**High dietary protein decreases fat deposition induced by high fat and high sucrose diet in rat**

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Abbreviations: High Protein (HP), Fatty Acid (FA), Triglyceride (TG), High Fat (HF), Protein (P), carbohydrates (C), Fat (F), Normo Protein (NP), High Sucrose (HS), Adipose Tissue (AT), Very-Low-Density Lipoprotein (VLDL), Body weight (BW), Oral glucose tolerance test (OGTT), Subcutaneous Adipose Tissue (SCAT), Retroperitoneal Adipose Tissue (RAT), Epididymal Adipose Tissue (EAT), and Mesenteric Adipose Tissue (MAT), White adipose Tissue (WAT), β–hydroxybutyrate (β-HB), Free Fatty Acids (FFA), Area Under the Curve (AUC)

**Abstract**

High protein diets are known to reduce adiposity in context of high carbohydrate and western diet. However few studies have investigated the specific high protein effect on lipogenesis induced by high sucrose diet or fat deposition induced by high fat feeding. We aimed to determine the effects of a high-protein intake in the development of fat deposition and partitioning in response to high-fat and/or high sucrose feeding. Thirty adult male Wistar rats were assigned to one of six dietary regimen with low and high protein, sucrose and fat contents for 5 weeks. Body weight and food intake were measured weekly. Oral glucose tolerance tests and meal tolerance tests were performed after 4 and 5 weeks of regimen respectively. At the end of the study, the rats were killed two hours after ingestion of a calibrated meal. Blood, tissues and organs were collected for analysis of circulating metabolites and hormones, body composition and mRNA expression in liver and adipose tissues. . No changes were observed in cumulative energy intake and body weight gain after 5 weeks of dietary treatment. However, high protein diets reduced by 20% the adiposity gain induced by high-sucrose and high-fat high-sucrose diets HS-HF. Gene expression and transcriptomic analysis suggested that high-protein intake reduced liver capacity for lipogenesis by reducing mRNA expression of Fasn, Acaca, Acacb and Srebf-1c. Moreover, ketogenesis, as indicated by plasma β-hydroxybutyrate (β-HB) levels, was higher in high-fat high-sucrose fed mice that were also fed high protein levels HS-HF. Taken together these results suggest that high-protein diets may reduce adiposity by inhibiting lipogenesis and stimulating ketogenesis in the liver.

**Introduction**

Increasing dietary protein reduces lipid accumulation in liver [30](#_ENREF_30); [6](#_ENREF_6); [13](#_ENREF_13); [23](#_ENREF_23) and in adipose tissue (AT) [6](#_ENREF_6); [24](#_ENREF_24). These effects have been attributed to a reduced caloric intake due to the satiating effect of proteins [11](#_ENREF_11); [1](#_ENREF_1) but it was also observed, in pair-fed rats [2](#_ENREF_2) and in *ad libitum* fed mice [30](#_ENREF_30), that a reduced fat mass can be observed without differences in caloric intake. Accordingly, the decrease in adiposity was also attributed to a specific lowering effect of dietary protein on both *de novo* fatty acid (FA) synthesis and direct dietary fatty acid accumulation as triglycerides (TG) in liver and adipose tissue. Results from different studies showed that increasing protein at the expense of carbohydrate reduces expression of Fatty Acid Synthase (Fasn) in the rat and of acetyl CoA carboxylase (Acc) in AT in rats and pigs [28](#_ENREF_28); [3](#_ENREF_3); [2](#_ENREF_2); [26](#_ENREF_26). Moreover, increasing the protein content of the diet in rats and mice fed high-fat and high-sucrose diets reduces body weight and adiposity and decreases hepatic TG accumulation by enhancing liver lipid secretion into very-low density lipoprotein (VLDL) particles [21](#_ENREF_21); [30](#_ENREF_30). In this context, the aim of the present study was to investigate the effect of, increasing the protein content of the diet on the deposition of fat originating from diet or lipogenesis. For this purpose this study evaluated in a rats the effect of increasing protein intake in rats fed a high sucrose (HS) or a high-sucrose high-fat (HS-HF) diet on food intake, lipid accumulation, glucose tolerance and gene expression in the liver and adipose tissue and signaling in the hypothalamus. In order to characterize the mechanisms involved in fat deposition and partitioning, these effects were investigated during the development of fat deposition.

**Experimental methods**

*Experimental design and diets*

The protocol was approved by the French ethical committee (avis saisine Comethea12-020). Thirty adult male Wistar rats (Harlan, Ganat, France) initially weighing 300-350g were housed in individual stainless steel cages in a temperature (23 ± 1°C) and humidity (55 ± 3 %) controlled room with an artificial 12:12 h light-dark reverse cycle (light off at 10:00). Rats were adapted to the laboratory conditions and the diet protocol (with normoprotein diet (NP)) for the first 10 days. Rats were accustomed to receiving their food according to a pattern that consisted of a calibrated meal of 87-88kJ between 09:00 and 10:00 and free access to food between 11:00 and 18:00. This pattern trained the animals to eat a standard meal within one hour, 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 meal test and at the end of the experiment, the day of euthanasia. The animals were then weight-matched and assigned (n=5 per group) to one of six dietary regimens: Two control (C) regimens with either a normal protein (NP) or high protein (HP) content (NP-C, HP-C), two high sucrose (HS) regimens with either a NP or HP content (NP-HS, HP-HS) and two high-fat high-sucrose (HS-HF) regimens with either a NP or HP content (NP-HS-HF, HP-HS-HF) (table 1). The animals were fed with their respective diet for 5 weeks with free access to water.

*Measure of food intake and body weight*

The diet was moistened to minimize spillage and the food containers were refilled daily with fresh food. Food intake was measured by the difference in food cup weight before and after the feeding period (11:00 – 18:00). Five days before exposure to respective diets, food intake was measured to obtain baseline values. Food intake was measured 5 days per week from one week before then during 3 weeks after exposure to the respective diets. Body weight (BW) was measured 5 days per week in the morning before giving the calibrated meal during the 5 weeks of the experiment.

*Oral glucose tolerance test (OGTT)*

The oral glucose tolerance test was performed on week 4th. The day of the test, after an overnight fast, rats received orally 100µl/100g of BW of glucose solution at a concentration of 1 g/L. Blood samples were collected from the tail vein before the test and 15, 30, 60, 90 and 120 min after administration of glucose solution. Blood glucose and plasma insulin were measured as described in analytical procedures and determination. [17](#_ENREF_17)

*Meal tolerance test*

Meal tolerance tests (MTT) were conducted between 09:00and 13:00 on the day of euthanasia. Rats were fasted overnight and a calibrated meal of their respective diet was given immediately after blood sampling. Blood glucose, plasma biochemical and hormonal changes in response to the meal were monitored for 2 hours after the meal at 30, 60 and 120 min. Data are presented as as means ± SD for fasting values and area under the curve for kinetics.

*Tissue sampling and body composition analysis*

At the end of the experiment, rats were euthanized two hours after ingestion of their calibrated meal. Rats were deeply anesthetized with an overdose of pentobarbital (50mg/kg) and decapitated. The main tissues and organs (liver, spleen, kidneys, brain, heart, skin, subcutaneous (SCAT), retroperitoneal (RAT), epididymal (EAT), and mesenteric (MAT) adipose tissues) were collected, blotted dry and weighed to the nearest 0.01g. Liver, hypothalamus and TAE aliquot were snap frozen in liquid nitrogen and stored at -80°C until RNA extraction.

### *Analytical procedures and determination*

The blood samples were collected on EDTA, immediately centrifuged, and the plasma aliquots for biochemical or hormonal analysis were stored at −70°C until assayed. All plasma biochemical analyses (albumin, Lactate, cholesterol (Cs), HDL cholesterol (HDL), triglycerides (TG), free fatty acid (FFA), glycerol and β-hydroxybutyrate (β-HB) were performed on an Olympus AU 400 robot specifically calibrated for rats’ assays (Centre d’ Explorations Fonctionnelles Intégrées (CEFI), Bichat, France). Plasma insulin was detected using enzyme linked immunoassay (Mercodia Rat Insulin). Plasma glucagon, leptin and ghrelin concentrations were assessed by Luminex (Bio-Plex Pro TM rat standard -BIO-RAD). Blood glucose was determined immediately after sampling with a blood glucose meter (Accu Check Go). Liver TG levels were determined using an enzymatic assay (TG –TIGS-, Randox kit).

*RNA extraction and Real-time RT-PCR analysis*

Total RNA was extracted from liver, adipose tissue and hypothalamus tissue with TRIzol reagent (Invitrogen, Breda, the Netherlands). Concentrations of RNA samples were determined on a NanoDrop ND-1000 UV-Vis spectrophotometer (Isogen, Maarssen, the Netherlands). RNA integrity was confirmedby ethidium bromide staining. 0.4µg of total RNA in a final volume of 10µl was reversely transcribed using High Capacity cDNA Archive Kit Protocol (Applied Biosystems). mRNA concentration was determined by Real Time on the ABI 7300 (Applied Biosystems) using Power Sybr Green PCR master mix (Applied Biosystems) as previously described [28](#_ENREF_28). For each run, the efficiency and the possible contaminations were checked. The primer sequences of targets genes were detailed in Supplemental Table 1. Gene expression was calculated as: 2-ΔΔCT relatively to NP-C group and 18S RNA was used as the housekeeping gene. Data are presented as means ± SD.

*Rat liver transcriptome: Microarray analysis*

After extraction, total RNA was purified using SV total RNA isolation system (Promega, Leiden, the Netherlands). Total purified liver RNA of 5 rats per group was subsequently pooled at a concentration of 1500 ng/rat. Quality of the pooled RNA was assessed on Agilent 2100 Bioanalyzer with RNA 6000 Nano Chips using the Eukaryote Total RNA Nano assay (Agilent Technologies, Amsterdam, the Netherlands). RNA quality was considered as sufficient for array hybridisation if RNA integrity number was ≥ 7.0. The Ambion WT Expression kit (Life Technologies, Bleiswijk, The Netherlands) in conjunction with the Affymetrix GeneChip WT Terminal Labelling kit (Affymetrix, Santa Clara, CA) was used for the preparation of labelled cDNA from 100ng of total RNA without rRNA reduction. RNA (100 ng) was labelled with the Whole Transcript Sense Target Assay (Affymetrix, Santa Clara, CA). Hybridisation, washing and scanning of AffymetrixRat Gene 1.0 ST array was carried out according to standard Affymetrix protocols. Quality control and normalisation were performed using Bioconductor packages integrated in an on-line pipeline [14](#_ENREF_14). Probe sets were redefined according to Dai et al.(2005) [5](#_ENREF_5). Normalised expression estimates of probe sets were computed by the robust multiarray (RMA) analysis algorithm available in the Bioconductor library AffyPLM using default settings [10](#_ENREF_10). Array data have been submitted to the Gene Expression Omnibus and is available under accession number GSE47570. To detect an an effect of the increased protein content of the diets, fold changes of signal intensities were calculated as signal log ratios (HP-NP) for each of the background diets. Fold changes of > 0.25 or < -0.25 were considered as biological relevant.

*Statistics*

Data are expressed as means ± SD in table or SEM in figures for kinetics. The effect of the amount of protein in the diet (normal vs high) and the interaction with dietary lipid and sucrose levels (low vs high) were analyzed by ANOVA using the GLM procedure of SAS (version 9.1 SAS, Cary, NC). Kinetics were analyzed using SAS mixed models for repeated measurements. The effects of dietary fat (F) or sucrose (S) levels and their interaction or their interaction with protein and the effect of Time (t) are indicated when significant. *P* <0.05 was considered significant and 0.05<*P*<0.1 was considered to indicate a tendency. *Post-hoc* Tukey tests for multiple comparisons were performed to make pair-wise comparisons.

**Results**

*Effect of HP intake on food intake, body weight, body composition*

No difference in cumulative food intake over 5 weeks of diet and no differences in final BW were observed between groups (data not shown). However, the protein content of the diets significantly affected the evolution ofrats’ BW since BW was lower in groups fed HP diets between the second and third weeks of regimen (figure 1). Moreover, the effect of the protein depended on the sucrose content of the diet since the BW level was greater in the groups fed a HS diet (P x S x days *P*=0.007) than control group.

Analysis of body composition showed no effect on liver weight (Figure 2A) whereas the weight of all dissected fat pads was significantly reduced in rats fed HP diets (Figure 2 B,C). These effects were also observed for the ratio of AT/weight on total body weight (WAT *P*=0.01, MAT *P*= 0.0005; SCAT *P*=0.02, a tendency for EAT *P*=0.06 and RAT *P*=0.06).

*Effect of HP intake on plasma Ghrelin and leptin*

In the fasted state, the HP diets did not affect plasma ghrelin but reduced plasma leptin in HS-HF fed rats (Table 2). In response to the meal test, HP diets reduced plasma leptin and ghrelin under HS and HF conditions (Figure 3A and 3B).

*Effect of HP intake on glucose homeostasis*

In the fasted state, no effect of the composition of the diet was observed on glycaemia, insulinemia and glucagonemia (Table 2). After the meal, glycaemia blood glucose was higher in HS groups than in C groups (sucrose effect *P*= 0.02 for glycaemia kinetic) (Figure 3A) without any effect on plasma insulin and glucagon (Figure 3B and 3C). Nevertheless, the insulin to glucagon ratio (IG) was 70% higher (*P*=0.01 for sucrose effect) in NP-HS than in NP-C group (0.07±0.025 and 0.114±0.042, in NP-C and NP-HS group, respectively). Blood glucose tended to be lower in HP than in NP groups (*P*=0.08 for kinetics and *P*=0.054 for AUC) (Figure 4A and 4D) whereas the protein content of the diet had no effect on plasma insulin (Figure 4B and 4E). In contrast to plasma insulin, glucagon were higher in the HP groups (*P*=0.02 for kinetics). In accordance with the inhibitory effect of glucose on glucagon secretion, the difference in glucagon concentrations between HP and NP groups was more important when the sucrose content of the diet was lower (Figure 4C and 4F).

In response to the oral glucose tolerance test (OGTT) no major effect of dietary macronutrient composition was observed on glucose and insulin kinetics, despite different basal values and a lower increase in blood glucose and plasma insulin in HP-HS-HF groups than the NP-HS-HF group (supplemental Figure 1). No differences were observed for the area under the curve.

*Effect of HP intake on plasma lipids and liver TG*

The fat or sucrose content of the diets had no major effect of plasma biochemical parameters in the fasted state (Table 2). However, after the meal, plasma TG (+85%), FFA (+69%) and β-HB (+36%) were significantly higher in the NP-HS-HF group than in the NP-C group (Table 3). These results are in accordance with the increase in dietary lipids intake of the NP-HS-HF group. Plasma lactate was significantly lower in the HS-HF groups than in C and HS ones (*P*=0.02), which was probably due to the lower carbohydrate content of the HS-HF diet.

In the fasted state, the increase of protein content of diet had also no major effect on most of plasma biochemical parameters studied except for TG and HDL (Table 2). Plasma TG was lower in HP groups (*P*<0.001) whereas HDL was greater in HP-C and HP-HS-HF (P<0.05) than NP groups (Table 3). Plasma TG concentration was 37%, 25% and 47% lower in HP-C, HP-HS and HP-HS-HF respectively, than in the respective NP groups (Table 3). Plasma β-HB was greater in all HP groups with the highest value observed for HP-HS-HF (+87% in comparison with NP-HS-HF).

Liver TG content was not significantly modified by the diets but there was a tendency for a more liver TG in the HS-HF groups (*P*=0.07) and less liver TG in HP-C and HP-HS groups than in NP-C and NP-HS groups (p=0.08). In contrasts liver was similar in HP- HS-HF and NP- HS-HF groups (Table 3).

*Effect of HP intake on gene expression in liver, adipose tissue, muscle and hypothalamus (qPCR - analysis)*

To assess the changes in the control of lipogenesis, the expression of acetyl-CoA carboxylase (Acaca and Acacb), fatty acid synthase (Fasn) and sterol regulatory element binding transcription factor 1 (Srebf-1c) was measured in the liver and in epididymal adipose tissue (Table 4). In the liver, mRNA encoding Acaca and Srebf-1c were significantly lower in HP than in NP groups (Table 4). Fasn expression was reduced also less in HP than in NP groups.. In adipose tissue, Fasn mRNA (*P*=0.04) was significantly lower in rats fed the HF-HS diet than in those fed the HC diets but was not different between rats fed HP and NP diets. These results suggest that HP feeding decreased lipogenesis in both the liver and the adipose tissue. Moreover, no change on adipose tissue leptin mRNA levels was observed which suggests that the lower plasma leptin levels in HP groups would rather relate to the lower adiposity in these groups.

In the liver, there was no effect of the protein content of the diet on mRNA encoding proteins involved in lipolysis and ß-oxidation (hormone sensitive lipase (Lipe) in adipose tissue and carnitine palmitoyltransferase (Cpt1a), carnitine palmitoyltransferase 2 (Cpt2) and uncoupling protein 2 (Ucp2) in the liver (Table 4). In parallel, expression of Pck1 (encoding PEPCK enzyme) was induced by HP diets, whatever the lipids or sucrose content of the diet.

Concerning the main hypothalamic neuropeptides controlling food intake, the mRNA coding for the anorexigenic neuromediator POMC was significantly lower in rats fed with the HP diets (Table 4) but no effect of the composition of the diets on NPY expression was observed.

*Effect of HP intake on hepatic gene expression profile (Microarray analysis)*

The influence of HP content on gene expression profile in the liver was determined by comparing each background diets (C, HS or HS-HF) with their HP counterpart.

Analysis of the expression profile of genes encoding for amino-acids transporters and enzymes which metabolise amino-acids for energy supply suggest an induction of gluconeogenesis by HP feeding. For instance we observed a strong up-regulation of the expression of SLC1a4, which transports glutamate as well as transporters for cationic and neutral AA under all HP conditions (Figure 5A). On the other hand, most aminotransferases showed increased expression values under all HP (Figure 5B). Mitochondrial glutaminase 2 (Gls2) expression was mostly increased by HP under the two low fat background diets (C and HS) but less under HS-HF conditions. A similar pattern was observed for serine dehydratase (Sds), proline dehydrogenase (Prodh) and methylcrotonoyl-CoA carboxylase 2 beta (Mccc2). Finally, Pck1 expression, a key regulator of gluconeogenesis, was increased in livers of HP fed rats as already detected by qPCR. Expression of glucose-6-phosphatetase (G6pc) was induced by HP under HS-HF conditions.

Genes encoding key enzymes of fatty acid synthesis were down-regulated by HP diets under all background diets (Figure 5D). Expression of genes coding for enzymes involved in the control of triglyceride synthesis was less modulated, except for mitochondrial glycerol-3-phosphate acyltransferase (Gpam) and acylglycerol-3-phosphate O-acyltransferase 1 (Agpat1) that showed an increased expression but only under HS and HS-HF. It is however more difficult to conclude on the effects of HP diets on the regulation of β-oxidation because the picture (Figure 5E) suggested either an increased translocation of fatty acids into mitochondria for oxidation (up-regulation of Acls3, Crat, Cpt1a and Cpt1b) and a lower oxidation of long chain fatty acids (down-regulation of Hadhb).

HP diet also increased expression of key genes of ketogenesis (Figure 5F) but expression of acetyl-coenzyme A acetyltransferase 1 (Acat1) involved in ketone body degradation was inhibited. In general, highest changes in gene expression by HP were mostly found in the HS-HF group, which is in agreement with increased plasma β-HB levels.

Expression of genes encoding enzymes in cholesterol metabolism was almost exclusively up-regulated by the HP diets (Figure 5G). This up-regulation was highest when rats were fed a C and HS diets. Also gene expression of enzymes catalysing bile acid synthesis was only slightly changed (Figure 5H).

Relevant pathways are summarised in Figure 5I. A complete list of genes in relevant pathways can be found in the supplemental figure 2 and 3.

**Discussion**

The objective of this work was to assess the effect of high protein diets on fat deposition induced by high sucrose and high-fat high-sucrose diets. For that purpose, rats were fed diets containing normal (C) or high amounts of sucrose (HS) or of either fat and sucrose (HS-HF), each of these diets with a normal or high protein content (NP or HP). Weight gain and adiposity did not differ for HS-HF and HS diets after 5 weeks of dietary treatment. HP diets limited adiposity gain induced by both the HS and the HS-HF diet which cannot be attributable to a decrease in food intake since no change were observed between the different diets despite a decrease in plasma ghrelin level in the HP groups. Gene expression analysis showed that HP intake reduced expression of genes involved in lipogenesis and stimulated ketone body production by liver, in particular when HP was associated with HS-HF intake. Taken together this suggests that under HP feeding the induction of ketogenesis channels the excess of AA and fat to indirect oxidation thereby lowering dietary lipid deposition and adiposity gain.

As expected, a reduction of food intake was observed during the 3 first days after the transition to HP diet [1](#_ENREF_1); [12](#_ENREF_12) but we also observed no decrease of cumulative energy intake after 5 weeks, which is in accordance with other results in mice [30](#_ENREF_30). Leptin is an anorexigenic hormone secreted by the adipose tissue in proportion to its mass and signals to the hypothalamus the energy stores status . Leptin induces opposite effects on two ARC neuronal subpopulations by inhibiting the orexigenic NPY/AgRP neurons and stimulating the activity of anorexigenic POMC neurons [4](#_ENREF_4); [22](#_ENREF_22). Thus, the decrease of POMC mRNA observed in the present experiment, two hours after ingestion of a HP meal, could be related to the decreased levels of plasma leptin we observed in HP fed rats. However, in a previous experiment, we observed that a HP diet (50% protein-enriched diet) increased POMC mRNA levels in both *ad libitum* fed and 12 h fasted rats [7](#_ENREF_7); [25](#_ENREF_25). The discrepancies could be explained by a different time of hypothalamic collection (2h after ingestion of meal given after an overnight fast versus in this study vs *ad libitum conditions* previously) and the duration of the HP diet longer in this study than in previous ones). Ghrelin is an orexigenic gut-peptides that generally stimulates food intake by targeting neural pathways in the central nervous system including the hypothalamus and in the long-term increases fat mass [29](#_ENREF_29). It has been reported that intracerebroventricular administration of ghrelin did not induced hyperphagia when rats fed a high fat diet and gained adiposity[20](#_ENREF_20). Thus, in our study, the no effect of the decrease in plasma ghrelin on food intake could be related to the adiposity. Moreover, the role of ghrelin in the increase of food intake in response to high fat diet remained to be clarified given that the ablation of ghrelin cell in transgenic mice did not decrease food intake [15](#_ENREF_15). It can therefore be concluded that the observed decrease in adiposity induced by the HP diets in this study was not due to the effect of proteins on food intake.

Body FA primarily stored in adipose tissues originate from dietary TG and FA, and from *de novo* synthesis of FA in the liver and adipose tissue. Between meals, when glucose availability decreases, stored TG are lipolyzed by LPL to release FA to cover energy requirements. Results from targeted gene expression analysis and gene expression profiling showed that a main process involved in the reduced adiposity of HP fed rats is a reduced hepatic lipogenesis (Fasn, Acaca, Acacb, Srebf-1c) from carbohydrates whereas adipose LPL expression was not affected. The signals at the origin of this lower expression of lipogenic enzymes could be related to a lower increase in post-prandial glycaemia but also to other amino acid and hormone-derived signaling pathways [28](#_ENREF_28); [3](#_ENREF_3); [2](#_ENREF_2); [26](#_ENREF_26). No major changes were observed for insulin sensitivity during OGTT. However some insulin sensitivity indices (ISIMatsuda , 1/I, G/I and QUICKI[17](#_ENREF_17)) tended to be higher in NP-HS-HF group than the other groups (data not shown) which suggests that an increase of the fat content of the diet decreases whole-body insulin sensitivity and that this increase was prevented by increasing the protein level of the diet. This result is also in line with the lower plasma TG levels observed in the HP fed rats of this study. Interestingly, we observed that lipogenesis in adipose tissue was also lower in high sucrose HP fed rats compared to NP fed animals, conditions where lipogenesis was induced, but this effect of HP feeding was not observed in HS-HF fed animals because lipogenesis is low under HF feeding. Taken together, these observations indicate that lipogenesis is mainly lowered by protein in the liver whereas in adipose tissue it is lowered by both fat and protein.

In response to protein excess, the protein pool remains unchanged because of the regulation of protein homeostasis that maintains the stability of lean mass. These regulations include amino acid oxidation and interconversions as indicated by the increased expression of several transaminases and a general up-regulation of genes encoding enzymes involved in gluconeogenesis. However, the size of the glycogen pool is limited which limits the possibility to convert amino-acids to glucose and push the conversion of ketogenic amino acids towards ketone production [8](#_ENREF_8); [27](#_ENREF_27); [28](#_ENREF_28). Accordingly we observed an increase in plasma ketone body concentration in all HP groups, more drastically when HP was associated with HF intake. This result is in favor of the role of ketogenesis in energy homeostasis maintenance in the context of HP feeding. However, there is no clear induction of key genes of ketogenesis suggesting that this metabolic process is controlled by short term processes. According to this hypothesis, the intake of a diet rich in ketogenic essential amino acids inhibits hepatic lipogenesis and avoids the development of the hepatic steatosis [19](#_ENREF_19); [18](#_ENREF_18). Moreover, HP diet (60 % in casein) increases "Branched Chain α-cetoacids " in the blood and in the organs [9](#_ENREF_9); The conversion of amino acids into ketone bodies or cetoacids could thus prevent the storage of amino acids carbon skeletons as lipids. Indeed, the stimulation of ketogenesis following a HP diet with a low or high content of fat represents a way of by-passing storage of the energy and thus prevents adiposity. Recent data of plasma metabolomic analysis of rats confirmed an increase of β-hydroxybutyrate as biomarkers of high protein intake [16](#_ENREF_16). However, there is no clear induction of key genes of ketogenesis suggesting that this metabolic process is controlled by short term post-transcriptionnal processes.

In conclusion, HP intake may reduce *de novo* synthesis of FA through a decrease in the expression of enzymes involved in lipogenesis but also through a poor utilization of AA-derived carbon skeleton in lipogenic pathways. These results suggest that the increase of protein intake reduced fat deposition through the inhibition lipogenesis in the liver and adipose tissue. In parallel, the increase in plasma β-HB concentration which suggests a stimulation of liver ketogenic pathways in which AA but also FA-derived carbon skeletons could be channeled at the expense of FA synthesis and deposition.

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**Conflict of interest:** C. Chaumontet, P. C. Even, , Schwarz J., Simonin-Foucault A., J. Piedcoq, G. Fromentin, D. Azzout-Marniche and D. Tomé have no conflicts of interest.

**Authorship:** DT and GF: designed research, CC, PE, ASF, JP and DAM: conducted research; JS: realised transcriptomique analysis; DAM and JS: analyzed data; DAM, JS, CC and DT wrote the paper; DAM and DT: had primary responsibility for final content. All authors have read and approved the manuscript submission.

**References**

1. Bensaid A, Tome D, L'Heureux-Bourdon D *et al.* (2003) A high-protein diet enhances satiety without conditioned taste aversion in the rat. *Physiol Behav* **78**, 311-320.

2. Blouet C, Mariotti F, Azzout-Marniche D *et al.* (2006) The reduced energy intake of rats fed a high-protein low-carbohydrate diet explains the lower fat deposition, but macronutrient substitution accounts for the improved glycemic control. *J Nutr* **136**, 1849-1854.

3. Brito MN, Brito NA, Brito SR *et al.* (1999) Brown adipose tissue triacylglycerol synthesis in rats adapted to a high-protein, carbohydrate-free diet. *Am J Physiol* **276**, R1003-1009.

4. Cowley MA, Smart JL, Rubinstein M *et al.* (2001) Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* **411**, 480-484.

5. Dai M, Wang P, Boyd AD *et al.* (2005) Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. *Nucleic Acids Res* **33**, e175.

6. Della-Pace ID, Rambo LM, Ribeiro LR *et al.* (2013) Triterpene 3beta, 6beta, 16beta trihidroxilup-20(29)-ene protects against excitability and oxidative damage induced by pentylenetetrazol: the role of Na(+),K(+)-ATPase activity. *Neuropharmacology* **67**, 455-464.

7. Faipoux R, Tome D, Gougis S *et al.* (2008) Proteins activate satiety-related neuronal pathways in the brainstem and hypothalamus of rats. *J Nutr* **138**, 1172-1178.

8. Fromentin C, Azzout-Marniche D, Tome D *et al.* The postprandial use of dietary amino acids as an energy substrate is delayed after the deamination process in rats adapted for 2 weeks to a high protein diet. *Amino Acids*.

9. Hutson SM & Harper AE (1981) Blood and tissue branched-chain amino and alpha-keto acid concentrations: effect of diet, starvation, and disease. *Am J Clin Nutr* **34**, 173-183.

10. Irizarry RA, Hobbs B, Collin F *et al.* (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**, 249-264.

11. Jean C, Rome S, Mathe V *et al.* (2001) Metabolic evidence for adaptation to a high protein diet in rats. *J Nutr* **131**, 91-98.

12. Journel M, Chaumontet C, Darcel N *et al.* (2012) Brain responses to high-protein diets. *Adv Nutr* **3**, 322-329.

13. Kawai HD, La M, Kang HA *et al.* (2013) Convergence of nicotine-induced and auditory-evoked neural activity activates ERK in auditory cortex. *Synapse* **67**, 455-468.

14. Lin K, Kools H, de Groot PJ *et al.* (2011) MADMAX - Management and analysis database for multiple ~omics experiments. *J Integr Bioinform* **8**, 160.

15. McFarlane MR, Brown MS, Goldstein JL *et al.* (2014) Induced ablation of ghrelin cells in adult mice does not decrease food intake, body weight, or response to high-fat diet. *Cell Metab* **20**, 54-60.

16. Mu C, Yang Y, Luo Z *et al.* (2015) Metabolomic analysis reveals distinct profiles in the plasma and urine of rats fed a high-protein diet. *Amino Acids*.

17. Muniyappa R, Lee S, Chen H *et al.* (2008) Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. *Am J Physiol Endocrinol Metab* **294**, E15-26.

18. Nishikata N, Shikata N, Kimura Y *et al.* (2011) Dietary lipid-dependent regulation of de novo lipogenesis and lipid partitioning by ketogenic essential amino acids in mice. *Nutr Diabetes* **1**, e5.

19. Noguchi Y, Nishikata N, Shikata N *et al.* Ketogenic essential amino acids modulate lipid synthetic pathways and prevent hepatic steatosis in mice. *PLoS One* **5**, e12057.

20. Perez-Tilve D, Heppner K, Kirchner H *et al.* (2011) Ghrelin-induced adiposity is independent of orexigenic effects. *Faseb J* **25**, 2814-2822.

21. Pichon L, Huneau JF, Fromentin G *et al.* (2006) A high-protein, high-fat, carbohydrate-free diet reduces energy intake, hepatic lipogenesis, and adiposity in rats. *J Nutr* **136**, 1256-1260.

22. Qiu J, Fang Y, Ronnekleiv OK *et al.* (2010) Leptin excites proopiomelanocortin neurons via activation of TRPC channels. *J Neurosci* **30**, 1560-1565.

23. Raymond F, Wang L, Moser M *et al.* (2013) Consequences of exchanging carbohydrates for proteins in the cholesterol metabolism of mice fed a high-fat diet. *PLoS One* **7**, e49058.

24. Rietman A, Schwarz J, Tome D *et al.* (2014) High dietary protein intake, reducing or eliciting insulin resistance? *Eur J Clin Nutr* **68**, 973-979.

25. Ropelle ER, Pauli JR, Fernandes MF *et al.* (2008) A central role for neuronal AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) in high-protein diet-induced weight loss. *Diabetes* **57**, 594-605.

26. Schwarz J, Tome D, Baars A *et al.* (2012) Dietary protein affects gene expression and prevents lipid accumulation in the liver in mice. *PLoS One* **7**, e47303.

27. Stepien M, Gaudichon C, Azzout-Marniche D *et al.* Postprandial nutrient partitioning but not energy expenditure is modified in growing rats during adaptation to a high-protein diet. *J Nutr* **140**, 939-945.

28. Stepien M, Gaudichon C, Fromentin G *et al.* Increasing protein at the expense of carbohydrate in the diet down-regulates glucose utilization as glucose sparing effect in rats. *PLoS One* **6**, e14664.

29. Theander-Carrillo C, Wiedmer P, Cettour-Rose P *et al.* (2006) Ghrelin action in the brain controls adipocyte metabolism. *J Clin Invest* **116**, 1983-1993.

30. Xiao F, Huang Z, Li H *et al.* (2011) Leucine deprivation increases hepatic insulin sensitivity via GCN2/mTOR/S6K1 and AMPK pathways. *Diabetes* **60**, 746-756.

*Table 1:* Composition of the diets

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | NP-C1 | HP-C1 | NP-HS | HP-HS | NP-HS-HF | HP-HS-HF |
| g.kg DM-1 |
| Protein1 | 140 | 530 | 140 | 484 | 160 | 580 |
| Sucrose  | 100.3 | 45.7 | 361.35 | 189.35 | 291.3 | 80 |
| Cornstarch | 622.4 | 287 | 361.35 | 189.35 | 391.4 | 80 |
| Soya oil | 40 | 40 | 40 | 40 | 40 | 40 |
| Palm oil | 0 | 0 | 0 | 0 | 120 | 122.82 |
| Minerals (AIN-93M)2 | 35 | 35 | 35 | 35 | 35 | 35 |
| Vitamins (AIN-93M)3 | 10 | 10 | 10 | 10 | 10 | 10 |
| Cellulose | 50 | 50 | 50 | 50 | 50 | 50 |
| Choline | 2.3 | 2.3 | 2.3 | 2.3 | 2.3 | 2.3 |
| % of energy |
| Total protein  | 14.7 | 55.6 | 14.7 | 52.4 | 14.5 | 52.4 |
| Total fat  | 9.4 | 9.4 | 9.4 | 9.4 | 32.7 | 33.1 |
| Total carbohydrate  | 75.8 | 34.9 | 75.8 | 39.8 | 52.8 | 14.4 |
| Metabolizable energy (kJ.gDM-1) | 15.95 | 15.95 | 15.95 | 15.95 | 18.46 | 18.52 |

1 Dietary treatment: C: control, HF: High Fat, HP: High protein, HS: High sucrose, NP: Normal protein

2 Total milk protein isolate, purchased from Nutrinov, Rennes, France

3,4 ICN biochemicals, Cleveland, OH

5 DM : dry matter

*Table 2:* Effect of High protein diet on plasma parameters in fasted state

|  |  |  |  |
| --- | --- | --- | --- |
|  | NP1 | HP1 | Statistical Effect 3  |
|  | C1 | HS1 | HS-HF1 | C1 | HS1 | HS-HF1 |
| Glycaemia (g.l-1) | 1.04±0.87 | 1.10±0.11 | 1.12±0.37 | 1.06±0.13 | 1.04±0.08 | 1.10±0.13 | NS |
| Glycerol (µM.l-1) | 305.9±33.2 | 315.8±90,0 | 300.0±36.3 | 266.2±40.9 | 271.8±52.2 | 295.8±60.7 | NS |
| Triglyceride (mM.l-1) | 0.94±0.18 | 1.03±0.44 | 1.08±0.32 | 0.59±0.08 | 0.64±0.13 | 0.63±0.12 | P (<0.001) |
| Albumin (g.l-1) | 39.5±2.5 | 41.2±1.7 | 40.1±1.1 | 39.8±1.6 | 39.8±1.5 | 37.7±0.9 | NS  |
| Lactate (mM.l-1) | 2.72±0.79 | 2.90±0.94 | 2.98±1.45 | 3.10±1.67 | 2.30±0.60 | 3.28±2.20 | NS  |
| Cholesterol (mM.l-1) | 1.81±0.18 | 1.86±0.27 | 1.77±0.15 | 2.04±0.49 | 1.70±0.46 | 2.10±0.46 | NS |
| HDL (mM.l-1) | 1.18±0.13 | 1.20±0.17 | 1.18±0.11 | 1.38±0.30 | 1.19±0.31 | 1.42±0.27 | P (<0.05) |
| β-Hydoxybutyrate (mM.l-1) | 0.59±0.24 | 0.55±0.21 | 0.67±0.33 | 0.66±0.26 | 0.71±0.24 | 0.69±0.20 | NS |
| NEFA (mM.l-1) | 1.08±0.38 | 0.68±0.39 | 0.83±0.47 | 0.52±0.31 | 0.97±0.28 | 0.879±0.33 | NS |
| Glucagon (ng.l-1) | 40.4±13.6 | 41.3±26 | 40.9±19.3 | 57.8±6.9 | 43.9±12.7 | 48.2±29.0 | NS |
| Insulin (µg.l-1) | 1.28±0.77 | 3.03±1.66 | 1.28±0.77 | 1.13±0.81 | 1.81±1.11 | 2.21±1.64 | NS |
| Leptin (µg.l-1) | 1.14±0.57 | 1.34±1.13 | 1.44±1.16 | 1.01±0.42 | 0.81±0.41 | 0.64±0.19 | PxF (<0.05) |
| Ghrelin (µg.l-1**)** | 1.28 ±0.46 | 0.88±0.61 | 0.49±0.32 | 0.63±0.54 | 0.45±0.17 | 0.39±0.19 | NS |

1 Dietary treatment: C: control, HF: High Fat, HP: High protein, HS: High sucrose, NP: Normal protein

2Data are expressed as mean ± SD (n=5)

3The p value is indicated when the difference is or tended to be significant. P: protein effect, F: fat effect, S: Sucrose effect, PxF interaction between NP-HS-HF and HP-HS-HF, PxC: interaction between NP-HS and HP-HS. NS indicate no significant statistical effect (>.0.05). The different letters indicate the results of pair-wise comparisons.

 *Table 3:* Effect of High protein diet on plasma parameters after meal test AUC (Area Under the Curve) and liver TG content

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | NP1 |  |  | HP1 |  | Statistical Effect 2 |
|  | C1 | HS1 | HS-HF1 |  | C1 | HS1 | HS-HF1 |
| Glycaemia (g.h.l-1) | 31.1±2.05 | 31.3±4.63 | 34.6±4.16 |  | 31.7±2.90 | 34.0±1.27 | 31.2±3.15 | P (=0.054) F (<0.02) |
| Glycerol (µM.h.l-1) | 24.2±3.1 | 26.2±9.1 | 35.5±7.5 |  | 23.9±5.1 | 23.6.±2.3 | 30.8±4.4 | NS |
| Triglyceride (mM.h.l-1) | 114.8±26.2 | 107.2±37.1 | 212.8±61.8 |  | 72.4±17.2 | 79.7±18.2 | 111.6±18.9 | P(<0.0001) F(<0.0001) |
| Albumin (g.h. l-1) | 474.1±27.9 | 493.6±15.5 | 477.0±18.9 |  | 469.0±16.6 | 475.4±13.8 | 456.4±8.4 | NS |
| Lactate (mM.h.l-1) | 406.5±78.4 | 551.7±210.9 | 378.3±76.2 |  | 371.1±131.9 | 387.6±56.8 | 346.2±105.5 | F (<0.02) |
| Cholesterol (mM.h.l-1) | 205.6±21.6 | 222.0±30.1 | 211.3±15.7 |  | 230.5±58.3 | 212.4±49.6 | 232.4±38.0 | NS |
| HDL (mM.h.l-1) | 128.4±16.8 | 146.9±14.2 | 135.6±15.5 |  | 156.2±36.8 | 145.2±29.2 | 156.5±24.5 | P (=0.055) |
| β-Hydoxybutyrate (mM.h.l-1) | 15.7±16.8 | 14.1±14.2 | 21.4±15.5 |  | 22.1±36.8 | 23.0±29.2 | 40.2±24.5 | P (<0.02),F (=0.07) |
| NEFA (mM.h.l-1) | 54.8±9.0 | 63.5±26.4 | 92.9±21.0 |  | 62.9±16.5 | 61.5±7.2 | 93.9±23.4 | F(<0.001)  |
| Liver TG (mg.g-1) | 24.1±5.4 | 21.1±7.3 | 24.2±4.3 |  | 18.1±3.4 | 18.6±2.2 | 23.7±5.8 | F (=0.07)P (=0.08) |

1 Dietary treatment: C: control, HF: High Fat, HP: High protein, HS: High sucrose, NP: Normal protein

2Data are expressed as mean ± SD (n=5)

3The p value is indicated when the difference is or tended to be significant. P: protein effect, F: fat effect, S: Sucrose effect, PxF interaction between NP-HS-HF and HP-HS-HF, PxC: interaction between NP-HS and HP-HS. NS indicate no significant statistical effect (>0.05).

*Table 4:* Effect of High protein diet on hepatic, adipose and hypothalamic mRNA encoding keys proteins involved in energy metabolism

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Relative expression** | **NP1** |  |  | **HP1** |  | Statistical Effect3 |
|  | C**1** | HS**1** | HS-HF**1** |  | C**1** | HS**1** | HS-HF**1** |
| **Liver**AcacaAcacbSrebf1cFasnCpt1aCpt2Ucp2Pck1 | 1.0±0.31.0±0.51.0±0.51.0±0.91.0±0.71.0±0.31.0±0.51.0 ±0.3 | 1.1±0.41.8±1.11.0±0.70.7±0.51.5±1.11.3±0.21.7±0.91.2±0.2 | 0.9±0.52.0±1.40.7±0.40.8±0.50.9±0.41.0±0.31.1±0.61.4±0.7 |  | 0.8±0.52.3±0.80.5±0.20.7±0.51.3±0.81.3±0.61.5±0.92.5±1.5 | 0.6±0.21.9±0.60.3±0.10.4±0.21.0±0.51.3±0.21.2±0.32.0±0.9 | 0.3±0.11.6±0.50.3±0.10.4±0.31.0±0.71.3±0.41.0±0.22.7±1.4 | P (<0.05)NSP (<0.05)NSNSNSNS P (p=0.09) |
| **Adipose Tissue**FasnAcacaHsl / LipeLplLeptin  | 1.0±0.51.0±0.51.0±0.51.0±0.31.0±0.3 | 0.7±0.50.6±0.50.4±0.30.8±0.90.6±0.4 | 0.3±0.30.5±0.20.6±0.30.7±0.10.6±0.1 |  | 0.4±0.40.6±0.41.8±2.00.8±0.30.8±0.4 | 0.6±0.40.7±0.51.6±1.30.9±0.50.8±0.4 | 0.3±0.20.2±0.10.7±0.40.7±0.30.5±0.2 | F (<0.05)NSNSNSS (=0.09) |
| **Hypothalamus**NpyPomc | 1.0±0.71.0±0.7 | 1.1±0.30.9±0.6 | 0.8±0.20.6±0.4 |  | 0.8±0.430.3±0.2 | 1.0±0.50.4±0.2 | 1.0±0.60.3±0.1 | NSP (<0.05) |

1 Dietary treatment: C: control, HF: High Fat, HP: High protein, HS: High sucrose, NP: Normal protein

2Data are expressed as mean ± SD (n=5). Gene expression was calculated as: 2-ΔΔCT relatively to NP-C group and expressed as relative values normalised with 18S.

3The p value is indicated when the difference is or tended to be significant. P: protein effect, F: fat effect, S: Sucrose effect, PxF interaction between NP-HS-HF and HP-HS-HF, PxC: interaction between NP-HS and HP-HS. NS indicate no significant statistical effect (>0.05).

**Figures legends:**

**Figure 1:** **Effect of the increased protein content of the diet on the ability of sucrose and/or fat to increase (A and B) body weight and (C) food intake in rats**. Rats were fed a normal protein diet (NP) (white bars) or a high protein diet (HP) (black bars) for five weeks, containing a normal amount of sucrose and fat (Control: C), high sucrose (HS) or high sucrose and high fat (HS-HF) (see "Experimental Procedures" for details of diet composition). The body weight and food intake was followed during the experiment (A and C) from one week before the beginning of the experiment until the 4th week and was measured at the end of the experiment (B). Results are given as mean ± SD (n=5). The main statistical effects are indicated on each graph: The p value is indicated when the difference is or tended to be significant. P: protein effect, F: fat effect, S: Sucrose effect, PxF interaction between NP-HS-HF and HP-HS-HF, PxC: interaction between NP-HS and HP-HS. NS indicate no significant statistical effect (>.0.05).

**Figure 2: Effect of high protein intake on body composition.** (A) The weight of liver, (B) total adiposity and (C) each fat pad was measured at the end of the experiment by body composition analysis. Results are given as mean ± SD (n=5). The main statistical effects are indicated on each graph: The p value is indicated when the difference is or tended to be significant. P: protein effect, F: fat effect, S: Sucrose effect, PxF interaction between NP-HS-HF and HP-HS-HF, PxC: interaction between NP-HS and HP-HS. NS indicate no significant statistical effect (>.0.05).

**Figure 3: Effect of high protein intake on glucose homeostasis.** On days 33 to 37, rats were fed a calibrated meal and blood was sampled at t0 (fasted state) and 30, 60 and 120 min after the meal. Results are given as mean ± SEM for (A)glycaemia, (B) insulinemia and (C) glucagonemia. Kinetics for HP groups were compared to NP groups for control (C), high sucrose (HS) or high sucrose and high fat (HS-HF), respectively. Results for AUC are given as mean ± SD for glycaemia (D), insulinemia (E) and glucagonemia (F). The main statistical effects are indicated on each graph: The p value is indicated when the difference is or tended to be significant. P: protein effect, F: fat effect, S: Sucrose effect, PxF interaction between NP-HS-HF and HP-HS-HF, PxC: interaction between NP-HS and HP-HS. NS indicate no significant statistical effect (>.0.05).

**Figure 4: Effect of high protein intake on leptin and ghrelin kinetics in response to the meal intake.** On days 33 to 37, rats were fed a calibrated meal and blood was sampled at t0 (fasted state) and 30, 60 and 120 min after the meal. Results are given as mean ± SEM for (A) leptin and (B) ghrelin kinetics. HP groups were compared to NP groups under control (C), high sucrose (HS) or high sucrose and high fat (HS-HF) conditions. The main statistical effects are indicated on each graph: The p value is indicated when the difference is or tended to be significant. P: protein effect, F: fat effect, S: Sucrose effect, PxF interaction between NP-HS-HF and HP-HS-HF, PxC: interaction between NP-HS and HP-HS. T indicate the effect of time. NS indicate no significant statistical effect (>.0.05).

**Figure 5: Heatmaps of genes encoding (A)amino acids transporters, (B) enzymes involved in metabolism of amino acids for energy supply, (C) gluconeogenesis, (D) fatty acid and triglyceride synthesis, (E) fatty acid oxidation, (F) ketogenesis, (G) cholesterol metabolism and (H) bile acid synthesis.** Fold changes are based on signal log ratios for each of the background diets (HP-NP). Values >0 represent increase in gene expression under HP compared to NP conditions while values <0 represent decrease in gene expression under HP conditions.