

The role of amino acids in liver protein metabolism under a high protein diet : identification of amino acids signal and associated transduction pathways

Nattida Chotechuang

▶ **To cite this version:**

Nattida Chotechuang. The role of amino acids in liver protein metabolism under a high protein diet : identification of amino acids signal and associated transduction pathways. Food and Nutrition. AgroParisTech, 2010. English. NNT : 2010AGPT0026 . pastel-00610998

HAL Id: pastel-00610998
https://pastel.hal.science/pastel-00610998

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THESIS

submitted to obtain the degree of

Doctor of Philosophy

at

L'Institut des Sciences et Industries du Vivant et de l'Environnement (AgroParisTech)

Speciality: Nutrition Science

Presented and defended in public by

Nattida CHOTECHUANG

on 22nd March 2010

THE ROLE OF AMINO ACIDS IN LIVER PROTEIN METABOLISM UNDER A HIGH PROTEIN DIET: IDENTIFICATION OF AMINO ACIDS SIGNAL AND ASSOCIATED TRANSDUCTION PATHWAYS

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Acknowledgement

I would like to express my gratitude to all those who gave me the chance to complete my PhD.

Firstly, I would like to thank Prof. Daniel TOME, my thesis director, for giving me the great opportunity to conduct research in his lab and offering me the chance to do pre-doctoral research in Japan. This PhD opened a lot of doors for my career.

I wish to thank the members of the jury, Dr. Pierre FAFOURNOUX and Dr. Claude FOREST for accepting being the reporter as well as Pr. Bernard PORTHA and Dr. Tatiana STEILER for accepting being the examiners of my thesis.

I am heartily thankful to my supervisor Dr. Dalila AZZOUT-MARNICHE, whose help, guidance and support from the start to the finish enabled me to develop an understanding of the subject, whose encouragement helped me during the period of research and later of thesis writing, and also her patience to work with a Thai who could not understand French very well.

I acknowledge the Danone for their financial support to this project and the Royal Thai Government for my scholarship and funding to study in France. I am especially thankful to the office of Educational Affairs at Royal Thai embassy in Paris for their unwavering administrative help and support.

I am particularly indebted to Catherine CHAUMONTET for her help and suggestions in the research and writing of this thesis. I want to thank all my colleagues from the PNCA, especially Gilles Fromentin, Claire Gaudichon and Cécile Bos for all their help, support, interest and valuable hints, and the internship students: Stephanie, Catherine and Wafa, for their help.

In addition, I want to thank to my office mates Anne-Sophie, Fanny, Jessica, Magda, Wahiba and Nad (post-doctoral fellow at AgroParisTech) for their great moral support and the good time that we spent together.

Last, but not least, I would like to say a big thank you to my family and friends, especially my mother, for supporting and encouraging me to pursue this degree. I am also grateful to my grandmother and my father, who encouraged my interest in science starting from an early age. Even though they are not with us, they will forever occupy a special place in my heart. Moreover, I especially want to thank to Itthikorn, Wanalee, Darin, Pete, Pimpavadee, Wanrug, Pijika, Dalad, Areeya, Karima, Patharawan, Bruno, Guillaume, and all my Thai friends for their support, great company and all the fun I had outside work.

“The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.” William Bragg, Sr.

Abstract

High Protein (HP) intake improves glucose homeostasis and reduces weight gain, body fat mass, white adipose tissue and adipocyte size in rats. The metabolic adaptation is characterized by at least a decrease in hepatic lipogenesis and an increase in hepatic amino acid (AA) conversion into glycogen. However, the role of amino acids (AAs) in the control of these metabolic adaptations has not been studied, and the transduction pathways involved in the sensing of the increase in AA supply remain unclear. Therefore, the aim of our study was to understand the effect of AAs on translation and on proteolysis, to identify the transduction pathways involved in AA signaling and the AA or the groups of AAs involved in these effects, using both *in vivo* and *in vitro* approaches. Western blot analysis was performed on protein extracts to examine the phosphorylation state of the mammalian target of rapamycin (mTOR), adenosine monophosphate-activated protein kinase (AMPK) and general control non-depressible kinase 2 (GCN2) transduction pathways which may be involved in AA sensing and in the control of translation in liver. This study demonstrated that adaptation to HP diet was characterized by the stimulation of translation, at least for the initiation step in the liver. Using primary culture of hepatocytes, we showed that this activation required both high AA levels (at least for leucine alone or a branched-chain AA mixture) and insulin, as indicated by the increase of mTOR, 4E-BP1 and S6 phosphorylation and the decrease of AMPK and GCN2 phosphorylation. Using AICAR (AMPK activator) and rapamycin (mTOR inhibitor), we demonstrated that mTOR might not be the only regulator of 4E-BP1 and S6K1 (downstream targets of mTOR) in high AA conditions and that AMPK may also play an important role in their control. Moreover, the HP diet induced the inhibition of protein breakdown in the liver and these results were concomitant with a decrease of gene expression of the major components for both autophagy and the ubiquitin-proteasome system in liver. Subsequently, ubiquitinated protein in the liver was lower and both AAs and insulin were required for the down-regulation of ubiquitination. Indeed, mTOR and AMPK were also involved in the control of the ubiquitin proteasome system in the liver in response to the increase in AA and insulin concentrations. These results suggested that the control of the catabolic and anabolic pathways of protein metabolism was regulated by the same set of signals and mediated by the same transduction signaling pathways.

Resumé

La consommation d'un régime hyper protéique (HP) améliore l'homéostasie glucidique, le gain de poids, l'adiposité, en réduisant le tissus adipeux blanc et la taille des adipocytes. Les adaptations métaboliques dues à l'augmentation de l'apport protéique sont au moins caractérisées, au niveau du foie, par la diminution de la lipogenèse et l'augmentation de la conversion des acides aminés (AA) en glycogène. Cependant, le rôle des acides aminés dans le contrôle de ces adaptations métaboliques et des voies de transduction responsables de la transmission du signal « acides aminés » n'ont pas encore été élucidés. L'objectif de notre étude a été de déterminer l'effet de l'augmentation de l'apport en acides aminés sur la traduction et la protéolyse, et d'identifier les voies de signalisation impliquées dans la détection des acides aminés ainsi que l'acide aminé ou le groupe d'acide aminés responsable de ces effets, en utilisant des approches *in vivo* et *in vitro*. Les extraits protéiques ont été analysés par western blots pour examiner l'état de phosphorylation des protéines impliquées dans les voies de signalisation qui participent à la détection des AAs et à la régulation de la traduction, à savoir les voies: « mammalian target of rapamycin » (mTOR), « adenosine monophosphate-activated protein kinase » (AMPK) et « general control non-depressible kinase 2 » (GCN2). Cette étude a montré que l'adaptation à un régime de HP est caractérisée par la stimulation de la traduction dans le foie, au moins au niveau de l'étape d'initiation. Cette activation requiert à la fois la présence de fortes concentrations en AA (au moins la leucine ou des AAs à chaîne branchée) et d'insuline, comme l'indique l'augmentation de la phosphorylation de mTOR, 4E-BP1 et S6 et la diminution de la phosphorylation de l'AMPK et GCN2. L'utilisation de l'AICAR (activateur de l'AMPK) et de la rapamycine (inhibiteur de mTOR) nous a permis de montrer qu'en présence de fortes concentrations en AA et d'insuline, mTOR n'est pas le seul régulateur de 4E-BP1 et de la S6K1 (cibles de mTOR) et que l'AMPK peut également jouer un rôle important dans la régulation de leur état de phosphorylation. En outre, l'augmentation de l'apport protéique provoque une inhibition de la dégradation des protéines dans le foie et une diminution de l'expression des gènes codant les principales protéines du système autophagie et de l'ubiquitine-protéasome. En conséquence, les protéines sont moins ubiquitinées, donc moins dégradées. Les AAs et l'insuline semblent être les principaux régulateurs de la voie de protéolyse ubiquitine-protéasome et les voies mTOR et AMPK seraient les médiateurs des effets acides aminés et de l'insuline. Ces résultats suggèrent que le contrôle des voies cataboliques et anaboliques du métabolisme des protéines sont régulées par les mêmes signaux et font intervenir les mêmes voies de signalisation.

Key words:

Translation, Protein breakdown, Ubiquitination, amino acids, Leucine, Branched chain amino acids, insulin, AMPK, mTOR, GCN2, 4E-BP1, S6K1, S6 and eIF2

Mots clés :

Traduction, Protéolyse, Ubiquitination, acide aminés, Leucine, acide aminés à chaîne branchée, insuline, AMPK, mTOR, GCN2, 4E-BP1, S6K1, S6 et eIF2 .

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List of Abbreviations

4E-BP1:	Ekaryotic initiation factor 4E binding protein 1
4E-BP2:	Ekaryotic initiation factor 4E binding protein 2
4E-BP3:	Ekaryotic initiation factor 4E binding protein 3
5'-TOP mRNAs:	5' terminal oligopyrimidine tract
AA:	Amino acid
AAs:	Amino acids
AARE:	Amino acid response element-dependent transcription
AT :	Active transport
AAT:	Amino acid transporter
aa-tRNA:	Amino acyl-tRNA
ACC:	Acetyl CoA carboxylase
AICAR:	5-ainoimidazole-4-carbomide-1-β-D-ribofuranoside
Akt:	Also called Protein kinase B
AMP:	Adenosine monophosphate
AMPK:	AMP–activated protein kinase
AMPKα2-CA:	A constitutively active from of AMPKα2
ANS:	Anthranilic acid
ATF:	Activating transcription factor
ATF2:	Activating transcription factor 2
ATF3:	Activating transcription factor 3
ATF4:	Activating transcription factor 4
Atg:	Autophagy-related gene product
ATG:	Autophagy-related gene
Atg1:	Autophagy-related gene product 1
Atg3:	Autophagy-related gene product 3
Atg4:	Autophagy-related gene product 4
Atg5:	Autophagy-related gene product 5
Atg7:	Autophagy-related gene product 7
Atg8:	Autophagy-related gene product 8
Atg10:	Autophagy-related gene product 10

Atg12: Autophagy-related gene product 12

Atg13: Autophagy-related gene product 13

Atg16: Autophagy-related gene product 16

ATP: Adenosine triphosphate

AVO: Adheres voraciously to TOR2

AVO1: Adheres voraciously to TOR2 1

AVO2: Adheres voraciously to TOR2 2

AVO3: Adheres voraciously to TOR2 3

BCAA: Branched chain amino acids

BCH : α-aminoendobicyclo[2,2,1]heptane-2-carboxlic acid

BH_4: Tetrahydrobiopterin

CaM: Ca^{2+}/calmodulin

CaMKK2: Ca^{2+}/calmodulin-dependent protein kinase kinase2

cAMP: cyclic AMP

CBS: Crystathionine-β-synthase

CD98/LAT1: L-type amino acid transport 1

ChREBP: Carbohydrate response element-binding protein

CNS: Central nervous system

CO: Carbon monoxide

CoA: Coenzyme A

CP: Carbamoylphosphate

CPT-1: Carnitine palmitoyltransferase-1

CREB2: cAMP-response element binding protein 2

CVD: Cardiovascular disease

Cvt: Cytosol to vacuole targeting pathway

DCSAM: Decarboxylated S-adenosylmethionine

DNA: Deoxyribonucleic acid

E1: Ubiquitin-activating enzyme

E2: Ubiquitin conjugating enzyme

E3: Ubiquitin ligase

E4: Specific multiubiquitin chain-assembly factor

eEFs: Eukaryotic elongation factors

eEF1A: Eukaryotic elongation factor 1A

eEF1B: Eukaryotic elongation factor 1B

eEF2: Eukaryotic elongation factor 2

eEF2K: eEF2 kinase

eIFs: Eukaryotic initiation factors

eIF1: Eukaryotic initiation factor 1

eIF1A: Eukaryotic initiation factor 1A

eIF2: Eukaryotic initiation factor 2

eIF3: Eukaryotic initiation factor 3

eIF4A: Eukaryotic initiation factor 4A

eIF4B: Eukaryotic initiation factor 4B

eIF4E: Ekaryotic initiation factor 4E

eIF4F: Eukaryotic initiation factor 4F

eIF4G: Eukaryotic initiation factor 4G

eIF5: Eukaryotic initiation factor 5

EPN: Epinephrine

ER: Endoplasmic reticulum

eRFs: Eukaryotic release factors

eRF1: Eukaryotic release factor 1

eRF3: Eukaryotic release factor 3

FAS: Fatty acid synthase

GβL: G protein β-subunit-like protein

G6Pase: Glucose 6-phosphatase

GABA: γ-aminobutyric acid

Gap: General amino acid permease

Gap1p: General amino acid permease 1p

GCN2: General control nonderepressible 2

GDP: Guanosine diphosphate

GFR: Growth factor receptor

GS: Glycogen synthase

GSK3: Glycogen synthase kinase3

GTP: Guanosine triphosphate

GTPase: Guanosine triphosphatase

Gtr1p: Yeast Ras-like small GTPase 1

Gtr2p: Yeast Ras-like small GTPase 1

HMB: β-hydroxy-β-methylbutyrate

HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A reductase

HP: High protein

hVps15: Mammalian vascuolar protein sorting 15

hVps34: Mammalian vascuolar protein sorting 34

i.p.: intraperitoneal

ICE: Interleukin-β-converting enzymes.

IGF: Insulin-like growth factor

IR: Intracellular receptor

IRS-1: Insulin receptor substrate-1

KOG1: Kontroller of Growth-1

LCMT1: Leucine carboxyl methyltransferase 1

LKB1: Tumor suppressor LKB1 kinase

LST8: Lethal with SEC13 protein 8

LYAATs: Lysosomal amino acid transporters

MCD: Malonyl-CoA decarboxylase

MeAIB: α-methylaminoisobutyric acid

Met-tRNAi: Initiator methionyl-transfer RNA

mRNA: Messenger RNA

mSin1: Mammalian stress-activatied protein1

mTOR: Mammalian target of rapamycin

mTORC1: mTOR complex 1

mTORC2: mTOR complex 2

NAD: Nicotinamide adenine dinucleotide

NADP: Nicotinamide adenine dinucleotide phosphate

NAG: N-acetylglutamate

NAS: N-acetylserotonin

NEPN: Norepinephrine

NMDA: N-methyl-D-aspartate

NO:	Nitric oxide
NOS:	NO synthase
NP:	Normal protein
ODC:	Ornithine decarboxylase
P-4E-BP1	4E-BP1 phosphorylation
P-ACC	ACC phosphorylation
P-AMPK	AMPK phosphorylation
P-eIF2α	eIF2α phosphorylation
P-GCN2	GCN2 phosphorylation
P-mTOR	mTOR phosphorylation
P-S6	S6 phosphorylation
P5C:	Pyrroline-5-carboxylate
PABP:	Poly (A) binding protein
PATs:	Proton assisted amino transporters
PB:	Protein breakdown
PE:	Phosphatidylethanolamine
PEPCK:	Phosphoenolpyruvate carboxykinase
PI3K:	Class I phosphoinositide 3-kinase
PIP_3:	Phosphatidylinositol 3,4,5-triposphate
PKA:	Protein kinase A
PKB:	Protein kinase B
PME:	Protein phosphatase methylesterase
PP:	Protein phosphatase
PP1:	Protein Phosphatase 1
PP2A:	Protein phosphatase-2A
PP2C:	Protein phosphatase-2C
PRAS40:	Proline-rich Akt/PKB substrate 40kDa
PRR5:	Protein rich 5 [renal]
PS:	Protein synthesis
PTG:	Protein target to glycogen
Rab:	Small GTP binding protein isolated from rat brain
Rab5:	Rab GTPase 5

Rab7:	Rab GTPase 7
Rags:	Small GTPases in the Ras superfamily
Ras:	Small GTP-binding protein Ras
Rheb:	Ras homolog enriched in brain
RNA:	Ribonucleic acid
RTKs:	Receptor tyrosine kinases
S6:	Ribosomal protein S6
S6K1:	p70 ribosomal S6 protein kinase
SEC:	*S. cerevisiae* protein involved in secretion
siRNA:	Small interfering RNA
SNAT2:	Sodium-coupled neutral amino acid transporter 2
SREBP-1c:	Sterol regulatory element-binding protein-1c
Tau-Cl:	Taurine chloramine
TOR:	The Ser/Thr kinase target of rapamycin
TOR1:	The Ser/Thr kinase target of rapamycin 1
TOR2:	The Ser/Thr kinase target of rapamycin 2
TORC2:	CREB activity2
tRNA:	Transfer RNA
TSC:	Tuberous sclerosis complex
TSC1:	Tuberous sclerosis complex 1
TSC2	Tuberous sclerosis complex 2
TZDs:	Thiazolidinedioned
Vid:	Vacuolar import and degradation pathway
ZMP:	AMP analogue 5-aminoimidazole-4-carbonmide riboside

List of amino acids

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Cys	Cysteine
Gln	Glutamine

Glu	Glutamic acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Tau	Taurine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

Publications and Communications

Publications

Publication 1: mTOR, AMPK and GCN2 coordinate the adaptation of hepatic energy metabolic pathways in response to protein intake in the rat. (2009)

AJP Endocrinol. Met., 297: E1313–E1323.

Nattida Chotechuang, Dalila Azzout-Marniche, Cécile Bos, Catherine Chaumontet , Nicolas Gausserès, Tatiana Steiler**,** Claire Gaudichon and Daniel Tomé.

Publication 2: Down-regulation of the ubiquitin-proteasome proteolysis system in response to amino acids and insulin involves the AMPK and mTOR pathways in rat liver hepatocytes. *Submitted to PloS ONE.*

Nattida Chotechuang, Dalila Azzout-Marniche, Cécile Bos, Catherine Chaumontet, Claire Gaudichon, and Daniel Tomé.

Scientific communications

Oral Communications

- **The down regulation of ubiquitin-proteasome proteolysis system in response to amino acids and insulin involves AMPK and mTOR pathways in rat liver hepatocytes.**

  Nattida Chotechuang, Dalila Azzout-Marniche, Cécile Bos, Catherine Chaumontet, Claire Gaudichon, and Daniel Tomé.

  Oral communication at Experimental Biology Annual Meeting, April 2010, California, USA. (Abstract published in The FASEB Journal 2010 ; 24, 97.3).

- **mTOR, AMPK and GCN2 coordinate the adaptation of hepatic energy metabolic pathways in response to amino acids and insulin.**

  Daniel Tomé, **Nattida Chotechuang**, Dalila Azzout-Marniche, Catherine Chaumontet, Cécile Bos, Nicolas Gausserès and Claire Gaudichon.

  Oral communication at Experimental Biology Annual Meeting, April 2009, New Orleans, USA. (Abstract published in The FASEB Journal 2009 ; 23, 228.2).

- **Both stimulation of mTOR and inhibition ofGCN2 and AMPK are involved in the stimulation of protein translation in response to high protein diet**.

  Nattida CHOTECHUANG, Dalila Azzout-Marniche, Cécile Bos, Catherine Chaumontet, Nicolas Gausserès, Sophie Vinoy, Daniel Tomé and Claire Gaudichon

  Oral communication at 10th European Nutrition Conference, July 2007 in Paris, France. (Abstract published in Annual Nutrition Metabolism 2007 ;51:62)

Poster Comunications

- **Both branched-chain amino acids and insulin are required for stimulation of translational control in rat primary hepatocyte culture.**

  Nattida Chotechuang, Dalila Azzout-Marniche1, Catherine Chaumontet,

  Daniel Tomé and Claire Gaudichon.

  Poster communication at 19th International Congress of Nutrition, October, 2009, Bangkok, Thailand

- **mTOR, AMPK and GCN2 coordinate the adaptation of hepatic energy metabolic pathways in response to amino acids and insulin.**

  Nattida CHOTECHUANG, Dalila Azzout-Marniche, Catherine Chaumontet, Nicolas Gausserès, Tatiana STEILER, Claire Gaudichon and Daniel Tomé.

  Poster communication at Biochemical Society Focused Meeting “mTOR signaling, Nutrients and Disease”, September 2008, Oxford, United Kingdom.

- **AMPK phosphorylation is decreased in response to amino acids and glucose in Caco-2 intestinal cells**.

  Nattida chotechuang, Catherine Chaumontet, Dalila Azzout-Marniche[1], Claire Gaudichon[1], Nicolas Gausserès, Sophie Vinoy and Daniel Tomé

  Poster communication at Experimental Biology Annual Meeting, April, 2007, Wahington DC, USA. (Abstract published in The FASEB Journal 2007; 21, 289.7)

- **Un régime riche en protéines induit une diminution des taux de synthèse et dégradation protéiques hépatiques sans effet majeur sur les autres tissus chez le rat.**

  Nattida Chotechuang, Cécile Bos, Dalila Azzout-Marniche, Laure Chevalier, Catherine Luengo, Daniel Tomé, Claire Gaudichon.

  Poster communication at 6èmes Journées Francophones de Nutrition, 2007, Nice, France.

GENERAL INTRODUCTION

General Introduction

Previous work in our lab has shown that a high-protein (HP) diet (50% energy as protein) improved glucose homeostasis and reduced weight gain, body fat mass, white adipose tissue and adipocyte size (Lacroix et al. 2004 and Blouet et al. 2006) whereas no change was observed for lean mass (Jean et al. 2001 and Pichon et al. 2006). The modulation of both energy metabolism and protein kinetics by a HP diet is mainly attributable to adaptations in hepatic protein metabolism (Blouet et al. 2006; Azzout-Marniche et al. 2007 and Chevalier et al. 2009). These adaptations were characterized by a decrease in hepatic lipogenesis (Pichon et al. 2006) and an increase in hepatic amino acid (AA) conversion into glycogen (Azzout-Marniche et al. 2007). These effects are at least due to the ability of high physiological concentrations of amino acids (AAs) to regulate gene expression of gluconeogenic enzymes (Azzout-Marniche et al. 2007). Tissue-specific differences in both protein contents and fluxes revealed an enlarged hepatic protein pool in HP rats which was associated with a strong fed-state inhibition of synthesis (-39%) (Chevalier et al. 2009).

However, even if the metabolic consequences of HP diet changes have been studied, the role of AAs in the control of these metabolic adaptations remains unclear. It was reported that AAs can control the protein phosphorylation cascades of some signaling pathways and regulate the gene expression at the level of transcription, translation and post-translational steps (Wu 2009). However, the involvement of these transduction pathways in response to the increase of AA supply in the metabolic processes remains to be determined. Moreover, AAs are important regulators of both protein synthesis and degradation but little is known about the effect of increase of dietary protein intake and thus AA supply on protein breakdown as well as the molecular mechanisms involved in the control of proteolysis.

This present study investigated the molecular mechanisms involved in the effect of a HP diet on protein metabolism in the liver. We aimed to investigate the precise effect of high AA levels on translation and proteolysis. Furthermore, we tried to determine the transduction

pathways involved in AA signaling and the AA or the groups of AAs involved in these effects. In the first part of the manuscript, we present a review of the literature. In the second part, we present the results we obtained and finally we discuss and conclude about the significance of our findings.

PART I: SCIENTIFIC BACKGROUND

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I: Amino acid and Protein Metabolism

1. Amino acid metabolism

1.1. Digestion of dietary proteins

After feeding, the dietary protein was digested in the stomach by pepsin which hydrolysed ingested protein at the peptide bonds to cleave the polypeptide chains into a mixture of smaller peptides. The digestion continues in the small intestine by trypsin, chymotrypsin, carboxypeptidase A and B and aminopeptidase. The free AAs or small peptides then enter the blood capillaries in the villi and travel to the liver (Freeman et al. 1979).

The intestine is the primary site of AAs and peptide absorption. AAs are transported into enterocytes by facilitated diffusion or specific transport systems with sodium as a carrier, that take up AAs against a concentration gradient (Stevens et al. 1984; Lerner 1987). The transport systems are specific to groups of AAs rather than to individual AAs. In addition to the AA transporters, enterocytes have an active transport system for di- and tri-peptides, independent of the one for free AAs (Adibi et al. 1976; Webb 1990). AAs arising from dietary protein digestion or from protein degradation are constantly re-synthesized or used in protein turnover. Unrequired or damaged proteins are targeted for destruction. Some AAs are used for protein synthesis in the liver (constitutive or exported plasma proteins). The branched chain amino acids (BCAA) are, along with other AAs, required for the stimulation of total liver protein synthesis (Anthony et al. 2001b). However, when protein intake increased, no change in protein synthesis rate was observed despite an increase in the tissue protein pool (Chevalier et al. 2009). The effect on protein breakdown remained unknown. Furthermore, the AA surplus may be used as metabolic fuel or converted to other compounds. The main fates of the carbon skeletons remaining from AA deamination may be their use for cellular respiration, fatty acids or ketone bodies synthesis, or gluconeogenesis

whereas the amino groups are used for the biosynthesis of AAs, nucleotides and biological amines or are converted to urea for excretion through the urea cycle (Figure 1) (Shambaugh 1977; Brosnan 2003). Ureogenesis is an important process to protect the body from potentially toxic ammonium (Shambaugh 1977; Dimski 1994; Ding et al. 1997).

1.2. Interorgan amino acid metabolism

The functions of interorgan AA traffic are to maintain the relatively constant extracellular AA concentration in which tissues are bathed and to provide AAs for protein synthesis and those used in specific functions. AAs serve as the building blocks for proteins and some of them that exceed the body's needs for protein synthesis undergo oxidative degradation through transamination and desamination (Brosnan 2003). The liver is the main organ where many different proteins are synthesized (Brosnan 2003). For example, albumin is synthesized in the liver of a healthy adult human and 20g/day is secreted. This albumin is catabolized in the peripheral tissues, suggesting that about 20g of AAs are made available each day as a result of albumin catabolism (Maxwell et al. 1990).

The rate of AA uptake by tissues or organs depends on the activity of several transporters. In mammals, there are different AA transporters which are referred to as transport systems**.** Free AAs are transported across membranes through Na^+-independent (facilitated transport) or Na^+-dependent (secondary active transport) systems. Nomenclature of AA transport systems have letter designations based on their preferred AA substrates and the presence or absence of the requirement for sodium ion activation and co-transport (Mailliard et al. 1995), as shown in Table 1.

Table 1. AA transport systems of mammalian cells (Hyde et al. 2003)

(ai) Neutral-amino-acid transporters: sodium-dependent

System	Protein	Gene	Amino acid substrates (one-letter code)	Notes
A	SNAT1	SLC38A1	Gly, Ala, Ser, Cys, Gln, Asn, His, Met, Thr, Me-AIB, Pro, Tyr, Val	Short-chained-neutral-amino-acid transport.
	SNAT2	SLC38A2	Gly, Pro, Ala, Ser, Cys, Gln, Asn, His, Met, Me-AIB	Sensitive to low pH. Ubiquitous expression.
	SNAT3	SLC38A4	Gly, Pro, Ala, Ser, Cys, Asn, Met, His, Lys, Arg	SNAT3 may also function as a Na^+-independent cationic amino acid transporter.
ASC	ASCT1	SLC1A4	Ala, Ser, Cys	High-affinity short-chain-amino-acid exchanger. Ubiquitous expression.
	ASCT2	SLC1A5	Ala, Ser, Cys, Thr, Gln	
B^o	ASCT2	SLC1A5	Ala, Ser, Cys, Thr, Gln, Phe, Trp, Tyr	Broad substrate specificity. Expressed on apical surface of many epithelia.
BETA	GAT1	SLC6A1	GABA	Widely expressed Cl^--dependent GABA, betaine and taurine
	GAT2	SLC6A13	GABA, betaine, Pro, β-Ala transporters.	
	GAT3	SLC6A11	GABA, betaine, Tau	
	BGT1	SLC6A12	GABA, betaine	
	TAUT	SLC6A6	Tau	
Gly	GLYT1	SLC6A9	Gly, sarcosine	
	GLYT2	SLC6A5	Gly, sarcosine	Na^+- and Cl^--dependent high-affinity glycine transport. Expressed in brain and some non-neural tissues.
IMINO	–	–	Pro	Na^+-dependent epithelial proline transporter, inhibited by Me-AIB.
N	SN1	SLC38A3	Gln, Asn, His	Li^+-tolerant transport of Gln, Asn and His. H^+ antiport.
	SN2	SLC38A5	Gln, Asn, His, Ser, Gly	Li^+-intolerant variants described
N^m	–	–	Gln, Asn, His	
N^b	–	–	Gln, Asn, His	
PHE	–	–	Phe, Met	Brush-border transporter for Phe and Met
PROT	PROT	SLC6A7	Pro	Proline transporter in central nervous system.

(aii) Neutral-amino-acid transporters: sodium-independent

System	Protein	Gene	Amino acid substrates	Notes
asc	Asc1	SLC7A10	Gly, Ala, Ser, Cys, Thr	Small neutral AA exchanger.
	Asc2		Gly, Ala, Ser, Thr	
imino	PAT1/LYAAT1	SLC36A1	Pro, Gly, Ala, β-Ala, GABA, Me-AIB	H^+-coupled transport of small neutral amino acids. Inhibited by Me-AIB.
	PAT2/LYAAT2	SLC36A2	Pro, Gly, Ala, β-Ala, GABA Me-AIB	
L	LAT1	SLC7A5	His, Met, Leu, Ile, Val, Phe, Tyr, Trp, Gln	Ubiquitously expressed exchanger for large hydrophobic amino acids.
	LAT2	SLC7A8	Ala, Ser, Cys, Thr, Asn, Gln, His, Met, Leu, Ile, Val, Phe, Tyr, Trp	
T	TAT1	SLC16A10	Phe, Tyr, Trp	Aromatic-amino-acid transporter. H^+/monocarboxylate transporter family – insensitive to pH, however.

(bi) Anionic-amino-acid transporters: sodium-independent

System	Protein	Gene	Amino acid substrates	Notes
X^-_{AG}	EAAT1	SLC1A3	Glu, Asp	Widespread Glu and Asp transporter. K^+ antiport.
	EAAT2	SLC1A2	Glu, Asp	Substrate-dependent uncoupled anion flux.
	EAAT3	SLC1A1	Glu, Asp, Cys	Lack of stereospecificity toward Asp.

	EAAT4	SLC1A6	Glu, Asp	
	EAAT5	SLC1A7	Glu, Asp	

(bii) Anionic-amino-acid transporters: sodium-independent

System	Protein	Gene	Amino acid substrates	Notes
x^{-c}	xCT	SLC7A11	Glu, Cystine	Electroneutral Glu/cystine exchanger.
–	XAT2	–	Glu, Asp	Non-functional upon 4F2hc/rbAT heavy-chain co-expression. Predicted to associate with a novel glycoprotein.

(ci) Cationic-amino-acid transporters: sodium-dependent

System	Protein	Gene	Amino acid substrates	Notes
$B^{o,+}$	ATB(o,+)	SLC6A14	Lys, Arg, Ala, Ser, Cys, Thr, Asn, Gln, His, Met, Ile, Leu, Val, Phe, Tyr, Trp	Blastocysts and possibly brush-border membrane. Broad specificity for neutral and cationic amino acids. Accepts BCH.
y^+L	y+LAT1	SLC7A7	Lys, Arg, Gln, His, Met, Leu	Na^+-dependent cationic/neutral-amino-acid exchanger.
	y+LAT2	SLC7A6	Lys, Arg, Gln, His, Met, Leu, Ala, Cys	Electroneutral.

(cii) Cationic-amino-acid transporters: sodium-independent

System	Protein	Gene	Amino acid substrates	Notes
$b^{o,+}$	b(o,+)AT	SLC7A9	Lys, Arg, Ala, Ser, Cys, Thr, Asn, Gln, His, Met, Ile, Leu, Val, Phe, Tyr, Trp, Cystine	Broad-specificity cationic- and neutral-amino-acid exchanger.
y^+	Cat-1	SLC7A1	Arg, Lys, His	Cationic-amino-acid (and Na^+-dependent neutral-amino-acid) transport. Variable degree of *trans*-stimulation.
	Cat-2	SLC7A2	Arg, Lys, His	
	Cat-3	SLC7A3	Arg, Lys	
	Cat-4	SLC7A4	Unknown	

The interconversion of AAs trough transamination is an important process in the transport of ammonium and to maintain acid-base balance (Wu 2009). These processes converge on the central catabolic pathways, with the removal of the α-amino groups from the carbon skeleton. This was catalyzed by enzymes called aminotransferases or transaminases and then the carbon skeletons of most AAs found their way to the citric acid cycle (Metzler et al. 1982). Almost all AAs can be metabolized in the liver and it is the organ with urea cycle (Brosnan 2003).

Several non-essential AAs, including glutamine, glutamate and aspartate, are oxidized by epithelial cells in the mammalian small intestine and they do not enter the portal vein (Stoll et al. 1998; Wu 1998). The small intestine uptakes the glutamine as the major fuel and nitrogenous products derived from glutamine metabolism are released into the portal vein. These include the alanine and proline which are metabolized by the liver. Moreover, the output from the small intestine also includes citrulline which is taken up and converted to

arginine in the kidney (Wu 1997; Wu and Morris 1998) (Figure 2). The kidney plays a major role in the interorgan metabolism of citrulline, arginine, glycine, and glutamine. It takes up glycine and releases serine. In addition, the kidney uptakes glutamine which is the substrate for urinary ammonia production and it contributes in this way to the maintenance of acid-base homeostasis (Brosnan 2003) (Figure 2).

Glutamate and glutamine play critical roles in these transaminations. In the cytosol of hepatocytes, the amino groups from most AAs are transferred to α-ketoglutarate to form glutamate. The glutamate serves as an amino group donor for biosynthetic pathways or excretion pathways that lead to the elimination of nitrogenous products. Glutamate is then transported from cytosol into mitochondria, where the amino group is removed to form NH_4^+ via oxidative deamination promoted by L-glutamate dehydrogenase (Figure 3). The α-ketoglutarate formed from glutamate deamination can be used for energy production in the citric acid cycle and for glucose synthesis in the liver and kidney (Brosnan 2003). The ammonium ion is converted to urea for excretion through the urea cycle, which is distributed between the mitochondrial matrix and cytosol of hepatocytes (Shambaugh 1977).

The AAs in the liver can be transaminated and degraded to other citric acid cycle intermediates and acetyl CoA or oxaloacetate which can in turn be oxidized for energy supply or converted to glucose or fat (Figure 4). However, the oxidation of AAs produces much more ATP than the liver could actually use. Therefore, it seems that the carbon skeletons of these AAs are not completely oxidized in the liver and are converted to glucose via gluconeogenesis even in the fed state (Jungas et al. 1992). During starvation, hepatic gluconeogeneis plays an important role in the production of glucose for the brain and other glucose dependent organs and the AAs from the muscle proteolysis are the major precursors for this process (Brosnan 2003). The liver also synthesizes glutathione from glutamate, glycine, and cysteine for use by extrahepatic cells (including immunocytes) and tissues (Figure 2) (Wu 2009).

Given that the bulk of the body's protein is in the form of muscle proteins, this tissue will apparently play a critical role in the interorgan AA metabolism. The skeletal muscle is the major organ for the catabolism of BCAA and released both alanine and glutamine from BCAA

and α-ketoglutarate. This alanine is taken up by the liver and converted to glucose (the glucose-alanine cycle). Thus alanine is one of the important molecules in the transport of amino groups to the liver without increasing blood ammonia concentrations (Figure 2 and 4) (Brosnan 2003; Wu 2009)

2. Protein metabolism and regulation by nutritional conditions

Protein and AA metabolism is a large, dynamic and regulated process that accomplishes a variety of physiological functions. In adult humans, some 300 g of new protein is synthesized per day for maintenance, and an equivalent amount of protein is degraded to their constituent AAs. In eukaryotes, the half-lives of proteins vary from minutes to many days (Goldberg and St John 1976; Mayer and Doherty 1986). For example, in the rat liver, proteins might turn over once every one to two days, while some regulatory enzymes have half-lives only 15 minutes. Furthermore, the more stable proteins, such as actin and myosin in skeletal muscle, might turn over once every one or two weeks. In human, hemoglobin can remain for the entire lifetime of an erythrocyte (3 months) (Lecker et al. 1999). The overall process of protein synthesis and protein degradation is referred to as protein turnover. The rates of protein turnover may vary depending on the intracellular and extracellular environmental conditions, including the availability and balance of nutrients to which cells are exposed, and the hormones and the peptide factors that bind to receptors on cell surfaces or within the cell. It has been established that alterations in dietary macronutrient intake greatly affected the balance between tissue protein synthesis and protein degradation (Darmaun 1999). Since AAs serve as the currency of protein metabolism, they are hydrolyzed from protein via proteolysis systems and serve as the building blocks for new protein synthesis. Therefore, protein cell homeostasis is maintained by a precise balance between the overall rates of synthesis and degradation (Lecker et al. 1999).

In mammals, changes in nutrient availability induce changes in the levels of hormones to adapt the metabolism. Protein synthesis requires both AAs, both as precursors, and a substantial amount of metabolic energy. Maintaining the essential AA supply is

necessary to maintain the optimal rate of protein synthesis in both the liver and skeletal muscle. Deprivation of even a single essential AA causes a decrease in the cellular protein synthesis by inhibition of the initiation phase of mRNA translation (Kimball 2002).

There is evidence that protein synthesis was stimulated in muscle and in liver by 38 and 41%, respectively when rats were fed a diet containing 20% protein whereas no change was observed in rats fed no added protein (Yoshizawa et al. 1998). Moreover, plasma insulin concentrations were the same in rats fed either diet, suggesting that feeding-induced changes in plasma insulin are not sufficient to stimulate protein synthesis. Both dietary protein and insulin may be required to stimulate translation initiation (Yoshizawa et al. 1998). However, Kimball reported that insulin alone can activate the translation at the initiation step (Kimball and Jefferson 2006a). Moreover, AAs, especially BCAA, stimulated the protein synthesis in primary hepatocytes (Dubbelhuis and Meijer 2002; Ijichi et al. 2003) whereas in the livers of rats fed a high protein diet for 2 weeks, the protein synthesis rate was decreased (Chevalier et al. 2009) A slight inhibition of synthesis rates after the high protein diet was observed in the kidney while protein synthesis rates were significantly increased in stomach and skin. These results suggested that the adaptation to high protein diet was tissue specific (Chevalier et al. 2009). Furthermore, the reduction of protein levels in diets (20.7%, 16.7% or 12.7%) decreased the protein synthesis in the pancreas, liver, kidney and muscle in piglets receiving these diets for 2 weeks (Deng et al. 2008). Muscles play a role as a protein reservoir. Skeletal muscle is also the main organ of BCAA catabolism. There is evidence that carbohydrate restricted-with high protein diet, during 7 days, stimulated muscle protein synthesis (Harber et al. 2005). In humans, increasing protein ingestion resulted in an increase in protein synthesis (up to 20%) and a decrease in protein breakdown after adaptation for 7 days to higher protein intake (Motil et al. 1981; Hoerr et al. 1993; Gibson et al. 1996; Fereday et al. 1998; Forslund et al. 1998; Harber et al. 2005). However, the acute protein intake resulted in only slight increase of protein synthesis (around 8%) and greater decrease in its breakdown (Gibson et al. 1996; Forslund et al. 1998; Cayol et al. 1997; Fereday et al. 1998). Essential AAs and BCAA (especially leucine) specifically modulate protein synthesis by activating the initiation of translation (Anthony et al. 2001a; Anthony et al. 2001b; Yoshizawa 2004; Crozier et al. 2005). In rats *in vivo*, infusion of the BCAA

stimulated muscle protein synthesis and essential AAs maintained this effect (Kobayashi et al. 2006).

Protein degradation is also regulated by nutrition (Kettelhut et al. 1988). High amino acid concentrations and insulin are the main inhibitors of protein degradation, whereas glucagon and low concentrations of amino acids are the principal stimulators (Gelfand and Barrett 1987; Flakoll et al. 1989; Mortimore et al. 1989; Kadowaki et al. 1992; Blommaart et al. 1997; Boirie et al. 1997; Balage et al. 2001; Kanazawa et al. 2004; Waterlow 2006; Capel et al. 2008). Under acute feeding, proteolysis is inhibited while a chronic increase in protein intake, induced proteolysis in the fed state (Price et al. 1994; Forslund et al. 1998). In the post-absorptive state, whole body protein degradation varies only very slightly (Price et al. 1994; Forslund et al. 1998). In specific tissues, only a few studies have examined the response of protein degradation to increased protein intakes (Taillandier et al. 1996; Bolster et al. 2002; Harber et al. 2005). Liver proteolysis is known to be inhibited by insulin (Duckworth et al. 1994; Hamel et al. 1997; Bennett et al. 2000; Bennett et al. 2003; Kanazawa et al. 2004) and stimulated by glucagon (Schworer and Mortimore 1979; Mortimore et al. 1989). AAs also act as a negative feed- back regulator for proteolysis in the perfused rat liver (Poso et al. 1982; Mortimore et al. 1989; Kadowaki et al. 1992; Miotto et al. 1992) and isolated hepatocytes (Mortimore and Schworer 1977; Seglen et al. 1980). In muscle, numerous publications have described the stimulation of muscle protein breakdown in response to fasting, followed by an inhibition after re-feeding with a normal diet. Muscle protein breakdown is activated in response to one or more days of starvation (Medina et al. 1991; Wing and Banville 1994; Wing et al. 1995). Several AAs also have a direct regulatory affect on proteolysis: Leu, Gln, Tyr, Phe, Pro, Met, Trp and His in the liver and Leu in the skeletal muscle (Kadowaki and Kanazawa 2003; Meijer and Dubbelhuis 2004; Oshiro et al. 2007).

2.1. Protein synthesis

Protein synthesis is one of the most complex biosynthetic processes. In eukaryotes, almost 300 macromolecules cooperate to synthesize polypeptides. These macromolecules consist of over 70 different ribosomal proteins, 20 or more enzymes to activate the AA precursors, a dozen or more auxiliary enzymes and other protein factors for the initiation, elongation and termination of polypeptides, perhaps 100 additional enzymes for the final processing of different proteins and 40 or more kinds or transfer and ribosomal RNAs.

First, the production of polypeptides follows the process of transcription, the production of messenger RNA (mRNA) from a gene's nucleotide sequence which involves several steps. It consists of transcription initiation, elongation, RNA–processing reactions, e.g. capping and splicing, and termination. Second, the transcription is followed by the transport of mRNA to the cytosol where mRNA is decoded into protein by the translation. The mRNA transported the genetic code into the cytosol in the form of codon which is a triplet of nucleotides that codes for a specific AA. A specific first codon in the sequence of mRNA establishes an open reading frame. The reading frame is set when translation of an mRNA molecule begins and is maintained as the synthesis machinery reads sequentially from one triplet to the next. Several codons serve special functions such as the initiation codon, AUG, which signals the beginning of a polypeptide in all cells, in addition to coding for methionine residues of polypeptides. Moreover, there are the termination codons (also called stop codons or nonsense codons), UAA, UAG and UGA, which normally signal the end of polypeptide synthesis.

The three major stages of translation are:

1. initiation
2. elongation
3. termination

2.1.1. Initiation (Figure 5)

In eukaryotes, the initiation begins by the assembly of a complex from initiator methionyl-transfer RNA (met-tRNAi), 40S and 60S ribosomal subunits, with the aid of eukaryotic initiation factors (eIFs), into an 80S ribosome at the initiation codon of mRNA.

In the first step of translation initiation, the eukaryotic initiation factor 2 (eIF2) binds GTP and met-tRNAi, selected from the pool of tRNAs, to form the ternary complex (eIF2●GTP●met-tRNAi) and then binds to the 40S ribosomal subunit with other eIFs (eIF1, eIF1A, eIF3 and eIF5) to form the 43S preinitiation complex (Kapp and Lorsch 2004) (Figure 5). The eIF1, 1A and 3 promote the dissociation of 80S ribosomes (Kapp and Lorsch 2004). The eIF4F, including eIF4A, eIF4B, eIF4E and eIF4G, bind mRNA by a mechanism involving the initial recognition of the m7G cap at 5'-end of mRNA by eIF4E (Gingras et al. 1999). Then, mRNA binds to the 43S complex by the association of eIF3 and eIF4G (Gross et al. 2003; Prevot et al. 2003). The 43S complex scans along the mRNA in a 5' to 3' direction towards the initiation codon, base-paired with the anti-codon of met-tRNAi (Lopez-Lastra et al. 2005). Moreover, eIF1 interacts with the eIF1A to promote scanning of the start codon (Pestova and Kolupaeva 2002). There is another factor, the poly (A) binding protein (PABP), facilitating mRNA binding to the 43S complex. PABP interacts with eIF4G to circularise mRNA by linking the 5' cap and poly(A) tail in a "closed loop" (Figure 5). This association stimulates mRNA binding to the 43S complex, by enhancing eIF4F binding to the capped 5' end of mRNA (Kahvejian et al. 2005). Initiation ends when the initiation factors are released from the complex and the 60S ribosomal subunit joins to form the 80S ribosomal (initiation complex) and leave met-tRNAi in the ribosomal P site (Lopez-Lastra et al. 2005).

2.1.2. Elongation (Figure 6)

The elongation phase involves three distinct steps that are repeated many times during the formation of a polypeptide chain. The order of AAs is specified by the sequence of codons in the mRNA. Moreover, each AA is specific to its cognate tRNA to form amino acyl-tRNA (aa-tRNA) for which there is one aa-tRNA synthase per aa-tRNA pair (Guth and

Francklyn 2007). The elongation cycle requires the eukaryotic elongation factors (eEFs), including eEF1and eEF2, to catalyse this process. First, the eEF1A picks the aa-tRNA in the presence of GTP, and then the aa-tRNA●eIF1A●GTP complex enters the empty A-site on a ribosome. The anticodon of the incoming aa-tRNA needs to be matched against the mRNA codon positioned in the A-site. As the three bases in the codon can be arranged in 64 different combinations, the translational machinery must be able to select the aa-tRNA carrying the matching anticodon. When the correct three-base anticodon forms a complementary base pair with the codon on mRNA, the GTP is hydrolyzed leading to eEF1A●GDP dissociating from aa-tRNA. The resulting of eEF1A●GDP binds to eEF1B complex which facilitates exchange of GDP to GTP on eEF1A. The eEF1A●GTP now is ready to accept the next aa-tRNA. In the second step, a peptidyl transferase reaction catalysed by the ribosome itself occurs immediately after the accommodation of the correct aa-tRNA in the ribosomal A-site. The growing polypeptide in the ribosomal P-site is linked to the new AA in the A-site via a peptide bond. The reaction leaves an empty tRNA in the ribosomal P-site and the new peptidyl-tRNA in the A-site. The last step is the translocation, which promotes the ribosome's translocation along the mRNA by the length of one codon. Translocation is catalysed by the eEF2 and subsequent GTP hydrolysis. After the translocation, the ribosome is in the position of having an empty tRNA in the E site, the peptidyl-tRNA in the P site, and the next codon of mRNA in the A site, available for interaction with a new aa-tRNA. These reaction steps are repeated until the ribosome encounters an in-frame stop-codon. At this point, the translation is terminated (Kasinath et al. 2006; Frank et al. 2007; Groppo and Richter 2009; Ling et al. 2009).

2.1.3. Termination (Figure 6)

The final step is termination which involves the release of the polypeptide chain from mRNA. The three stop codons (UAA, UAG and UGA), the eukaryotic release factors (eRFs) and one GTP are required. The eRF1 recognizes one of three stop codons and binds to the ribosome in the place of a tRNA (Kisselev et al. 2003). This event along with binding of the eRF3, facilitates eRF1 stop codon recognition and stimulates GTP hydrolysis to release the polypeptide chain (Salas-Marco and Bedwell 2004; Fan-Minogue et al. 2008).

2.1.4. Regulation of translation at the initiation step

Translation is an important regulatory step in cellular protein synthesis. It is not only a metabolic pathway, but also a signaling pathway because most regulation of protein synthesis occurs at translation. In addition, the dominant mechanism of control of global protein synthesis occurs via the phosphorylation/dephosphorylation of the translation components, primarily of initiation and elongation factors (Sonenberg and Hinnebusch 2009). It is established that AAs are important factors in the regulation of intracellular signal transduction pathways involved in the control of translation. The essential AAs have been found to regulate signaling which modulate mRNA translation through the binding of met-tRNAi to the 40S ribosomal subunit to form the 43S preinitiation complex and the binding of mRNA to the 43S preinitiation complex (Kimball and Jefferson 2005).

The first regulated step of translation at the initiation step involves the binding of met-tRNAi to the 40S ribosomal subunit to form the 43S preinitiation complex by the phosphorylation of the α-subunit of eIF2. In the later step of initiation, the bound GTP of eIF2 is hydrolyzed to GDP, and the eIF2●GDP binary complex is released from the ribosome. To reform the active ternary complex, eIF2 binds to met-tRNAi, and the GDP is exchanged to GTP. This guanine nucleotide exchange reaction is catalyzed by a second initiation factor eIF2B. The mechanism for regulating eIF2B activity is through phosphorylation of the α-subunit of eIF2. The phosphorylation of eIF2α converts it from a substrate into a competitive inhibitor of eIF2B, effectively sequestering eIF2B into an inactive complex. Because translation of essentially all mRNA begins with met-tRNAi, the phosphorylation eIF2α results in a decline in the synthesis of almost all proteins (Kimball 2002).

The second regulated step in translation initiation involves the binding of mRNA to 43S ribosomal subunit, a reaction mediated by the initiation factor referred to as eIF4F. The active complex of eIF4F at 5' cap of the mRNA is regulated by the reversible interaction of eIF4E and one of its binding proteins (4E-BP1, 4E-BP2 or 4E-BP3) (Raught et al. 2000). For example, the binding of 4E-BP1 and eIF4E to eIF4G is mutually exclusive. Because the binding

of eIF4E to the 40S ribosomal subunit occurs through its interaction with eIF4G, binding of 4E-BP1 to eIF4E prevents binding of the eIF4E-mRNA complex to the 40S ribosomal subunit. The association of 4E-BP1 with eIF4E is controlled by phosphorylation of 4E-BP1. The phosphorylated 4E-BP1 does not bind to eIF4E. Thus the decrease of the association of eIF4E with eIF4G represses protein synthesis (Kimball 2002).

The latter regulated step involves the p70 ribosomal S6 protein kinase (S6K1), which phosphorylates the ribosomal protein S6 (S6), a component of the 40 ribosomal subunit. The function of S6 phosphorylation is thought to be in promoting the translation of the set of mRNAs that posses a 5' terminal oligopyrimidine tract (5'-TOP mRNAs). The 5' TOP encodes the translation machinery components such as ribosomal proteins and other translation factors and up–regulates their translation, thus the phosphorylation of S6 probably leads to an increase in the capacity of intracellular protein synthesis (Meyuhas 2000; Proud 2004).

2.2. Protein Degradation

The regulation of protein metabolism is essential for proper cellular function, and is a balance between synthesis and degradation. All cells possess multiple pathways for protein degradation. However, in the liver, there are at least two major degradation pathways: lysosomal system and the ubiquitin proteasome pathway.

2.2.1. Lysosomal Pathway

Long-lived proteins and some organelles are believed to be degraded within lysosomes (Dunn 1994; Klionsky and Ohsumi 1999). These lysosomes contain several acid-optimal proteases, including cathepsins B, H and D, and many other acid hydrolases (Lecker et al. 1999). There are at least three different pathways for lysosomal protein degradation: Cvt (cytosol to vacuole targeting pathway), Vid (vacuolar import and degradation pathway) and autophagy (Yang et al. 2005). Autophagy is a ubiquitous physiological process that occurs in all eukaryotic cells (Reggiori and Klionsky 2002). There are three primary forms of

autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (Klionsky 2005). Macroautophagy is the most prevalent form. It involves the formation of cytosolic double membrane vesicles. Initially a “C” shape double membrane structure appears in the cytoplasm, and then both ends of this membrane grow and close to form the vesicle, termed an autophagosome, that wraps the bulk of the cytoplasm and some organelles. Then, the autophagosome fuses with the lysosomal membrane, resulting in an inner vesicle (autophagic body) entering the lysosome/vacuole. The autophagic body is delivered into the lumen of the degradative compartment to degrade and carry constituent components that can be recycled (Yoshimori 2004; Klionsky 2005). Microautophagy has not been well characterized in mammalian cells. In this pathway, the lysosomal membrane itself invaginates, and then finally pinches off to form an internal vacuolar vesicle containing the materials derived from cytoplasm (Mortimore et al. 1988). Chaperone-mediated autophagy involves direct translocation of the targeted proteins (only cytosolic proteins) across the lysosomal membrane without vesicle-mediation (summarized in Figure 7) as performed in the other two processes. It is also a secondary response to starvation (Massey et al. 2004).

Autophagy is basically a non-specific degradation process. However, it may select to eliminate its targets in some cases, such as injured or excrescent peroxisomes, endoplasmic reticulum (ER) and mitochondria (Elmore et al. 2001). Molecular genetic studies in yeast have identified some of the components required for autophagosome formation (Klionsky et al. 2003). The autophagy-related genes and the products of these genes are named ATG and Atg, respectively. Around 31 genes involved in the autophagy has been identified (Suzuki et al. 2007), among of which 18 ATG genes, mostly conserved in the higher eukaryotes i.e. mammals (Yoshimori and Noda 2008), are essential for the autophagosome formation (Noda et al. 2009). The Atg1 is a serine/threonine protein kinase and its activity is essential for autophagy even though its substrate is not known (Yang et al. 2005). Moreover, Atg13 is hyperphosphorylated under growing conditions and dephosphorylated form can bind to Atg1 to simulated its kinase activity, which is a good candidate to induce autophagy, although no mechanism has been reported (Kamada et al. 2000). The other Atg proteins, involved in autophagosome formation are the two ubiquitin-like conjugation systems: Atg8-phosphatidylethanolamine (PE) and Atg12-Atg5. In fact one half of Atg genes are involved in these conjugation systems and these two systems are well conserved in eukaryotes. The

Atg8-PE system includes 4 Atg proteins: Atg3, Atg4, Atg7 and Atg8. The processed Atg8 is activated by Atg7, an ubiquitin-activating E1 like enzyme, and then is transferred to Atg3, an ubiquitin-activating E2-like enzyme. Finally the C-terminal glycine of Atg8 is conjugated to the amino group of PE. The Atg8-PE is deconjugated by Atg4 (Ichimura et al. 2000; Noda et al. 2009) (Figure 9). The model of Atg12-Atg5 conjugation comprised 5 proteins: Atg5, Atg7, Atg10, Atg12 and Atg16. Atg12 is activated by Atg7 as in the Atg8, and is then transferred to Atg10, an ubiquitin conjugating E2-like enzyme (Mizushima et al. 1998; Shintani et al. 1999). In the final step, the C-terminal glycine in Atg12 is conjugated to the side chain of Lysine-149 of Atg5 and the Atg12-Atg5 further form the complex with a multimeric protein, Atg16 with the Atg5 noncovalent interaction (Mizushima et al. 1999; Mizushima et al. 2003) (Figure9). It seems that this ubiquitin-like system is essential to autophagosome formation and is a constitutive process, since the formation of the Atg12-Atg5 conjugate is not dependent upon the starvation or other autophagy-inducing conditions (Mizushima et al. 1999).

2.2.2. The ubiquitin Proteasome System

The ubiquitin proteasome pathway is the other major piece of cytosolic protein degrading machinery responsible for the breakdown of most short- and long-lived proteins in mammalian cells (Rock et al. 1994) and plays an important role in the control of the degradation of specific proteins (Kadowaki and Kanazawa 2003). This pathway includes two main steps. The first step is the covalent attachment of the polyubiquitin chain to the substrate followed by the specific recognition of this signal and degradation of the ubiquitinylated protein by the 26S proteasome (Attaix et al. 2001).

Ubiquitin is a 76-amino-acid globular protein that is highly conserved throughout eukaryotes, with only three amino-acid changes from yeast to humans (Weissman 2001). Ubiquitination is a multiple step process (Ciechanover et al. 2000; Jesenberger and Jentsch 2002). Firstly, ubiquitin is activated to a high energy thiol ester intermediate by the ubiquitin-activating enzyme (E1), then E1 transfers ubiquitin to one of the ubiquitin conjugating enzymes (E2), which also forms a thiol ester linkage between the active site

cysteine and ubiquitin. Ubiquitin ligases (E3) catalyse the transfer ubiquitin from E2 to the substrate and builds up an isopeptide linkage between the activated C-terminal glycine residue of ubiquitin and the ε-amino group of lysine residue of an accepter protein. The formation of polyubiquitin chains is usually achieved by the transfer of additional activated ubiquitin moieties to the Lys48 of the precedent conjugated ubiquitin molecule (Attaix et al. 2001; Weissman 2001). The specific multiubiquitin chain-assembly factor (E4) has been described recently and is involved in polyubiquitin chain elongation (Figure10) (Koegl et al. 1999). Finally, the polyubiquitinated substrate of the 26S proteasome is composed of two 19S regulatory complexes attaches at each side to the barrel shaped 20S catalytic complex. The polyubiquitinated substrate binds to the ubiquitin receptor subunit of 19S and then is degraded to short peptides with the release of free and reusable ubiquitin (Ciechanover et al. 2000). In some cases, E2 binds the first ubiquitin molecule to protein substrates, resulting in the monoubiquitinylated protein, which is usually not a degradation target of the proteasome but it is targeted for endocytosis, resulting in proteolysis in the lysosome(Hicke 1999). Moreover, this alternative type of ubiquitin modification can regulate the mammalian receptors at the plasma membrane, transcriptional control and DNA repair (Hicke 1999; Pickart 2001; Weissman 2001).

Degradation of protein substrates by the ubiquitin system appears to be mediated by specific degradation signals. Previous study revealed that the substrate recognition of ubiquitination process might be explained by the specific interaction of substrate with the E2/E3 complex like the recognition of appropriate tRNAs by the aminoacyl-tRNA synthetases (Laney and Hochstrasser 1999). Another study explained that the E3 ligases recognized its substrates based on the presence of a specific ubiquitination signal at the N-terminal of protein(Varshavsky 1997; Pickart 2001). Furthermore, it is established that the protein substrates were targeted for ubiquitin protein ligases through the phosphorylation via the specific signal transduction cascades in response to environmental status (Yaron et al. 1997; Winston et al. 1999).

Moreover, recent findings indicate that nutrients, such as AAs and fatty acids, and hormones have effects on this protein degradation pathway (Hamel et al. 2004). In long-term control, AAs and hormones, such as glucocorticoids, well known to cause catabolic

breakdown, regulate the expression of ubiquitin system components. AAs downregulate the mRNA levels of the essential components, such as E2 and the proteasome subunit C9 (Adegoke et al. 2003). Conversely, glucocorticoids increase the expression of ubiquitin (Marinovic et al. 2002) and a number of 19S regulatory complexes (Combaret et al. 2004). However, the Ub proteasome pathway can also be regulated in the short-term, involving the regulation of proteasome activity, to respond more quickly to nutritional states and hormone levels (Hamel et al. 2004).

2.2.3 Other cytosolic proteolytic systems

The ubiquitin proteasome pathway is the major proteolysis process in cytosol, however other cytosolic proteolytic systems also exist in mammalian cells. One is the Ca^{2+} activated (ATP-independent) proteolytic process, which involves the cysteine proteases, termed calpains (Mellgren 1987). There are two types of calpain, one fully active at Ca^{2+} concentrations below 1 mM (calpain type I) and the other which requires at least 1 mM Ca^{2+} (calpain type II) for full activity (Mellgren 1980; Kishimoto et al. 1981). Other important cytosolic proteases are the caspases or interleukin-β-converting enzymes (ICE). The various caspases were activated in response to a variety of toxic stimuli (e.g. DNA damage), leading to programmed cell death (Salvesen and Dixit 1997). These enzymes cleave proteins after aspartic residues. Moreover, it is established that calpains and caspases were shown to be activated to degrade the cytoskeletal protein, i.e. the neuronal cytoskeletal protein αII-spectrin (Warren et al. 2007).

II: Amino acids and their sensing elements

1. Functions of amino acids

Besides the role as building blocks of proteins and polypeptides, some AAs regulate key metabolic pathways, which are essential to maintenance growth, reproduction and

immunity in organisms. Therefore, maximizing the efficiency of food utilization enhances protein accretion, reduces adiposity and improves health. (Wu et al. 2007b; Wu et al. 2007a; Suenaga et al. 2008; Wu 2009). High physiological concentrations of portal blood AAs are able to counteract glucagon-induced liver glucose 6-phosphatase (G6Pase) but not phosphoenolpyruvate carboxykinase (PEPCK) gene expression (Azzout 2007). These suggest that AAs are not only nutrients but are also the cell signaling molecules which can control the protein phosphorylation cascades and the gene expression (Wu 2009) (Table 2). AAs regulate the gene expression at several steps, including transcription, translation and post-translational modifications (Figure 11) (Wu 2009). Moreover, AAs control translation on a global scale and also act to control preferential changes in the translation of mRNAs encoding particular proteins or families of proteins. However, AAs do not directly modulate the function of translation initiation and elongation factors, but they regulate signaling through the transduction pathways involved in mediating hormone action (Kimball and Jefferson 2006a) and it is also established that BCAA are particularly effective in promoting protein synthesis (Kimball and Jefferson 2002).

Table2: Major metabolites and functions of AA in nutrition and metabolism(Wu 2009).

Amino acids	Products	Major functions
Amino acids	Directly	Protein synthesis; osmolytes; regulation of hormone secretion, gene expression and cell signaling
Alanine	Directly	Inhibition of pyruvate kinase and hepatic autophagy; gluconeogenesis; transamination; glucose–alanine cycle
β-Alanine	Directly	A component of coenzyme A and pantothenic acid
	Dipeptides	Carnosine (β-alanyl-L-histidine), carcinine (β-alanyl-histamine), anserine (β-alanyl-1-methyl-L-histidine), and balenine (β-alanyl-3-methyl-histidine) with antioxidative function
Arginine	Directly	Activation of mTOR signaling; antioxidant; regulation of hormone secretion; allosteric activation of NAG synthase; ammonia detoxification; regulation of gene expression; immune function; activation of BH_4 synthesis; N reservoir; methylation of proteins; deimination (formation of citrulline) of proteins[a]
	NO	Signaling molecule; regulator of nutrient metabolism, vascular tone, hemodynamics, angiogenesis, spermatogenesis, embryogenesis, fertility, immune function, hormone secretion, wound healing, neurotransmission, tumor growth, mitochondrial biogenesis, and function
	Agmatine	Inhibition of NOS, ODC, and monoamine oxidase; ligand for α_2-adrenergic and imidazoline receptors
	Ornithine	Ammonia detoxification; syntheses of proline, glutamate, and polyamines; mitochondrial integrity; wound healing
	Methylarginines	Competitive inhibition of NOS

Amino acids	Products	Major functions
Asparagine	Directly	Cell metabolism and physiology; regulation of gene expression and immune function; ammonia detoxification; function of the nervous system
	Acrylamide[b]	Oxidant; cytotoxicity; gene mutation; food quality
Aspartate	Directly	Purine, pyrimidine, asparagine, and arginine synthesis; transamination; urea cycle; activation of NMDA receptors; synthesis of inositol and β-alanine
Citrulline	Directly	Antioxidant; arginine synthesis; osmoregulation; ammonia detoxification; N reservoir
Cysteine	Directly	Disulfide linkage in protein; transport of sulfur
	Taurine	Antioxidant; regulation of cellular redox state; osmolyte
	H_2S	A signaling molecule
Glutamate	Directly	Glutamine, citrulline, and arginine synthesis; bridging the urea cycle with the Krebs cycle; transamination; ammonia assimilation; flavor enhancer; activation of NMDA receptors; NAG synthesis
	GABA	Excitatory neurotransmitter; inhibition of T-cell response and inflammation
Glutamine	Directly	Regulation of protein turnover through cellular mTOR signaling, gene expression, and immune function; a major fuel for rapidly proliferating cells; inhibition of apoptosis; syntheses of purine, pyrimidine, ornithine, citrulline, arginine, proline, and asparagines; N reservoir; synthesis of NAD(P)
	Glu and Asp	Excitatory neurotransmitters; components of the malate shuttle; cell Metabolism; ammonia detoxification; major fuels for enterocytes
	Glucosamine-6-P	Synthesis of aminosugars and glycoproteins; inhibition of NO synthesis
	Ammonia	Renal regulation of acid–base balance; synthesis of glutamate and CP
Glycine	Directly	Calcium influx through a glycine-gated channel in the cell membrane; purine and serine synthesis; synthesis of porphyrins; inhibitory neurotransmitter in CNS; co-agonist with glutamate for NMDA receptors
	Heme	Hemoproteins (e.g., hemoglobin, myoglobin, catalase, and cytochrome *c*); production of CO (a signaling molecule)
Histidine	Directly	Protein methylation; hemoglobin structure and function; antioxidative dipeptides; one-carbon unit metabolism
	Histamine	Allergic reaction; vasodilator; central acetylcholine secretion; regulation of gut function
	Urocanic acid	Modulation of the immune response in skin
Isoleucine	Directly	Synthesis of glutamine and alanine; balance among BCAA
Leucine	Directly	Regulation of protein turnover through cellular mTOR signaling and gene expression; activator of glutamate dehydrogenase; BCAA balance; flavor enhancer
	Gln and Ala	Interorgan metabolism of nitrogen and carbon
	HMB	Regulation of immune responses
Lysine	Directly	Regulation of NO synthesis; antiviral activity (treatment of Herpes simplex); Protein methylation (e.g., trimethyllysine in calmodulin), acetylation, ubiquitination, and *O*-linked glycosylation
	OH-lysine	Structure and function of collagen

Amino acids	Products	Major functions
Methionine	Homocysteine	Oxidant; independent risk factor for CVD; inhibition of NO synthesis
	Betaine	Methylation of homocysteine to methionine; one-carbon unit metabolism
	Choline	Synthesis of betaine, acetylcholine, phosphatidylcholine, and sarcosine
	Cysteine	Cellular metabolism and nutrition
	DCSAM	Methylation of proteins and DNA; polyamine synthesis; gene expression
	Taurine	Antioxidant; osmoregulation; organ development; vascular, muscular, cardiac, and retinal functions; anti-inflammation
	Phospholipids	Synthesis of lecithin and phosphatidylcholine cell signaling
Phenylalanine	Directly	Activation of BH_4 (a cofactor for NOS) synthesis; synthesis of tyrosine; neurological development and function
Proline	Directly	Collagen structure and function; neurological function; osmoprotectant
	H_2O_2	Killing pathogens; intestinal integrity; a signaling molecule; immunity
	P5C	Cellular redox state; DNA synthesis; lymphocyte proliferation; ornithine, citrulline, arginine and polyamine synthesis; gene expression; stress response
	OH-proline	Structure and function of collagen
Sarcosine	Directly	An intermediate in the synthesis of glycine from choline; possible treatment of certain mental disorders; a source of formaldehyde and H_2O_2; inhibition of glycine transport
Serine	Directly	One-carbon unit metabolism; syntheses of cysteine, purine, pyrimidine, ceramide and phosphatidylserine; synthesis of tryptophan in bacteria; gluconeogenesis (particularly in ruminants); protein phosphorylation
	Glycine	Antioxidant; one-carbon unit metabolism; neurotransmitter
	D-Serine[c]	Activation of NMDA receptors in brain
Theanine	Directly	An amino acid (glutamine analog) in tea leaves; antioxidant; increasing levels of GABA, dopamine, and serotonin in brain; neuroprotective effect
Threonine	Directly	Synthesis of the mucin protein that is required for maintaining intestinal integrity and function; immune function; protein phosphorylation and *O*-linked glycosylation; glycine synthesis
Tryptophan	Serotonin	Neurotransmitter; inhibiting production of inflammatory cytokines and superoxide
	NAS	Inhibitor of BH4 synthesis; antioxidant; inhibition of the production of inflammatory cytokines and superoxide
	Melatonin	Antioxidant; inhibition of the production of inflammatory cytokines and superoxide
	ANS	Inhibiting production of proinflammatory T-helper-1 cytokines; preventing autoimmune neuroinflammation; enhancing immune function
	Niacin	A component of NAD and NADP, coenzymes for many oxidoreductases
Tyrosine	Directly	Protein phosphorylation, nitrosation, and sulfation
	Dopamine	Neurotransmitter; regulation of immune response
	EPN and NEPN	Neurotransmitters; cell metabolism

Amino acids	Products	Major functions
	Melanin	Antioxidant; inhibition of the production of inflammatory cytokines and superoxide
Valine	Directly	Synthesis of glutamine and alanine; balance among BCAA
Arg and Met	Polyamines	Gene expression; DNA and protein synthesis; ion channel function; apoptosis; signal transduction; antioxidants; cell function; cell proliferation and differentiation
Arg, Met, and Gly	Creatine	Antioxidant; antiviral; antitumor; energy metabolism in muscle and brain; neurological and muscular development and function
Cys, Glu, and Gly	Glutathione	Free radical scavenger; antioxidant; cell metabolism (e.g., formation of leukotrienes, mercapturate, glutathionylspermidine, glutathione–NO adduct and glutathionylproteins); signal transduction; gene expression; apoptosis; cellular redox; immune response
Gln, Asp, Gly, and Ser	Nucleic acids	Coding for genetic information; gene expression; cell cycle and function; protein and uric acid synthesis; lymphocyte proliferation
	Uric acid	Antioxidant; the major end product of amino acid oxidation in avian species
Lys, Met, and Ser	Carnitine	Transport of long-chain fatty acids into mitochondria for oxidation; storage of energy as acetylcarnitine; antioxidant

ANS anthranilic acid, *BCAA* branched-chain AA, *BH* $_4$ tetrahydrobiopterin, *CNS* central nervous system, *CP* carbamoylphosphate, *CVD* cardiovascular disease, *DCSAM* decarboxylated *S*-adenosylmethionine, *EPN* epinephrine, *GABA* γ-aminobutyrate, *HMB* β-hydroxy-β-methylbutyrate, *NAG N*-acetylglutamate, *NAS N*-acetylserotonin, *NEPN* norepinephrine, *NOS* NO synthase, *ODC* ornithine decarboxylase, *P5C* pyrroline-5-carboxylate, *Tau-Cl* taurine chloramine
[a]Including myelin basic protein, filaggrin, and histone proteins
[b]Formed when asparagine reacts with reducing sugars or reactive carbonyls at high temperature
[c]Synthesized from L-serine by serine racemase

2. Amino Acid Sensing Pathways

2.1. The mTOR Transduction Pathway

2.1.1. The mTOR Signaling pathway

A well known example of an AA induced regulation of a signal transduction pathway is the mammalian target of rapamycin (mTOR) (Kimball and Jefferson 2006a). The mTOR was identified and cloned (Brown et al. 1994; Chiu et al. 1994; Sabatini et al. 1994) after the discovery of the two yeast genes, the Ser/Thr kinase target of rapamycin (TOR) 1 and TOR2, in the budding yeast *Saccharomyces cerevisae* during a screen for resistance to the immunosuppressant drug rapamycin (Helliwell et al. 1994). The molecular mechanisms by

which TOR proteins sense nutrient availability became clearer following the isolation of protein complexes associated with TOR1 and TOR2 from *S. cerevisiae* (Loewith et al. 2002). These complexes contain adheres voraciously to TOR2 (AVO) 1, AVO2 and AVO3, interacting with only TOR2, while lethal with SEC13 protein 8 (LST8) and Kontroller of Growth-1 (KOG1) interact independently with either TOR1 or TOR2 (Loewith et al. 2002). The phenotype of KOG1 deficiency in yeast resembles the phenotype of either TOR deficiency or rapamycin-treated cells, suggesting that KOG1 is a positive regulator of TOR (Loewith et al. 2002). Analysis of several LST8 mutants suggests that LST8 is also a positive regulator of mTOR (Chen and Kaiser 2003). The mammalian homologue of KOG1 is Raptor, the regulatory associated protein of mTOR, (Hara et al. 2002; Kim et al. 2002) and the mammalian homologue of LST8 is mLST8 (GβL, G protein β-subunit-like protein) (Kim et al. 2003). In mammalian cells, signaling of mTOR complex1 (mTORC1), comprises four known subunits: (i) mTOR, (ii) GβL, (iii) protein-riched Akt substrate of 40kDa (PRAS40) and (iv) the WD40 repeat containing subunit Raptor. The mTORC1 is nutrient-sensitive and inhibited by the bacterial macrolide rapamycin (Gwinn et al. 2008). A protein referred to as the rapamycin-insensitive companion of mTOR (rictor) is the mammalian homologue of AVO3 (Jacinto et al. 2004; Sarbassov et al. 2004). When associated with rictor, mTOR binds to GβL, mammalian stress-activatied protein1 (mSin1) and Protor, which is so called PRR5 (protein rich 5 [renal]), but not raptor and PRAS40 (Guertin and Sabatini 2007). This complex is termed mTOR complex 2 (mTORC2) which is neither nutrients nor inhibited by rapamycin, in addition, as in yeast, the mTORC2 does not phosphorylate 4E-BP1 or S6K1(Jacinto et al. 2004; Sarbassov et al. 2004; Guertin and Sabatini 2007). Moreover, mTORC2, could phosphorylate protein kinase Akt (also known as protein kinase B, PKB) on Ser473 in order to its full activation (Huang et al. 2008).

Raptor appears to serve as an adaptor protein that recruits mTOR substrates. It binds 4E- BP1 and p70S6k, both downstream targets of mTOR, and is necessary for the *in vitro* phosphorylation of 4E-BP1 by mTOR and for the efficient phosphorylation of S6K1 (Beugnet et al. 2003; Choi et al. 2003; Nojima et al. 2003; Schalm et al. 2003). Recent studies show that coexpression of GβL and mTOR results in increased kinase activity of mTOR toward 4E-BP1 and S6K1 compared with expression of mTOR alone. The reduction of GβL expression using siRNA represses leucine- and serum-induced phosphorylation of S6K1, suggesting that

GβL is involved in hormone and AA signaling through mTOR (Kim et al. 2003). It is established that the conformation of mTORC1 can be changed by nutrient conditions such as AA availability. This change in the conformation of the complex acts on the ability of mTOR for interacting with and phosphorylating its substrates (Hay and Sonenberg 2004). In AA deprivation, the mTORC1 rules out mTOR to bind to its substrates and/or prevents the access of mTOR (or mTOR associated kinase) to its substrates. In contrast, in the presence of AAs, a conformational change builds up potent interaction between raptor and mTOR substrates and/or increased accessibility of the mTOR substrates and its associated kinase (Hay and Sonenberg 2004).

The close upstream protein that has been identified in mTOR signaling pathways is the "Ras homolog enriched in brain (Rheb)". Rheb is a small GTPase that promotes phosphorylation of 4E-BP1, S6K1, and S6 in a mTOR-dependent manner when overexpressed (Castro et al. 2003; Garami et al. 2003; Inoki et al. 2003b; Li et al. 2004a; Shah et al. 2004; Tee et al. 2005). The activity of Rheb is controlled in part by a GTPase–activating protein referred to as tuberous sclerosis complex 2 (TSC2; also known as tuberin). TSC2 and its binding partner TSC1 (also known as harmartin) are encoded by the TSC II and TSC I genes, respectively, which are causative in the autosomal dominant syndrome tuberous sclerosis (Marygold and Leevers 2002; McManus and Alessi 2002; Inoki et al. 2003a; Krymskaya 2003; Kwiatkowski 2003; Sampson 2003; Li et al. 2004b). It is established that, in *Drosophila*, the normal role of these proteins is to restrict cell size and proliferation and act downstream of Akt but upstream of TOR (Tapon et al. 2001). Akt also directly phosphorylates TSC2 to repress the inhibitory action of the TSC1/TSC2 complex on mTOR signaling to 4E-BP1 and S6K1, down stream target of mTOR (Dan et al. 2002; Inoki et al. 2002) (Figure 12). However, there is paradoxical evidence showing that TSC1/TSC2 complex positively regulated mTORC2 to promote Akt activation in a manner independent of Rheb (Huang et al. 2008).

2.1.2. Effect of nutritional conditions on mTOR Transduction Pathways

mTOR signaling is stimulated by AAs, responding to intracellular AAs rather than to external AA levels and by growth factors, including insulin and insulin-like growth factor (IGF) (Proud 2004). Interestingly, AAs have been shown to modulate insulin signaling through mTOR-dependent effects on the insulin receptor substrate-1 (IRS-1) (Takano et al. 2001; Tremblay and Marette 2001). IRS-1 is phosphorylated by the insulin/IGF-I receptor tyrosine kinases (RTKs) on several tyrosine residues. The resulting phosphotyrosines initiate signal cascades by acting as binding sites for proteins containing src homology 2 domains, including the p85 regulator subunit of class I phosphoinositide 3-kinase (PI3K) (Manning 2004). The activation of PI3K by IGF-1 at the plasma membrane leads to generation of the lipid second messenger phosphatidylinositol 3,4,5-triposphate (PIP_3). PIP_3 recruits Akt to the membrane resulting in phosphorylation of Akt at Tyr 308.

Akt activates mTOR by inhibition of TSC2 through phosphorylation and subsequent activation of Rheb (Inoki et al. 2002; Manning et al. 2002). Moreover, it is also established that proline-rich Akt/PKB substrate 40kDa (PRAS40) is a novel mTOR binding partner which mediates Akt signaling to mTOR. When Akt is activated by insulin, PRAS40 is phosphorylated at Thr 246 and binds with 14-3-3. This 14-3-3 binding may lead to facilitated release of PRAS40 from mTOR, resulting in activation of mTORC1, and to inactivation of IRS-1 through negative feedback (Vander Haar et al. 2007). The mechanism of this feedback regulation has been attributed to the phosphorylation of serine residues on IRS-1 by mTORC1 and its downstream target S6K1, resulting in a decrease of IRS-1 protein stability. This feedback mechanism is most obvious in cell culture models with deficiency of the TSC/TSC2 complex, where mTORC1 and S6K1 are constitutively active, affecting the hyperphosphorylation and degradation of IRS-1 (Harrington et al. 2004; Shah and Hunter 2006).

Besides the insulin, AAs, especially leucine, positively regulate mTOR signaling (Kimball and Jefferson 2002; Proud 2002). Recent studies report that Rheb binds directly to mTOR in an AA-dependent manner and involves in AA signal transduction through mTOR because overexpressing of Rheb maintained the phosphorylation of S6K1 during AA

deprivation (Long et al. 2005b; Long et al. 2005a; Smith et al. 2005). Furthermore, in both *Drosophila* and mammalian cells, down-regulated expression of either TSC2 or TSC1 maintains S6K1 phosphorylation during AA starvation whereas the overexpression of TSC1 and TSC2 inhibits S6K1 activation by AAs (Gao et al. 2002; Tee et al. 2002; Smith et al. 2005). These suggest that TSC1 and TSC2 are involved in AA-induced signaling through mTOR (Kimball and Jefferson 2006a).

Although many recent studies have advanced our understanding of the molecular details of the mTOR pathway, the mechanism by which AA signal to mTORC1 remains unclear. Several studies have suggested that mTOR is associated with the intracellular membranes within the cytoplasm, including the endoplasmic reticulum, Golgi, endosome and mitochondrial membranes (Drenan et al. 2004; Liu and Zheng 2007; Mavrakis et al. 2007). Moreover, localization of Rheb to the endomembranes has shown to be important for its functions (Jean et al. 2001; Buerger et al. 2006). Furthermore, the Rag proteins, small guanosine triphosphatases (GTPases) in the Ras superfamily, have been identified as essential mediators of AA signaling to mTORC1 (Sancak et al. 2008; Shaw 2008). Sabatini and colleagues searched for proteins that immunoprecipitated with epitope-tagged Raptor in human embryonic kidney (HEK) 293 cells and found RagC (Sancak et al. 2008). Mammalian cells contain four members of the Rag subfamily of the Ras small GTPases (Sekiguchi et al. 2001). These proteins are homologues of the budding yeast Gtr1p and Gtr2p GTPases (Gao and Kaiser 2006), which interestingly have been shown to regulate microautophagy in concert with TOR signaling, even thought they were to function in parallel or downstream of TOR based on initial genetic studies (Dubouloz et al. 2005). RagA and RagB are mammalian homologues of Gtr1p and RagC and RagD are corresponding homologues of Gtr2p (Schurmann et al. 1995; Sekiguchi et al. 2001). The latest studies, using small interfering RNA (siRNA), demonstrated that the stimulation of mTORC1 by AAs required Rag GTPases in both *Drosophila* and mammalian cells. Given that the AA signaling is required for maximum stimulation of mTOR by insulin and growth factors, siRNA against Rag GTPases also decreased insulin-induced mTOR activation. Moreover, mutants of RagB locked in the GTP-bound (active) state presented resistance to AA deprivation in mammalian cells (Kim et al. 2008; Sancak et al. 2008), suggesting that the Rag GTPases were sufficient to pass AA signals to mTORC1 (Shaw 2008). The next question addresses to how Rag GTPases activate mTORC1. The Sabatini laboratory revealed that, unlike Rheb, Rag GTPases directly bind to Raptor and

showed that this association was stimulated by AAs. In spite of this direct association, Rag GTPases could not activate mTORC1 kinase activity *in vitro* (Sancak et al. 2008). Moreover, Rheb was either downstream or parallel to the Rag GTPases because a constitutively active Rheb allele was enough to promote cell growth in the absence of Rag GTPases, while a constitutively active Rag to promote cell growth required Rheb (Kim et al. 2008). The final important key was that Rag GTPases possibly regulated the intracellular localization of mTOR in response to AAs (Sancak et al. 2008). mTOR is predominantly cytoplasmic but associates with cellular membranes including those of the endoplasmic reticulum, Golgi apparatus and endosomes (Liu and Zheng 2007;Drenan et al. 2004; Mavrakis et al. 2007). In starved cells, mTOR was present in tiny puncta throughout the cytoplasm, while in AA-stimulated cells, mTOR relocated to the perinuclear region of the cell or to large vesicular structures overlapping with Rab7 (Rab GTPases belong to the RAS superfamily and are the key regulators of membrane traffic)(Sancak et al. 2008). This suggests that AAs stimulated mTOR localization. This translocation was confirmed by mTOR presenting on the Rab7-positive structures, where Rheb localizes in human cells (Saito et al. 2005; Buerger et al. 2006), even during AA deprivation in $RagB^{GTP}$ expressing cells (Sancak et al. 2008). Briefly, the Rags mediated mTORC1 relocation to the vesicle that might contain Rheb, and this association resulted in an increase of mTORC1 kinase activity. This provides a molecular explanation for why insulin signaling to mTOR required the AA signaling because the direct signaling of AAs to mTOR has the potential ability to relocate mTORC1 to the proper location to receive the growth-factor-mediated signal from Rheb (Shaw 2008)(Figure 12)**.**

Another component, which is mediated in part by AA activation of mTORC1, is mammalian vascuolar protein sorting 34 (hVps34) in mammalian cell culture (Byfield et al. 2005; Nobukuni et al. 2005). However, *in vivo* analysis in Drophophila with Vps34 mutants has not confirmed this role (Juhasz et al. 2008). hVps34 is in class III of the PI3K (phosphoinositide 3-kinase) family of lipid kinases, all of which phosphorylate the 3’ hydroxy position of the phosphatidylinositol ring (Fruman et al. 1998). The activity of hVps34 requires the presenting of an associated protein kinase, hVps15 (Stack et al. 1993). In mammalian cells, hVps34 has been shown to play a role in multiple vesicular trafficking pathways (Murray et al. 2002; Stein et al. 2005; Cao et al. 2007). Recent findings reveal that hVps34 was also involved in nutrient regulating pathways. In the presence of AAs, the ectopic

expression of hVps34 stimulates S6K1 activation and this effect is blocked by siRNA (Byfield et al. 2005; Nobukuni et al. 2005). Furthermore, in AA stimulated cells, the hVps34 activity is induced by increased production of phosphatidylinositol 3-phosphate (PI3P), which acts to recruit proteins, containing the PX or FYVE domains (phosphoinositide recognition domain) to the early endosome (Nobukuni et al. 2007). However, the mechanism by which PI3P signals to mTOR remains unclear. Nobukuni, *et al*. proposed that hVps34 possibly mediated AA signaling to mTOR through PX- or FYVE- domain -containing proteins (Nobukuni et al. 2007). Furthermore, a recent study revealed that AAs increased intracellular Ca^{2+}, inducing the direct binding of Ca^{2+}/calmodulin (CaM) to hVps34, through its conserved CaM-binding motif. This association is required for its lipid kinase activity and an increase of mTORC1 complex signaling (Gulati et al. 2008). Moreover, Gulati, *et al*. hypothesized that hVps34 interacted with mTOR, resulting in a conformation change and allowing activation of downstream targets such as S6K1.

Knowing that hVps34 played a role in multiple vesicular trafficking pathways, it was recruited to the early endosome by Rab5 (Murray et al. 2002) and it colocalized with Rab7 on the late endosome (Stein et al. 2003). Moreover, a recent study showed that overexpression of Rheb produces large Rab7/9-positive vesicles, suggesting a link between Rheb and endocytic trafficking (Saito et al. 2005). As described above, the Rags mediated mTORC1 relocation to the Rab7 positive vesicles that might contain Rheb, and this association results in increased mTORC1 kinase activity (Sancak et al. 2008). These showed that, in response to AA signaling, besides Rags, hVps34 was also a possible mediator of mTORC1 localization to the proper position, required for activation of downstream effectors, S6K1 or 4E-BP1 (Figure 12).

2.1.3. The role of mTOR in the regulation of translation

It is established that mTOR can regulate the translation through the phosphorylation and the inactivation of the translational machinery (Hay and Sonenberg 2004; Proud 2004). The targets of the mTOR pathway include several components of translational machinery (Proud 2002). The first step in translation which was shown to be regulated by mTOR was

the phosphorylation of 4E-BP1, a protein that binds to and represses the function of the cap-binding translation factor eIF4E (Figure 12 and 5). 4E-BP1 is phosphorylated at several sites *in vivo* and its phosphorylation is hierarchical, i.e., phosphorylation at some sites appears to be required for the subsequent modification of others (Hay and Sonenberg 2004; Proud 2004). mTOR directly phosphorylated the priming sites Thr37and Thr46 but might subsequently also phosphorylate the other sites. It is thus possible that several phosphorylation events, including Thr37, Thr 46, Ser65 and Thr70 cooperated to promote the decrease in 4E-BP1's affinity for eIF4E, leading to its dissociation from eIF4E, and allowing eIF4E to form the complexes with eIF4G (Figure 5) (Hay and Sonenberg 2004; Proud 2004). Phosphorylation of both Ser65 and Thr70 was abundantly increased by insulin and this was blocked by rapamycin. Thr37/46 were phosphorylated in the basal condition, i.e. in AA-treated cells, and this was further increased by insulin in a rapamycin-sensitive manner (Proud 2004).

The second step that was shown to be regulated by mTOR was the phosphorylation of S6K1. The activation of S6K1 phosphorylated its downstream target S6 to promote translation at the initiation step (Meyuhas 2000). These were activated by insulin and both its activation and phosphorylation by mTOR are blocked by rapamycin (Proud 2004).

2.1.4. The role of mTOR in the regulation of proteolysis

In parallel to the regulation of translation, mTOR also regulated proteolysis. It has been reported that mTOR suppressed the autophagy pathway (Shintani and Klionsky 2004), however, the involvement of mTOR in the regulation of the other proteolitic pathways has not yet been investigated and the mechanism involved in this inhibition is still largely unknown. In yeast, the inhibition of TOR may be required to stimulate Apg1 (now renamed as Atg) kinase activity, which is required for the formation of preautophagosomal membrane and also to increase the expression of Apg8, which is involved in the expansion step of the autophagosomal membrane (Meijer and Codogno 2004). The Apg1 activity is enhanced by starvation or rapamycin treatment. In addition, Apg13, which binds to and activates Apg1, is hyperphosphorylated in nutrient rich conditions and rapidly dephosphorylated in a Tor-dependent manner, reducing its affinity to Apg1 (Figure 13) (Kamada et al. 2000). Moreover,

S6K1, downstream target of mTOR in the translational control, was apparently required for autophagy (Scott et al. 2004). It is established that the phosphorylated-tail of S6K1, which is catalytically inactive, could have a structural role in autophagy, whereas S6K1 phosphorylated at Thr389 inhibited autophagy (Moller et al. 2004)(Figure12). In addition, in C2C12 myotubes, increasing AAs or leucine alone, act additively with insulin to down regulate proteolysis and expression of ubiquitin proteasome system components, 14-kDa E2 proteins and C2 subunit of 20S proteasome (Sadiq et al. 2007). Phosphorylation of mTOR was enhanced in response to BCAA, mainly leucine (Ijichi et al. 2003). These suggest that mTOR transduction pathway, besides the regulation of autophagy, is also involved in the control of the ubiquitin-proteasome system.

The hVps34 could also be the positive effector of autophagy since its activity was inhibited by the compound 3-methyl adenine (Eskelinen et al. 2002), a commonly used autophagy inhibitor and it potentially acted at a number of steps in autophagy (Backer 2008). Moreover, the hVps34 –Vps15 complex was also required to recruit the Atg12-Atg5 conjugated to the preautophagosome structure (Suzuki et al. 2001). Thus, even though, hVps34 was a positive regulator of mTOR, it played a critical role in autophagy which was paradoxical evidence (Petiot et al. 2000; Eskelinen et al. 2002; Qu et al. 2003).

In conclusion, mTOR is a well known signal transduction pathway involved in AA sensing. Both AAs and insulin can stimulate mTORC1 in divergent pathways. Once activated, mTOR stimulates the mRNA translation through the phosphorylation of 4E-BP1 and S6K1 and inhibited the proteolysis.

2.2 AMPK Transduction Pathway

2.2.1. AMPK signaling pathway

Adenosine monophosphate (AMP)–activated protein kinase (AMPK) is an important sensor for monitoring cellular energy status in response to nutritional environmental variation (Hardie and Sakamoto 2006; Viollet et al. 2007). AMPK is a heterotrimeric enzyme

complex consisting of a catalytic subunit α and two regulatory subunits β and γ. AMPK is activated following ATP depletion or a rise in the AMP: ATP ratio within the cell. AMP activates AMPK by directly binding to tandem repeats of crystathionine-β-synthase (CBS) domains in the γ subunit that result in a conformation change that exposes the activation loop in the α subunit, allowing it to be phosphorylated on Thr172 by upstream kinases (Viollet et al. 2007). It is principally performed in skeletal muscle and liver by tumor suppressor LKB1 kinase (LKB1) (Hardie 2004; Kahn et al. 2005; Woods et al. 2003; Sakamoto et al. 2005; Shaw et al. 2005) whereas in brain $Ca^{2+/}$calmodulin-dependent protein kinase kinase2 (CaMKK2) may play a more predominant role (Hawley et al. 2005; Anderson et al. 2008). Once activated, the AMPK regulates a variety of metabolic pathways, which results in the switching off of ATP-consuming pathways (such as fatty acid synthesis and cholesterol synthesis) and switching on of ATP-generating pathways (e.g. fatty acid oxidation and glycolysis) (Carling 2004; Viollet et al. 2007). These effects of AMPK are achieved by both short–term effects on phosphorylation of several downstream regulatory proteins and by long-term effects on gene expression (Xue and Kahn 2006). The AMPK phosphorylation is reversible and probably dephosphorylated by protein phosphtase-2C (PP2C) (Wang and Unger 2005; Sanders et al. 2007) and protein phosphatase-2A (PP2A) which inhibited AMPK (Wu et al. 2007c).

Several studies have reported that any stress which depletes cellular ATP such as metabolic poisons targeting mitochondria (arsenite, azide, oligomycin, antimycin A and 2,4-dinitrophenol), heat shock, hypoxia and two different antidiabetic drugs, metformin and thiazolidinedioned (TZDs), can activate AMPK by intracellular changes in AMP:ATP ratio (Zhou et al. 2001; Hardie 2004; Saha et al. 2004; Towler and Hardie 2007). Among chemical methods for activating AMPK in the liver, 5-aminoimidazole-4-carbomide-1-β-D-ribofuranoside (AICAR) is widely used both *in vivo* and *in vitro* (Corton et al. 1995; Pencek et al. 2005; Reiter et al. 2005). AICAR is taken up by the cells and via phosphorylation converted to the AMP analogue 5-aminoimidazole-4-carbonmide riboside (ZMP). The ZMP accumulation leads to AMPK activation by mimicking the effects of AMP on the upstream AMPK kinase (Corton et al. 1995). Although AICAR is widely used as the pharmacological activator of AMPK, some of its action could be independent of AMPK. ZMP can potentially influence enzymes whose activities are influenced by AMP, such as fructose 1, 6-bisphosphatases and 6-phosphofructose-2-kinase (Viollet et al. 2006).

2.2.2. Effect of nutritional conditions on the AMPK signaling pathway

AMPK plays a central role as the regulator of cellular energy status, and is inactivated during a decrease of the AMP:ATP ratio. It is established that hepatic AMPK could also be activated in response to physiological stimuli such as exercise(Hardie 2004). Moreover, recent studies demonstrate that AMPK is activated in the liver by dietary energy restriction (Jiang et al. 2008) and in a 24h fast (Munday et al. 1991; Winder et al. 1997).Conversely, AMPK phosphorylation is repressed by the nutrient availability, essential AAs and glucose (Fujita et al. 2007). However, there is a controversy about the data of AMPK in response to AAs. It was reported that AMPK activity is reduced with increased AA concentrations in β cells (Gleason et al. 2007) and in the hypothalamus (Ropelle et al. 2007) whereas, the study of Kimball described an induction of AMPK phosphorylation in hepatocytes by high AA levels (Kimball et al. 2004).

Moreover, AMPK also regulates food intake and energy balance by responding to hormonal and nutrient signals in the hypothalamus (Minokoshi et al. 2004). In the hypothalamus, leptin inhibits AMPK activity whereas adiponectin had also been shown to increase food intake by activating AMPK (Minokoshi et al. 2008). In contrast, leptin and adiponectin can stimulate the phosphorylation and activation of AMPK in skeletal muscle leading to stimulation of fatty acid oxidation. Indeed, the activation of AMPK by adiponectin reduces gluconeogenesis in the liver (Minokoshi et al. 2002; Yamauchi et al. 2002).

2.2.3. The role of AMPK in the regulation of translation

Protein synthesis is an anabolic pathway consuming high levels of cellular energy because the elongation step of translation needs 4 ATP equivalents per peptide bond. Given that AMPK is involved in the regulation of energy balance in the cell, it is logical that AMPK plays a role by suppressing protein synthesis (Viollet et al. 2006). Indeed, mTOR regulates protein synthesis at the mRNA translation level, thus raising the possibility that mTOR activity is linked to cellular energy status (Dennis et al. 2001). There is evidence that AMPK could inhibit translation through the mTOR pathway (Krause et al. 2002; Reiter et al. 2005). AMPK phosphorylates and activates TSC2

on Thr1227 and Ser1345 (Inoki et al. 2003a) to promote Rheb and mTOR inactivation (Figure 12). Indeed, it is established that Ser1387 of human TSC2 (Ser1345 in the rat cDNA) is phosphorylated by AMPK *in vivo*, and has been shown to serve as a priming site for additional phosphorylation by glycogen synthase kinase3 (GSK3) at Ser1383 and 1379 (Inoki et al. 2006). Furthermore, TSC2-deficient cells remained responsive to energy stress, admittedly less than in wild-type cells, suggesting that TSC2 was not the only substrate of AMPK but also that there were additional AMPK substrates which might modulate mTORC1 activity (Gwinn et al. 2008). Interestingly, there is evidence that AMPK also directly phosphorylated raptor, the mTOR binding partner, on Ser722 and Ser 792 to induce 14-3-3 binding to raptor and inhibited mTORC1 activity (Gwinn et al. 2008). Moreover, AMPK also directly phosphorylated mTOR on Thr2446 leading to its inactivation (Cheng et al. 2004).

The AMPK also regulated mRNA translation at the elongation step. The rate elongation step of translation can be regulated via eukaryotic elongation factor 2 (eEF2). eEF2 activity is inhibited through phosphorylation on Thr56 by eEF2 kinase (eEF2K) because it is unable to bind ribosomes (Ryazanov et al. 1988; Browne and Proud 2002). The phosphorylation of eEF2 is decreased by insulin and certain stimuli through signaling events that require mTOR (Browne and Proud 2004). Moreover, in hepatocytes, the activation of AMPK by AICAR due to increase eEF2 phosphorylation was known to inhibit its activity (Horman et al. 2002). eEF2K was inhibited through phophorylation on Ser366 by S6K1, the downstream target of mTOR, whereas AMPK has been demonstrated to activate eEF2K by phosphorylating Ser398 to prevent the activation of eEF2 (Browne et al. 2004). These suggested that AMPK also inhibits translation at elongation step.

2.2.4. The role of AMPK in the regulation of proteolysis

In yeast, AMPK activation stimulated autophagy (Wang et al. 2001). The mechanism by which AMPK activated autophagy reliesd either on eEF2 kinase, which was essential for autophagy (Wu et al. 2006) or mTOR signaling pathway via the phosphorylation of the TSC1/TSC2 complex, a negative regulator of mTOR. The phosphorylation of TSC2 by AMPK led to its activation (Inoki et al. 2003a) in order to inhibit the S6K1 phosphorylation at

Thr389, a negative regulator for autophagy (Moller et al. 2004)(Figure 12). In contrast with yeast, the activation of AMPK by AICAR inhibited autophagy in hepatocytes (Samari and Seglen 1998). However, recent literature revealed that the inhibition of autophagy by AICAR was not related to its ability to activate AMPK (Meley et al. 2006; Viana et al. 2008) because this effect was still presented in the fibroblasts infected by a dominant negative form of AMPK (Viana et al. 2008). AICAR inhibited the autophagy by impairing the formation of the functional complex of beclin-1, involved in the autophagosome formation (Liang et al. 1999), with class III PI3-kinase, which is the positive regulator of autophagy (Viana et al. 2008). However, Meley and colleagues concluded that AMPK was required for autophagy in both yeast and mammalian cells since the inhibition of AMPK activity by the compound C, a specific inhibitor of AMPK(Zhou et al. 2001), or by the transfection with an AMPK dominant negative form inhibited autophagy (Meley et al. 2006).

Furthermore, AMPK had the critical role in the down-regulation of proteasome degradation. The activation of AMPK either by AICAR or metformin induced an inhibition of proteasome activity. This effect is AMPK dependent because it is absent in the cells without the two catalytic subunits of AMPK (Viana et al. 2008).

2.3. GCN2 Transduction Pathway

2.3.1. The GCN2 signaling pathway

The pathway involved in the sensing of AA deprivation is the general control nonderepressible 2 (GCN2) phosphorylation, which was first identified in yeast (Dever and Hinnebusch 2005; Hinnebusch 2005). As with other kinases, autophosphorylation of the activation loop is additionally required to activate GCN2. Thr882 and Thr887 are the sites of autophospshorylation by GCN2 *in vitro*, and mutating them to Ala at Thr882 or Thr887 impaired or abolished GCN2 function, respectively (Romano et al. 1998). In the earliest steps leading to the initiation of mRNA translation, AAs are acylated to tRNA by their cognate aa-tRNA synthase enzymes, a process termed tRNA charging. Even if non essential AAs are available, a decrease in essential AAs will trigger tRNA deacylation. The uncharged tRNA is

known to initiate signaling for AA deficiency *in vivo* in single cell systems, such as yeast (Hinnebusch and Natarajan 2002). The GCN2 contains a domain related to histidyl-tRNA synthetase (HisRS), located C-terminally to the kinase domain, which mediates GCN2 activation in response to starvation for different AAs (Wek et al. 1995) and which binds different uncharged tRNAs *in vitro* with higher affinity than the corresponding charged tRNA (Dong et al. 2000). Binding of uncharged tRNA to the HisRS domain is thought to activate the adjacent protein kinase domain which phosphorylates a specific residue of the GTP binding translation initiation factor eIF2α that can modulate translation (Harding et al. 2003; Wek et al. 2006).

2.3.2. Effect of nutritional conditions on GCN2 signaling pathway

Nutrient control of gene expression in mammalian cells is an important aspect of regulating cellular responses to changes in the environment. The changes in AA availability, in addition to humoral and neuronal factors, have effects on many aspects of cell function, including regulation of cell signaling, gene expression as well as transport and metabolism of AAs themselves (Fafournoux et al. 2000; van Sluijters et al. 2000; Kilberg et al. 2005). Given that the accumulation of uncharged tRNA activates the GCN2 activity, this suggests that the GCN2 signaling pathway is designed to detect the AA insufficiency. Recent studies demonstrated that GCN2 in the mouse brain restricted intake of diets lacking essential AAs (Hao et al. 2005; Maurin et al. 2005). Moreover, leucine restricted diet for 6 days, in liver, resulted in the decrease of protein synthesis in liver of mice which is concomitant with the decrease of 4E-BP1 and S6K phosphorylation, translation regulators controlled nutritionally by mTOR, and an increase of eIF2 phosphorylation, the initiation factor regulated by GCN2. In mice lacking GCN2, eIF2 phosphorylation was decreased in response to dietary leucine restriction for 6 days whereas 4E-BP1 and S6K1 phosphorylation was not decreased. These suggested that GCN2 was involved in the sensing of AA depletion and it could also contribute to nutritional regulation of mTOR pathway (Anthony et al. 2004).

2.3.3. The role of GCN2 in the regulation of translation.

The GCN2 can regulate the translation at the initiation step through eIF2 in response to the nutritional change (Figure 12). The eIF2 forms a ternary complex with GTP and the met-tRNAi and delivers met-tRNAi to the small ribosomal subunit in the first step of translation initiation. The resulting ribosomal complex binds to mRNA, scans to an AUG initiation codon, and releases eIF2-GDP after GTP hydrolysis. The guanine nucleotide exchange factor eIF2B recycles eIF2•GDP to functional eIF2•GTP, and this reaction is blocked by eIF2α phosphorylation (Dever and Hinnebusch 2005). Accordingly, GCN2 ensures that all AAs are available to support cell growth and function. Thus, phosphorylation of eIF2α at Ser51 by GCN2 leads to the inhibition of translation initiation of most cellular proteins and a global reduction of protein synthesis(Harding et al. 2003; Dever and Hinnebusch 2005). However, translation of a small subset of mRNAs including AA biosynthetic enzymes and AA transporters was activated by the same eIF2α modification in response to AA starvation (Kilberg et al. 2005). In yeast, phosphorylation of the α subunit of eIF2 on serine 51 required GCN2 and was enhanced in cells grown under AA starvation conditions (Dever et al. 1992). In this condition, the translation of the transcription factor GCN4 was activated, resulting in a subsequent increase of expression of genes encoding AA biosynthetic enzymes that allows the yeast to survive in conditions lacking AAs (Hinnebusch 2005 576). The mammalian counterpart of GCN4 is the activating transcription factor 4 (ATF4, also known as CREB2), increasing the synthesis of non essential AAs and the capacity of cell to take up both non-essential and essential AAs. (Harding et al. 2000; Jousse et al. 2004; Kimball and Jefferson 2005; Lopez et al. 2007; Malmberg and Adams 2008). AA deprivation increased levels of GCN4 or ATF4 mRNA, additionally, the ATF4 was induced through the activation of GCN2 by uncharged tRNA and the eIF2α phosphorylation (Figure 12) (Siu et al. 2002; Hinnebusch 2005; Chaveroux et al. 2009). It might be concluded that the result of the GCN2 pathway was the activation of genes that corrected AA deficiencies and restored homeostasis (Malmberg and Adams 2008).

There is evidence that ATF4 was involved in the transcriptional regulation of the ATF3, C/EBP homologous protein (CHOP) and asparagine synthase in response to AA

limitation (Drenan et al. 2004; Jousse et al. 2004; Stipanuk 2007; Chaveroux et al. 2009). Normally, ATF3 was expressed at low levels in the cells and was rapidly induced in response to various stress signals (Jiang et al. 2004). Another component involved in the control of ATF3 transcription was ATF2, which was phosphorylated upon AA depletion. ATF4 and ATF2 were coordinated to induce the ATF3 transcription through the AA response element-dependent transcription (AARE), which located in the promoters of ATF3 gene. These suggest that in response to AA limitation, besides the control of translation, GCN2 is involved in the transcriptional control (Figure 12)(Chaveroux et al. 2009).

Moreover, a recent study also demonstrated that the GCN2 pathway is the major signaling pathway involved in the up- and down-regulation of gene expression in response to AA starvation whereas rapamycin, the mTORC1 inhibitor, regulates the expression of a set of genes that only partially overlaps with the set of genes regulated by leucine starvation (Deval et al. 2009). Thus, it has been hypothesized that AA deprivation may not inhibit activity of mTORC1 to the same extent as inhibition by rapamycin or it may control the gene expression via mTORC1-independent mechanisms (Deval et al. 2009).

2.3.4. The role of GCN2 in the regulation of proteolysis

As for the mTOR and AMPK signaling pathways, GCN2 signaling pathway is involved in the regulation of proteolysis. GCN2 positively controls autophagic sequestraion in mammalian cells in response to nutrient deprivation. In murine embryonic fibroblasts (MEFs), the phosphorylation of eIF2α serine 51 was required for AA starvation-induced autophagy because this effect was inhibited in mutant eIF2α S51A, where serine was replaced by alanine (Talloczy et al. 2002). However, future work is still required to determine whether the GCN2 acts as the upstream component of eIF2α during starvation-induced autophagy as well as to know the role of this signaling pathway in the regulation of the other proteolytic pathways.

3. Amino acid transporters and their role as nutrient sensors

It has been established that particular AA transporters have a key role in the maintenance of free AA concentrations in animal cells (Hyde et al. 2005; Evans et al. 2007), suggesting that the signaling pathways regulated by intracellular AA concentration may be linked to AA transporter activity and some AA transporters expressed at the surface of mammalian cells may also function directly as extracellular AA sensors (Taylor 2009).

The System L transporter is the basic route for indispensable large neutral AAs to enter the cell. Knowing that leucine could be a potent activator of the mTOR signaling pathway (Ijichi et al. 2003) and the overexpression of the system L in Xenopus oocytes increased the phosphorylation of S6K1, the well known downstream target of TOR (Christie et al. 2002), suggesting that the System L transporter could be a potential modulator of AA sensitive mTOR signaling. The System L operates as an 1:1 AA exchanger which can couple the cellular uptake of indispensable AAs with the outflow by heteroexchange of cytoplasmic AAs (Meier et al. 2002), thus the total AA concentrations on either side of the cell membrane could be without any overall change.

Additionally, the concentration gradients of the exchange AA substrates could be generated by the secondary AA transporters which are the sodium-dependent system, such as the System A. The sodium-coupled neutral AA transporter2 (SNAT2) is the principle System A isoform in most extraneural tissues and it is unidirectional Na^+- AA coupled transport cycle which is able to concentrate intracellular AA substrates (Mackenzie and Erickson 2004). Moreover, the AA substrate ranges of the Systems A and L overlap. The coupling of AA substrate flows through these transporter systems, termed tertiary active AA transport, may indirectly extend the scope for sensing nutrient abundance (Chillaron et al. 1996; Meier et al. 2002; Verrey et al. 2000). These systems generated cellular accumulation of leucine (System L substrate) which is the activator of mTOR signaling pathway, in exchange for cytoplasmic AAs i.e., glutamine (an AA substrate of both the Systems L and A) (Figure 14) (Taylor 2009) which is unable to directly activate TOR by itself when injected into oocytes (Christie et al. 2002). It is established that the expression of both the System L

(CD98/LAT1; L-type AA transport 1) (Liu et al. 2004; Edinger and Thompson 2004) and System A (SNAT) (Peyrollier et al. 2000) showed positive correlation with the activation of TOR pathway, and their functional coupling might help to explain why glutamine exerted a similar effect as leucine to TOR (Xia et al. 2003) pathway in circumstances or why it is, at least, required for the leucine effect (Fumarola et al. 2005).

Given a suitable mechanism for transducing the AA ligand-binding event to a change in activity of a downstream intracellular signaling cascade, the substrate binding sites of AA transporters were well positioned to act as direct sensors of AA availability at the cell surface. The transporters exhibit a dual function of transporter and receptor were called "transceptors" (Taylor 2009). In yeast**,** the AA permease stimulated the protein kinase A (PKA) signaling pathway and gave rise to changes in the metabolism and expression of stress-responsive genes (Donaton et al. 2003).

Although transporters in several classes have been linked to AA sensing, AA transporters which lie upstream of the TOR signaling pathway in *Drosophila* have been identified, such as the proton assisted amino transporters (PATs). PATs interacted with components of the PI3K/Akt and TOR signaling cascades and thus might be involved in the transduction of AA signals (Goberdhan et al. 2005). The PATS were first identified on the surface of mammalian lysosomes, which led to them being named as lysosomal AA transporters (LYAATs) (Sagne et al. 2001) and also as proton-dependent AA transporters which is classified in the system imino (Boll et al. 2002). In yeast, PATs were related to AA vascuolar transporters, which can transport out of the vacuole, a lysosomal structure (Russnak et al. 2001). It is now know that PATs are also found at the plasma membrane and in the endosomal compartments. First, PATs have been suggested to transport AAs from the apical surface to the gut (Thwaites and Anderson 2007). However, some PATs were expressed throughout the body (Bermingham and Pennington 2004), suggesting that they were likely to have much more widespread functions (Goberdhan et al. 2009). Given that AAs promoted the relocation of mTOR into Rheb-containing late endosome via Rag dependent mechanism (Sancak et al. 2008; Shaw 2008), proposed that Rags were involved in the regulation of the transmitting an AA dependent signal from a sensor at the cell surface to the intracellular compartments (Goberdhan and Boyd 2009). Moreover, in yeast, the

homologues of Rags were found in the late endosome (Nakashima et al. 1999) and were required for proper relocation of the general AA permease (Gap) 1p (Gao and Kaiser 2006), suggesting that membrane protein trafficking could be involved in their functions. These might be explain why hVps34, involved in the membrane trafficking (Murray et al. 2002), has also been implicated in AA sensing (Goberdhan and Boyd 2009). The PATs was a good candidate AA transporter to fulfill this shuttling role of AA sensing because they were found at the cell surface and in the endosomes and lysosomes and they can also function in acidic compartments i.e. the late endosome (Figure 15). Moreover, Rag could regulate the relocation of the PATs to the late endosomal which could help to recuit TOR (Goberdhan and Boyd 2009) (Figure 15). In addition, PATs also activated TOR by transporting low levels of AAs to a complex at a specific subcellular site (Goberdhan et al. 2005) and PATs would also provide a means to utilizing late endosomal rather than extracellular AAs (Goberdhan and Boyd 2009).

Another transporter which acted as the transreceptor is SNAT2. AA deprivation induces the expression of the cellular SNAT2 gene, termed “adaptive regulation” in a manner that can be repressed by re-supply of a System A’s substrate. Synthesis of SNAT2 mRNA increased within 1-2 h after AA removal from human hepatoma cells (HepG2) (Palii et al. 2006). The induction of transport system A for neutral AAs led to the recovery of AA levels once extracellular AA availability was restored. The efficiency of repressive effect by re-supply of AA substrates for System A was correlated directly with the transport K_m for system A’s substrates, including α-methylaminoisobutyric acid (MeAIB), a specific non-metabolizable substrate of System A. Furthermore, the transcription of a SNAT2 reporter gene was increased by SNAT2 gene silencing but decreased by the SNAT2 overexpression, suggesting that SNAT2 repressed its own expression via a signal at least partly responsive to the occupancy of the substrate binding site on SNAT2 (Hyde et al. 2007). The adaptive regulation of SNAT2 was apparently regulated by a GCN2-dependent mechanism. The increase in system A transport activity and SNAT2 mRNA levels upon AA starvation were abolished in cells with a mutant eIF2α (the downstream target of GCN2) that cannot be phosphorylated. This might help to adjust the gain of AA sensing in response to altered external AA availability (Gaccioli et al. 2006). Additional evidence , indicating that SNAT2 might also operate as a transceptor, was SNAT2 gene silencing in L6 skeletal muscle cells

appearing to stimulate proteolysis, impair insulin signaling through PI3K (Evans et al. 2008), and consequently potentially to down-regulate the signaling through mTOR to S6K1, S6, and 4E-BP1, leading to impairment of translation (Evans et al. 2007). Moreover, SNAT2 was also able to signal directly to TOR signaling pathway, since MeAIB could promote the phosphorylation of S6K1 in MCF7 cells (Taylor 2009).

It might be concluded that AA transporters are required to deliver AAs to intracellular AA sensors such as the mTOR and GCN2 signaling transduction pathways and may also regulate at different levels nutrient signaling in animal cells under normal circumstances (Figure 16).

III: Relation between protein metabolism and regulation in energy metabolism

It is established that the metabolic adaptations by HP diet are at least characterized by a down-regulation of lipogenesis from glucose (Blouet et al. 2006; Pichon et al. 2006), an up-regulation of AA catabolic pathways (Fafournoux et al. 1990; Moundras et al. 1993), AA transfer to protein synthesis (Taillandier et al. 1996; Yoshizawa et al. 1998) and gluconeogenic pathways (Azzout-Marniche et al. 2007). Moreover, it has been shown that AAs are able to transduce a signal, together with insulin and glucagons in order to control metabolic orientation in the liver (Azzout-Marniche et al. 2007). Given that the mTOR, AMPK and GCN2 transduction pathways act as intracellular sensors for AAs, to regulate the downstream components of translation and proteolysis, we aimed to analyze in this part of the introduction whether these signaling pathways are also involved in the control of energy metabolism such as fatty oxidation, lipogenesis, glycolysis and gluconeogenesis.

1. The role of mTOR in the regulation of the energy metabolism

Besides the control of translation and proteolysis, mTOR also involved in the control of fatty acid metabolism. In hepatocytes, the inhibition of mTOR by rapamycin for 18-48

hours, increased fatty acid oxidation (46-100 %) and reduces lipid synthesis (40-60%) Moreover, rapamycin decreased expression of genes encoding acetyl-CoA carboxylase 1 (ACC1) and mitochondrial glycerol phosphate acyltransferase (Brown et al. 2007) .The increase fatty acid oxidation rate was also observed in hepatocytes in the S6K1 $^{-/-}$ mice, one of the well known downstream targets of mTOR, against obesity (Um et al. 2004). The 4E-BP1 gene knockout studies in mice have also revealed a reduction in body fat content (Tsukiyama-Kohara et al. 2001). The reduction in the levels of the 4E-BP2 isoform by the antisense oligonucleotide treatment of obese mice lowered body fat content which associated with a reduction in liver lipogenic gene, fatty acid synthase (FAS) (Yu et al. 2008). However, the 4E-BP1 and 4E-BP2 double KO mice revealed a reduction in lipolysis and an increase in adipogenesis and insulin resistance which is controversial when compared with the preceding studies (Le Bacquer et al. 2007).

Furthermore, mTOR is involved in the control of glucose metabolism. The non-insulin glucose transport and the incorporation of glucose into glycogen were decreased by 20% and 30%, respectively, although glucose utilization was unchanged in the hepatocytes treated with rapamycin. This suggested that a decrease in glycogen synthesis without a change in glucose utilization would be reflected in a decrease in net glucose uptake (Brown et al. 2007). The prolonged (48 hours) rapamycin treatment significantly reduced the phosphorylation on Akt, indicating a decreased activation of that signaling kinase, a critical component of the insulin signaling pathway. The phosphorylation of mTOR and S6K1 were significantly decreased in rapamycin-treated hepatocytes, confirming the blockage of mTOR signaling in those cells (Brown et al. 2007). However, the S6K1 $^{-/-}$ mice increased whole-body insulin sensitivity, suggesting that the effects of rapamycin on glucose metabolism are mediated in part by a S6K1-independent mechanism (Um et al. 2004). Additionally, the G6Pase and PEPCK gene expression were decreased in the obese mice treated with an antisense oligonucleotide of 4E-BP2 (Yu et al. 2008). Taken together, these revealed that the mTOR signaling pathway is involved in the control of energy metabolism.

2. The role of AMPK in the regulation of energy metabolism

Given that AMPK acts as the cellular energy sensor maintaining energy balance within the cell, it is logical that AMPK is involved in the control of energy metabolism such as lipid metabolism and glucose metabolism. The liver has an important role in the control of whole body metabolism of energy nutrients and is the major site for storage and release of carbohydrates and for fatty acid synthesis. During the postprandial period, net glucose input permits the repletion of hepatic glycogen storage then excess dietary carbohydrates are converted into triglycerides to store as long-term energy (Owen et al. 1979). By contrast, during fasting, modifications of hepatic metabolism induce net glucose output and lipid breakdown in the liver to maintain whole body homeostasis. Moreover, activation of hepatic AMPK results in the increase of fatty acid oxidation and simultaneously reduces hepatic lipogenesis, cholesterol synthesis and glucose production (Viollet et al. 2006; Viollet et al. 2009). AMPK phosphorylates multiple downstream targets in the liver to switch on alternative catabolic pathways and switch off anabolic pathways. ACC and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), key enzymes in fatty acid and cholesterol synthesis, respectively, were the first enzymes shown to be downstream targets for AMPK (Hardie 2004). In liver, the major isoforms of ACC, ACC1 and ACC2, are encoded from two separate genes. While the malonyl-CoA synthesized by ACC1 is used in fatty acid synthesis, the malonyl-CoA produced by ACC2 is involved in the control of fatty acid oxidation (Abu-Elheiga et al. 2001). ACC is an important rate-limiting step enzyme for the synthesis of malonyl-CoA, which is both a critical precursor in lipogenesis and an inhibitor of mitochondrial fatty acid oxidation through the allosteric regulation of carnitine palmitoyltransferase-1 (CPT-1), an enzyme that controls the entry of long-chain fatty acyl-CoA into mitochondria (Viollet et al. 2006). AMPK inhibits ACC activity by phosphorylating Ser79 (Figure 17) (Munday et al. 1988). Thus, malonyl-CoA concentration is decreased, leading to subsequent reduction in fatty acid synthesis and increase in mitochondrial fatty acid oxidation (Viollet et al. 2006) (Figure 17). The recent findings showed that, in re-fed rats, ACC activity increased in 1-3 h after re-feeding, which was due to changes in the hepatic malonyl-CoA level and was associated with a decrease of AMPK activity, which remains low for at least 24h (Assifi et al. 2005). Moreover, in response to energy depletion, AMPK also

activated malonyl-CoA decarboxylase (MCD), an enzyme involved in the turnover of malonyl-CoA, to reduce malonyl-CoA content and increase fatty acid oxidation (Figure 17) (Assifi et al. 2005). Furthermore, AMPKα2 $^{-/-}$ mice and liver specific AMPKα2 $^{-/-}$ mouse models showed hypertriglyceridaemia (Viollet et al. 2003). Moreover, the expression of a constitutively active from of AMPKα2 (AMPKα2-CA) in the liver by adenovirus-mediated gene transfer, which encoded a constitutive active AMPK catalytic subunit α2, reduced the re-feeding-induced transcriptional activation lipogenic genes and their upstream regulators, sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate response element-binding protein (ChREBP), resulting in a decrease circulating lipid levels (Foretz et al. 2005). Indeed, AMPK activation led to ChREBP phosphorylation on Ser568, causing a decrease in its DNA binding activity and subsequent transcriptional inhibition of glucose responsive genes (Kawaguchi et al. 2002). These suggest that, in liver, AMPK plays an important role in the control of lipid homeostasis by regulating the activity of key enzymes in lipid metabolism.

Besides the role in the control of lipid metabolism, AMPK is also involved in the control of hepatic glycolysis and glycogenogenesis (Figure 17). In hepatocytes, the activation of AMPK inhibited glycolysis. There is evidence that the activation of AMPK decreased the gene expression of glucose transporter Glut2 (Leclerc et al. 2001). The incubation of hepatocyte suspensions from rats with glucose (15 mM) and AICAR led to a decrease in glucose-6-phosphate concentration and the inhibition of 6-phosphofructo-2-kinase activity (Vincent et al. 1992). In rat hepatoma FTO2B cells, AICAR inhibited glycolysis by decreasing fructose-1,6-biphosphate, indicating that 6-phosphofructo-1-kinase was inhibited (Javaux et al. 1995). Moreover, AMPK also has a long-term effect on glycolysis by suppressing L-pyruvate kinase expression (Leclerc et al. 1998; Leclerc et al. 2001; Kawaguchi et al. 2002) and reduced hepatic glycogen synthesis (Foretz et al. 2005).

For hepatic gluconeogenesis pathways, it was reported that the activation of AMPK by AICAR and metformin was able to suppress glucose production in the liver of normal and insulin-resistant obese rats (Bergeron et al. 2001; Zhou et al. 2001). This is confirmed by the results from primary cultures of hepatocytes treated with adenovirus expressing AMPKα2 – CA, resulting in the inhibition of glucose production (Foretz et al. 2005). Moreover, recent findings also demonstrated that hepatic AMPK had a physiological role in controlling whole-

body glucose homeostasis. In diabetic murine models, short-term activation of AMPK by AMPKα2 –CA adenovirus, was adequate to control hypoglycemia (Foretz et al. 2005). In addition, in the liver of LKB1-deficient mice, in which AMPK activity was low, there was an increase in fasting blood glucose levels (Shaw et al. 2005). By contrast, liver-specific AMPKα2 $^{-}/^{-}$ mouse models presented hyperglycemia, glucose tolerance and increased fasted hepatic glucose production (Andreelli et al. 2006). Most of these results suggest that hepatic AMPK had an important role in the inhibition of hepatic glucose production and the control of blood glucose level (Viollet et al. 2006). AMPK control gluconeogenesis through the inhibition of the activity of fructose -1,6-biphosphatase in hepatocytes (Vincent et al. 1991) and suppression of the gene expression of PEPCK and G6Pase in hepatocytes (Barthel et al. 2002; Koo et al. 2005; Shaw et al. 2005) and in rat hepatoma cells (Lochhead et al. 2000; Barthel et al. 2002). Moreover, AMPK regulated gluconeogenic gene expression through the transducer of regulated CREB (cyclic AMP response element binding protein) activity2 (TORC2). TORC2 is essential to control hepatic gluconeogenesis and it is regulated by phosphorylation of Ser171 (Screaton et al. 2004; Koo et al. 2005). Recent finding demonstrated that activation of AMPK by AICAR in rat primary cultures of hepatocytes led to phosphorylation of TORC2, which prevented its nuclear entry and blocks the expression of PEPCK and G6Pase (Koo et al. 2005) (Figure 17). These findings suggested that TORC2 was an important downstream target of AMPK's effect of down–regulating gluconeogenic gene expression thereby suppressing hepatic gluconeogenesis (Figure 17) (Xue and Kahn 2006). The gluconeogenic metabolic pathway consumes much energy. Thus, the inhibition of gluconeogenesis by AMPK has physiological significance because of its activation against the AMPK's role in the preservation of cellular energy status.

In conclusion, in the liver, when AMPK is activated, the ATP-consuming pathways (gluconeogenesis, lipogenesis, and cholesterol synthesis) are inhibited while the ATP generating pathways (such as fatty acid oxidation) are activated. These effects are controlled through AMPK by both short–term effects on phosphorylation of several downstream regulatory proteins (ACC, HMG-CoA reductase) and by long-term effects on gene expression (inhibition of L-pyruvate kinase, FAS, PEPCK and G6Pase expression).

3. The role of GCN2 in the regulation of energy metabolism

Little is know about the role of GCN2 in the control of energy metabolic pathways. Apart from being the sensor of AA deprivation that triggers a repression of global protein synthesis and regulates the expression of genes responding to AA deficiency, GCN2 also plays a more expansive role in controlling lipid homeostasis in response to AA deprivation (Towle 2007). In mice fed a leucine-restricted diet, triglyceride synthesis, expression of lipogenenic genes such as FAS and its activity, in liver, were repressed and lipid stores in adipose tissue were mobilized. However, GCN2 deleted mice fed a leucine depleted diet displayed a paradoxical increase in liver lipogenesis, including the lipogenic genes, such as FAS, and the liver exhibited steatosis. The accumulation of triglycerides in the liver correlated with an increase of SREBP-1c expression, a key transcriptional activator of the *de novo* lipogenic pathway (Guo and Cavener 2007). Hence, limitation of even a single AA can modulate a global response that included lipid synthesis.

Conclusion:

The effect of high protein intake on the metabolism is improved glucose homeostasis, reduced weight gain, body fat mass, white adipose tissue and adipocytes size (Lacroix et al. 2004; Blouet et al. 2006), stimulated protein synthesis (Ijichi et al. 2003) and decreased proteolysis (Mortimore et al. 1989; Mortimore et al. 1983). The liver plays a central role in the ability of omnivorous species such as rats or humans to adapt energy and AA metabolism to environmental nutritional conditions. Additionally, AAs are not only nutrients but are also the cell signaling molecules which can control the protein phosphorylation cascades and the gene expression (Wu 2009), however it remains unclear how AAs can signal to the cell. Thus, the mTOR, AMPK and GCN2 transduction pathways are excellent candidates for the intracellular sensor for AAs, to control the metabolism such as translation and proteolysis in liver. However, if the role of each signaling pathway in the transduction of AA signals has been studied, no study has investigated the role of these three pathways simultaneously. Moreover, the role of GCN2 in the detection of the increase of dietary AAs has not been examined. Finally, mainly the lysosomal proteolytic pathway has

been studied in liver whereas the nutritional conditions that affect the ubiquitin-proteasome in hepatic proteolysis remain unclear.

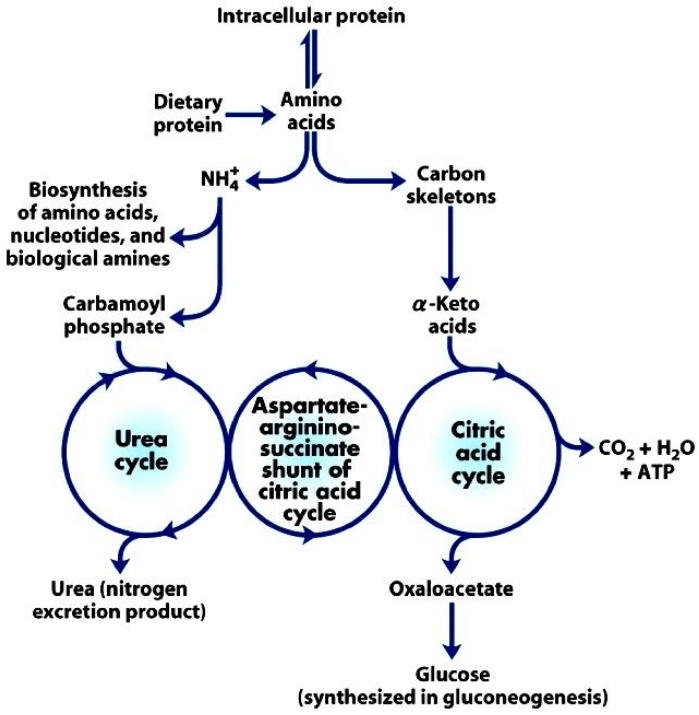


Figure 1. Overview of the catabolism of amino acids.
Amino acid catabolism include a key step in which the α-amino group is separated from the carbon skeleton and shunted into the pathways of amino group metabolism. The amino groups are used for the biosynthesis of AAs, nucleotides and biological amines or are converted to urea for excretion through the urea cycle whereas the main fates of the carbon skeletons are the conversion to oxaloacetate or citric acid cycle intermediates which can in turn be oxidized for energy supply or converted to glucose (Nelson and Cox 2000).

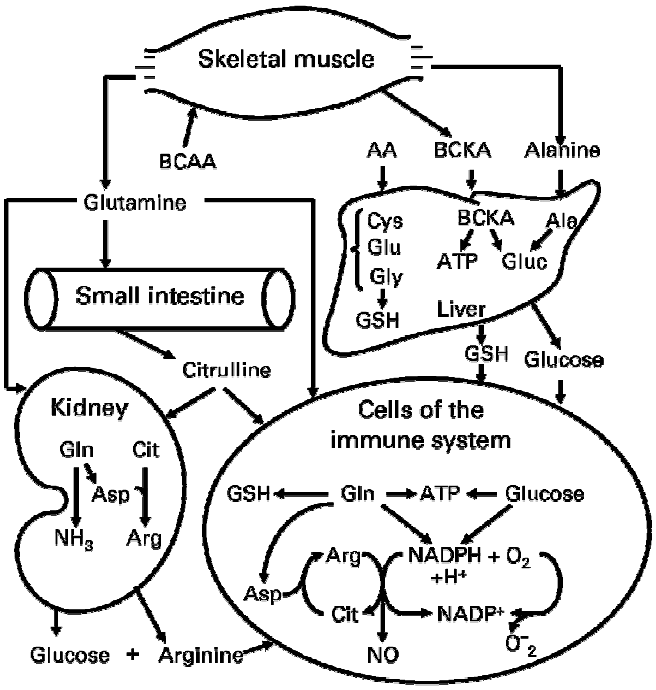


Figure 2. Interorgan metabolism of branched-chain amino acids (BCAA), glutamine and arginine and its role in immune function. Skeletal muscle takes up BCAA from the arterial blood, synthesizes both alanine and glutamine from BCAA and α-ketoglutarate, and releases these two amino acids into the circulation. The small intestine utilizes glutamine to synthesize citrulline, which is converted into arginine in kidneys, cells of the immune system, and other cell types. The liver is the primary organ for the synthesis of glutathione from glutamate, glycine, and cysteine and of glucose from alanine for use by extrahepatic cells (including immunocytes) and tissues. *Arg* arginine, *Asp* aspartate, *Cit* citrulline, *BCKA* branched-chain α-ketoacids, *Gluc* glucose, *GSH* glutathione. (Li et al. 2007)

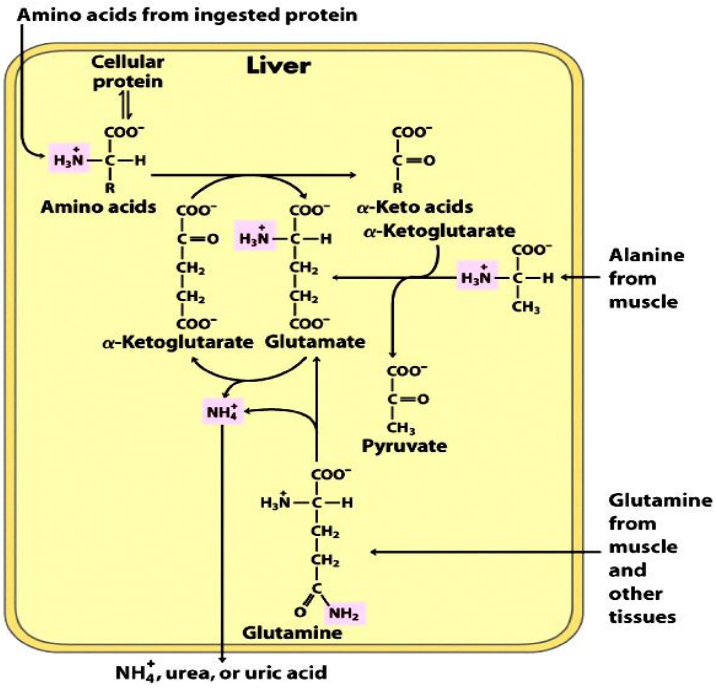


Figure 3. **Amino group catabolism in liver.** The amino group is transferred to the α-ketoglutarate o form glutamate. Glutamate is then transported from cytosol into mitochondria, where the amino group is removed to form NH_4^+. Excess is secreted as ammonia, urea or uric acid (Nelson and Cox 2000).

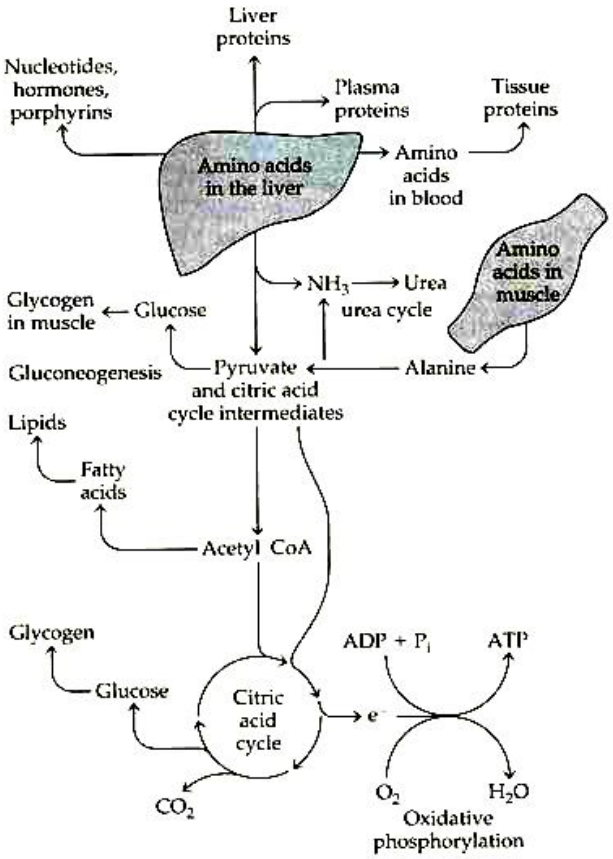


Figure 4. Role of Liver in the amino acid metabolism control. Liver is the site of synthesis of many different proteins from amino acids. Amino acids in liver can also be converted into non-protein products (nucleotides, hormones, and porphyrins) and can be transaminated and degraded to acetyl coA and other Krebs cycle intermediates, and these can in turn be oxidized for energy or converted to glucose or fat. Glucose formed from gluconeogenesis can be transported to muscle for use by that tissue, and synthesized fatty acids can be mobilized to adipose tissue for storage or used as fuel by muscle. Hepatocytes are exclusive site for formation of urea, the major excretory form of amino acid nitrogen (Nelson and Cox 2000).

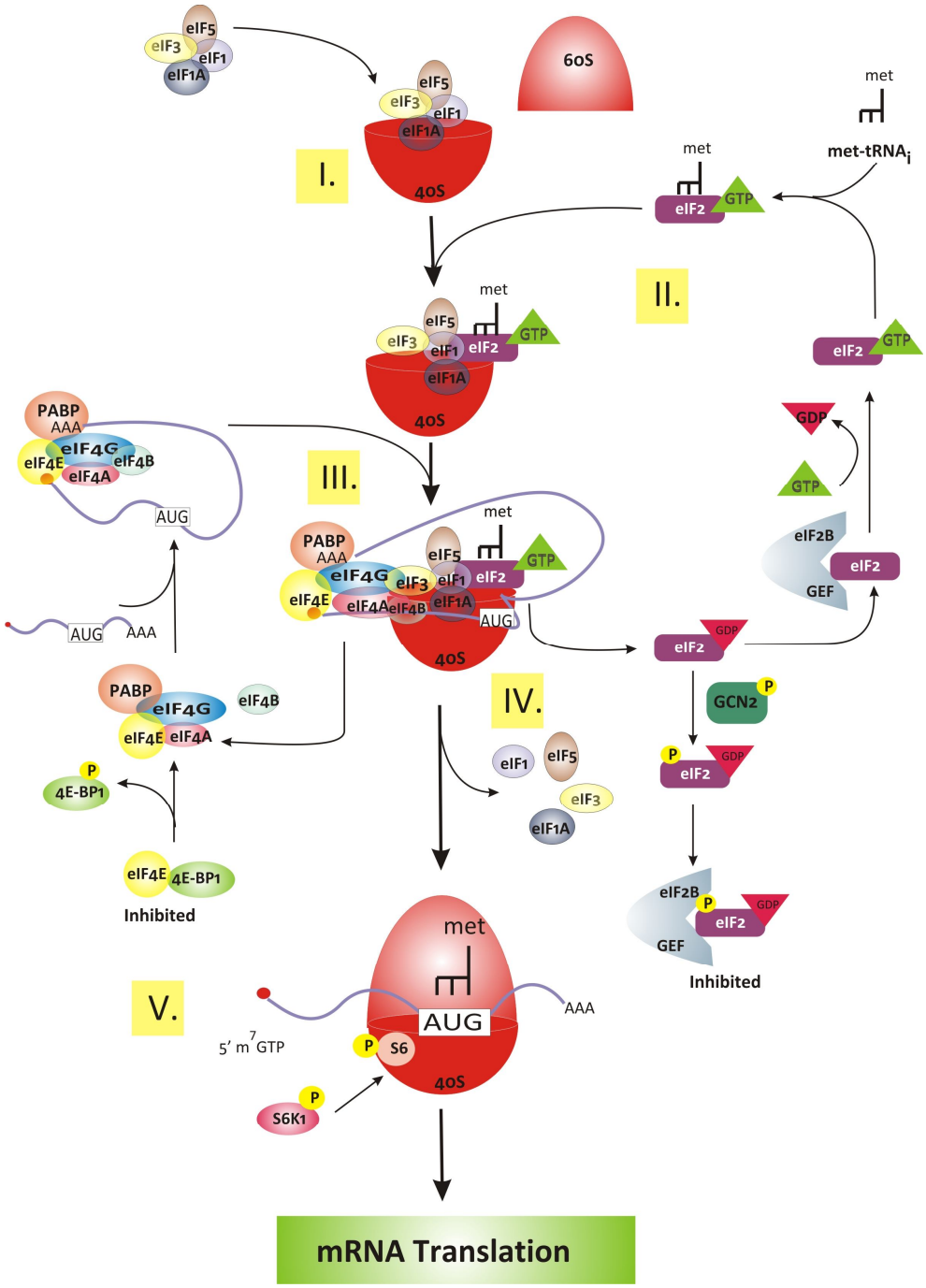
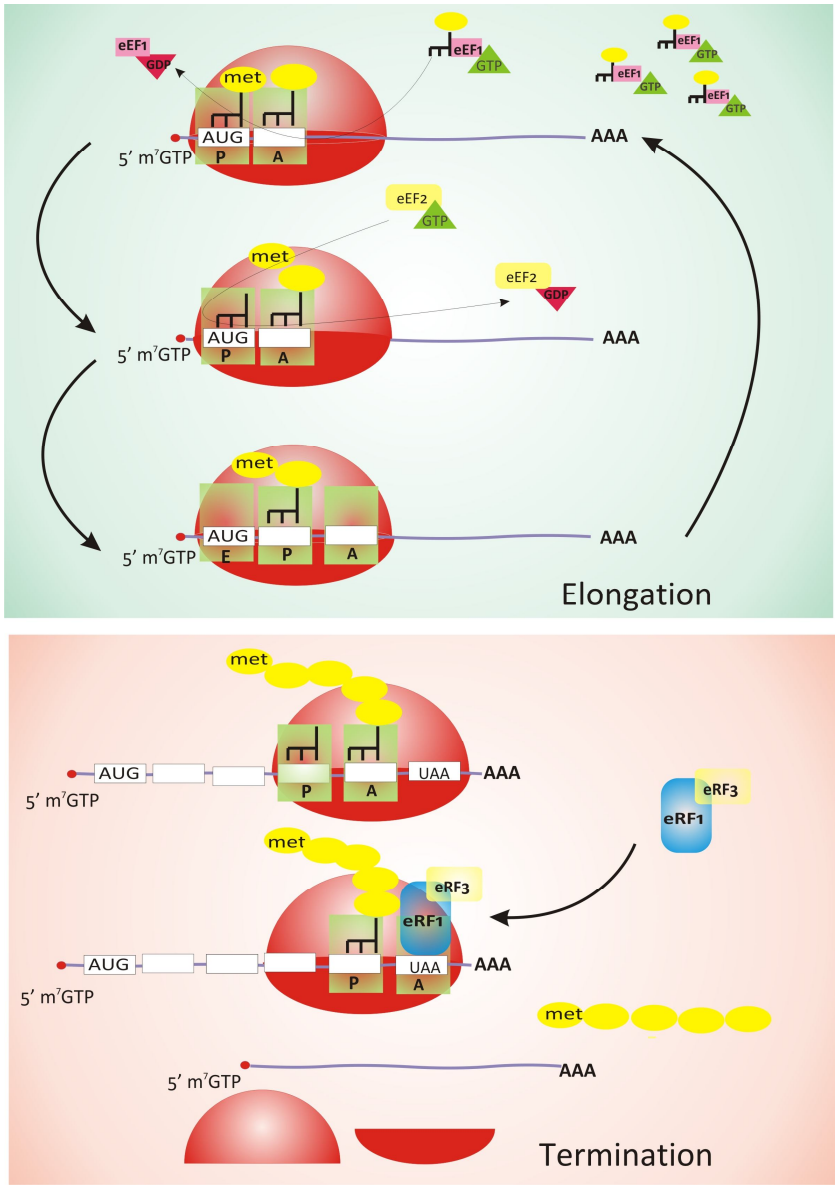


Figure 5. The initiation step of translation. First, the eukaryote initiation factors (eIF) including, eIF1, 1A and 3 promote the dissociation of 80S ribosomes and the eIF2 binds GTP and met-tRNAi, selected from the pool of tRNAs, to form the ternary complex (eIF2●GTP●met-tRNAi) and then binds to the 40S ribosomal subunit with other eIFs (eIF1, eIF1A, eIF3 and eIF5) to form the 43S preinitiation complex (step I. and II). The eIF4A, eIF4B, eIF4E and eIF4G, bind mRNA, subsequently, the interaction with the 43S complex through the eIF3 and eIF4G association (step III.). The 43S complex scans along the mRNA in a 5' to 3' direction towards the initiation codon (Step III.). At that moment, eIFs are released from the complex (step. IV.) and the 60S ribosomal subunit joins to form the 80S ribosomal (initiation complex) (Step V.) (Adapted from (Kimball and Jefferson 2002; Sonenberg and Hinnebusch 2009))



<u>Figure 6.</u> The elongation and the termination step of translation. The eukaryotic factor1 (eEF1) picks the aminoacyl-tRNA (aa-tRNA) in the presence of GTP, and then the aa-tRNA●eIF1A●GTP complex enters the empty A-site on a ribosome. The aa-tRNA anticodon of the incoming needs to be matched against the mRNA codon positioned in the A-site. Afterwards, the new amino acid at the A-site link to the growing polypeptide via the peptide bond which catalysed by peptidyl transferase. This reaction leaves an empty tRNA in the ribosomal P-site and the new peptidyl-tRNA in the A-site. Then, the ribosome translocates along the mRNA by the length of one codon which catalysed by the eEF2 and subsequent GTP hydrolysis. After the translocation, the ribosome is in the position of having an empty tRNA in the E site, the peptidyl-tRNA in the P site, and the next codon of mRNA in the A site, available for interaction with a new aa-tRNA. The elongation process repeates until the ribosome encounters an in-frame stop-codon including UAA, UAG and UGA. At this point, the translation is terminated. Consequently, the polypeptide chain releases from mRNA which requires eukaryotic release factors (eRFs). The eRF1 recognizes one of three stop codons and binds to the ribosome at the A-site. This event along with binding of the eRF3 which facilitates the recognition of stop codon by eRF1 and stimulates GTP hydrolysis to release the polypeptide chain from mRNA and ribosome.

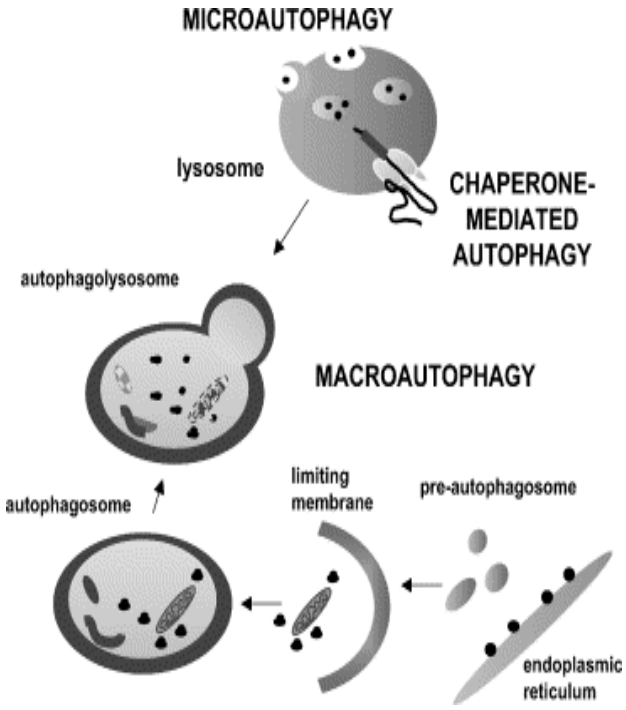
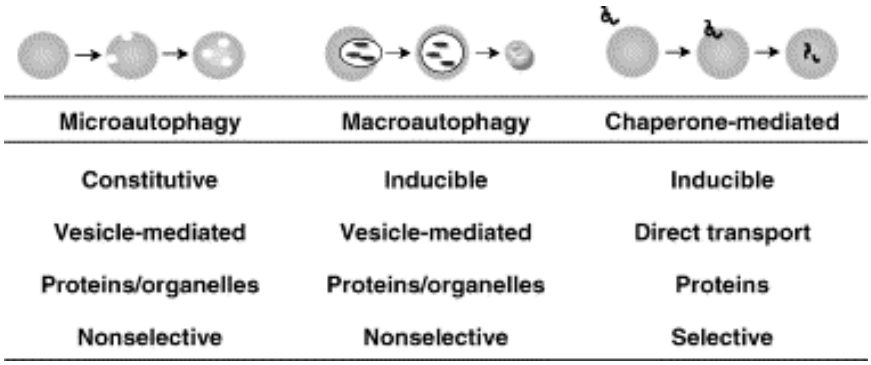


Figure 7. Schematic model of the three forms of autophagy in mammalian cells. The macroautophagy involves the formation of cytosolic double membrane vesicles first into autophagosomes that fuse then with lysosomes. The microatuphagy involves the direct invagination of lysosomal membrane to form an internal vacuolar vesicle containing the materials derived from cytoplasm. The chaperone-mediated autophagy processes through the selective uptake of targeted proteins (only cytosolic proteins) across the lysosomal membrane without vesicle-mediation (Massey et al. 2004)



Microautophagy	Macroautophagy	Chaperone-mediated
Constitutive	Inducible	Inducible
Vesicle-mediated	Vesicle-mediated	Direct transport
Proteins/organelles	Proteins/organelles	Proteins
Nonselective	Nonselective	Selective

Figure 8. Main characteristics of macroautophagy, microautophagy and chaperone-mediated autophagy. These characteristics refer to general macro- and microautophagy (Massey et al. 2004).

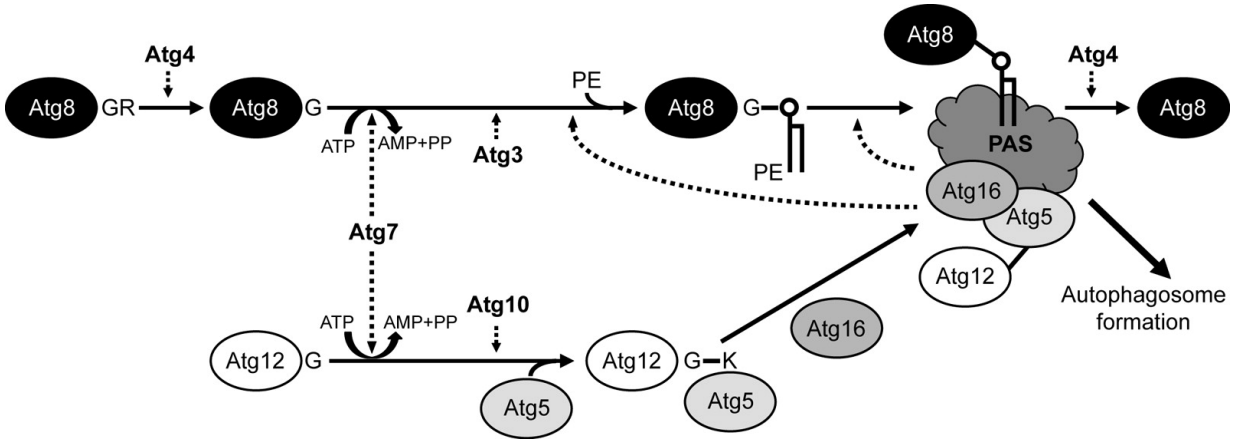


Figure 9. Atg conjugation systems. Atg12 is irreversibly conjugated to Atg5, and the Atg12sAtg5 conjugate further forms a complexwith Atg16, whereas Atg8 is reversibly conjugated to phosphatidylethanolamine (PE). Both the Atg12sAtg5-Atg16 complex and Atg8sPE localize to the pre-autophagosomal structure (PAS), where most Atg proteins colocalize (Noda et al. 2009).

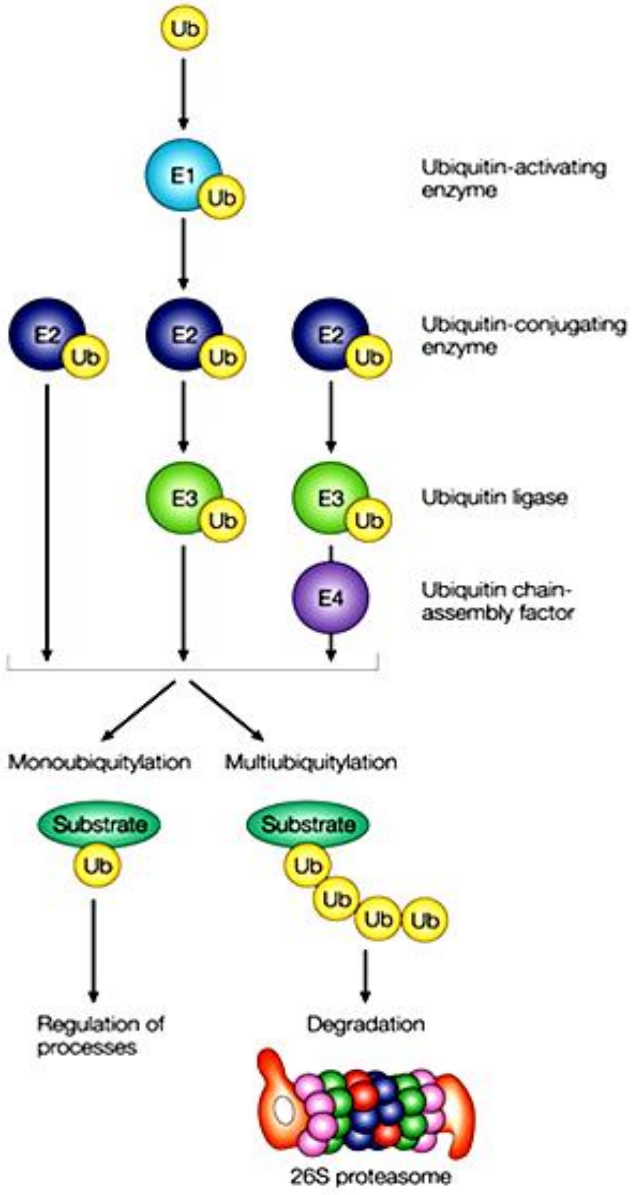


Figure 10. The ubiquitinqtion mechanisms. Free ubiquitin (Ub) is activated in an ATP-dependent manner by the activity of a ubiquitin-activating enzyme (E1), which hydrolyses ATP and forms a complex with ubiquitin. Subsequently, ubiquitin is transferred to one of many distinct ubiquitin-conjugating enzymes (E2s). In some reactions, E2s can directly ubiquitinylate substrates, whereas others require the help of ubiquitin ligases (E3s). Usually, several ubiquitin molecules, in the form of a multiubiquitin chain, are conjugated to a substrate. This reaction sometimes requires a specific multiubiquitin chain-assembly factor (E4). Multiubiquitylation serves mainly, but not exclusively, to label the substrate for degradation, whereas monoubiquitylation regulates several processes, such as endocytosis, DNA repair and transcriptional regulation (Jesenberger and Jentsch 2002).

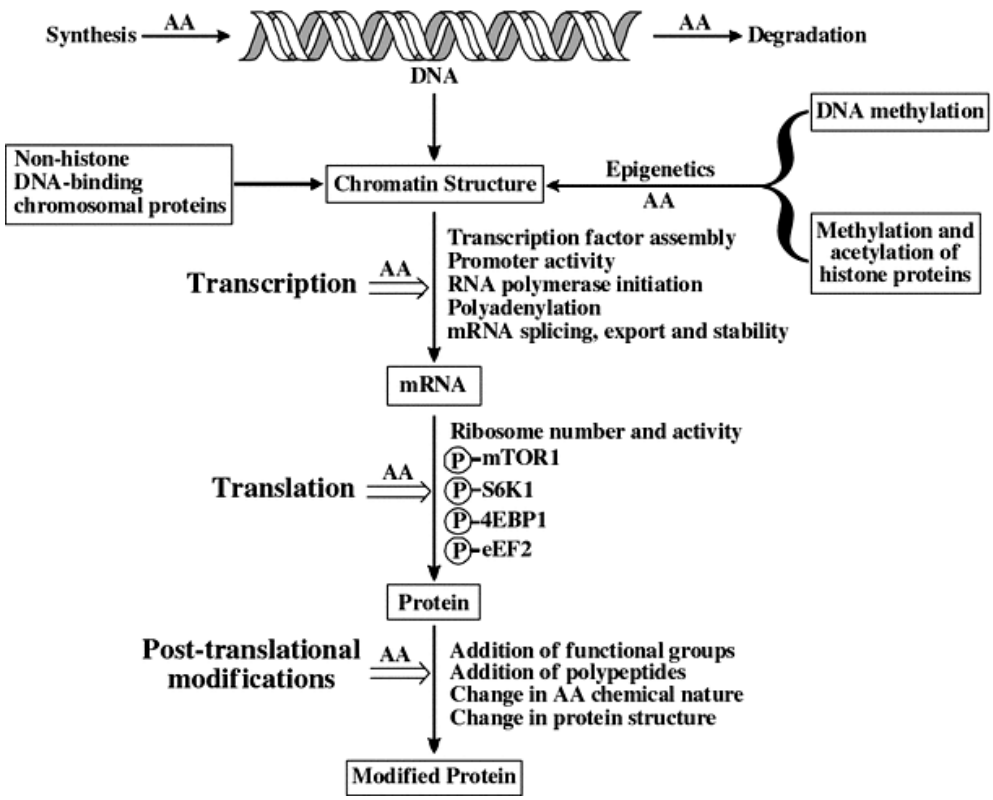
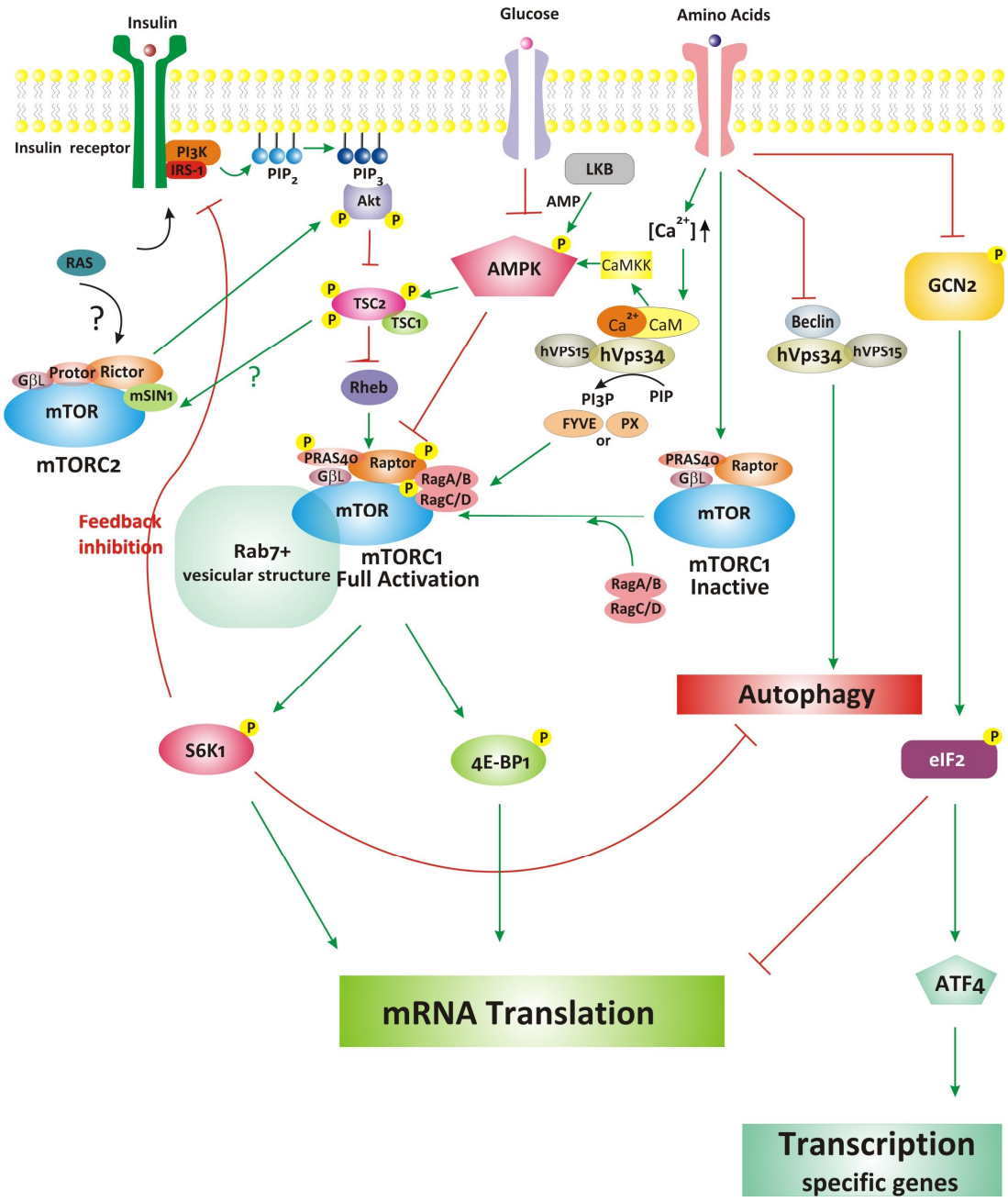
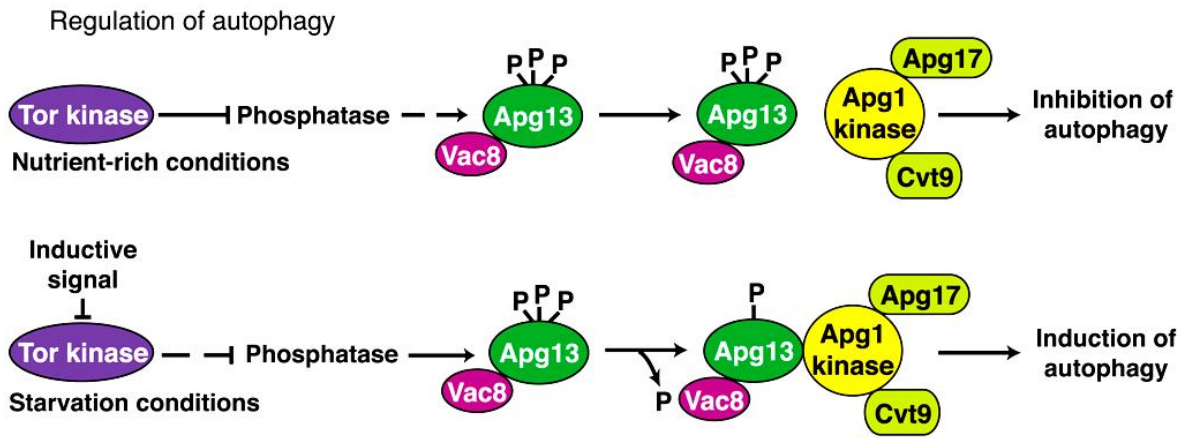


Figure 11. Possible mechanisms responsible for AA regulation of gene expression in cells. AA may regulate gene expression in animal cells at the levels of transcription, translation, and post-translational protein modifications. Post-translational protein modifications include acetylation, ADP-ribosylation, biotinylation, γ-carboxylation, disulfide linkage, flavin attachment, glutamylation, glycation, glycosylation, glycylation, heme attachment, hydroxylation, methylation, myristoylation, nitrosylation, oxidation, phosphorylation, palmitoylation, proteolytic cleavage, racemization, selenoylation, sumoylation , sulfation, and ubiquitination. (Wu 2009).



<u>Figure 12</u>. mTOR, AMPK and GCN2 transduction pathways in the control of mRNA translation and autophagy in response to nutrients, i.e. amino acids and glucose and insulin.

The mTORC1 was stimulated by insulin and AAs through PI3K/Akt pathway and Rags or hVps34, respectively. When mTORC1 was full activation, it could phosphorylate its downstream target, S6K1 and 4E-BP1 in order to activate translation. However, mTOR and S6K1could inhibit the IRS-1 called feedback inhibition. Moreover, S6K1 also was the negative regulator of autophagy whereas hVps34 was the positive regulator of autophagy. AMPK acted as the cellular energy sensor and its activity was inhibited by glucose. AMPK was phosphorylated by LKB1 or CaMKK and then it could phosphorylate TSC2 Raptor or mTOR to inhibit mRNA translation. GCN2 was activated by AA deprivation. When GCN2 was activated, it phosphorylated eIF2 in order to inhibit translation. GCN2 also activated the transcription of some specific genes through ATF4 (Adapted from (Dever and Hinnebusch 2005; Hardie et al. 2006; Um et al. 2006; Nobukuni et al. 2007; Gulati et al. 2008; Huang et al. 2008; Shaw 2008; Chaveroux et al. 2009)).



<u>Figure 13.</u> The Tor kinase exerts a negative regulatory effect on autophagy when cells are growing under nutrient-rich conditions. When starvation occurs, the Tor kinase is inactivated, and the negative regulation is relieved resulting in induction of autophagy. Most of the proteins required for autophagy are constitutively expressed and are used for biosynthetic import through the cytoplasm to vacuole targeting pathway under these conditions. The downstream effectors of Tor are likely to include phosphatases and kinases that modulate the phosphorylation state of Apg13. An inductive signal such as carbon or nitrogen starvation inactivates Tor and results in partial dephosphorylation of Apg13. This form of Apg13 associates more tightly with the Apg1 kinase and stimulates its activity. The function of Apg1 kinase is required for autophagosome formation. (Klionsky and Emr 2000)

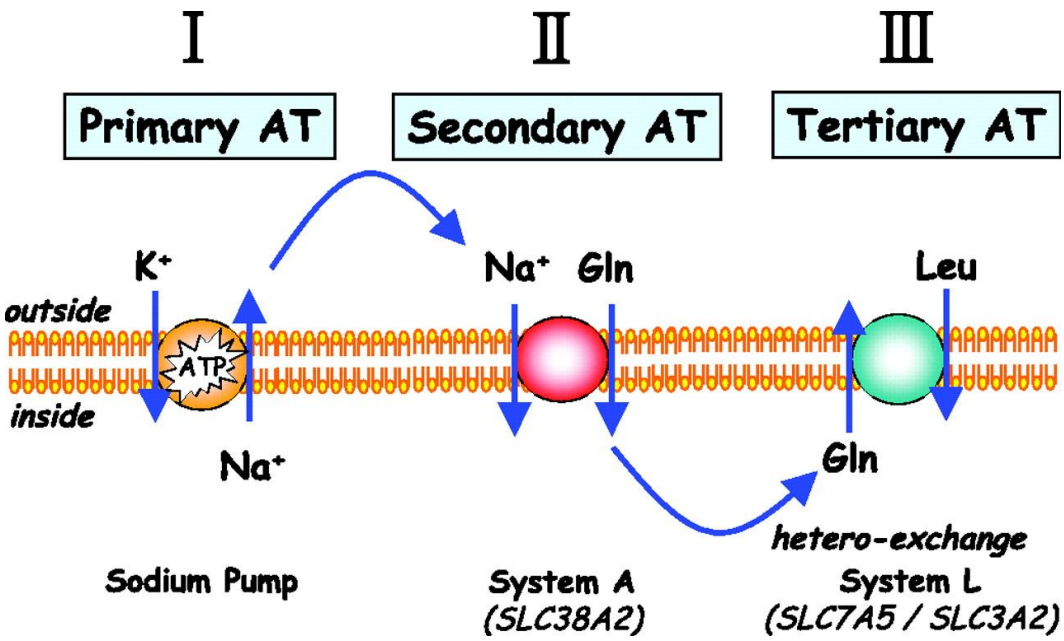
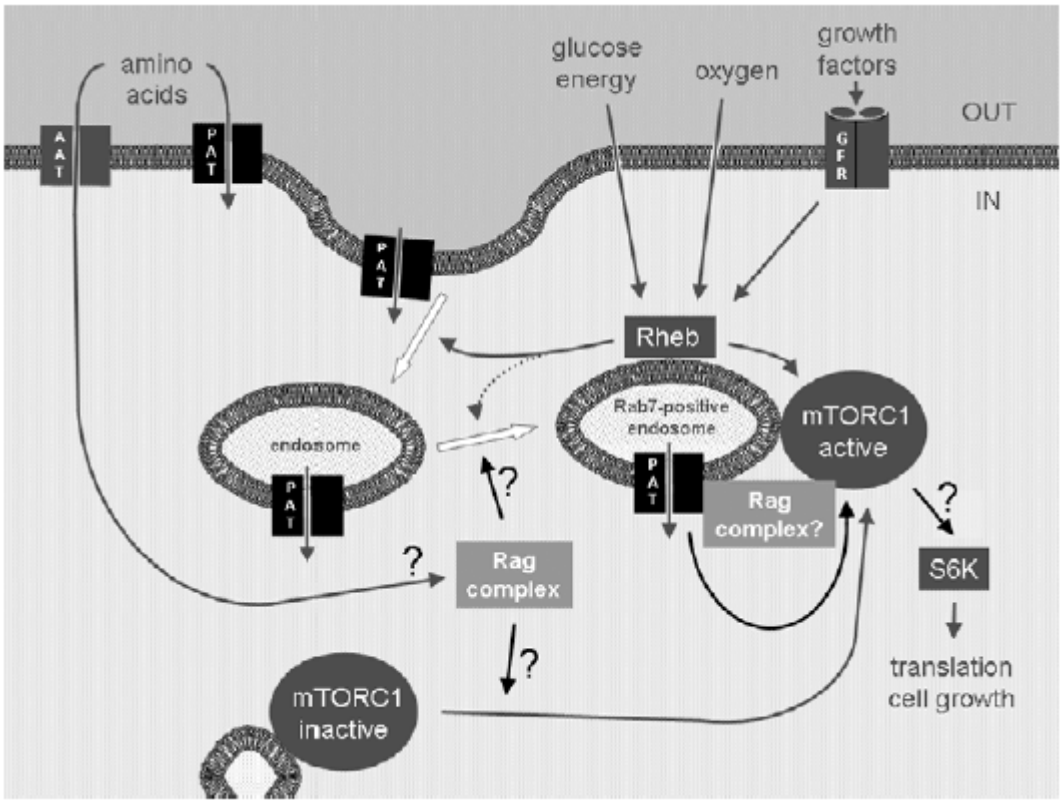


Figure 14. Integration of primary (I), secondary (II), and tertiary (III) active transport (AT) mechanisms may affect transmembrane distribution of particular AAs. Secondary active transporters (e.g., System A/SNAT2) generate net movement of AA from the extracellular to the intracellular pool, whereas tertiary active transport through exchangers such as LAT1 (System L) allows for redistribution of individual AAs without affecting total pool sizes (Hundal and Taylor 2009).



<u>Figure 15.</u> The role of PATs.
Rags are required to activate mTOR in response to amino acids and for mTOR to be shuttled to late (Rab7-positive)endosomes, which also contain themTOR activator Rheb. The PATs are TOR regulators that can potentially operate in endosomal and lysosomal compartments, as well as at the cell surface. In the present review, we suggest that the Rags might play a role in shuttling the PATs to late endosomes, from which they can help to recruit TOR, presumably leading to the activation of S6K (S6 kinase). The PATs would provide a means of utilizing late endosomal rather than extracellular amino acids, which may be particularly important under nutrient-deprived conditions. AAT, amino acid transporter; GFR, growth factor receptor; mTORC1, mTOR complex 1. (Goberdhan and Boyd 2009)

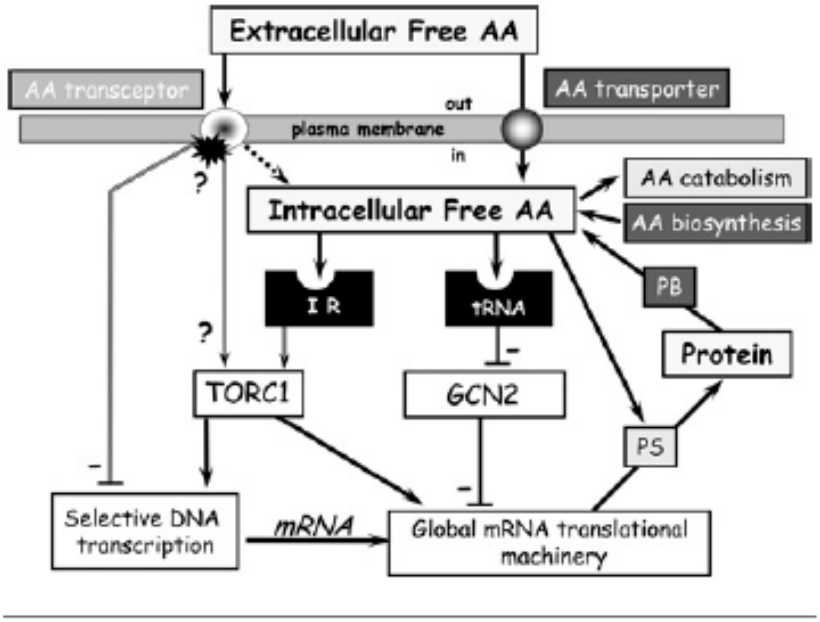


Figure 16. Schematic diagram illustrating processes which contribute to establishment of 'steady-state' intracellular amino acid (AA) concentrations and their interactions with major intracellular nutrient signalling pathways.

The size of intracellular free AA pool is determined by the balance between AA flows through underlying processes which include (i) AA transport across the cell membrane, (ii) protein synthesis and protein breakdown and (iii) AA biosynthesis and catabolism. Therefore, a key role for particular AA transporters in the maintenance of "steady-state" free AA concentrations and subsequently, implied that signaling pathways regulated by intracellular AA concentration may be intrinsically linked to AA transporter activity.

Dark-grey boxes indicate processes adding to the intracellular free AA pool, and medium-grey boxes indicate those taking away from the pool.

IR, intracellular receptor; PB, protein breakdown; PS, protein synthesis. (Taylor 2009)

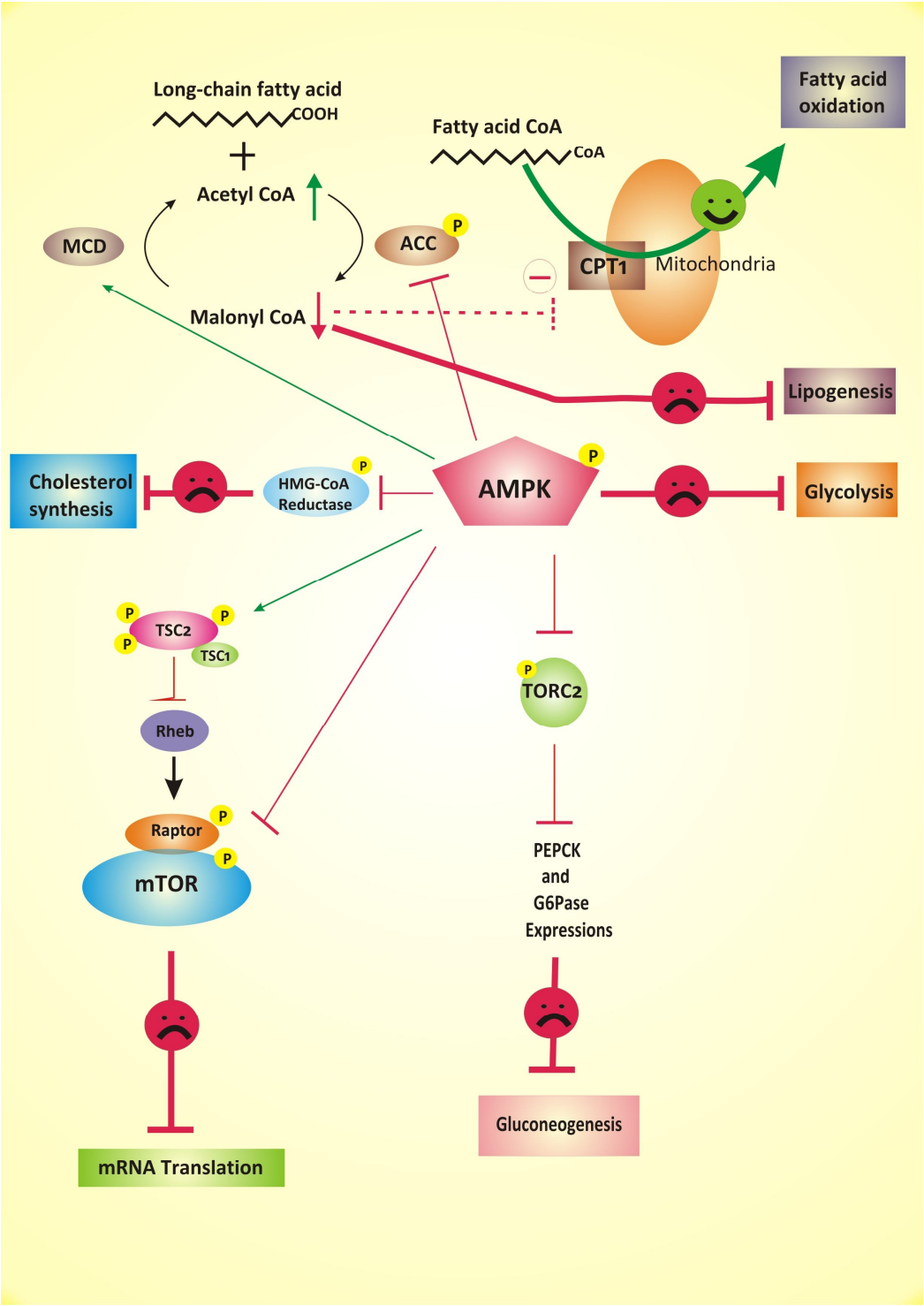


Figure17. The role of AMPK in the regulation of energy metabolism. When AMPK is activated, the ATP-consuming pathways (gluconeogenesis, translation, lipogenesis, and cholesterol synthesis) are inhibited while the ATP generating pathways (such as fatty acid oxidation) are activated. These effects are controlled through AMPK by both short–term effects on phosphorylation of several downstream regulatory proteins (ACC, HMG-CoA reductase, TSC2 and Raptor) and by long-term effects on gene expression (inhibition of L-pyruvate kinase, FAS, PEPCK and G6Pase expression). However, AMPK is also inhibited the glycolysis in the liver. (Adapted from (Viollet et al. 2006)).

PART II: PERSONAL WORK

PART II: Personal Work

Protein consumption largely exceeds recommendations in most developed countries (Hu et al. 1999; Volatier and Verger 1999; Harnack et al. 2000) and can reach extreme levels in specific populations using HP diets for dieting or muscle inflation purposes. However, unlike the effects of low protein diets which have been extensively investigated, little is known about the adaptation of protein and energy metabolism to the increase in protein intake, in particular in individual tissues. It has been observed that shifting from a high-carbohydrate to a HP diet induced important modifications in AA and energy nutrient handling and metabolism. As a consequence, HP intake improved body composition through stabilization of lean mass and a decrease of fat tissue (Jean et al. 2001; Pichon et al. 2006). These effects were ascribed both to the satiating effect of protein and macronutrient composition of the diet (Bensaid et al. 2002; Bensaid et al. 2003; Blouet et al. 2006). Metabolic adaptation to HP intake was characterized by a downregulation of lipogenesis from glucose (Blouet et al. 2006; Pichon et al. 2006), an upregulation of AA catabolic pathways (Fafournoux et al. 1990; Moundras et al. 1993), AA transfer to gluconeogenic pathways (Azzout-Marniche et al. 2007), and protein synthesis (Taillandier et al. 1996; Yoshizawa et al. 1998). We have reported previously that the effect of HP diet on gluconeogenesis, lipogenesis, and protein metabolism was attributable mostly to hepatic metabolism adaptation (Blouet et al. 2006; Azzout-Marniche et al. 2007; Chevalier et al. 2009). Moreover, it has been shown that AAs are able to transduce a signal, which controls, together with insulin and glucagon, metabolic orientation in the liver. Thus, we aimed to investigate:

1. The effect of AA levels on translation.
2. The effect of AA levels on proteolysis.
3. The transduction pathways involved in AA signaling
4. The AA or the groups of AAs involved in these effects

We chose to work in the primary culture of hepatocytes which is an *in vitro* approach with some limits in its utilisation, but, by contrast to *in vivo* study, this model allows investigation into the respective effects of different effectors, i.e. AAs, glucose, insulin, AICAR or rapamycin.

I. The effect of amino acid levels on translation and the transduction signaling pathways involved in these effects in the liver.

AAs have been demonstrated to act as important cellular signals and mTOR, AMPK and GCN2 are important candidates as transduction pathways involved in the control of metabolic response to high protein feeding in the liver. It remained to be determined:

- AMPK, mTOR and GCN2 transduction pathways activation state in response to changes in HP intake and AA concentrations in liver.
- Which AA or the group of AAs can be a signal on these transduction pathways.
- The effect of AICAR (AMPK activator) and rapamycin (inhibitor of mTOR) in the stimulation of translation in primary hepatocyte culture**.**

1. Respective role of amino acids, glucose and insulin in the effect of a HP diet on translation and the identification of the signaling pathways involved in these effects in rat liver:

Publication 1: Nattida Chotechuang, Dalila Azzout-Marniche, Cécile Bos, Catherine Chaumontet, Nicolas Gausserès, Tatiana Steiler**,** Claire Gaudichon and Daniel Tomé (2009)
mTOR, AMPK and GCN2 coordinate the adaptation of hepatic energy metabolic pathways in response to protein intake in the rat. *AJP Endocrinol. Met., 297: E1313–E1323.*

We first aimed to examine the effect of HP intake and the respective role of AAs, glucose and insulin on the phosphorylation state of proteins involved in mTOR, AMPK, and GCN2 transduction signaling pathways in the liver and in primary culture of hepatocytes. It has been reported that mTOR phosphorylation was enhanced in isolated hepatocytes in response to BCAA, mainly leucine (Ijichi et al. 2003). AMPK phosphorylation is repressed by an increase in AA concentrations in β cells (Gleason et al. 2007) and the hypothalamus (Ropelle et al. 2008)

whereas it was induced in hepatocytes (Kimball et al. 2004). However, the effect of AAs on AMPK phosphorylation and activation remains unclear (Kimball et al. 2004; Gleason et al. 2007; Ropelle et al. 2008). The GCN2 transduction pathway in liver has been shown to be involved in the sensing of leucine deficiency (Anthony et al. 2004; Guo and Cavener 2007) but its role in the detection of increases in AA concentrations has not been addressed.

In order to understand the role of these transduction pathways in the control of hepatic metabolic process in response to HP feeding, we conducted *in vivo* and *in vitro* experiments. First we examined the effect of HP diet on rats compared with rats fed a normal protein (NP) diet for 14 days. The animals were euthanazied at the end of the experiment, in the fasted or the fed state (2 hours after the beginning of a calibrated meal). Liver was taken and gene expression and the phosphorylation state of the key proteins involved in these transduction pathways and their downstream targets were determined by Western blot analysis. We used the anti-phospho AMPKα (Thr 172) catalytic subunit, the anti-AMPKα catalytic subunit, anti-phospho mTOR (Ser2448), anti-mTOR, anti-phospho 4E-BP1 (Thr 37/46), anti-4E-BP1, anti-phospho GCN2 (Thr898), anti GCN2, anti-phospho eIF2α (Ser51), anti-eIF2α, anti-phospho ACC (Ser79) and anti-ACC (Cell Signaling Technology, Beverly, MA, USA). All antibodies are diluted to 1/1000.

In the liver of HP fed rats, the phosphorylation of mTOR (P-mTOR) and 4E-BP1 (P-4E-BP1), the well known downstream target of mTOR, were increased (Figure 1a and 1b publication 1) whereas the phosphorylation of AMPK (P-AMPK), GCN2 (P-GCN2) and eIF2α (P- eIF2α) were decreased (Figure 1c, 1d and 1e publication 1). Moreover, the gene expression of AMPKα1 catalytic subunit, AMPKα2 catalytic subunit, mTOR and GCN2 was also determined in the liver in both the fasted and fed states in NP and HP rats, respectively (Figure 2 publication 1). No changes were observed in gene expression encoding mTOR, GCN2, AMPKα1 and AMPKα2 between HP and NP fed rats, which was in line with no changes in the total protein level observed in Western blot. This suggested that the differences in the phosphorylation state were not due to changes in total protein level. However, we were unable to measure total protein

level of GCN2. The similar level of mRNA between HP and NP fed rats indicated that there was no effect of protein intake on GCN2 gene expression.

To determine whether AAs, glucose and insulin are involved in the changes of phosphorylation states of these transduction pathways in the liver of HP fed rats, we performed an *in vitro* study, using primary culture of hepatocytes. The hepatocytes were cultured in the presence of low or high glucose or AA concentrations with or without insulin for 60 minutes. High glucose concentrations induced a decrease of P-AMPK while no changes were observed in the phosphorylation of mTOR, 4E-BP1 and GCN2 (Figure 3 publication 1). High AA levels also reduced AMPK phosphorylation (Figure 4c publication 1). Interestingly, even if glucose and AAs reduced P-AMPK, ACC phosphorylation, a well known target of AMPK, was decreased only by high glucose concentration (Figure 6 publication 1), whereas no effect of the increase of AAs concentration on ACC phosphorylation was observed. Thus, to confirm the effect of AAs on AMPK dephosphorylation in hepatocytes, immunohistochemical analyses of P-AMPKα were performed under both low and high AA concentrations. Hepatocytes were incubated with low AA concentrations, bright fluorescence was observed, whereas a reduction in fluorescence was clearly seen after cell exposure to high AA concentrations for 60 minutes (Figure 7 publication 1).

Either high AA levels or insulin induced an increase of mTOR phosphorylation (Figure 4a publication 1) whereas both signals were required to enhance P-4E-BP1 (Figure 4b publication 1). In particular, the increase in the concentration of BCAA or leucine was sufficient to stimulate the phosphorylation of both mTOR and 4E-BP1 in the presence of insulin (Figure 5 publication 1). The presence of insulin or high AA concentrations alone had no effect on P-GCN2 but the presence of both decreased P-GCN2 phosphorylation (Figure 4d publication 1).

In conclusion, the mTOR, AMPK and GCN2 transduction pathways and their downstream targets 4E-BP1, ACC and eIF2α, play a key role in nutrient sensing and in the regulation of protein and energy metabolism. Taken together, our results agree with the idea that AA sensing

in the liver involves coordinated action of these transduction pathways to ensure the adaptation of liver protein and energy metabolism to the availability of AAs and energy nutrients. Moreover, the stimulation of translation required both high AA levels and insulin. These were associated with the increases of P-mTOR and P-4E-BP1 and the decreases of P-AMPK and P-GCN2. Interestingly, the present study highlighted that 4E-BP1 might not always be the downstream target of mTOR since the increase of P-4E-BP1 was not always related to the increase of P-mTOR. Similarly, the phosphorylation state of ACC was not always related to the phosphorylation state of AMPK.

PUBLICATION 1

Nattida Chotechuang, Dalila Azzout-Marniche, Cécile Bos, Catherine Chaumontet, Nicolas Gausserès, Tatiana Steiler, Claire Gaudichon and Daniel Tomé
Am J Physiol Endocrinol Metab 297:1313-1323, 2009. First published Sep 8, 2009;
doi:10.1152/ajpendo.91000.2008

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Am J Physiol Endocrinol Metab 297: E1313–E1323, 2009.
First published September 8, 2009; doi:10.1152/ajpendo.91000.2008.

mTOR, AMPK, and GCN2 coordinate the adaptation of hepatic energy metabolic pathways in response to protein intake in the rat

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Submitted 15 December 2008; accepted in final form 6 September 2009

Chotechuang N, Azzout-Marniche D, Bos C, Chaumontet C, Gausserès N, Steiler T, Gaudichon C, Tomé D. mTOR, AMPK, and GCN2 coordinate the adaptation of hepatic energy metabolic pathways in response to protein intake in the rat. *Am J Physiol Endocrinol Metab* 297: E1313–E1323, 2009. First published September 8, 2009; doi:10.1152/ajpendo.91000.2008.—Three transduction pathways are involved in amino acid (AA) sensing in liver: mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), and general control nondepressible kinase 2 (GCN2). However, no study has investigated the involvement of these signaling pathways in hepatic AA sensing. To address the question of liver AA sensing and signaling in response to a high-protein (HP) dietary supply, we investigated the changes in the phosphorylation state of hepatic mTOR (p-mTOR), AMPKα (p-AMPKα), and GCN2 (p-GCN2) by Western blotting. In rats fed a HP diet for 14 days, the hepatic p-AMPKα and p-GCN2 were lower ($P < 0.001$), and those of both the p-mTOR and eukaryotic initiation factor 4E-binding protein-1 phosphorylation (p-4E-BP1) were higher ($P < 0.01$) compared with rats receiving a normal protein (NP) diet. In hepatocytes in primary culture, high AA concentration decreased AMPKα phosphorylation whether insulin was present or not ($P < 0.01$). Either AAs or insulin can stimulate p-mTOR, but this is not sufficient for 4E-BP1 phosphorylation that requires both ($P < 0.01$). As expected, branched-chain AAs (BCAA) or leucine stimulated the phosphorylation of mTOR, but both insulin and BCAA or leucine are required for 4E-BP1 phosphorylation. GCN2 phosphorylation was reduced by both AAs and insulin($P < 0.01$), suggesting for the first time that the translation inhibitor GCN2 senses not only the AA deficiency but also the AA increase in the liver. The present findings demonstrate that AAs and insulin exert a coordinated action on translation and involved mTOR, AMPK, and GCN2 transduction pathways.

mammalian target of rapamycin; adenosine 5′-monophosphate-activated protein kinase; general control nondepressible kinase 2; transduction pathways; translation; high-protein diet

DIFFERENT TRANSDUCTION PATHWAYS sensitive to energy, nutrient sufficiency, and hormones continuously adapt protein and energy metabolic pathways to maintain an energy and nutrient balance in cells. These pathways include adenosine 5′-monophosphate-activated protein kinase (AMPK), which is the downstream component of a protein kinase cascade acting as an intracellular energy sensor (17, 53), the mammalian target of rapamycin (mTOR), which is the downstream component of a nutrient-sensing kinase cascade able to phosphorylate two regulatory proteins, the p70 ribosomal S6 protein kinase ($p70^{S6K}$) and the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), that stimulate translation initiation (42), and the general control nondepressible 2 kinase (GCN2), which is sensitive to amino acid (AA) deprivation and inhibits translation via the phosphorylation of eukaryotic initiation factor (eIF)2α (1, 14, 50).

The liver plays a central role in the ability of omnivorous species such as rats or humans to adapt energy and AA metabolism to environmental nutritional conditions. It has been observed that shifting from a high-carbohydrate to a high-protein (HP) diet induces important modifications in AA and energy nutrient handling and metabolism. As a consequence, HP intake improves body composition through a stabilization of lean mass and a decrease of fat tissue (29, 41). These effects were ascribed both to the satiating effect of protein and macronutrient composition of diet (4, 5, 7). Metabolic adaptation to HP intake was characterized by a downregulation of lipogenesis from glucose (7, 41), an upregulation of AA catabolic pathways (16, 38), AA transfer to gluconeogenic pathways (2), and protein synthesis (48, 56). We have reported previously that the effect of HP diet on gluconeogenesis, lipogenesis, and protein metabolism was attributable mostly to hepatic metabolism adaptation (2, 7, 12).

AAs have been demonstrated to act as important signals, and AMPK, mTOR, and GCN2 are important candidates as transduction pathways involved in the control of metabolic response of the liver to HP feeding. AMPK phosphorylation is repressed by an increase in AA concentrations in β-cells (19) and the hypothalamus, (43) whereas it is induced in hepatocytes (34). Phosphorylation of mTOR was enhanced in isolated hepatocytes in response to branched-chain AAs (BCAA), mainly leucine (26). Interestingly, AMPK has been demonstrated to repress the mTOR signaling pathway (11, 32, 33, 36) and to prevent the activation of eukaryotic elongation factor 2 (9, 25) and $p70^{S6K}$ (15) in the liver. However, the effect of AAs on AMPK phosphorylation and activation remains unclear (19, 34, 43). The GCN2 transduction pathway in liver has been shown to be involved in sensing leucine deficiency (1, 21), but its role in liver AA sensing and in the detection of increases in AA concentration has not been addressed.

This study aims to evaluate whether the interplay between the transduction pathways AMPK, mTOR, and GCN2 can participate specifically in the sensing of the AA level provided to the liver and participate in hepatic metabolic responses to protein and energy intake in adapted rats. For this purpose, the phosphorylation state of mTOR, AMPK, and GCN2 transduction pathways was determined in the liver of rats fed a HP or normal protein (NP) diet. The specific role of these transduc-

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tion pathways in sensing AAs, glucose, and insulin was also determined in isolated hepatocytes. The results showed the involvement of mTOR, AMPK, and GCN2 in AA sensing and in coordinating energy supply and protein metabolism in the liver.

MATERIALS AND METHODS

Animals. The animal experimental protocols used in these studies were carried out in accordance with the guidelines of the French Committee for Animal Care and the European Convention of Vertebrate Animals Used for Experimentation. Male Wistar rats ($n = 24$), initially weighing 175–200 g, were purchased from Harlan (Horst, The Netherlands). They were housed under a 12:12-h light-dark cycle (12-h dark period starting at 2000) and were allowed free access to a commercial laboratory chow diet and water for 6 days before the initiation of any dietary adaptations or in vitro study.

In vivo study design in NP- and HP-fed rats. Rats were allocated to receive a NP or HP diet for 14 days. The rats' initial body weights did not differ between the groups or between experiments. NP and HP diets were formulated to be isoenergetic (14.7 kJ/g) and provided 13 and 48% of energy as protein, 12 and 14% of energy as fat, and 75 and 38% of energy as carbohydrate, respectively (Table 1). Rats were accustomed to receiving their food according to a pattern that consisted of a small meal of 6 g of dry matter between 0900 and 1000 and free access to food between 1400 and 1800. This pattern was adopted to train the animals to eat a standard meal within 1 h so as to standardize both the amount of energy ingested and the physiological state of animals that were to be studied in a fed state on the day of the experiment. The rats were allowed free access to water. At the end of this experiment, the rats were anesthetized with pentobarbital sodium (50 mg/kg body wt). After incision of the abdomen, the rats' livers were rapidly harvested under sterile conditions, frozen in liquid nitrogen, and stored at −70°C until further analysis for gene expression measurements and Western blots.

Primary culture of rat hepatocytes. Prior to the experiments, the rats used for in situ liver perfusion studies were allowed free access to a commercial laboratory chow diet and water for at ≥1 wk. Hepatocytes were isolated from the liver of fed rats using the collagenase method (6), as described previously (2). Cell viability was assessed by the trypan blue exclusion test and was always >85%. Hepatocytes were seeded at a density of 7×10^6 cells/dish in 100-mm petri dishes in M199 medium with Earle's salts (GIBCO, Invitrogen, Cergy Pontoise, France) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1% (wt/vol) BSA, 2% (vol/vol) Ultrocer G (Pall Life Sciences, East Hills, NY), 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO), 1 nM insulin (Sigma-Aldrich), and 100 nM triiodothyronine (Sigma-Aldrich). After cell attachment (4 h), the medium was replaced by M199 medium salts supplemented with 5.5 mM glucose and the AA concentration found in the portal vein of NP-fasted rats (low AA concentration), as described previously (2). Hepatocytes were then incubated overnight (18–24 h) at 37°C. The following day, the cells were cultured for a further 2 h in the same fresh medium and then incubated under various conditions, as described in the figure legends. Each treatment was performed in duplicate. In primary culture of hepatocytes, the cells do not keep the initial phenotype of the rat liver but are influenced by the composition of the medium.

Table 1. *Composition of diets*

	NP Diet	HP Diet
g/kg DM^{-1}		
Protein*	116.0	424.0
Sucrose	99.8	45.5
Cornstarch	548.0	253.0
Lactose	7.0	26.5
Soya oil	40.0	40.0
Dairy fat	2.8	10.6
Minerals (AIN-93M)†	35.0	35.0
Vitamins (AIN-93M)†	10.0	10.0
Cellulose	50.0	50.0
Choline	2.3	2.3
%Energy		
Total protein, %energy	13.0	48.0
Total fat, %energy	12.0	14.0
Total carbohydrate, %energy	75.0	38.0
Metabolizable energy, kJ/g DM^{-1}	14.8	14.7

NP, normal protein; HP, high protein; DM, dry matter. *Total milk protein isolate, purchased from Nutrinov, Rennes, France. †Purchased from ICN Biochemicals, Cleveland, OH.

Western blot analysis. Frozen liver (100 mg) or 7×10^6 hepatocyte cells were homogenized in a lysis buffer containing 40 mM Tris·HCl (pH 7.5), 300 mM sodium chloride, 2 mM EDTA, 2 mM EGTA, 100 mM sodium fluoride, 2 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 2 mM sodium orthovanadate, and 1% (vol/vol) Triton X-100 supplemented with a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The samples were centrifuged for 10 min at 10,000 *g* at 4°C, and the protein concentration was determined in the supernatant using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). An equivalent of 200 μg for liver or 70 μg for hepatocytes of protein per sample was resolved with Criterion XT gel Bis-Tris 4–12% (Bio-Rad Laboratories). After electrophoresis, the proteins were transferred overnight at 4°C to a nitrocellulose membrane (Millipore, Bedford, MA). The quality of transfer was verified by Coomassie gel and Ponceau S membrane staining. The membrane was then blocked for 1 h at room temperature in a solution of 5% (wt/vol) low-fat milk powder in 10 mM Tris·HCl (pH 7.4), 0.5 M NaCl, and 0.1% (vol/vol) Tween-20 (TBST) and then washed with TBST at room temperature. Incubation with the primary antibody (Cell Signaling Technology, Beverly, MA) was performed overnight at 4°C. The primary antibodies (dilution 1/1,000) used were the anti-phospho AMPKα (Thr^{172}) catalytic subunit, the anti-AMPKα catalytic subunit, anti-phospho-mTOR (Ser^{2448}), anti-mTOR, anti-phospho-4E-BP1 ($Thr^{37/46}$), anti-4E-BP1, anti-phospho-GCN2 (Thr^{898}), anti-GCN2, anti-phospho-eIF2α (Ser^{51}), anti-eIF2α, anti-phospho-acetyl-CoA carboxylase (ACC; Ser^{79}), and anti-ACC. At the end of incubation, the blots were washed extensively with TBST at room temperature and incubated with a secondary antibody conjugated to horseradish peroxidase (1/2,000; Santa Cruz Biotechnology, Santa Cruz, CA) for 90 min at room temperature. After further washing with TBST, the blots were developed using an enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL). To achieve further hybridization, the membrane was stripped in stripping buffer (pH 6.8) containing 62.5 mM Tris·HCl, 2% (wt/vol) SDS, and 100 mM β-mecaptoethanol at 60°C for 15 min.

Analysis of gene expression by real-time PCR. Total RNA was extracted from 0.05 to 0.1 g of frozen liver using Trizol Reagents (Invitrogen, Carlsbad, CA), and its amount was quantified at 260 nm. The quality and integrity of total RNA were assessed on 1% agarose gels containing ethidium bromide. First-strand cDNA was synthesized from 400 ng of total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) with a random hexamer. Real-time PCR was performed on 10 ng of cDNA using the power SYBR Green PCR master mix (Applied Biosystems) and a 7300 real-time PCR system (Applied Biosystems). All PCR reactions were carried out as follows: denaturation at 95°C for 10 min, 40 amplification cycles, with each cycle consisting of 15 s at 95°C, followed by 1 min at 60°C. The primers were designed using Oligo Explorer 1.1.0 software. The sequences of PCR primers used were 5′-TGTGGCTCGCCCAATTATG-3′ (up) and 5′-GACCCCGCTGCTCCAGAT-3′ (down) for AMPKα1 (NM_019142), 5′-TGGAGGTGAATTGTTCGACTACAT-3′ (up) and 5′-ACAGTAGTCCACGGCAGACAGA-3′ (down) for AMPKα2 (NM_023991), 5′-TGGAGGGAG-

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AGCGTCTGAGA-3′ (up) and 5′-TGATGTGCCGAGGCTTTGT-3′ (down) for mTOR (NM_019906), 5′-CTGCGGGTCCCTTTTGC-3′ (up) and 5′-AATCGGTCTAACTTCCTAGGTCTGAA-3′ (down) for GCN2 (NM_013719), and 5′-GGGAGCCTGAGAAACGGC-3′ and 5′-GGGTCGGGAGTGGGTAATTT-3′ for 18S.

For each run, a melt curve was performed to analyze the products generated and controlled for possible contamination resulting from residual genomic DNA amplification (using control without reverse transcriptase) and/or from primer-dimer formation (controls with no DNA template and no reverse transcriptase). The cycle threshold (C_T) for each sample was determined at a constant fluorescence threshold line. Ribosomal 18S RNA amplifications were used to account for variability in the initial quantities of cDNA, and interplate variations were corrected using an RT calibrator. Gene expression was determined using the $2^{-\Delta C_T}$ formula, where 2 represents the optimum efficiency (E) of PCR, which is $E = 2$ and $\Delta C_T = (C_{T\ \text{target gene}} - C_{T\ 18S})$. PCR efficiency was determined for each gene using a serial dilution of reverse-transcribed RNA.

Immunohistochemistry. For immunohistochemistry analyses, hepatocytes were seeded onto Lab-Tek Chamber Slide culture chambers (Nunc, Naperville, IL). Twenty-four hours after seeding, the cells were treated for 0, 30, and 60 min with low or high AA concentra-

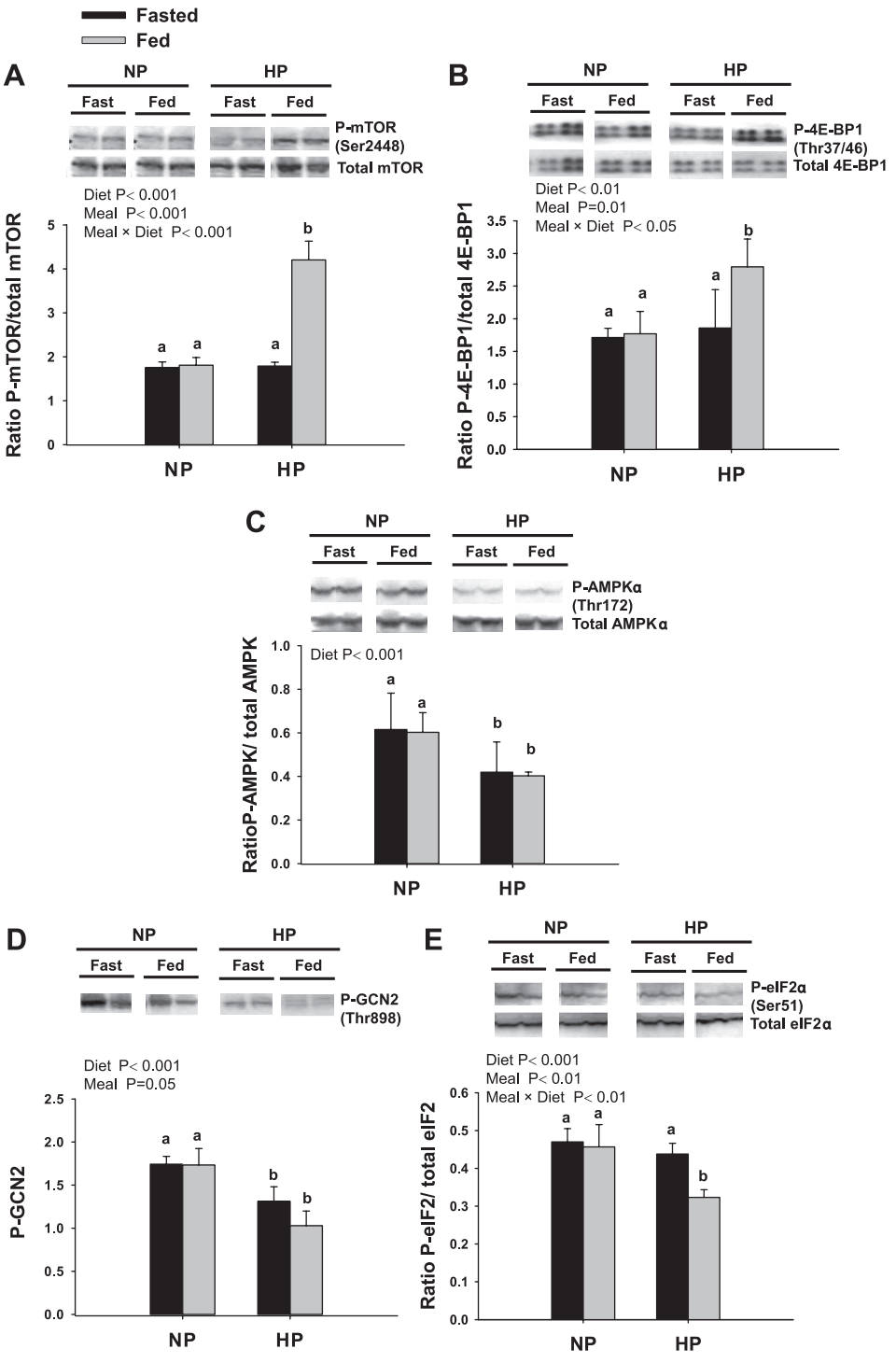


Fig. 1. Effects of normal- (NP) and high-protein (HP) diets on mammalian target of rapamycin (mTOR), eukaryotic initiation factor (eIF) 4E-binding protein-1 (4E-BP1), AMP-activated protein kinase (AMPK), general control nonderepressible kinase 2 (GCN2), and eIF2α phosphorylation in rat. Protein extracted from the livers of rats adapted to NP or HP diets for 14 days (n = 24) and euthanized in the fasted or fed state was processed for Western blot analysis as described in MATERIALS AND METHODS. *A–E*: representative Western blots (2 from 6 animals) of mTOR phosphorylation (p-mTOR) and total mTOR (*A*), 4E-BP1 phosphorylation (p-4E-BP1) and total 4E-BP1 (*B*), AMPKα phosphorylation (p-AMPKα) and total AMPKα (*C*), GCN2 phosphorylation (p-GCN2; *D*), and eIF2α phosphorylation (p-eIF2α) and total eIF2α (*E*). The results are expressed as means ± SD. The effects of diet (NP or HP) or meal (fasted or fed) were assessed by 2-way ANOVA. Bars with different letters within a graph are statistically significantly different (post hoc Tukey tests for multiple comparisons, $P < 0.05$).

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tions. They were then washed briefly three times with PBS, permeabilized with 0.25% Triton X-100 in 4% paraformaldehyde for 2 min, and fixed with 4% paraformaldehyde in PBS for 30 min. Cells were prehybridized for 1 h with 2% bovine serum albumin in PBS and then incubated overnight at 4°C with anti-phospho AMPKα (Thr^{172}) catalytic subunits (dilution 1/100) (Cell Signaling Technology) of rabbit monoclonal antibody. The membranes were then hybridized with secondary rabbit antibody conjugated to fluorescein isothiocyanate (FITC; dilution 1/200) (Santa Cruz Biotechnology). After mounting in Vecta-Shield (Vector Laboratories, Biovalley, Marne la Vallée, France), the samples were observed (×400 magnification) using a microscope equipped with epifluorescence and a camera (Axio Imager Z.1; Zeiss, Göttingen, Germany).

Statistics. The data are shown as means ± SD. The effects of diets (NP vs. HP) and meals (fasted vs. fed state) and their interactions were tested by two-way ANOVA (SAS 9.1; SAS Institute, Cary, NC). Post hoc Tukey tests for multiple comparisons were performed to make pairwise comparisons. Differences were considered significant at $P < 0.05$. For in vitro studies, differences from controls were analyzed using a paired *t*-test, comparing different treatments with the control. The level of significance was set at $P < 0.05$.

RESULTS

Liver mTOR, AMPK, and GCN2 phosphorylation and gene expression in NP and HP rats. The rats received the NP and HP diets for 2 wk. The liver was then removed in either the fasted or fed state to measure the phosphorylation state of mTOR, AMPK, and GCN2 and their downstream targets 4E-BP1 and eIF2α (Fig. 1). The results showed that HP feeding in the fed state upregulated mTOR and downregulated both the AMPK and GCN2 signaling pathways. Under these nutritional conditions, translation initiation was stimulated, as indicated by the increase of p-4E-BP1 and the decrease of p-eIF2α. In the HP-fasted state, no changes were observed for the mTOR pathway, whereas p-AMPKα and p-GCN2 decreased. These changes in the AMPKα and GCN2 phosphorylation state affected neither 4E-BP1 nor eIF2α phosphorylation. More precisely, both the mTOR (meal × diet effect, $P < 0.001$; Fig. 1*A*) and 4E-BP1 (meal × diet effect, $P < 0.05$; Fig. 1*B*) phosphorylation states were increased in the fed state in HP rats. The phosphorylation states of AMPKα and GCN2 were more influenced by diet than by nutritional state (diet effect, $P < 0.001$, meal effect not significant). As shown in Fig. 1*C*, HP-fed rats were characterized by lower levels of AMPKα phosphorylation (diet effect $P < 0.001$) in both the fasted and fed states when compared with NP rats. GCN2 phosphorylation was lower in HP rats and very low in HP-fed rats (diet effect, $P < 0.001$; Fig. 1*D*). The phosphorylation of eIF2α (Fig. 1*E*), the downstream target of GCN2, followed the same variations as p-GCN2 (meal × diet effect, $P < 0.01$).

The gene expression of AMPKα1 catalytic subunit, AMPKα2 catalytic subunit, mTOR, and GCN2 was also determined in the liver in both the fasted and fed states in NP and HP rats, respectively (Fig. 2). mTOR gene expression was affected in the liver neither by diet (NP or HP) nor by nutritional state (fasted or fed). GCN2 gene expression was not affected by diet (NP or HP) in the fasted state or by nutritional state (fasted or fed) in NP rats. By contrast, in HP rats, GCN2 gene expression was significantly higher (20%) in the fasted state than in the fed state (meal × diet effect, $P < 0.01$). For AMPK, and to distinguish between the AMPKα1 and AMPKα2 catalytic subunits, we examined mRNA levels for each isoform. AMPKα1 was significantly affected by diet ($P < 0.01$) and induced at the same level in both the fasted and fed states (37.5%) in HP rats. AMPKα2 gene expression was affected by neither diet nor nutritional conditions. The

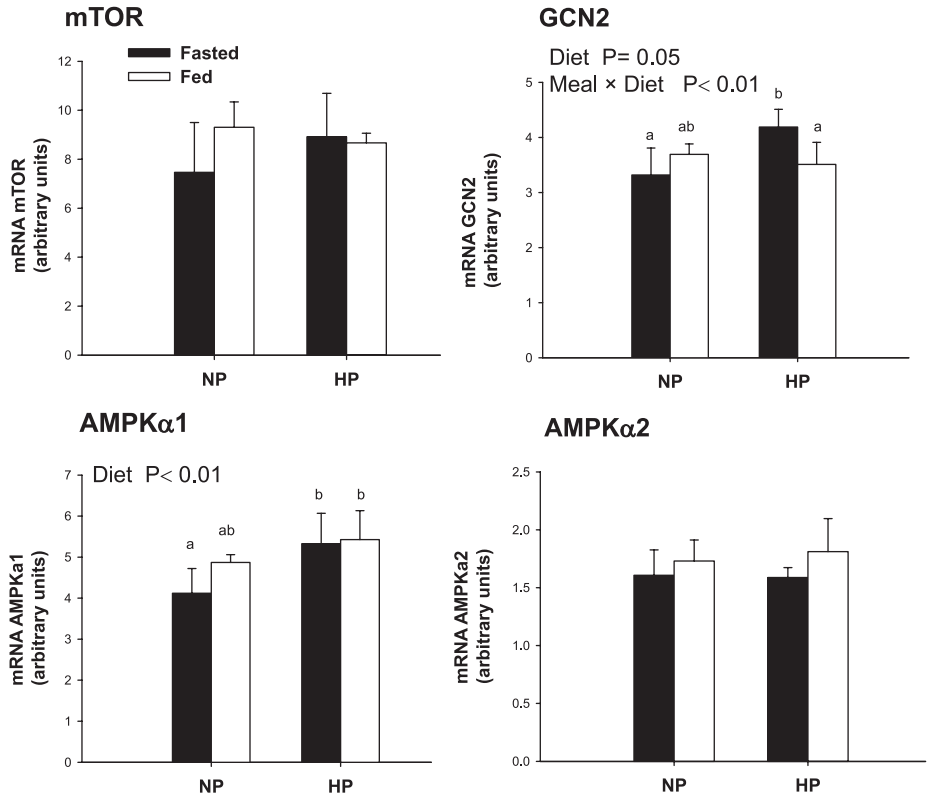


Fig. 2. Relative expression of genes encoding mTOR, GCN2, AMPKα1, and AMPKα2. Hepatic gene expression was measured using real-time PCR in rats adapted to NP or HP diets for 14 days ($n = 20$) and euthanized in the fasted or fed state (5 animals in each condition). The results are expressed as means ± SD. The effects of diet (NP or HP) or meal (fasted or fed) were assessed by 2-way ANOVA. Bars with different letters within a graph are statistically significantly different (post hoc Tukey tests for multiple comparisons, $P < 0.05$).

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results of gene expression in HP-fed state followed the same variation as protein, at least for mTOR and AMPK, compared with NP-fed rats. For GCN2, we were unable to measure total protein level. The similar level of mRNA between HP- and NP-fed rats indicated that there was no effect of protein intake on GCN2 gene expression.

Influence of AAs, glucose, and insulin on mTOR, AMPK, and GCN2 transduction pathways in vitro on isolated rat hepatocytes. To investigate whether AAs, glucose, or insulin are involved in the changes of mTOR, AMPK, and GCN2 phosphorylation state in the liver of HP-fed rats, we performed an in vitro study using primary culture of hepatocytes. Cells were incubated in the presence of low or high AA concentration [corresponding to the concentration measured in the portal vein of NP-fasted rats or HP-fed rats, respectively, as described previously (2)] in the presence or absence of insulin (100 nM). Hepatocytes were also incubated at low or high concentrations of glucose (5 or 25 mM of glucose).

The effects of glucose (Fig. 3), AAs, and insulin (Figs. 4 and 5) on the phosphorylation state of mTOR, AMPKα, GCN2, and 4E-BP1 were determined in vitro in primary cultures of isolated rat hepatocytes. As reported in Fig. 3, the increase of glucose concentration from 5.5 to 25 mM in the culture medium for 60 min did not modify the phosphorylation state of mTOR, 4E-BP1, and GCN2 (Fig. 3, *A*, *B*, and *D*) but significantly reduced the phosphorylation state of AMPKα (Fig. 3*C*) ($P < 0.001$). High AA concentrations or insulin enhanced mTOR phosphorylation ($P < 0.001$; Fig. 4*A*), but both signals were required to induce 4E-BP1 phosphorylation ($P < 0.01$; Fig. 4*B*). In particular, the increase in the concentration of BCAA or leucine was sufficient to stimulate the phosphorylation of both mTOR and 4E-BP1 in the presence of insulin (Fig. 5). AMPKα phosphorylation was lower in the presence of a high AA concentration whether insulin was present or not ($P < 0.01$; Fig. 4*C*). Insulin or high AA concentrations alone had no effect on p-GCN2, but the presence of both decreased p-GCN2 phosphorylation ($P < 0.0001$; Fig. 4*D*).

To confirm the effects of glucose and AAs on AMPKα, we examined whether the decrease in AMPKα phosphorylation was associated with changes in ACC phosphorylation. Surprisingly, we found that, after 60 min, AMPKα phosphorylation was decreased in the presence of either high glucose concentrations (25 mM, $P < 0.01$; Fig. 6*A*) or high AA concentrations (Fig. 6*B*) ($P < 0.001$), whereas ACC phosphorylation was decreased with high glucose concentration (Fig. 6*A*) ($P < 0.01$) but was not affected by high AA concentrations (Fig. 6*B*). Moreover, to confirm the effect of AAs on AMPK dephosphorylation in hepatocytes, immunohistochemical analyses of p-AMPKα were performed under both low and high AA concentrations. As shown in Fig. 7, under negative control

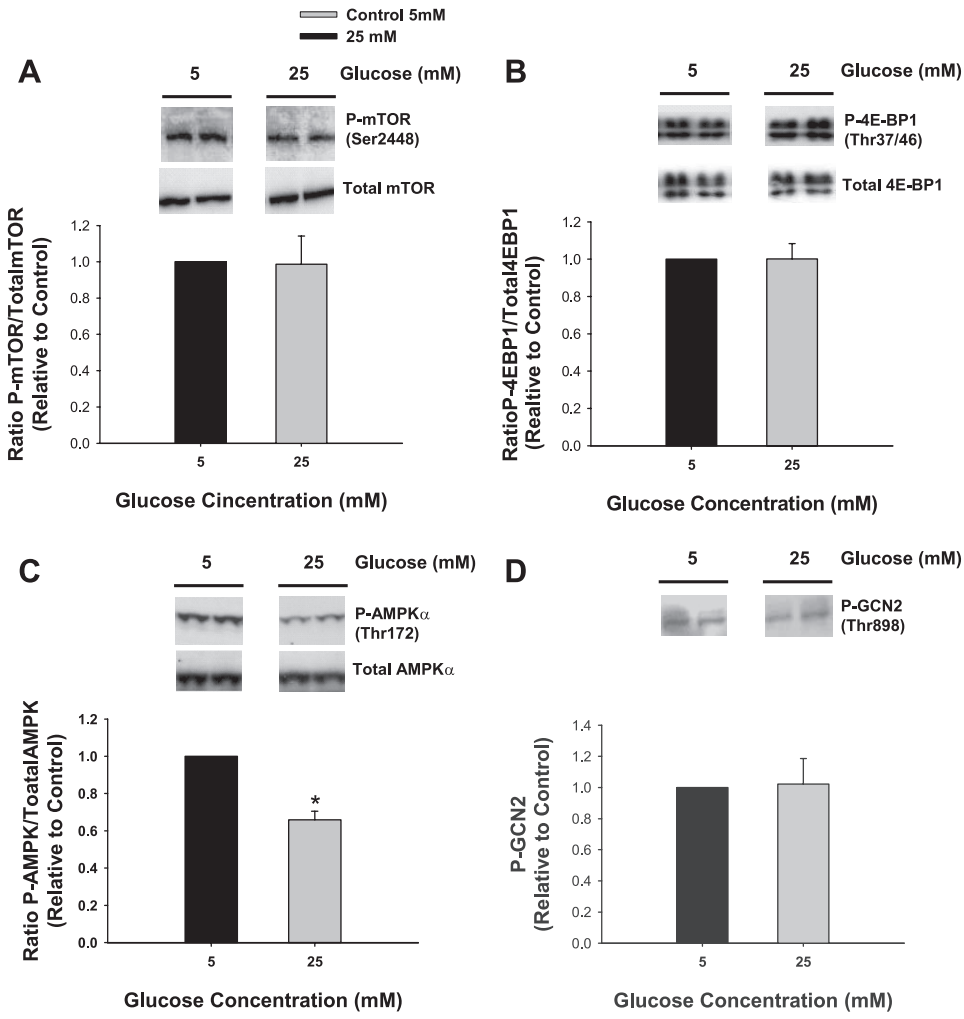


Fig. 3. Effect of glucose on mTOR, 4E-BP1, AMPK, and GCN2 in a primary hepatocyte culture. Hepatocytes were incubated for 60 min in M199 salt medium containing a low amino acid concentration plus glucose at 5 or 25 mM. Protein extracts were processed for Western blotting, as described in MATERIALS AND METHODS. Results are representative of at least 2 separate experiments. Western blots of p-mTOR and total mTOR (*A*), p-4E-BP1 and total 4E-BP1 (*B*), p-AMPKα and total AMPKα (*C*), and p-GCN2 (*D*) are represented. The graphs represent the results of phosphorylated protein and the total protein ratio for the 4 samples from 2 separated cultures, except for GCN2. The results are expressed as means ± SD; $n = 4$. Statistically significant differences from controls were determined using a paired *t*-test, comparing different treatments with the controls, and are represented in the graphs (*$P <$ 0.01).

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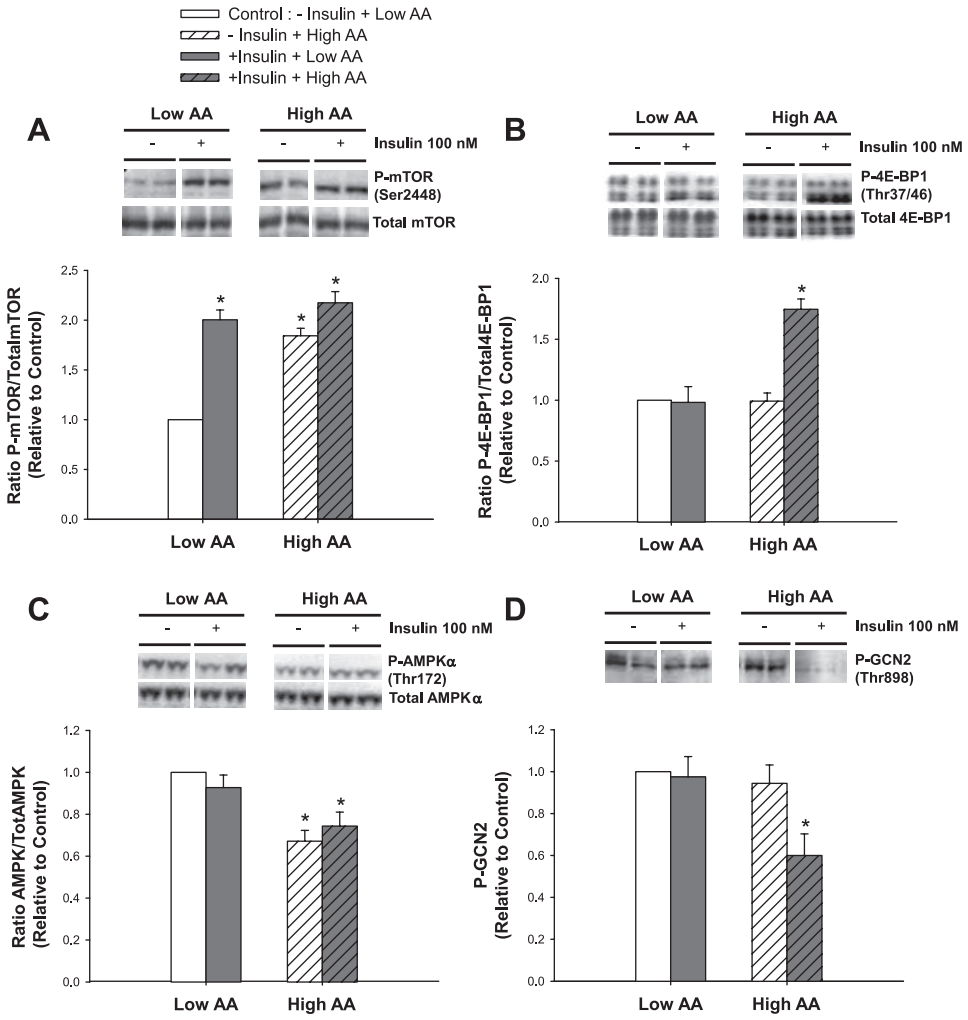


Fig. 4. Effect of amino acids (AA) and insulin on mTOR, 4E-BP1, AMPK, and GCN2 in a primary hepatocyte culture. Hepatocytes were incubated for 60 min in M199 salt medium containing 5 mM glucose supplemented with amino acids at low (low AA) or high amino acid (high AA) concentrations with or without insulin. Protein extracts were processed for Western blot analyses, as described in the MATERIALS AND METHODS. Results are representative of at least 2 separate experiments. Western blots of p-mTOR and total mTOR (*A*), p-4E-BP1 and total 4E-BP1 (*B*), p-AMPKα and total AMPKα (*C*), and p-GCN2 (*D*) are represented. The graphs represent the results of phosphorylated protein and the total protein ratio for the 4 samples from 2 separated cultures, except for GCN2. The results are expressed as means ± SD; $n = 4$. Statistically significant differences from controls were determined using a paired *t*-test, comparing different treatments with the controls, and are represented in the graphs (*$P < 0.01$).

conditions (without anti-p-AMPKα Thr^{172} antibody), no emission of fluorescence could be detected. In cells incubated with low AA concentrations, bright fluorescence was observed, whereas a reduction in fluorescence was seen clearly after cell exposure to high AA concentrations for 60 min. At 30 min, there was no difference in fluorescence emission under low and high AA concentrations.

DISCUSSION

The mTOR, AMPK, and GCN2 transduction pathways and their downstream targets 4E-BP1, ACC, and eIF2α play a key role in nutrient sensing and in the regulation of protein and energy metabolism. Taken together, our results agree with the idea that amino acid sensing in the liver involves coordinated action of these transduction pathways to ensure the adaptation of liver protein and energy metabolism to the availability of amino acids and energy nutrients. A proposed scheme for the control of liver metabolism by AMPK, mTOR, and GCN2 according to protein level in the diet is shown in Fig. 8. The results showed that, at high glucose levels, AMPK phosphorylation was inhibited, whereas p-GCN2 and p-mTOR were not affected. At high amino acid levels, p-AMPK and p-GCN2

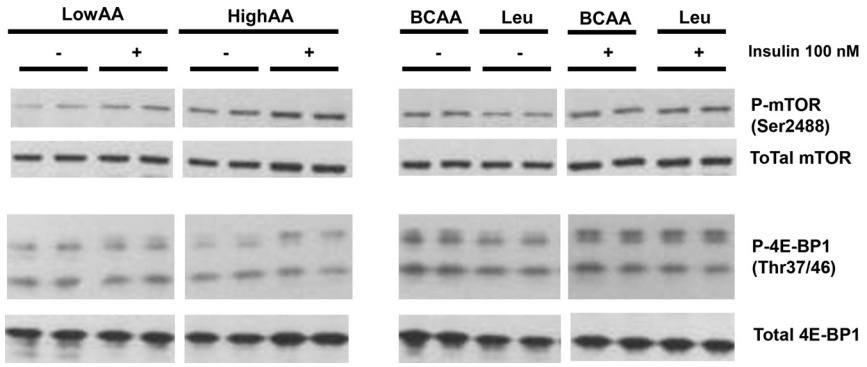


Fig. 5. Effect of branched chain amino acids (BCAA), leucine (Leu), and insulin on mTOR and 4E-BP1 phosphorylation in a primary hepatocyte culture. Hepatocytes were incubated for 60 min in M199 salt medium containing 5 mM glucose supplemented with AA at low or high AA concentrations with or without insulin. To study the effect of BCAA and Leu, the medium containing low AA was supplemented with BCAA or Leu at the same level as high AA. Protein extracts were processed for Western blot analyses, as described in the MATERIALS AND METHODS. Results are representative of at least 2 separate experiments.

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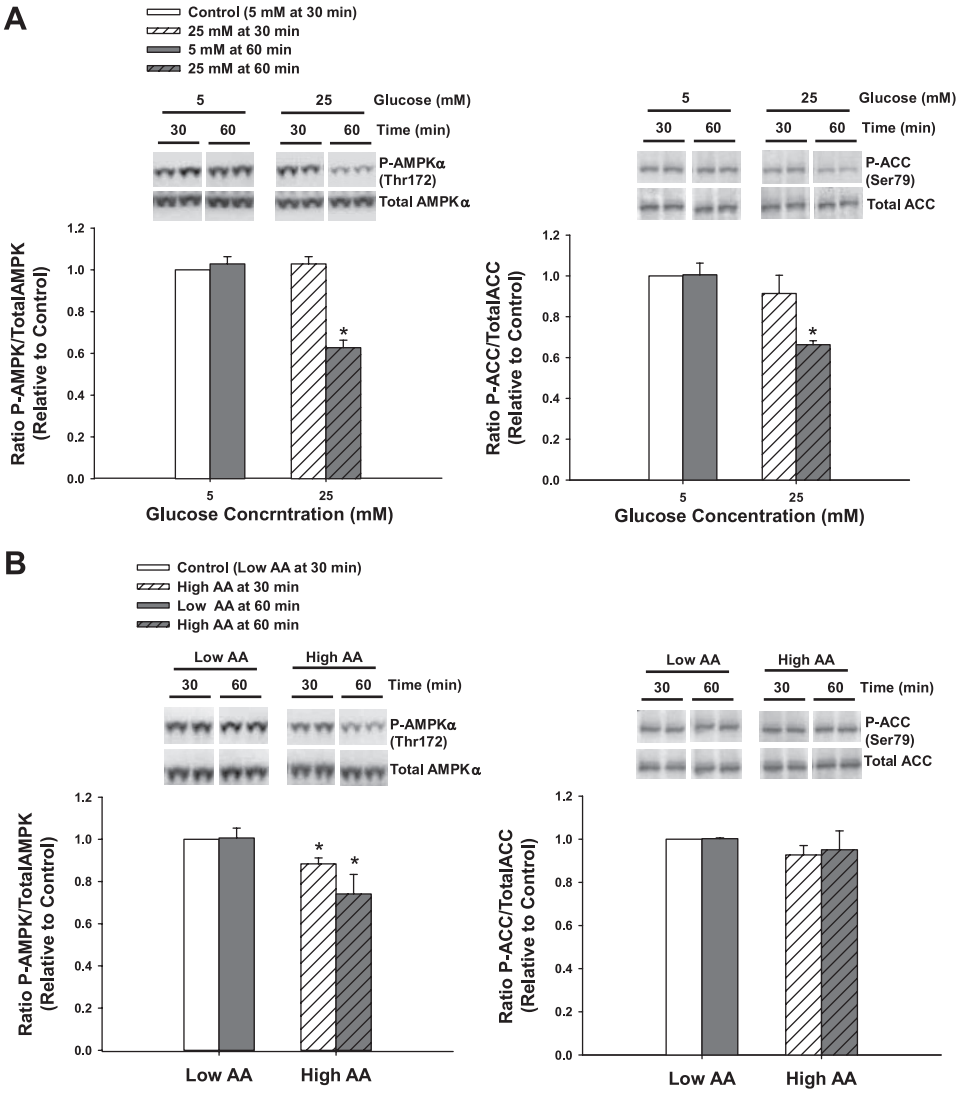


Fig. 6. Effects of glucose and AAs on AMPK and acetyl-CoA carboxylase (ACC) in primary hepatocytes. Hepatocytes were treated with various conditions: 5 or 25 mM glucose or low or high AA concentrations for 30, 60, and 90 min. Cells were processed for Western blotting, as described in MATERIALS AND METHODS. The results are representative of at least 2 separate experiments. Western blots of p-AMPKα and total AMPKα and p-ACC and total ACC in response to glucose (*A*) and amino acids (*B*), respectively, are represented. The graphs represent the results of phosphorylated protein and the total protein ratio for the 4 samples from 2 separated cultures. The results are expressed as means ± SD; $n = 4$. Statistically significant differences from controls were determined using a paired *t*-test, comparing different treatments with the controls, and are represented in the graphs (*$P <$ 0.01).

decreased, and p-mTOR was enhanced. One important result was the demonstration that GCN2 is able to sense an increase in amino acid levels in the liver in the presence of insulin. Another important result was that amino acid and insulin levels could modify the phosphorylation state of 4E-BP1 in response to mTOR and AMPK. A third important observation was that glucose but not amino acids decreased p-ACC through the decrease of p-AMPK.

This study provides evidence that in rats adapted to a HP diet, the increase of protein intake induces a metabolic adaptation characterized in the liver by a concomitant increase of mTOR phosphorylation and a decrease of both AMPK and GCN2 phosphorylation compared with a NP diet. It also appears that amino acids together with glucose and insulin are involved in the control of mTOR, AMPK, and GCN2 phosphorylation in the liver. The availability of energy substrates, i.e., glucose or amino acids, through the increase of ATP/AMP cellular ratio could act directly as a signal into the cells. Indeed, the decrease in p-AMPK in response to the increase of either glucose or amino acids observed in the present study confirmed this hypothesis. Moreover, in rats fed a HP diet, the plasma insulin was at the same level as that seen in rats fed an NP diet (2, 7), which suggests that insulin could be an important regulatory factor in both NP and HP conditions. These results are in line with an increase of p-mTOR, which required insulin or high amino acid concentration, as observed previously in other tissues (18, 23, 52). Interestingly, GCN2 has been reported to be involved in the control of translation during amino acid deprivation (14, 50), and the present results showed that GCN2 phosphorylation was reduced by both amino acids and insulin, suggesting that GCN2 senses not only the amino acid deficiency but also the amino acid increase in the liver. Moreover, our findings also demonstrated that an increase in 4E-BP1 phosphorylation and a decrease in GCN2 phosphorylation required both amino acids and insulin. Finally, no changes were observed in gene expression encoding mTOR, GCN2, AMPKα1, and AMPKα2, between HP- and NP-fed rats, which was in line with no changes in the total protein level observed. This suggests that the differences in the phosphorylation status were not due to changes in total protein level.

The mTOR, AMPK, and GCN2 transduction pathways and their downstream targets play key roles in the response of liver energy metabolism to HP feeding conditions. These nutritional conditions were previously characterized by both lower lipo-

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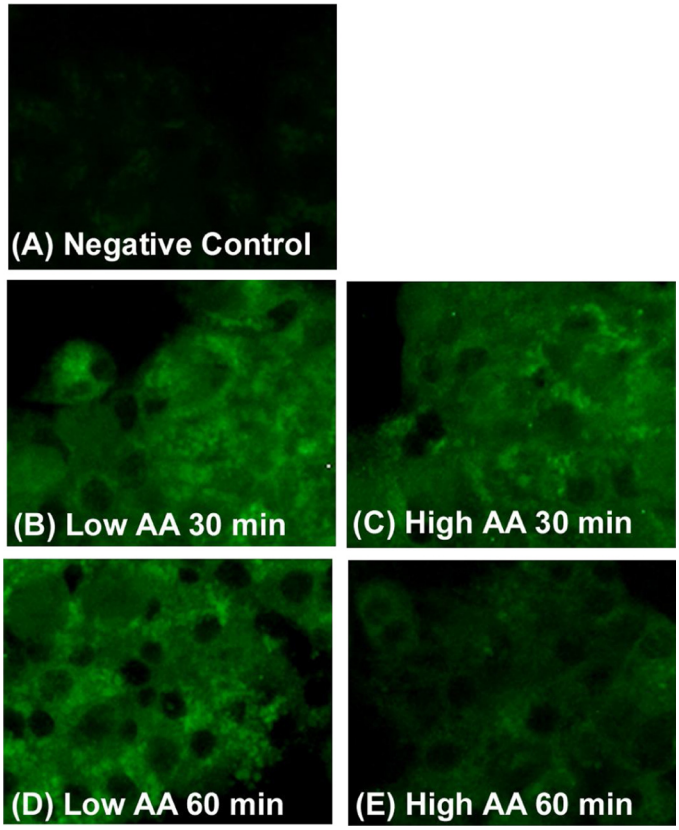


Fig. 7. Immunofluorescence of AMPK phosphorylation in control cells and cells treated with low or high AA concentrations for 30 and 60 min. Cells were plated on epoxy-treated slides, and AMPK phosphorylation was detected using p-AMPKα (Thr^{172}) antibody followed by treatment with a 2nd FITC-conjugated antibody. These results are representative of 2 separate primary hepatocyte cell cultures. Samples were observed (×400 magnification) using a microscope equipped with epifluorescence and a camera (Axio Imager Z.1; Zeiss, Göttingen, Germany).

genesis and higher gluconeogenesis in the liver (2, 7). Interestingly, the present results showed that p-AMPK levels were decreased by amino acids or glucose, whereas p-ACC was reduced only by glucose (but not by amino acids) in hepatocytes. As a consequence, liver lipogenesis could be stimulated in the presence of high glucose concentration but not in the presence of high amino acid concentration. It has been established that AMPK phosphorylates and inactivates ACC, a key regulatory enzyme of the biosynthesis of fatty acids and a regulator of fatty acid oxidation through the formation of malonyl-CoA. In addition, mTOR inhibition increases fatty acid oxidation and reduces lipid synthesis (8). Data arising from gene knockout studies have also suggested that a reduction in p-GCN2 and an increase in p-4E-BP1 should induce lipogenesis. In mice lacking GCN2, lipogenic genes such as fatty acid synthase and sterol regulatory element-binding protein-1c are induced, and the liver exhibits steatosis (21). Moreover, 4E-BP1 gene knockout studies in mice have also revealed a reduction in body fat content (51). Reduction in the levels of the 4E-BP2 isoform by the antisense oligonucleotide treatment of obese mice lowered body fat content associated with a reduction in liver lipogenic (fatty acid synthase) and gluconeogenic (glucose-6-phosphatase and phospho*enol*pyruvate carboxykinase) gene expression (57). In agreement with our results, Baquet et al. (3) reported that amino acids exerted no effect on malonyl-CoA concentrations in incubated hepatocytes in the absence of glucose, whereas lipogenesis was induced at high concentration of glucose and amino acids, suggesting that glucose is the principal regulator of ACC phosphorylation. Thus, in contrast with previous scientific consensus views on the effect of AMPK on the control of ACC activity, these data suggest that the effect of glucose involves at least two steps: first, a decrease in AMPK phosphorylation and second, a dephosphorylation of ACC, which are additional. However, amino acids only exert their effects on the first step by decreasing AMPK phosphorylation and activity to produce a permissive effect on translation rather than lipogenesis. The difference in the cellular action of glucose and amino acids may result from the activation of different serine/threonine protein phosphatases or from different substrate specificity of one of these phosphatases. In addition, the specific effect of amino acids on AMPK phosphorylation, comparatively with glucose effect, was in line with a stimulation of liver gluconeogenic pathways with HP feeding in the rat (2) since the stimulation of AMPK downregulated hepatic glucose production (31). As a consequence, the decrease of AMPK phosphorylation induced by the increase in amino acid concentration could stimulate liver gluconeogenesis.

This study also showed that the control of translation in the liver by an HP diet requires energy, amino acids, and insulin, all sensed by AMPK, mTOR, and GCN2. Through mTOR activation and AMPK inhibition, HP diet could stimulate liver translation at the initiation step of mRNA 5′-cap recognition via 4E-BP1. Amino acids can stimulate p-$p70^{S6K}$ and/or p-4E-BP1, downstream targets that are representative of mTOR activity (26, 35). Despite much evidence of direct phosphorylation of 4E-BP1 by mTOR (54), the present findings indicate that in hepatocytes, either amino acids or insulin can stimulate p-mTOR, but this is not sufficient for 4E-BP1 phosphorylation that requires both. As expected, BCAA or leucine stimulated the phosphorylation of mTOR (26), but without any effect on 4E-BP1 phosphorylation. Both insulin and BCAA or leucine are required for 4E-BP1 phosphorylation. These results are in agreement with the idea that amino acids and insulin exert a coordinated action on translation (35). The stimulation of mTOR by insulin through the class I phosphoinositide 3-kinase/Akt pathway inactivates the TSC complex (tuberous sclerosis complex of TSC1/TSC2, the negative regulator of mTOR) (28, 33, 36, 37) leading to the activation of Rheb (27, 33). Furthermore, mTOR activation required that both mTOR complex 1 (mTORC1) formation (mTOR, raptor, PRAS40, mLST8) and amino acids stimulate the relocation of mTORC1 mediated by Rags [small guanosine triphosphatases (GTPases) in the Ras superfamily] to the proper location, which is the perinuclear region of the cell, or to large vesicular structures overlapping with Rab7 (Rab GTPases belong to the RAS superfamily and are the key regulators of membrane traffic) (45). This proper location might contain Rheb (45, 46), a Ras family GTPase that is upstream of the mTOR transduction pathway (27), and this association increases mTORC1 kinase activity and phosphorylates its downstream targets, i.e., 4E-BP1 and $p70^{S6K}$. Thus, amino acids has a potential ability to relocate mTORC1 to the proper location to receive the growth factor-mediated signal from Rheb (45, 46). Another component, which is mediated in part by amino acid activation of

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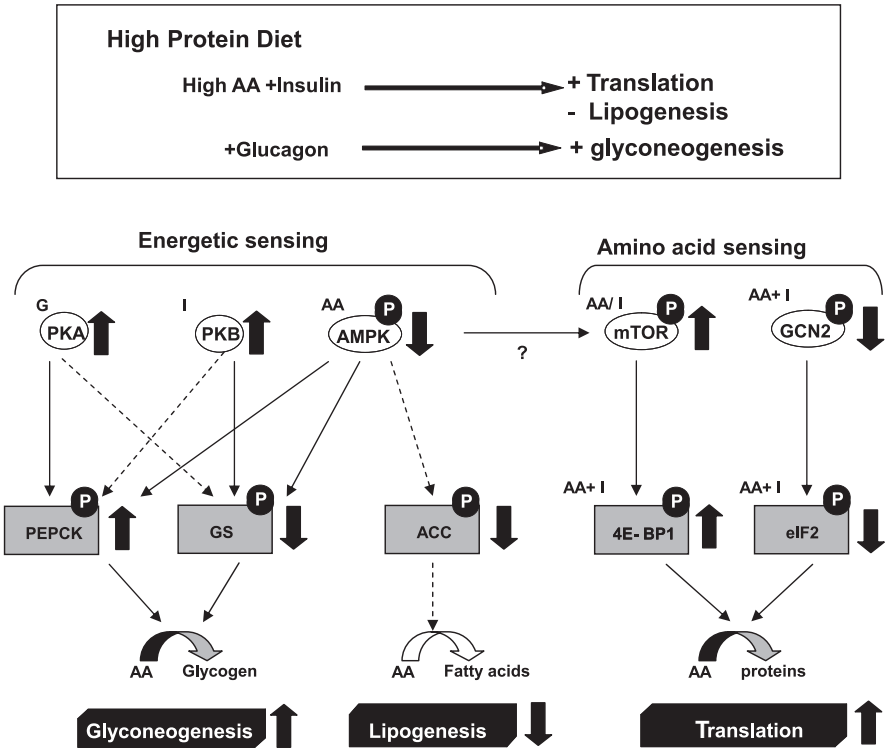


Fig. 8. Proposed scheme for the effects of nutrients on translation and lipogenesis in the liver. AMPKα senses energetic nutrients, whereas mTOR and GCN2 act as AA and insulin intracellular signal sensors. A HP diet stimulates both translation and glyconeogenesis and inhibits lipogenesis in the liver. Both high AA levels and insulin (I) are required to stimulate translation because it is associated with an increase in p-mTOR and p-4EBP1 and a decrease in p-GCN2 and p-eIF2α. In addition, high AA levels reduce p-AMPK and exert no effect on p-ACC, as shown by the dashed line. For glyconeogenesis pathways, AAs together with glucagon (G) stimulate gluconeogenesis at the level of phospho*enol*pyruvate carboxykinase (PEPCK), but the glucose 6-phosphate produced may be directed to glycogen synthesis because of the inhibition of glucose-6-phosphatase. The decrease in AMPK phosphorylation may be involved in this effect through removing inhibition of PEPCK and glycogen synthase (GS; as presented by dashed line). Insulin has no effect on the inhibition of PEPCK (dashed line) but acts together with AAs to stimulate glycogen synthesis.

mTORC1, is mammalian vascuolar protein sorting 34 (hVps34) (10, 40). Amino acids through the increase of intracellular Ca^{2+} induced the direct binding of Ca^{2+}/calmodulin to hVps34, which is required for an increase of mTORC1 complex signaling (20). Gulati et al. (20) additionally hypothesized that hVps34 interacts with mTOR, resulting in a conformation change and allowing activation of downstream targets. Moreover, hVps34 plays a role in multiple vesicular trafficking pathways, and it is recruited to the early endosome by Rab5 (39) and colocalizes with Rab7 on the late endosome (47). Overexpression of Rheb-eGFP constructs produces large Rab7/9-positive vesicles, suggesting a link between Rheb and endocytic trafficking (44). These findings suggest that, in response to amino acid signaling, besides Rags, hVps34 is also a possible mediator involved in the mTORC1 localization to the proper position. Moreover, AMPK can phosphorylate mTOR directly on Thr^{2446}, leading to its inactivation (11), or indirectly via the phosphorylation of the TSC2 gene product and raptor (22). Thus, the decrease in p-AMPK by amino acids observed in the present study suggests an additional cooperative effect between insulin and amino acids to stimulate the mTORC1 complex.

In addition to mTOR and AMPK effect through 4E-BP1, translation was also controlled at the level of binding Met-$tRNAi^{Met}$ to the 40S subunit, a process that is controlled indirectly by the phosphorylation state of eIF2α. The phosphorylation of eIF2α on serine 51 inhibits guanine nucleotide exchange on eIF2, converting it to a competitive inhibitor of eIF2B, and restrains general and specific mRNA translation (13, 14). eIF2α phosphorylation is under the control of GCN2, which is known to be activated by uncharged tRNA accumulation during amino acid limitation (24, 55). Our findings show that, in response to an increase in both amino acids and insulin, the decrease in phosphorylation of GCN2 and its downstream target eIF2α could be involved in the stimulation of the second step of translation, thereby enhancing general or specific mRNA translation (13). The physiological consequences of the changes in the factors that control translation require further investigations. Interestingly, in vitro, hepatocytes quickly respond (within 1 h) to the increase of amino acid concentration, suggesting that liver cells have the ability to sense the changes of environmental exposure. However, in vivo, the behavior and food intake parameters were disturbed during the first day of HP feeding, and then most parameters returned to the basal values by the 2nd day (5). Ropelle et al. (43) have reported that AMPK phosphorylation decreased in the brain during the first 3 days of HP feeding and increased progressively from the 5th to the 21st day, whereas mTOR downstream targets followed an inverse profile. Thus, we can hypothesize that many changes in signaling events could occur during acute exposure to a HP diet in vivo. Taken together, these data suggest that there is a progressive adaptation to HP intake through earlier (during the first 5 days) sensing events probably involving the gut-brain axis and later responses (after 1 wk) after a stabilization of blood nutrients and hormones that affect physiological responses through progressive metabolic changes. The role of amino acids in this progressive adaptation to HP intake remains to be determined.

In conclusion, this study provides evidence that the mTOR, AMPK, and GCN2 transduction pathways are key actors in liver protein and energy metabolic pathway responses, according to nutritional conditions, in adapted rats. These transduction pathways are important elements for the modulation of glycolysis, lipogenesis, glycogenogenesis, gluconeogenesis, and protein synthesis, depending on the different energy nutrient profiles induced by nutritional conditions. It appeared in our study that, regardless of the nutrient profile in the fed state (i.e., high glucose or high amino acid release from the meal),

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AMPK was depressed, thus playing a major role as a ubiquitous energy sensor. By contrast, other sensing systems such as mTOR and GCN2 were more nutrient specific and modulated the metabolic effects of AMPK. At high glucose levels, both AMPK and mTOR were inhibited and participated together with insulin in upregulating glycolysis, glycogenogenesis, and lipogenesis to use glucose as a fuel and store the energy excess provided by glucose as fatty acids. By contrast, at high amino acid levels, AMPK and GCN2 were inhibited, whereas mTOR was activated, and this mechanism participated with glucose, insulin, and glucagon in upregulating gluconeogenesis and protein synthesis to produce glucose from amino acids and store the excess of amino acids as liver protein (mainly albumin) (49), whereas the lipogenesis arising from glucose was inhibited to spare glucose (7, 30, 41). These complex signaling pathways would likely allow liver cells to adapt to changes in nutrient supply.

DISCLOSURES

No conflicts of interest are declared by the author(s).

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2. Complementary results

The results from the first publication demonstrated that both high AA levels and insulin were required to stimulate translation. Moreover, the stimulation of translation by AAs and insulin was associated with the increase in mTOR phosphorylation and a decrease in AMPK and GCN2 phosphorylation. We performed experiments in order to identify the AA signal and to investigate the role of mTOR and the AMPK signaling pathway in the control of translation in response to insulin and AAs using AICAR and rapamycin.

2.1. Identification of the amino acid signals

It was previously reported that mTOR phosphorylation was enhanced in isolated hepatocytes in response to BCAA, mainly leucine (Leu) (Ijichi et al. 2003). Thus, we aimed to investigate whether BCAA or Leu had a major role in these signaling pathways. Primary cultures of hepatocytes were performed and the phosphorylated protein was analysed by Western blot. The hepatocytes were incubated in low or high AA concentrations, or BCAA or Leu alone, at the same level as high AA concentrations in the presence or absence of insulin.

As we previously reported in the first publication, the results showed that mTOR phosphorylation was enhanced by high AA concentrations and 100 nM of insulin (Figure 18a). Interestingly, we observed that BCAA or Leu were sufficient to stimulate the phosphorylation of mTOR at a level which is similar to that observed with high AA (Figure 18a). Similarly to mTOR, the increase in AA levels, BCAA or Leu, in the presence of insulin induced the 4E-BP1 phosphorylation (Figure 18b). We also observed that the phosphorylation of S6, the downstream target of S6K1 (another well known downstream target of mTOR), was increased either by insulin or by high AA concentrations, and the effect of high AA levels was reproduced by BCAA or Leu (Figure 18c). Moreover, AMPK phosphorylation was not significantly reduced by insulin whereas this decrease was more pronounced by high AA concentrations, BCAA and Leu (Figure 19d). The GCN2 phosphorylation was dramatically decreased by both high AA levels,

BCAA or Leu and insulin (Figure 19e).

These results demonstrated that BCAA or Leu had the same effect as high AA concentrations. These suggested that at least BCAA or Leu were the AA signals and the increase of the concentration of these AAs, together with insulin was sufficient to stimulate the translation in primary culture of hepatocytes. Moreover, the stimulation of translation by BCAA or Leu in the presence of insulin involved the same signaling pathways as those observed at high AA concentrations in the presence of insulin, and was characterized by an increase in mTOR phosphorylation and a decreased in both AMPK and GCN2 phosphorylation.

2.2. Effect of AICAR and Rapamycin on the mTOR, AMPK and GCN2 transduction pathways

In order to understand the role of mTOR and AMPK transduction pathways in AA signaling and the relation between the two signaling pathways, we performed *in vitro* experiment using AICAR, an activator of AMPK, or rapamycin, an inhibitor of mTOR. The hepatocytes were incubated in low or high AA concentrations in the presence of insulin with or without AICAR or rapamycin for 60 minutes. At the end of incubation, cells were lysed and protein extracts were analysed by Western blot.

First, this study showed that mTOR phosphorylation was slightly decreased by AICAR and more reduced by rapamycin in both low and high AA concentrations in the presence of insulin (Figure 20a). Moreover, AMPK phosphorylation was strongly induced by AICAR in both low and high AA concentrations in the presence of insulin whereas rapamycin had no effect on P-AMPK (Figure 21d). Surprisingly, AICAR induced a dramatic decrease of 4E-BP1 phosphorylation, one of the well known downstream targets of mTOR, at both low and high AA concentrations in the presence of insulin (Figure 20b). Rapamycin had no effect on 4E-BP1 phosphorylation at low AA concentrations whereas at high AA concentrations, rapamycin induced a decrease in P-4E-BP1 (Figure 20b). In addition, either AICAR or rapamycin decreased S6 phosphorylation in both low

and high AA levels in the presence of insulin (Figure 20c). For GCN2, AICAR induced a slight increase in P-GCN2 however this effect was not significant. We also observed that rapamycin had no significant effect on GCN2 phosphorylation (Figure 21e).

These results demonstrated that the stimulation of translation in hepatocytes required the activation of mTOR and the inhibition of AMPK. This was associated with an increase of P-4E-BP1 and P-S6. These results also confirmed that the stimulation of translation by AAs and insulin required the coordination of mTOR, AMPK and GCN2 transduction pathways. Moreover, because AICAR strongly decreased 4E-BP1 phosphorylation without any effect on mTOR and because S6 phosphorylation was decreased in the presence either rapamycin or AICAR, our results suggest that AMPK was involved in the control of translation through 4E-BP1 and/or S6K (one of the S6 regulators).

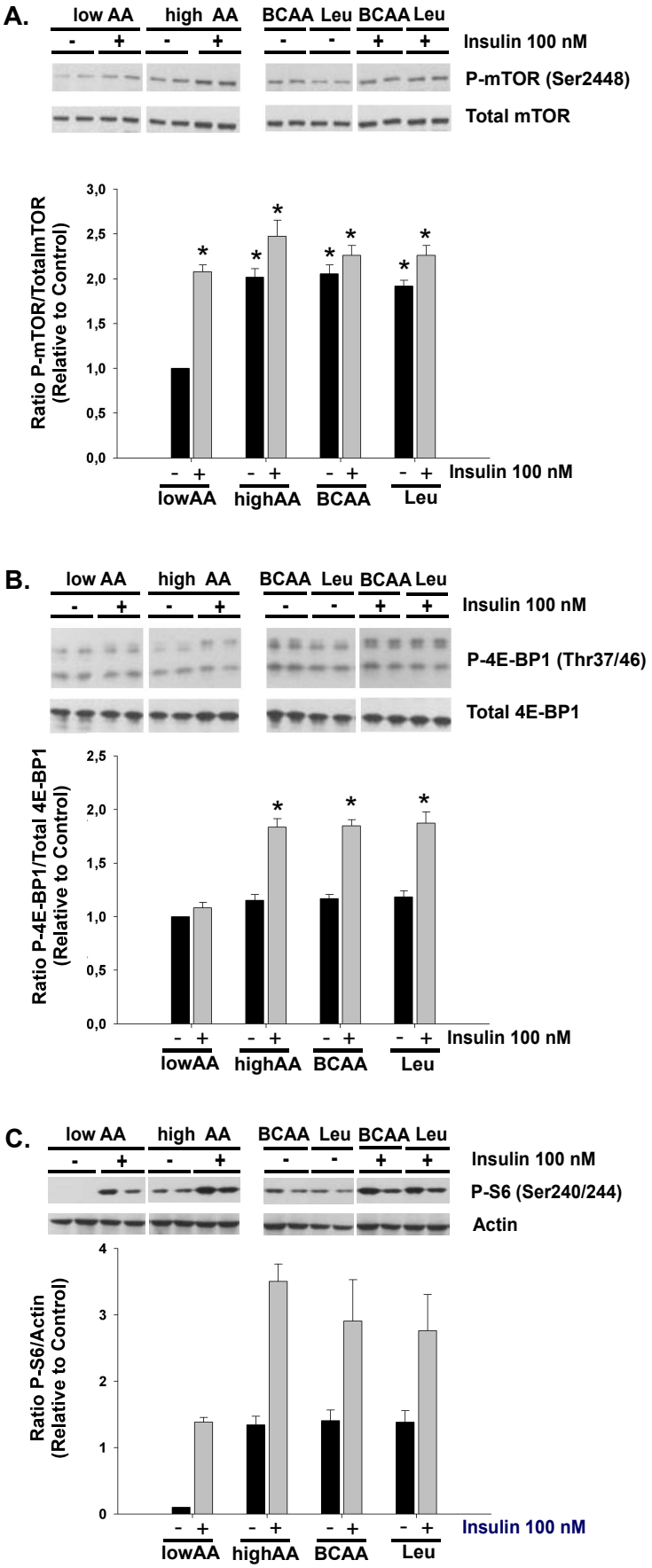


Figure 18 : Effect of Branched chain amino acids (BCAA), leucine (Leu) and insulin on mTOR, 4E-BP1, and S6 phosphorylation in a primary hepatocyte culture. Hepatocytes were incubated in a M199 salt medium containing 5 mM glucose supplemented with amino acids at low (lowAA) or high amino acid (highAA) concentrations, BCAA or Leu at the same level as highAA with or without insulin. Protein extracts were processed for Western blot analysis. Results are representative of at least two separate experiments. A, B and C are representative of the Western blots of mTOR phosphorylation (P-mTOR), total mTOR (Total mTOR), 4E-BP1 phosphorylation (P-4E-BP1), total 4E-BP1(Total 4E-BP1), S6 phosphorylation (P-S6) and Actin, respectively. The graphs represent the results of the four samples from two separated cultures. The results are expressed as means ±SD, for n=4. Statically significant differences from control (lowAA without insulin) were determined using a paired *t-test* comparing different treatments with the controls and are represented in the graphs (* P<0.001).

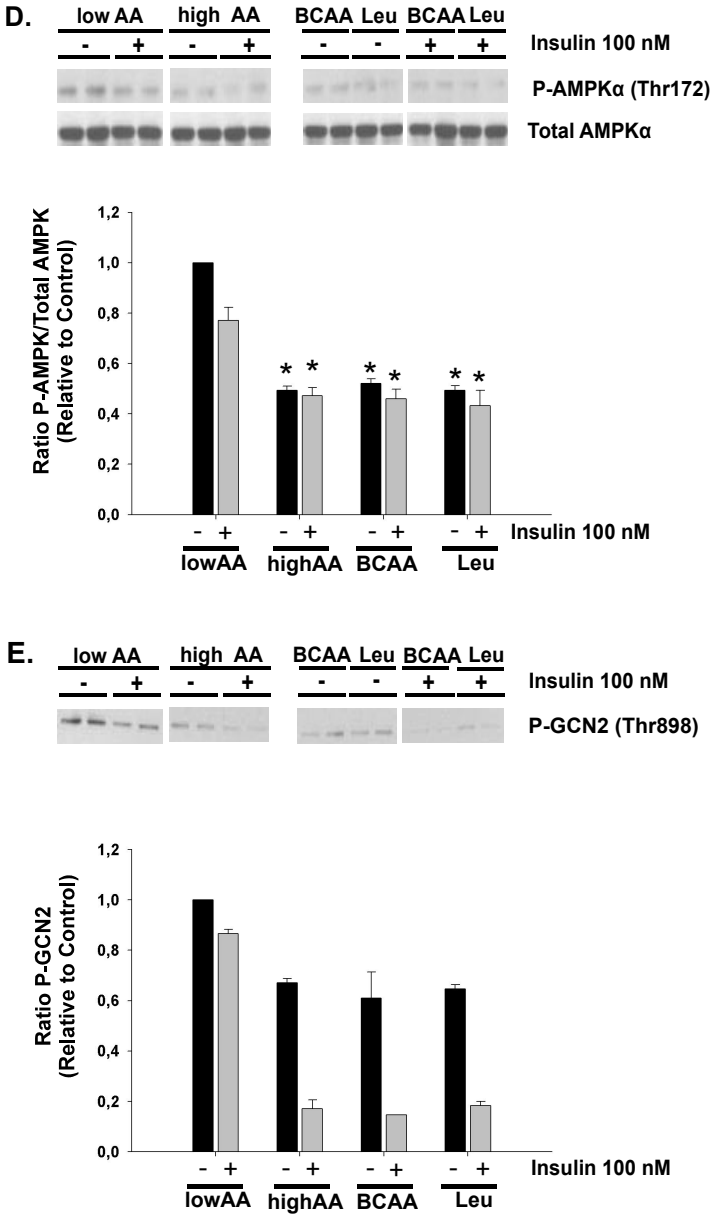


Figure 19 : Effect of Branched chain amino acids (BCAA), leucine (Leu) and insulin on AMPKα and GCN2 phosphorylation in a primary hepatocyte culture. Hepatocytes were incubated in a M199 salt medium containing 5 mM glucose supplemented with amino acids at low (lowAA) or high amino acid (highAA) concentrations, BCAA or Leu at the same level as highAA with or without insulin. Protein extracts were processed for Western blot analysis. Results are representative of at least two separate experiments. D and E. are representative of the Western blots of AMPK phosphorylation (P-AMPK), total AMPK (Total AMPK) and GCN2 phosphorylation (P-GCN2), respectively. The graphs represent the results of the four samples from two separated cultures. The results are expressed as means±SD, for n=4. Statically significant differences from control (lowAA without insulin) were determined using a paired *t-test* comparing different treatments with the controls and are represented in the graphs (* P<0.001).

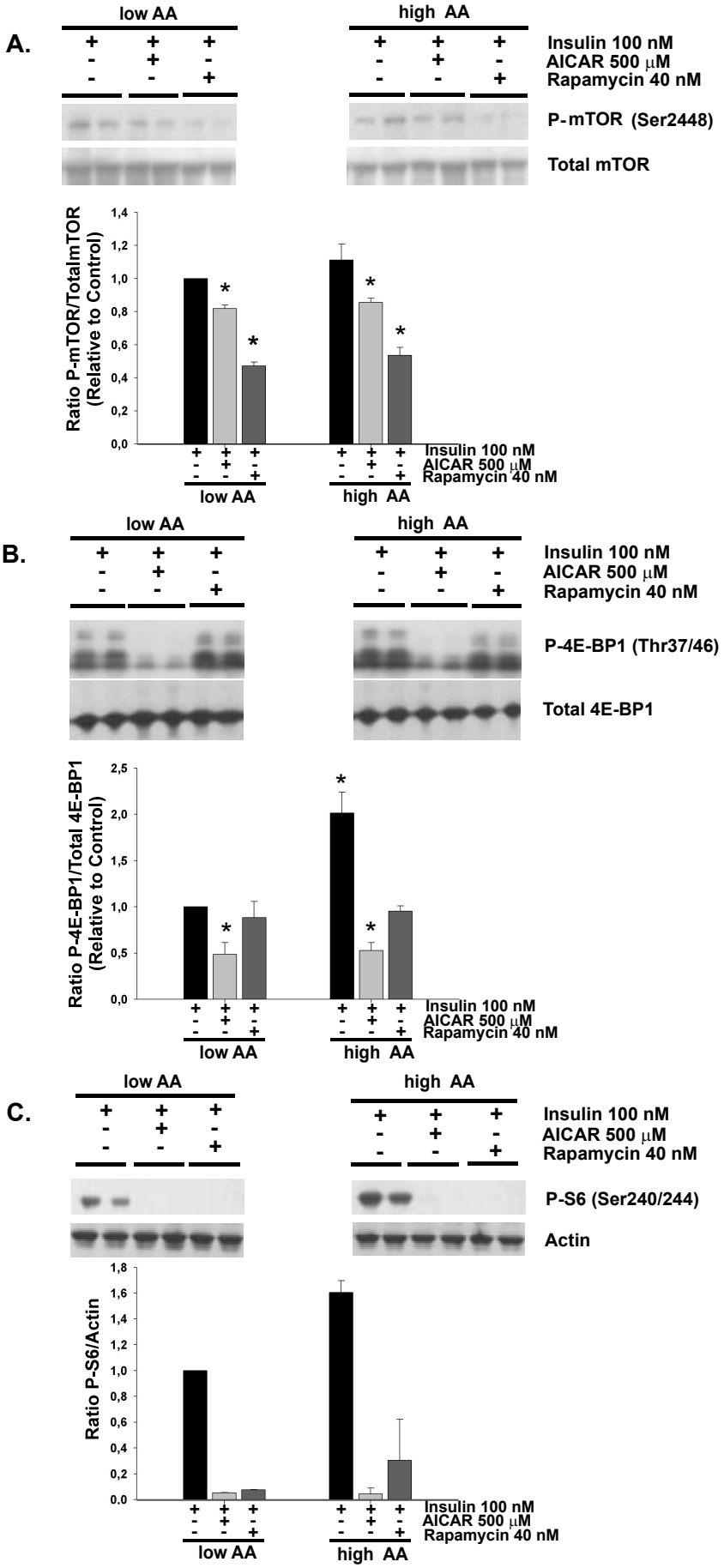


Figure 20 : Effect of AICAR and Rapamycin on mTOR, 4E-BP1 and S6 phosphorylation in a primary hepatocyte culture. Hepatocytes were incubated in a M199 salt medium containing 5 mM glucose supplemented with amino acids at low (lowAA) or high amino acid (highAA) concentrations, with or without insulin or AICAR or Rapamycin. Protein extracts were processed for Western blot analysis. Results are representative of at least two separate experiments. A, B and C are representative of the Western blots of mTOR phosphorylation (P-mTOR), total mTOR (Total mTOR), 4E-BP1 phosphorylation (P-4E-BP1), total 4E-BP1 (Total 4E-BP1), S6 phosphorylation (P-S6) and Actin, respectively. The graphs represent the results of the four samples from two separated cultures, except for S6. The results are expressed as means ± SD, for n=4. Statically significant differences from control (lowAA with insulin) were determined using a paired *t-test* comparing different treatments with the controls and are represented in the graphs (* P<0.05).

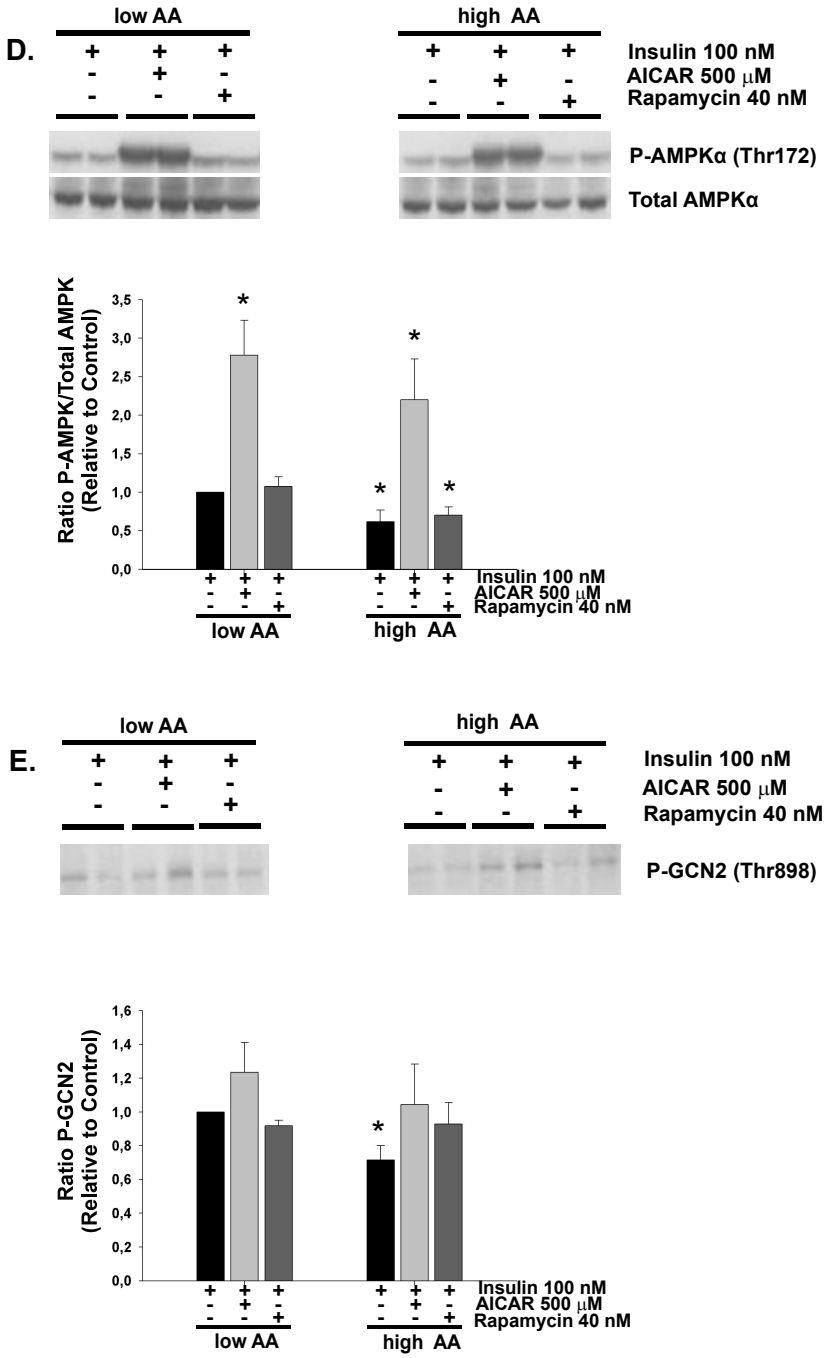


Figure 21 : Effect of AICAR and Rapamycin on AMPK and GCN2 phosphorylation in a primary hepatocyte culture. Hepatocytes were incubated in a M199 salt medium containing 5 mM glucose supplemented with amino acids at low (lowAA) or high amino acid (highAA) concentrations, with or without insulin or AICAR or Rapamycin. Protein extracts were processed for Western blot analysis.Results are representative of at least two separate experiments. D and E are representative of the Western blots of AMPK phosphorylation (P-AMPK), total AMPK (Total AMPK) and GCN2 phosphorylation (P-GCN2), respectively. The graphs represent the results of the four samples from two separated cultures. The results are expressed as means ±SD, for n=4. Statically significant differences from control (lowAA with insulin) were determined using a paired *t-test* comparing different treatments with the controls and are represented in the graphs (* P<0.05).

II. Effect of high protein intake and amino acids on proteolysis.

Protein synthesis and protein degradation are equally important processes that contribute to protein turnover in the bodies of living organisms. However, the proteolytic mechanisms are much less understood than protein synthesis.

The stimulation of protein degradation rates by short term or long term fasting, has been studied (Nair et al. 1987) whereas the understanding of the regulation of proteolysis by the nutrient supply and associated hormonal changes is far from complete. We know that during acute feeding, proteolysis is inhibited (Waterlow 2006) and this effect is mainly mediated by insulin (Gelfand and Barrett 1987), but is also under the influence of AAs (Flakoll et al. 1989; Balage et al. 2001; Capel et al. 2008) To our knowledge, individual tissue breakdown rates have never been measured in this condition. This present study aimed to determine the effect of HP diet on proteolysis rates in the liver, kidney and muscle and examined which proteolysis pathways respond to HP diet. Finally, we investigated whether mTOR and AMPK, involved in the translation control in liver, could also be involved in the control of hepatic protein ubiquitination in response to the increase in AA concentrations in primary hepatocyte culture.

1. Effect of HP diet, amino acids and insulin on proteolysis and protein ubiquitination in liver

Publication 2: Nattida Chotechuang, Dalila Azzout-Marniche, Cécile Bos, Catherine Chaumontet, Claire Gaudichon, and Daniel Tomé. (2010) **Down-regulation of the ubiquitin-proteasome proteolysis system in response to amino acids and insulin involves the AMPK and mTOR pathways in rat liver hepatocytes.** *Submitted to PloS ONE.*

In humans, whole-body protein turnover studies revealed that an increase in protein intake was associated with increases in AA oxidation whereas the protein breakdown rate was

stimulated in the fasted state and was strongly inhibited in the fed state (Pacy et al. 1994; Forslund et al. 1998; Harber et al. 2005). However, the tissue localization, physiological implications and mechanisms of proteolysis underlying the effect of HP diet have not been established.

We aimed to investigate the effect of HP on the proteolysis rate in liver, kidney and muscle of rats receiving a NP or HP diet for 14 days, both in the fasted and the fed state. Firstly, we observed that the protein content was significantly increased in hepatocytes in HP rats when compared to NP rats whereas there were no changes in the protein content in kidney and muscle (Table 1 publication 2). Moreover, tissue breakdown rates were examined by measuring the release of valine, α-amino N and phenylalanine from the *ex vivo* incubation of hepatocytes, kidney slices and muscle, respectively (Figure 1 publication 2) in the presence of a protein synthesis inhibitor (cycloheximide). The release of valine from incubated hepatocytes was more strongly inhibited by the meal in HP rats than in NP rats. Kidney breakdown rates were differently affected by meal ingestion in NP and HP rats. In HP rats, the fed state breakdown rate measured in the kidneys only reached 56% of that measured in NP rats, although this difference was not significant. Muscle protein degradation rates were influenced neither by the diet nor by the meal.

The gene expression of proteins involved in tissue proteolysis, including cathepsinD, Atg3, ubiquitin, 14-kDa E2 enzyme, C2 and m-calpain, were then measured by Real-Time PCR in liver, kidney and muscle. HP diet mainly affected ubiquitin and 14-kDa E2 gene expression in the liver, which encoded key enzymes of the ubiquitin proteasome proteolysis pathway. Hepatic ubiquitin mRNA was significantly inhibited (-35%) by the meal in the HP group, whereas no effect was observed in the NP group (Figure 2 publication 2). The cathepsinD mRNA in the liver were also significantly influenced by the meal in the HP group, but not in the NP group. In the kidneys, no significant difference in mRNA levels was observed. In muscle, inverse variations were observed for ubiquitin and E2 enzyme mRNA. mRNA encoding ubiquitin increased after

meal intake whereas E2 mRNA level decreased. However, the change of protein intake levels has no effect on both ubiquitin and E2 expression (Figure 1 publication 2).

In order to confirm the effect of HP diet on the ubiquitin-proteasome system in liver, we examined whether the decrease of ubiquitin mRNA was associated with the changes in ubiquitinated proteins. We performed a postprandial time course of protein ubiquitination at 0, 30, 60 and 120 minutes after the beginning of the standardized meal in rats receiving each diet. Zero minute time corresponds to overnight fasted rats. Rats were euthanized, the livers were quickly harvested and protein extracts were analyzed by Western blot. No change between NP and HP groups in the fasted state (Figure 3 publication 2) was observed since proteins between 216 and 62 kDa were similarly stained. In contrast, the ubiquitinated proteins were reduced (less stained) in HP fed rats at 30, 60 and 120 min compared with the fasted state, whereas no change appeared over time in the NP group (Figure 3 publication 2).

Furthermore, we investigate the respective role of AAs and insulin in the down-regulation of the ubiquitin-proteasome system by HP diet in liver. Western blot was performed with protein extracts from primary hepatocyte culture. We observed that high AA concentrations reduced the ubiquitinated protein (less stained) when compared with low AA concentrations without insulin (Figure 4 publication 2). In the presence of insulin, high AA levels more strongly reduced ubiquitinated protein (Figure 4 publication 2) whereas insulin, at low AA concentrations, had no significant effect. These results suggest that both high AA levels and insulin were required for down-regulation of ubiquitin-proteasome systems by HP diet in liver.

1.1.Role of mTOR and AMPK signaling pathways in the control of the ubiquitin proteasome pathway in response to the increase of amino acid concentrations and insulin

It was proposed that the dynamic control by AAs of their anabolic and catabolic action may be coordinated by the same signaling pathway and may involve the mTOR pathway

(Blommaart et al. 1995). We therefore aimed to determine whether AMPK and mTOR were involved in the control of the ubiquitin-proteasome systems. Hepatocytes were cultivated under low or high AA concentrations with or without insulin, AICAR (the activator of AMPK) and/or rapamycin (the inhibitor of mTOR). First, we observed that AMPK phosphorylation was decreased by high AA concentrations and AICAR induced an increase of P-AMPK in both low and high AA concentrations (Figure 5a publication 2). For mTOR, either high AA levels or insulin increase mTOR phosphorylation. In the presence of rapamycin alone, P-mTOR decreased in both low and high AA concentrations (Figure 5b publication 2). The presence of both, AICAR and rapamycin, increased P-AMPK with the same intensity as AICAR alone, while P-mTOR decreased slightly (Figure 5a and 5b publication 2). Furthermore, we studied the effect of rapamycin and/or AICAR on the ubiquitinated protein. Surprisingly, rapamycin increased the level of ubiquitinated protein and this effect was abolished in the presence of AICAR. However, AICAR alone had no effect on the protein ubiquitination at low AA levels whereas AICAR reversed the inhibition of ubiquitination at high AA levels in the presence of insulin. (Figure 5c publication 2). These results suggest that mTOR and AMPK are involved in the control of protein ubiquitination in hepatocytes.

In conclusion, the HP fed rats were characterized by a decrease in protein degradation rate, mainly in liver. This inhibition was associated with the decrease of ubiquitin mRNA and protein ubiquitination. Moreover, both high AA levels and insulin were required for the down-regulation of ubiquitin-proteasome systems by HP diet in liver. Furthermore, mTOR and AMPK were involved in the control of protein ubiquitination in hepatocytes. These results suggested that in hepatocytes, the presence of both AAs and insulin, besides controlling translation, were also involved in the control of protein degradation, in particular the ubiquitin-proteasome system, through the AMPK and mTOR signaling pathways.

PUBLICATION 2

Down-regulation of the ubiquitin-proteasome proteolysis system in response to amino acids and insulin involves the AMPK and mTOR pathways in rat liver hepatocytes

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Abstract

BACKGROUND:

Protein synthesis and protein degradation are equally important processes that contribute to the body protein turnover in living organisms. However, the proteolytic mechanisms are much less understood than protein synthesis. Protein degradation systems and proteolysis are sensitive to the nutritional state and high amino acid (AA) concentrations and insulin are the main inhibitors of protein degradation.

METHODOLOGY/PRINCIPLE FINDING:

This study aimed to examine whether changes to dietary protein levels could elicit differential responses of tissue proteolysis and the pathway involved in this response. In rats fed a high protein diet (55%) for 14 days, the liver was the main organ where adaptations occurred, characterized by an increased protein pool and a strong, meal-induced inhibition of the protein breakdown rate (-42%), compared to the normal protein diet (14%). This was associated with the decrease in the gene expression of key-proteins in the ubiquitin-proteasome and autophagy pathway and the level of hepatic ubiquitinated protein. In hepatocytes, the ubiquitin-proteasome pathway was down-regulated by an AA increase, but this inhibition was stronger in the presence of insulin. Interestingly, AICAR, an adenosine monophosphate-activated protein kinase (AMPK) activator, reversed the inhibition of protein ubiquitination induced by insulin at high AA concentrations whereas rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, reversed the insulin effect at both high and low AA concentrations. The presence of both AICAR and rapamycin, AICAR reversed the effects of rapamycin.

CONCLUSION/SIGNIFICANCES

These results demonstrated that an increase in high dietary protein induced a decrease in proteolysis through the down-regulation of ubiquitin proteasome system in the liver and AMPK inhibition and the mTOR activation may involve in the down-regulation of protein ubiquitination in response to high AA concentrations and insulin.

Key word: High protein diet, protein degradation, liver, AMPK, mTOR,

Introduction

In mammalian tissues, protein degradation involves three main pathways; i.e. the ubiquitin-proteasome, lysosomal and the Ca^{2+}-dependent systems. The ubiquitin-proteasome system is the principal machinery degrading cytosolic protein and is responsible for a considerable proportion of cellular protein degradation, mainly of short-lived or abnormal proteins (Hamel et al. 2004). Protein ubiquitination is a multiple step process mediated by the ubiquitin enzymes E1, E2 (14kDa-E2) and E3 (Attaix et al. 2001). Proteins labeled by ubiquitin are delivered to the proteasome or lysosomes for degradation, depending on the number of ubiquitins attached to the protein and the lysyl residues to which subsequent ubiquitins are attached (Hicke and Dunn 2003; Elsasser and Finley 2005). The lysosomal system (also called autophagy) degrades long-life proteins and organelles, including the mitochondrion and peroxisomes. This system mainly acts through a complex cellular process of macroautophagy that involved cytoplasm sequestration into vesicles and delivery to a degradative lysosome that contains a range of hydrolases. Of these hydrolases, cathepsin is able to degrade proteins and requires the involvement of autophagy-related genes (Atg) for autophagosome formation (Klionsky and Emr 2000; Levine and Klionsky 2004). The third pathway is the calcium-dependent proteolytic system that involves calpain as the proteinase (Ilian and Forsberg 1992; Goll et al. 2003; Costelli et al. 2005). Calpain may also be required for macroautophagy in mammalian cells (Otani et al. 2004; Demarchi et al. 2006).

Protein degradation systems and proteolysis are sensitive to the nutritional state (Kettelhut et al. 1988). Proteolysis is increased in the post-absorptive state and during fasting and inhibited under feeding and an increase in habitual protein intake induces both more pronounced fasted state increase and more pronounced meal-induced inhibition of proteolysis, respectively (Price et al. 1994; Forslund et al. 1998). High amino acid concentrations and insulin are the main inhibitors of protein degradation, whereas glucagon and low concentrations of amino acids are the principal stimulators (Gelfand and Barrett 1987; Flakoll et al. 1989; Mortimore et al. 1989; Kadowaki et al. 1992; Blommaart et al. 1997; Boirie et al. 1997; Balage et al. 2001; Kanazawa et al. 2004; Waterlow 2006; Capel et al. 2008). In muscle, inhibition of the ubiquitin-proteasome pathway appears to be responsible for the postprandial inhibition of proteolysis in mature rats (Combaret et al. 2005), and during fasting there is an increase in ubiquitin expression that is associated with elevated levels of ubiquitinated proteins in parallel with the rate of proteolysis (Medina et al. 1991). These changes were reversed by refeeding and may at least be due to a direct effect of insulin on 14-kDa ubiquitin-conjugating enzyme expression (Wing and Banville 1994). Lysosomal pathways account for approximately only 10–20% of total skeletal muscle proteolysis, under short-term starvation (Mitch and Goldberg

1996; Attaix et al. 1998). Starvation has been shown to induce macroautophagy in the liver, (Mortimore et al. 1983; Mortimore et al. 1989; Bleiberg-Daniel et al. 1994) and to increase the expression of 14-kDa ubiquitin-conjugating enzyme in the liver and kidney (Wing and Banville 1994). Furthermore, in rat hepatocytes, adenosine monophosphate-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR) transduction pathways are involved in the control of autophagic proteolysis (Kanazawa et al. 2004; Meley et al. 2006).

The aim of the present study was to further evaluate individual tissue responses to protein feeding which may differ from each other or from the global trend. Indeed, these individual responses were rarely investigated or limited to skeletal muscle for evident physiological interest and despite the importance of other organs such as the liver in protein metabolism (Chevalier et al. 2009). In addition, the AMPK and mTOR transduction pathways have previously been shown to be involved in the control of liver translation in response to a protein intake in the rat (Chotechuang et al. 2009), but whether they also control protein degradation was not addressed. In order to better understand these processes, the present study determined the consequence of high protein feeding on proteolysis and proteolysis pathways in both the fasted and the fed state in the liver, kidney and muscle of the rat, and also examined whether amino acids, insulin and the AMPK and mTOR transduction pathways are involved in the control of liver protein degradation in response to protein feeding.

Materials and Methods

Animals

Male Wistar rats (n=74) were first of all purchased from Harlan (Horst, The Netherlands) and were used according to the guidelines of the French National Animal Care Committee. The rats were placed under a reversed light rhythm (lights from 20:00 to 08:00) and adapted to these experimental conditions for one week. They were then randomly allocated to receive either a normal protein (NP) or a high protein (HP) diet (The composition of the diets are detailed on supplemental data). Both diets were iso-energetic (14.89 kJ/g). The rats were accustomed to receiving their food in two meals: an initial, small meal of 6 g dry matter (DM) between 09:00 and 10:00, and a second meal with a free access to food between 14:00 and 18:00. After the first week, the rats had become used to finishing their first meal within one hour. All animals had free access to water.

Experimental design

Four separate experiments were carried out in accordance with the guidelines of the French Committee for Animal Care and the European Convention on Vertebrate Animals Used for Experimentation.

Experiment 1: Measurement of ex vivo tissue proteolysis (n=26).

On day 15, half of the rats in each group were studied in the fasted state (16h fast) and half in the fed state (2h after the start of the meal). The animals were anesthetized with an IP injection of sodium pentobarbital (50 mg/kg BW). Hepatocytes were prepared as previously described (Azzout-Marniche et al. 2007). Protein degradation fluxes were measured on Hepatocytes (8.10^6), kidney slices and soleus muscle by determining the release of an indispensable amino acid in an amino acid-free medium in the presence of a protein synthesis inhibitor (cycloheximide, Sigma-Aldrich, St. Louis, MO, USA) (Tischler et al. 1982). At the end of incubation, cells or tissues were rapidly separated from their incubation medium by spinning in a micro centrifuge (1s), frozen in liquid nitrogen and stored at –20°C for the assessment of *in vitro* tissue protein degradation and the analysis of nitrogen and amino acids as described in supplemental data.

Experiment 2: Analysis of the gene expression of key proteolysis enzymes using Real-Time PCR (n=24)

Rats were studied in the fasted or fed state, as described in experiment 1. After incision of the abdomen, the liver, kidneys and soleus muscles were quickly harvested under sterile conditions, frozen in liquid nitrogen and stored at -70°C until analysis. Total RNA were extracted and reverse transcribed and the cDNA were used to measure the expression of cathepsin D, Atg3, ubiquitin 14kDa E2 enzyme, C2 subunit of 20S proteasome, m-calpaine and 18S. The analysis of gene expression and the primers used are detailed in supplemental data.

Experiment 3: Postprandial time course of protein ubiquitination (n=24)

After an overnight fast, six rats previously adapted to the NP or HP diet were sacrificed in the fasted state and used as controls. The other nine rats receiving each diet were divided into three groups that were sacrificed 30, 60 or 120 minutes after the beginning of their standardized meal (n=3 per group on each diet). The livers were quickly harvested and frozen in liquid nitrogen and stored at -70°C until Western blot analysis as described in supplemental data.

Experiment 4: Effect of amino acids, insulin, AICAR and rapamycin on protein-ubiquitination in rat hepatocytes.

Prior to the experiments, the rats used for *in situ* liver perfusion studies were allowed free access to a commercial laboratory chow diet and water for at least one week. Hepatocytes were isolated from the livers of fed rats as described in *experiment 1* and were seeded at a density of 7×10^6 cells/dish in 100-mm Petri dishes. After cell attachment (4 h) and overnight incubation, the medium was replaced a fresh one corresponding to M199 medium salts supplemented with 5.5 mM glucose and the amino acid (AA) concentration found in the

portal vein of NP fasted rats, as previously described (Azzout-Marniche et al. 2007). The hepatocytes were then incubated for 60 minutes in low or high amino acid concentrations (corresponding to the concentrations measured in the portal vein of NP fasted rats or HP fed rats, respectively, as previously described (Azzout-Marniche et al. 2007) with or without insulin (100nM) (Sigma-Aldrich), AICAR (500 µM) and/or rapamycin (40 nM) (Cell Signaling Technology, Beverly, MA, USA). Each treatment was performed in duplicate. At the end of the incubation, the hepatocytes were lysed and the proteins extracted were subsequently analyzed using Western blot.

Statistics

Data are expressed as means ± SD. The effects of diet (NP vs. HP) and meal (fasted vs. fed state) or amino acids (low AA vs. high AA) and conditions (with vs. without insulin, AICAR and/or rapamycin) and their interactions were analyzed using 2-way ANOVA (SAS 9.1, SAS Institute, Cary, USA). Post-hoc Tukey tests for multiple comparisons were performed to enable pair-wise comparisons. Differences were considered to be significant at $P<0.05$.

Results

Influence of HP feeding on tissue protein contents and tissue protein degradation rates in vivo.

The protein content was significantly increased in hepatocytes in HP rats as compared to NP rats whereas no changes were observed regarding hepatocyte mass (Table 1). The kidney mass was slightly higher in rats fed a HP diet when compared with NP rats, but the protein content was similar whatever the diet (Table 1). In muscles there were no changes to either protein content or mass (Table 1). Tissue breakdown rates, measured from the *ex vivo* incubation (Figure 1), showed that the release of valine from incubated hepatocytes was more strongly inhibited by the meal in HP rats (10.0 ± 2.6 and 5.3 ± 2.7 nmol/gP/h in the fasted and fed states, respectively) than in NP rats (10.9 ± 2.6 and 9.2 ± 2.8 nmol/gP/h in the fasted and fed states, respectively) in the fasted and fed states, respectively). Kidney breakdown rates were differently affected by meal ingestion in NP and HP rats (diet x meal interaction: $P<0.05$). In HP rats, the fed state breakdown rate measured in the kidneys only reached 56% of that measured in NP rats, although this difference was not significant. Muscle protein degradation rates were influenced neither by the diet nor by the meal.

Influence of HP feeding on the gene expression of proteins involved in tissue proteolysis and on ubiquitinated proteins in liver.

As reported in Figure 2, the expression of ubiquitin and 14-kDa enzyme in the liver was significantly influenced by the diet ($P<0.05$) and the meal, which translated into a significant inhibition of hepatic ubiquitin expression by the meal in the HP group, whereas no effect was observed in the NP group (Figure 2). Cathepsin D mRNA were also decreased in HP-fed rats and there was a significant interaction between the diet and the meal. In the kidneys, the only effect was that the expression of Atg3, an E2-like enzyme responsible for autophagosome formation, was significantly influenced by the meal but not the diet. In muscle, inverse variations were observed regarding the expression of ubiquitin and E2 enzymes. mRNA encoding for ubiquitin increased after meal intake whereas E2 mRNA levels decreased. However, the level of protein intake had no effect on either ubiquitin or E2 expression. In order to confirm the effect of the HP diet on the ubiquitin-proteasome system in the liver, we examined whether this decrease in ubiquitin expression was associated with changes to ubiquitinated proteins. No difference was found between the NP and HP groups in the fasted state (Figure 3). By contrast, ubiquitinated proteins were reduced (less markedly stained) in HP-fed rats at 30, 60 and 120 minutes when compared with the fasted state, whereas no changes over time appeared in the NP group.

Effect of amino acids and insulin on ubiquitinated proteins in primary rat hepatocytes.

We had previously reported that the HP diet was characterized by an increase in portal amino acid levels; while plasma insulin levels were the same level as those seen in rats fed an NP diet (Azzout-Marniche et al. 2007). We therefore investigated the respective roles of amino acids and insulin in down-regulation of the ubiquitin-proteasome system in the liver by the HP diet, using primary hepatocyte culture experiments. We observed that a high amino acid concentration reduced the quantity of ubiquitinated protein (less markedly stained) when compared with low amino acid concentrations without insulin (Figure 4) (amino acid effect $P<0.0001$ and insulin effect $P<0.001$). In the presence of insulin, high amino acid levels more markedly reduced ubiquitinated protein levels ($P<0.05$) (Figure 4), whereas low amino acid concentration had no significant effect.

Role of AMPK and mTOR transduction pathways in the control of protein ubiquitination in primary rat hepatocytes.

In order to investigate the role of AMPK and mTOR in control of the ubiquitin-proteasome systems, hepatocytes were cultivated with or without AICAR (the activator of AMPK) and/or rapamycin (the inhibitor of mTOR). We checked first of all on the effect of amino acids and insulin on mTOR and AMPK phosphorylation state (P-mTOR and P-AMPK, respectively). The results showed a reduction in P-AMPK at a high AA concentration (Figure 5A), while either high AA levels or insulin increased mTOR phosphorylation (Figure 5B), which is in line with our previous report (Chotechuang et al. 2009). Secondly, we studied the effect of AICAR and rapamycin on AMPK and mTOR phosphorylation state. As expected, P-AMPK increased in the presence of AICAR (Figure 5A), and P-mTOR decreased under both low and high amino acid concentrations in the presence of rapamycin (Figure 5B). Rapamycin had no effect on P-AMPK (Figure 5A) while AICAR decreased P-mTOR under both low and high AA levels in the presence of insulin (Figure 5B). Both AICAR and rapamycin induced an increase in P-AMPK which was similar to that observed with AICAR alone, while P-mTOR inhibition was reversed by AICAR (Figure 5A and B). Thirdly, we studied the effect of rapamycin and/or AICAR on ubiquitinated protein. Our results showed that rapamycin strongly increased ubiquitinated protein ($P<0.01$) under both low and high AA concentrations in the presence of insulin (Figure 5C). AICAR exerted no significant effect on protein ubiquitination at a low AA concentration, while it reversed the inhibition of protein ubiquitination induced by insulin at a high AA concentration, (Figure 5C). Surprisingly, we observed that the effect of rapamycin on ubiquitinated proteins was reversed by AICAR (Figure 5C) and returned to the level observed after inhibition by either high AA and/or insulin.

Discussion

This study assessed the adaptation to a high dietary protein intake of protein degradation in three tissues that play major roles in protein metabolism in rats, with a focus on the transition between the fasted and fed states. The results demonstrated tissue-specific responses of proteolysis to high protein diets. In the liver, the HP diet was associated with an increase in the tissue protein pool, with a general trend for a more pronounced meal-induced inhibition of protein breakdown. By contrast, in peripheral tissues, no such changes were observed.

Previous studies described a stimulation of proteolysis over a long period of fasting of at least 24 hours and an inhibition after feeding at the whole body level (Waterlow 2006), a

regulation that is common to both muscle (Wing and Goldberg 1993; Wing and Banville 1994; Busquets et al. 2002) and liver (Mortimore et al. 1983; Mortimore et al. 1989; Bleiberg-Daniel et al. 1994). In the present study, when rats were fed a normal protein diet no significant meal-induced inhibition of protein degradation was observed after overnight fasting in the liver, kidney or muscle. Moreover, the significant meal-induced inhibition of proteolysis in the livers of rats fed the HP diet was in line with previous studies on whole-body protein turnover responses to increased protein intakes in humans (Pacy et al. 1994). Measures of whole body protein fluxes integrate different tissue fluxes, which may diverge one from another (Nair et al. 1995; Thivierge et al. 2005). The present results provide new insight by showing that tissue-specific responses to an HP diet can be observed using an *ex vivo* incubation method (Tischler et al. 1982) to measure these fluxes. The results indicated that the increased amplitude in diurnal N cycling observed with elevated protein intakes is more likely to concern specific protein pools such as hepatic proteins whereas muscle proteolysis is not significantly affected. The present study also indicates that the increased protein content in the liver previously observed in HP-fed rats that could not be explained by an effect on liver protein synthesis was paradoxically reduced (Chevalier et al. 2009) is in contrast directly related to a more pronounced meal-induced inhibition in protein breakdown.

The modulations affecting tissue protein degradation rates in response to an HP diet are consistent with the changes observed to the expression of major components in the proteolytic pathways, pointing to crucial roles for both autophagy and the ubiquitin-proteasome system in liver proteolysis. In the liver, ubiquitin and E2 enzyme expression was significantly inhibited by meal intake in HP-fed rats but not in NP-fed rats. As a consequence, liver proteins were less ubiquitinated and this process occurred as early as 30 minutes after the HP meal. The autophagic pathway may also be involved in these adaptations since cathepsin D expression was significantly lower in HP-fed rats than in HP-fasted rats. However, these variations did not differ from those seen in NP rats. These observations are in agreement with the role of the ubiquitin-proteasome dependent proteolytic pathway as a major cell catabolic process responsible for protein breakdown during short-term physiological and nutritional changes such as food deprivation (Ding et al. 1997). In the liver, the lysosomal pathway is generally considered to predominate (Bleiberg-Daniel et al. 1994; Blommaart et al. 1995; Del Roso et al. 2003; Kadowaki and Kanazawa 2003; Kanazawa et al. 2004), and only one study has reported a stimulation of hepatic ubiquitin expression after one or two days of starvation (Wing and Banville 1994). In contrast to the liver, the ubiquitin-proteasome dependent proteolytic pathway is believed to play a major role in regulating protein degradation in muscle (Medina et al. 1991; Wing et al. 1995; Hamel et al. 2003), even if some recent data have suggested that autophagy and/or Ca2+ proteolysis are responsible for the postprandial

inhibition of muscle protein breakdown (Capel et al. 2008). In muscle, moderate variations were seen to affect the expression of genes encoding key ubiquitin proteins, which was in line with the results regarding muscle protein breakdown. Numerous publications have described a stimulation of muscle protein breakdown in response to fasting, followed by an inhibition after re-feeding with a normal diet. The ubiquitin-proteasome pathway is the principal route of muscle protein breakdown that is activated in response to one or more days of starvation (Medina et al. 1991; Wing and Banville 1994; Wing et al. 1995) Given that glucocorticoids are the main activators of the ubiquitin-dependant proteolytic system (Wing and Goldberg 1993; Marinovic et al. 2007), and that this hormone is released in response to long-term fasting, it could be expected that under short-term fasting, muscle proteolysis may not be activated as it was in the present study. In the kidney, a marked inhibition of Atg3 expression was observed in NP-fed rats that did not translate into consistent changes to the rate of proteolysis. Atg3 is one component in a large family of autophagy-related genes implicated in autophagosome formation (Levine and Klionsky 2004) a complex process that involves more than 16 components. The inhibition of Atg3 observed in the study, together with the absence of changes to the expression of other components in the autophagy pathway, suggests that Atg3 alone may not be a good predictor of proteolytic fluxes.

Insulin is known to be involved in the fed-state inhibition of protein breakdown (Gelfand and Barrett 1987), and liver proteolysis is known to be inhibited by insulin and stimulated by glucagon (Schworer and Mortimore 1979; Mortimore et al. 1989). Plasma insulin levels are generally lower or similar in the fed state in rats or humans subjected to a high protein/low carbohydrate diet, whereas glucagon concentrations is increased (Lacroix et al. 2004; Harber et al. 2005; Baum et al. 2006; Blouet et al. 2006; Azzout-Marniche et al. 2007). Thus the stronger inhibition of hepatic proteolysis induced by an HP diet could not be ascribed directly to insulin. Plasma amino acid and branched-chain amino acid concentrations are increased by HP diets in the fed state (Forslund et al. 1998; Blouet et al. 2006; Azzout-Marniche et al. 2007). Moreover, the results showed that the increased amino acid concentration in the media was sufficient to induce a decrease in ubiquitinated protein in a primary culture of hepatocytes, and that this inhibition was stronger in the presence of insulin. This result agrees with previous findings which demonstrated an inhibitory effect of different amino acid mixtures on protein degradation in the perfused rat liver (Poso et al. 1982; Mortimore et al. 1989; Kadowaki et al. 1992; Miotto et al. 1992) and isolated hepatocytes (Seglen et al. 1980).

It has been proposed that control of the anabolic and catabolic pathways by amino acids may be coordinated by the same signaling system and might involve the mTOR pathway (Blommaart et al. 1995). Moreover, it has previously been demonstrated that both amino

acids and insulin are required to stimulate translation and are involved at least in the mTOR, AMPK and GCN2 transduction pathways (Chotechuang et al. 2009). We observed that rapamycin, the mTOR inhibitor, stimulated protein ubiquitination under both low and high amino acid levels in the presence of insulin. It has been reported that in hepatocytes, rapamycin prevents the inhibition of autophagic proteolysis by insulin and amino acids during a lengthy exposure (60 and 90 minutes) (Blommaart et al. 1995; Kanazawa et al. 2004). This suggests that in the liver, the mTOR transduction pathway, as well as regulating autophagy, is also involved in controlling the ubiquitin-proteasome system in response to a high protein intake. Surprisingly, we observed that AICAR reversed the inhibition of protein ubiquitination induced by insulin under high amino acid levels, while it had no effect at low amino acid levels. Moreover, the effect of rapamycin on protein ubiquitination was abolished in the presence of AICAR. These findings suggest that the inhibition of AMPK and the activation of mTOR transduction pathways were required for the down-regulation of protein ubiquitination in response to high amino acid and insulin concentrations. It is clear that the modification made to mTOR activity by rapamycin had no effect on AMPK phosphorylation state. By contrast, it not clear how mTOR phosphorylation was affected by AMPK activation, because we observed that the latter reversed mTOR inhibition by rapamycin. AMPK can directly phosphorylate mTOR on Thr^{2446}, leading to its inactivation (Cheng et al. 2004), or indirectly via phosphorylation of the tuberous sclerosis complex (TSC) 2-gene product (TSC2) and raptor (Gwinn et al. 2008). Furthermore, mTOR full activation requires mTORC1 complex formation (mTOR, raptor, PRAS40, mLST8), and amino acids stimulate the relocation of mTOC1 to its correct site (Sancak et al. 2008; Shaw 2008). However, we cannot exclude the possibility that the activation of AMPK targets a protein downstream in mTOR signaling. It remains necessary to clarify whether AMPK is coordinated with mTOR in the regulation of protein ubiquitination, as both are known to act with GCN2 in order to control translation (Chotechuang et al. 2009).

The present results thus provide evidence of a major role for liver proteolysis in the control of protein accretion in response to an increased dietary protein intake in rats. The ubiquitin-proteasome system and autophagy are the principal pathways involved in this process. Furthermore, in hepatocytes, an increase in the amino acid concentration was sufficient to down-regulate the ubiquitin-proteasome pathway, but this inhibition was stronger in the presence of insulin. The mTOR signaling pathway may be involved in the down-regulation of protein ubiquitination in response to a high protein diet. A proposed scheme for the coordinated control by insulin and amino acids of liver ubiquitin protein breakdown as a function of the protein level in the diet, is shown in Figure 6. In many tissues, proteolysis

assumes an essential cellular homeostatic or housekeeping function, removing damaged or unwanted organelles and proteins. It has been proposed that the homeostatic function of autophagy represents an anti-aging mechanism and may be involved in the conserved effect of protein caloric restriction (Del Roso et al. 2003). A clearer understanding of the nutritional control of protein breakdown regulation, and particularly the effect of protein intake, would be of importance in the context of preserving muscle mass during aging.

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Figure legends

Figure 1. Protein degradation rates in liver, kidneys and muscle (soleus). Protein degradation rates were expressed as the amounts of amino acid released by tissue mass in rats adapted to NP or HP diets for 14 days (n=26) and sacrificed in the fasted or fed state. Tissues were incubated in the presence of cycloheximide as described in *"Experimental procedures"*. Values are means ±SD. The effects of diet (NP or HP) or meal (fasted or fed) were assessed by 2-way ANOVA. Bars with different letters within a graph are statistically different (post-hoc Tukey tests for multiple comparisons, $P<0.05$).

Figure 2. Relative expression levels of genes encoding for the main proteolytic pathways in liver, kidneys and muscle (soleus). The expression of mRNAs encoding for cathepsin D, Atg3, ubiquitin, 14-kDa E2 enzyme, C2 subunit of the proteasome and m-calpain were measured by Real-Time PCR in rats adapted to NP or HP diets for 14 days (n=24) and sacrificed in the fasted or fed state. Values are means ±SD. The effects of Diet (NP or HP) or Meal (fasted or fed) were assessed by 2-way ANOVA. Bars with different letters within a graph are statistically different (post-hoc Tukey tests for multiple comparisons, $P<0.05$).

Figure 3. Time course effect of NP and HP meals on protein ubiquitination. Protein extracted from the livers of rats adapted to NP or HP diets for 14 d (n=24) and was processed for Western blot analysis as described in *"Experimental procedures"*. The upper Western blot shows ubiquitinated protein in liver and the bottom blot shows the Western blot for actin.

Figure 4. **Effect of amino acids (AA) and insulin on protein ubiquitination in a primary hepatocyte culture.** Hepatocytes were incubated for 60 minutes in M199 salt medium containing 5 mM glucose plus low AA or high AA concentrations, with or without insulin. Protein extracts were processed for Western blot analyses. Results are representative of four separate experiments. The graphs represent the ratio of ubiquitinated protein and actin for eight samples from four separated cultures. The results are expressed as means ± SD, for n=8. The effects of AA (low AA or high AA) or insulin (with or without insulin) were assessed by 2-way ANOVA. Bars with different letters within a graph are statistically significantly different (*post-hoc* Tukey tests for multiple comparisons, $P<0.05$).

Figure 5. The role of AMPK and mTOR in the control of protein ubiquitination in a primary hepatocyte culture. Hepatocytes were incubated for 60 minutes in M199 salt medium containing 5 mM glucose plus low or high amino acid (AA) concentrations, with or without insulin, AICAR and/or rapamycin. Protein extracts were processed for Western blot

analysis. **A, B, and C** are representative of the Western blots for AMPKα phosphorylation (P-AMPKα) and total AMPKα (Total AMPKα) , mTOR phosphorylation (P-mTOR) and total mTOR (mTOR), ubiquitinated protein and actin, respectively. Results are representative of two separate experiments. The graphs represent the ratio of ubiquitinated protein and actin for four samples from two separate cultures. The results are expressed as means ± SD, for n=4. The effects of AA (Low AA or High AA) or conditions (with or without insulin, AICAR and rapamycin) were assessed by 2-way ANOVA. Bars with different letters within a graph are statistically significantly different (*post-hoc* Tukey tests for multiple comparisons, $P<0.05$).

Figure 6. Proposed scheme for the coordinated action of insulin and amino acids on hepatic ubiquitin-dependent proteolysis. When the AA concentration is low, insulin stimulates the phosphorylation of mTOR but without any significant effect on the proteolysis rate and the ubiquitin protein breakdown pathway. When the amino acid concentration increases, ubiquitinated protein is reduced when compared with a low amino acid concentration and this effect is stronger in the presence of insulin. The mTOR transduction pathway plays a key role in control of the ubiquitin-proteasome system. The inhibition of mTOR by rapamycin reverses the inhibition of protein ubiquitination under low and high amino acid levels in the presence of insulin, which suggests that stimulation of the mTOR pathway is required for inhibition of the ubiquination process. The effects of insulin and amino acids on mTOR full activation require: i) inactivation by insulin of the TSC complex (tuberous sclerosis complex of TSC1/TSC2), the negative regulator of mTOR, ii) mTORC1 complex formation (mTOR, raptor, PRAS40, mLST8) and iii) the amino acid stimulation of mTORC1 relocation. Moreover, the AMPK transduction pathway is also involved in this process. AMPK activation by AICAR reverses inhibition of the ubiquitination process induced by an increase in the AA concentration whereas it has no significant effect at a low AA concentration in the presence of insulin. Interestingly, AICAR reverses the effect of rapamycin with both low and high AA concentrations, suggesting that a decrease of P-AMPK is required for activation of the ubiquitination proteolysis pathway by mTOR inhibition. Moreover, mTOR phosphorylation is affected by AICAR, which suggests that AMPK controls mTOR phosphorylation, either directly by phosphorylation or indirectly through subcellular localization of the mTOR complex.

Table 1. Tissue mass and composition in rats fed NP or HP diets for 14 days

	NP		HP		Stat effect[1]
	Fasted	Fed	Fasted	Fed	
Hepatocytes					
Mass (g)	9.3 ± 1.0	10.4 ± 0.8	10.1 ± 1.3	10.3 ± 1.3	NS
Protein [2] (g/100g tissue)	6.20 ± 1.35 [a]	6.57 ± 1.44 [a,b]	8.24 ± 1.32 [b]	7.62 ± 0.92 [a,b]	D
Kidneys					
Mass (g)	2.17 ± 0.39	2.15 ± 0.44	2.43 ± 0.39	2.45 ± 0.45	D
Protein (g/100g tissue)	10.4 ± 0.9	10.6 ± 1.1	10.4 ± 0.4	10.5 ± 1.5	NS
Soleus muscles					
Mass (g)	0.258 ± 0.024	0.267 ± 0.037	0.272 ± 0.033	0.265 ± 0.034	NS
Protein (g/100g tissue)	18.9 ± 0.8	19.0 ± 1.0	19.0 ± 0.7	18.4 ± 1.0	NS

Values are means ± SD, n=24, NS: not significant
[1]Results from 2-way Anova with D: diet effect (NP vs. HP); M: meal effect (fasted vs. fed), PxM: diet by meal interaction. NS: non significant. Means with a different superscript within a row are significantly different (post-hoc Tukey tests for multiple comparisons).

Figure 1.

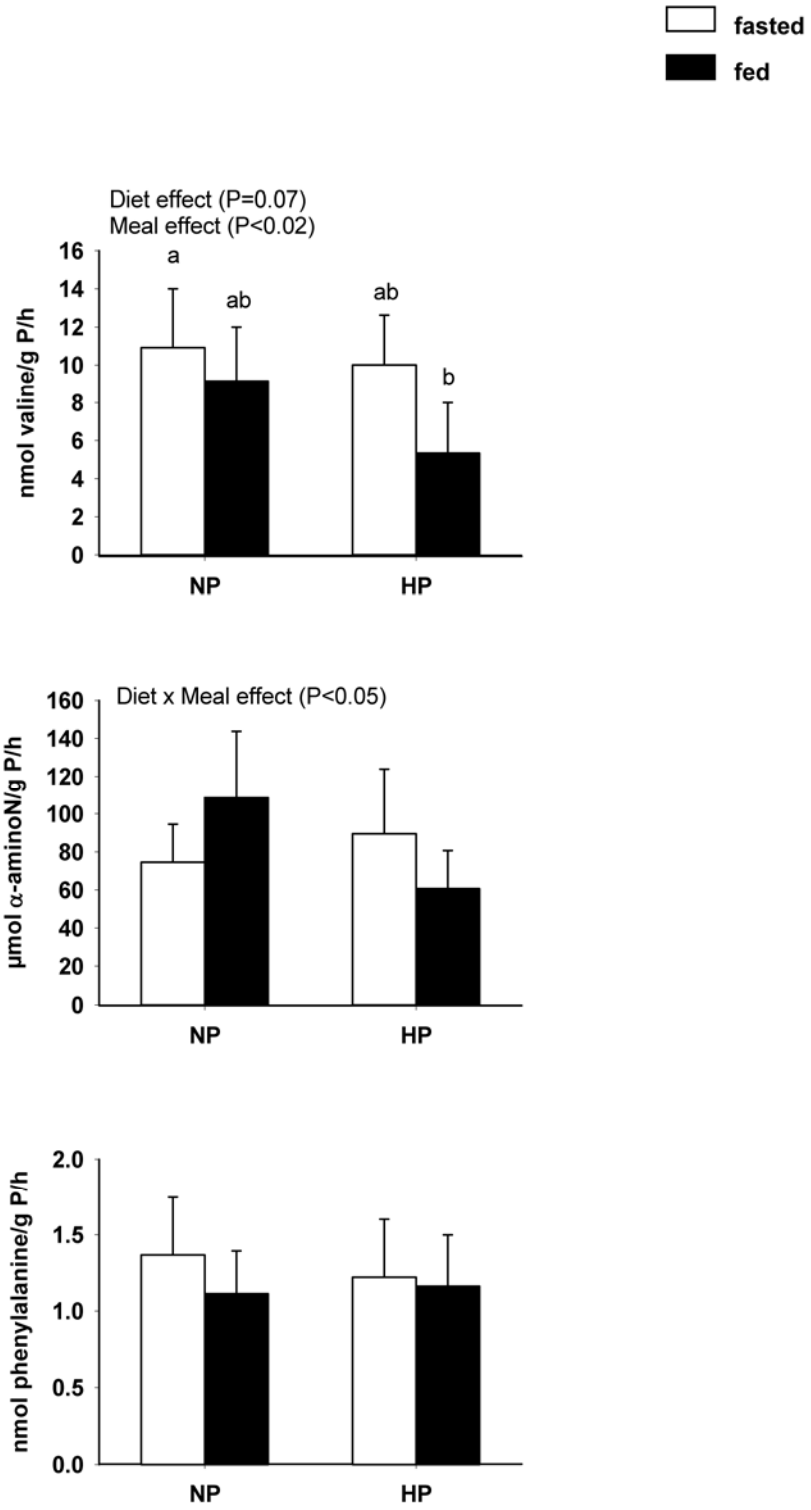

fasted
fed
Diet effect (P=0.07)
Meal effect (P<0.02)
nmol valine/g P/h
a
ab
ab
b
NP
HP
Diet x Meal effect (P<0.05)
µmol α-aminoN/g P/h
nmol phenylalanine/g P/h

Figure 2.

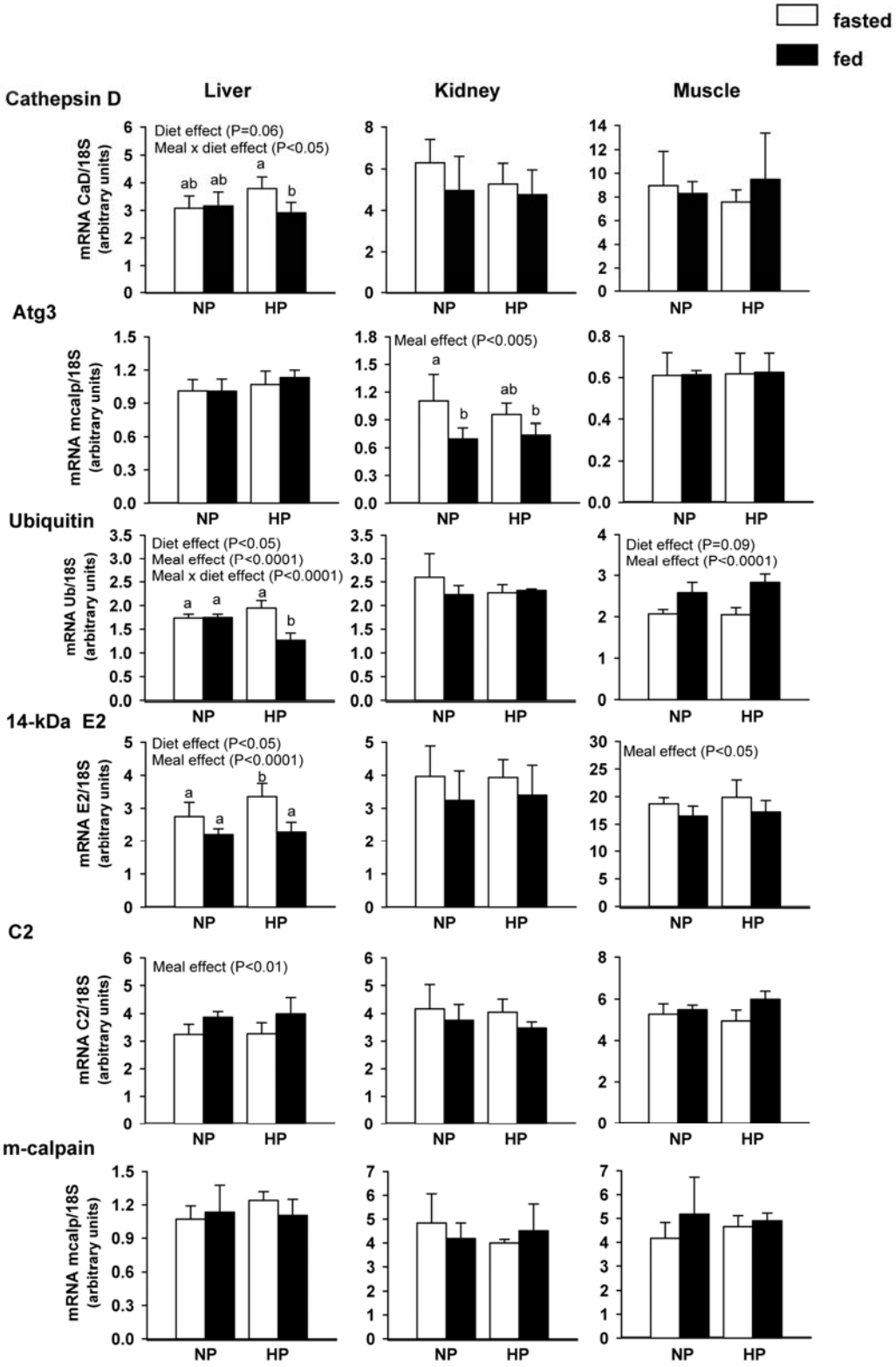

fasted
fed
Liver
Kidney
Muscle
Cathepsin D
mRNA CaD/18S (arbitrary units)
Diet effect (P=0.06)
Meal x diet effect (P<0.05)
Atg3
mRNA mcalp/18S (arbitrary units)
Meal effect (P<0.005)
Ubiquitin
mRNA Ub/18S (arbitrary units)
Diet effect (P<0.05)
Meal effect (P<0.0001)
Meal x diet effect (P<0.0001)
Diet effect (P=0.09)
Meal effect (P<0.0001)
14-kDa E2
mRNA E2/18S (arbitrary units)
Diet effect (P<0.05)
Meal effect (P<0.0001)
Meal effect (P<0.05)
C2
mRNA C2/18S (arbitrary units)
Meal effect (P<0.01)
m-calpain
mRNA mcalp/18S (arbitrary units)
NP
HP

Figure 3.

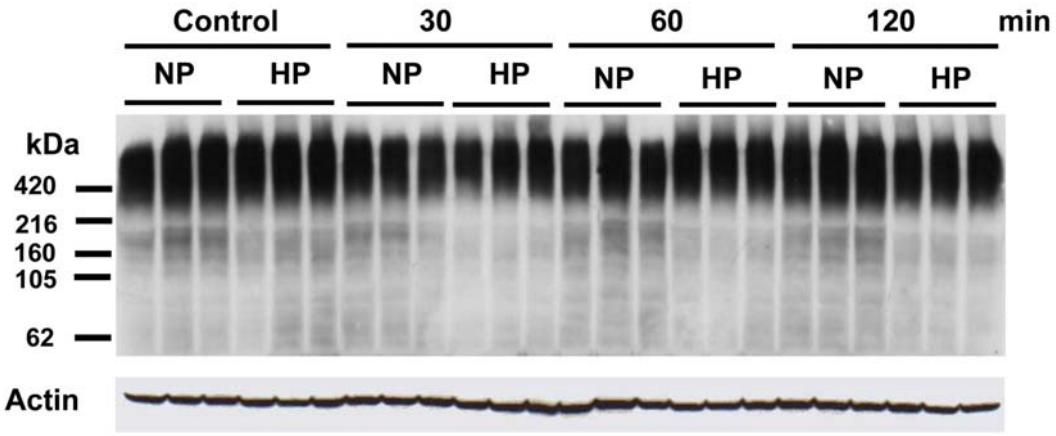

Control
30
60
120
min
NP
HP
NP
HP
NP
HP
NP
HP
kDa
420
216
160
105
62
Actin

Figure 4.

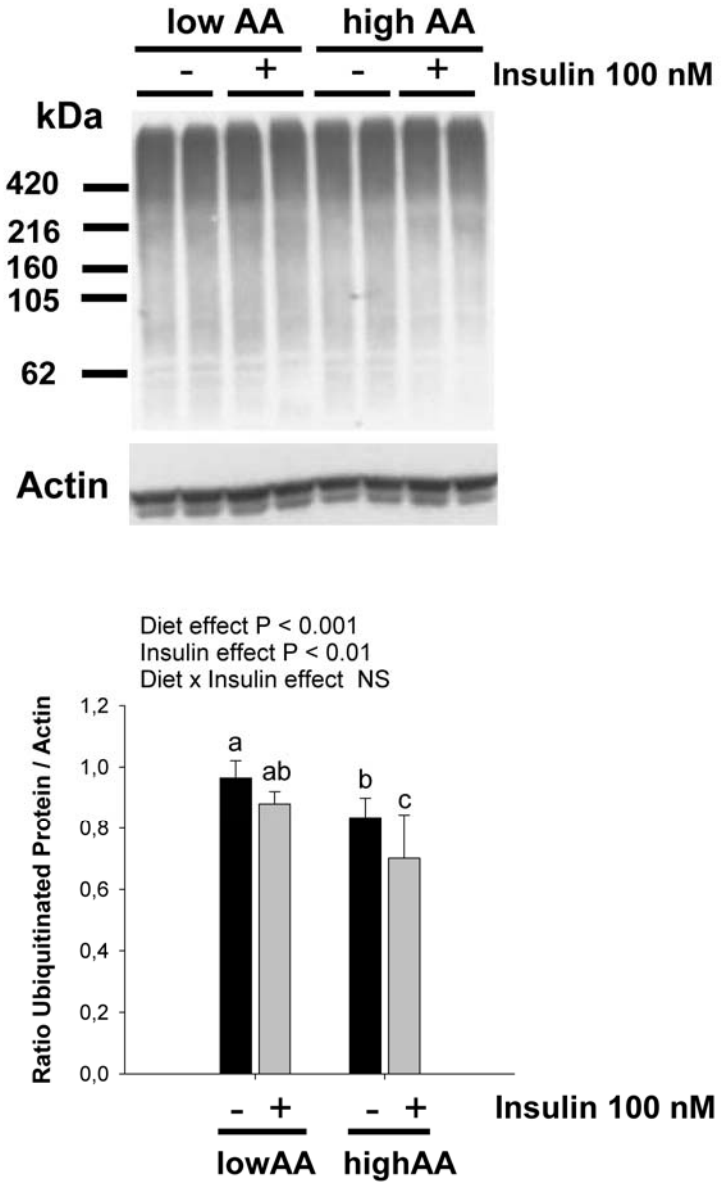

low AA
high AA
- + - +
Insulin 100 nM
kDa
420
216
160
105
62
Actin
Diet effect P < 0.001
Insulin effect P < 0.01
Diet x Insulin effect NS
Ratio Ubiquitinated Protein / Actin
1,2
1,0
0,8
0,6
0,4
0,2
0,0
a
ab
b
c
- + - +
Insulin 100 nM
lowAA
highAA

Figure 5.

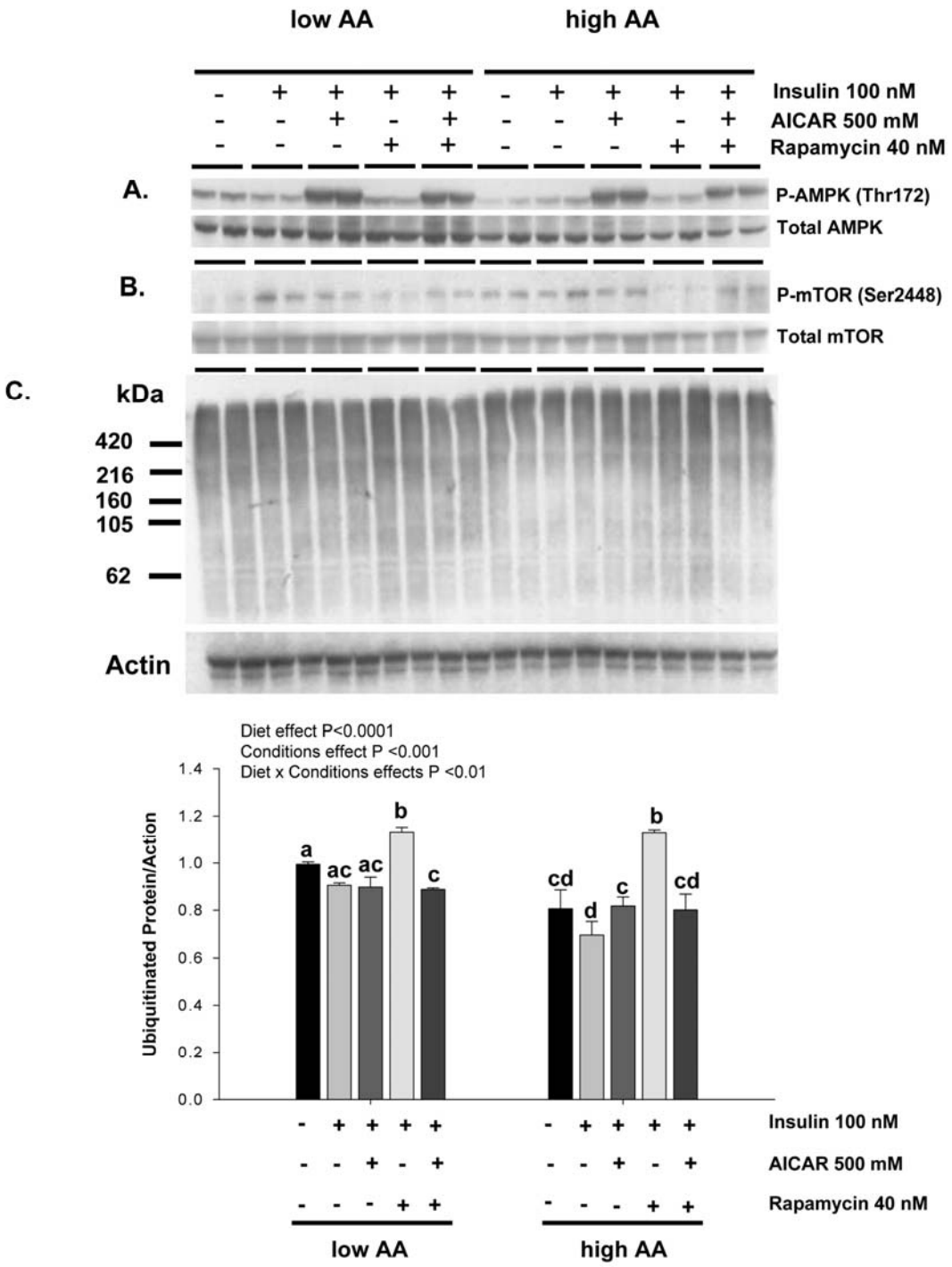
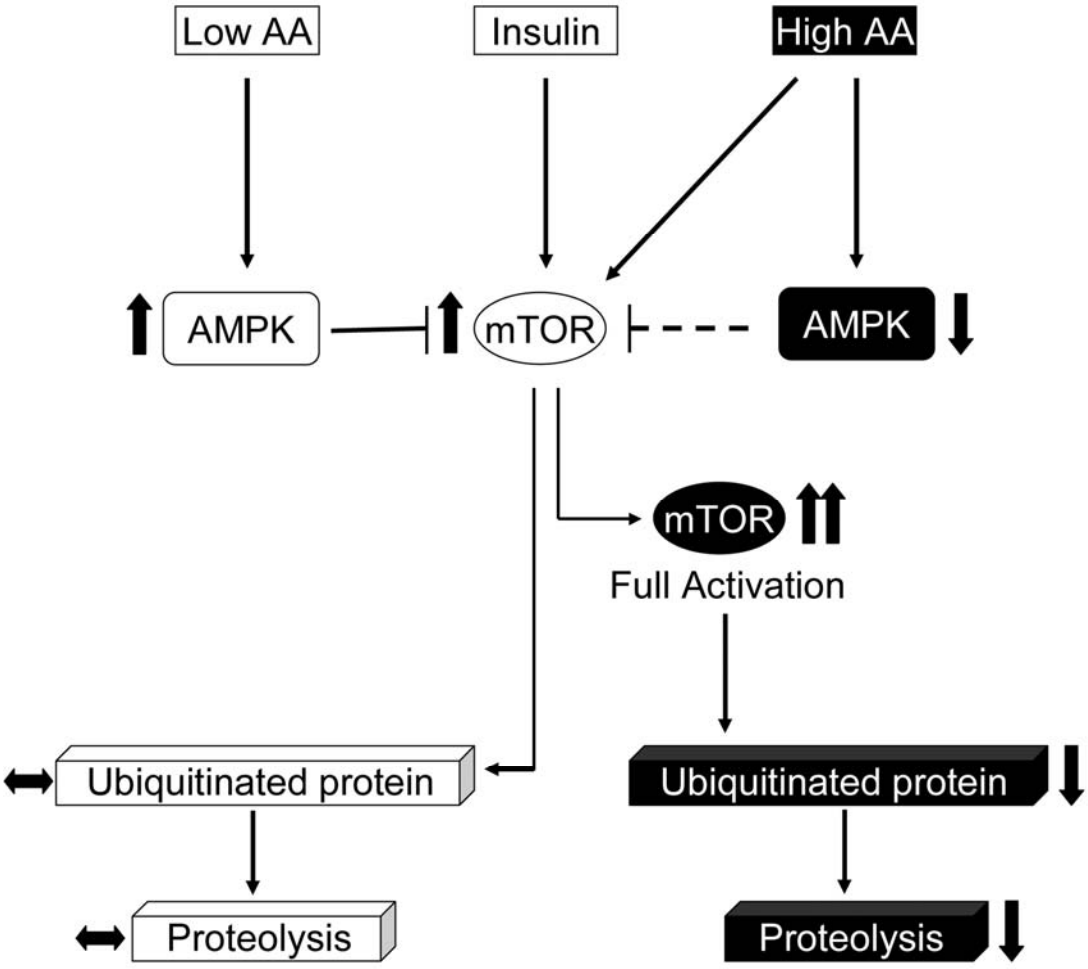

low AA
high AA
Insulin 100 nM
AICAR 500 mM
Rapamycin 40 nM
A.
P-AMPK (Thr172)
Total AMPK
B.
P-mTOR (Ser2448)
Total mTOR
C.
kDa
420
216
160
105
62
Actin
Diet effect P<0.0001
Conditions effect P <0.001
Diet x Conditions effects P <0.01
Ubiquitinated Protein/Action
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
ac
ac
b
c
cd
d
c
b
cd
Insulin 100 nM
AICAR 500 mM
Rapamycin 40 nM
low AA
high AA

Figure 6.


Low AA
Insulin
High AA
AMPK
mTOR
AMPK
mTOR
Full Activation
Ubiquitinated protein
Ubiquitinated protein
Proteolysis
Proteolysis

Supplemental data

Assessment of in vitro tissue protein degradation

Hepatocytes (8.10^6), kidney slices and soleus muscle (placed in extension on non-oxidizable pieces of metal to prevent proteolysis) were pre-incubated in a Krebs–Henseleit bicarbonate buffer for 20 min and then incubated for 60 min in 3 mL (hepatocytes and kidney slices) or 6 mL (soleus) of Krebs–Henseleit bicarbonate buffer supplemented with 10 mM sodium Hepes, 5 mM glucose and cycloheximide at pH 7.4, in an atmosphere of O_2/CO_2 (19:1), as described elsewhere (39,40). Cycloheximide (10 µM for hepatocytes and kidney slices; 500 µM for soleus muscles) was added to inhibit protein synthesis. The respective releases of valine by hepatocytes and phenylalanine by muscle were considered to constitute an index of protein degradation because these amino acids are poorly metabolized by these tissues and their use for protein synthesis is prevented by the action of cycloheximide. For kidneys, we chose to express α-amino N as an index of protein degradation rates; these rates were expressed as mmolAA/g tissue/h.

Nitrogen and amino acid analyses

The protein content of tissues was determined as described (33). Tissues and digesta were freeze-dried and their total N content was assessed by the Dumas method using an elemental analyzer (NA 1500 Series 2, Fisons) with atropina as standard. Amino acid concentrations were analyzed in the incubation media of hepatocytes, kidney and soleus muscle on deproteinized samples by HPLC after separation on cation exchange resin and post-column ninhydrin derivatization (Biotek Instruments, St Quentin-en-Yvelines, France). Norvaline and amino-guanidopropionic acid were used as internal standards to correct for amino acid loss between sampling and preparation for the analyses. Gamma-amino-butyric acid was added just prior to the analysis in order to control the injection volume.

Analysis of gene expression by Real-Time PCR

Total RNA (0.05 to 0.1 g) were extracted from frozen tissue using Trizol® reagents (Invitrogen, Carlsbad, CA, USA) and gene expression was analyzed by real-time PCR on 10 ng of cDNA using the Power Syber Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on a 7300 real-time PCR system (Applied Biosystems), as previously described (34). Primers were designed using Primer Express software and the sequences are described in supplemental data. Gene expression was determined using the $2^{-\Delta Ct}$ formula where 2 represents the optimum efficiency (E) of PCR which is $E = 1$ and $\Delta Ct = (Ct_{\text{target gene}} - Ct_{18S})$. PCR efficiency was determined in each plate using a serial dilution of reverse transcribed RNA.

The sequences of the PCR primers used were: 5'-ACACTGGCTCCTCAACCTG -3' (up) and 5'-TCCACCTTGATACCTCCTAAG-3' (down) for cathepsin D (NM_134334), 5´-TATCTACCAACAGGGAAACAG-3´ (up) and 5´-CCATCACCATCATCTTCTTC-3´ (down) for Atg3 (NM_134394) 5'-CGCACCCTCTCTGACTACA-3' (up) and 5'-GCCCTCTTTATCCTGGATCT-3' (down) for ubiquitin (Ubb, NM_138895), 5'-GGAAAACAAACGGGAGTATG -3'(up) and 5'-ACACAACGACGATGGAAAG-3' (down) for 14kDa E2 enzyme (M62388), 5'-AGAAAGACCACAGAGAAAAGC-3'(up) and 5'-TACCACCCAATACATTACAGC-3' (down) for C2 subunit of 20S proteasome (NM_017278), 5'-GCTGGAGGAAGAAGATGAAG -3'(up) and 5'-GAAGTAGAAGAAGGAGGTCG-3' (down) for m-calpaine (NM_017116), 5'-GGGAGCCTGAGAAACGGC-3' and 5'-GGGTCGGGAGTGGGTAATTT-3' for 18S.

Western blot analysis

Frozen liver (100 mg) or 7×10^6 hepatocyte cells were homogenized in a lysis buffer and an equivalent of 70 µg of proteins per sample were analyzed by Western blot as previously described (34). The antibodies used are detailed in supplemental data. The blots were developed using an enhanced chemiluminescence kit (Pierce Biotechnology, Inc, Rockford, IL, USA.). The bands were quantified by densitometric analysis of the Western blots using

the alphaImager 2200 (Alpha Innotech Corporation). For polyubiquitinated proteins, the bands between 216 and 62 kDa were quantified.

The primary antibodies (dilution 1/1000) used were the anti-phospho AMPKα (Thr 172) catalytic subunit, the anti-AMPKα catalytic subunit, anti-phospho mTOR (Ser2448), anti-mTOR, anti-ubiquitin (Cell Signalling Technology) and anti-actin (Santa Cruz Biotechnology, Inc, CA USA), The secondary antibody used is conjugated to horseradish peroxidase (dilution 1/10000) (Jackson Immunoresearch Laboratories, Inc, West Grove, PA, USA).

Table 2: Composition of the NP and HP diets

	P14	**P55**
		g/kg diet
Total milk protein [1]	140.0	530.0
Cornstarch [2]	622.4	277.0
Sucrose [3]	100.3	50.0
Soybean oil [4]	40.0	40.0
AIN 93M mineral mix [5]	35.0	35.0
AIN93V vitamin mix [5]	10.0	10.0
Cellulose [6]	50.0	50.0
Choline [5]	2.3	2.3
Metabolizable energy *kJ/g*	14.6	14.6
P/E (%)	14.0	55.0
G/E *(%)*	76.0	5.0
L/E *(%)*	10.0	40.0

***** [1] IDI ;Arras, France; [2] Cerestar, Haubourdin, France; [3] Eurosucre, Paris, France; [4] Bailly SA, Aulnay-sous-bois, France; [5] ICN Biochemicals, Ohio, USA (see Reeves et al. 1993 for composition); [6] Medias filtrants Durieux, Torcy, France. P/E, G/E, L/E: percentage of dietary energy provided by protein, carbohydrates and lipids, respectively.

PART III: DISCUSSION AND CONCLUSION

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1. General discussion

This study assessed the adaptation to a high dietary protein intake of protein metabolism in the liver, that plays a major role in the control of metabolism in the whole body, using different *in vivo* and *in vitro* experiments: characterization of translation by focusing on the activation state of key proteins involved in the transduction signaling pathways involved in the translational control using both *in vivo* and *in vitro* approaches and characterization of protein degradation through the measurement of proteolytic rate in *ex vivo* experiments, determination of the expression of key proteins of the proteolytic pathways and investigation of ubiquitinated protein pattern. The results of this work demonstrated that HP feeding induced hepatic metabolic adaptations which were characterized by an increase of mTOR phosphorylation and the decrease of both AMPK and GCN2 phosphorylation, suggesting that the increase of protein intake stimulated the translation at the initiation step. Moreover, our findings also revealed that the stimulation of the translation at the initiation step by a HP diet required both high AA levels and insulin as suggested by the increase mTOR, 4E-BP1 and S6 phosphorylation and the decrease of AMPK and GCN2 phosphorylation. Our study also demonstrated that HP diet in the fed state induced the inhibition of hepatic protein breakdown whereas no significant changes were observed in this state for kidney and muscle. This results were concomitant with the decrease of gene expression of the major components for both autophagy and ubiquitin-proteasome systems in liver. As a consequence, ubiquitinated proteins in the liver were lower when compared to a NP diet and this process occurred as early as 30 minutes after a HP meal. The autophagic pathway may also be involved in these adaptations since the cathepsin D expression was significantly lower in the HP fed state than in the HP fasted state. However, these variations did not significantly differ from those seen in a NP diet. Furthermore, the down-regulation of ubiquitination in the liver required high AA levels and this process was more inhibited in the presence of insulin. Finally, this work also revealed that mTOR and AMPK were involved in the control of the down-regulation of ubiquitination in liver in

response to the increase in high protein intake.

Effect of HP diet on the protein metabolism in liver

The present study reported that the increase in the protein intake resulted in the increase of the protein content, the stimulation of the translation and the decrease of the protein breakdown in the liver. In parallel, other studies performed in our lab, using the flooding dose method, have shown that the rate of protein synthesis in the liver was decreased in rats adapting to HP feeding for 2 weeks (Chevalier et al. 2009). These results seem inconsistent with the stimulation of the translation as shown in our study which suggests a stimulation of protein synthesis. It would be possible that the measurement of protein synthesis rate in liver using the flooding dose method would not reflect the hepatic synthesis rate because some protein synthesized in the liver are exported in plasma. However, approaches using dietary AA labelled with ^{13}C and ^{15}N, have shown that plasma proteins did not incorporate more dietary AAs when protein intake was increased above normal (Stepien 2010), which confirmed that the rate of hepatic synthesis is slower in HP fed rats. However, the results of Chevalier *et al.* indicated that protein synthesis was activated which is in line with the stimulation of translation but the rate of new protein synthesized was slower when protein intake rises. A likely explanation for this discrepancy is that the stimulation of the translation at the initiation step may be not enough to stimulate the hepatic protein synthesis rate in the liver since the latter processes, such as elongation and termination of translation, the post-translation steps and the polysome formation, are also critical steps in the control of hepatic protein synthesis. Another explanation is that the protein synthesis is a dynamic process depending on the physiological circumstances. It was reported that high AA levels stimulated protein synthesis in 1 hour (Dubbelhuis and Meijer 2002) and leucine stimulated the albumin synthesis after 6 hours (Ijichi et al. 2003) in hepatocytes. As a consequence, results of the protein synthesis rate obtained at 2 hours after the meal may be different from those obtained at a different time, such as 1 hour or 6 hours and needed to be clarified. It remained also to be determined the relation between the activation state and the rate of protein synthesis. Moreover, the quantity of synthesized protein was related to the quantity of mRNA, which itself depended on the transcription. Under HP

condition, fewer genes might be expressed when compared with NP condition (Blouet et al. 2006), resulting in less mRNA translated. It would be interesting to examine the quantity of synthesized protein under HP condition. Finally, given that mTOR, AMPK and GCN2 were key regulators of translation initiation and signaling pathways that control not only protein metabolism but also energy metabolism adaptation, we could postulate that in the case of the increase in protein intake, their main function in this case would be to coordinate metabolic adaptation.

Control of both translation and protein degradation by the same nutritional signals and the same transduction pathways

This study demonstrated that both AAs and insulin are required to stimulate the translation, at least for the initiation step, and to down-regulate the ubiquitin-proteasome system in the liver. These results suggested that the control of protein metabolism in both anabolic and catabolic pathways in the liver, in response to HP feeding, utilized the same signals, at least for AAs and insulin. Because the increase of BCAA or Leu levels was sufficient to stimulate the phosphorylation of mTOR, 4E-BP1 and S6 and to induce the decrease of the phosphorylation of AMPK and GCN2, our findings also suggested that BCAA, especially Leu, play a crucial role in AA sensing in the liver, which is in agreement with an earlier study (Ijichi et al. 2003). Our results also revealed that in high AA conditions, beside mTOR, AMPK may play an important role in the control both of 4E-BP1 and S6K1 since both P-4E-BP1 and P-S6 were dramatically decreased by AICAR whereas rapamycin induced a dramatic decrease only in P-S6. Additionally, the phosphorylation state of 4E-BP1 was not always related to the P-mTOR as both AAs and insulin were required for P-4E-BP1 increase while either AAs or insulin are sufficient to increase P- mTOR.

Based on the results obtained in the present thesis, we could postulate that the mTOR, AMPK and GCN2 transduction pathways coordinated to control the translation in response to AAs and insulin as shown in the figure 22. The mTOR pathway acts as the sensor of insulin and

AAs. On the other hand, our results revealed that the increase of P-mTOR by either insulin or AAs alone was not enough to phosphorylate 4E-BP1. These were in line with the previous studies showing that AAs and insulin were necessary for full activation of mTOR. Insulin activated mTOR through PI3K/Akt pathways (Inoki et al. 2002; Manning et al. 2002; Li et al. 2004b; Kimball and Jefferson 2006b) and AAs stimulated the relocalization of mTORC1 mediated by Rags to the proper localization, contained Rheb in order to receive the growth-factor-mediated signal (Sancak et al. 2008; Shaw 2008). Moreover, besides the Rags, hVps34 involved in the activation of mTOR through the association and/or the relocation in response to AAs (Nobukuni et al. 2007; Gulati et al. 2008; Murray et al. 2002; Stein et al. 2003). These suggested that the full activation of mTOR by both insulin and AAs was required to translational activation as demonstrated by the increase of both S6 and 4E-B1 phosphorylation (Figure 22). AMPK acts as an energy sensor (Viollet et al. 2006) which was consistent with our results showing the decrease of AMPK phosphorylation by nutrients, i.e. glucose and AAs. Thus, AMPK could be “a guard" and allow a stimulation of translation, if energy was available whereas, if it is not, AMPK shuts off the pathway. The latter signaling pathway, GCN2, acts as an AA sensor. It was previously reported that GCN2 was activated by AA deprivation (Anthony et al. 2004; Dever and Hinnebusch 2005). The present study demonstrated that GCN2 also sensed AA availability and insulin in the liver.

The previous observation proposed that the control of the anabolic and catabolic pathways by AAs may be coordinated by the same signaling system and might involve the mTOR pathway (Blommaart et al. 1995). mTOR was involved both in the stimulation of translation though S6K and 4E-BP1 (Hay and Sonenberg 2004; Proud 2004) and in autophagic control with largely unknown mechanism (Shintani and Klionsky 2004). Moreover, hVps34, the positive regulator of mTOR, was also involved in the control of autophagy (Petiot et al. 2000; Eskelinen et al. 2002; Qu et al. 2003). Similarly, AMPK also inhibited the translation through the phosphorylation of TSC2, raptor, mTOR and eEF2K (Inoki et al. 2003a; Gwinn et al. 2008; Cheng et al. 2004; Browne et al. 2004) and it was involved in autophagy control and proteasome activity (Meley et al. 2006; Viana et al. 2008). The latter transduction pathway, GCN2, controlled the translation and autophagy through the eIF2 phosphorylation (Dever and Hinnebusch 2005;

Towle 2007; Talloczy et al. 2002). As described, the decrease of proteolysis in liver of HP fed rats was associated with the down-regulation of hepatic protein ubiquitination induced by AAs and insulin. Our findings also demonstrated that mTOR and AMPK regulated hepatic proteolysis through the down-regulation of the ubiquitin proteasome pathway in response to high protein intake. Rapamycin reversed the inhibition of ubiquitination of protein at both low and high AA levels in the presence of insulin whereas AICAR reversed the inhibition of ubiquitination only at high AA levels in the presence of insulin. Moreover, in the presence of both AICAR and rapamycin, AICAR reversed the effect of rapamycin on the ubiquitinated protein. The present findings revealed that, besides the control of autophagy, mTOR and AMPK also coordinated to control the hepatic protein ubiquitination. Furthermore, our results demonstrated that AAs and insulin had an additive effect on ubiquitinated proteins in hepatocytes.. We could hypothesize that the full activation of mTOR may play a crucial role in the down-regulation of ubiquitinqtion in response to the additive effect of AAs and insulin (Figure 23).

Finally, we might conclude that, after HP feeding, the AAs and insulin were sensed by mTOR, AMPK and GCN2 transduction pathways in hepatocytes. Afterward, the AA and insulin signals were transduced in order to stimulate the translation, at least at the initiation step, and, at the same time and to down-regulate the protein degradation through the ubiquitin-proteasome system. However, the role of GCN2 in the down-regulation of ubiquitination in response to the increase of protein intake remained to be determined.

Upstream components of the transduction pathways involved in the amino acid sensing

1 AA transporters

The liver is the major site of AA and carbohydrate metabolism in the body. Transport of an individual AA into hepatocytes may present the rate-limiting step in its own metabolism and may therefore be an important point for regulation. For example, hepatic transport of alanine mediated by the System A can be a rate-limiting step for further alanine metabolism

(Fafournoux et al. 1990; Fafournoux et al. 1983). The System A transporters which transport neutral AAs, such as the SNAT1, SNAT2 , SNAT3 and SNAT4, are expressed in the liver (Gu et al. 2003; Gu et al. 2001a; Gu et al. 2000; Gu et al. 2001b; Gu et al. 2005; Hyde et al. 2003). Moreover, our previous study demonstrated that the AA concentrations found in the portal vein of rats fed a HP diet for 2 weeks were increased only for some AAs, i.e. neutral AAs and BCAA (Azzout-Marniche et al. 2007). This suggested that these AAs could have a determining role in signaling to the liver the increase of dietary protein. Thus, the AA transporters for BCAA might have a crucial role in the cellular AA sensing in the liver. Furthermore, the AA transporters also function for AA exchange, such as the system A and L, termed as tertiary active exchangers (Figure 14) (Hundal and Taylor 2009). The system L is expressed ubiquitously in mammalian tissues and mediates the cellular uptake of indispensable AAs, such as BCAA (Hundal and Taylor 2009). Indeed, because the AA substrate ranges of the Systems A and L overlap, the coupling of AA substrate flows through these transporter systems generates cellular accumulation of leucine (System L substrate) which is the activator of the mTOR signaling pathway, by exchanging with cytoplasmic AAs i.e., glutamine (an AA substrate of both the Systems L and A) (Taylor 2009; Hundal and Taylor 2009). Additionally, the AA transporters, such as SNAT2 and PATs, have been linked to the AA sensing pathways, i.e. mTOR and GCN2 (Goberdhan et al. 2005; Goberdhan and Boyd 2009; Gaccioli et al. 2006; Evans et al. 2007; Taylor 2009). Given that AA transporters have been demonstrated as dual transporter and receptor function, so called "transcepter", these suggested that AA transporters could play a function as first sensor for AA availability and then translate these signals to the transduction signaling pathways to regulate metabolic orientation. Further studies are required to investigate the role of AA transporters as the upstream components of transduction signal of AAs.

2 Protein phosphatases

The phosphorylation state of the functional proteins in the cascade pathways is a crucial point to control their activity in response to transduction signals. Apart from mTOR, most of the upstream proteins involved in AA signaling pathways, i.e. AMPK and GCN2, are less phosphorylated in response to the increase of AA concentration. Thus, along with the protein kinases, the protein phosphatases could play an important role in the control of transduction

signaling pathways in response to AA signals. The family of serine/threonine protein phosphatases (PP) includes PP1, PP2A, the Ca^{2+}-dependent PP2B (calcineurine), the Mg^{2+}-dependent PP2C, PP4 (PPX) which are PP2A-like phosphatases, PP5 and PP7. The PP1 and PP2A are the most abundant of protein phosphatases and their activity is regulated by the nutrition status (Kabashima et al. 2003; Wu et al. 2007).

The PP2A comprise 3 subunits: a catalytic subunit (PP2AC) and two regulatory subunits including a subunit A or PR65 and a subunit B. The activity of PP2A was regulated via the subunit B. The recruitment of the regulatory subunit B depended on the phosphorylation or methylation state at the C-terminal of PP2AC. The PP2A activity was inhibited by the phosphorylation of Tyr307 on PP2AC. Nevertheless, the protein kinase which phosphorylated the PP2AC had been not identified. Moreover, the PP2A was activated through the methylation of PP2AC at the carboxyl group of Leu309 via S-adenosyl-methyl-dependent leucine carboxyl methyltranferase (LCMT1) and this methyl group can be demethylated by the protein phosphatase methylesterase (PME-1). Although, the methylation of PP2AC did not seem to directly modify the PP2A activity, it was required for the formation of trimers (Longin et al. 2008). PP2A was activated by glucose and dephosphorylated its downstream targets (Kabashima et al. 2003) and by palmitate in aortic endothelial cells (Wu et al. 2007). Additionally, AMPK and ACC were dephosphorylated by PP2A (Wu et al. 2007; Gaussin et al. 1996). Besides the PP2A, AMPK was also dephosphorylated by PP2C (Sanders et al. 2007). These data were in agreement with our findings showing that AMPK and ACC phosphorylation were decreased by glucose. PP2A was also able to dephosphorylate S6K1 *in vitro* and in mammalian cells (Ferrari et al. 1991;Westphal et al. 1999; Peterson et al. 1999). Moreover, previous studies have reported that the mTOR-dependent phosphorylation of S6K1 and 4E-BP1 was an indirect result of the inhibition of PP2A (Gingras et al. 2001a; Gingras et al. 2001b), suggesting that S6K1and 4E-BP1 were also substrates of PP2A (Bishop et al. 2006).

The PP1 is an ubiquitous expression. The PP1 corresponds to the catalytic sub-unit which interacts with the PP1-targeting protein. The PP1-targeting proteins are anchor proteins which regulate PP1 activity by a sub-cellular localisation of the enzyme to its substrate (Ceulemans and

Bollen 2004). PP1 was regulated by insulin and glucose, and glycogen metabolism was a well known target of PP1 (Ceulemans and Bollen 2004). The PP1 activated the GS activity through its dephosphorylation and formed the GS-PP1-PTG (Protein target to glycogen) complex in the presence of insulin and glucose (Brady and Saltiel 2001). Moreover, eIF2α and S6 were also the downstream targets of PP1 (Ceulemans and Bollen 2004).

These literatures revealed that AMPK, 4E-BP1, S6K, S6 and eIF2 were the downstream targets of protein phosphatases. However, the role of protein phosphatases on the mTOR and GCN2 dephosphorylation remained to be identified. Moreover, the effect of the increase in AA availability on the activation state of these protein phosphatases has not been investigated. We hypothesize that these protein phosphatases will be good candidates to transduce AA signal upstream to mTOR, AMPK and GCN2.

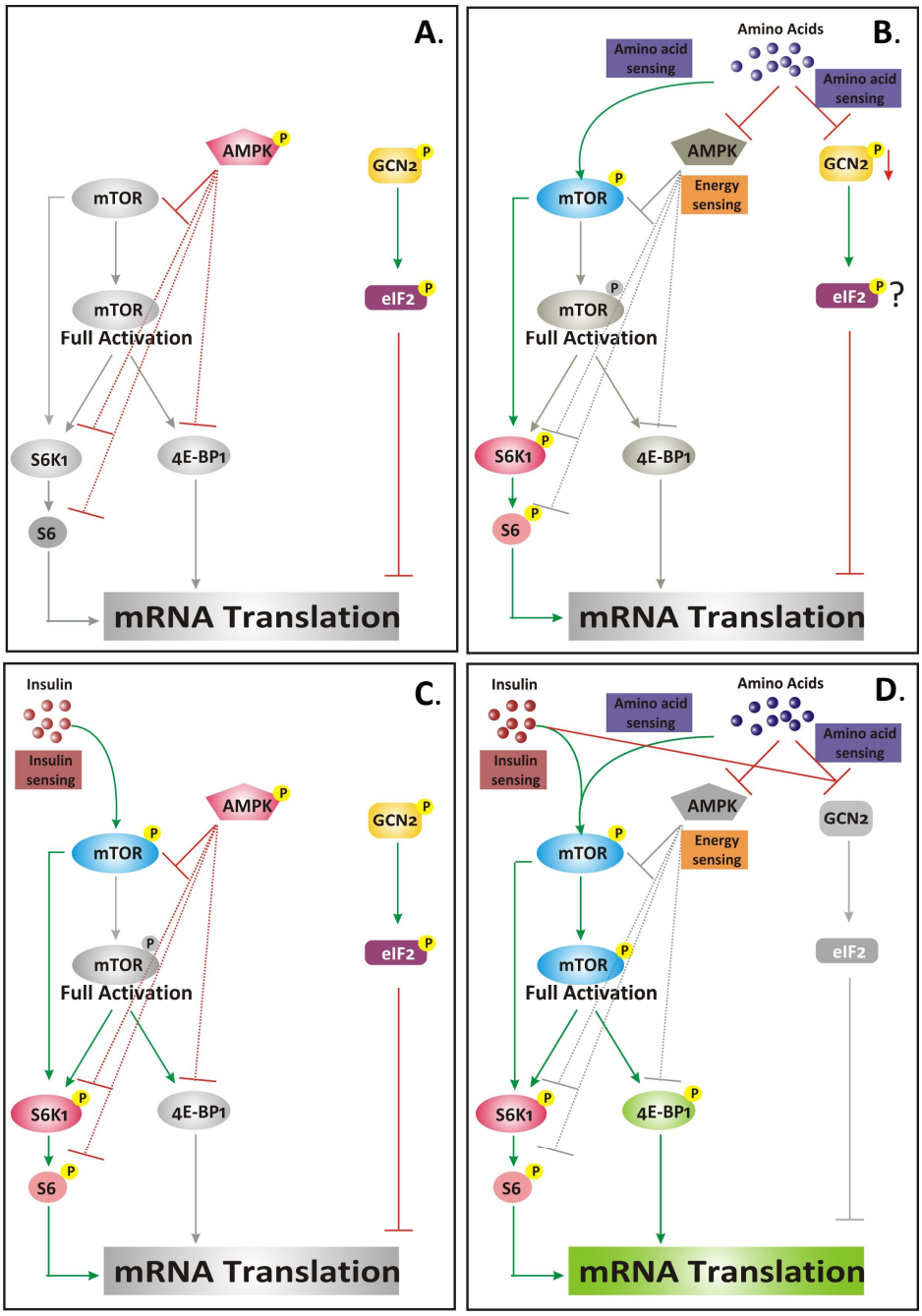
2. General conclusion

In conclusion, this study demonstrated that the adaptation to a high dietary protein diet was characterized by the stimulation of translation, at least at the initiation step, and the decrease of proteolysis in the liver. We have shown that both AAs, especially BCAA or Leu, and insulin act as the important signals to stimulate the translation and mTOR, AMPK and GCN2 transduction pathways coordinated to control the translation in response to HP intake. AMPK may play a central role in the regulation of translation through 4E-BP1 and S6K. Additionally, we confirmed that the HP diet induced an inhibition of protein breakdown in the liver and concomitantly decreased gene expression of the major components for both autophagy and the ubiquitin-proteasome system in the liver. Therefore, ubiquitinated protein in the liver was lower when compared to NP diet and the ubiquitination down-regulation required both AAs and insulin. Interestingly, we have shown that mTOR and AMPK were also involved in the control of ubiquitin proteasome system in hepatocytes in response to the increase in AA and insulin concentrations. These results suggested that the control of the catabolic and anabolic pathway of protein metabolism was regulated by the same signals and mediated by the same transduction signaling pathways.

3. Perspectives

This work presented for the first time that the same signals and the same transduction pathways were involved in the control of translation at the initiation step and protein ubiquitination in the liver in response to HP feeding. However, to clarify the discrepancy between the stimulation by a HP meal of the translation at the initiation step observed in our study and the decrease in the hepatic protein synthesis rate at 2 hours after a HP meal (Chevalier et al. 2009), the measurement of the protein synthesis rate at different times postprandially in the liver as well as in hepatocytes in response to insulin and to the increase of AA concentration will be necessary. Moreover, along with the protein synthesis rate, the quantity of synthesized protein should be determined by using ^{35}S-methionine and proteomics.

Additionally, it would be interesting to further identify the upstream components of AMPK, mTOR or GCN2 signaling pathways, such as AA transporters and protein phosphatases, and to elucidate the role of AMPK in the control of mTOR and GCN2 activation state. The effect of a HP diet on the protein phosphatase and AA transporter activity *in vivo* should be assessed. Furthermore, to investigate the respective effect of AAs and insulin on protein phosphatase and the AA transporter activity, *in vitro* approaches using primary culture of hepatocytes could be used. Finally, it will be interesting to use gene silencing approaches to demonstrate the role of each component of AA signal both *in vitro* and *in vivo*.



<u>Figure 22.</u> The stimulation of translation by amino acids (AAs) and insulin through mTOR, AMPK and GCN2 transduction pathways in liver. In low amino acid (AA) concentration and in the absence of insulin, mTOR was dephosphorylated whereas AMPK and GCN2 were phosphorylated, resulting in the non-activation of translation, as shown in A. In low AA and in the presence of insulin, P-mTOR and P-S6 were increased, , however, it was not sufficient to phophorylate 4E-BP1. P-AMPK and P-GCN2 were not changed. Theses results suggested that insulin alone was not sufficient to stimulate translation as shown in B. In the presence of High AA concentration, P-mTOR and P-S6 were increased but no changes was observed in P-4E-BP1. Moreover, P-AMPK was decreased and P-GCN2 was slightly decreased by the increase in AAs concentration. In this case, the translation was not activated as shown in C. In the presence of both High AA levels and insulin, the P-mTOR, P-S6 and P4E-BP1 were increased whereas the P-AMPK and GCN2 were decreased, suggesting that the translation was stimulated as shown in D. These results confirmed that there were the AAs, insulin and energy sensing and AAs and insulin were essential to stimulate the full activation of mTOR and the translation as shown in D.

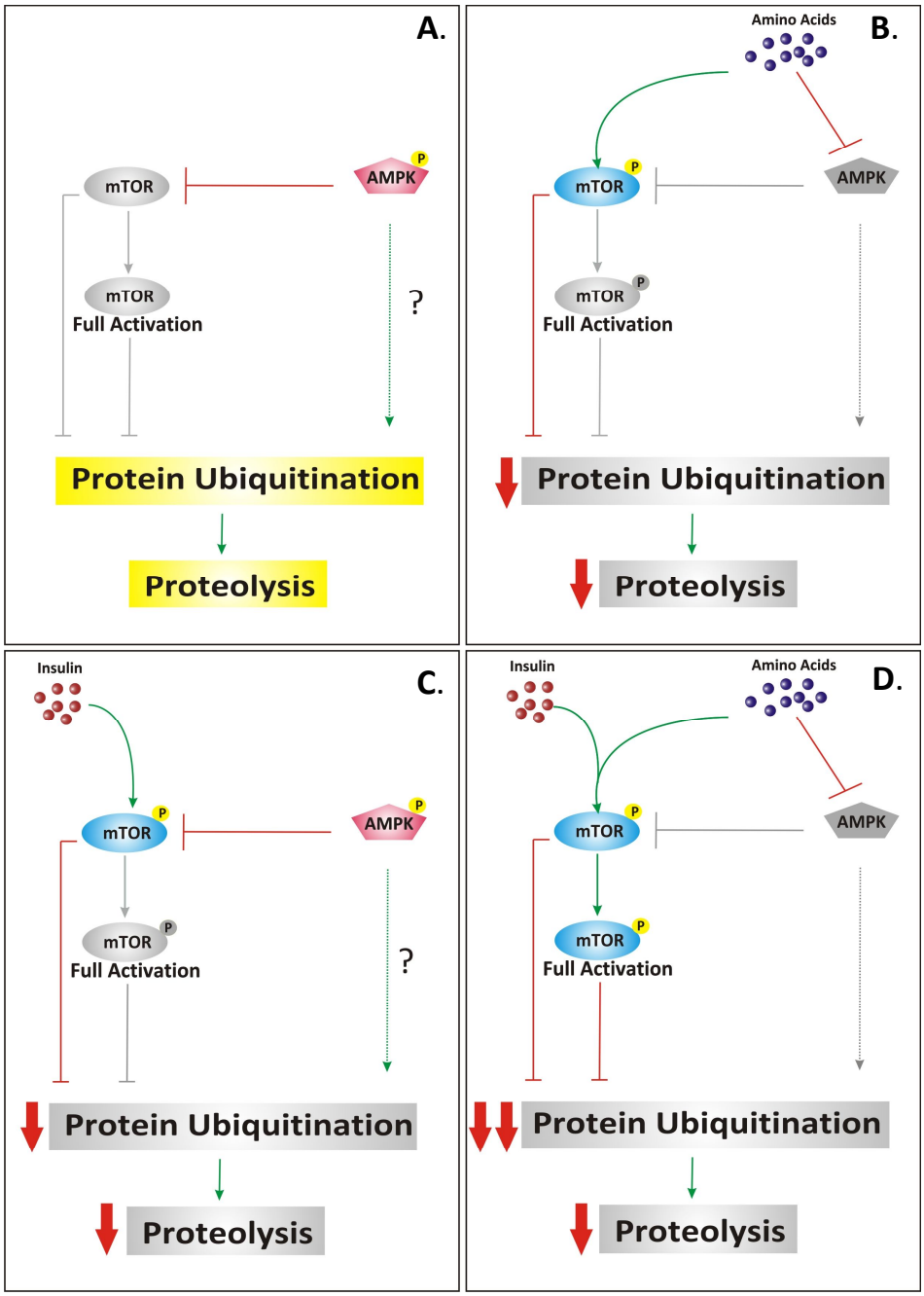


Figure 23. The down-regulation of protein ubiquitination by amino acids (AAs) and insulin through the mTOR and AMPK in liver. In Low amino acid (AA) concentration and in the absence of insulin we observed that the ubiquitination and proteolysis were not inhibited, and the mTOR was dephosphorylated while P-AMPK was increased, as shown in A. In the presence of insulin alone, the ubiquitination was slightly decreased and P-mTOR was increased whereas no change was observed with P-AMPK as shown in B. In the presence of High AAs, the ubiquitination was also slightly decreased and P-mTOR was increased while P-AMPK was decreased as shown in C. In the presence of both AAs and insulin, the ubiquitinated proteins were more decreased and P-mTOR was increased whereas P-AMPK was decreased as shown in D. Taken together, these results suggested that High AAs and insulin had an additive effect in the down-regulation of ubiquitination and these signals were required for mTOR full activation. These results suggested also that the activation of mTOR and the inhibition AMPK were required for the down-regulation of ubquitination in liver.

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