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Mitochondrial oxidative metabolism during respiratory infection in riboflavin deficient mice

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Studies in children and mice have shown that respiratory infection alters riboflavin metabolism, resulting in increased urinary loss of this vitamin. This could be due to mobilization of riboflavin from the liver to blood because liver Flavin adenine dinucleotide (FAD) levels were lowered in the mice during infection. To understand the functional implications of lowered hepatic FAD levels during respiratory infection, flavoprotein functions such as oxidative phosphorylation and β-oxidation of the liver mitochondria were examined during infection in mice. Weanling mice were fed either riboflavin-restricted or control diet for 18 days and then injected with a sublethal dose of Klebsiella pneumoniae. *During infection, the state 3 respiratory rate with palmitoyl-L-carnitine and glutamate were significantly lowered (27–29%) in the riboflavin-restricted group, whereas in the control group 10% reduction was observed with palmitoyl-L-carnitine as substrate. A 22% reduction in the respiratory control ratio with palmitoyl-L-carnitine as substrate was observed during infection in the riboflavin-restricted group. The β-oxidation of palmitoyl-L-carnitine was significantly lowered (29%) in the riboflavin-restricted infected group. The results of the study suggest that the effects of infection on vital physiologic functions were more pronounced in the riboflavin-restricted mice than in the control mice. © Elsevier Science Inc. 1999* (J. Nutr. Biochem. 10:728–732, 1999) *© Elsevier Science Inc. 1999. All rights reserved.*

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Introduction

There is high incidence of subclinical riboflavin deficiency among low-income women and children in India as judged by the activation of erythrocyte glutathione reductase with its coenzyme FAD[1–3] [erythrocyte glutathione reductase activation coefficient (EGR-AC)]. Studies in these children suggested that the etiology of riboflavin deficiency in this population may be more complex and, apart from low-dietary intake of riboflavin, repeated respiratory infections also may play a role.[3] A control study in preschool children with respiratory infections or measles showed a transient increase in urinary and erythrocyte total flavin levels and a reduction in EGR-AC (improved riboflavin status). However, after recovery there was a deterioration of riboflavin status as judged by the above-mentioned biochemical parameters.[4] Based on these studies, it was postulated that respiratory infections mobilize riboflavin from the tissues to blood and urine, leading to deterioration of riboflavin status in a population whose dietary intake of this vitamin is low. This hypothesis was verified using mouse as the experimental animal and *Klebsiella pneumoniae* as the infecting organism.[5] The results of the above study confirmed the earlier observation in children with respiratory infection regarding blood and urinvary levels of flavins and EGR-AC. In addition, this study showed a reduction in the liver FAD levels and an increase in erythrocyte FAD levels, thereby supporting the above-mentioned hypothesis.

Infections are known to enhance energy requirement[6] and FAD-dependent enzymes play a major role in energy production via oxidative phosphorylation and β-oxidation of substrates. To understand the functional implications of lowered hepatic FAD levels during respiratory infection, oxidative phosphorylation and β-oxidation of liver mitochondria were examined using mouse as the animal model and *K. pneumoniae* as the infecting organism.

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Materials and methods

Male weanling mice of the Swiss/NIN strain were divided into two groups containing 32 controls and 32 low riboflavin fed mice. The animals were housed individually in screen-bottomed cages at optimal temperature (22–25°C) and humidity (55±10%) and a 12-hour light-dark cycle. They were fed ad libitum a purified diet containing (g/kg): sucrose 700, vitamin-free casein (Sigma Chemical Co., St. Louis, MO USA) 200, peanut oil 50, salt mixture 40, and vitamin mixture 10. The composition of the salt and vitamin mixture was the same as that described by Lakshmi et al.[7] The riboflavin content of the low riboflavin diet was 0.5 mg/kg. This level of riboflavin would be expected to simulate the human situation in developing countries such as India where riboflavin is one of the limiting nutrients. The control diet contained 13.3 mg riboflavin/kg diet.

After 18 days of feeding the mice with the experimental diets, 16 animals in each group were infected with a sublethal dose (6.15×10^6 cells) of *K. pneumoniae* in 0.5 mL distilled water, administered intraperitoneally. The remaining mice in both the groups received 0.5 mL of distilled water intraperitoneally and they served as uninfected controls.

K. pneumoniae was chosen as a representative organism for respiratory infection because the mouse was found to be sensitive to it. This organism was isolated from the lungs of the rat (Wistar/NIN strain) and purified as described by Domenico et al.[8] In a preliminary study mice were infected with different doses of *K. pneumoniae* and the number of surviving mice over a period of time was recorded. These data were used for calculating the LD_{50}.[9]

At 72 hours after injection of the organism, the animals showed signs of acute infection, such as loss of appetite and reddening of the snout. At this stage half the number of animals from each group were sacrificed after withdrawing a sample of blood from the ocular plexus. The remaining animals were sacrificed 15 days later when the infected animals had recovered completely. The liver was perfused with cold saline to remove blood completely and then excised.

The riboflavin status of the mice was assessed by the sensitive biochemical parameter EGR-AC. Glutathione reductase is a FAD-dependent enzyme. EGR-AC is the ratio of FAD stimulated to unstimulated activity. During deficiency state, enhanced stimulation occurs with the in vitro addition of FAD, resulting in increased EGR-AC. Activity of this enzyme was measured as described by Bayoumi and Rosalki[10] without any modification.

Mitochondria were prepared by centrifuging the supernatant at 8,000 rpm after removal of cell debris according to the method of Hoppel et al.[11] Its purity and intactness were checked by measuring the citrate synthase activity.[12]

Oxidation experiments

The oxidation experiments were carried out using Clark-type oxygen electrode attached to a recorder (Gilson Model 5/6H Oxygraph). The oxygen electrode was calibrated according to the method of Robinson and Cooper.[13] Oxidation experiments were carried out by the method described by Hoppel et al.[11] The incubation medium contained 80 mM KCl, 50 mM Mops, 5 mM Pi, 1 mM EGTA, and 1 mg defatted BSA/mL; pH of the medium was adjusted to 7. Palmitoyl-L-carnitine, a fatty acid substrate, was used at 4 mM concentration, and glutamate, a representative of nonfatty acid substrate, was used at 1 M concentration. Respiration was initiated by the addition of substrate to the incubation medium containing mitochondria. "State 3" represented adenosine diphosphate (ADP)-stimulated respiration, and "state 4" represented ADP-limited respiration, and these are similar to those described by Chance and Williams.[14] Respiratory control ratios (RCR) and ADP/O ratios were determined by the method of Estabrook.[15]

For β-oxidation, the incubation medium was similar to that described above except that it contained 1.5 mg defatted BSA/mL and 5 mM malonate to ensure complete oxidation to acetoacetate, which directly measures the flux through β-oxidation.[11] The substrate used was palmitoyl-L-carnitine. The rate of oxygen consumed was expressed as nanograms of oxygen atoms consumed per minute per milligram of protein.

β-Oxidation of palmitoyl-L-carnitine was also measured following the reduction of cytochrome C.[11] The reaction rate was expressed as nmol of cytochrome C reduced per milligram of protein at 37°C.

Protein was estimated by the Folin-Lowry method.[16]

Statistics

Values are presented as means with their standard errors. Homogeneity of variances was tested using the Bartlett test. If this test was found to be significant ($P < 0.05$), then the actual values were transformed into logarithmic values and tested by means of analysis of variance with the least significant difference multiple range test.[17] Comparisons were made between infected and uninfected mice in the same period (period I) and during infection (period I) and after recovery from infection (period II) within the same group.

Results

There was a nonsignificant reduction in feed intake on days 1 and 2 after infection. Infection did not significantly affect the body weights of the low riboflavin and control groups. However, riboflavin restriction per se reduced weight gain (*Table 1*).

EGR-AC values increased significantly in the low riboflavin fed group, confirming riboflavin deficiency. In the low riboflavin fed infected group, EGR-AC values were lower than the respective uninfected group ($P < 0.05$). After recovery from infection the values were similar to those observed in the corresponding uninfected group. Infection had no effect on the EGR-AC values in the control group (*Table 1*).

Citrate synthase activity was similar in all groups and the values were comparable to previously reported results,[11,18] suggesting that the mitochondrial preparations were pure and deficiency or infection did not affect the intactness of the mitochondria (*Table 1*).

Oxidative phosphorylation

State 4 respiration was not affected by either riboflavin deficiency or infection with both palmitoyl-L-carnitine (*Table 2*) and glutamate (*Table 3*). However, state 3 respiration and RCR were significantly ($P < 0.05$) reduced by riboflavin deficiency, regardless of the substrate used (*Tables 2 and 3*). Infection produced further reduction in state 3 respiration and RCR in the riboflavin-restricted group. Similar changes were not observed in the control group, except for a slight decrease in state 3 respiration with palmitoyl-L-carnitine as the substrate (*Tables 2 and 3*). The ADP:O ratio was not altered either by deficiency or by infection (*Tables 2 and 3*). After recovery (period II), the state 3 respiratory rate and RCR in the low riboflavin fed group were similar to the corresponding uninfected group.

The β-oxidation of palmitoyl-L-carnitine was signifi-

Table 1 Effect of infection on body weight, EGR-AC and citrate synthase activity

	Low riboflavin group		Control group	
	Uninfected	Infected	Uninfected	Infected
1. Body weight (g)				
Period I	$18.2^{ax} \pm 0.43$	$17.8^{ax} \pm 0.61$	$24.8^{bx} \pm 0.73$	$23.6^{bx} \pm 0.75$
Period II	$21.6^{ay} \pm 0.63$	$20.8^{ay} \pm 0.62$	$28.5^{by} \pm 0.75$	$27.8^{by} \pm 0.69$
2. EGR-AC				
Period I	$1.28^{b} \pm 0.04$	$1.08^{ax} \pm 0.03$	$1.10^{a} \pm 0.04$	$1.08^{a} \pm 0.05$
Period II	$1.31^{b} \pm 0.05$	$1.26^{by} \pm 0.06$	$1.07^{a} \pm 0.05$	$1.05^{a} \pm 0.06$
3. Citrate synthase (n mol/min/mg protein)	185 ± 12.2	197 ± 15.3	201 ± 11.7	191 ± 14.7

Values are mean ± SEM of 8 observations.
Values not sharing a common superscript (a, b, c) are significantly different ($P < 0.05$) by analysis of variance (ANOVA) and least significant difference (LSD) multiple range test in a period.
Values not sharing a common superscript (x, y) are significantly different ($P < 0.05$) by ANOVA and LSD multiple range test between periods in a group.
EGR-AC–erythrocyte glutathione reductase activation coefficient. Period I – peak period of infection. Period II – after recovery.

cantly reduced in riboflavin deficiency as well as during infection in this group as judged by the rate of oxygen consumption per milligram protein or cytochrome C reduction. Though similar changes were observed in the control infected group, the reduction in oxygen consumption per milligram of protein was not statistically significant (*Table 4*). After recovery from infection (period II), β-oxidation of fatty acid was similar to that observed in the respective uninfected group (*Table 4*).

Discussion

The reduction in EGR-AC observed in the low riboflavin fed group during infection confirmed earlier findings in children with respiratory infection and in mice infected with *K. pneumoniae*.[4,5] This reduction could be due to transient increase in erythrocyte FAD level during infection[5] caused by the mobilization of riboflavin from the liver to blood.[19] Increased leukocytic glutathione reductase activity has been reported in rats during phagocytosis and during treatment with hydrogen peroxide.[20] Increased activity of this enzyme in blood cells during infection may enhance the formation of reduced glutathione and protect the cells from hydrogen peroxide and free radicals generated during bactericidal activity. The mice in the control group were adequately nourished with riboflavin, glutathione reductase was saturated with its coenzyme FAD, and hence infection did not have any effect on EGR-AC.

The lower state 3 respiratory rate with palmitoyl-L-carnitine and glutamate as substrates in the low riboflavin fed group confirmed the earlier observations in riboflavin-deficient animals.[11,18,21] Hoppel et al.[11] observed a reduction in state 3 and state 4 respiratory rates in riboflavin-deficient rats using palmitoyl-L-carnitine and glutamate as substrates, but RCR was reduced only with glutamate. With other substrates, such as succinate and α-ketoglutarate, some researchers observed a decrease in the state 3 respiratory rate[11,18,21,22] whereas others did not observe similar

Table 2 Effect of infection on oxidation of Palmitoyl-L-Carnitine

	Low riboflavin group		Control group	
	Uninfected	Infected	Uninfected	Infected
State 4 (ng atoms 0/min/mg protein)				
Period I	16.96 ± 1.03	15.73 ± 1.33	18.41 ± 0.84	18.76 ± 0.73
Period II	18.92 ± 1.29	18.95 ± 1.55	20.06 ± 1.80	21.03 ± 1.69
State 3 (ng atoms 0/min/mg protein)				
Period I	$36.75^{b} \pm 2.01$	$26.95^{ax} \pm 1.34$	$62.03^{dx} \pm 2.70$	$55.77^{cx} \pm 1.86$
Period II	$38.28^{a} \pm 4.39$	$39.69^{ay} \pm 2.11$	$72.49^{by} \pm 3.75$	$67.28^{by} \pm 3.30$
RCR				
Period I	$2.20^{b} \pm 0.18$	$1.71^{a} \pm 0.08$	$3.42^{c} \pm 0.14$	$2.99^{c} \pm 0.15$
Period II	$1.71^{a} \pm 0.17$	$2.13^{a} \pm 0.22$	$3.63^{b} \pm 0.23$	$3.27^{b} \pm 0.20$
ADP/O				
Period I	2.38 ± 0.16	2.46 ± 0.20	2.40 ± 0.10	2.21 ± 0.12
Period II	2.21 ± 0.09	2.43 ± 0.11	2.20 ± 0.13	2.36 ± 0.12

Values are mean ± SEM of 6–8 observations.
Values not sharing a common superscript (a, b, c, d) are significantly different ($P < 0.05$) by analysis of variance (ANOVA) and least significant difference (LSD) multiple range test in a period.
Values not sharing a common superscript (x, y) are significantly different ($P < 0.05$) by ANOVA and LSD multiple range test between periods in a group.
Period I – peak period of infection. Period II – after recovery. RCR–respiratory control ratio. ADP/O–micromoles ADP added/microatoms oxygen utilized.

Table 3 Effect of infection on oxidation of glutamate

	Low riboflavin group		Control group	
	Uninfected	Infected	Uninfected	Infected
State 4 (ng atoms 0/min/mg protein)				
Period I	19.02 ± 1.29	16.96 ± 0.72	17.89 ± 0.87	16.45 ± 1.36
Period II	15.52 ± 1.21	14.29 ± 2.07	15.21 ± 1.57	16.02 ± 1.91
State 3 (ng atoms 0/min/mg protein)				
Period I	40.84^{b} ± 3.64	29.33^{ax} ± 2.03	65.23^{c} ± 5.92	65.06^{c} ± 3.33
Period II	44.49^{a} ± 2.35	44.30^{ay} ± 2.71	63.14^{b} ± 4.21	70.90^{b} ± 3.16
RCR				
Period I	2.17^{ax} ± 0.21	1.74^{ax} ± 0.16	3.64^{b} ± 0.30	4.05^{b} ± 0.33
Period II	2.90^{ay} ± 0.15	3.20^{ay} ± 0.37	4.29^{b} ± 0.42	4.42^{b} ± 0.58
ADP/O				
Period I	2.65 ± 0.11	2.47 ± 0.14	2.54 ± 0.10	2.71 ± 0.25
Period II	2.43 ± 0.11	2.69 ± 0.11	2.62 ± 0.09	2.60 ± 0.11

Values are mean ± SEM of 6 to 8 observations.
Values not sharing a common superscript (a, b, c) are significantly different ($P < 0.05$) by analysis of variance (ANOVA) and least significant difference (LSD) multiple range in a period.
Values not showing common superscript (x, y) are significantly different ($P < 0.05$) by ANOVA and LSD multiple range test between periods in a group.
Period I–peak period of infection. Period II–after recovery. RCR–respiratory control ratio. ADP/O–micromoles ADP added/microatoms oxygen utilized.

changes.[23,24] It has been suggested that the lowered rate of glutamate oxidation in riboflavin deficiency is due to reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase activity because the flux through complex I in the respiratory chain is considered the rate limiting step.[18]

The reduction in state 3 respiratory rate in response to *K. pneumoniae* infection suggests that the oxidative capacity of liver mitochondria is lowered especially in the low riboflavin fed group. The unaltered inorganic phosphate-to-oxygen consumption (P:O) ratio during the oxidation of palmitoyl-L-carnitine or glutamate in riboflavin deficiency and infection suggests that the phosphorylating capacity per unit of oxygen consumed is not affected under these conditions.

The lower β-oxidation rate during deficiency and infection could be due to reduced acylcoenzyme A (acyl-CoA) dehydrogenase activity. Several earlier studies reported lower activity of this enzyme in riboflavin-deficient rats and mice.[18,21,25–28] The addition of FAD to the hepatic mitochondrial preparation of riboflavin-deficient rats restored only 10 to 25% of the lost enzyme activity.[26,29] Hence, it was suggested that the loss of activity was due to loss of apoenzyme. It later was demonstrated that the mature acyl-CoA dehydrogenase binds to FAD in the mitochondria and this stabilizes the enzyme.[30]

The results of the present study show that the reduction in liver FAD levels due to *K. pneumoniae* infection impairs vital biochemical functions such as oxidative phosphorylation and β-oxidation. These effects were more marked in the riboflavin-restricted mice. Riboflavin deficiency is rampant in developing countries such as India.[2,3] However, contrary to some micronutrient deficiencies, it fails to invite attention. Acute respiratory infection is a common cause of

Table 4 β-oxidation during infection in mouse liver mitochondria

	Low riboflavin group		Control group	
	Uninfected	Infected	Uninfected	Infected
Palmitoyl-L-canine (ng atoms 0/min/mg protein)				
Palmitoyl-L-carnitine				
Period I	37.67^{b} ± 2.17	27.04^{ax} ± 1.74	66.32^{c} ± 3.70	56.91^{cx} ± 4.96
Period II	33.02^{a} ± 4.13	38.52^{ay} ± 5.45	70.49^{b} ± 4.44	77.56^{by} ± 4.75
Cytochrome C (nmol reduced/min/mg protein)				
Period I	31.73^{b} ± 1.56	22.94^{ax} ± 0.84	46.68^{d} ± 2.16	37.36^{cx} ± 2.03
Period II	29.95^{a} ± 1.86	32.08^{ay} ± 1.88	53.05^{b} ± 3.06	53.14^{by} ± 2.93

Values are mean ± SEM of 6 to 8 observations.
Values not sharing a common superscript (a, b, c, d) are significantly different ($P < 0.05$) by analysis of variance (ANOVA) and least significant difference (LSD) multiple range in a period.
Values not sharing a common superscript (x, y) are significantly different ($P < 0.05$) by ANOVA and LSD multiple range test between periods in a group.
Period I–peak period of infection.
Period II–after recovery.

morbidity in children and also causes mortality. The protective effects of riboflavin supplementation during acute respiratory infection needs to be investigated.

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