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, Gaussères 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 AMPK 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 repressed 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

DIFERENT 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 (p70S6K) 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 p70S6K (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-
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 isonitrogenous 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 acclimated 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 −80°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, 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) Ultradish 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.

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 of 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 (Thr172) catalytic subunit, the anti-AMPKα catalytic subunit, anti-phospho-mTOR (Ser2448), anti-mTOR, anti-phospho-4E-BP1 (Thr70/e), anti-4E-BP1, anti-phospho-GCN2 (Thr898), anti-GCN2, anti-phospho-4E-BP2 (Ser65), anti-4E-BP2, and anti-phospho-ACC (ACC, Ser79), 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 hors eradish 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 6.25 mM Tris·HCl, 2% (wt/vol) SDS, and 100 mM β-mecaptopropanol 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, 20 s at 55°C, and 20 s at 72°C. The primers were designed using Oligo Explorer 1.1.0 software. The sequences of PCR primers used were 5′-TGTGGCTCCTGCGAGCTCCATTAG-3′ (up) and 5′-GACCCCGCT-GCTCCAGAT-3′ (down) for AMPKα1 (NM_019142), 5′-TGGAG- GTGAATTTGTGACTACAT-3′ (up) and 5′-ACAGATGTCACAGAGACACAGA-3′ (down) for AMPKα2 (NM_023991), 5′-TGGAGGGGAG-

Table 1. Composition of diets

<table>
<thead>
<tr>
<th>g/kg DM</th>
<th>NP Diet</th>
<th>HP Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein*</td>
<td>116.0</td>
<td>242.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>99.8</td>
<td>45.5</td>
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<tr>
<td>Cornstarch</td>
<td>548.0</td>
<td>253.0</td>
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<tr>
<td>Lactose</td>
<td>7.0</td>
<td>26.5</td>
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<tr>
<td>Soya oil</td>
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<td>40.0</td>
</tr>
<tr>
<td>Dairy fat</td>
<td>2.8</td>
<td>10.6</td>
</tr>
<tr>
<td>Minerals (AIN-93M)†</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Vitamins (AIN-93M)†</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Choline</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>%Energy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein, %energy</td>
<td>13.0</td>
<td>48.0</td>
</tr>
<tr>
<td>Total fat, %energy</td>
<td>12.0</td>
<td>14.0</td>
</tr>
<tr>
<td>Total carbohydrate, %energy</td>
<td>75.0</td>
<td>38.0</td>
</tr>
<tr>
<td>Metabolizable energy, kcal/g DM</td>
<td>14.8</td>
<td>14.7</td>
</tr>
</tbody>
</table>

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

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AGCGTCTGAGA-3' (up) and 5'-TGATGGCCGCGCTTGTGTG-3' (down) for mTOR (NM_019906), 5'-CTGCGGTCCTCTTTTTGC-3' (up) and 5'-AATCGGTCTAATCTTCAGTGTCGAA-3' (down) for GCN2 (NM_013719), and 5'-GGGACCCTGAGAAACGGC-3' and 5'-GGGTGGAGGTGGTGGTATTT-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 (CT) 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\(^{-\Delta\Delta CT}\) formula, where 2 represents the optimum efficiency (E) of PCR, which is E = 2 and ΔCT = (CT target gene - CT 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 concentrations.

**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 nondepressible 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).

**E1315**

**AMINO ACID SENSING BY HEPATOCYTES**

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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 (Thr172) 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 VectaShield (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 4E-BP1 and eIF2α (meal effect, *P* < 0.001; Fig. 1A) and eIF2α phosphorylation state 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. 1C, 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. 1D). The phosphorylation of eIF2α (Fig. 1E), 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 effect, *P* < 0.01; Fig. 1C). GCN2 expression was significantly different 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 diets (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).
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. 3C) (P < 0.001). High AA concentrations or insulin enhanced mTOR phosphorylation (P < 0.001; Fig. 4A), but both signals were required to induce 4E-BP1 phosphorylation (P < 0.01; Fig. 4B). 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. 4C). 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. 4D).

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. 6A) or high AA concentrations (Fig. 6B) (P < 0.001), whereas ACC phosphorylation was decreased with high glucose concentration (Fig. 6A) (P < 0.01) but was not affected by high AA concentrations (Fig. 6B). 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

![Graphs showing the effects of glucose, AAs, and insulin on mTOR, AMPK, and GCN2 phosphorylation in hepatocytes.](http://ajpendo.physiology.org/)

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).
conditions (without anti-p-AMPKα 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.

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).

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.
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-
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 glycogen synthesis 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 phosphoenolpyruvate carboxykinase) gene expression (57). In agreement with our

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 (Thr172) 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).
mTORC1, is mammalian vescular 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-tRNA$^{\text{Met}}$ to the 40S subunit, a process that is controlled indirectly by the phosphorylation state of eIF2$\alpha$. The phosphorylation of eIF2$\alpha$ on serine 51 inhibits guanine nucleotide exchange on eIF2, converting it to a competitive inhibitor of eIF2B, and restraints general and specific mRNA translation (13, 14). eIF2$\alpha$ 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 eIF2$\alpha$ 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 vivo, 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),
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 glutagoc 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).

REFERENCES


