Hepatic Drug Hydroxylation and Lipid Peroxidation in Riboflavin-Deficient Rats*

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(Received 9 November 1973)

The effect of riboflavin deficiency and phenobarbital pretreatment on drug hydroxylation and lipid peroxidation was investigated. A significant decrease in aniline and acetanilide hydroxylation as well as NADPH-linked and ascorbate-induced lipid peroxidation was observed during 4- and 7-week riboflavin deficiency in both adult male and adult female rats. The drug-hydroxylation and lipid-peroxidation activities were further lowered with the increase in riboflavin deficiency. The phenobarbital pretreatment induced aniline and acetanilide hydroxylase activity even in riboflavin-deficient animals. Drug hydroxylation inhibits lipid peroxidation in both deficient and normal rats. The administration of riboflavin was followed by a significant increase in drug hydroxylation and lipid peroxidation.

Many foreign lipid-soluble compounds are metabolized by the liver microsomal enzyme system in the presence of NADPH and O₂ (Quinn *et al.*, 1958; McMahon, 1966). Lipid peroxidation was also observed in microsomal suspensions in the presence of NADPH, O₂ and various free radicals such as Fe²⁺ and vitamin C (Schneider *et al.*, 1964; Hochstein & Ernster, 1963; Wills, 1969a). It has been also observed that drugs undergoing hydroxylation inhibit lipid peroxidation (Orrenius *et al.*, 1964). A number of investigators have reported that the activities of drug-metabolizing enzymes and concentrations of electron-transport components vary with several factors such as dietary status (Marshall & McLean, 1971; Catz *et al.*, 1970; Patel & Pawar, 1973; Wade *et al.*, 1972; Zannoni *et al.*, 1972; Becking, 1973). Sufficient information is not available on the effect of riboflavin deficiency on lipid peroxidation and drug hydroxylation and therefore the present investigation was undertaken to study this effect.

Materials and Methods

Haffkine-strain adult albino male and female rats, weighing 130–140g, were obtained from the Haffkine Institute, Bombay, India. They were housed in individual cages at room temperature and kept away from exposure to pharmacologically active compounds such as insecticides and smoke. All animals were fed on a synthetic diet for 1 week before initiation of the

experiments. Rats were then allotted to one of two groups and pair-fed for 7 weeks on the following diets. Diet A (normal) contained 13% casein, 45% corn starch, 31% sucrose, 6% groundnut oil, 4% salt mixture [the salt mixture was that of Hegsted *et al.* (1941) with the following modifications: CuSO₄·5H₂O, 3.266g; CoCl₂·6H₂O, 2.16g; NaF, 0.432g] and 1% vitamin mixture [the vitamin mixture was that of Schultze (1956): 100i.u. of vitamin A, 20i.u. of vitamin D and 0.5mg of vitamin E/rat per day were also added to the diet as well as groundnut oil] (125µg of riboflavin/rat per day). Diet B (riboflavin-deficient) contained the same ingredients except that casein was replaced by vitamin-free casein (obtained from Calbiochem, Lucerne, Switzerland, and containing 0.5µg of riboflavin/g), and riboflavin-free vitamin mixture was used.

Riboflavin administration

Riboflavin (125µg/rat per day) was administered orally to 7-week riboflavin-deficient rats. The effect of riboflavin administration on various parameters was investigated at the end of 5 and 15 days.

Phenobarbital pretreatment

At the end of 4 and 7 weeks as well as during re-feeding studies the rats were injected intraperitoneally with sodium phenobarbital (50mg/kg body weight) for 5 successive days.

Tissue preparation

The animals were starved for the last 12h and killed by decapitation, 24h after the last injection. The livers were excised, weighed, washed in ice-cold

* Dedicated to Professor Ronald W. Estabrook, Professor and Chairman, Department of Biochemistry, University of Texas, Southwestern Medical School at Dallas, Texas, U.S.A., as a mark of respect and whose inspiration is responsible for the work.

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Table 1. *Effect of riboflavin deficiency and phenobarbital pretreatment on liver weight, liver protein content and hepatic drug hydroxylation in adult male and female rats*
The results are means $\pm$ S.E.M. (10 rats in each group). Rats in group A were fed on the normal diet; rats in group B received the riboflavin-deficient diet.

	Feeding period (weeks)	Group A						Group B					
		4			7			4			7		
		Control	Phenobarbital-treated	Phenobarbital-treated	Control	Phenobarbital-treated	Phenobarbital-treated	Control	Phenobarbital-treated	Phenobarbital-treated	Control	Phenobarbital-treated	Phenobarbital-treated
Liver weight (g)	Sex												
	Male	7.2 $\pm$ 0.2	10.2 $\pm$ 0.7	6.8 $\pm$ 0.3	11.2 $\pm$ 0.5	5.7 $\pm$ 0.1	11.1 $\pm$ 0.6	4.8 $\pm$ 0.3	7.7 $\pm$ 0.4				
	Female	4.1 $\pm$ 0.2	7.3 $\pm$ 0.3	4.6 $\pm$ 0.1	6.2 $\pm$ 0.4	4.5 $\pm$ 0.4	7.5 $\pm$ 0.3	3.7 $\pm$ 0.2	7.6 $\pm$ 0.3				
Liver protein content (mg/g of liver)	Sex												
	Male	227 $\pm$ 7	261 $\pm$ 5	250 $\pm$ 9	285 $\pm$ 5	232 $\pm$ 10	270 $\pm$ 9	290 $\pm$ 7	312 $\pm$ 5				
	Female	205 $\pm$ 7	233 $\pm$ 3	217 $\pm$ 4	247 $\pm$ 4	226 $\pm$ 6	260 $\pm$ 9	231 $\pm$ 7	269 $\pm$ 4				
Aniline hydroxylation (nmol/min per mg of protein)	Sex												
	Male	3.8 $\pm$ 0.1	8.2 $\pm$ 0.4	5.3 $\pm$ 0.2	10.8 $\pm$ 0.5	2.4 $\pm$ 0.2	5.0 $\pm$ 0.1	2.0 $\pm$ 0.1	4.2 $\pm$ 0.2				
	Female	2.6 $\pm$ 0.1	4.9 $\pm$ 0.2	5.0 $\pm$ 0.1	8.7 $\pm$ 0.3	2.0 $\pm$ 0.2	3.5 $\pm$ 0.1	1.4 $\pm$ 0.1	2.7 $\pm$ 0.1				
Acetanilide hydroxylation (nmol/min per mg of protein)	Sex												
	Male	3.4 $\pm$ 0.1	8.7 $\pm$ 0.1	5.5 $\pm$ 0.1	9.4 $\pm$ 0.2	2.1 $\pm$ 0.2	5.1 $\pm$ 0.3	2.0 $\pm$ 0.1	4.0 $\pm$ 0.1				
	Female	2.9 $\pm$ 0.1	5.1 $\pm$ 0.1	4.9 $\pm$ 0.2	9.0 $\pm$ 0.3	1.8 $\pm$ 0.1	3.4 $\pm$ 0.2	1.5 $\pm$ 0.1	2.7 $\pm$ 0.1				

Table 2. *Effect of riboflavin deficiency and phenobarbital pretreatment on lipid peroxidation in adult male and female rats*

The lipid-peroxidation results are means $\pm$ S.E.M. (10 rats in each group).

Group	Feeding period (weeks)	Sex	Rate of NADPH-linked lipid peroxidation (nmol of malonaldehyde formed/min per mg of protein)			Percentage of difference due to:			Rate of ascorbate-induced lipid peroxidation (nmol of malonaldehyde formed/min per mg of protein)			Percentage difference due to:		
			Phenobarbital-treated			Phenobarbital-treated			Phenobarbital-treated			Phenobarbital-treated		
			Control	Phenobarbital-treated	Diet	Control	Phenobarbital-treated	Diet	Control	Phenobarbital-treated	Diet	Control	Phenobarbital-treated	Diet
A	4	Male	13.8 $\pm$ 0.9	10.2 $\pm$ 0.5	100	-26†	11.3 $\pm$ 0.8	9.8 $\pm$ 0.4	100	-24†				
	7	Male	16.3 $\pm$ 0.7	13.7 $\pm$ 0.3	100	-16†	13.4 $\pm$ 0.5	10.5 $\pm$ 0.3	100	-22†				
B	4	Male	11.3 $\pm$ 0.5	8.4 $\pm$ 0.3	-18†	-26†	9.7 $\pm$ 0.3	7.8 $\pm$ 0.5	-14	-20†				
	7	Male	12.7 $\pm$ 0.7	9.0 $\pm$ 0.4	-38*	-29†	10.0 $\pm$ 0.4	8.2 $\pm$ 0.5	-25†	-18†				
A	4	Female	9.6 $\pm$ 0.3	7.0 $\pm$ 0.3	100	-27†	8.7 $\pm$ 0.1	6.2 $\pm$ 0.2	100	-29†				
	7	Female	11.8 $\pm$ 0.5	9.3 $\pm$ 0.2	100	-21†	10.0 $\pm$ 0.2	8.5 $\pm$ 0.3	100	-15				
B	4	Female	6.3 $\pm$ 0.2	5.0 $\pm$ 0.3	-34*	-21†	6.0 $\pm$ 0.2	4.5 $\pm$ 0.2	-31*	-25†				
	7	Female	7.9 $\pm$ 0.3	5.8 $\pm$ 0.2	-33*	-27†	6.3 $\pm$ 0.2	5.2 $\pm$ 0.1	-37*	-18†				

* Indicates significant difference ($P < 0.001$).

† Indicates significant difference ($P < 0.01$).

0.9% NaCl and minced. The tissues were homogenized (1:10, w/v) in ice-cold 0.25M-sucrose with a Teflon-pestle glass homogenizer. All tissue preparations were made at 0–2°C. The homogenates were centrifuged at 9000g for 20min in a refrigerated centrifuge at –2°C. Protein was determined by the biuret method (Gornall *et al.*, 1949).

Drug-hydroxylation activity

Measurement of hydroxylation was done in a medium containing 50mM-Tris-HCl buffer, pH7.4, 5mM-MgCl₂, 50μl of NADPH (50mg/ml), 2mg of protein (9000g supernatant)/ml and aniline and acetanilide as substrates (8mM each) in a final volume of 15ml.

The incubations were carried out under air at 37°C for 15min in a Dubnoff metabolic shaker. Under these assay conditions linearity was observed. Aniline and acetanilide hydroxylations were measured by following the formation of *p*-aminophenol (Brodie & Axelrod, 1948) and *p*-hydroxyacetanilide (Weisburger & Goodall, 1968) respectively. Activities were expressed as nmol of product formed/min per mg of protein.

Lipid peroxidation

NADPH-linked lipid peroxidation was assayed by the method of Ernster & Nordenbrand (1963) in a medium containing 50mM-Tris-HCl buffer, pH7.4, 10μM-MnCl₂, 50mM-nicotinamide, 0.5mM-NADP⁺, 0.5mM-sodium isocitrate, 50μl of isocitrate dehydrogenase, 0.2mM-ADP, 30μM-Fe²⁺ and 2mg of protein (9000g supernatant)/ml in a final volume of 15ml. The incubations were carried out under air in a Dubnoff metabolic shaker at 37°C for 15min.

Ascorbate-induced lipid peroxidation was carried out in the same medium, but nicotinamide was

omitted and the NADPH-generating system was replaced by 1mM-ascorbate. Malonaldehyde formation was measured by the thiobarbituric acid reaction (Bernheim *et al.*, 1948).

O₂ uptake

O₂ uptake was measured by using Warburg's (1957) method in a medium containing 50mM-Tris-HCl buffer, pH7.4, 0.5mM-NADPH and 5mg of protein in a final volume of 3.0ml. The reactions were carried out at 37°C for up to 30min.

Results

Effect of riboflavin deficiency on liver weight and liver protein content

The liver weights of the riboflavin-deficient male rats were significantly decreased compared with controls after both 4 weeks and 7 weeks of deficiency. However, in riboflavin-deficient female rats a slight increase was observed after 4 weeks, followed by a marked decrease at the end of the 7th week as compared with controls. In all groups liver weights were lower in female than in male rats.

Phenobarbital pretreatment produced significant induction of liver protein synthesis even in the riboflavin-deficient animals. This was independent of sex and the degree of riboflavin deficiency. At the end of the 7th week, the induction in riboflavin-deficient female rats was higher than in all other animals. The liver protein content was increased in riboflavin-deficient male and female rats compared with their respective controls. Phenobarbital pretreatment significantly increased the liver protein content even in deficient animals. The degree of induction was greater in control male rats than in control female

Table 3. Effect of riboflavin deficiency and phenobarbital pretreatment on O₂ consumption in adult male and female rats

The results are means ± S.E.M. (10 rats in each group).

Group	Sex	Feeding period (weeks)	O ₂ consumption (nmol/min per mg of protein)		Percentage difference due to diet	Percentage of individual control due to phenobarbital treatment
			Control	Phenobarbital-treated		
A	Male	4	23.5 ± 3.0	30.0 ± 3.0	100	127*
		7	33.7 ± 2.0	40.2 ± 2.0	100	119*
	Female	4	20.2 ± 1.0	27.6 ± 1.0	100	136*
		7	29.6 ± 2.0	31.7 ± 1.0	100	106
B	Male	4	30.7 ± 3.0	37.5 ± 3.0	+30.6	121*
		7	32.0 ± 3.0	40.0 ± 2.0	–5.2	125*
	Female	4	25.2 ± 2.0	32.1 ± 1.0	+24.7*	127*
		7	21.7 ± 1.0	30.2 ± 3.0	–26.8*	110

* Indicates significant difference ($P < 0.01$).

rats. The degree of induction was higher in riboflavin-deficient female rats than in riboflavin-deficient male rats.

Drug-hydroxylation activity

Table 1 presents results for aniline hydroxylase activity and acetanilide hydroxylase activity. These activities were lower in livers from riboflavin-deficient animals (both male and female) and decreased further with an increase in the degree of deficiency. Activities were always lower in livers from female rats than in those from male rats.

Phenobarbital pretreatment, irrespective of sex and degree of deficiency, caused a marked stimulation of hydroxylase activity in riboflavin-deficient animals.

Lipid peroxidation

Table 2 gives the rate of lipid peroxide formation in control and riboflavin-deficient male and female rats. NADPH-linked lipid peroxidation was low in riboflavin-deficient rats. The percentage decrease was 18 and 34% in male and female rats respectively at the end of 4 weeks, and 38 and 33% at the end of 7 weeks. Thus it can be inferred that there was a pronounced decrease in NADPH-linked lipid peroxidation resulting from chronic riboflavin deficiency.

Ascorbate-induced lipid peroxidation was also low in riboflavin-deficient rats. The percentage decrease was 14 and 31% in male and female rats respectively at the end of 4 weeks, and 25 and 37% at the end of 7 weeks. The decrease in ascorbate-induced lipid peroxidation was relatively greater in all rats during chronic deficiency of riboflavin.

Effect of phenobarbital pretreatment on lipid peroxidation

Phenobarbital pretreatment caused a significant decrease in NADPH-linked and ascorbate-induced lipid peroxidation in all animals irrespective of sex and extent of riboflavin deficiency. The decrease in NADPH-linked lipid peroxidation in normal rats (both male and female) was greater at the end of the 4th week than at the end of the 7th week, whereas in all riboflavin-deficient animals NADPH-linked lipid peroxidation decreased progressively during riboflavin deficiency. A similar decrease was observed in ascorbate-induced lipid peroxidation.

O₂ consumption

Table 3 shows the effect of riboflavin deficiency and phenobarbital pretreatment on O₂ consumption. At the end of the 4th week the O₂ consumption was increased significantly in both male and female rats and decreased at the end of the 7th week.

Table 4. *Effect of riboflavin administration on liver weight, liver protein content and hydroxylation of aniline and acetanilide in adult male and female rats*

The results are means $\pm$ S.E.M. (10 rats in each group).

	Sex	Re-feeding period (days) ...	Group A				Group B			
			5		15		5		15	
			Control	Phenobarbital-treated	Control	Phenobarbital-treated	Control	Phenobarbital-treated	Control	Phenobarbital-treated
Liver weight (g)	Male		7.0 $\pm$ 0.2	12.0 $\pm$ 0.4	9.5 $\pm$ 0.3	10.5 $\pm$ 0.3	5.0 $\pm$ 0.2	9.0 $\pm$ 0.5	5.0 $\pm$ 0.1	9.0 $\pm$ 0.2
	Female		4.0 $\pm$ 0.4	6.0 $\pm$ 0.3	5.0 $\pm$ 0.1	9.7 $\pm$ 0.2	4.0 $\pm$ 0.2	6.0 $\pm$ 0.7	3.5 $\pm$ 0.3	6.0 $\pm$ 0.2
Liver protein content (mg/g of liver)	Male		266 $\pm$ 3	286 $\pm$ 4	272 $\pm$ 5	299 $\pm$ 4	297 $\pm$ 5	310 $\pm$ 4	285 $\pm$ 5	300 $\pm$ 8
	Female		216 $\pm$ 5	249 $\pm$ 5	232 $\pm$ 4	264 $\pm$ 5	245 $\pm$ 6	264 $\pm$ 5	230 $\pm$ 5	252 $\pm$ 6
Aniline hydroxylation (nmol/min per mg of protein)	Male		5.4 $\pm$ 0.2	10.4 $\pm$ 0.5	6.2 $\pm$ 0.2	12.0 $\pm$ 0.4	3.0 $\pm$ 0.1	5.0 $\pm$ 0.2	5.8 $\pm$ 0.2	10.7 $\pm$ 0.3
	Female		5.0 $\pm$ 0.3	8.9 $\pm$ 0.2	6.1 $\pm$ 0.5	10.7 $\pm$ 0.3	2.5 $\pm$ 0.1	4.7 $\pm$ 0.2	5.4 $\pm$ 0.1	9.4 $\pm$ 0.4
Acetanilide hydroxylation (nmol/min per mg of protein)	Male		5.3 $\pm$ 0.1	9.7 $\pm$ 0.5	6.0 $\pm$ 0.2	11.9 $\pm$ 0.5	2.5 $\pm$ 0.2	4.7 $\pm$ 0.1	5.7 $\pm$ 0.1	9.9 $\pm$ 0.4
	Female		4.8 $\pm$ 0.3	9.2 $\pm$ 0.4	5.7 $\pm$ 0.1	11.0 $\pm$ 0.3	2.3 $\pm$ 0.2	4.5 $\pm$ 0.2	5.6 $\pm$ 0.2	9.5 $\pm$ 0.3

Table 5. Percentage increase in lipid peroxidation caused by administration of riboflavin in adult male and female rats

Lipid peroxidation	Sex	Percentage decrease in malon- aldehyde formation at the end of 7th week of riboflavin deficiency compared with control	Percentage in malonaldehyde formation during various re-feeding periods				
			Days ...	5	7	10	15
NADPH-linked	Male	−38.2		+16.2	+18.8	+25.8	+29.0
	Female	−33.1		+8.0	+13.1	+17.1	+17.8
Ascorbate-induced	Male	−25.4		+1.0	+5.0	+11.8	+22.1
	Female	−42.7		+7.5	+10.5	+14.5	+18.4

Phenobarbital pretreatment caused a significant increase in O₂ consumption in the animals irrespective of sex and feeding period.

Re-feeding studies

Effect of riboflavin administration on liver weight and liver protein content. After the scheduled period of the experiment, when normal diet was fed to both the control rats and the riboflavin-deficient rats, an increase in liver weights and a decrease in liver protein content were observed in riboflavin-deficient rats, whereas in the control group of rats of both sexes the liver weights and liver protein content increased normally. The phenobarbital-induced effect was observed in all groups, but the percentage of induction was slightly lower in riboflavin-deficient male rats than in riboflavin-deficient female rats.

Effect of riboflavin administration on drug-hydroxylation activity. There was a significant increase in drug hydroxylation that was due to the administration of riboflavin in both male and female rats during the various re-feeding periods (Table 4). The increase in the activities of aniline hydroxylase and acetanilide hydroxylase was more rapid for riboflavin-deficient animals during the re-feeding periods than for controls. Riboflavin administration caused an almost 60% reversal of drug-hydroxylation activity in both deficient male and female rats as compared with the values at the end of the 7th week.

Effect of riboflavin administration on lipid peroxidation. Table 5 gives the rates of NADPH-linked and ascorbate-induced lipid peroxidation and the percentage increase owing to riboflavin administration to the chronic riboflavin-deficient rats. Lipid peroxidation increased with the increase in riboflavin-administration period in both male and female rats. The total recovery, at the end of the 15th day of riboflavin administration, in NADPH-linked lipid peroxidation was about 70% in male rats and 55% in female rats. In the ascorbate-induced system the percentage increase was about 88 and 40% in male and female rats respectively. The response to riboflavin administration was higher in male than in female rats.

Discussion

Catz *et al.* (1970) and McMurray & Rogers (1973) have noted a significant increase in the concentration of cytochrome *P*-450 in riboflavin-deficient mice. Earlier we observed a marked decrease in the activities of drug-metabolizing enzymes and lipid peroxidation in growing rats during riboflavin deficiency (Patel & Pawar, 1973). Patel & Pawar (1974) have also shown a significant decrease in the activity of NADPH-cytochrome *c* reductase, cytochrome *P*-450 content and the concentrations of flavins (FMN, FAD) in adult rats, resulting from chronic riboflavin deficiency. It is known that FAD is the active prosthetic group of NADPH-cytochrome *c* reductase in the mixed-function oxidases. The dietary deficiencies could alter the morphological and structural features of smooth and rough endoplasmic reticulum, which could be a possible reason for the decreased activities of the drug-metabolizing enzymes. The ultrastructural changes in the hepatocytes of riboflavin-deficient mice have been investigated by Tandler *et al.* (1968). They have also reported evidence for changes in the endoplasmic reticulum.

The classic experiments of Wills (1969a) have significantly contributed towards the understanding of NADPH-linked and ascorbate-induced lipid peroxidation in microsomal fractions. Wills (1969b) has also reported that liver microsomal fractions clearly contain an iron component essential for the formation of lipid peroxides in the presence of ascorbate or NADPH. The source of inhibition of lipid peroxidation during drug hydroxylation has already been traced (Orrenius *et al.*, 1964) to the competition between the two pathways for the common flavoprotein NADPH-cytochrome *c* reductase. The decrease in the activities of drug-metabolizing enzymes and lipid peroxidation could be due to the decreased concentrations of haem, NADPH-cytochrome *c* reductase, FMN, FAD, and/or the loss of the structural integrity of the microsomal electron-transport system. The altered composition of the membrane phospholipids could be an additional cause for the reported changes in the enzyme activities. The observed decrease in lipid peroxidation could be

due to the displacement of iron from its active sites during riboflavin deficiency. It is also possible that the riboflavin deficiency could cause lowering of the concentrations of free iron (non-haem) and total iron.

The observed increase in the activities of drug-metabolizing enzymes and lipid peroxidation resulting from the administration of riboflavin to chronic riboflavin-deficient animals could be due to the resulting higher concentrations of electron-transport components. It is also possible that the integrity of the system is partially repaired during re-feeding of riboflavin.

This work was supported by a grant (1971-1973) from the Indian Council of Medical Research. We are grateful to Dr. V. G. Ganla and Professor M. B. Gharpure, of the Government Medical College, Aurangabad, for providing the animal-house facilities. We also thank Miss Surinder Makhija and Miss Suniti Javalgekar for their assistance in preparing the manuscript.

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