A long-term high-protein diet markedly reduces adipose tissue without major side effects in Wistar male rats

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Lacroix, Magali, Claire Gaudichon, Antoine Martin, Céline Morens, Véronique Mathé, Daniel Tomé, and Jean-François Hunault. A long-term high-protein diet markedly reduces adipose tissue without major side effects in Wistar male rats. Am J Physiol Regul Integr Comp Physiol 287: R934–R942, 2004. First published May 20, 2004; 10.1152/ajpregu.00100.2004.—Although there is a considerable interest of high-protein, low-carbohydrate diets to manage weight control, their safety is still the subject of considerable debate. They are suspected to be detrimental to the renal and hepatic functions, calcium balance, and insulin sensitivity. However, the long-term effects of a high-protein diet on a broad range of parameters have not been investigated. We studied the effects of a high-protein diet in rats over a period of 6 mo. Forty-eight Wistar male rats received either a normal-protein (NP: 14% protein) or high-protein (HP: 50% protein) diet. Detailed body composition, plasma hormones and nutrients, liver and kidney histopathology, hepatic markers of oxidative stress and detoxification, and the calcium balance were investigated. No major alterations of the liver and kidneys were found in HP rats, whereas NP rats exhibited massive hepatic steatosis. The calcium balance was unchanged, and detoxification markers (GSH and GST) were enhanced moderately in the HP group. In contrast, HP rats showed a sharp reduction in white adipose tissue and lower basal concentrations of triglycerides, glucose, leptin, and insulin. Our study suggests that the long-term consumption of an HP diet in male rats has no deleterious effects and could prevent metabolic syndrome.

Keywords: hormones; glucose; body composition; histology

DESPITE THE EPIDEMIOLOGICAL evidence that protein intake increased in industrialized countries since three decades (25, 66), and reached about two times the recommended intakes (72), the question of potential adverse effects of a high-protein (HP) diet remains highly controversial. It is well established now that HP diets induce early marked metabolic changes in human and animal models, especially when the diet contains at least 50% of energy as protein (13, 17, 40, 42). Nevertheless, the physiological and functional consequences of a long-term HP diet have not been explored fully.

Because of the prevalence of overweight and obesity, especially in Western countries (41), HP diets are often considered as an alternative and successful strategy to perform efficient and long-term weight loss (62). In fact, HP diets are currently known to induce feeding-suppressive effects in rats (5, 46) and in humans (30, 70). Low-calorie high-protein diets may be successfully used for the treatment of obese subjects (3, 70).

However, the medical use of a restricted-restricted diet for weight loss does not necessarily lead to a high amount of protein intake. When dealing with spontaneous dietary intake, the contribution of high-protein intake in lowering overweight prevalence is not entirely consensual, since two epidemiological studies found a positive association between protein intake and corpulence in early childhood (52, 64).

Among the suspected adverse effects of HP diets, impairment of hepatic and renal functions is often argued because of their central role in nitrogen metabolism. The liver and kidney size has been shown to be enhanced after 2 wk of HP diet in rats (24, 43). Moreover, the glomerular filtration rate (GFR) has been proved to be increased by high-protein intake (58, 73) or amino acid infusion (9, 21), but the concomitant impairment of renal function in healthy conditions is not demonstrated. Long-term protein intake has also been suspected to promote insulin resistance (24, 35) and to impair cortisol secretion (60). Other adverse effects have also been advanced, such as an increase of urinary calcium excretion and a possible bone resorption (31, 34), an enhancement of oxidative stress (47), and a modification of detoxification enzymes (49).

Given the difficulty to realize long-term interventional studies in human subjects and the lack of complete toxicological studies on high-protein diet effects, we investigated on a wide range of biochemical, anatomical, and histological parameters whether long-term ingestion of a HP diet could have adverse and/or beneficial effects in an obesity-prone strain of rats. For this purpose, male Wistar rats were given either a normal protein (NP) diet or a HP diet for 6 mo. Detailed body composition, biomarkers of oxidative stress [reduced (GSH) and oxidized (GSSG) glutathione, thiobarbituric acid reactive substances (TBARS), expression of γ-glutamyl-cysteine-ligase (γ-GCL)] and detoxification function [glutathione-S-transferase (GST)], plasma hormones (insulin, cortisol, and leptin), and liver and kidney histopathology were investigated. Calcium balance was also assessed after 4 mo of an NP or an HP diet.

MATERIALS AND METHODS

Animals and diets. All animals used in these experiments were cared for in accordance with criteria outlined in the European Convention for the Protection of Vertebrate Animals. Experiments were carried out on Male Wistar rats (Harlan, Horst, The Netherlands) weighing 180–200 g at the beginning of the experiment (n = 48). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
animals were housed in individual stainless steel wire cages in a room with controlled temperature (22 ± 1°C) and humidity and a 12:12-h light-dark cycle (light 8:00 PM-8:00 AM). Rats were fed a standard chow diet during the first 3 days [Harlan Teklad TRM 9608 rat/mouse diet (18% protein), Harlan, Gännstatt, France]. They were then randomly assigned to three groups of 16 rats each. In each group, eight rats were given a standard NP diet containing 14% total milk protein (TMP) in dry matter (DM), and eight rats were given an HP diet containing 50% TMP in DM. The composition of the experimental diets produced by the Atelier de Production des Aliments Experiments (INRA, Jouy-en-Josas, France) is shown in Table 1. The NP diet was an AIN-93 modified diet: instead of casein and cysteine, this diet contained 140 g TMP/kg food. The amounts of energy, fat, cellulose, minerals, and vitamins were similar in both diets; the HP diet was lower in carbohydrate (33.6% of DM) than the NP diet (63.6% of DM). Both diets were moistened (water/powdered diet: 1:2 for the NP and HP groups, respectively) to prevent spillage. The food thus had the same consistency and palatability in both groups. Fresh food was provided daily, at the beginning of the dark phase (8:30 AM). Water levels were checked one time a day, and the supply was renewed every 2 days. During the whole experiment, the rats had free access to tap water and food. The HP and NP groups were killed 15 days, 3 mo, and 6 mo after the beginning of the experimental diet period. The animals were weighed 5 days/wk during the first 2 mo and then two times monthly until the end of the experiment. To determine food consumption, cups were weighed 5 days/wk for 1 mo and then by periods of 5 days every 3 wk until the end of the experiment.

Experimental procedure and sample collection. After 3 and 6 mo of ingesting either an NP diet or an HP diet, and 1 wk before death, peripheral blood was taken from the cranial branch of the medial saphenous vein of overnight-fasted rats. Glyceremia was measured immediately using a portable refractometer (Encore glucometer; Bayer Diagnostics, Puteaux, France). After blood sampling, the rats received their regular meal.

After 15 days, 3 mo, and 6 mo of the experimental diet, animals fasted overnight were weighed, anesthetized with pentobarbital sodium via the intraperitoneal route at a dose of 30 mg/kg (Sano, 5,000 IU heparin (Laboratoires LEO, Saint-Quentin-en-Yvelines, France). The abdomen was opened, and the blood was removed after rupture of both the abdominal aorta and vena cava. Blood samples were centrifuged, and the plasma was frozen at −80°C for subsequent analyses of plasma total proteins, amino acids (AA), insulin, leptin, and cortisol. Simultaneously, the liver, kidneys, and muscles were removed promptly, washed free of gross blood with a 0.9% NaCl solution, and weighed. The liver was sliced and immediately frozen at either −20°C (enzyme or biochemistry assays) or −196°C in liquid nitrogen (molecular biology assays) and then stored at −80°C. One-quarter of the liver and one of the kidneys were fixed in a 2% formalin, 5% acetic acid, and 75% ethyl alcohol solution (FAA), embedded in paraffin, and stained with hematoxylin-eosin-saffron (HES) for subsequent histological analyses. These three dyes reveal the nucleus, the cytoplasm, and collagen, respectively. In addition, period acid-Schiff reagent (PAS) was used on kidneys for demonstration of basement membrane. Paraffin sections were 4 μm thick for kidney and liver. Urine was tapped directly into the bladder and immediately frozen at −20°C. Four deposits of white adipose tissue (WAT) and epididymal, mesenteric (including omental tissue), and subcutaneous tissue were removed and weighed, as well as the skin. The other abdominal and thoracic organs were discarded, and the distal parts of the limbs, the head, and the tail were stripped to determine the weight of this so-called ‘striped’ carcass. In some of the 6-mo animals (6 rats in the NP group and 5 rats in the HP group), the carcasses were frozen to determine the weight of the skeletons at a later date. Briefly, the carcasses were boiled, and the muscles and tendons were removed carefully with a scalpel blade; the remaining tissues were sifted to recover small bones (caudal and dorsal vertebrae, ribs). For all animals in the 6-mo group, the heart, adrenal glands, and interscapular brown adipose tissue were also weighed.

An oral glucose tolerance test was performed after an overnight fast in a complementary study performed on 12 rats (n = 6/group) after 3 mo of experiment. Fasted rats received a 1 g/kg oral glucose load, and blood glucose levels were measured sequentially. The results are expressed as a percentage of the difference between basal and each time point. To quantify the calcium balance, after 4 mo of the experimental diet, the 6-mo rats were housed for 7 days in individual metabolism cages. Urine and feces were collected daily; crystalized thymol was added to the urine collection tubes to prevent bacterial proliferation. Sampled urine and feces were stored at −20°C at the end of each day of experimentation.

Analytic methods. With the exception of solvents and alcohols purchased from Carlo Erba (Val de Reuil, France), all chemicals used as reagents, in solutions or buffers, and all enzymatic assays were obtained from Sigma-Aldrich Chimie (Saint-Quentin Fallavier, France). Urinary and plasma urea concentrations were determined by an enzymatic assay (Urea Nitrogen endpoint kit); albumin was revealed by its reaction with bromocresol purple (Albumin kit BCP). GST activity was measured according to the method of Habig et al. (23). Briefly, GST conjugates with 1-chloro-2,4-dinitrobenzene and the enzyme activity in the presence of this aromatic substrate is determined spectrophotometrically. For oxidized (GSSG) and total (GSH) glutathione, samples were homogenized in 9 vol iced 0.9% NaCl. Once filtered, 50 μl of the solution were frozen at −20°C to perform protein quantitative analyses, and 300 μl were sampled mixed with 100 μl of 20% sulfosalicylic acid and centrifuged (3,000 g, 15 min, 4°C). The supernatant was collected and used to determine GSH and GSSG levels according to the method described by Anderson (2) and TBARS levels according to the technique of Satoh (55).

Liver protein concentrations were determined using the bicinchoninic acid (BCA) method (Fierce BCA-200 Protein Assay kit; Perbio Science, Bezons, France). For AA and plasma protein analysis, 1 ml plasma was deproteinized with 40 μl of 100% sulfosalicylic acid, stored at 4°C for 1 h, and then centrifuged (3,000 g, 4°C, 15 min). The supernatant was dried and resuspended in a lithium citrate buffer (pH 2.2) for analysis. The plasma protein pellet was lyophilized. Total nitrogen in plasma proteins was measured with an elemental nitrogen analyzer (EA5NA 1500 II; Fisons Instruments, Manchester, UK) with atropine (Carlo Erba Instruments, Arceuil, France) as a standard, as previously described (18). Protein concentrations were calculated using a conversion factor of 6.25, originating from the standard amount of nitrogen in protein, i.e., 16%. Plasma amino acid concentrations were determined using an HPLC system (Bio-Tek Instruments, St. Quentin en Yvelines, France) combined with postcolumn amino acid derivatization. Elution was performed on a cation exchange resin (Bio-Rad Dowex AG-50X8; Interchim, Montluçon, France). All amino acids were detected at 540 nm, except for proline (440 nm). γ-Aminobutyric acid was used as an internal standard.

Table 1. Composition of diets

<table>
<thead>
<tr>
<th>Nutrient, g/kg</th>
<th>Normal Protein (14% Protein)</th>
<th>High Protein (50% Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total milk protein</td>
<td>140</td>
<td>500</td>
</tr>
<tr>
<td>Pregelatinized cornstarch</td>
<td>569.9</td>
<td>312.7</td>
</tr>
<tr>
<td>Sucrose</td>
<td>92.8</td>
<td>50</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Mineral mixture (AIN93-M)</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mixture (AIN93-V)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>α-Cellobiose</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Choline</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Total</td>
<td>1,000</td>
<td>1,000</td>
</tr>
<tr>
<td>Energy, kJ/g</td>
<td>15.9</td>
<td>15.8</td>
</tr>
</tbody>
</table>
Plasma creatinine, triglycerides, and total and high-density lipoprotein-cholesterol assays were performed using a Mascott + 3× spectrophotometer (Lisabio, Pouilly-en-Auxois, France).

Calium levels in the urine and feces (and in drinking water and food) were determined using a flame atomic absorption spectrometer (model SpectrAA 50; Varian, Les Ulis, France) with an air-acetylene burner (reducing flame). Before analysis, liquid samples were diluted 1:100 in a spiking solution containing 5 g/l lanthanum chloride, 1 g/l calcium chloride, and 0.2 g/l potassium chloride. Solid samples (feces and diets) were mineralized by heating for 2 h (100°C) in 25 vol of 2 N nitric acid. The wavelength of the cathode lamp was 422.7 nm.

To measure changes in γ-GCL mRNA expression levels after 3 mo of the experimental diet, we chose a semiquantitative RT-PCR protocol. RNA extraction was carried out on liver samples from four animals in each group using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions. The following primers (Genset Oligos, La Jolla, CA) were used to amplify γ-GCL mRNA using a one-step RT-PCR kit (Qiagen, Courtaboeuf, France) and [33P]dATP (Perkin-Elmer Life Science and Products, Courtaboeuf, France) according to the manufacturer’s instructions. The PCR products were then separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and quantified using a digital imaging system (Alpha Innotech, San Leandro, CA). Concurrently, quantification was also performed by measuring the amount of [33P]dATP recovered in the amplification products using liquid scintillation counting. Briefly, ethidium bromide-stained spots were punched and solubilized in chaotropic buffer (QX1; Quiagen, Courtaboeuf, France) and 3 ml of a liquid scintillation cocktail (Ultima Gold; Perkin-Elmer Life Science and Products, Courtaboeuf, France): 5'-CAAGAACACACCATCTCCGTTG-3' (forward) and 5'-ACTGTACCTCCATTGGTCGGAAC-3' (reverse). Running PCR without prior reverse transcription controlled the absence of DNA contamination. Amplification was performed in a PTC-200 thermocycler (MJ Research, Watertown, MA) for 24 cycles.

Statistics. The results were expressed as means ± SE. The effects of diet and age were tested by two-way ANOVA, using the general linear model procedure of SAS (version 6.11: SAS, Cary, NC). An α-level of 0.05 was set to determine significance.

RESULTS

Energy intake, body weight, body composition, and calcium balance. Whatever the diet, food intake varied from one day to another, but no refusal or unexpected reduction in food intake was observed during the 6-mo period. The mean daily food intake after 6 mo of experiment differed significantly between HP rats (348.8 ± 3.1 kJ) and NP rats (388.2 ± 6.8 kJ). The energy intake in HP rats was 87% of that seen in NP rats. After 6 mo of the experimental diet, a significant reduction in body weight was observed in HP rats compared with NP rats. The weight of HP rats was 82% of that seen in NP rats.

Body weight, WAT, and stripped carcass weights increased significantly with age (P < 0.05). After 15 days of an experimental diet (results not shown), body compositions exhibited no differences between the groups except regarding the kidneys (1.99 ± 0.11 and 2.29 ± 0.25 g for NP and HP groups, respectively, P < 0.05). However, after 6 mo of the experimental diet, the body composition showed marked differences.

Table 2. Body weight and body composition of rats after 3 mo and 6 mo ad libitum consumption of either an NP (14% of protein) or HP (50% of protein) diet

<table>
<thead>
<tr>
<th></th>
<th>3 Month</th>
<th></th>
<th>6 Months</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP</td>
<td>HP</td>
<td>NP</td>
<td>HP</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>519.6 ± 22.9</td>
<td>465.5 ± 12.0</td>
<td>620.1 ± 34.4</td>
<td>510.63 ± 15.3</td>
</tr>
<tr>
<td>Liver± †</td>
<td>12.6 ± 0.5</td>
<td>12.0 ± 0.3</td>
<td>14.3 ± 0.7</td>
<td>11.8 ± 0.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.5 ± 0.1</td>
<td>2.8 ± 0.10</td>
<td>2.6 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.7 ± 0.0</td>
<td>0.8 ± 0.0</td>
<td>0.8 ± 0.0</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Gut</td>
<td>11.2 ± 0.47</td>
<td>11.1 ± 0.3</td>
<td>10.6 ± 0.4</td>
<td>10.7 ± 0.3</td>
</tr>
<tr>
<td>White adipose tissue, g</td>
<td>105.6 ± 11.9</td>
<td>64.3 ± 2.1</td>
<td>170.6 ± 20.1</td>
<td>98.8 ± 12.3</td>
</tr>
<tr>
<td>Expressed as %body wt †</td>
<td>20.0 ± 1.5</td>
<td>13.8 ± 0.3</td>
<td>27.0 ± 1.7</td>
<td>19.0 ± 2.0</td>
</tr>
<tr>
<td>Epidymal †</td>
<td>20.2 ± 1.9</td>
<td>13.5 ± 0.6</td>
<td>27.4 ± 2.8</td>
<td>18.3 ± 2.5</td>
</tr>
<tr>
<td>Retroperitoneal †</td>
<td>23.6 ± 2.0</td>
<td>15.6 ± 0.9</td>
<td>35.1 ± 3.6</td>
<td>20.8 ± 2.5</td>
</tr>
<tr>
<td>Subcutaneous †</td>
<td>41.4 ± 6.4</td>
<td>23.6 ± 1.2</td>
<td>80.3 ± 12.0</td>
<td>42.7 ± 6.1</td>
</tr>
<tr>
<td>Mesenteric †</td>
<td>20.3 ± 3.6</td>
<td>11.5 ± 0.6</td>
<td>27.7 ± 2.1</td>
<td>17.0 ± 1.9</td>
</tr>
<tr>
<td>Brown adipose tissue†</td>
<td>ND</td>
<td>ND</td>
<td>4.4 ± 0.3</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>Skin, g</td>
<td>80.4 ± 3.7</td>
<td>69.8 ± 1.7</td>
<td>86.5 ± 3.2</td>
<td>83.1 ± 4.7</td>
</tr>
<tr>
<td>Expressed as %body wt ‡</td>
<td>15.5 ± 0.2</td>
<td>15.0 ± 0.4</td>
<td>14.0 ± 0.3</td>
<td>16.2 ± 0.7</td>
</tr>
<tr>
<td>“Stripped carcass,” g*</td>
<td>200.9 ± 7.1</td>
<td>195.9 ± 6.9</td>
<td>224.7 ± 9.1</td>
<td>206.5 ± 5.8</td>
</tr>
<tr>
<td>Expressed as %body wt ‡</td>
<td>38.9 ± 1.2</td>
<td>42.0 ± 0.6</td>
<td>36.4 ± 0.7</td>
<td>40.7 ± 1.5</td>
</tr>
<tr>
<td>Skeletons, g</td>
<td>ND</td>
<td>ND</td>
<td>11.56 ± 1.25</td>
<td>11.70 ± 0.90</td>
</tr>
</tbody>
</table>

Values are means ± SE. NP, normal protein; HP, high protein; stripped carcass is cleaned out carcass, without skin, tail, head, and distal part of the limbs. P < 0.05, (ANOVA), significant effect of age (*), diet (†), and age × diet (‡). ND, not determined.
in WAT, since this represented 27.5% of body weight in NP rats but only 19.3% in HP rats (Table 2). Subcutaneous WAT in NP rats was double that in HP rats, whereas the respective stripped carcass weights were similar in NP and HP rats. Therefore, WAT was the main contributor to the increased body weight of NP rats compared with HP rats. The brown adipose tissue weight was two times as high in NP rats as in HP rats. No differences between groups were observed with respect to skeleton weight after 6 mo of adaptation.

Quantifications of calcium intake (food and water) after 4 mo (2.549 ± 70 and 2.900 ± 64 mg·animal⁻¹·wk⁻¹ in NP and HP rats, respectively; P < 0.05) and calcium excretion in feces (887 ± 51 and 1.413 ± 91 mg·animal⁻¹·wk⁻¹ in NP and HP rats, respectively; P < 0.05) and in the urine (22 ± 2 and 38 ± 5 mg·animal⁻¹·wk⁻¹ in NP and HP rats, respectively; P < 0.05) led to a positive balance in both groups, with no significant difference between NP and HP rats (1.640 ± 79 and 1.448 ± 99 mg·animal⁻¹·wk⁻¹ in NP and HP rats, respectively; not significant).

**Plasma hormones, glucose, proteins, amino acids, and lipids.** Fasting plasma glucose, insulin, leptin, and cortisol concentrations were significantly higher in NP rats than in HP rats (Table 3). There was also a significant effect of age on blood leptin, insulin, and sugar levels, with a dramatic increase in leptin with age (P < 0.0001). Moreover, glucose tolerance tended to be improved in HP rats (Fig. 1). The areas under the three curves were 103.8 ± 12.3 Δmmol × min⁻¹ in the HP group and 138.3 ± 14.1 Δmmol × min⁻¹ in the NP group. However, these values did not differ significantly (P = 0.09).

Plasma urea was systematically higher in HP rats than in NP rats (P < 0.01), whatever the duration of the experiment (4.5 ± 0.2 vs. 3.4 ± 0.2 mmol/l for HP and NP rats after 6 mo of experiment; Table 3). Plasma creatinine tended to be higher in NP rats but did not reach the significance when all groups (15 day, 3 mo, and 6 mo) were taken in account (P = 0.053). A significant difference was observed in rats slaughtered after 3 mo. Plasma homocysteine levels remained unchanged throughout the experiment. Plasma albumin, when expressed either as an absolute value or as a percentage of total plasma proteins and plasma protein, varied with age (P < 0.01), but the diet had no effect. Circulating amino acid values showed few differences dependent on age or diet. Indispensable amino acids decreased significantly with age. Leucine and isoleucine concentrations were significantly higher in the HP group, whereas threonine concentrations were lower (data not shown).

**Plasma cholesterol and triglycerides varied significantly with age (Table 3).** Furthermore, triglyceride levels were significantly higher in NP rats (P = 0.0009), whereas diet had no effect on total and high-density lipoprotein cholesterol.

**Oxidative stress-related parameters and histological study.** Hepatic oxidative stress was quantified by measuring TBARS and glutathione levels (Table 4). Oxidized glutathione concentrations were negligible. Total liver glutathione levels rose with age in HP rats but not in NP rats (age × regime effect, P < 0.004). TBARS liver concentrations did not differ in HP rats when compared with NP animals. GST activity markedly

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**Table 3. Fasting plasma levels of hormones, glucose, proteins, amino acids, homocysteine, urea, creatinine, and lipids in rats after 15 days 3 mo, and 6 mo ad libitum consumption of either an NP (14% of protein) or HP (50% of protein) diet**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NP 15 Days</th>
<th>HP 15 Days</th>
<th>NP 3 Months</th>
<th>HP 3 Months</th>
<th>NP 6 Months</th>
<th>HP 6 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin, mIU/l†‡</td>
<td>75.2 ± 2.4</td>
<td>62.6 ± 8.0</td>
<td>75.8 ± 5.9</td>
<td>66.5 ± 4.1</td>
<td>122.8 ± 21.4</td>
<td>90.3 ± 3.5</td>
</tr>
<tr>
<td>Cortisol, µg/l†‡</td>
<td>21.0 ± 1.6</td>
<td>14.4 ± 0.1</td>
<td>22.3 ± 3.1</td>
<td>15.0 ± 1.7</td>
<td>23.8 ± 2.5</td>
<td>14.2 ± 1.0</td>
</tr>
<tr>
<td>Leptin, µg/l†‡</td>
<td>9.0 ± 2.2</td>
<td>6.8 ± 1.3</td>
<td>34.2 ± 4.9</td>
<td>17.2 ± 1.1</td>
<td>46.9 ± 4.0</td>
<td>26.4 ± 3.7</td>
</tr>
<tr>
<td>Glycemia, mmol/l†‡</td>
<td>ND</td>
<td>ND</td>
<td>5.33 ± 0.17</td>
<td>4.44 ± 0.17</td>
<td>4.99 ± 0.17</td>
<td>4.11 ± 0.11</td>
</tr>
<tr>
<td>Urea, mmol/l†</td>
<td>3.8 ± 0.5</td>
<td>5.0 ± 0.4</td>
<td>3.5 ± 0.2</td>
<td>6.6 ± 1.0</td>
<td>3.4 ± 0.2</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>Creatinine, µmol/l†‡</td>
<td>35.4 ± 5.3</td>
<td>32.0 ± 4.0</td>
<td>47.0 ± 6.7</td>
<td>38.3 ± 3.2</td>
<td>54.8 ± 7.6</td>
<td>52.5 ± 5.8</td>
</tr>
<tr>
<td>Protein, g/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein*</td>
<td>61.0 ± 1.3</td>
<td>58.0 ± 1.8</td>
<td>65.4 ± 0.9</td>
<td>63.6 ± 2.9</td>
<td>55.2 ± 1.9</td>
<td>56.4 ± 1.1</td>
</tr>
<tr>
<td>Albumin*</td>
<td>42.6 ± 1.9</td>
<td>41.3 ± 1.6</td>
<td>42.2 ± 2.2</td>
<td>43.6 ± 1.1</td>
<td>49.1 ± 1.3</td>
<td>46.5 ± 1.2</td>
</tr>
<tr>
<td>Amino acids, µmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indispensable*</td>
<td>1,166 ± 74</td>
<td>1,202 ± 162</td>
<td>959 ± 52</td>
<td>892 ± 47</td>
<td>926 ± 53</td>
<td>828 ± 36</td>
</tr>
<tr>
<td>Dispensable</td>
<td>2,217 ± 105</td>
<td>2,260 ± 317</td>
<td>2,070 ± 106‡</td>
<td>1,776 ± 85‡</td>
<td>2,049 ± 144</td>
<td>2,090 ± 84</td>
</tr>
<tr>
<td>Neoglucogenic</td>
<td>1,628 ± 66</td>
<td>1,892 ± 271</td>
<td>1,953 ± 93</td>
<td>1,548 ± 63</td>
<td>1,335 ± 93</td>
<td>1,913 ± 83</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>6.9 ± 1.0</td>
<td>6.6 ± 1.2</td>
<td>8.7 ± 1.3</td>
<td>8.8 ± 1.1</td>
<td>6.5 ± 1.0</td>
<td>8.8 ± 0.9</td>
</tr>
<tr>
<td>Lipids, mmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides†</td>
<td>0.76 ± 0.09</td>
<td>0.56 ± 0.08</td>
<td>0.86 ± 0.10</td>
<td>0.66 ± 0.10</td>
<td>1.28 ± 0.21</td>
<td>0.68 ± 0.07</td>
</tr>
<tr>
<td>Total cholesterol*</td>
<td>1.79 ± 0.13</td>
<td>1.53 ± 0.13</td>
<td>1.54 ± 0.16</td>
<td>1.53 ± 0.17</td>
<td>2.14 ± 0.18</td>
<td>1.89 ± 0.12</td>
</tr>
<tr>
<td>HDL-cholesterol*</td>
<td>0.90 ± 0.07</td>
<td>0.77 ± 0.08</td>
<td>0.53 ± 0.03</td>
<td>0.58 ± 0.10</td>
<td>0.76 ± 0.06</td>
<td>0.77 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE. HDL, high-density lipoprotein. P < 0.05 (ANOVA), significant effect of age (*) and diet (†).
increased with age ($P < 0.0001$) and was higher in HP rats ($P = 0.0006$).

RT-PCR products are visualized on ethidium bromide-stained agarose gel (Fig. 2). The quantification of γ-GCL mRNA expression after 3 mo of experiment did not differ between groups, whatever the method (ratio: 0.10 ± 0.03 vs. 0.09 ± 0.01 in HP and NP rats, respectively, in the radioactivity assay and 0.10 ± 0.00 vs. 0.11 ± 0.00 in HP and NP rats, respectively, in the agarose gel assay).

No serious histological lesions were observed in either group after 3 or 6 mo of experiment. Figure 3 shows typical histological sections of liver (HES stain). All NP rats systematically exhibited marked hepatic micro- and macrovacuolar steatosis, which was probably related to their excessive adiposity (Fig. 3A, A1 and A2). In contrast, no steatosis was found in HP rats (Fig. 3B). Minor, nonpathological inflammatory lesions were found in the liver of two rats in the HP group after 6 mo of the experimental diet (data not shown). Figure 4 shows histological kidney glomeruli sections (PAS stain). Despite the relative thickness of the sections (4 μm), focus adjustment permitted careful examination of renal tissues. The capillary loops of the glomeruli were well defined and thin. No nephrocalcinosis, area of collagenous sclerosis, or hypercellular glomerulus was detectable. Endothelial and mesangial cells were normal and so were the surrounding tubules. Similar to PAS stain, HES stain of kidneys (data not shown) did not reveal any noticeable lesion. However, one rat of the HP group exhibited a localized and limited interstitial lymphocytic inflammatory nodule and another HP rat showed a minor kidney lesion indicative of moderate inflammation of the renal papilla (data not shown).

### DISCUSSION

The aim of this study was to estimate the consequences of a long-term intake of high levels of protein in an obesity-prone rat strain. The results showed that the long-term ad libitum consumption of a high-protein diet markedly reduced food intake and lowered WAT, in accordance with several midterm studies. Concomitantly, basal blood insulin, leptin, and triglyceride levels and glucose tolerance were improved. Calcium balance was unaffected by a high intake of milk proteins. Moreover, in contrary to what has generally been admitted, no adverse effects of the HP diet were reported, particularly regarding hepatic and renal histopathology.

After 6 mo of the experiment, the body weight of rats fed the HP diet was 18% lower than that of rats fed the NP diet. Body composition measurements revealed remarkable differences between the two groups, especially concerning the subcutaneous fat pad. These results are in agreement with those obtained during short-term studies (3 wk; see Refs. 39, 29, and 42) and can be explained to some extent by the reduction in caloric intake of 13%. This decrease in energy consumption was most probably the result of the greater sensory-specific satiety of protein compared with carbohydrate (5, 37, 65). The ratio of energy intake to body mass was higher in NP rats (0.95 kJ/g) than in HP rats (0.68 kJ/g), whereas the ratio of energy intake to lean body mass is similar in both groups (1.72 kJ/g in NP rats vs. 1.69 kJ/g in HP rats). In consequence, the surplus of energy intake in NP rats has probably been used to generate fat tissues. However, the subsequent reduction of 58% in WAT could also arise from an increased thermogenic response to a protein meal intake (61). HP diets are good candidates for weight reduction, especially in obese female subjects (44). A 6-mo randomized trial concerning a protein vs. carbohydrate diet (45) resulted in a significant weight loss (3.2 ± 0.2 kg) greater than with an NP diet (59). However, some studies have reported no specific effect of HP diets on reducing body weight or body fat in obese subjects (3, 48).
In the present study, the weight reduction in rats fed the HP diet was strongly associated with lower basal blood sugar and insulin levels, as previously described (68), and improved glucose tolerance. Because the isocaloric exchange between HP and NP diets was performed on carbohydrate, the flattening effect of the HP diet on insulin and glucose basal levels could be attributed to its reduced carbohydrate content. This contrasts with the results of many studies that have acknowledged the fact that high protein intakes induce an increase in glucose and insulin concentrations (7, 35, 54). The oxidative conversion of amino acids to glucose resulting from HP feeding has been postulated to exert a negative metabolic effect on the glycolysis/gluconeogenesis balance in the liver, through a modification of the activity of key enzymes involved in glucose metabolism (8, 45). This assumption forms the scientific basis for the theory of amino acid-glucose competition as a factor in insulin resistance. However, many of the previous data do not fully support this theory, since they were obtained either with a simultaneous load of glucose and amino acids or without strictly controlled diets. In contrast, the results of a randomized controlled trial on obese glucose-tolerant subjects showed that a low-calorie HP diet could improve insulin sensitivity after 21 days of experiment (48) and achieve a normalization of insulin levels within 4 wk (3). Although the present study does not explore insulin sensitivity, it shows that the long-term isocaloric exchange between protein and carbohydrate is favorable regarding the basal concentrations of insulin and glucose and tends to improve glucose tolerance. This is in accordance with the fact that weight loss is often associated with improved glucose tolerance and insulin sensitivity related to enhanced glucose oxidation (15, 16, 20). Moreover, rats fed an HP diet seemed to develop less leptin resistance with age than rats fed an NP diet, which is in line with their marked reduction of adipose tissue (12, 27).

Many studies in obese and overweight hyperinsulinemic humans have shown a decrease in basal plasma cholesterol levels associated with weight loss, irrespective of the type of diet (10, 44). Although a hypercholesterolemic effect of casein has often been suspected, especially when compared with a legume protein source (22, 38), we did not find any increase in circulating cholesterol in HP rats consuming three times more milk protein than NP rats. In agreement with studies performed in humans (14, 33), we also reported a stabilization of serum triacylglycerol concentrations with age in HP rats, whereas triglycerides increased from 15 days to 6 mo in NP rats. This difference was probably related to the lower carbohydrate content of the HP diet.

Given the roles of the liver and kidneys in amino acid and nitrogen metabolism, HP diets have been suspected of having potentially deleterious effects on the structure and function of

Fig. 3. Liver histology sections in rats after 6 mo ad libitum consumption of either an NP (14% of protein) or HP (50% of protein) diet. A1: liver section of a rat after 6 mo consumption of an NP diet; shown is the microvacuolar and macrovacuolar steatosis that was observed in the 8 rats in this group. A2: detail of macrovacuolar steatosis (note the nucleus pushed aside of the cell membrane). B: liver section of one rat after 6 mo ingestion of an HP diet.
term, and these are important findings. In the same way, the glomerular hyperfiltration induced by elimination of the end products of amino acid metabolism has led to the conclusion that HP diets could be detrimental to renal function. Basic amino acid loads have been shown to increase water consumption in rats (1), and, indeed, we also found a 1.4-fold rise in water consumption in rats receiving the HP diet (data not shown). A higher water consumption is likely to increase glomerular filtration rate, and this could explain the trend of a lower plasma creatinine that we observed in HP rats. To our knowledge, the only study that ever documented a toxic effect of an HP diet is that of Stonard et al. (63), who reported that a moderate HP diet (33%) was associated with nephrocalcinosis and tubular damage in a specific strain of female rats (Alderley Park). However, this significant occurrence of renal damage was observed in rats fed single-cell proteins but not in rats fed casein, suggesting a specific effect of this protein source. A moderate restriction of dietary protein has also been demonstrated to slow the progression of renal impairment in rats (6, 11, 50) and in human patients (19, 53, 75) but did not provide any benefit in healthy individuals (67). On the other hand, a prospective trial on the effects of protein restriction on the progression of chronic renal insufficiency in 456 adult patients showed no correlation between a worsening of renal failure and the protein catabolic rate (36). Nevertheless, none of these findings contradicts the absence of pathological findings in our study as we explored the potential adverse effects of HP diets on the renal function of healthy male animals. Although these results are hardly transposable to humans, especially because the capacity to concentrate urea is severalfold higher in rodents than in humans, it is interesting to note that a recent study showed that dietary intervention for 6 mo in 65 obese subjects consuming a 25% protein diet caused adaptive changes in renal size and glomerular filtration rate without any indications of adverse effects (58). To further conclude the absence of renal toxicity of HP diets, a longer-term study should be necessary as well as a study on rat females in which lesions can be produced more readily, as mentioned in the work of Stonard et al. (63).

Excess dietary protein has also been suspected of generating large quantities of acids (phosphates and sulfates) excreted by the kidneys to maintain pH homeostasis. This increase in net acid excretion is coupled with calciuria, with the skeleton thus supplying a buffer by bone resorption (71). Some authors have postulated that HP diets cause a pathological impairment of calcium homeostasis because the long-term consequences of even a small change in the calcium balance are substantial (4, 28). However, there is no clear evidence that HP diets alone cause osteopenia. Recently, Skov et al. (57) found that a 6-mo HP intake was associated with a slowing in bone mineral loss in obese subjects. Calcium excretion and markers of bone turnover were reported to be unchanged in overweight subjects consuming a 15 or a 27% protein diet for 16 wk (14). In our study, the increased calcium losses observed in the HP group were balanced by higher intakes resulting from the association of calcium and caseinates. In consequence, no alteration to the calcium balance was observed, in accordance with the similar skeleton weights observed in both groups. However, it must be pointed out that calcium intake in the HP group was important because of the use of milk protein as the sole protein source.
and calcium balance may have been different using another protein source.

We performed this study to explore whether or not HP diets might be detrimental to healthy animals over a long period representing a quarter of their life span. It would be almost impossible to perform a study on an equivalent period in humans. We can conclude that, in male rats, a protein intake of three times the requirements did not produce any adverse effects on the renal and hepatic functions, on oxidative stress, or on calcium balance. On the contrary, exchange of carbohydrates for proteins was beneficial regarding body composition, basal triglycerides, glucose, leptin, and insulin plasma concentrations. The results of the present study agree with the idea that long-term dietary management is of major importance to preventing obesity and its correlative X syndrome. Further research is required to specify the respective effects of weight loss and high-protein intake on glucose and lipid metabolism.

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REFERENCES

SIDE EFFECTS OF THE LONG-TERM CONSUMPTION OF A HIGH-PROTEIN DIET


