Long-Term High Protein Intake Does Not Increase Oxidative Stress in Rats

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ABSTRACT The maximum dietary protein intake that does not cause adverse effects in a healthy population is uncertain. We tested whether a high protein intake enhances oxidative stress. Adult rats were adapted to different casein-based diets containing either an adequate (13.8%; AP), medium (25.7%; MP), or high (51.3%; HP) level of crude protein; a fourth group received a HP diet but no *RRR*-α-tocopherol acetate (HP-toc). After 15 wk of feeding, plasma protein carbonyl concentration, liver lipid peroxide levels [thiobarbituric acid-reacting substances (TBARS)], reduced glutathione (GSH) status and leucine kinetics ([1-^{13}C]leucine) were measured. Higher concentrations of protein carbonyls and TBARS were found in rats fed the AP and the HP-toc diets compared with those fed the MP and HP diets ($P < 0.05$). GSH concentrations in plasma did not differ but total blood GSH concentrations were significantly ($P < 0.05$) lower in rats fed the HP-toc diet compared with those fed the AP, MP and HP diets. Liver GSH concentrations were significantly ($P < 0.01$) lower in rats fed the AP diet compared with the other groups. Rates of postabsorptive leucine oxidation (LeuOX) and flux (Q_{Leu}) were positively correlated with the dietary protein level (for AP, MP, and HP, respectively: LeuOX, 74.9 $\pm$ 28.5, 109 $\pm$ 35.2, 142.3 $\pm$ 38.4 μmol/(kg $\cdot$ h); Q_{Leu}, 425 $\pm$ 102, 483 $\pm$ 82, 505 $\pm$ 80 μmol/(kg $\cdot$ h). Only HP-toc resulted in a significantly greater protein breakdown (PB_{Leu}) and Q_{Leu}. No difference was seen in nonoxidative leucine disposal. Long-term intake of high protein diets did not increase variables of oxidative stress, in contrast to our initial hypothesis. An unexpected finding was that adequate protein feeding (AP) may in fact induce oxidative stress. J. Nutr. 130: 2889–2896, 2000.

KEY WORDS: • *rats* • *high protein diets* • *oxidative stress* • *leucine flux* • *vitamin E deficiency*

Dietary protein provides 12–15% of the total energy intake of adults consuming Western diets. This corresponds to a daily intake of ~1.5 g protein/kg which considerably exceeds the recommended intake of 0.75 g/kg (FAO/WHO 1985). To date, there are no proven or adequately confirmed benefits and possibly some risks of a chronic high protein intake (Durnin et al. 1999). However, the maximum protein intake that will not cause adverse effects to almost all of the healthy population ingesting that level (Institute of Medicine 1999) is uncertain, although undesirable metabolic effects have been shown with an intake of ~1.6 times or more the recommended intake (Metges and Barth 2000).

Chronic high dietary protein intake or intake of dietary amino acids in excess or above actual needs leads to an increase in amino acid oxidation to maintain amino acid homeostasis (Harper 1994). This catabolic process involves the mitochondrial redox chain, which is a physiologic source of free oxygen radicals. Thus, we hypothesized that a high protein diet may contribute to enhanced mitochondrial oxygen radical generation, resulting in oxidative stress (Petzke et al. 1999). Free radical generation during mitochondrial oxygen reduction may lead to oxidative stress if the antioxidant potential is insufficient to quench the extra free radical production. This is reflected in changes of thiol concentrations in body tissues and fluids (Bray and Taylor 1993, Lu 1999), generation of peroxides due to damage of polyunsaturated fatty acids (e.g., thiobarbituric acid-reacting substances, TBARS)[2] (Benzie 1996) or oxidative modification of protein-bound amino acids (e.g., carbonyl formation) (Chao et al. 1997, Grune et al. 1997, Levine et al. 1994).

At present it is not clear whether a high protein diet causes physiologic oxidative stress. An increase in liver protein damage was found in growing rats when the protein intake was increased from a subadequate level of 5 to 20% casein (Youngman et al. 1992). Generally, the role of free oxygen radicals in pathologic processes is well documented. Therefore, it is important to clarify whether the chronic intake of a high protein diet contributes to oxidative stress.

We recently reported that chronic consumption of a high protein diet does not enhance protein carbonyl concentration in rat plasma (Petzke et al. 1999). However, we also found that after feeding rats a 60% casein diet for only 1 wk, oxidative modified protein-bound amino acids were significantly increased compared with rats fed 15 and 30% casein diets (Petzke et al. 1999). Surprisingly, rats fed a 15% casein diet for

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[2] Abbreviations used: AP, adequate protein level; APE, atom percent excess; DTT. dithiothreitol; GC/MS, gas chromatography/mass spectrometry; GSH, reduced glutathione; HP, high protein level; HP-toc, high protein diet without α-tocopherol acetate; KIC, 2-ketoisocaproate; LeuOX, leucine oxidation; MP, medium protein level; MPE, mole % excess; NOLD, nonoxidative leucine disposal; PB_{Leu}, leucine appearance via protein breakdown; $PROX_{KIC}$, [^{13}C]KIC-derived protein oxidation; Q_{Leu}, leucine flux; SSA, 5-sulfosalicylic acid; TBARS, thiobarbituric acid-reacting substances.

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14 wk showed plasma protein carbonyl levels as high as those in rats that consumed a vitamin E–deficient 60% casein diet. Because the meaning of this finding was unclear, we measured additional variables to further describe the oxidative status of these animals.

MATERIALS AND METHODS

Animals and diets. Male adult Wistar rats (body weight ~230 g, ~3 mo of age, Tierzucht Schönwalde GmbH, Germany) were housed individually in wire-bottomed cages in a room with controlled humidity (60%) and temperature (23°C), and a fixed 12-h light:dark cycle (light 0700 to 1900 h). Before the feeding experiment, all rats had consumed ad libitum a nonpurified pelleted stock diet (Altromin, Lage, Germany; crude protein 190 g/kg, crude fat 40 g/kg, metabolizable energy 11.9 MJ/kg).

Rats ($n = 10$ per group) were randomly assigned to experimental diets containing three different levels of protein. These diets were consumed ad libitum for 18 wk. The composition and components of the test diets are shown in **Table 1**. The experimental diets contained 13.8% (adequate protein; AP), 25.7% (medium protein; MP) or 51.3% (high protein; HP) crude protein with casein as the only protein source. Casein was exchanged isoenergetically by wheat starch. The diets were supplemented with 0.35 g/100 g DL-methionine. The AP, MP and HP diets contained 0.109, 0.109 and 0.119 mg/g *RRR*-α-tocopherol acetate, respectively, which was not significantly different among diets. In addition, a fourth group, which consumed the HP diet but without the *RRR*-α-tocopherol acetate supplement (HP-toc), was included in the feeding experiment to subject the rats to oxidative stress in vivo. The purpose was to compare and contrast rats suffering from oxidative stress due to the lack of a well-documented antioxidative protection factor with rats fed high protein diets that we hypothesized would cause oxidative damage. In wk 18, mean leucine intakes of rats were 582, 982, 1865 and 1855 mg/(kg · d) for the groups fed AP, MP, HP and HP-toc, respectively. Drinking water was consumed ad libitum. Food intake was monitored daily and body weight was monitored weekly. Blood samples were drawn after 15 and 17 wk of feeding the experimental diets. In wk 18, a 4-h [1-^{13}C]leucine infusion study was performed in food-deprived rats, which were subsequently killed (between 1200 and 1400 h) by decapitation during sedation by ether inhalation. Blood and tissue samples were obtained as described below.

TABLE 1

Composition, protein, energy and tocopherol contents of purified test diets

	Adequate protein	Medium protein	High protein	High protein without tocopherol
	g/100 g			
Casein[1]	15	30	60	60
Wheat starch[2]	58	43	13	13
Saccharose[3]	10	10	10	10
Palm kernel fat[4]	3	3	3	3
Soybean oil[5]	2	2	2	2
Cellulose[6]	5	5	5	5
Mineral mixture[7]	5	5	5	5
Vitamin mixture[8]	2[9]	2[9]	2[9]	2
Crude protein	13.8	25.7	51.3	51.3
Gross energy, *kJ/g*	16.8	17.9	19.9	19.9
	mg/100 g			
α-Tocopherol acetate[10]	10.9	10.9	11.9	0
α-Tocopherol[10]	0.11	0.05	0.07	0.04
β+γ-Tocopherol[10]	0.91	0.65	0.39	0.55
δ-Tocopherol[10]	0.42	0.36	0.26	0.33

[1] Dauermilchwerk Peiting GmbH, Landshut, Germany, contained 86% crude protein (% N × 6.38).
[2] Heller u. Strauß, Berlin, Germany.
[3] Nordzucker GmbH, Uelzen, Germany.
[4] Union Deutsche Lebensmittelwerke, Hamburg, Germany.
[5] Kunella-Feinkost GmbH, Cottbus, Germany.
[6] Rettenmeier, Ellwangen, Germany.
[7] Mineral mixture per 100 g diet: Ca, 930 mg; P, 730 mg; Mg, 80 mg; Na, 440 mg; K, 710 mg; S, 170 mg; Cl, 360 mg; Fe, 20 mg; Mn, 10 mg; Zn, 3 mg; Cu, 800 mg; J, 40 mg; F, 400 mg; Se, 20 mg; Co, 10 mg (Altromin GmbH, Lage, Germany).
[8] Tocopherol-deficient vitamin mixture containing 17.5 g/100 g DL-methionine, vitamin content in 100 g diet: A, 0.45 mg; Cholecalciferol, 1.3 mg; K_3, 1 mg; thiamine, 2 mg; riboflavin, 2 mg; B-6, 1.5 mg; B-12, 3 mg; niacin, 5 mg; pantothenate, 5 mg; folic acid, 1 mg; biotin, 20 mg; choline chloride, 100 mg; *p*-aminobenzoic acid, 10 mg; inositol, 10 mg (Altromin GmbH, Lage, Germany).
[9] Addition of *RRR*-α-tocopherol acetate to the tocopherol-deficient vitamin mixture.
[10] Values are means of two extractions each injected twice [taken from Petzke et al. (1999)].

The study was approved by the Ethical Committee on the Use of Animals as Experimental Subjects of the Ministry of Nutrition, Agriculture and Forestry, state Brandenburg, Germany.

Blood and tissue sampling. For the determination of plasma protein carbonyl content after 15 wk of feeding the experimental diets, postabsorptive blood samples were drawn between 0800 and 1000 h into chilled heparinized tubes from the retroorbitalis vein during slight sedation by ether inhalation. Plasma was obtained by centrifugation at 4°C for 10 min at 3000 × *g* (Biofuge fresco, Heraeus Instruments, Osterode, Germany).

For the analysis of plasma and whole-blood total thiol concentration, blood was drawn into prechilled prepared microvessels containing EDTA (potassium salt, Sarstedt AG, Nürnbrecht, Germany) from the retroorbitalis vein after 17 wk of feeding the test diets. An aliquot of 200 μL EDTA-blood was added to prechilled microvessels containing 1 mL hemolysis solution (Sarstedt AG), 60 μL of 0.6 mmol/L dithiothreitol (DTT) and 240 μL dimethyl sulfoxide. Samples were deproteinized after 5 min using 5-sulfosalicylic acid (SSA). After vortexing, incubation for 20 min in the dark at room temperature and centrifugation at 8000 × *g* (4°C, 5 min), 100 μL of the supernatant fraction was stored in liquid nitrogen until analysis. For the determination of free thiols in plasma, EDTA-blood was centrifuged immediately at 4°C for 1 min at 8000 × *g*. About 4 min after blood drawing, 200 μL of plasma was added into chilled tubes containing SSA. Samples were vortexed, stored on ice for 5 min and centrifuged at 8000 × *g* at 4°C for 5 min. The supernatant (100 μL) was stored immediately in liquid nitrogen until analysis.

After the rats were killed (wk 18), blood samples were collected from the trunk into chilled heparinized tubes and centrifuged at 4°C for 10 min at 3000 × *g*. Plasma was stored in liquid nitrogen until analysis of α-tocopherol concentrations, [1-^{13}C]2-ketoisocaproate (KIC) enrichment and amino acid concentrations. Liver samples were removed and immediately stored in liquid nitrogen until analysis of TBARS and reduced glutathione (GSH).

[1-^{13}C]leucine infusion study. Rats were placed into metabolic chambers to allow a constant infusion of [1-^{13}C]leucine into a lateral tail vein. Movement was restricted by a wire frame that prevented the rats from turning around. The rats usually slept during the infusion period between 0800 and 1200 h and were in the postabsorptive state. Air, flowing at a rate of 0.5 L/min, was passed through each of four parallel chamber lines by vacuum pumps (model N035AN.18, KNF Neuberger, Freiburg, Germany). The chamber system consisted of a flowmeter (Bayley Fischer Porter, Göttingen, Germany), a CO_2 trap (filled with breath lime, Zeneca GmbH, Plankstadt, Germany), the metabolic chamber, a gas meter and a second flowmeter to measure air flow and volume and to eliminate CO_2 from the inflowing air. Breath samples for determination of $^{13}CO_2$ production and ^{13}C-enrichment from each chamber were collected in triplicate into evacuated tubes (Labro Limited, Bucks, UK). Baseline samples were taken at −30, −15 and 0 min before and then at 15-min intervals during the [1-^{13}C]leucine infusion. After the rat was placed into the

wire frame, the catheter was placed into a lateral tail vein and the catheter tube was attached via a chamber port to the infusion pump (Lineomat, MLW Medizinische Geräte, Chemnitz, Germany).

After the bicarbonate pool was primed with 100 μL of ^{13}C-sodium bicarbonate solution (3.73 μmol/kg$^{0.75}$, 99 atom%, Cambridge Isotope Laboratories, Andover, MA), a primed ([1-^{13}C]leucine, 99 atom%; MassTrace, Woburn, MA, 5.79 μmol/kg$^{0.75}$) constant intravenous infusion of [1-^{13}C]leucine [7 μmol/(kg$^{0.75}$ · h); 1.2 mL/h] dissolved in physiologic saline was given. The precise tracer amount infused was determined by weighing the syringe before and after termination of the infusion.

Assays and calculations. [1-^{13}C]KIC enrichment was measured as the quinoxalinol-*N*-methyl-*N*-(*tert*-butyldimethylsilyl) derivative (El-Khoury et al. 1994, Matthews et al. 1982) using gas chromatography/mass spectrometry (GC/MS) (SSQ 710, Finnigan MAT GmbH, Bremen, Germany) coupled with a GC Varian 3400 (Varian Chromatography Systems, Walnut Creek, CA). Sample (1 μL, splitless injection) separation was performed on a DB-5 capillary column (30 m, 0.25 mm, 0.25 μm; J&W Scientific, Folsom, CA), temperature programmed from 100°C (0.5 min) to 280°C (30°C/min, 4.5 min isothermic). Natural and [1-^{13}C]KIC signals appeared after 6.3 min and were monitored at *m/z* 259 and 260 by selected ion monitoring. Tracer/tracee ratios were calculated from m + 1 and m + 0 area ratios. Graded mixtures of [1-^{13}C]KIC and unlabeled KIC over a 0–10 mol fractional range corrected for baseline were used for calibration purposes.

Breath samples were analyzed by isotope ratio MS (Breath MAT, Finnigan MAT GmbH, Bremen, Germany). The measurement of CO_2 production (V_{CO2}) was based on the CO_2 concentration (%CO_2) of each breath sample (Breath MAT) and the chamber air flow rate. CO_2 was separated from N_2 and O_2 using a 2-m HayeSep-D packed steel column. CO_2 peak areas (*V*s) were converted into %CO_2 using a calibration curve corrected by a factor based on a previously measured external standard (%$CO_{2\ corr}$). Further, CO_2 concentration was converted into standard volume $CO_{2\ stand}$ (L/min) using the following equation:

$$CO_{2\,stand} = (\%CO_{2\,corr} \times FR/10^2) \times (T_{stand} \times P)/[P_{stand} \times (T_{stand} + T)] \times Rf$$

where *FR* is the flow rate through the metabolic chamber (L/h), *P* is the air pressure (torr), P_{stand} is 760 Torr, *T* is the room temperature on the day of experiment (°C) and T_{stand} is 273.16 °C. *Rf* is the chamber specific recovery factor for CO_2, which was determined experimentally by injecting known amounts of CO_2 (50, 60 and 70 mL) into the chamber. The mean *Rf* was 67.84 ± 1.83% and was independent of the amount of CO_2 injected (Thielecke 1997). The standard volume $CO_{2\ stand}$ was converted into V_{CO2} [μmol/(kg · h) as follows:

$$V_{CO2} = (CO_{2\,stand}/22.26) \times 10^3/BM$$

where 22.26 is the molar volume of CO_2 (L), and BM is the body mass (kg).

Leucine oxidation (LeuOX) and leucine flux (Q_{Leu}), nonoxidative leucine disposal (NOLD) and protein breakdown (PB_{Leu}) were calculated as described (El-Khoury et al. 1995) as

$$\text{LeuOX}\ [\mu\text{mol/(kg} \cdot \text{h)}] = {}^{13}CO_2\ \text{production}\ [\mu\text{mol/(kg} \cdot \text{h)}] \times 10^2/E_{KIC}$$

where E_{KIC} is the ^{13}C plasma KIC enrichment (MPE, mole % excess) and

$$^{13}CO_2\ \text{production}\ [\mu\text{mol/(kg} \cdot \text{h)}] = V_{CO2}\ [\mu\text{mol/(kg} \cdot \text{h)}] \times {}^{13}CO_2\ \text{enrichment (APE, atom \% excess} \times 10^3) \times 10^{-5} \times R^{-1}$$

where V_{CO2} and $^{13}CO_2$ enrichment (corrected for $^{13}CO_2$ background enrichment) were averaged during plateau ^{13}C enrichment of breath (75–240 min). Plateau enrichment was defined by the absence of a significant slope. For *R* (the ^{13}C fraction recovered in breath during the experimental time when ^{13}C bicarbonate was infused), a value of 0.79 was taken from the literature (El-Khoury et al. 1995, Leijssen and Elia 1996). E_{KIC} above baseline enrichment was determined in plasma collected at the end of the 240-min infusion study. Baseline [1-^{13}C]KIC tracer/tracee ratios were measured in plasma of postabsorptive rats subjected to the same feeding regimen but without receiving the [1-^{13}C]leucine tracer. Leucine flux Q_{Leu} was calculated as

$$Q_{Leu}\ [\mu\text{mol/(kg} \cdot \text{h)}] = i/(E_i/E_{KIC} - 1)$$

where *i* is the rate of [1-^{13}C]leucine infused [μmol/(kg · h)] and E_i is the tracer enrichment (atom %).

Nonoxidative leucine disposal (NOLD) via protein synthesis was computed as follows:

$$\text{NOLD}\ [\mu\text{mol/(kg} \cdot \text{h)}] = Q_{Leu} - \text{LeuOX}$$

and leucine appearance via protein breakdown PB_{Leu} was calculated as

$$PB_{Leu}\ [\mu\text{mol/(kg} \cdot \text{h)}] = Q_{Leu} - i$$

Leucine oxidation was converted to whole-body protein oxidation ($PROX_{KIC}$) assuming 7.6% leucine in whole-body mixed proteins of rats (Obled and Arnal 1991) using the following equation:

$$PROX_{KIC}\ [\text{mg/(kg} \cdot \text{h)}] = \text{LeuOX}\ [\text{mg/(kg} \cdot \text{h)}] \times 100/7.6$$

α-Tocopherol concentrations in plasma and food samples were analyzed after extraction with hexane by HPLC analysis as described earlier (Schultz et al. 1995).

Lipid peroxidation was estimated in liver and plasma samples as the concentration of TBARS using a modification of the method of Ohkawa et al. (1979). Frozen liver tissue was pulverized in liquid nitrogen. Samples of 10–30 mg powdered liver or 50 μL plasma were added to 100 μL of 0.28 mol/L sodium dodecylsulfate, 1 mL of 3.33 mol/L acetic acid (adjusted to pH 3.5 using sodium hydroxide) and 1 mL of 46.5 mmol/L thiobarbituric acid (aqueous solution). The mixture was vortexed vigorously and incubated at 95°C for 60 min. After cooling, 2 mL was added to a mixture of *n*-butanol and pyridine (15:1, v/v) and vortexed vigorously. After centrifugation at 3000 × *g* for 10 min at 20°C, the organic layer was taken and its absorbance difference was measured at 535 and 510 nm (UVIKON 932, Kontron Instruments GmbH, Neufahrn, Germany) as the TBARS value using 1,1,3,3-tetraethoxypropane as an external standard.

Carbonyl concentrations in plasma proteins were determined on the basis of a modified HPLC gel filtration procedure of Levine et al. (1994) using 2,4-dinitrophenylhydrazine (10 mmol/L) dissolved in guanidine hydrochloride buffer solution as eluent essentially as described (Petzke et al. 1999).

The measurement of reduced thiol (GSH, cysteine) concentrations in plasma, whole-blood and liver tissue samples was performed using monobromobimane according to Jahoor et al. (1995) with some modifications. SSA-treated plasma and whole-blood samples were prepared as described above. For derivatization with monobromobimane, 100 μL of SSA-treated plasma was mixed with 450 μL of 25 mmol/L Tris-HCl buffer (pH 8.0 at 20°C), 30 μL of 1 mol/L sodium hydroxide, 10 μL of 1.38 mol/L SSA and with 10 μL of 20 mmol/L monobromobimane in acetonitrile. Before the monobromobimane solution was added, the mixture was checked to be slightly above neutral pH (Slordal et al. 1993). Frozen liver tissue was powdered in liquid N_2 and aliquots (0.05–0.1 g) were added to 1 mL of Tris-HCl buffer (25 mmol/L, pH 8.0 at 20°C, 5 mmol/L EDTA, 20 mmol/L DTT, 140 mmol/L dimethyl sulfoxide). Then the samples were vortexed (5°C, 15 min, 1000 min^{-1}, Thermomixer comfort, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) and 100 μL of 1.97 mol/L SSA was added; after vortexing, the mixture was centrifuged (4°C, 10000 × *g*, 10 min). The supernatant was frozen in liquid N_2 until analysis. SSA-treated whole-blood samples or liver tissue extract (10 μL) was mixed with 580 μL 25 mmol/L Tris-HCl buffer (pH 8.0 at 20°C) and with 10 μL of 20 mmol/L monobromobimane in acetonitrile. The derivatization mixtures (20 μL) were injected and

TABLE 2

Body weight gain and liver weight, gross energy and nitrogen intake, and protein concentrations in liver and muscle of rats fed diets with different protein concentration for 18 wk[1,2]

	Adequate protein	Medium protein	High protein	High protein without tocopherol
Body weight gain, *g*	239 ± 29[ab]	251 ± 44[ab]	254 ± 21[b]	226 ± 25[a]
Total gross energy intake, *MJ*	44.4 ± 3.5[a]	45.4 ± 5.1[ab]	51.8 ± 1.5[c]	49.8 ± 5.5[bc]
Total nitrogen intake, *g*	57.2 ± 4.6[a]	102.4 ± 11.6[b]	210.3 ± 6.3[c]	201.3 ± 22.1[c]
Energy efficiency,[3] *g/MJ*	5.37 ± 0.53[b]	5.49 ± 0.50[b]	4.90 ± 0.36[a]	4.56 ± 0.44[a]
Liver weight, *g*	10.32 ± 1.03[a]	10.48 ± 1.39[ab]	11.61 ± 1.14[b]	10.99 ± 0.91[ab]
Liver protein, *g*	2.14 ± 0.22[a]	2.35 ± 0.29[ab]	2.54 ± 0.21[b]	2.43 ± 0.23[b]
Liver protein, *g/100 g liver*	20.76 ± 0.82[a]	22.46 ± 0.65[b]	21.94 ± 0.61[b]	22.14 ± 0.62[b]
Liver protein, *g/kg body*	4.62 ± 0.34[a]	4.87 ± 0.44[a]	5.40 ± 0.54[b]	5.37 ± 0.32[b]
Skeletal muscle protein, *g/100 g muscle*	21.33 ± 0.29[a]	21.91 ± 0.43[b]	21.15 ± 0.36[a]	21.66 ± 0.50[ab]

[1] Values are means ± SD, $n = 10$. Within a row, values without a common superscript differ, $P < 0.05$.
[2] For food composition see Materials and Methods, and Table 1.
[3] Body weight gain (g) divided by energy intake (MJ).

measured between 30 min and 4 h after the addition of monobromobimane by HPLC (System Gold, Beckman Instruments GmbH, Munich, Germany) equipped with a fluorescence detector (RF-551, Shimadzu Deutschland GmbH, Duisburg, Germany) and an ODS Hypersil column, 5 μm, 4.6 × 200 mm (Hewlett-Packard GmbH, Waldbronn, Germany). Elution of thiols was accomplished over 35 min by a linear gradient of 3% acetonitrile to 13.5% acetonitrile in 1% acetic acid in water between 1 and 26 min and was held 8 min at 13.5% acetonitrile (flow rate 1 mL/min). Retention time of reduced GSH under these conditions was 24 min. The detector was operated at an excitation wavelength of 375 nm and emission wavelength of 465 nm.

Plasma free amino acid concentrations were analyzed by ion-exchange chromatography with postcolumn ninhydrin detection (Trione ninhydrin reagent, Pickering Laboratories, Mountain View, CA) using HPLC units (Beckman Instruments GmbH), equipped with a high efficiency analytical column (3 × 150 mm, Pickering Laboratories) and a step-change elution method using lithium eluents (Laborservice Onken, Gründau, Germany).

Colorimetric and enzymatic standard methods were used for the determination of plasma creatinine, glucose, triglycerides, cholesterol, total protein and albumin in plasma (Cobas Mira S, Hoffmann-La Roche AG, Grenzach-Whylen, Germany).

Nitrogen content of diets was determined by a standard micro-Kjeldahl method (AOAC 1990, Proll et al. 1998) and a protein conversion factor of 6.38 was used. Gross energy of diets was determined by means of an adiabatic bomb calorimeter (IKA-Calorimeter C4000, Janke & Kunkel, IKA Analysentechnik, Heitersheim, Germany).

Chemicals were purchased from several suppliers (Sigma-Aldrich Chemie GmbH, Deisenhofen; Merck KGaA, Darmstadt, Germany; Fluka Chemie AG, Buchs, Switzerland) unless otherwise stated and were all of analytical grade.

Statistical analysis. Data are reported as means ± SD. Differences between mean values were determined by ANOVA followed by comparisons using the Newman-Keuls multiple range test (Weber 1972). Differences with $P < 0.05$ were considered significant.

RESULTS

Characteristics of animals. Rats fed high protein levels (MP, HP) did not gain significantly more body weight than rats fed the adequate protein diet (AP) (**Table 2**). Depending on the different protein-containing diets, the protein efficiency calculated (mean g body weight gain/g of mean dietary nitrogen intake) was 4.2, 2.5, 1.2, and 1.1 for AP, MP, HP and HP-toc fed groups, respectively. Energy efficiency (g body weight gain/MJ food energy consumed) was significantly lower in rats fed both HP diets compared with those fed the AP and MP diets. Gross energy intake was higher in rats fed the HP diet compared with those fed the other diets. Liver weight and liver protein concentrations were slightly but significantly higher at higher dietary protein concentrations. The 3.7 times higher nitrogen intake by rats fed the HP diet compared with the AP diet corresponded to a 20% higher total liver protein mass (g/kg body). The α-tocopherol–deficient diet, HP-toc, resulted in a significantly lower weight gain relative to the HP diet–fed rats but liver weight and liver and muscle protein concentrations did not differ. Muscle protein concentration was slightly but significantly higher in rats fed the MP diet.

Plasma creatinine, glucose and triglyceride concentrations in the AP group did not differ from the MP and HP groups (data not shown). However, plasma total protein and albumin concentrations were significantly lower in rats fed the HP diet compared with those fed the AP and MP diets. There was a trend ($P = 0.07$) for lower total plasma cholesterol concentrations in the vitamin E–deficient rats compared with those fed the HP diet (1.09 ± 0.34 vs. 1.39 ± 0.36 mmol/L, respectively).

Variables of oxidative status. The lipid peroxide levels in liver and the reactive carbonyl concentration in plasma protein were significantly higher in the group consuming the AP diet in comparison to the MP and HP diets (**Table 3**). The reactive carbonyl residue concentration in plasma proteins was as high as that in rats fed HP-toc. The liver lipid peroxide level was enhanced by ~80% in the HP-toc group compared with the rats fed the MP and HP diets. The concentration of free plasma cysteine was significantly higher in rats fed the HP diet. The total plasma GSH concentrations were not significantly different among the experimental groups. However, the total blood GSH concentrations were significantly lower by ~15% in rats fed the HP-toc diet compared with those fed the AP, MP and HP diets. Liver GSH concentrations were significantly lower in rats fed the AP diet.

Plasma amino acid concentrations. Most of the dispensable plasma amino acid concentrations were significantly lower in rats fed the MP diet compared with those fed the AP diet in the postabsorptive state (**Table 4**). With the exception of glycine, citrulline and histidine, a further increase in the dietary protein level (HP) had no significant effect on the amino acid concentrations in relation to the MP protein diet. Among the indispensable amino acid concentrations, only

TABLE 3

Lipid peroxide, reactive carbonyl residues, α-tocopherol, and thiol concentrations in rats fed diets with different protein concentrations for 18 wk[1,2]

	Adequate protein	Medium protein	High protein	High protein without tocopherol
Lipid peroxides				
Liver, *nmol/g*	376.9 ± 74.9[b]	263.6 ± 70.8[a]	296.7 ± 87.4[a]	496.6 ± 149.3[c]
Plasma, *mmol/L*	10.3 ± 2.6	9.0 ± 1.1	9.0 ± 2.9	10.2 ± 2.6
Reactive carbonyl residues				
Plasma protein, *μmol/g*	3.72 ± 1.56[b]	2.43 ± 1.06[a]	2.55 ± 0.77[a]	3.75 ± 1.68[b]
α-Tocopherol[3]				
Plasma, *μmol/L*	27.0 ± 4.5[b]	27.5 ± 5.7[b]	31.3 ± 4.8[b]	3.9 ± 2.4[a]
Thiols				
Plasma free cysteine, *μmol/L*	18.2 ± 4.5[a]	18.5 ± 4.4[a]	23.1 ± 3.1[b]	22.1 ± 3.3[ab]
Plasma GSH,[4] *μmol/L*	14.2 ± 4.3	15.3 ± 3.6	17.1 ± 3.5	15.3 ± 3.2
Whole blood GSH, *μmol/L*	421 ± 57[b]	453 ± 51[b]	427 ± 67[b]	372 ± 42[a]
Liver GSH, *μmol/g*	4.44 ± 0.63[a]	5.30 ± 0.54[b]	5.38 ± 0.71[b]	5.24 ± 0.63[b]
Total liver GSH, *μmol*	46.0 ± 8.7[a]	55.6 ± 9.7[b]	62.8 ± 12.7[b]	57.6 ± 8.0[b]

[1] Values are means ± SD, $n = 10$. Within a row, values without a common superscript differ, $P < 0.05$.
[2] For food composition see Materials and Methods, and Table 1.
[3] Taken from Petzke et al. (1999).
[4] GSH, reduced glutathione.

cystine and tyrosine were significantly higher at the highest dietary protein level (HP). Arginine concentrations followed the same pattern. Plasma lysine concentrations were significantly higher at both the lower (AP) and high (HP) dietary protein levels in comparison to rats fed the MP protein diet. The α-tocopherol–deficient diet did not significantly affect the postabsorptive amino acid concentrations in plasma at the high dietary protein level of 51.3% casein. The plasma concentrations of aspartic acid, asparagine, glutamic acid, proline, isoleucine, phenylalanine, tryptophan, taurine and urea were not different among diet groups (data not shown).

Leucine kinetics. Postabsorptive leucine kinetic parameters (**Table 5**) were calculated on the basis of $^{13}CO_2$ production rate, [1-^{13}C]leucine tracer infusion rate and [1-^{13}C]KIC enrichment (data not shown). Breath $^{13}CO_2$ enrichment increased with increasing dietary protein (leucine) content (4.83 ± 1.37, 6.28 ± 1.49 and 7.00 ± 1.70 APE × 10^3, for AP, MP and HP, respectively). The corresponding plasma [1-^{13}C]KIC enrichment decreased slightly ($P = 0.10$) with increasing dietary casein concentration (2.01 ± 0.36, 1.79 ± 0.31, 1.62 ± 0.22 MPE × 10^2, for AP, MP and HP, respectively).

As expected, leucine and protein oxidation rates were significantly enhanced by increasing concentrations of casein in the diets (Table 5). Leucine flux and PB_{Leu} showed a tendency to increase with higher protein levels, whereas NOLD was not different among the AP, MP and HP groups. Compared with the diet group with an adequate α-tocopherol intake (HP), there was a striking increase in postabsorptive leucine oxidation, flux and NOLD rates, respectively, with administration of the HP-toc diet.

TABLE 4

Postabsorptive plasma free amino acid concentrations of rats fed diets with different protein concentrations for 18 wk[1,2]

	Adequate protein	Medium protein	High protein	High protein without tocopherol
		μmol/L		
Threonine	263 ± 60[b]	163 ± 20[a]	146 ± 16[a]	152 ± 17[a]
Lysine	351 ± 39[b]	296 ± 35[a]	334 ± 38[b]	332 ± 53[ab]
Valine	212 ± 41[a]	238 ± 26[ab]	246 ± 30[ab]	252 ± 33[b]
Leucine	177 ± 29[a]	199 ± 16[ab]	200 ± 24[ab]	209 ± 32[b]
Cystine	30 ± 5[a]	33 ± 4[ab]	35 ± 4[b]	35 ± 4[b]
Methionine	39 ± 8[ab]	36 ± 4[a]	42 ± 6[b]	41 ± 4[b]
Tyrosine	41 ± 7[a]	42 ± 5[a]	51 ± 9[b]	50 ± 7[b]
Histidine	65 ± 7[b]	62 ± 4[b]	56 ± 3[a]	58 ± 5[a]
Serine	223 ± 33[b]	173 ± 19[a]	165 ± 20[a]	170 ± 16[a]
Glutamine	788 ± 87[b]	689 ± 66[a]	654 ± 84[a]	658 ± 68[a]
Glycine	302 ± 78[c]	198 ± 29[b]	171 ± 24[a]	172 ± 22[a]
Alanine	209 ± 49[a]	206 ± 42[a]	237 ± 38[ab]	259 ± 53[b]
Citrulline	93 ± 19[c]	70 ± 15[b]	57 ± 5[a]	55 ± 6[a]
Ornithine	45 ± 13[c]	29 ± 2[b]	24 ± 10[b]	23 ± 3[a]
Arginine	86 ± 11[a]	85 ± 9[a]	97 ± 9[b]	104 ± 20[b]

[1] Values are means ± SD, $n = 10$. Within a row, values without a common superscript differ, $P < 0.05$.
[2] For food composition see Materials and Methods, and Table 1.

TABLE 5

Postabsorptive leucine kinetic parameters in rats fed diets with different protein concentrations for 18 wk[1,2]

	Adequate protein	Medium protein	High protein	High protein without tocopherol
Leucine oxidation, *μmol/(kg · h)*	74.9 ± 28.5[a]	109.9 ± 35.2[b]	142.3 ± 38.4[b]	186.9 ± 43.7[c]
Leucine flux, *μmol/(kg · h)*	425 ± 102[a]	483 ± 82[a]	505 ± 80[a]	675 ± 220[b]
Leucine oxidation, *% flux*	17.8 ± 6.4[a]	23.7 ± 7.3[ab]	28.1 ± 6.2[b]	29.0 ± 6.8[b]
Non-oxidative leucine disposal, *μmol/(kg · h)*	350 ± 95	369 ± 86	363 ± 63	488 ± 188
Leucine appearance via protein breakdown, *μmol/(kg · h)*	417 ± 101[a]	475 ± 82[a]	497 ± 80[a]	666 ± 220[b]
Protein oxidation, *mg/(kg · h)*	124 ± 47[a]	182 ± 58[b]	235 ± 63[b]	309 ± 72[c]

[1] Values are means ± SD, $n = 10$. Within a row, values without a common superscript differ, $P < 0.05$.
[2] For food composition see Materials and Methods, and Table 1.

DISCUSSION

In this study, the long-term intake of high protein diets (MP, HP) did not lead to an increase in variables of oxidative stress (Table 3). This is in contrast to our hypothesis. We assumed that higher rates of substrate (amino acid) oxidation caused by an intake that considerably exceeded the actual needs is related to an increased electron flow along the mitochondrial respiratory chain, resulting in oxidative stress (Petzke et al. 1999). Nevertheless, as expected, amino acids taken up in excess (MP, HP diets) were oxidized (Harper 1994, Moundras et al. 1993, Petzke et al. 1986). We observed significantly higher rates of leucine oxidation as the protein (or leucine) component of the diets was increased above requirement, which was similar to previous studies in postabsorptive rats using continuous ^{14}C leucine infusion (Harper and Benjamin 1984, Laurent et al. 1984, Yagi and Walser 1990).

The lower energy efficiency (Table 2) during the HP diet in contrast to the AP diet may indicate a less efficient ATP production per liter of oxygen consumed or an increase in the thermogenic response due to uncoupled respiration. Unfortunately, calorimetric data were not available, but other studies have shown increased energy expenditure and postprandial thermogenesis during acute feeding of protein or administration of amino acids relative to other nutrients (Crovetti et al. 1997, Tappy et al. 1993, Westerterp et al. 1999). Both the increase in amino acid oxidation and the reduced energy efficiency support our hypothesis concerning the higher rates of substrate oxidation caused by excessive intake of protein; however, our results do not show an increase in oxidative stress variables (TBARS, reactive carbonyl concentration in plasma proteins) or a compromised concentration of GSH when an adequate amount of antioxidants is supplied. It has been discussed that a redox energy dissipating proton leak down the electrochemical gradient across the inner mitochondrial membrane may control the free radical production (Rolfe and Brand 1997, Rolfe et al. 1999, Waterlow 1999), but it remains to be elucidated whether this mechanism is activated during adaptation to a high protein intake.

Surprisingly, chronic feeding of the AP diet resulted in significantly higher values of oxidative stress indicators (TBARS concentration in liver, reactive carbonyl concentrations in plasma protein) and a lower liver GSH concentration. This was an unexpected finding because the AP diet should provide an adequate amount of protein for adult rats (NRC 1995). Although the mechanism for this effect remains unclear, it cannot be ruled out that the AP diet providing 13.8% crude protein as casein and supplemented with 0.35% DL-methionine was insufficient to meet the metabolic needs for amino acids with respect to maintenance of oxidative stress defense. However, our growth data (Table 2) indicate that the rats in the AP group gained weight continuously and in a manner comparable to the other groups during the whole experimental period. In earlier rat studies, it was shown that a dietary protein concentration of 10–15% is required for maximum growth in young rats when a low fiber diet with a balanced amino acid pattern and an adequate fat content is fed (NRC 1995). Later, it was demonstrated that 19% unsupplemented casein (17% crude protein) in the diet was necessary to give 95% of the maximum growth response. For maintenance, ~5% high quality protein was suggested to be sufficient for rats (NRC 1995). However, when variables other than growth are used to judge adequacy of protein intake in rats, such as a component of the antioxidative system, tissue GSH concentrations and plasma GSH turnover rates were lower with a protein intake <20% (Darmon et al. 1993, Deneke et al. 1983, Hum et al. 1992, Rana et al. 1996). Further, enhanced TBARS concentrations and reduced activities of antioxidant enzymes were determined under conditions of feeding low protein diets (5–8% lactalbumin or casein) (Huang et al. 1992, Rana et al. 1996, Tandon et al. 1998). In this context, it is interesting to note that the GSH concentration was depressed drastically in protein-energy malnourished rats (Goss et al. 1994) and that the classic clinical features of protein-energy malnutrition were suggested to result from a peroxidative damage to biomembrane integrity due to low GSH concentrations (Golden and Ramdath 1987). Thus, an adequate dietary protein supply for adult rats might be in the range of 13.8–25.7% protein when judged on the basis of oxidative/antioxidative balance or on liver GSH concentration. This relates to the suggestion made by Reeds and Hutchens (1994) that functional variables are more relevant than nitrogen balance in judging dietary protein adequacy in adult animals.

Reactive plasma protein carbonyl concentration and the concentration of liver TBARS were similarly enhanced after feeding the diet HP-toc as with the AP diet (Table 3). The effect seen in the HP-toc diet was presumably due to the impaired antioxidant status in chronic vitamin E deficiency. The effect was also confirmed by significantly lower whole-blood GSH concentrations in the groups fed the HP-toc diet in comparison to the groups fed the AP, MP and HP diets. As shown (Table 3), the plasma α-tocopherol concentration in rats fed the vitamin E–inadequate diet for 18 wk was only 15% that of the vitamin E–adequate diet groups. Modifications of protein molecules and increased TBARS concentration in tissues were described previously as a consequence of oxidative

stress due to vitamin E deficiency (Awad et al. 1994, Ibrahim et al. 1997, Palamanda et al. 1993).

We also found that vitamin E deficiency (HP-toc) led to significantly greater leucine kinetic variables (Table 5) than those of the HP group. This might indicate that modifications of protein molecules due to oxidative stress could be a signal to trigger an increase in protein turnover. This view is supported by studies showing an enhanced susceptibility of oxidatively modified proteins to proteolytic digestion (Ayala et al. 1996, Grune et al. 1997, Nagasawa et al. 1997). Further, in vitamin E–deficient rabbits, an increase in protein turnover, measured by the incorporation of ^{14}C leucine and ^{14}C glycine in muscle, liver and plasma proteins, was observed (Diehl 1986, Diehl and Delincée 1986). As shown in vitro and in vivo, oxidative modifications of protein-bound amino acid residues caused by various free radical–mediated reactions resulted in changes of physicochemical properties of proteins and loss of protein functions (Chao et al. 1997, Daneshvar et al. 1997, Grune et al. 1997). In fibroblasts, exposure to hydrogen peroxide increased protein turnover and reduced oxidatively modified proteins (Sitte et al. 1998) Therefore, increased protein turnover rates during conditions of oxidative stress, such as during chronic vitamin E deficiency, may be interpreted as an efficient component of the defense system to keep the concentration of oxidatively damaged proteins at a relatively low level.

In conclusion, our results show that chronic ingestion of high protein diets well above requirement (25.7 or 51.3% crude protein, casein) does not lead to oxidative stress in adult rats when diets are adequate in antioxidants, in contrast to chronic feeding of an adequate protein diet (13.8% crude protein). Further, an increased protein turnover rate may counteract the in vivo accumulation of oxidatively damaged protein molecules at relatively high dietary protein intakes.

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