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Long-term intake of a high-protein diet increases liver triacylglycerol deposition pathways and hepatic signs of injury in rats☆,☆☆

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Abstract

Intake of high-protein (HP) diets has increased over the last years, mainly due to their popularity for body weight control. Liver is the main organ handling ingested macronutrients and it is associated with the beginning of different pathologies. We aimed to deepen our knowledge on molecular pathways affected by long-term intake of an HP diet. We performed a transcriptome analysis on liver of rats chronically fed with a casein-rich HP diet and analyzed molecular parameters related to liver injury. Chronic increase in the dietary protein/carbohydrate ratio up-regulated processes related with amino acid uptake/metabolism and lipid synthesis, promoting a molecular environment indicative of hepatic triacylglycerol (TG) deposition. Moreover, changes in expression of genes involved in acid–base maintenance and oxidative stress indicate alterations in the pH balance due to the high acid load of the diet, which has been linked to liver/health damage. Up-regulation of immune-related genes was also observed. In concordance with changes at gene expression level, we observed increased liver TG content and increased serum markers of hepatic injury/inflammation (aspartate transaminase, C-reactive protein and TNF-alpha). Moreover, the HP diet strongly increased hepatic mRNA and protein levels of HSP90, a marker of liver injury. Thus, we show for the first time that long-term consumption of an HP diet, resulting in a high acid load, results in a hepatic transcriptome signature reflecting increased TG deposition and increased signs of health risk (increased inflammation, alterations in the acid–base equilibrium and oxidative stress). Persistence of this altered metabolic status could have unhealthy consequences.
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1. Introduction

Obesity and related comorbidities, such as metabolic syndrome and cardiovascular diseases, are a major public health concern in Western countries. Intake of diets with a high percentage of protein replacing carbohydrates is one of the main dietary strategies for weight loss purposes and also to maintain normal body weight [1,2]. In fact, intake of high-protein (HP) diets as daily dietary practice and/or as part of a life style pattern is increasing [3]. HP diets have been associated with body weight loss and with positive metabolic effects, such as control and normalization of some risk factors related to metabolic syndrome, like hyperglycaemia, hypercholesterolemia and hypertriglyceridemia, in obese people [2]. However, there is a scientific controversy on the effects of long-term consumption of these diets, and it has recently been reported that high protein intake is related with a higher risk of weight gain and with increased risk of fatal and non-fatal outcomes [4]. Although there are many human and animal studies focused on the physiological, biochemical and/or pathological effects induced by specific nutrients and dietary factors, there have been relatively few studies investigating safety and potential adverse effects of HP diets. Potential deleterious effects of these diets may be associated with the nonspecific effects of individual amino acids and/or generated metabolites in the different steps of amino acid metabolism [5], as well as to alterations in the acid–base balance due to increased dietary acid load [6]. There is evidence indicating that HP diets could be related to impairment of kidney and liver function [7,8], but diets with a high protein content are generally considered safe and healthy in subjects without any pathology [9].

Liver is a key tissue involved in dietary nutrient handling. Metabolic alterations in this organ resulting from the intake of unbalanced diets constitute a starting point for numerous pathologies [10]. Thus, molecular studies in liver are very useful for understanding metabolic effects generated by the intake of diets with an altered macronutrient composition. Furthermore, transcriptome analysis

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using whole genome microarrays can provide an unbiased overview at the molecular level of alterations that can occur [11]. For this reason, we assessed the global effects of HP diets on liver gene expression in healthy adult male Wistar rats to better understand the long-term effects of such diets on health.

2. Materials and methods

2.1. Animals

All animal experimental procedures followed in this study were reviewed and approved by the Bioethical Committee of the University of the Balearic Islands, and guidelines for the use and care of laboratory animals of the University were followed. Six-month-old male Wistar rats (Charles River Laboratories España, SA, Barcelona, Spain) single-housed at 22 °C with a period of light/dark of 12 h (light on at 8:00 pm) were examined. At the age of 2 months, the animals were stratified on body weight into two groups: a control group (n=7) and a pair-fed high-protein group (HP, n=6), as part of a larger animal study [12]. Sample size was selected based on similar experimental designs, which show that this size is adequate to detect statistical differences. Control animals were fed a normolipidic diet (D12450B; Research Diets) containing 70% of energy (kcal) from carbohydrates, 10% from fats and 20% from proteins. HP animals were fed a high-protein diet (Research Diets) containing 45% of energy from carbohydrates, 10% from fats and 45% from proteins (mainly casein). Diets (detailed composition in Table 1) were purchased from Brogaarden (Gentofte, Denmark) and were given isocalorically for 4 months. Food intake was recorded daily to calculate the daily and cummulative caloric intake; body weight was recorded three times a week. Our HP-fed animals ingested 2.3 times higher amount of protein than controls; this overconsumption is similar to the extent of overconsumption observed for humans following high-protein weight loss diets [13]. Animals were sacrificed in the fed state at the beginning of the light cycle by decapitation and truncal blood was collected from the neck to collect serum. Liver was rapidly removed, weighed, snap-frozen in liquid nitrogen and stored at −70 °C until further analysis. One week prior to sacrifice, animals were submitted to nocturnal 14-h fasting to collect serum in fasted conditions to analyze the HOMA-IR score using the formula of Matthews et al. [14].

2.2. Estimation of dietary acid load

The estimation of cumulative dietary acid load was calculated based on the diet composition as shown in Table 1, using two different approaches: the estimated net endogenous acid production (NEAP) from the potassium and protein content of the diet according to the formula of Frassetto et al. [15,16] [NEAP (mEq/day)=(54.5×protein (g/day)/potassium (mEq/day))−10.2], and the estimated diet-induced potential renal acid load (PRAL) directly calculated from dietary intakes applying the formula reported by Remer et al. [17] [PRAL (mEq/day)=0.49×protein (g/day)+0.037 phosphorus (mg/day)−0.021 potassium (mg/day)−0.026 magnesium (mg/day)−0.013 calcium (mg/day)].

Table 1
Diet composition

	Control diet		HP diet	
	g%	kcal%	g%	kcal%
Protein	19.2	20	43.2	45
Carbohydrate	67.3	70	43.3	45
Fat	4.3	10	4.3	10
Energy (kcal/g)	3.85		3.85	
Ingredients				
Casein	200	800	**450**	**1800**
L-Cystine	3	12	6	24
Corn starch	**315**	**1260**	62	248
Maltodextrin	35	140	35	140
Sucrose	350	1400	350	1400
Soy bean oil	25	225	25	225
Lard	20	180	20	180
Cellulose	50	0	50	0
Mineral Mix, S10026 [a]	10	0	10	0
Dicalcium phosphate [a]	13	0	13	0
Calcium carbonate [a]	5.5	0	5.5	0
Potassium citrate [a]	16.5	0	16.5	0
Vitamin Mix, V10001	10	40	10	40
Choline bitartrate	2	0	2	0

Control and high-protein (HP) diets were from Research Diets, were purchased from Brogaarden (Gentofte, Denmark) and served as dry pellets. The macronutrient percentage is given in g (grams) and in kcal. The energy content is given in kcal/g of food. Diet ingredients composition is also given in g/kg and in kcal/kg. Bold font highlights the main differential contributors to the two type of diets used. The control diet was rich in corn starch, while the HP diet was rich in casein; both diets had identical amounts of sucrose, fat, cellulose, vitamins and minerals, coming from the same sources.
[a] 1 kg of diet contains 2.96 g phosphorus, 6.3 g potassium; 0.5 g magnesium and 0.2 g calcium (both diets).

2.3. Quantification of circulating parameters

Circulating glucose was measured using an Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). ELISA kits were used to measure serum insulin (DRG Instruments, Marburg, Germany), TNF-alpha (R&D Systems Europe, Minneapolis, MN, USA) and C-reactive protein levels (eBioscience, Affymetrix, San Diego, CA, USA). Other serum parameters were measured using the following enzymatic kits: nonesterified free fatty acids (NEFAs; Wako Chemicals, Neuss, Germany), triacylglycerols (TGs; Sigma Diagnostics, Madrid, Spain), total circulating cholesterol and urea (BioSystems, Barcelona, Spain) and aspartate transaminase (AST; Bioo Scientific, Austin, TX, USA).

2.4. Determination of lipids and glycogen in liver

Lipid extracts were obtained from liver as previously described [18] and used to measure TG content using the Serum Triglyceride Determination Kit (Sigma Diagnostics), lipid content using the Folch method [19] and glycogen content using an anthrone method [20].

2.5. Liver histological analysis and Oil Red O staining

Liver tissue samples were embedded in paraffin blocks for light microscopy, and 5-μm-thick sections were mounted on slides and counterstained with hematoxylin/eosin. To determine the presence of hepatic steatosis, we analyzed the entire area (~2 cm^2) of histological stained liver sections from animals of the different conditions, and searched for the presence of fatty vesicles. For lipid highlight, 15-μm cryostat sections were fixed 1 h in 4% paraformaldehyde, washed in distilled water and stained whit Oil Red O Stain Kit (Abcam, Cambridge, United Kingdom) following the manufacturer's instructions. Briefly, sections were incubated whit Oil Red O for 6 min, washed in distilled water, counterstained whit Mayer hematoxylin and mounted in aqueous medium (Mount Quick aqueous; Bio-Optica, Milan, Italy). All groups were examined in a blind manner.

2.6. Total RNA isolation

Total RNA from liver samples was extracted using Tripure Reagent (Roche Diagnostic) and purified with Qiagen RNeasy Mini Kit spin columns (Izasa SA, Barcelona, Spain). RNA yield was quantified on a Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and its integrity was measured on an Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Agilent Technologies, South Queensferry, United Kingdom).

2.7. Microarray processing

For the microarray analysis, a total of 13 individual liver RNA samples from the control (n=7) and HP (n=6) groups were used, which were part of a larger set of dietary groups that were simultaneously processed. Samples were randomized before hybridization and all arrays were hybridized simultaneously. Labeling and microarray processing (hybridization, washing and scanning) were performed as previously described [21]. Briefly, 1 μg RNA of each sample was reverse transcribed, half was labeled with Cy5 and the other half with Cy3. After purification and quantification, Cy3-labeled cRNA samples were pooled on an equimolar basis. Each individual Cy5-labeled sample was hybridized with Cy3 pool on 4x44K G4131F rat whole genome Agilent microarrays (Agilent Technologies).

2.8. Microarray data analysis

Quantification and quality control of the array data was performed as previously described [22]. In total, about 54% of all probes were considered to be expressed (22,765 out of 41,012 probes). The data were deposited in NCBIs Gene Expression Omnibus and are accessible through GEO Series accession number GSE57858.

Statistical differences between the HP group *vs.* the control group were assessed by Student's t test in GeneMaths XT. Fold change equals HP/control ratio in the case of increase or equals −1/ratio in the case of decrease. For pathways analysis using Metacore (Thomson Reuters, St. Joseph, MI, USA), a threshold of P<.001 was selected. Subsequently, a statistically generated list of genes was manually analyzed in regard to their biological information, obtained with the use of available databases (Rat Genome Database, Genecards, KEGG, NCBI, Reactome, UniProt, USCN, WikiPathways and PubMed) based on key biological domains, such as molecular function and biological process. Some of these processes overlapped; thus, they were collected and renamed and all the unique genes were assigned into several biological processes according to their function. We manually supplemented the significantly enriched biological processes with nonannotated genes from the selected gene set using biological databases (Biocarta, SOURCE, GenMAPP and KEGG) and scientific literature. As processes overlap, we bundled some processes and renamed them. In some cases, we annotated probes manually in order to have the best annotated list of probes.

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