Acyl-CoA dehydrogenase activity in the riboflavin-deficient rat

Effects of starvation

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Riboflavin deficiency in weanling rats causes a metabolic disorder characterized by failure to oxidize fatty acids. The disorder is similar to that seen in several human diseases, some of which are responsive to pharmacological doses of riboflavin. Previous analysis of the riboflavin-deficient rat has shown that the failure of fatty acid oxidation is due to a decrease in the activity of the acyl-CoA dehydrogenases of β -oxidation. The activity of these flavoenzymes in liver rapidly decreases when a riboflavin-deficient diet is initiated. The objectives of these experiments were to analyse the effects of starvation on liver mitochondria isolated from the riboflavin-deficient rat. Our studies show that the decreased mitochondrial fatty acid oxidation induced by riboflavin deficiency is partially reversed by starvation. The extent of this reversal is proportional to the duration of starvation. The starvation-associated increase in fatty acid oxidation is mediated by an increase in the mitochondrial short-chain acyl-CoA dehydrogenase activity. The activity of this enzyme is increased such that the ratio of short-chain acyl-CoA dehydrogenase apoenzyme to holoenzyme does not change. We conclude that short-chain acyl-CoA dehydrogenase activity is limiting for fatty acid oxidation when its activity falls below a critical point. The increased mitochondrial specific activity of short-chain acyl-CoA dehydrogenase during starvation may result from an increased availability of flavin coenzyme or an increase in enzyme catalytic efficiency.

INTRODUCTION

Riboflavin deficiency in weanling rats causes a characteristic clinical syndrome and an associated metabolic disorder (Hopfel & Tandler, 1978). Of particular interest are specific changes in hepatic mitochondrial oxidative function which begin within 24 h after the introduction of a riboflavin-deficient diet. The ability of liver mitochondria to oxidize fatty acid substrates is decreased within 24 h of the initiation of such a diet. At that point the defect is specific; non-lipid substrates are oxidized normally. Control weanling rats that are starved do not display this abnormality. Thus absence of dietary riboflavin in a weanling rat causes specific disruption in the development of the hepatic mitochondrial fatty acid oxidation pathway.

The defect has been characterized further by assay of the enzymes which comprise the mitochondrial β -oxidation system. This analysis revealed a decrease in the activity of the acyl-CoA dehydrogenases, enzymes which catalyse the first step of β -oxidation (Hopfel *et al.*, 1979). They belong to a family of flavoprotein (FAD) dehydrogenases that have differing acyl-group specificities (Green *et al.*, 1954; Hauge *et al.*, 1956; Ikeda *et al.*, 1985). The three dehydrogenases involved in mitochondrial β -oxidation are termed short-chain (EC 1.3.99.2), medium-chain and long-chain acyl-CoA dehydrogenases (EC 1.3.99.3), to reflect their chain-length specificities (Ikeda *et al.*, 1985). The rapid and relatively specific decrease in the activity of these enzymes during riboflavin deprivation is probably due to the interaction between the dehydrogenase apoenzyme, its flavin coenzyme and the amount of dietary riboflavin required to assure adequate supply of coenzyme.

Riboflavin deficiency also produces a characteristic

dicarboxylic aciduria in rats (Goodman, 1981) resulting from the metabolism of incompletely oxidized fatty acyl groups. A similar dicarboxylic aciduria has been described in children with deficiency of medium-chain acyl-CoA dehydrogenase activity (Stanley *et al.*, 1983). In addition, a kindred has been described with a disorder similar to glutaric aciduria type II with multiple acyl-CoA dehydrogenase deficiency (Harpey *et al.*, 1983). This syndrome is characterized by the same type of dicarboxylic aciduria seen in riboflavin deficiency. In these patients, the clinical syndrome can be ameliorated by a diet rich in riboflavin. A riboflavin-responsive lipid myopathy has also been reported, but urinary organic acids were not measured in this patient (Carroll *et al.*, 1981). The precise nature of the defect in these cases has not been characterized. However, the requirement for large amounts of riboflavin suggests a disorder either in the synthesis of flavin nucleotide from riboflavin or in the association of flavin coenzyme with apoprotein. A patient with deficiency of short-chain acyl-CoA dehydrogenase activity also presented with lipid-storage myopathy (Turnbull *et al.*, 1984). Riboflavin therapy was not reported in this case.

The present experiments were designed to develop further the model of riboflavin deficiency in the rat as a tool for the analysis of these clinical syndromes of abnormal fatty acid oxidation. The results reported here show that a specific starvation-associated increase in mitochondrial short-chain acyl-CoA dehydrogenase activity occurs in riboflavin-deficient rats, and is accompanied by a proportionate increase in the capacity of the β -oxidation pathway in liver mitochondria. Portions of this work were presented at the meeting of the American Society of Biological Chemists (Ross & Hopfel, 1984).

METHODS

Animals

Male Wistar weanling rats (21 days old) weighing 40–60 g were obtained from Charles River (Wilmington, MA, U.S.A.). On arrival, rats were separated into two groups. Group I was fed *ad libitum* with a diet deficient only in riboflavin (Teklad Test Diet, Madison, WI, U.S.A.) (Brady & Hoppel, 1985). These rats were housed in wire-bottom cages either individually or four to a cage. The type of housing did not affect experimental results. Rats in Group II were housed individually in wire-bottom cages and pair-fed with a control diet identical with that given to the deficient group except for the addition of 22 mg of riboflavin/kg of diet. This involved feeding the control rats with a quantity of diet equal to the mean daily intake of the rats given the riboflavin-deficient diet. Several studies were done with rats fed *ad libitum* on the control diet. The results from these animals were identical with those obtained from Group II.

Experimental protocols

The rats were maintained on their assigned diet for 4 weeks. At that point, experiments were performed with each group on animals that were fed, starved for 24 h or starved for 48 h. The animals were killed between 06:00 and 07:00 h on the day of the experiments. Blood was collected in heparinized beakers, and from it plasma was prepared and frozen at -70°C . Hepatic mitochondria were prepared from both experimental groups. Mitochondrial O_2 consumption was measured as previously described in a 0.5 ml chamber (Hoppel *et al.*, 1979). Maximal oxidation rates were measured in the presence of excess ADP. An enzymic assay was used to measure plasma β -hydroxybutyrate (Olsen, 1971). Carnitine palmitoyltransferase (Bieber *et al.*, 1972) and citrate synthase (Srere, 1969) were assayed by published methods. Acyl-CoA dehydrogenase activity was measured at 37°C as described by Hoppel *et al.* (1979), with butyryl-CoA (200 μM), octanoyl-CoA (200 μM) and palmitoyl-CoA (50 μM) as substrates. Activity was measured in both the absence and the presence of 0.1 mM-FAD.

Materials

Materials were purchased or synthesized as described previously (Hoppel *et al.*, 1979). Acyl-CoA compounds were purchased from P-L Biochemicals. In addition, butyryl-CoA was also synthesized in our laboratory by the anhydride method (Simon & Shemin, 1953), and was identical with the compound commercially obtained when analysed spectrophotometrically or by h.p.l.c.

Statistics

The data were analysed by analysis of variance and linear regression analysis when appropriate (Dixon, 1981): $P < 0.05$ was considered significant.

RESULTS

The plasma concentration of β -hydroxybutyrate was identical in riboflavin-deficient and pair-fed control rats, respectively 0.13 ± 0.01 mM and 0.14 ± 0.01 mM in the fed state and 1.36 ± 0.35 mM and 1.5 ± 0.45 mM after 48 h starvation. The similarity of riboflavin-deficient to control rats during starvation was somewhat surprising.

Fig. 1. Mitochondrial State-3 oxidation rates for liver mitochondrial isolated from pair-fed control (●, ▲, ■) and riboflavin-deficient (○, △, □) rats

Measurements were made in fed rats (○, ●) and in rats starved for 24 h (△, ▲) or 48 h (□, ■). The rates are expressed as ng-atoms of O/min per mg of mitochondrial protein. The substrates used were 40 μM -palmitoylcarnitine (a), 40 μM -octanoylcarnitine (b) and 10 mM-glutamate (c). Palmitoylcarnitine and octanoylcarnitine oxidations were done in the presence of 10 mM-malate as a source of oxaloacetate. There was a significant starvation-induced increase in oxidation rate ($P < 0.05$) for palmitoylcarnitine and octanoylcarnitine in the deficient rats only.

Table 1. Acyl-CoA dehydrogenase activity in rat liver mitochondria at 4 weeks of riboflavin deficiency

The substrates used were butyryl-CoA for short-chain, octanoyl-CoA for medium-chain and palmitoyl-CoA for long-chain acyl-CoA dehydrogenase activities. The activities are expressed as nmol of cytochrome *c* reduced/min per mg of mitochondrial protein.

Acyl-CoA dehydrogenase		Rats . . . Control	Riboflavin-deficient
Short-chain	+ FAD	126.6 ± 16.3	9.9 ± 1.3
	– FAD	86.7 ± 9.6	4.3 ± 1.0
Medium-chain	+ FAD	56.6 ± 3.7	12.7 ± 2.2
	– FAD	37.8 ± 2.6	8.9 ± 0.9
Long-chain	+ FAD	78.7 ± 6.6	28.8 ± 2.2
	– FAD	77.1 ± 4.6	26.3 ± 2.3

Plasma β -hydroxybutyrate concentration is the net result of synthesis, primarily in liver, and peripheral utilization. In light of the known defect in fatty acid oxidation caused by riboflavin deficiency, we decided to analyse liver mitochondria isolated from riboflavin-deficient and pair-fed control rats starved for up to 48 h.

The quality of the isolated liver mitochondria from both controls and riboflavin-deficient rats, as estimated by measuring respiratory control ratios (Estabrook, 1967), was good. The respiratory control ratio with glutamate as substrate was lower (5.0 ± 0.6 versus 12.1 ± 3.8) in the riboflavin-deficient group, owing to a

Fig. 2. Short-chain acyl-CoA dehydrogenase activity during starvation

Symbols are the same as in Fig. 1. Points connected by continuous lines are measurements made in the presence of FAD; those connected by broken lines were made in the absence of FAD. There was no significant change in the pair-fed control group. The activity in the deficient groups significantly increased ($P < 0.05$) during starvation measured either with or without FAD.

decreased State 3 (ADP-stimulated) rate of oxidation. With fatty acid substrates, respiratory control ratios could not be determined, because of the markedly diminished State 3 rates.

Fatty acid oxidation was measured with palmitoyl-carnitine and octanoylcarnitine as substrates by using freshly isolated mitochondria from rats that had been fed or starved for 24 or 48 h. In the riboflavin-deficient rats, the oxidation rate increased 2-fold at 24 h and approx. 4-fold at 48 h of starvation when compared with the fed state (Fig. 1). The mitochondrial fatty acid oxidation rate was about 8-fold greater in the fed controls than in the fed riboflavin-deficient rats. In the controls, there was no change in mitochondrial fatty acid oxidation during starvation. Glutamate oxidation was 2-fold higher in control than in riboflavin-deficient mitochondria, and was unchanged by starvation in either experimental group. Therefore starvation induced a specific increase in mitochondrial oxidation of both long- and medium-chain fatty acids in riboflavin-deficient rats.

Decreased fatty acid oxidation by hepatic mitochondria from riboflavin-deficient rats was shown previously to result from decreased activity of acyl-CoA dehydrogenase (Hoppel *et al.*, 1979). The activities of all three acyl-CoA dehydrogenases involved in β -oxidation were decreased in riboflavin deficiency, but the short-chain acyl-CoA dehydrogenase was most severely affected (Table 1).

Fig. 3. Activity of mitochondrial carnitine palmitoyltransferase (a) and citrate synthase (b)

Although there are significant differences between control and deficient rats, no starvation-associated changes are noted. Symbols are the same as in Fig. 1.

The acyl-CoA dehydrogenases exist either coupled with FAD as the holoenzyme, or without FAD as the apoenzyme (Ikeda *et al.*, 1985). In our mitochondrial preparations, the holoenzyme activity can be measured directly, and the presence and amount of apoenzyme can be inferred by the addition of FAD to the assay. The ratio of activity measured in the presence of FAD to that in its absence (activation coefficient) reflects the amount of apoenzyme present.

The activities of the three acyl-CoA dehydrogenases were measured with and without FAD in mitochondria from fed control and deficient rats as shown in Table 1. As previously reported (Hoppel *et al.*, 1979), the activities of the dehydrogenases in the absence of added FAD are markedly decreased in riboflavin-deficient rats. The total activity (measured in the presence of FAD) is also decreased in the deficient group. The resultant lack of change in the ratio of apoenzyme to holoenzyme suggests that there is no significant accumulation of apoenzyme during riboflavin deficiency.

Short-chain acyl-CoA dehydrogenase activity measured with and without exogenous FAD in the starved riboflavin-deficient and control rats is shown in Fig. 2. There was no change in enzyme activity under either assay condition during starvation in the control group. In contrast, there is a 2-fold increase in activity of 24 h starvation and a 4-fold increase in activity at 48 h starvation in the deficient group. Furthermore, the activation coefficient remains constant during this period. In contrast, the decreased activities of the medium- and long-chain acyl-CoA dehydrogenases in the fed state (Table 1) are unaffected by starvation. To characterize further the mitochondrial changes during starvation, the mitochondrial-inner-membrane enzyme carnitine palmitoyltransferase and the matrix enzyme citrate synthase were measured (Fig. 3). No significant change in the activity of either occurs during starvation. Thus starvation in the riboflavin-deficient rat is associated with a specific increase in short-chain acyl-CoA dehydrogenase activity.

Fig. 4. Comparison of palmitoylcarnitine oxidation and short-chain (butyryl-CoA) dehydrogenase activity in liver mitochondria isolated from riboflavin-deficient rats

The correlation is highly significant ($P < 0.05$, with $r = 0.87$). Symbols are the same as in Fig. 1.

DISCUSSION

Riboflavin deficiency in the rat leads to a rapid and marked decrease in hepatic mitochondrial fatty acid oxidation (Hoppel *et al.*, 1979). We therefore predicted that starvation-induced ketosis would not develop in the riboflavin-deficient rat. However, when starved, these rats developed plasma β -hydroxybutyrate concentrations identical with those of their pair-fed controls.

We examined fatty acid oxidation in isolated liver mitochondria from starved riboflavin-deficient rats. With either octanoylcarnitine or palmitoylcarnitine as substrate, the State-3 rates of oxidation were markedly decreased after 4 weeks of deficiency. An increase in the oxidation rates of these substrates was observed after 24 h of starvation, with a further increase at 48 h. This contrasts with the absence of change in fatty acid oxidation in control rats. The changes are identical with either long-chain (palmitoylcarnitine) or intermediate-chain (octanoylcarnitine) fatty acid substrate. These data suggest that the changes that occur affect fatty acid oxidation in a manner independent of chain length.

The primary defect in fatty acid oxidation induced by riboflavin deficiency is a decrease in the activity of the three acyl-CoA dehydrogenases of fatty acid β -oxidation (Hoppel *et al.*, 1979). The most dramatic decrease is seen in the short-chain acyl-CoA dehydrogenase. During starvation the activity of this enzyme increased, whereas long-chain and medium-chain acyl-CoA dehydrogenases did not change. In the riboflavin-deficient group, the capacity for mitochondrial fatty acid oxidation and the activity of the short-chain acyl-CoA dehydrogenase increase in parallel (Fig. 4). There is an excellent correlation between these two measurements ($r = 0.87$), which is consistent with the hypothesis that the increase in mitochondrial fatty acid oxidation capacity is due to an increase in short-chain acyl-CoA dehydrogenase activity.

It has been shown previously that certain intermediates of β -oxidation are inhibitors of acyl-CoA dehydrogenase activity, and thus of fatty acid oxidation (Davidson & Schulz, 1982). Some of these intermediates are removed

from intact mitochondria by treatment with carnitine (Bremer & Wojtczak, 1972). In liver mitochondria isolated from riboflavin-deficient rats, the decrease in the rate of oxidation is as great with palmitoyl-CoA plus carnitine as substrate as when palmitoylcarnitine is the substrate (Brady & Hoppel, 1985). Therefore the decreased oxidation rates cannot be attributed to inhibition by carnitine-extractable intermediates of β -oxidation.

Short-chain acyl-CoA dehydrogenase is encoded by the nuclear genome, synthesized in the cytosol and transported into the mitochondrial matrix by a leader-sequence-dependent mechanism (Ikeda *et al.*, 1984). The role of FAD in these processes is unknown. Possible mechanisms by which mitochondrial activity of this enzyme increases during starvation include increased synthesis, decreased degradation, increased transport, increased catalytic efficiency or intracellular redistribution of FAD allowing formation of short-chain acyl-CoA dehydrogenase holoenzyme from apoenzyme. This last possibility is unlikely, in view of the unchanged activation coefficient during starvation. The data thus far obtained do not allow distinction among the other possible mechanisms.

Our results show that (1) the defect in mitochondrial fatty acid oxidation caused by riboflavin deficiency is reversible in a time-dependent manner by starvation, (2) this reversal is accompanied by a specific starvation-associated increase in activity of the short-chain acyl-CoA dehydrogenase, and (3) flux through the mitochondrial fatty acid oxidation pathway is limited by the short-chain acyl-CoA dehydrogenase when its activity falls below a critical point.

This work was supported by the VA Medical Research Service and the National Institute of Health (AM-15804). We thank Dr. R. M. Miller and Dr. A. Zinn for their review of the manuscript, and Cherice Edmonds for word-processing services. N.S.R. was an Associate Investigator of the Veterans Administration at the time of these studies.

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Received 31 December 1986; accepted 6 February 1987