Mechanism of the Inhibition of Cholesterol Absorption by DL-Melinamide: Inhibition of Cholesterol Esterification

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Accepted September 1, 1986

Abstract—In order to elucidate the mechanism of action of DL-melinamide [DL-MA, N-(α -methylbenzyl)linoleamide], an inhibitor of cholesterol absorption, the effect of DL-MA on esterification of cholesterol in the mucosa of rabbit small intestine was studied. DL-MA inhibited acyl CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) activity in the mucosal microsomes, with 50% inhibition occurring at approximately 0.5 μ M. On the other hand, DL-MA had no effect on the cholesterol esterase (EC 3.1.1.13) activity in the mucosal cytosol. Kinetic studies indicate that DL-MA is an uncompetitive inhibitor of ACAT. D-MA, one of the two optical isomers of DL-MA, was found to be a more effective inhibitor of ACAT than L-MA, another isomer. This finding indicates that the inhibition of cholesterol absorption by DL-MA depends on the inhibition of ACAT by this compound, in view of the fact that D-MA is a more effective inhibitor of cholesterol absorption than L-MA.

It has been reported that DL-melinamide (DL-MA, Artes®), N-(α -methylbenzyl)-linoleamide, has a marked cholesterol-lowering and anti-atherogenic effect in several kinds of cholesterol-fed animals (1–4). The cholesterol-lowering effect of DL-MA is thought to be due to interference with the intestinal absorption of cholesterol, as is the case with β -sitosterol (5, 6), but the detailed mechanism has not yet been clarified.

Fukushima et al. (6) reported that after administering DL-MA along with [3 H]-cholesterol to thoracic-duct fistula rats, the total [3 H]cholesterol level (free+ester) and its ester ratio (ester/total) in the lymph were markedly depressed. Considering that cholesterol is absorbed as the free form (7) and is predominantly esterified before entering the lymph (8), the findings of Fukushima et al. (6) suggest that DL-MA suppresses cholesterol absorption by inhibiting esterification of cholesterol in the intestinal mucosa.

There are two major cholesterol esterification enzymes in the small intestine,

cholesterol esterase (CEase, EC 3.1.1.13) (9) and acyl CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) (10). However, which esterifying enzyme is responsible in vivo is still unclear (11–20). The effect of DL-MA on each of these two enzymes can be studied independently because CEase is found in the cytosol (21) while ACAT is found in the microsomes (10, 22).

In order to elucidate the mechanism of action of DL-MA, the present investigation was undertaken to find out the effects of DL-MA on esterification of cholesterol in the intestinal mucosa in vitro and to compare its effects with those of compound 57–118 (23) and β -sitosterol (5), inhibitors of cholesterol absorption.

Materials and Methods

Chemicals: [4 - 14 C]cholesterol (60 mCi/mmol) and [1 - 14 C]oleoyl CoA were obtained from Daiichi Pure Chemicals (Tokyo, Japan), and β -sitosterol was obtained from Nakarai Chemicals, Ltd. (Kyoto, Japan). DL-MA and two optical isomers of DL-MA (D-MA and

L-MA) were synthesized in our laboratories. Optical rotations of these isomers were: $[\alpha]_D^{26.5} = +49.4$ for the D-isomer and $[\alpha]_D^{26.5} = -51.4$ for the L-isomer. N-(1-oxo-9-octadecenyl)-DL-tryptophan (Z) ethyl ester was synthesized in our laboratories. All other chemicals used were standard high purity materials.

Animals: Male, Japanese white rabbits (Kitayama Labs., Inc., Kyoto, Japan) weighing 2.0–2.5 kg were used. They were housed in cages and allowed free access to rabbit pellet chow, CR-1 (Clea Japan, Inc., Tokyo) and water for two weeks before the study.

Preparation of cytosol and microsomes: Five rabbits were used in this preparation. The microsomal and cytosol fractions from the mucosa of rabbit small intestine were isolated from a homogenate prepared in 0.154 M phosphate buffer (pH 6.2) as described by Heider et al. (23). The cytosol was used for CEase assay, and the microsomal fraction was resuspended in 0.154 M phosphate buffer (pH 7.4) for use in the ACAT and triglyceride-synthesis assays.

ACAT assay: The incorporation of $[1-^{14}\text{C}]$ -oleoyl CoA into cholesteryl ester was investigated mainly by the method of Heider et al. (23). The total volume for each assay was 0.5 ml consisting of 10 nmol of BSA (assuming a mol wt of 60,000), 5 μl of acetone solution of each test compound, and 10 nmol of $[1-^{14}\text{C}]$ -oleoyl CoA (6 mCi/mmol) in 0.154 M phosphate buffer (pH 7.4). The control samples received 5 μl of acetone alone. The mixture was incubated at 37°C for 5 min before the addition of the microsomal fraction (0.05 to 0.1 mg of protein) to start the reaction. Then the incubation mixtures were further incubated for 4 min at 37°C.

CEase assay: CEase activity was determined mainly by the method of Heider et al. (23). The volume of the mixture or preincubation was 0.75 ml consisting of 2.0 to 3.0 mg of cytosol protein and 5 μl of acetone solution of each test compound in 0.154 M phosphate buffer (pH 6.2). The control samples received 5 μl of acetone alone. After the mixture was incubated for 5 min at 37°C, substrates and other materials were added to give final concentrations of 3.9 mM $[4-^{14}\text{C}]$ -

cholesterol (0.051 mCi/mmol), 11.6 mM oleic acid, 8.2 mM sodium taurocholate, and 3 mg of BSA per ml in 1 ml of 0.154 M phosphate buffer (pH 6.2) by the addition of 0.25 ml of the substrate mixture prepared by the method of Borja et al. (12). Then the samples were incubated for 3 hr at 37°C.

Assay of triglyceride-synthesis activity: The incorporation of $[1-^{14}\text{C}]$ -oleoyl CoA into triglyceride (TG) was followed mainly by the method of Ross et al. (24). The incubation mixture consisting of 0.5 ml of 0.154 M phosphate buffer (pH 7.4) containing 10 nmol of BSA, 5 μl of acetone solution of each test compound, and 0.05 to 0.1 mg of microsomal protein was incubated at 37°C for 5 min before the addition of 10 nmol of $[1-^{14}\text{C}]$ -oleoyl CoA (6 mCi/mmol). The control samples received 5 μl of acetone alone. Then the samples were incubated at 37°C for 4 min.

Assay of plasma lecithin:cholesterol acyltransferase: The activity of lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) was determined mainly by the method of Bell and Hubert (25, 26). The final volume of the incubation mixture was 0.5 ml. To 425 μl of rabbit plasma, 0.2 mCi of $[4-^{14}\text{C}]$ -cholesterol (60 mCi/mmol) as an albumin-stabilized suspension (25) was added and permitted to equilibrate with lipoprotein cholesterol for 4 hr at 37°C during which time LCAT was inhibited by 5,5-dithio-bis-2-nitrobenzoic acid (0.7 mM). The reaction was initiated by adding β -mercaptoethanol (12 mM) to reverse the inhibition of LCAT. Each test compound was added to the incubation mixture dissolved in acetone (5 μl /0.5 ml of incubation mixture) immediately prior to adding β -mercaptoethanol. The control samples received 5 μl of acetone alone. Then the assay mixtures were incubated at 37°C for 1 hr.

Analyses: Lipids were extracted with a chloroform-methanol mixture as described by Taketani et al. (27), and the extracts were fractionated by thin-layer chromatography on silica gel G-coated glass plates (Merck, Darmstadt) in a solvent system consisting of n-hexane/diethylether/acetic acid (85:15:1, v/v/v) (24) in order to isolate the cholesteryl esters and triglycerides. The cholesteryl

esters and triglycerides were visualized by exposure of the chromatograms to I_2 vapor and then scraped from the plates into vials containing 10 ml of Scintisol AM-1 (Wako Pure Chemical Industry, Ltd., Osaka, Japan) for radioactivity assay in a Packard model TRICARB 460 CD-liquid Scintillation counter. Quench corrections were made using an external standard. Plasma free cholesterol was measured using Free Cholesterol C-Test Wako (Wako Pure Chemical Industries, Ltd.).

Protein was determined by the method of Lowry et al. (28).

Results

Effect on CEase activity: No effect was found for DL-MA, β -sitosterol and 57-118 on CEase activity over the concentration range of 0.1–10 μ M (Table 1).

Effect on ACAT activity: DL-MA was shown to be a potent inhibitor of ACAT, with 50% inhibition occurring at approximately 0.5 μ M and 90% inhibition at 10 μ M (Fig. 1). β -Sitosterol inhibited ACAT activity

much less effectively than DL-MA (Fig. 1, Exp. 1), while 57-118 inhibited ACAT activity in the same manner as DL-MA (Fig. 1, Exp. 2). The kinetic data with DL-MA concentrations of 0 (control) and 0.5 μ M were plotted according to Lineweaver-Burk (Fig. 2). The apparent K_m values were 18.8 and 3.2 μ M, and the apparent V_{max} values were 278 and 42 pmol·mg protein⁻¹·min⁻¹ at DL-MA concentrations of 0 and 0.5 μ M, respectively. These data suggest that DL-MA acts as an uncompetitive inhibitor of ACAT.

Fukushima et al. (6, 29) have shown that D-MA, one of the two optically active isomers of DL-MA, depresses the elevation of total cholesterol levels in the serum of cholesterol-fed rabbits and rats more effectively than L-MA, another isomer. Therefore, in order to determine whether the inhibition of cholesterol absorption by DL-MA depends on the inhibition of ACAT activity by DL-MA, the inhibitory effect of D-MA on ACAT activity was compared with that of L-MA. The results shown in Fig. 3 indicate that the inhibitory effect of D-MA is significantly greater than

Table 1. Effect of DL-MA, β -sitosterol, and 57-118 on CEase activity in the mucosal cytosol of the rabbit small intestine

Exp. 1		
Concentration (μ M)	CEase activity (% of control)	
	DL-MA	β -Sitosterol
0.1	106.7 $\pm$ 0.4	104.0 $\pm$ 1.7
1.0	104.8 $\pm$ 0.6	104.0 $\pm$ 2.1
3.0	104.5 $\pm$ 0.8	101.1 $\pm$ 1.2
10.0	101.6 $\pm$ 0.9	102.9 $\pm$ 1.0
Exp. 2		
Concentration (μ M)	CEase activity (% of control)	
	DL-MA	57-118
0.1	99.0 $\pm$ 1.2	92.8 $\pm$ 1.2
1.0	97.9 $\pm$ 3.2	94.3 $\pm$ 0.9
3.0	100.2 $\pm$ 1.3	98.1 $\pm$ 0.7
10.0	97.4 $\pm$ 2.1	97.0 $\pm$ 3.4

Each compound dissolved in 5 μ l of acetone was added to 1.0 ml (final volume) of the incubation mixture for CEase. The control mixtures received 5 μ l of acetone alone. Enzyme assays were performed as described in Methods. Reactions were initiated by the addition of the substrate mixture containing [4-¹⁴C]cholesterol and oleic acid, after a 5-min incubation of all other components at 37°C. The values are the mean of triplicate samples $\pm$ S.E.M. The control values in Exp. 1 and Exp. 2 were 0.96 $\pm$ 0.01 and 0.90 $\pm$ 0.04 nmol of [4-¹⁴C]cholesteryl oleate·mg of cytosol protein⁻¹·hr⁻¹, respectively.

Fig. 1. Effect of DL-MA, β -sitosterol and 57-118 on ACAT activity in the mucosal microsomes of rabbit small intestine. Each compound dissolved in 5 μ l of acetone was added to 0.5 ml of the incubation mixture for ACAT. The control mixtures received 5 μ l of acetone alone. Enzyme assays were performed as described in Methods. Reactions were initiated by the addition of the microsomal fraction, after a 5-min incubation of all other components at 37°C. Each value is the mean of triplicate assays $\pm$ S.E.M. The control values in Exp. 1 and Exp. 2 were 78.7 ± 4.3 and 88.3 ± 11.8 pmol of cholesteryl [1- 14 C]oleate:mg of microsomal protein $^{-1}$ ·min $^{-1}$, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control.

Fig. 2. Lineweaver-Burk plot of inhibition of ACAT activity by DL-MA. The enzyme assay conditions were as described in Methods except for the variations shown. Each value is the mean of triplicate assays. v , nmol of cholesteryl [1- 14 C]oleate formed:mg of microsomal protein $^{-1}$ ·min $^{-1}$; s , [1- 14 C]oleoyl-CoA μ M.

Fig. 3. Effect of D-MA and L-MA on ACAT activity. Enzyme assays were performed as described in Methods. The values are the means of triplicate samples $\pm$ S.E.M. The control activity of ACAT was 57.2 ± 2.3 pmol of cholesteryl [1- 14 C]oleate:mg of microsomal protein $^{-1}$ ·min $^{-1}$. * $P < 0.05$, ** $P < 0.01$, compared with the corresponding values of D-MA.

that of L-MA at levels of 0.5, 1.0 and 10 μ M.

Effect on TG-synthesis and LCAT activity:

In order to determine whether DL-MA is a selective inhibitor of ACAT, the effects of DL-MA on the TG-synthesis and LCAT activities were studied. DL-MA was shown to inhibit the TG-synthesis activity at 10 μ M (Fig. 4), but at least 60% activity remained at 10 μ M of DL-MA, which inhibited ACAT activity almost completely (Fig. 1). β -Sitosterol had a negligible effect on the TG-synthesis activity (Fig. 4, Exp. 1), and the effect of 57-118 on this activity was similar to that of DL-MA (Fig. 4, Exp. 2).

DL-MA, β -sitosterol and 57-118 had almost no effect on LCAT activity over the concentration range of 0.1–10 μ M (Fig. 5).

Discussion

The present study clearly shows that DL-MA is a potent and selective inhibitor of ACAT in the rabbit intestinal mucosa (Figs. 1, 4 and 5). DL-MA is thought to inhibit *in vivo*

Fig. 4. Effect of DL-MA, β -sitosterol, and 57-118 on the TG-synthesis activity in the mucosal microsomes of the rabbit small intestine. Each compound dissolved in 5 μ l of acetone was added to 0.5 ml of the incubation mixture for TG-synthesis. The control samples contained an equal volume of acetone (5 μ l). Enzyme assays were performed as described in Methods. Reactions were initiated by the addition of [14 C]oleoyl CoA, after a 5-min incubation of all other components at 37°C. Each value is the mean of triplicate assays $\pm$ S.E.M. The control values in Exp. 1 and Exp. 2 were 0.66 ± 0.01 and 0.60 ± 0.03 nmol [14 C]oleoyl-CoA incorporated into TG $\cdot$ mg of microsomal protein $^{-1} \cdot$ min $^{-1}$, respectively. * $P < 0.05$ ** $P < 0.01$, *** $P < 0.001$ vs. control.

ACAT activity in the mucosa of the small intestine because about 50% of orally administered DL-MA is absorbed in rabbits (30). Moreover, the inhibitory effect of D-MA on ACAT activity is significantly greater than that of L-MA (Fig. 3). It was found that both D-MA and L-MA were not metabolized in the incubation mixture in our preliminary study using 14 C-DL-MA (data not shown). Therefore, it may be due to the difference of the stereospecificity of these two isomers. Fukushima et al. (6) demonstrated that the inhibitory effect of D-MA on the transportation of [3 H]cholesterol, administered orally, into the lymph is greater than that of L-MA in thoracic-duct fistula rats. Therefore, it is suggested that the inhibitory effect of DL-MA on ACAT activity is closely related to the inhibition of cholesterol absorption by DL-MA.

Fig. 5. Effect of DL-MA, β -sitosterol, and 57-118 on LCAT activity in rabbit plasma. LCAT activity in rabbit plasma (425 μ l) was inhibited by the addition of DTNB, and lipoprotein cholesterol was permitted to equilibrate with [14 C]cholesterol as described in Methods. The enzyme assay conditions and chemical determination were performed as described in Methods. Reactions were initiated by adding β -mercaptoethanol to the incubation mixture to reverse the inhibition of LCAT activity. Each compound dissolved in 5 μ l of acetone was added to 0.5 ml (final volume) of the incubation mixture for LCAT just prior to adding β -mercaptoethanol. The control mixtures received 5 μ l of acetone alone. Each value is the mean of triplicate assays $\pm$ S.E.M. The control values in Exp. 1 and Exp. 2 were 65.9 ± 4.4 and 57.2 ± 4.4 pmol of [14 C]cholesterol ester formed $\cdot$ ml of plasma $^{-1} \cdot$ min $^{-1}$, respectively.

β -Sitosterol has almost no ability to inhibit ACAT activity as compared with DL-MA (Fig. 1). This finding supports the work of Field and Mathur (31) who showed that β -sitosterol does not interfere with nor compete with the esterification of cholesterol via ACAT. Therefore, it is suggested that β -sitosterol inhibits cholesterol absorption by mechanisms other than inhibition of ACAT activity. For example, the restriction of the micellar solubility of cholesterol by β -sitosterol in the intestinal lumen is proposed as a possible mechanism (32).

Recently, two long chain fatty acylamides were reported to inhibit both cholesterol absorption and ACAT activity. One is compound 57-118 which was reported by Heider et al. (23). They demonstrated that 57-118 inhibits cholesterol absorption in cholesterol-

fed rabbits and that this effect is correlated with the inhibition of ACAT activity in the intestinal mucosa. Our present data (Fig. 1, Exp. 2) confirm the inhibitory effect of 57–118 on ACAT activity observed by Heider et al. (23). DL-MA is similar to 57–118 with regard to the ability to inhibit both cholesterol absorption and ACAT activity. Furthermore, these two compounds have a similar chemical structure: long chain fatty acylamide. However, they have some different features. DL-MA is an uncompetitive inhibitor of ACAT, while 57–118 is a competitive inhibitor (23).

Another acylamide is compound 58–035, 3-(decyldimethylsilyl)-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide, which was studied by Ross et al. (24) and Clark and Tercyak (33). Ross et al. demonstrated that 58–035 is a potent inhibitor of ACAT in the microsomes of both rat liver and hepatoma cells, while it has a negligible effect on both acyl CoA:retinol acyltransferase and TG-synthesis activity. Furthermore, Clark and Tercyak (33) demonstrated that 58–035 greatly reduces the secretion of total cholesterol into the mesenteric lymph and its ester ratio in rats. Fukushima et al. (6) reported the same effect of DL-MA on the secretion of total cholesterol into the lymph and its ester ratio as that of 58–035.

The findings described above suggest that these three acylamides, DL-MA, 57–118, and 58–035, have a common action: inhibition of the transportation of total cholesterol into the mesenteric lymph by inhibiting ACAT activity in the intestinal mucosa. Therefore, it should be very interesting to clarify the relationship between the chemical structure of various acylamides and the inhibitory effects of these compounds on ACAT activity. Extensive studies on this problem are now in progress.

Acknowledgement: We thank Dr. Takenari Nakagome and Dr. Junki Katsube for helpful discussion and encouragement throughout this work.

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