Liver glyconeogenesis: a pathway to cope with postprandial amino acid excess in high protein fed rats?

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Running title: Amino acid excess and glyconeogenesis

Key words: high protein diet, glyconeogenesis, glucose 6-phosphatase, phosphoenolpyruvate carboxykinase, liver

Footnotes

1 Abbreviations: BCAA: Branched chain amino acids, DAA: Dispensable amino acids, IAA (Indispensable amino acids), GAA: Gluconeogenic amino acids, G6P: glucose 6-phosphate, G6Pase: glucose 6-phosphatase, G6PC1: gluconeogenic isoform of the glucose 6-phosphatase catalytic subunit; G6PC3: ubiquitous isoform of the glucose 6-phosphatase catalytic subunit; PEPCK: phosphoenolpyruvate carboxykinase.

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Abstract

This paper provides molecular evidence for a liver glyconeogenic pathway, i.e. a concomitant activation of hepatic gluconeogenesis and glycogenesis, which could participate in the mechanisms which cope with amino acid excess in high protein (HP) fed rats. This evidence is based on the concomitant up-regulation of phosphoenolpyruvate carboxykinase (PEPCK) gene expression, down-regulation of glucose 6-phosphatase catalytic subunit (G6PC1) gene expression, an absence of glucose release from isolated hepatocytes and restored hepatic glycogen stores in the fed state in HP fed rats. These effects are mainly due to the ability of high physiological concentrations of portal blood amino acids to counteract glucagon-induced liver G6PC1 but not PEPCK gene expression. These results agree with the idea that the metabolic pathway involved in glycogen synthesis is dependant upon the pattern of nutrient availability. This non-oxidative glyconeogenic disposal pathway of gluconeogenic substrates copes with amino excess and participates in adjusting both amino acid and glucose homeostasis. In addition, the pattern of PEPCK and G6PC1 gene expression provides evidence that neither the kidney nor the small intestine participated in gluconeogenic glucose production under our experimental conditions. Moreover, the main G6Pase isoform expressed in the small intestine is the ubiquitous isoform of G6Pase (G6PC3) rather than the G6PC1 isoform expressed in gluconeogenic organs.
Introduction

The consequences of high protein feeding on the regulation and pathways of glucose disposal through glycogen metabolism and gluconeogenesis remain unclear. High protein feeding induced both insulin and glucagon secretion in the fed state, associated with the repletion of glycogen stores (3,45,52), whereas excess dietary amino acids are metabolized within tissues and the derived nitrogen released into the circulation in the form of glutamine, alanine, serine, and glycine (28,56). These amino acids are subsequently taken up by the liver and the nitrogen is converted to urea and glutamine (12,28), the bulk of their carbon skeleton being converted to glucose through hepatic gluconeogenesis (8,29,37,51). This concomitant activation of both glycogen synthesis and gluconeogenesis in the fed state raises the question as to whether the glucose-6-P produced from gluconeogenic amino acid precursors could be directly channeled to glycogen through glycogenesis instead of being excreted as glucose from hepatocytes. This pathway of glyconeogenesis has been discussed over several decades but despite some evidence during refeeding (49), its significance and molecular basis remain poorly understood (4,30) and it has not previously been hypothesized under high protein feeding.

It is established that insulin stimulates glycogen synthesis and represses the gluconeogenic production of glucose, whereas glucagon represses glycogen synthesis and stimulates glucose production through glycogen hydrolysis and gluconeogenesis (48). Phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are the key regulatory enzymes of gluconeogenesis. Two isoforms of PEPCK have been described; they differ in terms of their intracellular distribution from species to species. In the rat, more than 90% of total activity is cytosolic, whereas in humans, the enzyme is distributed nearly equally in both cytosolic and mitochondrial compartments (11,44). The cytosolic phosphoenolpyruvate
carboxykinase (PEPCK) gene is expressed in multiple tissues and regulated in a complex tissue-specific manner. Two isoforms of G6Pase have been described. G6PC1 is mainly expressed in the gluconeogenic organs, i.e. liver and kidney, whereas G6PC3 (24), also called the glucose 6-phosphatase catalytic subunit-related protein (UGRP) (38) or G6Pβ (53), is ubiquitous and not directly related to gluconeogenesis.

The acute regulation of PEPCK and G6Pase is achieved by the modulation of gene transcription and mRNA stability. Major factors that increase PEPCK and G6Pase gene expression include cyclic AMP, glucocorticoids and thyroid hormone, whereas insulin inhibits this process (2). Glucose, in contrast to other factors, has opposing effects on PEPCK and G6PC1 gene expression since it down-regulates PEPCK (15) and up-regulates G6Pase (40). Glutamine has been shown to counteract the effect of glucose on PEPCK gene expression (35), but no study has examined the effect of a physiological increase of amino acid availability on the regulation of PEPCK and G6PC1 gene expression in gluconeogenic organs. There is however some evidence that glucose alone is not a good precursor for hepatic glycogen synthesis in vitro in perfused rat liver (26) or in isolated hepatocytes (21,31) and that the addition of modest amounts of gluconeogenic substrates in the culture medium (lactate or amino acids) results in markedly enhanced glycogenesis (7,47). Gluconeogenic substrates including amino acids may contribute to glycogen accumulation by exerting a regulatory effect on hepatic de novo glucose synthesis and glycogen metabolism (32,57).

The present study hypothesized that the increased post-prandial portal blood delivery of amino acids produced by high protein feeding could induce a liver glyconeogenic pathway among the metabolic pathways involved in the response enabling coping with the amino acid excess. In this context, we first determined the liver glycogen content and glucose production
by isolated hepatocytes in rats adapted to a normal or high protein diet. These results were further related to the liver expression of the two key gluconeogenic enzymes, i.e. PEPCK and G6PC, and the possible contribution of post-prandial increase in portal blood amino acid in the control of the expression of these two key enzymes. In addition, although the liver is considered as the principal gluconeogenic organ, we have also examined PEPCK and G6PC gene expression in kidney which is known as a second gluconeogenic organ (22) and in the intestine which has been described as a potential gluconeogenic organ after prolonged fasting (16,50) or in rats fed a high protein diet (41).

**Materials and methods**

**Animals, diet, and experimental design.** 40 male Wistar rats weighing 180-220 g (Harlan-France, Gannat, France) were used according to the guidelines of the French National Animal Care Committee. The rats were placed under a reversed light-dark cycle (lights on from 8:00 pm to 8:00 am) and adapted to the experimental conditions for one week. Experimental diet groups consisted of a normal protein (NP, 14% energy as protein) group and a high protein (HP, 50 % energy as protein) group, which for 15 days received a control diet (in g/kg of dry matter: 140 total milk protein, 100 sucrose, 622.4 Cornstarch, 40 soybean oil, 35 AIN 93M mineral mix, 10 AIN 93 vitamin mix, 50 α-cellulose and 2.3 choline) or a high protein diet (in g/kg: 530 total milk protein, 46.5 sucrose, 286.0 Cornstarch 40 soybean oil, 35 AIN 93M mineral mix, 10 AIN 93 vitamin mix, 50 α-cellulose and 2.3 choline). The two diets were isoenergetic (14.89 kJ/g) and all dietary components were purchased or prepared by the A.P.A.E. (Atelier de Preparation des Aliments Expérimentaux, French National Institute of Agronomic Research, INRA, Jouy en Josas, France). Rats were divided into two groups of 16 rats which received the experimental diet in two gauged meals consisting of a breakfast (6 g of food)
between 8:00 am and 9:00 am, and a lunch (30 g of food) between 14:00 pm and 18:00 pm. Firstly, eight rats from each NP and HP group, in either the fed state (30 minutes after the end of breakfast) or fasted state (after an overnight fast) were used for glucose production measurements and blood sampling. Secondly, eight rats from each NP and HP rat group were sacrificed in either the fed or fasted state for gene expression studies. A third group of four rats receiving a standard chow diet was used for the preparation of primary hepatocyte cultures. The animals were anesthetized with intraperitoneal pentobarbital (0.1ml/100g), the abdomen was opened, and blood was collected from the portal vein for amino acid measurements, and by intracardiac puncture for serum insulin and glucose determinations. Blood samples were collected in pre-chilled tubes containing 0.7% EDTA (and 0.014% aprotinin for insulin determinations) and centrifuged (3000 × g, 15 min, 4 °C). The resulting plasma was divided into aliquots and stored at -20° until later analysis. The liver, proximal small intestine and kidney were removed quickly, frozen in liquid nitrogen and stored at –80°C. The proximal small intestine was scraped to remove epithelial cells before being frozen.

**Glucose production by isolated rat hepatocytes.** Hepatocytes were isolated by the collagenase method (5). The liver was perfused with 300 mL of a perfusion medium containing (in mM): 118.9 NaCl, 4.76 KCl, 1.19 KH₂PO₄, 1.19 K₂HPO₄ and 10 HEPES, pH 7.4, supplemented with 2.4 mM CaCl₂ and 0.90 g collagenase H (Roche) to achieve liver digestion. When digestion was completed, the liver was excised and the cells disrupted. The cell suspension was filtered through nylon mesh filters (250 µm and 100 µm pore sizes) and washed twice by centrifugation for 3 min at 500 g. Cells were suspended in 50 mL of the perfusion medium. Cell viability was assessed using the trypan blue exclusion test and was always higher than 85%. Cells (8.10⁶) were incubated at 37°C in 4 ml Krebs–Henseleit bicarbonate buffer supplemented with 10 mM sodium Hepes, pH 7.4, in an O₂/CO₂ atmosphere (19:1). The cells
were incubated for 60 min in the absence or presence of gluconeogenic precursors (3 mM lactate/0.3 mM pyruvate, 3 mM alanine and 3 mM glycerol) (Sigma Chemical). Each measurement was performed in duplicate. At the end of incubation, the cells were rapidly removed from the incubation medium by spinning in a micro centrifuge (1s), frozen in liquid nitrogen and stored at –20°C. Glucose production was measured in the incubation medium. The rate of glucose production was determined by subtracting the amount of glucose produced in the absence of gluconeogenic precursors from the total amount of glucose produced after the addition of gluconeogenic precursors. Results were expressed per 10^6 cells.

**Primary culture of rat hepatocytes.** Hepatocytes were plated on 100-mm tissue culture dishes at a density of 7 x 10^6 cells in medium 199 (M199) with Earle's salts (Gibco/BRL, Paisley, United Kingdom) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 0.1% (wt/vol) bovine serum albumin, 2% (vol/vol) Ultroser G (Biosepra), 100 nM dexamethasone (Sigma), 1 nM insulin (Sigma), and 100 nM triiodothyronine (T3; Sigma). The cells were allowed to adhere to the culture dishes for 4-5 h and the medium was replaced with fresh M199 medium containing 5,5mM glucose and low amino acids concentration (corresponding to the portal amino acid concentrations measured in NP fasted rats) supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were maintained in this medium overnight (18-24 h) and then utilized during subsequent experiments. Primary rat hepatocytes were cultured overnight in the presence of 100 nM glucagon and then incubated for an additional 2 h with 100 nM glucagon supplemented with different concentrations of insulin (0, 10 or 100 nM) and amino acids (low or high concentrations of amino acids, corresponding to the portal amino acid concentrations measured in NP fasted and HP fed rats, respectively).

**Real-time PCR (RT-PCR).** Total RNA preparation and cDNA synthesis: 0.1 g frozen tissue was extracted using TRIzol® Reagent (Invitrogen). The amount of total RNA extracted was quantified at 260 nm. RNA integrity had been confirmed by ethidium bromide staining. 2µg
of total RNA in a final volume of 20µl was reversely transcribed using reverse transcriptase (MMLV-RT, Invitrogen). *Oligonucleotide primers:* Primers were designed using Oligo Explorer 1.1.0 software. In order to amplify cDNA but not genomic DNA the sense and antisense primers of each target gene were placed in two different exons of the gene. The sequences of PCR primers used were: 5’-GTATGGATTCCGGTGCTT-3’, and 5’-TGGAGTCTTGTCAGGCATT-3’ for G6PC1, 5’-TGAGATTGGTCAGGCAG-3’ and 5’-TCTAGCTGGTTATCCTTG-3’ for G6PC3, 5’-GAGACCACAGGATGAGGAA-3’ and 5’-ATGACCTTGCCCTTATGCT-3’ for PEPCK, 5’-GGGAGCCTGAGAAACGGC-3’ and 5’-GGGTCGGGAGTGGTAAATT-3’ for 18S.

*Quantification by real-time PCR:* Real-time PCR was carried out using the qPCR Core kit for SYBR® Green I No ROX Kit (Eurogentec SA, Belgium) with a MyiQ Real-Time PCR Detection System (Bio-Rad Laboratories Inc, USA) and SYBR GreenI (DNA binding dye) for the RNA extracted from frozen tissues. RNA extracted from primary hepatocyte cultures was analyzed by ABI 7300 (Applied Biosystems) using the power syber green PCR master mix (applied biosystems). Each PCR reaction was performed in a final volume of 20 µl, containing 5 µl of the RT reaction medium (diluted 10-fold). All PCR reactions were performed as follows: denaturation at 95°C for 10 min, 40 amplification cycles with each cycle, consisted of 15s at 95°C following by 1 min at 60°C. For each run, a melting curve was subsequently performed to detect any contamination resulting from residual genomic DNA amplification (using controls 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. Ribosomal 18 S 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^(-ΔΔC_T) formula where 2 represents the optimum efficiency (E) of PCR which is E = 2,
and \( \Delta Ct = Ct \text{ target gene} - \Delta Ct \text{ 18S} \) (\( \Delta Ct \text{ 18S} = Ct \text{ 18S calibrator} - Ct \text{ 18S sample} \)). PCR efficiency was determined in each plate using a serial dilution of reverse transcribed RNA. Data are presented as means ± SE of \( 10^{10} \times 2^{-\Delta Ct} \).

**Analytical procedures.** Liver glycogen contents were determined according to the method described by Bergmeyer (4). Glucose concentrations were measured by enzymatic spectrophotometric assay using the Glucose oxidase/Peroxidase (Sigma) enzymatic method. Serum insulin concentrations were assessed using a rat insulin ELISA kit (Mercodia kit, Sweden). Serum amino acid (AA) concentrations were determined using an HPLC system (Bio-Tek Instruments, St. Quentin en Yvelines, France) combined with post-column ninhydrin derivatization, and monitored using Kromasystem 2000 software. Separation was performed on a cation exchange resin (MCIGEL CK10F, Interchim, Montluçon, France). All amino acids were detected at 540 nm, except for proline (440 nm). \( \gamma \)-aminobutyric and \( \alpha \)-amino adipic acids were added to the aliquot to act as an internal standard, as previously described (9).

**Statistics.** The data are shown as mean values and standard error (± SE). Differences between groups were tested by two-way ANOVA using the general linear model procedure of SAS (version 6.11: SAS, Cary, NC). Statistical significance was set at \( P<0.05 \).

**Results**

**Plasma insulin and glucose, liver glycogen content, and glucose production**

Plasma insulin and glucose were determined in NP and HP rats in both the fasted and fed states (table 1). In the fed state, plasma glucose was slightly but significantly lower (\( P<0.05 \)) in HP rats compared to NP rats. The same trend was observed for plasma insulin, but the difference was not significant. The liver glycogen content was significantly higher in the fed
state than in the fasted state, whatever the diet (table 2). In the fed state, glycogen stores were similar in HP and NP rats. Moreover, glucose production by isolated hepatocytes from gluconeogenic substrates reached high levels in fasted rats but was markedly reduced in fed rats, without any difference between NP and HP rats (table 2).

**PEPCK and G6Pase gene expression in the liver**

To further investigate hepatic gluconeogenesis, the gene expression of the two key hepatic enzymes involved in gluconeogenesis, i.e. PEPCK and the catalytic subunit of G6Pase, was examined in the fasted and fed states in both NP and HP rats (figure 1). In NP rats, PEPCK gene expression was repressed in the fed state and strongly induced after overnight fasting (P<0.05) (figure 1A). By contrast, PEPCK gene expression in HP rats was induced at the same level in both the fasted and fed states. For the catalytic subunit of G6Pase (G6PC), expression of the two isoforms, i.e. G6PC1 and G6PC3, was determined (figures 1B and 1C). Firstly, the pattern of G6PC gene expression in the liver showed that G6PC1 was the principal subunit expressed in the liver. Secondly, in both NP and HP rats, G6PC1 gene expression was induced after overnight fasting and repressed under fed conditions.

**PEPCK and G6Pase gene expression in the kidney and intestine**

In the kidney, the abundance of PEPCK mRNA (figure 2A) was similar to that observed in the liver and significantly increased in HP fasted rats when compared with NP rats. G6PC3, the ubiquitous isoform of G6Pase, was ten times more strongly expressed in the kidney than G6PC1 (figures 2B and 2C). In the small intestine, PEPCK mRNA abundance did not differ between NP and HP adapted rats, in both the fasted and fed states. A slight increase was observed in the fed state compared to the fasted state, but was only significant in HP adapted rats (Figure 2D). However, regardless of the nutritional conditions, PEPCK gene expression
in the intestine remained very low, amounting only to 0.05% to 1% of that measured in the liver. As for PEPCK, the mRNA abundance of G6PC1 was very low and represented only 0.3% to 6.5% of that expressed in the liver. The results also indicated that G6PC3 was the main isoform of G6Pase expressed in the small intestine; mRNA abundance was 8 to 19 times higher than that of G6PC1. G6PC1 and G6PC3 were both induced in the fed state, whatever the protein content of the diet (figures 2E and 2F).

**Influence of amino acids and insulin on glucagon-induced PEPCK and G6PC1 gene expression in hepatocytes**

As reported in table 3, portal blood amino acid concentrations were significantly increased in HP rats compared to NP rats in the fed state (P<0.05), due to increases in indispensable amino acids (IAA), branched chain amino acids (BCAA) and some dispensable amino acids (DAA), i.e. proline, taurine and tyrosine. However, the pattern of gluconeogenic amino acid (GAA) variations depended upon the amino acid considered. Portal concentrations of alanine were enhanced in the fed state whatever the diet (P<0.0001), aspartate/asparagine increased in HP fed rats (P<0.005), and glycine and serine were more strongly influenced by the diet (P<0.0001 and P<0.05, respectively) whereas no variations were observed for glutamate/glutamine. In this context, the respective influences of amino acids and insulin on glucagon-induced PEPCK and G6PC1 gene expression were determined in vitro in a primary culture of rat hepatocytes (figure 3). The results showed that high but physiologically relevant concentrations of amino acids (corresponding to the portal amino acid levels of HP fed rats) significantly inhibited glucagon-induced G6PC1 (figure 3B) (p<0.0005), i.e. the last step of the glucagon-dependent induction of gluconeogenesis, but not PEPCK (Figure 3A) gene expression. These results also showed that the expression of G6PC1 was more responsive than that of PEPCK to insulin inhibition.
Discussion

The results of this study provide evidence for an indirect liver glyconeogenic pathway in the fed state in rats adapted to a HP diet. This pathway could participate in coping with amino acid excess in high protein fed rats. In HP rats, the fed state was characterized by high hepatic glycogen levels, equivalent to those found in NP fed rats, in agreement with the findings of other studies (3,45,52). Moreover, we found that glucose release from isolated hepatocytes is very low and is not different between NP and HP fed rats. There was a concomitant induction of PEPCK whereas no changes were observed for G6PC1 gene expression, which catalyzes the first step of cytosolic gluconeogenesis and the production of glucose from G6P, respectively. As a result, the newly synthesized G6P derived from gluconeogenetic precursors could be directed toward glycogen synthesis rather than being converted to glucose and subsequently released from hepatocytes.

In the present study, HP fed rats exhibited a slight decrease in plasma glucose, no change in plasma insulin and significantly increased levels of portal blood amino acids. Previous studies had reported a postprandial secretion of both insulin and glucagon in rats fed a HP diet (3,18). In that context, the respective influence of amino acids and insulin on glucagon-induced PEPCK and G6PC1 gene expression was investigated in vitro in primary cultures of isolated hepatocytes with constant concentration of glucose (5.5 mM). The present study showed that a portal blood amino acid excess was able to counteract the glucagon-induced expression of G6Pase but not PEPCK in the liver. These observations provide evidence for a role of amino acids in controlling the gene expression of G6C1 in the liver, and agree with the hypothesis that G6Pase suppression is sufficient to “push” G6P into glycogen synthesis (7,43). This
regulatory effect of amino acids on G6PC1 gene expression constitutes molecular evidence for possible orientation of the gluconeogenesis flux toward liver glycogen synthesis. An additional mechanism could also involve the activation of glycogen synthase (GS), which might also “pull” G6P into glycogen (27). Indeed, G6P not only constitutes the crossroads of several metabolic pathways, but also plays a key role as a signal molecule in regulating hepatic glucose and glycogen metabolism. An increase in G6P levels induces the activation of GS through cellular redistribution of the enzyme and activation of the phosphatase 1 proteins (PP1) that mediate GS dephosphorylation (19). Moreover, exogenous glucose and gluconeogenesis are equally effective in increasing intracellular G6P (33) and activating GS (23). For instance, it has been reported that G6P levels were increased in a proline perfused liver (7) and following the administration of casein hydrolysate to rats (20). There is also some evidence of a direct effect of amino acids on GS activity, probably through cell swelling (47). Furthermore, amino acids stimulate p70(s6k) and transiently inhibit glycogen synthase kinase 3 (GSK3), which in turn stimulates GS (1). According to the present and other studies, amino acids can thus regulate glycogen synthesis indirectly via G6P, and directly by inactivating GSK3 and activating PP2A.

The liver appeared to be the main organ involved in regulating glucose homeostasis under high protein feeding in the adult rat, whereas the kidney and intestine were only slightly or not involved in glucose production. Because G6PC1 gene expression in the kidney was not affected by either overnight fasting or HP feeding, renal gluconeogenesis did not seem to be involved in glucose homeostasis under our experimental conditions. In line with these results, other studies also showed that PEPCK and G6Pase expression was not induced in the kidney during short term fasting (13). However, the significant increase in renal PEPCK expression in HP fasted rats was consistent with the role of the kidney in the whole body acid–base
balance. Indeed, when dietary amino acid supplies increase, the renal catabolism of plasma glutamine produced by the muscles generates ammonium ions which are partially excreted in urine. Renal PEPCK transcription is stimulated in the event of metabolic acidosis (17) and is involved in glutamine extraction (54). The conversion of glutamine to serine generates bicarbonate ions (28) that neutralize any cation excess. The small intestine has also been suggested as a gluconeogenic organ after prolonged fasting or under HP feeding in the rat (41,42). However, in the present study, expression of the genes encoding the key gluconeogenic enzymes PEPCK and G6PC1 remained at very low levels in the intestinal mucosa when compared with the liver and kidney, whatever the nutritional conditions. In line with our results, other studies have also demonstrated very low or even undetectable levels of G6PC1 mRNA in the small intestine of adult rats (24,36). Moreover, both our results and those of others (10) showed that the principal G6PC isoform expressed in the small intestine in adult rats was the ubiquitous (G6PC3) isoform. Interestingly, we observed that the expression of intestinal G6PC1 and G6PC3 was stimulated by feeding, regardless of the diet. These results are consistent with a possible role of intestinal G6Pase in transepithelial transport of luminal glucose (14,25). In accordance with the view that the intestine was unlikely to produce glucose, and in line with the conclusions reached by Watford (55), the gluconeogenic capacity of this organ was recently refuted, based on the lack of glucose production by small intestine and as observed from in vivo and in vitro tracer studies (39). As for observations concerning the kidney, the stimulation of intestinal PEPCK expression with high protein feeding could have been related to extensive amino acid interconversion in this tissue and the production of bicarbonate ions to balance the cation excess (28).

This study questions the metabolic significance of the liver glyconeogenic pathway that confers the capacity to convert gluconeogenic precursors to glycogen in the fed state under
high protein feeding. Knowing that the control of G6Pase activity by nutrients has a profound impact on glucose homeostasis, we postulate for an adaptative adjustment between G6Pase activity and glycogen metabolism in rats fed a high protein diet. This non-oxidative glyconeogenic disposal pathway of gluconeogenic substrates copes with amino acid excess and participates in adjusting both amino acid and glucose homeostasis. This pathway may be involved in improving glucose disposal and reducing fat deposition, as previously observed in rats fed a high protein diet (6,34,46). Further investigations are however required to verify this hypothesis and to understand the regulatory role of amino acids in this process.
References


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Table 1. Plasma glucose and insulin in fasted (overnight fast) or fed (90 min after the beginning of the meal) rats (n= 4 to 6 per group) adapted for 15 days to a NP or HP diet. Statistical differences for Diet (D), Conditions (C) and interaction between the two factors (DxC) are reported (Two-way-ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>Insulin (pmol/L)</th>
<th>Glucose (mmol/L)</th>
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</thead>
<tbody>
<tr>
<td>NP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasted</td>
<td>170.9 ± 42.9a</td>
<td>7.4 ± 0.6a</td>
</tr>
<tr>
<td>Fed</td>
<td>595.3 ± 114.4b</td>
<td>7.1 ± 0.9a</td>
</tr>
<tr>
<td>HP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasted</td>
<td>140.1 ± 43.5a</td>
<td>6.2 ± 0.7ab</td>
</tr>
<tr>
<td>Fed</td>
<td>364.1 ± 47.6ab</td>
<td>5.9 ± 0.7b</td>
</tr>
</tbody>
</table>

Statistical effect (P)
- D NS <0.05
- C <0.001 NS
- D x C NS NS

The results are expressed as means ± SE.

Within each column, values with different letters are statistically different (P < 0.05).
Table 2. Liver glycogen content and glucose production by isolated hepatocytes in fasted (overnight fast) (n=5) or fed (90 min after the beginning of the meal) (n=5) rats adapted for 15 days to a NP or HP diet. Glucose production by isolated hepatocytes was measured after incubation for 60 min in the absence or the presence of 3 mM alanine, 3 mM lactate/0.3 mM pyruvate and 3 mM glycerol. The amount of glucose produced from gluconeogenic precursors is determined by subtracting the glucose production in the absence of gluconeogenic precursors. Statistical differences for Diet (D), Conditions (C) and interaction between the two factors (DxC) are reported (Two-way-ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>Glycogen (mg/g)</th>
<th>Glucose production (nmol/106 hepatocytes/hour)</th>
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<tr>
<td></td>
<td>Fasted</td>
<td>Fed</td>
</tr>
<tr>
<td>NP</td>
<td>0.86 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.91 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>1809.9±72.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>490.3±29.7&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>HP</td>
<td>4.32 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.41 ± 4.6&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>1869.7±160.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>534.5±64.6&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>NS</td>
<td>NS</td>
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<tr>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
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<tr>
<td>D x C</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

The results are expressed as means ± SE.

In each column values with different letter are statistically different (P < 0.05).
Table 3. Portal blood amino acid concentrations in fasted (overnight fast) or fed (90 min after the beginning of the meal) rats (n= 4 to 6 per group) adapted for 15 days to NP or HP diet. Statistical differences for Diet (D), Conditions (C) and interaction between the two factors (DxC) are reported (Two-way-ANOVA).

<table>
<thead>
<tr>
<th>Amino acids(µmol/l)</th>
<th>NP</th>
<th>HP</th>
<th>Statistical effect</th>
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<tr>
<td></td>
<td>Fasted</td>
<td>Fed</td>
<td>Fasted</td>
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<tr>
<td>Indispensable</td>
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<tr>
<td>Histidine</td>
<td>27 ± 5a</td>
<td>39 ± 3ab</td>
<td>36 ± 1a</td>
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<td>Isoleucine</td>
<td>39 ± 3a</td>
<td>37 ± 5a</td>
<td>57 ± 6a</td>
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<td>Leucine</td>
<td>73 ± 6a</td>
<td>68 ± 10a</td>
<td>105 ± 8a</td>
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<td>Lysine</td>
<td>269 ± 31a</td>
<td>235 ± 18a</td>
<td>296 ± 6a</td>
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<td>Methionine</td>
<td>26 ± 3a</td>
<td>28 ± 5a</td>
<td>29 ± 1a</td>
</tr>
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<td>Phenylalanine</td>
<td>38 ± 4a</td>
<td>39 ± 7a</td>
<td>49 ± 3ab</td>
</tr>
<tr>
<td>Threonine</td>
<td>163 ± 12a</td>
<td>163 ± 9a</td>
<td>107 ± 4a</td>
</tr>
<tr>
<td>Valine</td>
<td>91 ± 7a</td>
<td>98 ± 8a</td>
<td>127 ± 9a</td>
</tr>
<tr>
<td>Dispensable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>262 ± 17a</td>
<td>415 ± 12b</td>
<td>237 ± 11a</td>
</tr>
<tr>
<td>Aspartate/Asparagine</td>
<td>27 ± 4a</td>
<td>33 ± 2a</td>
<td>31 ± 2a</td>
</tr>
<tr>
<td>Arginine</td>
<td>52 ± 8</td>
<td>47 ± 11</td>
<td>68 ± 9</td>
</tr>
<tr>
<td>Citruline</td>
<td>53 ± 3</td>
<td>54 ± 8</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>Glutamate/Glutamine</td>
<td>286 ± 22</td>
<td>280 ± 16</td>
<td>264 ± 10</td>
</tr>
<tr>
<td>Glycine</td>
<td>224 ± 17a</td>
<td>179 ± 25b</td>
<td>129 ± 6a</td>
</tr>
<tr>
<td>Ornithine</td>
<td>22 ± 1</td>
<td>33 ± 12</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Proline</td>
<td>100 ± 9a</td>
<td>155 ± 29a</td>
<td>94 ± 4b</td>
</tr>
<tr>
<td>Sérine</td>
<td>179 ± 9a</td>
<td>159 ± 10b</td>
<td>116 ± 4b</td>
</tr>
<tr>
<td>Taurine</td>
<td>45 ± 4a</td>
<td>82 ± 27ab</td>
<td>123 ± 8a</td>
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<tr>
<td>Tyrosine</td>
<td>44 ± 3a</td>
<td>52 ± 7a</td>
<td>44 ± 3a</td>
</tr>
<tr>
<td>IAA</td>
<td>726 ± 61a</td>
<td>706 ± 62a</td>
<td>805 ± 31a</td>
</tr>
<tr>
<td>DAA</td>
<td>1285 ± 65a</td>
<td>1488 ± 101a</td>
<td>1174 ± 14a</td>
</tr>
<tr>
<td>BCAA</td>
<td>203 ± 16b</td>
<td>204 ± 22a</td>
<td>289 ± 23a</td>
</tr>
<tr>
<td>GAA</td>
<td>1142 ± 66ab</td>
<td>1228 ± 62a</td>
<td>884 ± 8a</td>
</tr>
<tr>
<td>TotalAA</td>
<td>2011 ± 125a</td>
<td>2194 ± 154a</td>
<td>1979 ± 33a</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE. In each column, values with different letters are statistically different (P < 0.05). IAA (Indispensable amino acids), BCAA ( Branched chain amino acids), GAA (Gluconeogenic amino acids), DAA (Dispensable amino acids)
Figure Legends

Figure 1. Changes in liver mRNA encoding PEPCK (A), G6PC1 (B) and G6PC3 (C) in rats fed an NP or HP diet, in the fasted state (overnight fast) or in the fed state (90 min after the beginning of the meal). Total RNA was extracted from frozen tissues and analyzed by real-time PCR, as described in the Methods. Ribosomal 18 S RNA was used as the internal control. The results are expressed as means ± SE for five different animals. The level of statistical significance was set at $P < 0.05$ and was reported in the graphs using different letters. Global statistical effects concerning diet (D), Conditions (C) and the interaction of the two factors (DxC) are shown at the top of each graph (Two-way-ANOVA).

Figure 2. Tissue-specific gene expression of PEPCK (A, D) G6PC1 (B, E) and G6PC3 (C, F) in the kidney and small intestine of rats fed an NP or HP diet, in the fasted state (overnight fast) or in the fed state (90 min after the beginning of the meal). Total RNA were extracted from the kidney (A, B, C) and the proximal part of the small intestine (D, E, F) and analyzed by RT-PCR. Ribosomal 18 S RNA was used as the internal control. The results are expressed as means ± SE for five different animals. The level of statistical significance was set at $P < 0.05$ and was reported in the graphs using different letters. Global statistical differences concerning diet (D), Conditions (C) and the interaction of the two factors (DxC) are shown at the top of each graph (Two-way-ANOVA).

Figure 3. Effects of amino acids on PEPCK (A) and G6PC1 (B) gene expression induced by glucagon in primary cultures of hepatocytes. Ribosomal 18 S RNA was used as the internal control. The results are expressed as fold increase of the basal value (first black bar) and are representative of two independent experiments. The level of statistical significance was set at
$P < 0.05$ and was reported in the graphs using different letters. Global statistical effects concerning amino acids (AA), insulin and the interaction of the two factors (AA x insulin) are shown at the top of each graph (Two-way-ANOVA).
Fig. 1

A

mRNA PEPCK/18S

D P<0.01, C P<0.005, DxC ns

B

mRNA G6PC1/18S

D ns, C P<0.005, DxC ns

C

mRNA G6PC3/18S

D ns, C ns, DxC P<0.005

NP

Fasted

Fed

HP

Fasted

Fed

ab

ab
Fig. 3

A

PEPCK relative expression

AA ns, insulin P<0.001, AAxinsulin ns

B

G6PC1 relative expression

AA P<0.0001, insulin P<0.0001, AAxinsulin P<0.0001

Insulin Glucagon

Low amino acid High amino acid