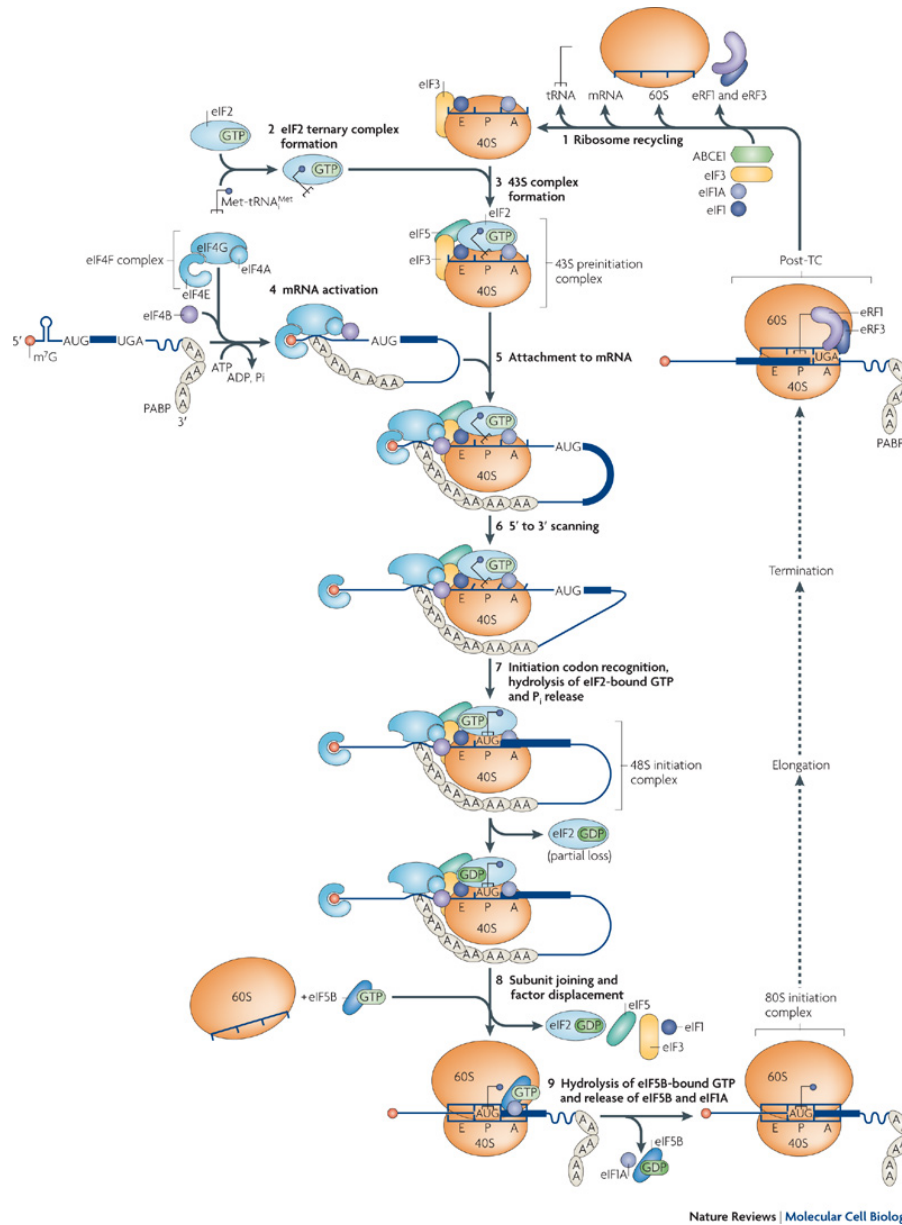
Fig 3. Schematic illustration of protein synthesis. The canonical pathway of eukaryotic translation initiation is divided into eight stages (**2**–**9**). These stages follow the recycling of post-termination complexes (post-TCs; **1**) to yield separated 40S and 60S ribosomal subunits, and result in the formation of an 80S ribosomal initiation complex, in which Met-tRNA$^{Met}_i$ is base paired with the initiation codon in the ribosomal P-site and which is competent to start the translation elongation stage. These stages are: eukaryotic initiation factor 2 (eIF2)–GTP–Met-tRNA$^{Met}_i$ ternary complex formation (**2**); formation of a 43S preinitiation complex comprising a 40S subunit, eIF1, eIF1A, eIF3, eIF2–GTP–Met-tRNA$^{Met}_i$ and probably eIF5 (**3**); mRNA activation, during which the mRNA cap-proximal region is unwound in an ATP-dependent manner by eIF4F with eIF4B (**4**); attachment of the 43S complex to this mRNA region (**5**); scanning of the 5′ UTR in a 5′ to 3′ direction by 43S complexes (**6**); recognition of the initiation codon and 48S initiation complex formation, which switches the scanning complex to a 'closed' conformation and leads to displacement of eIF1 to allow eIF5-mediated hydrolysis of eIF2-bound GTP and P$_i$ release (**7**); joining of 60S subunits to 48S complexes and concomitant displacement of eIF2–GDP and other factors (eIF1, eIF3, eIF4B, eIF4F and eIF5) mediated by eIF5B (**8**); and GTP hydrolysis by eIF5B and release of eIF1A and GDP-bound eIF5B from assembled elongation-competent 80S ribosomes (**9**). Translation is a cyclical process, in which termination follows elongation and leads to recycling (**1**), which generates separated ribosomal subunits. The model omits potential 'closed loop' interactions involving poly(A)-binding protein (PABP), eukaryotic release factor 3 (eRF3) and eIF4F during recycling, and the recycling of eIF2–GDP by eIF2B. Whether eRF3 is still present on ribosomes at the recycling stage is unknown.

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translation. However, it is still not known how amino acids influence mTOR signaling. Perhaps, various cells may use different mechanisms in such control events. Other cell signaling pathways, that influence translation is the RAS/MAPK pathway, which is responsible for phosphorylation of eIF4E and eIF4B (74, 75).

Branched chain amino acids (BCAA) are the most hydrophobic amino acids and inner parts of globular proteins mainly contain leucine, isoleucine and valine. Membrane proteins also need hydrophobic amino acids in transmemebrane domains for interactions with hydrocarbon chains in lipids. BCAA have other important functions in complex proteins as myosins, fibrinogen or keratins that contain coiled-coiled alpha helixes. Thus, many proteins in tissues and organs contain high proportions of BCAA and around 35% of our daily need of essential amino acids is BCAA, while most dietary proteins contain around 20% BCAA only (76). Branched chain amino acids, particularly leucine, are proposed to have special roles in amino acid signaling for protein synthesis as well as in other metabolic activities (77, 78). After digestion of food, BCAAs are preferentially taken up in skeletal muscles instead of being metabolized in the liver as most other amino acids. BCAA are catabolized by two common enzymatic steps of which the first is branched-chain aminotransferase (BCAT), which is low expressed in liver cells (79). Therefore, BCAAs appear in peripheral tissues in proportions to dietary intakes (80). BCAAs are rapidly oxidized in skeletal muscle cells and are more or less insulin independent for uptake and oxidation. Leucine may then be a metabolic alternative to glucose oxidation in conditions within severe insulin resistance (81).

Amino acid transporting

Translocation of amino acids into the cell from the interstitial fluid occurs via specific amino acid transporters (82) whereas the passage of amino acids from the blood stream to the interstitial fluid seems to be directed by diffusion, perhaps being a limiting step under some circumstances (83, 84). Functionally distinct amino acid transporting systems have been defined on the basis of amino acid selectivity. Some of the amino acid transporting systems are tissue specific but most of the transporters are ubiquitously expressed in cells (82). System A, Asc, N and L are the major amino acid transporting systems. Small, short chained amino acids are transported by system A or Asc, while branched chain and aromatic amino acids are transported by system L. Intracellular amino acid concentrations in skeletal muscle tissue are

higher than observed in extracellular fluids and active transport is used against amino acid concentration gradients into the cell (85, 86).

Cells can respond to changes in availability of nutritents by the control of activity in amino acid transporting systems. System A transporting can increase after both insulin and IGF-I stimulation and such hormone induced responses can be blocked by inhibitors of PI3K signaling (87). System A activity is also increased when cells are incubated in an amino acid free medium (87, 88). High concentrations of leucine in the medium can regulate system A transporting in the presence of other amino acids, but it requires at least 3 hours of incubation before system A transporting appears to be increased (89). Starvation induced increase of amino acid transporters usually results from increased Vmax with no change in Km of the transporters, where Vmax reflects the transporting capacity and Km the affinity to the transporter. Upregulation of transporting seems to be dependent on translocation of a pre-made pool of transporters to plasma membranes as well as depending on de novo synthesis (88). In the present studies we evaluated how provision of extracellular amino acids influenced mRNA content of the transporter Snat2. We found that the transcription of Snat2 (encoded by the gene slc38a2), was affected by refeeding muscle cells with amino acids in various combinations. Several amino acids such as glutamine, histidine, cystein and methionine are transported by Snat2, while branched chain- and the aromatic amino acids are transported by system L across cell membranes (82, 90). We found that Snat2 mRNA was increased in starved cells compared to cells refed by amino acids, consistent with increased system A transporting after amino acid withdrawal. Concentrations of Snat2 mRNA were decreased when cells were refed by groups of amino acids, as gln, his, lys, arg and thr only. Refeeding cells by branched chain amino acids also decreased Snat2 mRNA, although these amino acids are transported by system L, which is operating as an 1:1 amino acid exchanger that may couple influx of branched chain amino acids to efflux of cytoplasmatic amino acids such as glutamine (87). It is then possible that refeeding with branched chain amino acids caused efflux of other amino acids to the extent that extracellular concentrations of neutral amino acids increased enough to alter Snat2 mRNA levels. If so, Snat2 should be controlled by extracellular availability of either glutamine or histidine, since it was not changed by refeeding with cysteine or methionine, which are specific Snat2 substrates. Our present and others results, that Snat2 mRNA and transporting are up-regulated by amino acid deprivation, are however in contrast with a new report examining amino acid transporter expression in human skeletal muscle tissue after drinking a mix of essential amino acids. It was reported

that mRNA for amino acid transporters SNAT2/SLC38A2 and PAT1/SLC36A1 were transiently up-regulated one hour after ingestion of essential amino acids. SNAT2 mRNA expression was also followed by increased protein expression of SNAT2, 2-3 hours post ingestion of amino acids (91).

It has not yet been finally assessed how amino acids signal across cell membranes to elicit intracellular triggers for induction of translation initiation of proteins, although it has been discussed for decades whether there may be extracellular amino acid sensors or whether cells are sensitive to alterations of intracellular amino acid concentrations monitored in control of net protein balance. Phyllogenetically, it makes sense that extracellular amino acid concentrations should be communicated to the intracellular compartment when available for induction of protein synthesis. A simplistic model could be that amino acid transporters are sensitive to increasing levels of extracellular amino acids. If so, net influx will occur (6). This model is also proposed by Bohe who suggested that protein synthesis is regulated by extracellular and not intracellular amino acids based on the findings that intracellular levels of amino acids initially decreased when protein synthesis was stimulated (92). On the other hand, it has been suggested from cell culture experiments that intracellular amino acids, especially leucine levels, may be a trigger in cell signaling to protein synthesis (93). Recent research has however proposed amino acid transporters to function as hybrid transporter-receptors (tranceptors) whereby structural changes during amino acid transport cycles could be transduced to intracellular signaling pathways perhaps via PI3K kinase or JNK mediated signaling (85).

Clinical investigations

Even in the early era of clinical nutrition, it was observed that artificial nutrition was rather inefficient to promote re-synthesis of lost whole-body proteins, such as circulating albumin, intestinal mucosa, various immune proteins and skeletal muscles as well. Our own observations have particularly focused on the skeletal muscle compartments of chronically ill patients where cancer wastage represents a major group (2, 4, 94). Earlier, it was observed that both enteral and parenteral nutrition were inefficient to support net influx of amino acids across skeletal muscle beds (4, 95). These results were later on confirmed by findings that standard clinical nutrition effectively attenuated net break down of muscle proteins, although

it was considerably more difficult to initiate re-synthesis of overall whole-body proteins to net gains (5). In the light of such results amino acids may be a limiting component of intravenous feeding. If so, it is interesting to consider effects of different amino acid compositions in commercially available formulations. The scientific basis for such speculations is findings that amino acids in themselves stimulate muscle protein synthesis in skeletal muscles evaluated in human models (11, 96, 97). A potential role of amino acids was confirmed showing that primed constant infusions of amino acids at increasing dose rates were translated into increased net uptake of all amino acids across peripheral skeletal muscles beds in healthy volunteers. Such findings suggested that circulating arterial concentrations of amino acids were related to amino acid profiles in the infusion formulations as well as to dose rates of individual amino acids as significant factors. These observations may explain observed therapeutical inefficiency of clinical nutrition due to limitations in the amino acid availability (98). Based on this background it is important to consider how amino acid formulations should be designed to represent more optimal treatment (99). However, restricting prerequisites for industrial development of improved amino acid formulations are based on technical possibilities to create optimal amino acid formulations. Therefore, we decided to evaluate whether different commercially available amino acid solutions are actually translated into different amino acid profiles in arterial blood during infusion at steady-state conditions in critically ill patients. If not so, there should be little justification for modifications of amino acid profiles in parenteral feeding formulations.

Our results demonstrate that ICU patients had significantly lower over all amino acid blood concentrations in basal state conditions compared to fasted healthy volunteers (100). This may reflect that amino acid oxidation was significantly increased, since overall protein balance is usually negative in ICU patients (101). Thus, it obviously requires a considerable amount of amino acids to support both substrate oxidation and elevation of arterial concentrations to be high enough to switch net outflow of amino acids to net cellular inflow for protein synthesis, particularly in the large skeletal muscle pool. Amino acids were then provided at rates corresponding to 0.2 gN/kg/day. This rate has earlier been confirmed to switch net outflux to influx of amino acids across peripheral tissues in our healthy volunteers (6, 12). However, this dose rate of infusions to ICU patients did not increase overall blood amino acid concentrations higher than fasting plasma levels in healthy volunteers (Fig. 2, II). Therefore, it is not likely that such amino acid regimens should be enough to initiate translation of protein synthesis in skeletal muscles by amino acids only.

In our next study we evaluated initiation factor complexes after infusion of a constant and low dose of amino acids (~43 mg/kg/h) to patients scheduled for upper gastrointestinal surgery (18). Despite the fact that those patients had comparable plasma amino acid levels as our patients in study II they responded to the nutrition with alterations in initiation factor complexes and increased p70s6kinase phosphorylation. However, as discussed in the methods section, it may be questioned to what extent protein complex formation and protein phosphorylation changes really reflect protein synthesis activity. On one hand, numerous data from rats, mouse and pigs are available, which show that changes of initiation factors with increasing eIF4G·eIF4E and decreasing 4E-BP1·eIF4E complexes should be related to elevated protein synthesis. However, exceptions to these reports occur where increased and decreased protein synthesis was not reflected by changes of initiation factors in muscle tissue (102-104). Similar studies on human skeletal muscles are limited, but infusion of BCAA increased the amount of 4E-BP1 in the gamma form and increased phosphorylation of p70s6k without increased protein synthesis in skeletal muscles (105). However, infusion of mixed amino acids caused similar changes in 4E-BP1 phosphorylation simultaneously with increased protein synthesis (106). Therefore, it seems important to measure eIF4G complexes as performed in the present study.

In Study III we used a constant low dose amino acid infusion (~ 43 mg/kg/hr), which compares well with a low dose used by Bohé et al (43.5 mg/kg/hr) inducing a 30% increased mixed muscle protein synthesis (92). However, another study by Bohé et al indicated that muscle protein synthesis may became refractory despite continuous availability of amino acids (107). By contrast, our present results showed sustained activation of proteins for initiation of translation during 16 hours infusion of a low dose of amino acids (18). This difference may be explained by amino acid overloading secondary to a high dose infusion indicated by appearing ureagenesis as reported (107). Also, it is likely that activated phosphoproteins reflect continuously changing recruitment of various mRNAs for translation indicated by sustained formation of initiation complexes. Therefore, selection of investigative methods should always be considered with the specific aim in mind, where quantification of initiation complexes seems ideal for determination of directional changes of protein synthesis in non-steady state conditions particularly.

Insulin-like growth factor 1 (IGF-I)

Anabolic effects by IGF-I are well recognized, particularly such as stimulation of protein synthesis related to cell proliferation, tissue growth and cell differentiation (108, 109). The IGF system is comprised of two ligands, IGF-I and IGF-2, six binding proteins (IGFBPs 1-6) and the IGF-IR, which mediates most of the cellular signaling functions in this system (110). IGFs are transported in the circulation bound to the IGFBPs, which regulate its ability to bind to receptors. Following receptor binding, the receptor undergoes a conformational change leading to activation of its intrinsic tyrosine kinase, which autophosporylates additional tyrosines that act as docking site for IRS-1, a site for several signaling intermediates including p85; the regulatory subunit of PI-3-kinase (PI3K). PI3K activation leads to phospholipid generation in the plasma membrane, which recruits and activates AKT leading to activation of mTOR and p70s6kinase (p70s6K), which may induce increased protein synthesis. IGF-I ligand binding can also activate Shc/RAS/MAP kinase pathway leading to mitogenesis.

Original knockout studies of the IGFs showed that the development of most tissues and organs is controlled to some extent by the IGF system (111, 112). These studies showed that mice lacking either IGF-I or IGF-2 genes are born severely growth retarded, with bodyweight approximately ~60 % of wild-type mice. Mice lacking a functional IGF-IR were even smaller and died soon after birth (111). In the mid 1990s the Cre-lox P technique was introduced for inducible knockout of genes (113, 114) and made it possible to produce inducible tissue specific knockouts. In use of such mice it was found that the liver is the main producer of IGF-I in the circulation and that mice with post-natal hepatic deletion of the IGF-I gene grew almost normally, suggesting that local tissue IGF-I is the main determinant of post-natal body growth (19). Local IGF-I expression also seems to have a well-defined role in exercise related muscle growth and satellite cell proliferation (59, 108). Increased IGF-IR mRNA and IGF-I protein levels have been reported in response to exercise (115-117), although load-induced hypertrophy can occur in mice with non-functional IGF-IR (118), indicating that increased protein synthesis is not entirely dependent on signaling through the IGF-IR.

It has also been assumed that circulating IGF-I may be involved in promotion of diurnal alterations of muscle protein synthesis in response to meal feeding (119). Malnourished patients have usually decreased plasma IGF-I levels and both plasma IGF-I and muscle IGF-I mRNA can be influenced by diet (120). However, a complex situation, with protein bound

circulating and locally produced IGF-I makes it difficult to demonstrate defined roles for all these various factors, particularly when binding proteins themselves may have independent regulatory functions across muscle membranes (121, 122). Accordingly, earlier attempts to study nutritional effects by IGF-I have not provided unanimous and conclusive results (8, 11, 123). Injection of anti IGF-I antibodies before feeding attenuated a subsequent rise in protein synthesis by 25%, while ip. injections of IGF-I to overnight starved mice only increased protein synthesis marginally (11). Studies on genetically altered mice implied that fractional resynthesis of muscle protein was unrelated to circulating GH, blood IGF-I and locally tissue produced IGF-I mRNA (123). On the other hand, it has been reported that a one hour iv. infusion of IGF-I to overnight starved mice increased muscle protein synthesis to levels comparable to fed mice (124). Furthermore, reducing free plasma IGF-I to 50% of normal levels, by infusion of IGF binding protein 1, decreased muscle protein synthesis by 25% with decreased phosphorylation of p70s6k (125), and provision of IGF-I/IGFBP-3 complex improved sepsis induced muscle catabolism (126). Also, plasma IGF-I did not diverge from basal levels in humans who received a drink of essential amino acids at a time when muscle protein synthesis was stimulated by more than 100 % (127). By contrast, when IGF-I were infused directly into arm muscle beds, compared to systemically raised IGF-I, protein synthesis was only increased subsequently to locally infused IGF-I (128).

In our present experiments, we used liver IGF-I deficient mice to study activation of protein synthesis following feeding. We found no difference between liver IGF-I deficient and wild type mice regarding protein synthesis rate or translational control of protein synthesis in skeletal muscles. Both mouse groups showed increased protein synthesis rate after refeeding compared to overnight starvation and similar alterations in phosphorylation status of 4E-BP1, p70s6kinse, mTOR^{ser2448} and association of the 4E-BP1·eIF4E complex. Thus, effects by circulating IGF-I seemed less important for the control of translation initiation of protein synthesis compared to other possible factors such as plasma or extracellular amino acids. Both liver IGF-I knockouts and wild type mice showed increased mRNA expression of the IGF-IR during starvation. This increased receptor expression may thus reflect a positive feed back control of IGF-I signaling secondary to extra- or intracellular IGF-I close to membranes; a conclusion supported by observations in cultured cells with increased IGF-IR number and promoted amino acid transport in relationship to decreased extracellular concentration of amino acids, particularly of glutamine (6, 129). Consistent alterations of IGF-IR mRNA were observed in our experiments while IGF-I mRNA in skeletal muscle tissue seemed more

variable among starved and refed mice. Thus, IGF-IR expression may be more tightly controlled than tissue IGF-I expression in skeletal muscle metabolism in response to starvation – refeeding. There were no changes in either PI3K or AKT mRNA expression, while mTOR expression was up-regulated in starved IGF-I knockouts without apparent changes in protein phosphorylation. This up-regulation in starved knockouts may therefore reflect compensatory effects unrelated to phosphorylation of mTOR secondary to decreased circulating IGF-I. This condition would also explain why IGF-I expression did not decrease to the same extent in starved IGF-I knockouts as observed in wild type mice. Therefore, effects by circulating IGF-I seems less important for alterations of protein synthesis in response to feeding compared to other factors, for example increased flux of extracellular amino acids across cell membranes.

Insulin

For years it has been debated whether insulin is an important stimulator of overall protein synthesis in skeletal muscles, although it has been repeatedly shown in cell culture and animal experiments that supraphysiological levels of insulin stimulate protein synthesis independently of the presence of amino acids (130-132). Some human experiments have however made doubts on the role insulin may have in physiological settings to activate muscle protein synthesis (133-135). Also, we have earlier reported that knockout mice with overt diabetes displayed close to normal fractional synthesis rate of overall muscle proteins during re-feeding; observations that may also question an absolute or permissive role for insulin to support skeletal muscle protein synthesis (11). However, it is usually believed that insulin has a permissive role to support protein synthesis at feeding and other conditions as supported by a recent report on adult individuals, where reduced amino acid concentrations during increased nutrient and insulin availability blunted mTOR/S6K1 signaling in relationship to muscle protein synthesis (136-138). Therefore, at least amino acids are necessary for a physiological activation of mTOR/S6K1 and subsequent protein synthesis in human skeletal muscle (138).

The effect on protein synthesis by insulin was evaluated by different methods in our present studies. Incorporation experiments revealed that insulin at physiological levels did not clear cut stimulate overall phenylalanine incorporation into muscle proteins while pharmacological

concentrations (3 mU/ml) increased incorporation significantly, more so at normal than at low medium concentrations of amino acids, which was also confirmed by our Western blot analyses on initiation factors (17). These results indicate that pathways for initiation of translation of muscle proteins by insulin and amino acids may either in part be different (106, 139) or suggesting that insulin and amino acids may stimulate different proteins. Insulin may also control transcription either positively or negatively.

Mevalonate pathway and steroid hormones

Computer searches on Gene ontology (GO) categories revealed that amino acids had pronounced effects on steroid biosynthesis and lipid metabolism in skeletal muscle cells. Our present microarray experiments also indicated that a large number of transcripts (30%) across large parts of the genome were changed following amino acid provision. Cholesterol and steroid hormones are produced by enzymes in the mevalonate pathway (140) and it was interesting to find that most enzymes in this pathway were increased in skeletal muscle cells following amino acid provision to cell cultures. It was recently reported that skeletal muscle cells are capable of local synthesis of sex steroid hormones, where testosterone can be synthesized from dihydroepiandosterone (DHEA) in the circulation (141). Thus, testosterone may be synthesized from either cholesterol in cell membranes or through metabolism of DHEA by 17β-hydroxy-steroid dehydrogenases (140), which increased in L6 cells refed by amino acids. The expression of these hormones can also increase after exercise and may therefore represent an important part of anabolic responses to physical training in skeletal muscles (142). The first step in the mevalonate pathway is also a target in treatment with cholesterol lowering drugs as statins. Accordingly, lovastatin treatment of cells has been reported to inhibit protein synthesis rate and to induce phosphorylation changes of translation regulatory proteins (p70s6k, 4E-BP1 and eIF4E) (143). Well-known transcription factors for cholesterol and fatty acid metabolism, sterol regulatory element binding protein (SREBP) 1A and 1C, are also induced by activation of the PI3K/ AKT pathway or MAPkinase pathways by insulin and growth factors (144-147) This suggests additional functions in skeletal muscle cells at low rate of lipid synthesis (148, 149). Thus, amino acids may control skeletal muscle protein balance by several mechanisms related to both acute and more long-term alterations at feeding.

Concluding remarks

Our present studies represent different aspects with possible importance to the complex cell machinery for protein synthesis in skeletal muscles. Should there be any impact and possibility to improve nutritional treatment of patients in the need of nutritional support it must be possible to provide amino acids to skeletal muscles via the blood circulation in order to increase extracellular amino acid concentrations by the infused solutions as evaluated in our ICU and preoperative patients. However, not all provided amino acids appeared with increased concentrations in blood and some amino acids remained unchanged, although delivered at increased amounts. This shortcoming may hamper the effectiveness of intravenous nutrition. However, we confirmed that TPN infusions activated translation initiation of muscle proteins, which was indicated by changes of initiation factor complexes as well as increased p70s6 kinase phosphorylation as markers to reflect increased protein synthesis in muscle tissue. We also found that the amino acid transporter Snat2 mRNA was regulated by feeding cells with different combinations of amino acids. Changes in concentration of extracellular amino acids may thus be sensed by amino acid transporters, which may signal to muscle cells that extracellular amino acids are available for protein synthesis and intermediary metabolism.

IGF-I mRNA was measured as a representative for the influence of growth factors on protein synthesis in muscles. We found that IGF-I mRNA and IGF-IR mRNA were controlled by starvation-refeeding in muscle cells, but circulating IGF-I seemed to have a minor role in feeding induced upregulation of muscle protein synthesis. It was also possible to influence on cellular transcript levels of IGF-I by refeeding cultured L6 cells with amino acids (unpublished results) suggesting a possible link between extracellular amino acid concentrations and local IGF-I production within cells, although mechanisms are unclear. A most well-recognized signaling pathway for IGF-I effects is PI3K/AKT/mTOR. System A transporting is also connected to PI3kinase, supported by findings that pre-incubation of cells with a PI3kinase inhibitor prevented amino acid starvation induced changes in system A transporting (88). Likewise, leucine induced increase in system A transporting was possible to block by either PI3kinase or mTOR inhibitors (89). Also, in microarray experiments we found indications that mevalonate pathway metabolism was connected to the metabolism of amino acids in muscle cells. These observations support our speculations that anabolic reactions in

muscle tissue, secondary to amino acid metabolism, may be related to local muscle steroid production which makes sense.
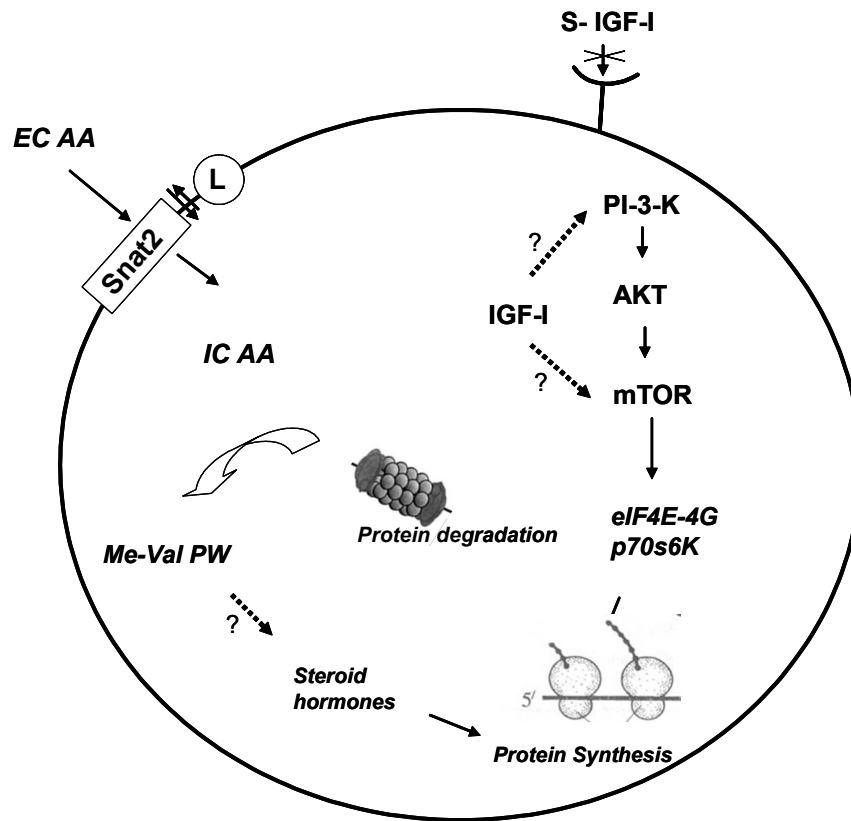

Fig 4. Possible interactions among amino acids, IGF-I and local muscle production of steroid hormones across well-recognized pathways for signalling to induce translation of initiation of muscle protein synthesis. Increased extracellular levels of amino acids interact with various amino acid transporters (Snat 2) for communication of altered flux of amino acids aimed to build proteins.

ACKNOWLEDGEMENTS

Min tid som doktorand har varit lång och varierande och min färd mot målet började delvis tidigare än jag formellt blev doktorand, då jag länge arbetat som laboratorieassistent på Kir Metabol Lab, där dessa studier utfördes. Många har bistått mig under den här tiden och jag är innerligt tacksam för all hjälp jag har fått.

Några personer vill jag dock ge ett speciellt tack. Först och främst min handledare, Professor **Kent Lundholm**, som uppmuntrade mig att bli doktorand. Trots min från början ganska avoga inställning till dessa studier, så upptäckte jag under tiden att jag tyckte det var rätt spännande och intressant trots allt. Tack, Kent, för all uppmuntran och alla kunskaper Du delat med dig av under åren. Jag vill också tacka **Elisabeth Svanberg**, som initialt var delaktig i mitt projekt, innan hon försvann mot vidare horisonter. Det var i Elisabeths sällskap jag steg in i initieringsfaktorernas förtrollande värld. Tack, Elisabeth, för många roliga diskussioner och stunder på lab. Många antikroppar gick åt innan det fungerade.

Tack, också till alla "korridorkollegor", såväl gamla som nya, för hjälp, vänskap och trevlig jobbsamvaro under åren. En hel del fikastunder, goda kakor och promenader i Botaniska trädgården hanns också med. Ett speciellt tack till; **Annika Gustafsson** och **Kristina Lagerstedt**, mina "doktorandkamrater", för frekvent lunchsällskap, många Genespring diskussioner och träningssällskap, när jag inte varit för lat; **Anita Olsson** och **Ulla Körner** för professionell hjälp med att hålla ordning på ord, siffror och patienter och dessutom för att Ni alltid har ett vänligt ord till övers; **Dick Delbro**, som alltid haft en bra historia att muntra upp mig med de gånger försöken inte gick så bra; **Karin Ahlman** för hjälp med qPCR och många muntra kommentarer; **Lilian Karlsson** för vänlig omtanke och sist men inte minst **Marianne Andersson** och **Christina Lönnroth**, mina mångåriga lab-kollegor, för mycket stöd och roliga stunder. Speciellt tack, för hjälp med microarrayerna.

Dessutom, ett varmt tack till mina medförfattare för bra samarbete, utan er hade det inte gått, då hade det varken blivit några patienter eller knockmöss i studierna.

Till sist, Tack, **Andreas, Rebecka och Matilda**. Vad vore jag utan Er…

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