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Studies on Hypolipidemic Agents. V.[1] Influence of 5-Tridecylpyrazole-3-carboxylic Acid, a New Hypolipidemic Agent, on Intestinal Cholesterol Absorption in Rats

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In order to clarify the inhibitory mechanism of 5-tridecylpyrazole-3-carboxylic acid (TDPC) on the absorptive process of cholesterol, the effects of this agent on micelle formation *in vitro* and intestinal uptake of cholesterol in rats as measured by the ligated-loop method *in situ* were investigated using β-sitosterol as a reference agent.

TDPC did not affect the micellar formation or distribution of micellar size, both of which are important determinants in the process of cholesterol uptake, but β-sitosterol increased the distribution of large-sized micelles (>450 nm).

TDPC significantly decreased the intestinal cholesterol uptake by 27%, 44% and 59% at the dose levels of 0.25, 0.50 and 1.0 mg, respectively. Similarly, β-sitosterol significantly decreased the cholesterol uptake by 79% at the dose of 1.0 mg.

It is apparent from the above data that the hypocholesterolemic activity of TDPC and β-sitosterol partly depends on interference with intestinal cholesterol uptake, though there is a difference between the two agents in terms of the effect on micellar size. It can be assumed that both energy-dependent and -independent systems are involved in the process of the intestinal cholesterol uptake, and the inhibitory effect of TDPC might be related to the energy-independent system rather than the energy-dependent system.

Keywords——pyrazole derivative; mucosal cholesterol uptake; micellar formation; micellar size

Introduction

It is well-known that the intestinal absorption of cholesterol plays an important role in the overall metabolism of sterols in the body.[2] Although the intestinal absorption of cholesterol has been a subject of interest to investigators for many years, it is not yet clear whether intestinal cholesterol absorption is dependent on a simple passive diffusion[3,4] or on an energy-dependent active transport process.[5] Consequently, the mechanism of the interfering effect of some hypolipidemic drugs on cholesterol absorption is not clear at present.

In the preceding paper,[1] we reported the effect on cholesterol metabolism of 5-tridecylpyrazole-3-carboxylic acid (TDPC) in dietary hypercholesterolemic rats and normal rats using the dual-isotope plasma ratio method of Zilversmit.[6,7] The results of these studies suggest that the inhibition of cholesterol absorption accounts for the major part of TDPC's cholesterol-lowering action in the hypercholesterolemic model. The present study was undertaken to investigate the effect of this agent on the cholesterol absorptive process. Micellar formation *in vitro* and the intestinal uptake of cholesterol as measured by the intestinal ligated-loop method were examined.

Results and Discussion

There is no conclusive evidence that the intestinal absorption of cholesterol is dependent on either a simple passive diffusion mechanism or an active transport process. In a recent study using the intestinal ligated-loop method, Watanabe *et al.*[8] demonstrated that the intestinal absorption of cholesterol occurs through an active transport system when a physiological dosage is given and by a passive diffusion mechanism when the dosage level is high.

The experimental conditions of the ligated-loop method *in situ* appear to be closer to physiological conditions than do other models used as assay systems for the effect of hypolipidemic agents on the absorptive process of cholesterol. In addition, this model is useful because of its high reproducibility and the short time it requires. Another advantage of this model is that 4—6 segments can be prepared from one animal. Accordingly, this model was used to study the mode of inhibitory action of TDPC on cholesterol absorption.

The coefficient of cholesterol uptake was measured by means of the ligated-loop method with the administration of 25 to 600 μg of cholesterol in 0.5 ml of dose solution. The intestinal cholesterol uptake increased linearly in proportion to the amount added in the range from 50 to 400 μg. However, the cholesterol uptake was clearly saturated in the range of 400 to 600 μg of cholesterol (not shown). These results strongly support the statement by Watanabe *et al.*[8] that a saturable phase exists in cholesterol absorption at the physiological level.

It is known that the amount of cholesterol absorbed through the intestinal tract increases remarkably in the presence of bile acids, and that bile acids are an essential component for the absorption of other nutrients. As shown in Fig. 1, the elevation of bile acid (sodium glycochenodeoxycholate) concentration in micellar solution increased micellar formation, and caused a decrease in the distribution of large-sized micelles, (>450 nm) and an increase in small micelles (<100 nm) as well. These changes were accompanied with marked changes in intestinal cholesterol uptake in the common bile-duct fistula in rats which were operated 24 h before beginning the experiment, to deplete endogenous bile acids. In other words, the cholesterol uptake clearly increased with elevation in bile acid concentration. This suggests that an increased cholesterol uptake results in an increase in small-sized micelles together with

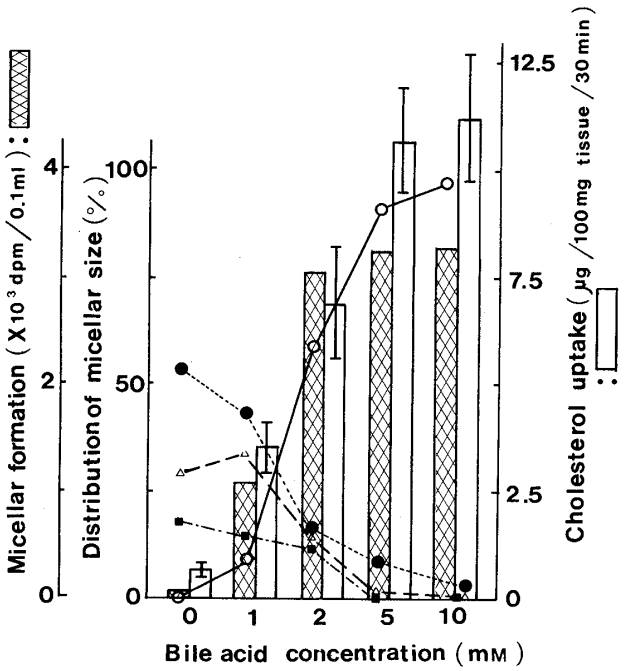

Fig. 1. Effect of Bile Acid Concentration on Micellar Formation, Micellar Size *in Vitro* and Cholesterol Uptake, in Common Bile Duct Fistula Rats

Micellar solution containing [4-^{14}C]cholesterol (0.01 μCi/0.5 ml) and non-radioactive cholesterol (100 μg/0.5 ml) were prepared by dissolving various concentrations of sodium glycochenodeoxycholate and 5 mM monooleoylglycerol in 50 mM sodium phosphate buffer (pH 6.3). Values of micellar formation are each the mean of duplicate determinations. The distributions of micellar size are >450 nm (●------●), <450—220 nm (△---△), <220—100 nm (■-·-■) and <100 nm (○——○). Values of cholesterol uptake are each the mean ± S.E. of triplicate determinations of the jejunal segments in bile duct fistula rats.

an elevation of bile acid concentration.

Since it is possible to determine suitable conditions for measuring cholesterol uptake by conducting a fundamental study, we examined the effect of TDPC in this model. Data relating to micellar formation, and the distribution of micellar size in dose solution measured by the micropartition system are shown in Table I. Micellar formation was not affected by treatment with TDPC or β-sitosterol. β-Sitosterol increased the distribution of large-sized micelles, while TDPC had hardly any effect on the distribution. Table I also shows the effects of TDPC and β-sitosterol on cholesterol uptake. TDPC decreased cholesterol uptake in a dose-dependent manner from 27% ($p<0.05$) at 0.25 mg to 44% ($p<0.01$) at 0.5 mg and 59% ($p<0.001$) at 1.0 mg per 0.5 ml of dose solution. As expected, cholesterol uptake was markedly reduced by β-sitosterol.

In order to examine the functional mode of the inhibitory action of TDPC on the cholesterol uptake, the time-course of cholesterol uptake by TDPC was measured in the presence or absence of 2,4-dinitrophenol (DNP), a typical uncoupler. As shown in Table II, the intestinal cholesterol uptake in the control and DNP groups increased linearly in proportion to the incubation time. These data indicate that the cholesterol uptake was not

TABLE I. Effect of TDPC on Micellar Formation, Micellar Size *in Vitro* and Cholesterol Uptake in Rats

Treatment		Micellar formation	Distribution of micellar sizes (%)				Cholesterol uptake
Agent	Dose (mg/0.5 ml)	(dpm/0.1 ml)	>450nm	<450—220nm	<220—100nm	<100nm	(μg/100 mg tissue/30 min)
Control	—	4652	6.2	0	6.0	87.7	15.2±0.7 [29]
TDPC	0.25	4900	16.1	1.1	4.3	78.4	11.1±1.4[a] (27%) [5]
	0.50	4766	11.3	0.6	4.2	83.9	8.5±2.6[b] (44%) [5]
	1.00	4877	10.7	0.3	2.0	87.1	6.2±2.5[c] (59%) [5]
β-Sitosterol	1.00	4669	93.4	0	2.3	4.2	3.2±0.8[c] (79%) [5]

The experimental conditions are given in the experimental section. Significantly different from the control by Student's *t*-test: *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$. Values in parenthesis represent the percentage reduction from the control level. Values in square brackets represent the number of segments.

TABLE II. Time Course of Effects of TDPC and DNP on Cholesterol Uptake

Treatment	Cholesterol uptake (μg/100 mg tissue)			
	15 min	30 min	45 min	60 min incubation
Control	5.0±1.2 (1.00)	16.5±1.7 (1.00)	26.5±2.5 (1.00)	32.0±3.0 (1.00)
TDPC 0.5 mg/0.5 ml	3.3±0.5 (0.66)	9.1±1.4[b] (0.55)	9.4±1.9[b] (0.35)	11.8±2.2[c] (0.37)
DNP 20 mM	1.5±0.4[b] (0.30)	6.0±0.7[c] (0.36)	17.0±2.1[a] (0.64)	22.4±2.5[a] (0.70)
TDPC 0.5 mg/0.5 ml + DNP 20 mM	1.0±0.2[c] (0.20)	3.6±0.8[c] (0.22)	5.0±1.2[c] (0.19)	7.0±1.5[c] (0.22)

Each value represents the mean ± S.E. of 4—5 segments. The values in parenthesis represent the relative ratio with respect to the corresponding control. Significantly different from the control by Student's *t*-test: *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

completely blocked by DNP, and that the effect of DNP decreased in proportion to the incubation time. These results strongly suggest that both energy-dependent and energy-independent systems are involved in the process of the intestinal cholesterol uptake at a physiological cholesterol level, and that the increase of the cholesterol uptake in proportion to the incubation time is dependent on the energy-independent system rather than the energy-dependent system. On the other hand, a significant reduction in the cholesterol uptake was found on treatment with TDPC alone, and the cholesterol uptake in the TDPC group reached a plateau after incubation for 30 min. From these results, it appears that the inhibitory effect of TDPC might be closely related to the energy-independent system rather than the energy-dependent system.

Using the intestinal ligated-loop method, Shidoji *et al.*[9] demonstrated that the intestinal cholesterol uptake clearly decreases when β-sitosterol (more than 200 μg) is added to micellar solution containing cholesterol at a low level (10 μg/0.5 ml). However, they did not examine in detail the mode of inhibitory action of β-sitosterol on intestinal cholesterol uptake. Therefore, it is not yet clear whether β-sitosterol inhibits the energy-dependent or energy-independent system in the cholesterol uptake process.

We found that β-sitosterol increased the distribution of large-sized micelles which contribute little to the intestinal cholesterol uptake. Consequently, the influence of β-sitosterol on the energy-dependent and -independent systems in the cholesterol uptake process could not be examined in this study. However, further observations are necessary to clarify in detail the mechanism of the inhibitory effects of TDPC and β-sitosterol on the intestinal cholesterol uptake in the ligated-loop method.

Recently, a great deal of attention has been focused on the role of mucosal acyl coenzyme A: cholesterol acyltransferase (ACAT) as a possible mediator in cholesterol absorption.[10-14] However, the inhibitory effect of TDPC on cholesterol uptake has almost no connection with the process of esterification of absorbed cholesterol since most of the radioactive cholesterol found within the intestinal segment in this experiment (1/2 h) was in the unesterified form (data not shown). Furthermore, TDPC does not affect the intestinal mucosal ACAT activity of rabbits. The details of these results will be presented in a subsequent paper.[15] Therefore, the inhibitory effect of TDPC on cholesterol uptake in the intestinal ligated-loop method is independent of esterification of cholesterol in the intestinal wall.

In conclusion, the present study demonstrates that the inhibitory effect of TDPC on cholesterol uptake accounts for at least part of the hypocholesterolemic action in dietary hypercholesterolemia, and shows that there is a substantial difference between TDPC and β-sitosterol with respect to their effects on micellar size distribution *in vitro*.

Experimental

Materials——Animals: Male Sprague–Dawley rats were purchased from Charles River Japan, Inc. The rats were allowed free access to a standard diet (CE-2, Nippon Clea) and tap-water unless otherwise noted.

Chemicals: TDPC was prepared by the method described in our previous paper.[16] Experimental materials were purchased from the following sources; cholesterol, β-sitosterol and sodium deoxycholate (Nakarai Chem. Co.); glycochenodeoxycholate, monooleoyl-glycerol and DNP (Sigma Chem. Co.); [4-^{14}C]cholesterol (s.a. 57.5 mCi/mmol), Aquasol-2 (scintillation solution) and Protosol (tissue solubilizer) [New England Nuclear].

Experiments——Preparation of Micellar Solution and Measurement of Micellar Sizes: The micellar solution was prepared according to the method of Shidoji *et al.*,[8,9] and consisted of 5 mM Na-glycochenodeoxycholate, 100 μg of non-radioactive cholesterol, 5 mM monooleoyl-glycerol and 0.01 μCi of [4-^{14}C]cholesterol in 0.5 ml of a 50 mM sodium phosphate buffer (pH 6.3). Agents were added to 0.5 ml of the micellar solution in amounts ranging from 0.25 to 1.0 mg. After replacement of the atmosphere in a screw-capped vial by N_2 gas, these solutions were incubated at 37 °C overnight in the dark. Aliquots (0.2 ml) of the micellar solutions were transferred to scintillation vials for the determination of radioactivity. This radioactivity value (dpm) was used as the index of micellar formation. Samples of micellar solution (0.2 ml) were filtered successively under centrifugation through filters of 450, 220 (FM-45, FM-22;

Fuji Film Co.) and 100 nm (Brunswick Tech. Co.) pore size in a micropartition system (MPS-1, Amicon Co.). The radioactivity values of each filter and the 100-nm filtrate were then determined in 4 ml of scintillation solution using a liquid scintillation spectrometer (Packard model LSC-900). The micellar size distribution (%) in the micellar solution was obtained by dividing each value by the sum of individual values.

Intestinal Cholesterol Uptake by the Ligated-Loop Method: Male rats, 7 weeks old, were put on an overnight fast prior to the experiment unless otherwise noted. The animals were well anesthetized with sodium pentobarbital, then 4 segments 4—6 cm in length were prepared in the jejunum, maintaining an intact blood supply. Before the segments were completely closed, 0.01 μCi of [4-^{14}C]cholesterol in 0.5 ml of the dose solution, prepared as described above, was injected through one end of each segment. The administered position of micellar solution into each of the four jejunal segments was based on a consideration of the differences of cholesterol uptake at different positions of the jejunum. In order to eliminate the influence of a drop in body temperature on the mucosal uptake, the animals were kept at body temperature by means of a floor heater during the experiment.

After a 30-min incubation, the segments were resected from the body and washed with 2 ml of ice-chilled physiological saline solution. The tissue was further washed 5 times with 2 ml of ice-chilled 5 mM sodium deoxycholate in saline solution to remove the radioactive cholesterol remaining on the mucosal surface. The washed tissue was weighed and transferred into a glass scintillation vial. The tissue was solubilized with 2 ml of Protosol and then decolorized with a small amount of hydroperoxide. Radioactivity was determined using 10 ml of scintillation solution. The mucosal uptake of non-radioactive cholesterol was calculated and expressed as μg/100 mg tissue/30 min.

In the experiment to study the combined effect of TDPC with DNP, and the effects of TDPC alone and DNP alone on the intestinal cholesterol uptake, 0.5 mg of TDPC and 20 mM DNP were included in the micellar solution which was then injected into different segments of the same rats. After incubation for 15, 30, 45 and 60 min, each segment was removed from the body and treated as described above.

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