[NAD+]/[NADH] redox-state metabolites of freezed-clamped livers of rats fed casein or gelatin diets

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Postprandial thermic effects of dietary protein meals have been hypothesized to be related to a diet-mediated increase in amino acid oxidation that may affect the in vivo redox states. The objective of the present study was to determine the effects of isoenergetic isonitrogenous restricted (80% of the ad libitum gross energy intake) diets containing either casein (30%) or gelatin (15% casein, and 12.7% gelatin) for 1, 3, or 28 days to Wistar rats on liver metabolites characterizing cytoplasmic and mitochondrial redox-states. Both casein and gelatin diets produced a progressive decline in the concentrations of lactate and an increase in the concentrations of acetoacetate with the duration of feeding. β-hydroxybutyrate concentrations were reduced on day 3 in relation to day 1 and were highest on day 28. Pyruvate levels changed moderately or stayed the same. The calculated redox ratios were increased in cytoplasm on day 3 and 28 in relation to day 1 and were insignificantly higher with the gelatin diet compared with the casein diet. The corresponding redox ratios in mitochondria were increased on day 3 and remained high on day 28 in relation to day 1 and were significantly higher on day 28 in the gelatin diet-fed group in relation to the casein diet-fed group. Adenine nucleotide content and energy charges were only slightly modified by the experimental conditions used. It is suggested that these results are in accordance with a stimulation of amino acid oxidation and reoxidation of reducing equivalents feeding the protein diets. (J. Nutr. Biochem 5:495–498, 1994.)

Keywords: liver metabolites; redox-state; protein diet; casein; gelatin; rats

Introduction

Compared with isonitrogenous and isoenergetic control diets, an experimental gelatin diet has a marked thermogenic postprandial effect, decreasing the nutritional efficiency of energy utilization.[1–3] This phenomenon has been related to amino acid catabolism but has yet to be satisfactorily explained.[4] Moreover, it is known that rat liver enzyme activities of the main glycine catabolic pathways are induced, and that the rate of glycine catabolism is stimulated when rats are fed high glycine (gelatin) diets.[5]

An increased degradation rate of glycine or other amino acids implies a higher rate of generation of reducing equivalents. Key enzymes in amino acid catabolism may be dependent on reoxidation rates of reducing equivalents. In the case of glycine, it is the glycine cleavage system (EC 2.1.2.10) that has a high adaptation potential and a strong dependence on the intramitochondrial redox-state whereby all conditions that contribute to an increased [NAD(P)+] to [NAD(P)H] ratio have a stimulating effect in vitro.[5,6] Comparable measures of glycine and serine catabolism were obtained in isolated rat liver cells in which the redox-state–dependent glycine cleavage enzyme system is involved [unpublished observations by Petzke].

The present study compares effects of a high glycine-containing gelatin diet (addition of 2.8% gelatin bound glycine, wt/wt) with an isonitrogenous 30% casein diet on the substrate concentrations in freezed-clamped rat liver samples used for the calculation of cytoplasmic and mitochondrial redox-states and energy charge.

It was hypothesized that a diet-mediated increase in amino acid oxidation affects in vivo redox-states. Because the experiments were performed mostly or exclusively in unadapted animals, we were also interested in changes of liver metabolite concentrations during adaptation to the respective diets used in this experiment.

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Methods and materials

Animals and diets

Male albino Wistar rats (Versuchstierproduktion, Schönwalde, Germany) were housed individually in wire-bottomed cages in a room with controlled humidity and temperature (23° C) and a fixed 12-hour light-dark cycle (0700 to 1900 light). Prior to the feeding experiment, all animals received a nonpurified pelleted diet supplied by Versuchstierproduktion Schönwalde. Depending on the study group to which they were assigned, animals received a control diet containing 30% casein (30C) or a purified isoenergetic isonitrogenous test diet in restricted amounts. In the test diet, 15C12.7G, half of the casein of the 30C diet was substituted isonitrogenously with gelatin. The composition and source of components of these diets were reported earlier[5] and are shown in *Table 1*. The dietary restriction was reduced from an *ad libitum* gross energy intake of 75 kcal/day, to 60 kcal/day. Nitrogen intake was 3.6 g/day. In the present study, rats were fed the casein diet or the gelatin diet for 1, 3, or 28 days to investigate time-dependent effects of protein quality.

Assays of liver metabolites

Levels of liver metabolites were determined using standardized enzymatic techniques.[7-9] All substrates, coenzymes, enzymes, and buffers were supplied by Boehringer Mannheim GmbH (Mannheim, Germany) or Serva Feinbiochemica GmbH & Co. KG (Heidelberg, Germany). A freeze-clamp technique was used to approximate in vivo concentrations of metabolites. Liver tissue from a single lobe was removed and immediately stored in liquid nitrogen. To extract the metabolites, the frozen tissue was pulverized in liquid nitrogen and proteins were precipitated with ice-cold perchloric acid. The precipitate was washed twice with perchloric acid and the supernatants (acid-soluble fraction) were combined, neutralized with K_2CO_3 using methylorange as indicator, and analyzed. The $[NAD^+]/[NADH]$ redox ratios of cytoplasmic and mitochondrial compartments were calculated according to Krebs and Veech,[10] and the energy charge according to Atkinson.[11]

Statistical analysis

Values are presented as the mean ± standard deviation. Statistical significance between two means was determined by Student's *t* test. Significance among more than two means was analyzed by applying the Newman-Keuls multiple-range test when the analysis of variance gave a significant F value ($P < 0.05$).

Table 1 Composition of the purified test diets

Ingredient	% composition by diet, wt/wt	
	30C (30% Casein)	15C12.7G (15% Casein/ 12.7% Gelatin)
Casein*	30	15
Gelatin†	—	12.7
Sunflower seed oil	3	3
Lard	12	12
Wheat starch	46	51.2
Microcrystalline cellulose	5	2.1
Salt mixture‡	4	4
Vitamins§	+	+

*Contained 85% crude protein (% N × 6.37).
†Contained 88% crude protein (% N × 5.55).
‡Composition in grams: NaCl, 50; $MgSO_4 \cdot 7H_2O$, 75; NaH_2PO_4, 130; KH_2PO_4, 250; $Ca(H_2PO_4)_2$, 16; calcium lactate, 375; $CaCO_3$, 70; KJ, 0.9; $CuSO_4 \cdot 5H_2O$, 0.3; $MnSO_4 \cdot 4H_2O$, 0.2; $ZnCO_3$, 0.2; and NaF, 0.03.
§Composition (per kilogram diet): thiamin · HCl, 5 mg; riboflavin, 5 mg; pyridoxine · HCl, 5 mg; nicotinic acid, 15 mg; Ca-D-pantothenate, 15 mg; folic acid, 5 mg; choline · Cl, 100 mg; retinyl acetate, 10,000 IU; cholecalciferol, 1000 IU; and DL-α-tocopherol, 50 mg.

Results

This study demonstrates that liver metabolite concentrations characterizing the redox ratios are changed measurably during adaptation to the protein diets used (*Table 2*). Both diets produced a decrease in the level of lactate and an increase in the level of acetoacetate. After feeding both casein and gelatin diets, β-hydroxybutyrate concentrations were reduced on day 3 in relation to day 1 and were highest on day 28. The feeding-time–dependent changes were in some cases significant ($P < 0.05$). Pyruvate levels changed moderately or stayed the same. The resulting redox ratios were increased in cytoplasm on days 3 and 28 in relation to day 1 of restrictive feeding (*Figure 1*). The values did not increase significantly in the gelatin-diet (15C12.7G) group compared with the casein-diet (30C) control group. The corresponding redox ratios in mitochondria (*Figure 2*) were drastically increased on day 3 of feeding and remained high on day 28 in relation to day 1 of feeding both protein diets. The values on day 28, in the adapted state, were significantly higher ($P < 0.01$) in the gelatin-diet group in relation to the casein-diet control group. Both protein diets produced relatively high standard deviations in the mitochondrial redox ratio on day 3. Therefore, the value was not significantly higher with the gelatin diet compared with the casein diet. There was no significant difference in the levels of liver ATP, ADP, and AMP and in the resulting energy charge between diet groups.

Discussion

The mean values measured for cytoplasmic redox ratios, ranging from 841 to 1,675, were consistent with those reported in the literature. Values between 725 and 1,845 have been previously reported.[12] In mitochondria, normal mean values have been reported ranging from 5.1 to 14.4.[12] In the present study, such values were found only in the adapted state measured on day 28 of feeding the protein diets. Furthermore, compared with day 1 and 28, relatively high values of the mitochondrial redox ratios were measured on day 3 after feeding the protein diets. It has been previously reported that hyperlipemic and hyperglycemic BHE rats fed a high carbohydrate diet produce values of the mitochondrial redox ratio ranging between 10 and 35.[13-15]

We suggest that the lower mean values for mitochondrial redox ratios on day 1 and the much higher values on day 3 resulted from a metabolic adaptation to a quantitatively and qualitatively altered protein consumption. This adaptation implies an induction of enzymes involved in amino acid degradation and an increase in amino acid oxidation for maintaining body amino acid homeostasis when the protein concentration in the diet is above the required value or when the amino acid composition is not optimal.[5,16] Both conditions were met by the experimental diets used. The 30C diet, containing 30% casein, is a relatively high protein diet, and the 15C12.7G diet was supplemented with the protein gelatin, whose amino acid composition is not optimal. This may explain why both protein diets gave princi-

Table 2 Effect of casein or gelatin diets fed for different time periods in restrictive amounts on rat liver metabolites (expressed as μmoles/g tissue) determining the cytosolic or mitochondrial [NAD⁺]/[NADH] redox ratio and the energy charge

Feeding period (d)	1	1	3	3	28	28
Diet	30C (30% casein)	15C12.7G (15% casein/ 12.7% gelatin)	30C (30% casein)	15C12.7G (15% casein/ 12.7% gelatin)	30C (30% casein)	15C12.7G (15% casein/ 12.7% gelatin)
Pyruvate	0.169 ± 0.037	0.154 ± 0.045	0.131 ± 0.039	0.160 ± 0.022	0.115 ± 0.020[A]	0.157 ± 0.063
Lactate	1.896 ± 0.503	2.031 ± 1.099	0.985 ± 0.253[A]	1.032 ± 0.175[A]	0.840 ± 0.166[A]	0.893 ± 0.216[A]
Acetoacetate	0.014 ± 0.009	0.022 ± 0.005	0.043 ± 0.031	0.046 ± 0.025[A]	0.071 ± 0.023[A]	0.083 ± 0.018[AB]
β-hydroxybutyrate	0.137 ± 0.025	0.204 ± 0.099	0.050 ± 0.039[A]	0.033 ± 0.035[A]	0.328 ± 0.094[AB]	0.234 ± 0.136[B]
ATP	2.80 ± 0.36	2.66 ± 0.47	2.92 ± 0.59	3.00 ± 0.22	2.51 ± 0.36	2.92 ± 0.34
ADP	1.48 ± 0.19	1.45 ± 0.20	1.26 ± 0.16[A]	1.35 ± 0.11	1.47 ± 0.29	1.19 ± 0.40
AMP	1.00 ± 0.20	0.95 ± 0.26	0.70 ± 0.21[A]	0.68 ± 0.08[A]	0.66 ± 0.24[A]	0.43 ± 0.23[A]
$\frac{[ATP] + 0.5 [ADP]}{[ATP] + [ADP] + [AMP]}$	0.67 ± 0.04	0.67 ± 0.05	0.73 ± 0.07	0.73 ± 0.02[A]	0.69 ± 0.07	0.71 ± 0.08

Values are means ± SD ($6 \leq n \leq 10$).
A: significantly different ($P < 0.05$) in relation to feeding period of 1 day of the respective diet group.
B: significantly different ($P < 0.05$) in relation to feeding period of 3 days of the respective diet group.
For composition of the diets see Methods and materials.

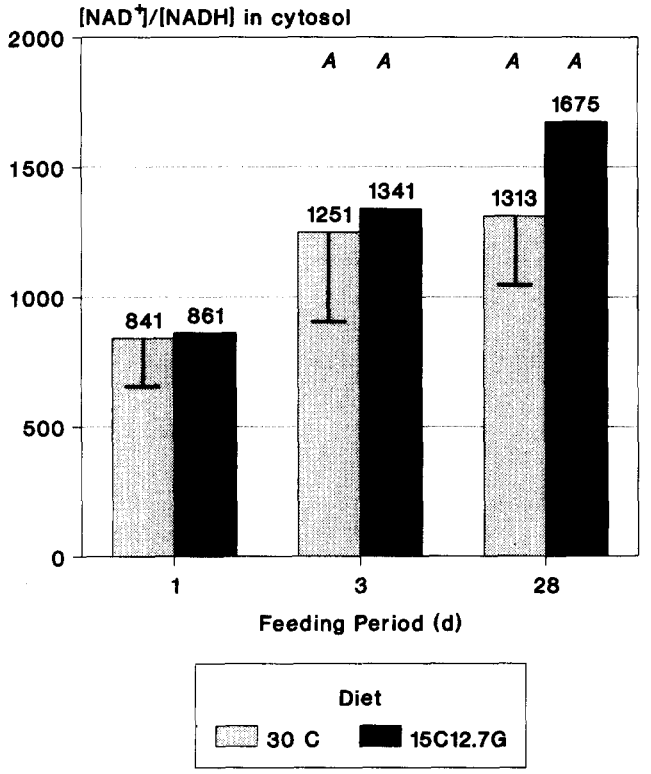

Figure 1 Effects of casein (30 C) or gelatin (15C12.7G) diets fed for different time periods in restrictive amounts on cytoplasmic [NAD⁺]/[NADH] redox ratios. Bars represent means − SD ($6 \leq n \leq 10$). A indicates statistical significance ($P < 0.05$) in relation to feeding period of 1 day of the respective diet group. For composition of diets see Methods and materials.

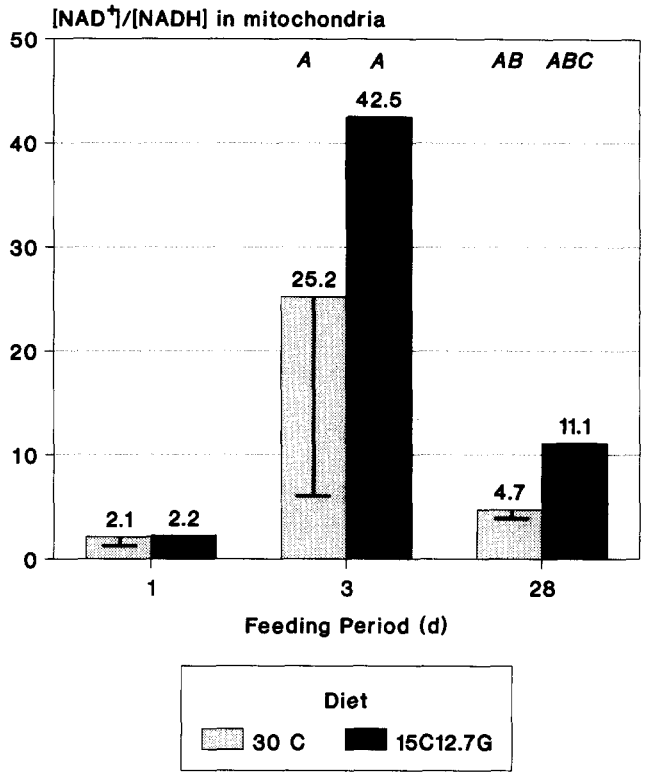

Figure 2 Effects of casein (30 C) or gelatin (15C12.7G) diets fed for different time periods in restrictive amounts on mitochondrial [NAD⁺]/[NADH] redox ratios. Bars represent means − SD ($6 \leq n \leq 10$). A indicates statistical significance ($P < 0.05$) in relation to feeding period of 1 day of the respective diet group. B indicates statistical significance ($P < 0.05$) in relation to feeding period of 3 days of the respective diet group. C indicates statistical significance ($P < 0.01$) in relation to the 30C diet. For composition of diets see Methods and materials.

pally the same results in the parameters measured during adaptation, although the effect for the gelatin-supplemented diet seems to be more pronounced.

"Thermic effects" after ingestion of different food stuffs have been well documented and were previously called "specific dynamic action."[17, 18] Despite overwhelming data and hypotheses addressing the biochemical nature of the food-induced increase in metabolic rate, particularly after protein meals, the exact mechanisms remain to be elucidated. However, different energy-dissipating mechanisms, which are related to the intermediary metabolism of amino acids, for example, amino acid transport and metabolism, detoxification of ammonia, or transport of reducing equivalents into the mitochondria where their reoxidation occurs are possible.[19–22] The question is reduced to processes that favor the oxidation of excessive amino acids and the reoxidation of the combined production of reducing equivalents. These processes are proposed to occur during substrate (amino acid) oxidation under conditions in which the demand for reducing equivalents may not be increased for biosynthetic systems. This implies that because of the link between the nicotine adenine dinucleotide system and the respiratory chain, either the futile cycle-linked ATP consumption is to be increased or the coupling degree is to be reduced, both leading to energy dissipation. The net result of such processes is an increase in heat production calorimetrically measurable and generally designated as thermogenesis. Recent findings confirmed the effect of the protein component of diets on bioenergetic functions. Toyomizu and Clandinin showed an impaired ADP to O value for cardiac mitochondria from weanling rats fed a high-protein diet for 23 days and that changes in ADP to 0 ratios were mainly due to changes in dietary protein rather than fat or carbohydrate level.[23] Moreover, in a study of Zaragoza et al. a long-term high-protein diet induced a significant increment in the density and size of liver cell mitochondria and the presence of megamitochondria in the periportal region.[24]

Further results presented earlier clearly show that experimental gelatin- or glycine-rich diets produce a more marked thermogenic effect in relation to other protein diets tested in vivo.[1–3] In the present study, both protein diets used gave similar results regarding changes in liver metabolite concentrations during adaptation. However, the calculated redox ratios were generally higher (significant in rat liver mitochondria on day 28 of feeding) in animals fed the gelatin diet. This describes a more (re)oxidized situation despite a proposed high generation rate of reducing equivalents during substrate (amino acid) oxidation.

The levels of adenine nucleotides and the energy charge were practically unchanged under our experimental conditions (*Table 2*), which is in accordance with the in vivo situation. Changes in the energy charge are to be expected either "in vitro" or under strong hypoxic conditions.[11]

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