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Influence of 5-Tridecylpyrazole-3-carboxylic Acid, a New Hypolipidaemic Agent, on Cholesteryl Ester Formation in Rabbit Intestinal Mucosa

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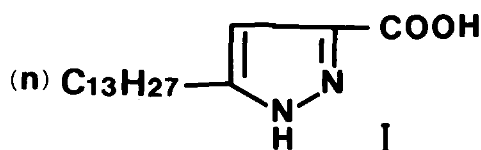
Abstract—The comparative effects of 5-tridecylpyrazole-3-carboxylic acid (TDPC), β-sitosterol and melinamide on the esterification of cholesterol (CH) have been investigated in rabbit intestinal microsomes and cytosol in-vitro. The three agents did not show an effect on cholesteryl ester formation by cholesterol esterase (CEase). TDPC and β-sitosterol did not affect cholesteryl oleate formation from oleoyl CoA by microsomal acyl CoA:cholesterol acyltransferase (ACAT), whereas melinamide significantly inhibited cholesteryl oleate formation. TDPC significantly inhibited the incorporation of oleic acid into cholesteryl oleate, which is associated with acyl CoA synthetase (ACS) plus ACAT in mucosal microsomes, at a concentration of 20–100 μM. On the other hand, 5-tridecylpyrazole-3-carbinol (TDPC-OH) a congener of TDPC, and β-sitosterol did not show any effect. From these results, it is demonstrated that carboxylic moiety of TDPC is necessary to inhibit ACS in-vitro. According to the kinetic analytical results, it is suggested that TDPC acts as a competitive inhibitor of ACS. These results suggest that the inhibitory effect of TDPC on cholesteryl ester formation may be mediated by an inhibition of ACS activity. It is apparent from the data presented that there are substantial differences between TDPC, β-sitosterol and melinamide with respect to their action on cholesteryl ester formation in rabbit intestinal mucosa.

It is well-known that esterification of cholesterol (CH) in the small intestine is a process in its absorption and in lipoprotein synthesis (Bennett Clark 1979; Field et al 1982). It has been suggested that two different enzymatic reactions are involved: a cholesterol esterase reaction catalysed by cholesterol esterase (CEase, EC 3.1.1.13) and an acyltransfer reaction catalysed by acyl CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26). An early study demonstrated that CEase activity and CH absorption decrease in rats with pancreatic fistulas (Borja et al 1964). Gallo et al (1980) reported that the CEase existing in the intestinal mucosa can be derived from pancreatic juice by immunocytochemical techniques. They also found a marked decrease in endogenous CH absorption in the small intestine of rats with no CEase, and with normal levels of ACAT activity, from which they determined the essential role of CEase in the process of CH absorption (Gallo et al 1984).

On the other hand, it has been found that ACAT exists in the intestine of many animals including man (Haugen & Norum 1976; Norum et al 1977, 1979; Field & Salome 1982), and that its activity is responsive to CH and fat feeding (Norum et al 1977; Drevon et al 1979; Field et al 1982); a strong correlation between CH absorption and the activity of intestinal ACAT has been observed.

Recently, Heider et al (1983) and Bennett Clark & Tercyak (1984) reported that specific inhibitors of ACAT cause an inhibition of CH absorption in the gut. They concluded that ACAT plays an essential role in the absorption of CH as reported by Haugen & Norum (1976), Norum et al (1979), Helgerud et al (1981), Bennett Clark (1979), Bennett Clark & Tercyak (1984) and Field et al (1982). Although the biochemical difference between CEase and ACAT is well understood (Hyun et al 1969; Haugen & Norum 1976; Norum et al 1979; Field & Mathur 1983), the relative and physiological role of both enzymes in the absorptive process of CH has not yet been clearly defined. Therefore, we have focused our attention on the relationship between esterification of CH in the small intestine and the action of drugs which inhibit its absorption. The present study was undertaken to study the effect of a new compound, 5-tridecylpyrazole-3-carboxylic acid (I; TDPC) which exerts hypolipidemic activity in hypercholesterolaemic rats as well as an inhibitory effect on CH absorption (Seki et al 1985), on esterification of CH in rabbit intestinal mucosa.



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Materials and Methods

Animals and materials

Male Japanese white rabbits, 2·2–2·4 kg, were caged individually and were maintained on rabbit chow (CR-3, Nippon Clea) with 1% cholesterol for 2 weeks. [1-^{14}C]Oleoyl CoA (55 mCi mmol^{-1}), [4-^{14}C] cholesterol (57·5 mCi mmol^{-1}), [1-^{14}C]oleic acid (53·8 mCi mmol^{-1}), [1α,2α-^{3}H]cholesteryl oleate (52 mCi mmol^{-1}) and Aquasol were purchased from New England Nuclear, Boston, MA, USA. Oleoyl CoA, sodium taurocholate, bovine serum albumin (essentially fatty acid-free), ATP (equine muscle 2Na), CoA and oleic

acid were obtained from Sigma Chem. Co. St. Louis, Mo, USA. All other chemicals used were standard commercial high purity materials. 5-Tridecylpyrazole-3-carboxylic acid (TDPC) was prepared by the method described by Seki et al (1984). Melinamide was isolated from a commercial drug by extraction with ethyl ether and vacuum distillation.

Preparation of subcellular fraction

The subcellular fraction was prepared according to Hashimoto et al (1974). The rabbits were killed between 0900 and 1000 h with rapid injection into the marginal vein of sodium pentobarbitone (200 mg/rabbit). The intestines were immediately removed and placed on ice. A section of small intestine, 15–90 cm from the stomach was taken, and after the lumen had been washed thoroughly with ice-cold saline solution, it was then opened longitudinally. Each segment was washed once with ice-cold saline solution, and the wash was absorbed on a filter paper. The intestinal mucosa was gently scraped with a microscope slide glass onto the surface of an ice-filled Petri dish and placed in 0·25 M sucrose solution. After gently mixing the suspension by inverting the stoppered centrifuge tube several times, it was centrifuged at 900 *g* for 10 min at 4 °C in a Hitachi SCR 20 BB centrifuge using a RPR-S 10 rotor. The resulting supernatant was discarded and the cells were gently resuspended in a final volume of 15 mL of 0·154 M phosphate buffer (pH 6·2). They were then centrifuged at 900 *g* for 10 min. The mucosal cells were resuspended in a final volume of 10 mL of phosphate buffer and homogenized in a motor-driven Teflon pestle (six passes with a loose-fitting pestle) in ice-water. The whole homogenate was centrifuged at 12 000 *g* for 15 min to sediment cellular debris, nuclei and mitochondria. The resulting supernatant was then centrifuged at 107 000 *g* for 30 min at 4 °C in a Hitachi 65 P ultracentrifuge with RP 65 rotor. This supernatant (cytosol fraction) was taken for cholesterol esterase assay and microsomes were resuspended in 0·154 M phosphate buffer (pH 7·4) for the ACAT and ACS plus ACAT assay. Each fraction was stored at −80 °C until it was used for enzyme assay. Protein was determined by the method of Lowry et al (1951) using bovine serum albumin (BSA) as reference standard.

Assay of ACAT activity

The activity of ACAT was determined by the formation of cholesteryl oleate from oleoyl CoA and endogenous cholesterol. The total volume of each assay was 0·5 mL of 0·154 M potassium phosphate buffer (pH 7·4) containing 18 nmol BSA, 1·8 nmol [1-^{14}C]oleoyl CoA (0·1 μCi) and 16·2 nmol oleoyl CoA. The reaction mixture was preincubated at 37 °C for 5 min. After the 5 min period, the reaction was started with 20 μL of microsomal fraction. Since dimethyl sulphoxide (DMSO) alone had no effect on ACAT activity, various concentrations of test agents (20–100 μM) were added to 5 μL of DMSO solution. Control samples contained 5 μL of DMSO alone. The reaction was stopped at 2 min with 7 mL of chloroform–methanol 2:1 (v/v) containing cholesteryl oleate (10 mg L^{-1}) as carrier. [1α,2α-^{3}H]cholesteryl oleate was added to the extraction mixture as an internal standard to calculate recovery. The lipids were extracted according to the procedure of Folch et al (1957) and the solvent was evaporated under nitrogen. The residue was dissolved in 60 μL of toluene and spotted on a thin-layer chromatography plate (LK 5F, whatmann). The chromatograms were developed in hexane–ethyl ether–acetic acid 80:20:1 (v/v). Lipids were visualized with iodine vapor and the band corresponding to cholesteryl ester was scraped directly into counting vials. Radioactivity was assayed by liquid scintillation counting with Aquasol in an Aloka model LSC-900 liquid scintillation counter. The esterification rate was calculated as pmol of cholesteryl oleate formed (mg microsomal protein)$^{-1}$ min^{-1}.

Assay of cholesterol esterase activity

The supernatant from the microsomal preparation was taken for the assay of cholesterol esterase activity essentially by the method of Gallo & Treadwell (1963) with some modification. An aliquot (0·375 mL) of cytosol in a 0·154 M phosphate buffer (pH 6·2) was preincubated at 37 °C for 10 min. After the addition of 0·125 mL of substrate mixture, the reaction mixture was incubated for 3 h. The final mixture contained 5 nmol [^{14}C]cholesterol (0·27 μCi), 1·95 μmol cholesterol, 5·8 μmol oleic acid, 4·1 μmol sodium taurocholate and 12·5 μmol of BSA in a final volume of 0·5 mL. The reaction was stopped at 3 h with 7 mL of chloroform–methanol 2:1 (v/v) containing cholesteryl oleate (10 mg L^{-1}). The extraction and determination of the cholesteryl ester formed were performed as described above of ACAT. The esterification rate was calculated as nmol of cholesteryl ester formed (mg cytosol protein)$^{-1}$ h^{-1}.

Assay of acyl CoA synthetase with addition of ACAT activities

Incorporation of oleic acid into cholesteryl oleate was determined by the method of Brecher & Chobanian (1974) with some modification. In a standard assay, reaction mixture containing 9 nmol [^{14}C]oleic acid (0·484 μCi), 9 nmol oleic acid, 3·75 μmol ATP, 0·3 μmol CoA, 3·75 μmol $MgCl_2$, 18 nmol BSA and 20 μL of microsomal fraction (0·284 mg as protein) in a final volume 500 μL was incubated at 37 °C for 20 min. The extraction and determination of cholesteryl oleate formed were performed as described above. The esterification rate was calculated as pmol of cholesteryl oleate formed (mg microsomal protein)$^{-1}$/20 min.

Results

Effect of TDPC on ACAT and CEase activities

Table 1 shows the results of TDPC and inhibitory agents of cholesterol absorption (β-sitosterol, melinamide) on microsomal ACAT and cytosol CEase activities. TDPC and β-sitosterol in the range of 20 to 100 μM did not have any effect on the activity of either enzyme, whereas melinamide significantly inhibited only ACAT in 20 and 100 μM ($P < 0·001$).

Effect on incorporation of oleic acid into cholesteryl oleate Optimal conditions of assay. Table 2 shows the effect of cofactors on oleic acid incorporation into cholesteryl oleate. The resulting amounts of cholesteryl oleate in the complete assay system containing cofactors (3·75 μmol ATP, 0·3 μmol CoA, 3·75 μmol $MgCl_2$) and in the incomplete assay system without cofactors were 69·9 ± 1·3 and 13·9 ± 1·3 (pmol (mg protein)$^{-1}$/20 min), respectively. The resulting amount of the

Table 1. The Effect of TDPC on cholesterol esterification in mucosal cells.

Treatment		Cholesterol esterification			
Agent	Concn[a] (μM)	Microsomes (ACAT)[b]		Cytosol (CEase)[c]	
Control	—	92·5±2·8[d]	(1·00)[e]	2·55±0·31	(1·00)[e]
TDPC	20	93·8±4·3	(1·01)	2·49±0·02	(0·98)
	50	95·1±6·6	(1·03)	2·62±0·02	(1·03)
	100	93·1±5·7	(1·01)	2·49±0·09	(0·98)
β-Sitosterol	20	98·3±4·5	(1·06)	2·64±0·10	(1·04)
	100	102·9±2·5	(1·11)	2·54±0·05	(1·00)
Melinamide	20	20·6±3·6	(0·22)	2·58+0·10	(1·01)
	100	16·4±1·2[f]	(0·18)	2·57±0·01	(1·01)

[a] The agent was added to the reaction mixture as a DMSO solution (5 μL). The control samples contained 5 μL of DMSO alone.
[b] Picomol cholesteryl oleate formed (mg microsomal protein)$^{-1}$ min^{-1}.
[c] Nanomol cholesteryl ester formed (mg cytosol protein)$^{-1}$ h^{-1}.
[d] Results are the mean±s.e. of three determinations, except control with five determinations.
[e] The value in parentheses indicates the relative ratio against the control.
[f] $P<0·001$ vs control.

Table 2. Cofactor requirement for the incorporation of oleic acid into cholesteryl oleate in microsomal fraction.

	Cholesteryl oleate formed (pmol (mg microsomal protein)$^{-1}$ /20 min)	
Complete assay system[a]	69·9±1·3[b]	(1·00)[c]
-ATP	12·7±1·1	(0·18)
-CoA	11·8±1·0	(0·17)
-$MgCl_2$	27·0±6·8	(0·39)
Incomplete assay system	13·9±1·3	(0·20)

[a] The complete assay system in a final volume of 0·5 mL contains 3·75 μmol ATP, 0·3 μmol CoA, 3·75 μmol $MgCl_2$, 18 nmol bovine serum albumin, 0·154 mM potassium phosphate buffer (pH 7·4) and 0·1% Triton X-100.
[b] Results are the mean±s.e. of the three determinations.
[c] The value in parentheses indicates the relative ratio against the control.

former was about 5-fold higher than that of the latter. The resulting amounts from which ATP, CoA and $MgCl_2$ were omitted singly from the complete system were reduced to 0·18, 0·17 and 0·39 times that of the complete assay system, respectively.

The influence of incubation time and protein content on the resulting amount of cholesteryl oleate is shown in Fig. 1. A linear relationship was found to exist between the resulting amounts of cholesteryl oleate and an incubation time for 2–30 min, protein content (from 0·143 to 0·855 mg). Therefore, subsequent experiments were performed with a 20 min incubation time and with a protein content of 0·285 mg per incubation vial under the complete assay system.

The inhibitory effect of TDPC. The effect of TDPC and its congener, 5-tridecylpyrazole-3-carbinol (TDPC-OH), β-sitosterol and melinamide on cholesteryl oleate formation is shown in Table 3. The resulting amount of cholesteryl oleate was inhibited by TDPC in a concentration-related manner, whereas no effect was found for TDPC-OH and β-sitosterol. Melinamide significantly inhibited the resulting

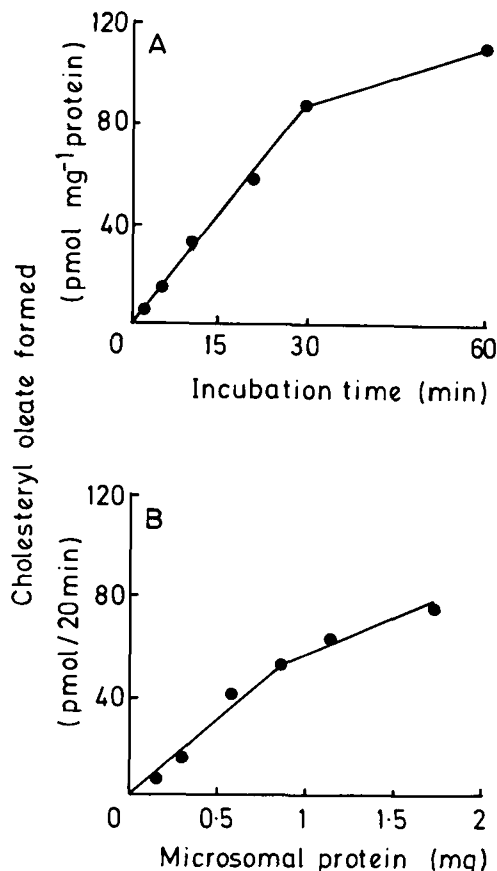

FIG. 1. Effect of incubation time (A) and microsomal protein (B) on the incorporation of oleic acid into cholesteryl oleate in microsomal fraction.

A. The incubation mixture of 0·48 mL of 0·154 mM potassium phosphate buffer (pH 7·4) in 0·1% Triton X-100 containing 3·75 μmol ATP, 0·3 μmol CoA, 3·75 μmol $MgCl_2$, 18 nmol BSA, 9 nmol [^{14}C]oleic acid and 9 nmol oleic acid was preincubated at 37 °C for 5 min before the addition of 20 μL of microsomal fraction (0·285 mg as protein), and incubated for 2–60 min after the addition of microsomes. Results are the mean of duplicate determinations.

B. The reaction mixture (0·5 mL) was preincubated at 37 °C for 5 min before the addition of 10–120 μL of microsomal fraction (0·143–1·71 mg as protein) and incubated for 20 min after the addition of microsomal fraction. Results are the mean of duplicate determinations.

amount of cholesteryl oleate in 100 μM. Furthermore, a significant difference was observed between the effects on cholesteryl oleate formation of TDPC and TDPC-OH in 100 μM.

Inhibitory mode of TDPC. We performed a kinetic study to find an inhibitory process of TDPC on cholesteryl oleate formation. Fig. 2 shows the double reciprocal relation between oleic acid concentrations (4·5–36 nmol) as substrate, and the resulting amounts of cholesteryl oleate. TDPC was found to act as a competitive inhibitor of oleic acid incorporation into cholesteryl oleate.

Discussion

In a previous paper (Seki et al 1985), we reported that TDPC is a new agent which has a hypocholesterolaemic effect on

Table 3. Inhibitory effect of TDPC on incorporation of oleic acid into cholesteryl oleate in microsomal fraction

Treatment		Oleic acid incorporation into cholesteryl oleate
Agent	Concn[a] (μM)	(pmol (mg microsomal protein) ^{1}/20 min)
Control	—	89·8±2·4 (1·00)
TDPC	10	78·2±7·6 (0·87)
	20	68·4±6·0[c] (0·76)
	50	57·0±3·1[d] (0·63)
	100	46·2±2·4[e] (0·51)
TDPC-OH	100	94·7±7·0[f] (1·05)
β-Sitosterol	100	85·5±2·0 (0·95)
Melinamide	100	60·3±4·2[c] (0·67)

The reaction mixture (0·5 mL of final volume) was preincubated at 37 °C for 5 min before the addition of microsomal fraction (0·285 mg as protein) and incubated for 20 min.
[a] The agent was added into the reaction mixture as a DMSO solution (5 μL).
[b] The value in parentheses indicates the relative ratio against the control.
[c] $P<0·05$ vs control, [d] $P<0·01$, vs control, [e] $P<0·001$ vs control.
[f] $P<0·001$ vs TDPC (100 μM).

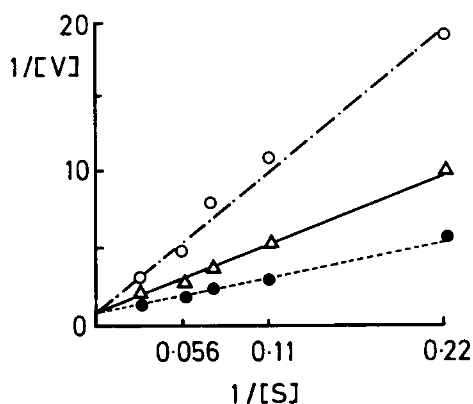

FIG. 2. Lineweaver-Burk plot of the effect of TDPC on incorporation of oleic acid into cholesteryl oleate in microsomal fraction. V = nmol cholesteryl oleate formed (mg microsomal protein)$^{-1}$ h^{-1}; S = nmol oleic acid/incubation mixture ●- - - -●: no TDPC △——△: 10 nmol TDPC, ○-·-·-○: 50 nmol TDPC. Results are the mean of duplicate determinations.

dietary hypercholesterolaemic rats and an inhibitory effect on CH absorption. In the present study, we investigated the effect of TDPC on esterification of CH in rabbit intestinal microsomes and cytosol to elucidate the functional mechanism behind inhibition of CH absorption. Since it has been demonstrated that ACAT activity changes according to CH content and fatty acid composition in microsomes (Field et al 1982), we conducted in-vitro tests to determine the direct action of these agents on enzyme activity. The experimental results demonstrated that TDPC and β-sitosterol did not have any effect on ACAT and CEase activity, whereas melinamide significantly inhibited only ACAT activity. Though TDPC was not found to exert direct action of ACAT, an interesting difference was observed among the inhibitors of CH absorption.

It is generally known that the substrates for ACAT are free CH and acyl CoA (mainly oleoyl CoA). It has also been reported that ACAT activity depends on the concentration of oleoyl CoA as substrate (Helgerud et al 1981). We attempted to determine whether TDPC has an effect on cholesteryl oleate formation, including the oleoyl CoA generating pathway, in microsomes. When oleic acid was used as the substrate for measuring cholesteryl oleate formation, the resulting amounts showed a remarkable increase in the presence of cofactors (ATP, CoA, Mg^{2+}), which increased in proportion to the incubation time and protein content. From these results, it can be assumed that cholesteryl oleate formation from oleic acid results from an energy-dependent enzyme reaction.

In the established assay system, TDPC was found to exert a significant inhibitory effect on cholesteryl oleate formation from oleic acid, with a direct relationship between this effect and the concentration of TDPC, whereas the 5-tridecylpyrazole-3-carbinol (TDPC-OH) congener of TDPC did not have any effect. This implies that the carboxylic moiety of TDPC is the ingredient necessary to elicit its activity. The finding of our preliminary study that the hypocholesterolaemic activity of TDPC-OH in hypercholesterolaemic rats was about 1/3 of that of TDPC, supports this finding. Taking into consideration the above results, it appears that the inhibitory effect of TDPC on cholesteryl oleate formation from oleic acid is closely related to its hypocholesterolaemic action.

The pathway of cholesteryl oleate formation from oleic acid in microsomes is associated with acyl CoA synthetase (ACS) and ACAT (Brecher & Chobanian 1974). It is likely TDPC's effect on cholesteryl oleate formation from oleic acid is based on the inhibition of acyl CoA formation by ACS since, as mentioned above, TDPC did not inhibit ACAT activity. Furthermore, it is suggested that the functional mode of TDPC on cholesteryl oleate formation is a competitive inhibition.

It can be assumed that inhibition of ACAT enlarged the pool size of acyl CoA, which made possible the development of the synthetic sthenia of triglyceride and phospholipid. It is therefore suggested that TDPC, which has an inhibitory action on oleoyl CoA formation compared with ACAT inhibitors, may have a hypolipidaemic action, including not only cholesteryl ester but triglyceride and phospholipid. This possibility is substantiated by the results of a subsequent experiment that showed TDPC to inhibit strongly the incorporation of labelled acetate into the triglyceride and phospholipid fraction in the liver in-vitro (unpublished data). However, further studies are necessary to clarify the difference of mode of TDPC and inhibitors of ACAT on the lipid metabolism in various tissues.

In conclusion, the present study demonstrated that TDPC is a hypolipidaemic agent, the functional mechanism of which is different from those of the known inhibitors of CH absorption (β-sitosterol and melinamide). The inhibition of cholesteryl oleate formation from oleic acid by TDPC may be at the least part of the mechanism whereby this agent inhibits CH absorption.

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