Studies on Riboflavin Tetranicotinate

III. Effect of Riboflavin Tetranicotinate on the Hepatic and the Serum Lipoperoxide Level of Rats Suffering From Fatty Liver¹

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(Received May 15, 1968)

Increase in the peroxide value was found in both fat-soluble and trichloroacetic acid-soluble fractions of fatty liver, which was induced by massive doses of vitamin B supplements on rats previously stunted with low protein, high carbohydrate diet with no B-vitamins. Administration of choline and myoinositol prevents the accumulation of fats in the liver, as well as the increase in TBA values of both the liver fractions, but at the same time, it seemed to induce the increase in the serum TBA value due probably to their acceleration of the removal of fats from the liver.

Although both riboflavin and B₂-Nic showed no beneficial effect on preventing the development of fat accumulation, either of them seemed to have some effect on preventing the increase in the lipoperoxide values both in the liver and in the serum. It was noted that by administering lipotropic agents and B₂-Nic simultaneously, both the fat accumulation in the liver and the increase in peroxide contents in the liver and in the serum were markedly prevented.

It is well-known that there are multiple causes that induce the accumulation of fat in the liver of experimental animal and, therefore, the elucidation of common mechanism of its induction has been considered to be difficult. Administration of chemicals such as ethanol (1-4), carbon tetrachloride (5-10), ethionine (11-13), orotic acid (14-16) and so on, or choline deficiency (17, 18), excess administration of fat or cholesterol (19, 20), vitamin imbalance (21), malnutrition due to the administration of low protein, high carbohydrate diet (22-25), all of which in fact induce the fat infiltration in the liver. Moreover, in view of the complexity of liver lipids and the fact that the liver is very active in lipid metabolism, it would not

¹ Following abbreviations are used: B₂-Nic, riboflavin-2',3',4',5'-tetranicotinate; TBA, thiobarbituric acid.

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be surprising that there are occurrences of many types of fatty liver.

Recently, however, it has been reported that the development of fatty liver induced by chemicals is always accompanied by the increase in the concentration of liver peroxide (4, 16, 26), and some antioxidants prevent the development of the fat accumulation of this kind (4). In view of these observations, there arises a possibility that the toxicity of peroxide (27-29) increasing in the liver would be a serious factor for the development of fatty livers (4, 16, 26).

To support this view further, it may be worthwhile to clarify the generality of the increase of peroxide in every type of fatty liver. Our recent study in fact has shown an increase of peroxide concentration in a fatty liver which was induced by stunting the experimental animals with low protein, high carbohydrate diet with no vitamin B supplements (22-25). Besides this discovery, the effects of the administration of lipotropic agents and of riboflavin or its derivative, B₂-Nic, on the liver-peroxide and the serum-peroxide concentration of rats suffering from this type of fatty liver are described herein.

MATERIALS AND METHODS

Male rats of Wistar strain weighing about 80-100 g were used. Free riboflavin was obtained from commercial source and used after being recrystallized from ethanol-water. Choline chloride was also obtained from commercial source and used without further purification. Myoinositol was kindly offered from the Central Laboratory of Toyo Koatsu Industry Co., and used without further purification. B₂-Nic was prepared according to Kotaki and Yagi (30). Other chemicals such as TBA and malonaldehyde (tetraethoxypropane) were obtained from commercial sources.

For the measurements of visible absorption spectra, a Beckman DK-2A spectrophotometer was used.

EXPERIMENTAL

1. *Feeding of Animals*

Rats were divided into 3 groups, A, B and C (see Table 1), and fed on high carbohydrate, low protein diet (23). In addition, group B was administered with free riboflavin, and group C with B₂-Nic. These supplements were given by mixing with the diet, respectively (see Table 1). Water was administered freely.

After stunting the rats upon these diets for 3 weeks in the individual cage maintained at 23°, each group was further divided into 5 groups, respectively. Groups A-II, B-II and C-II were then administered subcutaneously with vitamin B supplements (23) every day. Besides the vitamin supplements, groups A-III, B-III and C-III were administered subcutaneously with myoinositol; groups A-IV, B-IV and C-IV with choline; and groups A-V, B-V and C-V with myoinositol plus choline, as summarized in Table 1. After a week of these treatments, rats were sacrificed by decapitation under ether anesthesia, and bloods were collected and the livers removed rapidly.

2. *Measurements of Liver Total Lipids*

Measurements of liver total lipids were made according to the method described

TABLE 1
Diet and Supplements

Experimental group	Supplements ^a
A: Rats fed on high carbohydrate, low protein diet (23).	I: None II: Vitamins ^b III: Vitamins ^b +myoinositol (30 mg) IV: Vitamins ^b +choline (30 mg) V: Vitamins ^b +myoinositol (30 mg)+choline (30 mg)
B: Rats fed on high carbohydrate, low protein diet (23), containing 20 mg of riboflavin in 100 g	I: None II: Vitamins ^b III: Vitamins ^b +myoinositol (30 mg) IV: Vitamins ^b +choline (30 mg) V: Vitamins ^b +myoinositol (30 mg)+choline (30 mg)
C: Rats fed on high carbohydrate, low protein diet (23), containing 20 mg (with respect to riboflavin) of B ₂ -Nic in 100 g	I: None II: Vitamins ^b III: Vitamins ^b +myoinositol (30 mg) IV: Vitamins ^b +choline (30 mg) V: Vitamins ^b +myoinositol (30 mg)+choline (30 mg)

^a Injected subcutaneously to rats fed on the basal diets mentioned in the left column for three weeks.

^b A mixture of water soluble vitamins (23).

in the previous paper (31), except methanol-chloroform (1:3, v/v) was used for the extraction of liver lipids instead of ethanol-diethyl ether system used previously.

3. Measurements of Liver TBA Value

Measurements of TBA values of the liver lipid fraction were made according to Nishigaki, Ozawa and Yagi (32), and those of trichloroacetic acid-soluble fraction according to Tappel and Zalkin (33).

4. Measurements of Serum TBA Value

Serum TBA value was measured according to Yagi, Nishigaki and Ohama (34).

RESULTS

1. Effect of Administration of Free Riboflavin or B₂-Nic upon Development of Fatty Liver

The analytical data of liver total lipids of each experimental group are summarized in Table 2. While the results in fact indicate the lipotropic action of choline and myoinositol, neither riboflavin nor B₂-Nic shows preventing effect on the development of fat infiltration in the liver although rats were administered with either of them throughout the experimental period.

2. TBA Value of the Lipids of the Fatty Liver

As shown in Fig. 1, curve I, the lipids extracted from the fatty liver under argon gas flush (32), when reacted with TBA, showed the absorption spectrum identical with that of pinkish pigment obtained from the reaction of TBA with malonaldehyde (35) (Fig. 1, curve II). Since the formation of this pinkish colored substance from lipid and TBA could be attributed to the occurrence of lipoperoxide (32), it was considered to be valuable to estimate the TBA values of the extracted lipids of the livers of each experimental group, the results of which are summarized

TABLE 2
Analytical Data of Liver Total Lipids of Experimental Groups^a

Experimental group	Body weight	Liver wet weight	Liver total lipid	per cent ^b
	g	g	g	
A-I	67	2.0	0.20	10.0
A-II	100	5.1	0.73	14.3
A-III	100	5.5	0.47	8.6
A-IV	93	3.8	0.32	8.4
A-V	85	3.8	0.24	6.3
B-I	71	4.6	0.43	9.4
B-II	102	6.4	0.91	14.2
B-III	101	4.6	0.38	8.3
B-IV	105	7.1	0.64	9.0
B-V	100	5.5	0.36	6.6
C-I	68	2.7	0.26	9.6
C-II	107	7.5	1.07	14.3
C-III	98	4.9	0.38	7.8
C-IV	99	5.5	0.50	9.1
C-V	108	5.3	0.30	5.7

^a All figures are the averages of 5 animals.

^b Weight per cent of total lipids *versus* wet weight of liver.

in Table 3. The groups suffering from fatty liver (A-II, B-II and C-II) gave high TBA values than did the groups partly cured with myoinositol (A-III, B-III and C-III), choline (A-IV, B-IV and C-IV), or with both myoinositol and choline (A-V, B-V and C-V). From these observations, it is clear that the concentration of liver lipoperoxides increases when rats are suffering from fatty liver induced under the present experimental conditions. However, such increases in TBA value are not always in parallel with the fat accumulation in the liver, as deduced from the data reported in Tables 2 and 3. The lipid contents of the unit wet weight of the fatty livers (group A-II, B-II and C-II) are about 2-3 times higher than those of the livers cured with choline and myoinositol (group A-V, B-V and C-V), whereas as reported in Table 3, contents of TBA-reactive substances of the former are found to be more than 6 times higher than those of the latter (except group C). Data on group C indicate that the effect of B₂-Nic is noticeable; the TBA value of group A-II, which is administered with neither riboflavin nor B₂-Nic, is 6.5 times higher than that of group A-V which is cured by administration of choline and myoinositol, whereas the TBA value of group C-II, which is administered with B₂-Nic, is only 2.4 times higher than that of corresponding cured group (C-V). The result indicates that B₂-Nic plays a role in preventing the increase of lipoperoxide. Free riboflavin scarcely showed such effect as B₂-Nic did, and the effective action of B₂-Nic may be attributed to its high affinity with lipids.

3. TBA Value of the Trichloroacetic Acid Extract of the Fatty Liver

TBA develops orange-yellow color upon reacting with glucose (34); the absorption spectrum of the product is shown by curve III in Fig. 1. It shows a peak at 515 m μ , and a considerable absorbance at 532 m μ . Therefore, it is necessary to take the contribution of the color due to carbohydrate into account, when "TBA values" of samples containing some amount of carbohydrate such as water extracts of

FIG. 1 *Absorption Spectrum of Colored Substance Produced from the Liver Lipid Fraction and TBA*

Curve I: The lipid fraction extracted from 1 g of the liver of group A-II was heated at 100° for 60 minutes with 5 ml of $2.3 \times 10^{-2}M$ TBA in 50 % acetic acid. Colored solution was diluted 9 times with distilled water before measurement; Curve II: 4 ml of $4.8 \times 10^{-3}M$ tetraethoxypropane was heated at 100° for 60 minutes with 1 ml of $7 \times 10^{-2}M$ TBA; Curve III: 2 ml of $5.6 \times 10^{-2}M$ glucose mixed with 2 ml of 10 % trichloroacetic acid was heated at 100° for 60 minutes with 1 ml of $7 \times 10^{-2}M$ TBA.

of the substance which gives the same pigment, upon reaction with TBA, as that produced from malonaldehyde and TBA. However, when rats are administered with myoinositol or choline, a decrease in absorbance at $532 m\mu$ is observable as shown by curve II, suggesting the decrease of substances which give malonaldehyde-type color with TBA. It is also noted from the results of Table 3 that the action of flavins, especially B_2 -Nic, is remarkable in lowering also the TBA value of the trichloroacetic acid extracts of the fatty liver.

4. Effect of B_2 -Nic Administration on Serum TBA Value of Rats

Suppose that the effect of lipotropic agents consists in the acceleration of the removal of deposited fats from the liver, it may be possible to consider that by curing the fatty liver, lipoperoxide level in the serum lipoprotein fraction could rather be elevated. To test this, the serum TBA values of group A were measured

FIG. 2 *Absorption Spectra of Colored Substances Produced from Trichloroacetic Acid-Soluble Fraction of Rat Liver and TBA*

Curve I: 5 g of the liver of group A-II (rats suffering from fatty liver) was homogenized with 5 ml of 0.1 % NaCl at 0° and mixed with 10 ml of 10 % trichloroacetic acid at 0° . 4 ml aliquot of the supernatant was heated at 100° for 60 minutes with 1 ml of $7 \times 10^{-2}M$ TBA; Curve II: The same treatment as Curve I was made with the liver of group A-V (rats cured with choline and myoinositol).

organs are measured at $532 m\mu$. However, as shown by curve I in Fig. 2, the absorption spectrum of the TBA-reaction product of the trichloroacetic acid-soluble fractions of the fatty liver (livers of group A-II) showed the absorption maximum at $532 m\mu$, showing the presence

TABLE 3
Effect of Lipotropic Agents, Riboflavin and B₂-Nic on TBA Values of the Liver of Rats Fed on Low Protein, High Carbohydrate Diet^a

Experimental group	TBA value	
	Lipid fraction ^b	Trichloroacetic acid-soluble fraction
A-I	1.89	0.255
A-II	6.45	0.203
A-III	3.63	0.161
A-IV	1.19	0.148
A-V	1.00	0.150
B-I	0.88	0.215
B-II	6.16	0.155
B-III	1.21	0.145
B-IV	2.06	0.117
B-V	1.00	0.115
C-I	1.23	0.165
C-II	2.41	0.162
C-III	1.91	0.117
C-IV	0.77	0.122
C-V	1.00	0.125

^a All figures are the averages of 5 animals.

^b All figures in this column were expressed as relative values when TBA values of groups given both choline and myoinositol (A-V, B-V and C-V) are unity.

TABLE 4
Effect of B₂-Nic on the Serum TBA Value^a

Experimental group	TBA value
Control ^b	0.375
A-II	0.400
A-III	0.590
A-IV	0.862
A-V	1.345
C-II	0.158
C-III	0.138
C-IV	0.108
C-V	0.100

^a TBA values of groups A-I and C-I could not be measured because of their very low blood content.

^b Normal rats fed on CLEA (CE-2) diet obtained from Nippon Haigo-Shiryo Co.

according to Yagi *et al.* (34). Results are listed in Table 4. In support of the above-mentioned presumption, it is pointed out here that the serum TBA values of rats cured with lipotropic agents (group A-V) are considerably higher than those of rats suffering from fatty liver (group A-II). The result suggests that the lipotropic agents have no ability to decompose lipoperoxides to harmless substance in the living body; the only thing which they can do is probably their removal from the liver. Hence, it may be valuable to note that the use of lipotropic agents only is not always adequate for clinical purposes, in so far as the toxicity of lipoperoxides is taken into account. On the other hand, no elevation of serum TBA value was found when rats were previously fed on B₂-Nic (see Table 4, group C).

DISCUSSION

To measure the amount of lipoperoxides in the biological systems, methods of high specificity and high sensitivity are required because of the necessity of treating usually the objects of rather low lipoperoxide concentrations, in which in addition many disturbing substances would be present. Iodometry (36) may be the most standard method in determining the peroxide content, but it seems to have inadequate sensitivity for this purpose. The method of ultraviolet absorption (26, 37), on the other hand, considered to be suitable, though it is not a direct measure of peroxides but that of intermediates such as diene compounds.

In the present study, TBA method (38) was used because of its simplicity and high sensitivity, in spite of its low specificity. However, as was reported elsewhere (32), the substance in lipid that produces pinkish pigment upon reaction with TBA is considered to be lipoperoxide but not malonaldehyde, though the pigment produced is the same as that produced from malonaldehyde and TBA (35, 39). In addition, malonaldehyde was considered to be absent in the biological system as described by Recknagel and Ghoshal (26), because it, if actually exists, can be rapidly metabolized in mitochondria. Therefore, TBA value measured at 532 m μ probably reflect the amount of lipoperoxide not only in the lipids extracted but also in the trichloroacetic acid extract of the liver, for some amount of lipoperoxide or its low-molecular degradates could be extracted with acid aqueous solution. From these considerations, it may be possible to suppose that most of the TBA-reactive substances in the liver measured at 532 m μ are lipoperoxide, though the contribution of some other substances cannot be neglected. The same is true in the case of serum experiments; the device of Yagi *et al.* (34) for the determination of serum TBA value³ is principally based on these reasons. Therefore, the apprehension described by Recknagel and Ghoshal (26) that TBA reaction cannot be used as a tool to search the evidence for the peroxidation of lipids *in vivo* should be ruled out.

In accord with the observation reported in the literatures (4, 16, 26) that the fat accumulation in the liver due to the administration of chemicals is accompanied with the peroxidation of lipids, the present results in fact indicate the increase in the concentration of substances which react with TBA, in both the lipid- and the water-soluble fractions of the fatty liver induced by disturbing the nutritional balances. However, such an increase in TBA values of both fractions was apparently depressed by administering the lipotropic agents, and further with additional administration of the riboflavin derivative. This indicates that lipoperoxides can be removed from the liver by curing the fatty liver with choline and myoinositol, as long as the present results are concerned. In contrast, however, a significant increase of the TBA-reactive substances was found in the phosphotungstic acid precipitates (34) of sera of rats administered with these lipotropic agents. These results seem to suggest that peroxide-rich fats in the fatty liver move to the blood stream probably in the form of low-density lipoprotein, as a result of the lipotropic action of choline and myoinositol. If this presumption be valid, both choline and myoinositol have no ability to decompose lipoperoxide to harmless substance, but they

³ The method consists in the measurement of TBA values of phosphotungstic acid precipitates of sera in which the protein-bound lipids exist.

only accelerate the removal of lipoperoxide from the liver. To recover animals from the fatty liver in the true sense, it seems therefore necessary to use another substance which decomposes lipoperoxides, together with these lipotropic agents.

The present results have indicated that by using B₂-Nic together with these lipotropic agents, not only the TBA values of the liver fractions, but also the TBA values of serum can be lowered. In this respect it is worth noting the report of Yagi and his coworkers (40) on the antioxidant-like action of the fat-soluble riboflavin derivative; fat-soluble riboflavin derivative could react with lipoperoxide. In this connection may also be referred the reaction of the isoalloxazine ring of B₂-Nic with hydrogen peroxide in the presence of catalytic amount of ferric ion, through which the peroxide disappears (41).

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