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Research Communications

Plasma protein carbonyl concentration is not enhanced by chronic intake of high-protein diets in adult rats

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We tested the hypothesis of whether high dietary protein intake is linked to oxidative stress as measured by the concentration of reactive carbonyl residues in plasma proteins. Three groups of male Wistar rats (~230 g, n = *10) were fed either 15% (15C), 30% (30C), or 60% (60C) casein diets over a period of 18 weeks. For comparison, a vitamin E deficient diet (60C-E) based on diet 60C was given to an additional group to provoke oxidative stress. Concentrations of* α*-tocopherol in plasma and of reactive carbonyl residues in total plasma proteins were measured by high performance liquid chromatography using fluorescence and by diode array detection after 2,4-dinitrophenylhydrazine reaction, respectively. After 1 week the concentration of reactive carbonyl residues in plasma proteins was found to be significantly (*$P < 0.05$*) higher in the 60C and 60C-E groups (~2.7 nmol/mg protein) compared with the 15C and 30C groups (~1.7 nmol/mg protein). After 14 weeks the 15C (3.4 ± 1.2 nmol/mg protein) and 60C-E groups (3.9 ± 1.7 nmol/mg protein) showed a significantly increased concentration of reactive carbonyl residues in plasma protein compared with the 30C and 60C groups (2.5 ± 1.0 nmol/mg protein; 2.6 ± 0.8 nmol/mg protein). As expected, chronic vitamin E deficiency (60C-E) resulted in significantly decreased* α*-tocopherol concentrations (3.91 ± 2.42* μ*mol/mL vs. 31.3 ± 4.8* μ*mol/mL) and a higher concentration of reactive carbonyl residues in plasma proteins. These results do not support the hypothesis that a chronic intake of high-protein diets leads to oxidative stress in adult rats. However, in the non-adapted state (1 week) a high protein intake contributes to oxidative modifications of protein-bound amino acid residues.* (J. Nutr. Biochem. 10:268–273, 1999)

Keywords: rat; high-protein diet; vitamin E deficiency; oxidative stress; plasma protein oxidation; reactive carbonyl derivatives

Introduction

Nutrition societies are now compelled to define the maximum levels of nutrient intake that will not cause adverse effects to almost all of the healthy population ingesting that level.[1] For protein intake this is a difficult task because to date the scientific literature does not indicate unequivocally that there are detrimental health effects due to chronic dietary intake of amounts of protein well above defined requirements in healthy individuals.[2] In some pathologic conditions or critically ill patients (i.e., kidney disease) high protein intakes are known to have negative effects.[3,4]

It has been shown that an intake of dietary protein above the actual needs results in increased amino acid oxidation and urea synthesis.[5–7] Further, thermogenic effects have been observed after administration of high protein diets, which decrease the nutritional efficiency of energy utilization.[8–10] The reoxidation of reducing equivalents derived from amino acid oxidation is linked to the mitochondrial redox chain.[5] It is possible that free radical generation during mitochondrial oxygen reduction may lead to oxidative stress if the antioxidant potential is insufficient to

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Table 1 Composition of the purified test diets

	15C (15% casein)	30C (30% casein)	60C (60% casein)	60C-E (60% Casein, tocopherol deficient)
Ingredient				
Casein*	15	30	60	60
Wheat starch†	58	43	13	13
Saccharose‡	10	10	10	10
Palm kemel fat§	3	3	3	3
Soy bean oil‖	2	2	2	2
Cellulose**	5	5	5	5
Mineral mixture††	5	5	5	5
Vitamin mixture‡‡	2§§	2§§	2§§	2
Gross energy (kJ/g)	16.8	17.9	19.9	19.9
Crude protein (%) (% N × 6.38)	13.8	25.7	51.3	51.3

*Dauermilchwerk Peiting GmbH (Landshut, Germany) contained 86% crude protein (% N × 6.38).
†Heller u. Strauß (Berlin, Germany).
‡Nordzucker GmbH (Uelzen, Germany).
§Union Deutsche Lebensmittelwerke (Hamburg, Germany).
‖Kunella-Feinkost GmbH (Cottbus, Germany).
**Rettenmeier (Ellwangen, Germany).
††Mineral mixture per 100 g diet: calcium, 930 mg; phosphorous, 730 mg; magnesium, 80 mg; sodium, 440 mg; potassium, 710 mg; sulfur, 170 mg; chloride, 360 mg; iron, 20 mg; manganese, 10 mg; zinc, 3 mg; copper, 800 mg; J, 40 mg; fluoride, 400 mg; selenium, 20 mg; Co, 10 mg (Altromin GmbH, Lage, Germany).
‡‡Tocopherol-deficient vitamin mixture containing 17.5% DL-methionine, vitamin content in 100 g diet: A, 0.45 mg; D_3, 1.3 mg; K_3 1 mg; B_1, 2 mg; B_2, 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).
§§Addition of α-tocopheryl acetate to the tocopherol-deficient vitamin mixture. For content see *Table 2*.

quench the extra free radical production. One consequence is the oxidative modification of protein-bound amino acid residues caused by various kinds of free radical mediated reactions resulting in, for example, the formation of reactive carbonyl derivatives, changes in physicochemical properties of proteins, and losses of protein functions as shown in vitro and in vivo.[11–14]

In growing rats an increase of protein intake from a subadequate level of 5 to 20% casein fed for 12 weeks was accompanied by an increase of liver protein damage.[15] It is hypothesized that a dietary protein intake above actual needs may cause a situation of physiologic oxidative stress that leads to oxidative damage of macromolecules. The present study explores whether a high protein but otherwise adequate diet leads to oxidative modifications of plasma proteins in rats in vivo. For comparative purposes a vitamin E deficient diet with a high protein content was included in this study to weaken the antioxidative defense.

Methods and materials

Animals and diets

Male adult albino Wistar rats (body weight ~230 g; 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-hour light-dark cycle (7:00 AM to 7:00 PM light). Prior to the feeding experiment all animals had received a nonpurified pelleted stock diet (Altromin, Lage, Germany; crude protein 190 g/kg, crude fat 40 g/kg, metabolizable energy 11.9 MJ/kg) ad libitum. Depending on the study group to which the animals were randomly assigned ($n = 10$), animals received isoenergetic test diets ad libitum that contained either 15% (15C), 30% (30C), or 60% (60C) casein (*Table 1*). The diet was supplemented with 0.35% DL-methionine (w/w). An additional group (60C-E) was fed the 60C diet but did not receive α-tocopheryl acetate (*Table 2*). This test diet was included in the feeding experiment in order to subject the animals to oxidative stress in vivo. This was done to compare and contrast animals that suffered from oxidative stress due to the lack of an anti-oxidative protection factor to animals under conditions of high protein intake only. The food was mixed with two parts water (w/w) immediately before feeding. Drinking water was provided ad libitum. The experiment lasted for 18 weeks. Food intake was monitored daily and body weight was monitored weekly. The study was approved by the Ethical Committee on the Use of Animals as Experimental Subjects of the Ministry of Nutrition, Agriculture and Forestry of the Government of the Brandenburg Country in Germany.

Plasma collection

For the determination of plasma protein carbonyl content, blood was drawn from the retroorbitalis vein into chilled heparinized tubes by venipuncture during slight sedation by ether inhalation after 1 week and after 14 weeks of feeding the test diets between 8:00 AM and 10:00 AM when animals were in the fed state. Plasma

Table 2 Tocopherol content of diets*

	15C	30C	60C	60C-E
	(mg × 100 g^{-1})			
α-Tocopherol	0.11	0.05	0.07	0.04
β + γ-Tocopherol	0.91	0.65	0.39	0.55
δ-Tocopherol	0.42	0.36	0.26	0.33
α-Tocopheryl-acetate	10.90	10.90	11.90	0

*Values are means of two extractions each injected twice.

Table 3 Body mass, gross energy intake, and N intake of rats fed for 18 weeks with different test diets*

	15C	30C	60C	60C-E
Initial body mass (g)	231 ± 11	231 ± 9	231 ± 9	231 ± 9
Body mass increase (Δg/18 wk)	239 ± 29^{ab}	251 ± 44^{ab}	254 ± 21^{b}	226 ± 25^{a}
Total gross energy intake (MJ/18 wk)	44.4 ± 3.5^{a}	45.4 ± 5.1^{ab}	51.8 ± 1.5^{c}	49.8 ± 5.5^{bc}
Mean food efficiency (Δg/MJ)	5.37 ± 0.53^{b}	5.49 ± 0.50^{b}	4.90 ± 0.36^{a}	4.56 ± 0.44^{a}
Total N intake (g/18 wk)	57.2 ±4.6^{a}	102.4 ± 11.6^{b}	210.3 ± 6.3^{c}	201.3 ± 22.1^{c}

*Values are means ± SD, $n = 10$. Means within a row not sharing a superscript letter are significantly different, $P < 0.05$.

was obtained by centrifugation at 4°C for 10 minutes at 3,000 g (Biofuge fresco, Heraeus Instruments, Osterode, Germany). Animals were sacrificed at the end of the feeding trial after 18 weeks by decapitation and were exsanguinated in the fasted state between 12:00 PM and 2:00 PM. Blood samples were collected for the determination of α-tocopherol concentrations from the trunk into chilled heparinized tubes and centrifuged at 4°C for 10 minutes at 3,000 g (RT 6000 D, Sorvall GmbH, Bad Homburg, Germany). Plasma was stored in liquid nitrogen until analyzed.

Assays

Nitrogen content of diets was determined by a standard micro-Kjeldahl method.[16,17] The crude protein content was calculated by multiplying %N by the factor 6.38. Gross energy of diets was determined by the use of an adiabatic bomb calorimeter (IKA-Calorimeter C4000, Janke & Kunkel, IKA Analysentechnik, Heitersheim, Germany).

Tocopherol concentration in plasma and food samples was analyzed after extraction with hexane by high performance liquid chromatography (HPLC) analysis as described earlier.[18]

Carbonyls were determined by a modification of the HPLC gel filtration procedure described by Levine et al.[19] using 2,4-dinitrophenylhydrazine (DNPH; 10 mmol/L) dissolved in guanidine hydrochloride (GHCl) buffer solution as eluant. Proteins from 100 μL plasma were precipitated with 200 μL chilled 20% (w/v) trichloroacetic acid. Protein pellets were subsequently washed and vortexed twice with 1 mL 10% (w/v) trichloroacetic acid, twice with 1 mL deionized water, and once with 0.5 mL ethanol. Washings were achieved by gentle mechanical homogenization of the pellets in the washing solution, and re-pelleting by centrifugation at 8,000 g for 5 minutes at 4°C. The pellets were dried in a vacuum concentrator (Alpha-RVC, Christ Gefriertocknungsanlagen GmbH, Osterode, Germany) and, finally, were dissolved in 200 μL GHCl buffer solution (6.0 M GHCl, 0.5 M potassium phosphate, pH 2.5) under vortexing at 37°C for 30 minutes. Any insoluble material was removed by centrifugation. The HPLC (System Gold, pump 166, diode-array detector 168, Beckman Instruments, GmbH, München, Germany) was equipped with an autosampler (AS-950, Jasco Corporation, Tokyo, Japan) and a Zorbax Bio Series GF-450 column (6 μm, 250 × 9.4 mm). The following operation conditions were used: GHCl solution as the eluent, flow rate 2.0 mL/min, detection at 370 nm and 276 nm. $Area_{370}$ and $Area_{276}$ were integrated from the hydrazone derivatives peak and the protein peak, respectively. In the autosampler 60 μL of protein solution was mixed with 120 μL DNPH and a volume of 100 μL was injected after a derivatization time of 10 minutes. The amount injected per sample was 0.6 to 1.0 mg protein. The protein content was calculated using bovine serum albumin dissolved in GHCl as standard solution. A blank sample was measured in the same way except that GHCl was used instead of DNPH. One hundred microliters was injected by the autosampler 1 minute after mixing the protein solution with GHCl. A correction was made for $A_{370\ Blank}$ because the protein concentration slightly differs between sample and blank injections by approximately 3.7 ± 3.1% (mean of 40 injections ± SD) possibly due to high viscosity of protein solutions dissolved in GHCl. The carbonyl content (C) was calculated from its peak absorption at 370 nm using a molar absorption coefficient of $\epsilon = 22{,}000$ L mol^{-1} cm^{-1} and expressed as nmol carbonyl × mg $protein^{-1}$. Using the path length of the analytical flow cell of the diode-array detector ($P = 1$ cm), the flow rate (F = 2 mL/min), and the area units as milliabsorbance units (mAU) × seconds/C can be calculated by the following equation:

$$C\ (\text{nmol} \times \text{mg protein}^{-1}) = A_{370} \times F \times 10^6 \times e^{-1} \times P^{-1} \times 60^{-1} \times (\text{mg protein})^{-1};$$

where $A_{370} = [Area_{370\ Sample} - (Area_{370\ Blank} \times Area_{276\ Sample} \times Area_{276\ Blank}^{-1})]$.

Statistical analysis

Data are reported as means ± SD. Differences between mean values were determined by analysis of variance (ANOVA) followed by comparisons using the Newman-Keuls multiple range test.[20] A *P*-value of less than 0.05 was considered to be significantly different.

Results and discussion

Initial body mass, final body mass, and food intake is shown in *Table 3*. The body mass increase in animals fed the vitamin E deficient diet with a high protein content (60C-E) for 18 weeks was significantly lower than in the 60C group (*Table 3*). Comparison of gross energy intake to body mass change ratio (MJ/δg) showed similar values in the 15C and 30C groups. However, ratios were significantly higher in the 60C and 60C-E groups. Although carcass analysis was not performed and, therefore, body composition is not known, higher MJ/Δg values are indicative of a lower efficiency of food energy utilization resulting from a lower efficiency of ATP synthesis during the oxidative degradation of nutrients.[21] As would be expected, the N-intake clearly reflects the protein content of the diets (*Tables 1 and 3*).

After 1 week the reactive protein carbonyl concentration was significantly higher in total plasma proteins of groups fed the 60% casein diets (60C and 60C-E) compared with

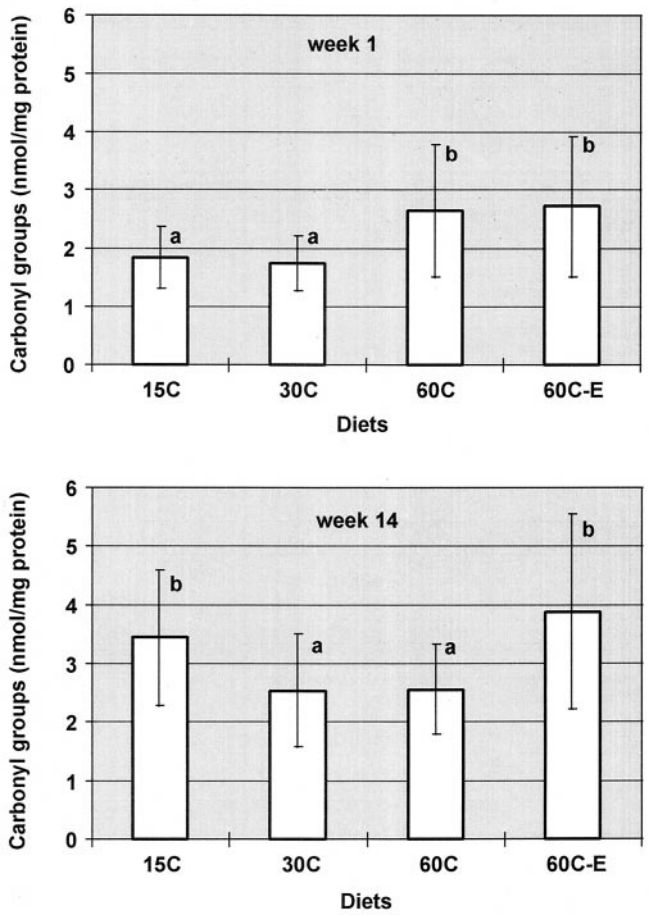

Figure 1 Concentration of reactive carbonyl residues in plasma proteins of adult rats fed either 15% (15C), 30% (30C), or 60% (60C) casein diets, or a vitamin E deficient diet (60C-E) based on diet 60C after 1 and 14 weeks. Bars represent means ± SD. Means not sharing the same letter are significantly different within experimental weeks ($P < 0.05$, $n = 10$). For composition of diets see Materials and methods.

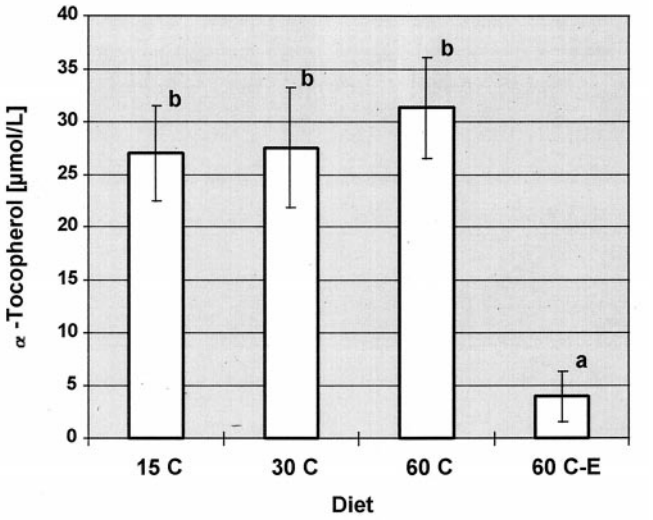

Figure 2 α-Tocopherol concentration in plasma of adult rats fed either 15% (15C), 30% (30C), or 60% (60C) casein diets, or a vitamin E deficient diet (60C-E) based on diet 60C for 18 weeks. Bars represent means ± SD. Means not sharing the same letter are significantly different within experimental week ($P < 0.001$, $n = 10$). For composition of diets see Materials and methods.

the 15C and 30C groups (*Figure 1*). However, after chronic intake of high protein diets, reactive plasma protein carbonyl concentration was found to be significantly higher in the diet group receiving the lowest amount of protein (15C) than in the 30C and 60C groups. Whether a 15% casein diet is insufficient to meet the actual needs for amino acids (i.e., sulfur amino acids to maintain tissue glutathione status in adult rats) is uncertain. Hum et al.[22] measured hepatic glutathione content and plasma glutathione turnover in rats adapted to diets containing 0 to 40% casein. As dietary casein increased from 0 to 20% there was an increase in both parameters, but no further significant increase was obtained for diets containing 40% casein. According to these results, one may speculate that a 15% casein diet could lead to conditions in which the oxidative/antioxidative balance is rendered more susceptible. Other studies have demonstrated reduced glutathione concentrations in tissues of rats and/or increased susceptibility to prooxidative conditions when diets below 25% casein were fed.[23,24] Furthermore, it has been shown that an 8% lactalbumin diet in comparison to a 20% lactalbumin diet aggravated the enhanced production of thiobarbituric acid-reacting substances and reduced activities of antioxidant enzymes in rats given highly polyunsaturated fat (up to 20% soybean oil) for 8 weeks.[25]

After week 14 higher levels of reactive protein carbonyls were found in group 60C-E than in the 60C and 30C groups, presumably due to the impaired antioxidant status in chronic vitamin E deficiency. The plasma α-tocopherol concentration in rats fed the vitamin E inadequate diet 60C-E for 18 weeks was only approximately 15% of the vitamin E adequate diet groups (*Figure 2*).

An increased reactive protein carbonyl content was assumed to be a reliable indicator of oxygen free radical mediated damage produced by either cellular metabolism or pathologic events during inadequacy of antioxidant mechanisms.[11] Among dietary experimental regimens reported to increase oxidative stress or free radical mediated oxidative protein modifications in vivo, a deficit in micronutrients such as selenium and vitamin E[26–28] or an overload of iron[28,29] are documented. Our observation of a significantly increased reactive protein carbonyl content in the vitamin E deficient group is consistent with those results. Furthermore, studies on mechanisms of aging suggest a role of calorie or protein restriction to be effective in lowering formation and accumulation of oxidatively modified proteins in different tissues of experimental animals during aging.[15,30,31] Youngman et al.[15] studied the accumulation of oxidized proteins of liver tissue in young weaning rats fed isoenergetic diets containing either 5%, 10%, or 20% casein for 12 weeks. The mean protein carbonyl content of liver tissue proteins differed significantly between groups and was determined to be 1.67, 2.03, and 2.94 nmol/mg with 5%, 10%, and 20% casein diets, respectively. However, it remains unclear why dietary protein restriction below requirement levels (5–10% casein)[32] should increase the capacity to quench free radicals because some studies

suggest that protein restriction reduces antioxidative capabilities.[21–24] Further, it was shown that dietary protein restriction increases oxygen consumption as discussed recently.[15] In this context, it is interesting to note that Golden and Ramdath[33] have suggested that the classic clinical features of kwashiorkor disease is a result of low concentrations of glutathione, which results in peroxidative damage to biomembrane integrity. In addition it is unclear whether the age of the animals plays a role in the present findings. It should be borne in mind that the animals in the present study had protein intakes considered adequate for growing rats (19% protein)[32] prior to the experiment. The feeding experiment started when the rats were approximately 3 months old (~230 g body weight) and rats were provided with high protein diets. This is in contrast to the experiment of Youngman et al.[15] who investigated young weaning rats for 12 weeks. Further, the diets fed in their study are not considered excessive in terms of protein intake for young rats.

We hypothesized that a diet that induces an increase in substrate (amino acid) oxidation may contribute to enhanced mitochondrial oxygen radical generation. Such a situation may be present, for example, during chronic high dietary protein intake because amino acids that are taken up in excess or above actual needs are catabolized to maintain amino acid homeostasis of the organism.[34–37] The oxidation of substrates is coupled to the production of reducing equivalents that are to be reoxidized in the mitochondrial redox chain. Thus, more reactive oxygen radicals may be generated during substrate (i.e., amino acid) oxidation mediated increased electron flow along the respiratory chain. This view is supported by studies showing that very high dietary protein intakes (more than 40% protein level) increased the thermogenic response, which was accompanied by a lower efficiency of food energy utilization, an increase in oxygen consumption, and impaired oxidative phosphorylation capacities (lower adenosine diphosphate oxygen values for nicotinamide adenine dinucleotide-linked substrates in mitochondria).[5,8,9,21,38,39]

In conclusion, our results do not unequivocally support the hypothesis that ingestion of high protein diets well above requirement leads to oxidative stress in adult rats fed adequate levels of protein from weaning up to 3 months of age prior to the experiment. In contrast, chronic feeding of low protein diet (15% casein diet) led to a higher concentration of reactive carbonyl residues in plasma proteins in comparison to 30% or 60% casein diets. However, in non-adapted animals consuming 60% casein diets, higher concentrations of plasma protein carbonyl residues were measured. Therefore, the concentration of reactive protein carbonyls in plasma proteins do not appear to serve as a physiologic parameter to establish maximum safe levels of protein intake.

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