Studies on Myoinositol

VI. Effect of Myoinositol on Plasma Lipoprotein Metabolism of Rats Suffering from Fatty Liver

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To approach the mechanism of the lipotropic effect of myoinositol, rates of incorporations of injected ¹⁴C-acetate and ¹⁴C-leucine into cholesterol and protein moiety respectively, of plasma lipoprotein of rats, partly recovered from Handler's fatty liver with this cyclitol, were investigated in comparison with those of fatty liver control. The results indicated that the incorporations of these isotopes are equally elevated by curing partly the fatty liver with myoinositol. In addition, higher incorporation of ¹⁴C-leucine into microsomal trichloroacetic acid-insoluble fraction was observed with rats injected with myoinositol. It was suggested from these results that myoinositol plays some role in the over-all mechanism of the synthesis and secretion of plasma lipoprotein in the liver.

It has been shown in the previous study (1), that myoinositol promotes the recovery of delayed transport of hepatogenic cholesterol to adipose tissues observed in rats suffering from fatty liver induced according to Handler (2). To approach further the mechanism of the lipotropic action of this cyclitol, therefore, it seems important to test whether or not its action is correlated with the turnover of plasma β -lipoprotein, chiefly because the principal mechanism by which the liver disposes of fats and cholesterol synthesized in this organ is considered to be their extrusion from the liver to the blood stream as constituents of β -lipoprotein (3), and the different steatogenic agents which induce fatty liver in experimental animals in fact interfere directly or indirectly with the synthesis and/or secretion of plasma low density lipoprotein (4-11). In addition, decrease in the plasma lipoprotein is observable in rats placed on a choline de-

ficient diet (12-14). Reported herein is a comparison *in vivo* of the rates of incorporations of injected ¹⁴C-acetate and ¹⁴C-leucine into cholesterol and protein moiety of plasma lipoproteins between rats suffering from Handler's fatty liver and those recovered partly from fatty liver with myoinositol. Moreover, additional comparison was made as to the incorporation of injected ¹⁴C-leucine into microsomal protein.

EXPERIMENTAL

1. Rearing of Animals

Male albino rats of Wistar strain weighing about 100 g were used throughout the experiment. Animals were housed in individual wire-bottomed cages in a room maintained at 23°. The animals were placed on a low-protein diet described by Handler (2) as reported previously (1), throughout the experimental period of 4 weeks. After 3 weeks of depletion, the

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animals were divided into 2 groups. One group (Group A) received vitamin B complex (1) containing no myoinositol and choline through subcutaneous injection daily. The other group (Group B) received myoinositol (30 mg/rat/day) together with the B vitamin complex. These treatments were continued for a week.

2. Administration of ^{14}C -Acetate

After a week's treatment of the animals with the B vitamins with or without myoinositol, each rat was injected intraperitoneally with 0.36 μmole of ^{14}C -acetate (41 mCi/mmole, purchased from Daiichi Pure Chemicals Co., Tokyo). The animal was then sacrificed by exsanguination under ether anesthesia at certain times after injection (1 and 2 hours) and blood and liver were collected. In another experiment, 1.2 μmole of ^{14}C -acetate (20.5 mCi/mmole, purchased from Daiichi Pure Chemicals Co., Tokyo) was injected in the same way as above.

3. Administration of ^{14}C -Leucine

Uniformly labeled ^{14}C -leucine (311 mCi/mmole) was obtained from the Radiochemical Centre, Amersham. This was diluted to 82 μCi /mmole with cold leucine and 2.5 μCi of the diluted was injected intraperitoneally to each rat of groups A and B. The animal was then sacrificed by exsanguination under ether anesthesia at certain times after injection (1, 2 and 4 hours) and blood and liver were collected.

4. Determination of Total Hepatic Lipids

To check the extent of fat accumulation in the liver, total hepatic lipids of rats injected with these isotopes were determined gravimetrically as reported previously (15).

5. Separation of Serum Lipoprotein

Separation of low density lipoproteins from rat serum was made by a modified method of Burstein and Samaille (16). One ml of the serum of each rat was mixed with 0.1 ml of 2% dextran sulfate 2000 (obtained from Nakarai Chemicals Co., Kyoto) and 0.2 ml of 1 M CaCl_2 . The resulting precipitates were collected by centrifugation and dissolved in 0.2 ml of 5% NaCl. The solution was then mixed with 0.8 ml of distilled water and 0.2 ml of 1 M CaCl_2 . Precipitates formed were collected again by centrifugation. About 60-70% of serum total cholesterol was concentrated into this lipoprotein dextran sulfate- Ca^{2+} complex fraction, which was used for further analysis without further purification.

6. Incorporation of ^{14}C -Acetate into Serum Lipoprotein-Bound Cholesterol

The serum lipoprotein-dextran sulfate complex fraction of rats injected with ^{14}C -acetate mentioned above was directly taken for saponification with 2 N

alcoholic KOH. Free cholesterol in the hydrolysate was separated on thin layer plate (Silica gel G, distributed from Merck AG, Darmstadt) using a mixture of benzene and ether (4:1, v/v) as a developer and eluted with chloroform-methanol (2:1, v/v). The solvent was then evaporated and the residue was redissolved in ether. An aliquot of the solution was used for estimation of cholesterol according to Zlatkis *et al.* (17). Another aliquot was used for the measurement of radioactivity by use of a liquid scintillation spectrometer of Kobe Industrial Co. Specific radioactivity was expressed as cpm/mg cholesterol. Scintillator used was prepared according to Bray (18).

7. Incorporation of ^{14}C -Leucine into Microsomal Protein

Liver microsomes of rats injected with ^{14}C -leucine were separated as follows: 1 g of fresh liver was minced with scissors, homogenized in 10 ml of 0.25 M sucrose and centrifuged at $13,000 \times g$ for 15 min to precipitate nuclei and mitochondria. The supernatant was recentrifuged at $105,000 \times g$ for 30 min to obtain a microsomal pellet. All operations were made at $0-2^\circ$. The microsomal pellet was resuspended in 10 ml of 0.25 M sucrose and mixed with an equal volume of 10% trichloroacetic acid. The precipitate formed was washed 3 times with 15 ml of 5% trichloroacetic acid, 2 times with water, and 2 times with 99.5% ethanol. The microsomal protein thus obtained was hydrolyzed with 6 N HCl in a evacuated sealed tube at 110° for 24 hours. The hydrolysate was evaporated to dryness *in vacuo* and the residue was dissolved in water. An aliquot was taken for the measurement of radioactivity and the other aliquot was, after mineralization, used for the determination of nitrogen content according to an indophenol method (19). Specific radioactivity was expressed as cpm/mg nitrogen of the hydrolysate.

8. Incorporation of ^{14}C -Leucine into Serum Lipoprotein

The serum lipoprotein of rats injected with ^{14}C -leucine, which was separated as dextran sulfate complex as mentioned above, was dissolved in 0.2 ml of 5% NaCl and applied on a column of Sephadex G-75 pre-equilibrated with 0.1 M phosphate buffer, pH 7.4, containing 5% NaCl, to eliminate free ^{14}C -leucine. The elution was made with the same buffer and the eluate was collected in quantities of 2.5 ml and protein fractions were pooled. Protein was then precipitated by mixing with an equal volume of 10% trichloroacetic acid and was further dealt in the same way as for the trichloroacetic acid-insoluble protein of microsomes.

RESULTS

1. Incorporation of Injected ^{14}C -Acetate into Cholesterol Moiety of Rat Plasma Lipoprotein Fraction

In accord with the previously reported histological and gravimetric results (1), myoinositol administered in addition to the B vitamin complex which is given to fatty liver controls obviously lowers the extent of fat accumulated in the liver (Table 1). To approach further the mechanism of such lipotropic action of myoinositol, our investigation has been initiated by a comparison between the rates of incorporation of injected $1\text{-}^{14}\text{C}$ -acetate into cholesterol moieties of plasma lipoprotein fractions of rats suffering from fatty liver and those treated with myoinositol. Obviously, as shown in Table 2, there is a significant difference in the rate of cholesterol labeling, and higher incorporation is found in rats treated with myoinositol. Since the liver is the principal site for cholesterol biosynthesis (20,21) it is rational to consider that most of isotopic cholesterol found in the plasma lipoprotein fraction is hepatogenic. If so, the difference found in the rate of cholesterol labeling would reflect some beneficial effect of myoinositol on the system of transporting cholesterol from the liver directly to the blood stream. A similar result is obtained when $2\text{-}^{14}\text{C}$ -acetate is used as substrate (Table 3). In this case the efficiency of labeling is considerably higher than that found with the use of $1\text{-}^{14}\text{C}$ -acetate, which is not inconsistent with values expected from established pathway of cholesterol biosynthesis (22).

2. Incorporation of Injected ^{14}C -L-Leucine into Protein Moiety of Rat Plasma Lipoprotein Fraction

As shown in Table 4, the effect of myoinositol on the labeling of protein moiety of plasma lipoprotein fraction with injected ^{14}C -leucine is likely to be in good correspondence to the labeling of cholesterol moiety with injected ^{14}C -acetate. The specific radioactivities of myoinositol-treated groups found 1 hour after injection are obviously higher than those of fatty liver controls. As fats and cholesterol

TABLE 1

Gravimetric results of hepatic total lipid of rats suffering from fatty liver and of those treated with myoinositol^a

Experimental group	Average body weight	Average liver weight	Lipid content
	g		mg/g tissue
Fatty liver controls	87	5.3	205.5
Myoinositol-treated	83	4.2	97.2

^a All the figures are the average values of 36 rats.

TABLE 2

Incorporation of $1\text{-}^{14}\text{C}$ -acetate into plasma lipoprotein-bound cholesterol of rats suffering from fatty liver and of those treated with myoinositol^a

Experimental group	Specific radioactivity	
	1 hour after injection	2 hours after injection
	cpm/mg	cpm/mg
Fatty liver controls	220	545
Myoinositol-treated	504	870

^a All the figures are the average values of 9 rats.

TABLE 3

Incorporation of $2\text{-}^{14}\text{C}$ -acetate into plasma lipoprotein-bound cholesterol of rats suffering from fatty liver and of those treated with myoinositol^{a,b}

Experimental group	Specific radioactivity	
	1 hour after injection	2 hours after injection
	cpm/mg	cpm/mg
Fatty liver controls	2,794	4,104
Myoinositol-treated	4,376	11,572

^a In this case, $25\text{ }\mu\text{Ci}$ of $2\text{-}^{14}\text{C}$ -acetate was injected to each rat.

^b All the figures are the average values of 9 rats.

were not taken by rats during the experimental period, it is expected that the production of chylomicra in lymph cells is so small that most of protein moiety of the plasma lipoprotein fractions is originated from the liver cells. If this presumption is valid, the observed result can be interpreted to mean that the rate of secretion of hepatogenic lipoproteins from the liver cells to the blood stream considerably decreases when rats are suffering from fatty liver, but the decrease is

TABLE 4

Incorporation of ^{14}C -leucine into plasma lipoprotein of rats suffering from fatty liver and of those treated with myoinositol^a

Exp. No	Group	Specific radioactivity		
		1 hour after injection	2 hours after injection	4 hours after injection
		<i>cpm/mg N</i>	<i>cpm/mg N</i>	<i>cpm/mg N</i>
1	Fatty liver controls	7,074	10,494	14,097
	Myoinositol-treated	13,515	16,144	11,000
2	Fatty liver controls	4,330	13,858	14,369
	Myoinositol-treated	10,311	16,649	7,969

^a All the figures are the average values of 9 rats.

reverted by the treatment with myoinositol.

Four hours after injection, however, specific radioactivity of myoinositol-treated groups begins to fall in contrast with the fatty liver controls in which it is still increasing. This fact would indicate that by curing the fatty liver with myoinositol the rate of turnover of hepatogenic lipoprotein increases.

3. Incorporation of ^{14}C -Leucine into Liver Microsomal Protein

Table 5 shows the specific radioactivity of trichloroacetic acid-insoluble protein of liver microsomes obtained 1 hour after injection. In good agreement with the fact observed in the rates of incorporations of ^{14}C -acetate and ^{14}C -leucine into plasma lipoprotein fraction, the specific radioactivity of microsomal proteins of rats treated with myoinositol is much higher than that of fatty liver controls. The result indicates that the hepatic protein synthesis, which is probably inhibited when rats are suffering from fatty liver, is increased by curing the fatty liver with myoinositol.

TABLE 5

Incorporation of ^{14}C -leucine into liver microsomal trichloroacetic acid-insoluble protein of rats suffering from fatty liver and of those treated with myoinositol^a

Exp. No	Group	Specific radioactivity ^b
		<i>cpm/mg N</i>
1	Fatty liver controls	3,448
	Myoinositol-treated	5,802
2	Fatty liver controls	3,352
	Myoinositol-treated	6,247

^a Figures are the average values of 9 rats.

^b Measured 1 hour after injection.

DISCUSSION

In the present study, the point to be emphasized is that the transport of hepatogenic cholesterol into the blood stream is considerably enhanced when fatty liver is cured by the action of injected myoinositol. Before discussing the significance of this enhancement, however, it may be valuable to consider about the principal route through which the present isotopic cholesterol emerges into the blood stream. Although direct identification of plasma lipoprotein bearing labeled cholesterol as constituent has not been made in the present study, several lines of evidence appear to support the idea that the main route is the direct secretion from the liver as a constituent of hepatogenic low density lipoprotein. Certainly, it is possible to suppose that another route such as the excretion through bile duct and reabsorption from small intestine is also working. However, the contribution of this route may not in fact be so large as that of direct secretion, because as reported in the previous paper (1) the removal of deposit fats and cholesterol in the liver as a result of myoinositol administration appears to occur predominantly from the central vein of each lobule, not from the peripheral region. In addition, as pointed above, the amount of chylomicron in the presently prepared lipoprotein fraction is considered to be very small because any fats and cholesterol were not added to the diet in order to minimize the chylomicron secretion from lymph cells and, hence, fractions collected as dextran sulfate complex are expected to be mainly hepatogenic

low density lipoprotein.

If the above consideration is the case, the present results indicate that the action of myoinositol is obviously in the direction to cease the decline of plasma low density lipoprotein secretion from the liver. Therefore, it is rational to take the myoinositol's effect into account in connection with the biosynthesis of plasma lipoprotein in the liver and/or with the mechanism of its secretion into the blood stream. Probably, the production of "transferable" phosphatidylinositol in the liver due to the enlargement of myoinositol pool (23,24) is intimately correlated with these mechanisms.

Several workers (12-14) have indicated that a considerable reduction occurs in the level of circulating low density lipoproteins when rats are placed on choline deficient diet for several days. This may be explained by the fact that the choline containing phospholipids are the main component of low density lipoprotein (25); they are probably essential for the binding between neutral lipids (fat and cholesterol) and protein.

Although the content of cephalin in the plasma low density lipoproteins was reported to be small (25), it is possible to consider that phosphatidylinositol is also an essential constituent to make up intact lipoprotein. If this is the case, myoinositol would also be an essential factor for the transport of neutral lipids from the liver. Besides this point, however, it seems inevitable to show how the lipoprotein thus produced is secreted into blood stream, in order to reach the full understanding of the over-all mechanism of fat extrusion from the liver. In this respect, it is worth to cite here the view of Hokin (26) on the role of phosphatidylinositol turnover in the mechanism of zymogen secretion in pancreas.

The present result has indicated that the incorporation of injected ^{14}C -leucine into the liver microsomal protein of rats suffering from fatty liver is considerably lower than that of rats of which liver condition is improved by the myoinositol treatment. Although this fact is not inconsistent with the increased incorporation of injected ^{14}C -leucine into plasma low

density lipoprotein of rats treated with myoinositol, it does not mean that only the plasma lipoprotein synthesis is depressed in the case of fatty liver. It is more likely that the protein synthesis in the endoplasmic reticulum is totally decreased, and this may be, even though partly, explained by the fact that the level of lipoperoxide, which is toxic for various enzymes (27), is high in the fatty liver of this kind (28).

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