Hepatic peroxisomal and mitochondrial fatty acid oxidation in the riboflavin-deficient rat

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(Received 2 April 1985; accepted 10 April 1985)

The effects of riboflavin deficiency on hepatic peroxisomal and mitochondrial palmitoyl-CoA oxidation were examined in weanling Wistar-strain male rats. The specific activities of peroxisomal catalase and palmitoyl-CoA-dependent NAD⁺ reduction were not affected by up to 10 weeks of riboflavin deficiency. In contrast, the specific activity of mitochondrial carnitine-dependent palmitoyl-CoA oxidation was depressed by 75% at 10 weeks of deficiency. The amount of peroxisomal protein per g of liver was not affected by riboflavin deficiency, whereas, expressed per liver, both riboflavin-deficient and pair-fed controls showed decreased peroxisomal protein compared with controls fed *ad libitum*. Hepatic mitochondria, but not peroxisomes, were sensitive to riboflavin deficiency.

The hepatic mitochondrial capacity to oxidize fatty acids has been examined in the riboflavin-deficient rodent (Hoppel & Tandler, 1975; Hoppel *et al.*, 1979; Olpin & Bates, 1982; Sakurai *et al.*, 1982). Within 1 week of consuming a riboflavin-deficient diet, male weanling Wistar rats exhibit a 20–25% decrease in their ability to oxidize palmitate (Hoppel *et al.*, 1979). Within 1–4 weeks, mitochondrial fatty acid oxidation rates are only 25% of the values obtained from riboflavin-supplemented animals. We originally documented the loss of acyl-CoA dehydrogenase activities with riboflavin deficiency as the biochemical event directly responsible for loss of mitochondrial capacity to oxidize fatty acids (Hoppel *et al.*, 1979). This finding has subsequently been confirmed (Sakurai *et al.*, 1982).

As a sequel to riboflavin deficiency, and presumably as a result of the alterations in the hepatic mitochondrial ability to oxidize fatty acids, changes are observed in hepatic fatty acid composition (Taniguchi & Nakamura, 1976), and unusual products of fatty acid oxidation are found in urine of riboflavin-deficient rats (Goodman, 1981; Gregersen & Kolvraa, 1982). Such products include C₆–C₁₀ dicarboxylic acids. Dicarboxylic acids are produced in several other pathophysiological

states. In the streptozotocin-diabetic rat liver, dicarboxylic acids are apparently produced by microsomal ω -hydroxylation of longer-chain fatty acids, followed by peroxisomal β -oxidation (Mortensen & Gregersen, 1982). Since there is no reason to propose an alternative pathway for dicarboxylic acid production in riboflavin deficiency, it may be hypothesized that peroxisomal capacity for β -oxidation is less affected by riboflavin deficiency than is the mitochondrial.

The peroxisomal system of β -oxidation was first documented by Lazarow & de Duve (1976). The peroxisomal reaction sequence is identical with the mitochondrial sequence in terms of the transformations of the fatty acid (Lazarow, 1978). However, the electron acceptor in the first step, catalysed by the acyl-CoA oxidase, is oxygen. Just like its mitochondrial counterpart, the oxidase requires an FAD cofactor.

The present study examines hepatic peroxisomal fatty acid oxidation in male weanling rats fed on a riboflavin-deficient diet for up to 70 days.

Experimental

Animals and diets

Male rats (Wistar strain, 21 days old) were purchased from a commercial supplier (Charles River Laboratories, Wilmington, MA, U.S.A.). On arrival, rats were weighed (initial body wt. 55.8 ± 0.6 g; mean $\pm$ S.E.M., $n = 81$) and assigned to

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one of three dietary regimens: (1) riboflavin-supplemented, fed *ad libitum*; (2) riboflavin-deficient, fed *ad libitum*; (3) riboflavin-supplemented, fed with a quantity of riboflavin-supplemented diet equivalent to the food consumption of the deficient rats in the previous 24 h (i.e. 'pair-fed'). The diets were commercially prepared to our specifications as 1.3 cm pellets (Teklad Test Diets, Madison, WI, U.S.A.). In brief, the diet contained (g/kg): vitamin-free casein, 200; DL-methionine, 3; sucrose, 557; corn starch, 150; corn oil, 50; mineral mixture, 35; vitamin mixture (riboflavin omitted), 5. This diet was supplemented with 22 mg of riboflavin/kg body wt. to create the riboflavin-supplemented diet. The rats were housed individually in wire-bottomed stainless-steel cages with access to water *ad libitum*.

At 1, 4 and 10 weeks of feeding, rats were killed by decapitation, and the liver was rapidly removed. A sample of whole blood was taken for determination of 'erythrocyte glutathione reductase activity coefficient' (EGR-AC) to provide evidence of riboflavin deficiency (Tillotson & Sauberlich, 1971). The EGR-AC is the ratio of activity of glutathione reductase obtained in the presence of exogenous FAD to that in the absence of exogenous FAD.

Mitochondria

Mitochondria were isolated by differential centrifugation as described previously (Hoppel *et al.*, 1979). Mitochondrial respiration rates were determined polarographically with a Clark-type oxygen electrode. State-3 (ADP-stimulated oxidation) rates were determined for palmitoyl-CoA plus carnitine and for palmitoylcarnitine in the presence of malate (Hoppel *et al.*, 1979). Oxidative rates were expressed per mg of mitochondrial protein, determined by the biuret method (Gornall *et al.*, 1949). Oxidative rates per g of liver and per total liver were calculated on the basis of citrate synthase activity (Srere, 1969) in homogenate and in the mitochondrial suspension. State-4 (ADP-limited oxidation) rates, respiratory control ratios and ADP/O were determined as quality-control measures (see Brady & Hoppel, 1983a).

Peroxisomes

Peroxisomes were isolated, with minor modifications, as described by Neat *et al.* (1981), by using a Percoll gradient. We have described the specific equipment used previously (Brady & Hoppel, 1983b). Ultracentrifugation was at 1850×10^7 – 1950×10^7 rad²/s. The peroxisomal peak was identified on the basis of catalase activity (Bock *et al.*, 1980). Palmitoyl-CoA-dependent NAD⁺ reduction was determined in the major peroxisomal

peak fraction, by using a dual-wavelength spectrophotometer (Aminco DW 2a; Travenol Laboratories, Chicago, IL, U.S.A.) at 340 nm with a 400 nm reference wavelength (Neat & Osmundsen, 1979). It was confirmed that the measured oxidation was cyanide-insensitive. Peroxisomal palmitate oxidation per g of liver and per total liver were calculated on the basis of catalase activity in the peroxisomal fraction and in the homogenate (Neat *et al.*, 1981). Cyanide-insensitive palmitoyl-CoA oxidation was determined in homogenates from rats fed for 10 weeks by the above method in the presence of 0.5 mM-KCN.

Protein in peroxisomal fractions was determined by the biuret method. To use this method in the presence of Percoll it was necessary to centrifuge the sample before reading the A_{540} . Bovine serum albumin standards were run in the presence and absence of Percoll. We attempted to use the Bradford (1976) method for protein determination, but, for reasons which remain uncertain, the method was unacceptably variable.

Statistical analysis

Data were analysed as a 3×3 factorial arrangement of treatments (Federer & Zelen, 1966). Where data were available at 10 weeks only, the data were analysed by analysis of variance as a completely randomized design (Steel & Torrie, 1960).

Results

Within 1 week, the riboflavin-deficient rats had erythrocyte glutathione reductase activity coefficients 60% greater than for either control group (Table 1). The values for the deficient rats further increased and were 2.0 at 4 and 10 weeks. Values greater than 1.2 have been considered indicative of riboflavin deficiency (Tillotson & Sauberlich, 1971). At 4 weeks the riboflavin-deficient and pair-fed control rats had comparable body weights; by 10 weeks of feeding with the diet, the pair-fed rats were heavier (218 ± 12 g; mean $\pm$ S.E.M.) than the riboflavin-deficient rats (152 ± 7 g). The controls fed *ad libitum* were considerably heavier throughout the feeding period and at 10 weeks weighed 426 ± 13 g. The liver weights were similar in the deficient and the pair-fed controls throughout the experiment, and both are significantly less than in the controls fed *ad libitum* (Table 1). To account for the differences in body weight, the liver weight was expressed per 100 g body wt. Both the riboflavin-deficient rats and their pair-fed controls had similar values at 1 and 4 weeks, whereas, by week 10, the percentage liver weight was slightly higher in the deficient rats than in both controls.

Table 1. *Effect of riboflavin deficiency on liver weight and peroxisomal catalase activity*
Data are presented as means $\pm$ S.E.M. for three rats at each time point. Data were analysed as a 3 $\times$ 3 factorial arrangement of treatments. Statistically significant effects ($P < 0.05$) are designated as 'R' for effect of dietary regimen, 'T' for temporal effect and 'I' for interaction of regimen and duration of feeding. Erythrocyte glutathione reductase-activity coefficient (EGR-AC) is the ratio of reductase activity with exogenous FAD to that without added FAD. Catalase units are expressed in terms of the first-order rate constant. Peroxisomal protein was determined from catalase recoveries.

Riboflavin regimen	...	Supplemented, fed <i>ad libitum</i>			Supplemented, pair-fed			Deficient, fed <i>ad libitum</i>			Significant effects
		1	4	10	1	4	10	1	4	10	
Period fed with diet (weeks)	...										
EGR-AC		1.00 $\pm$ 0.03	1.03 $\pm$ 0.02	1.04 $\pm$ 0.05	0.99 $\pm$ 0.09	1.08 $\pm$ 0.03	1.14 $\pm$ 0.07	1.60 $\pm$ 0.23	1.96 $\pm$ 0.28	1.93 $\pm$ 0.22	R
Liver wt.											
(g)		4.7 $\pm$ 0.3	12.1 $\pm$ 0.7	16.8 $\pm$ 0.8	3.7 $\pm$ 0.5	5.8 $\pm$ 0.7	7.8 $\pm$ 0.7	3.6 $\pm$ 0.3	5.9 $\pm$ 0.3	6.8 $\pm$ 0.4	R, T, I
(g/100g body wt.)		5.0 $\pm$ 0.1	5.0 $\pm$ 0.1	4.0 $\pm$ 0.1	4.8 $\pm$ 0.7	4.3 $\pm$ 0.2	3.6 $\pm$ 0.1	4.8 $\pm$ 0.3	4.7 $\pm$ 0.4	4.5 $\pm$ 0.3	—
Catalase activity											
(units/mg of peroxisomal protein)		2.6 $\pm$ 0.4	2.1 $\pm$ 0.3	4.6 $\pm$ 1.1	2.1 $\pm$ 0.3	3.2 $\pm$ 1.0	2.4 $\pm$ 1.7	2.6 $\pm$ 0.4	2.6 $\pm$ 1.8	1.9 $\pm$ 0.4	—
(units/g of liver)		59.1 $\pm$ 5.9	48.6 $\pm$ 7.7	21.4 $\pm$ 5.3	56.6 $\pm$ 16.7	43.9 $\pm$ 11.4	18.0 $\pm$ 8.8	58.8 $\pm$ 15.7	34.7 $\pm$ 15.4	16.7 $\pm$ 4.0	T
(units/liver)		305 $\pm$ 31	521 $\pm$ 44	383 $\pm$ 90	207 $\pm$ 61	224 $\pm$ 23	120 $\pm$ 39	174 $\pm$ 27	194 $\pm$ 82	102 $\pm$ 18	R
Peroxisomal protein											
(mg/g of liver)		24.5 $\pm$ 1.4	24.5 $\pm$ 5.0	8.0 $\pm$ 2.0	24.5 $\pm$ 6.5	14.4 $\pm$ 1.0	11.3 $\pm$ 2.8	22.2 $\pm$ 2.8	20.8 $\pm$ 5.6	9.1 $\pm$ 2.0	T
(mg/liver)		129 $\pm$ 11	269 $\pm$ 59	142 $\pm$ 82	87 $\pm$ 24	83 $\pm$ 36	93 $\pm$ 31	72 $\pm$ 3	118 $\pm$ 31	56 $\pm$ 9	R

Catalase activity was used as a marker for peroxisomes. In the peroxisomal fraction, catalase activity per mg of peroxisomal protein was not affected by dietary regimen or duration of feeding (Table 1). Catalase activity per g of liver decreased between 4 and 10 weeks in all these groups of rats. Activity per total liver was depressed in both the riboflavin-deficient and pair-fed rats relative to the controls fed *ad libitum*, reflecting the decreased total liver weight.

Peroxisomal protein was calculated by dividing the catalase activity per g of liver by the catalase activity per mg of peroxisomal protein. The amount of peroxisomal protein per g of liver was not affected by dietary regimen, but declined in all groups between 4 and 10 weeks, reflecting the corresponding decrease in catalase activity per g of liver (Table 1). Peroxisomal protein per total liver did not exhibit this temporal effect. However, in both the riboflavin-deficient and pair-fed rats, peroxisomal protein per liver was decreased compared with the controls fed *ad libitum*.

Carnitine-dependent palmitoyl-CoA-oxidation rates were depressed in liver mitochondria from riboflavin-deficient rats relative to either control within 1 week of feeding (Fig. 1a). This decrease progressed from about 25% at week 1 to 75% by week 10. When expressed per g of liver, carnitine-dependent palmitoyl-CoA oxidation was significantly decreased in riboflavin-deficient rats within 1 week of feeding (Fig. 1b), and the pattern was similar to that observed in mitochondria. The total hepatic capacity was decreased by 50% in the riboflavin-deficient rats compared with either control (Fig. 1c). However, the oxidative activity increased per liver in the controls fed *ad libitum*, so that it was significantly greater at 4 and 10 weeks than in the pair-fed controls (Fig. 1c).

The rate of palmitoyl-CoA-dependent NAD⁺ reduction in the peroxisomal fraction (per mg of protein) was not affected by treatment (Fig. 2a). All groups demonstrated an increased specific activity at 10 weeks. No significant effect was noted when activity was expressed per g of liver (Fig. 2b). At 10 weeks the lack of change reflects an increased specific activity per mg of peroxisomal protein, combined with a decrease in peroxisomal protein per g of liver. When peroxisomal activity is expressed per total liver, an effect of treatment was observed (Fig. 2c). At 10 weeks only, both pair-fed and deficient rats had capacities significantly lower than the controls fed *ad libitum*, which reflects the decreased total liver weight. The hepatic capacities in the pair-fed and deficient rats did not differ throughout the experiment (Fig. 2c). Cyanide-insensitive palmitoyl-CoA-dependent NAD⁺-reduction rates were measured in liver homogenates from the three groups at 10 weeks. As

Fig. 1. Mitochondrial palmitoyl-CoA oxidation rates. Each point is the mean for three rats; error bars are S.E.M. Dietary treatments are described in detail in the Experimental section: ●, riboflavin-supplemented, fed *ad libitum*; △, riboflavin-deficient, fed *ad libitum*; ▲, riboflavin-supplemented, pair-fed. Data were analysed by analysis of variance for a 3×3 factorial arrangement of treatments (Federer & Zelen, 1966). Statistical significance was determined at the 5% level. Mitochondrial activity is expressed as: (a) ng-atoms of O/min per mg of mitochondrial protein (significance = diet effect); (b) μg-atoms of O/min per g of liver (significance = diet effect); (c) μg-atoms of O/min per liver (significance = diet and time effects).

shown in Figs. 2(b) and 2(c), there is good agreement between this direct measurement and the values calculated by using catalase recovery.

Discussion

Although mitochondrial fatty acid oxidation was quite sensitive to riboflavin deficiency, we did not observe any effect on peroxisomal palmitate oxidation during 10 weeks of deficiency. However, we did observe that both riboflavin-deficient and pair-fed rats had significantly lower total hepatic peroxisomal palmitoyl-CoA oxidation rates than

Fig. 2. Peroxisomal palmitoyl-CoA oxidation rates. The protocol and notations are the same as in Fig. 1. Peroxisomal palmitoyl-CoA dependent NAD^+ reduction is expressed as: (a) nmol/min per mg of peroxisomal protein (no significant effect); (b) μmol/min per g of liver (no significant effect); (c) μmol/min per liver (significance = diet and time effect). H, activity of cyanide-insensitive palmitoyl-CoA dependent NAD^+ reduction measured in liver homogenates of rats at 10 weeks.

did the controls fed *ad libitum*. The difference represents the larger liver in these controls, because the peroxisomal specific activity and activity per g of liver were similar in all groups. Several investigators have reported changes in peroxisomal fatty acid oxidation rates with overt starvation (Ishii *et al.*, 1980; Thomassen *et al.*, 1982; Brady & Hoppel, 1983b). In the present study, food restriction decreased the total hepatic peroxisomal oxidative capacity. Another possible factor for the difference could be sexual maturation, which would occur between weeks 4 and 10 in this experiment, interacting with food restriction. Sakurai *et al.* (1982) have reported no effect of 4 weeks of riboflavin deficiency on peroxisomal palmitoyl-CoA oxidation expressed per g of liver.

Erythrocyte glutathione reductase-activity coefficients have been used as a quantitative measure

of riboflavin deficiency in man (Sauberlich *et al.*, 1972), rats (Tillotson & Sauberlich, 1971) and pigs (Brady *et al.*, 1979). The ratios encountered in the present study are larger than the values of 1.2–1.4 generally considered evidence of riboflavin deficiency (Tillotson & Sauberlich, 1971). However, the degree of riboflavin deficiency in our study was not as severe as is possible. Nonetheless, mitochondrial palmitoyl-CoA oxidation was significantly decreased within 1 week of feeding the riboflavin-deficient diet, although the degree of depression was not as great as previously observed with diet from another vendor (Hoppel *et al.*, 1979).

Thus hepatic mitochondria, but not peroxisomes, were sensitive to riboflavin deficiency. One possible consequence of this shift in mitochondrial to peroxisomal fatty acid oxidation is the appearance of dicarboxylic acids (C_6 – C_{10}) in urine in riboflavin deficiency (Goodman, 1981; Gregersen & Kolvraa, 1982). Data obtained primarily with diabetic rats indicate that C_6 – C_{10} dicarboxylic acids arise from chain shortening of C_{10} – C_{14} dicarboxylic acids (Petersen *et al.*, 1972). Previous studies have shown dicarboxylic acids to be a poor substrate for mitochondrial oxidation (Petersen, 1973). The mitochondrial oxidation rates of dicarboxylic acid were less than 5% the rate of palmitate oxidation. Mortensen *et al.* (1982) demonstrated cyanide-insensitive chain shortening of dicarboxylic acids, using liver homogenates. They have observed peroxisomal oxidation of dicarboxylic acids. We have observed that the rate of oxidation of hexadecanedioyl-CoA by rat liver peroxisomes is about 18% of the rate for palmitoyl-CoA oxidation (P. S. Brady, unpublished work). Although low, this oxidation rate in peroxisomes is considerably higher than the mitochondrial rates for the diacid. The riboflavin-deficient rat should prove to be a useful model in delineating the mitochondrial and peroxisomal contributions in hepatic fatty acid oxidation and, in particular, the consequences of an increased peroxisomal capacity for fatty acid oxidation relative to mitochondria.

This work was supported by the Veterans Administration and by grants from the National Institutes of Health (AM 15804 and the Metabolism Training Program AM 07319). We express our gratitude to Dr. Linda J. Brady for preparation and evaluation of mitochondria.

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