Effect of E5324, a Novel Inhibitor of Acyl-CoA:Cholesterol Acyltransferase, on Cholesteryl Ester Synthesis and Accumulation in Macrophages

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ABSTRACT—The in vitro potencies of a novel inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT), E5324 (n-butyl-N-[2-[3-(5-ethyl-4-phenyl-1H-imidazol-1-yl)propoxy]-6-methylphenyl]urea), were studied. E5324 was found to be a potent ACAT inhibitor in microsomes from a various tissues and in cultured cell homogenate, with IC₅₀ values in the range of 0.044 to 0.19 μM. The kinetic study on E5324 showed that the inhibition of rat intestine ACAT was competitive with respect to oleoyl CoA. E5324 inhibited [³H]oleate incorporation into cholesteryl [³H]oleate in phorbol ester-treated THP-1 cell lines (IC₅₀=0.44 μM). The rate of [³H]oleate incorporation into phospholipids and triglycerides was not affected by E5324. In an experiment with [³H]cholesterol as the substrate for ACAT, E5324 also inhibited [³H]cholesteryl ester synthesis (IC₅₀=0.41 μM). Furthermore, E5324 prevented accumulation of both esterified and total cholesterol in acetyl low density lipoprotein-loaded THP-1 cells. These results indicate that E5324 is a potent and selective ACAT inhibitor and prevents cholesteryl ester accumulation in macrophages.

Keywords: Acyl-CoA:cholesterol acyltransferase, Inhibitor, E5324, THP-1, Macrophage

Acyl-CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26), which catalyzes the intracellular formation of cholesteryl esters from fatty acyl-CoA and cholesterol, is a membrane bound enzyme present in various tissues such as the intestine, liver and arterial wall (1). In the intestine, ACAT facilitates the absorption of dietary cholesterol. In the liver, cholesterol generated by endogenous cholesterol biosynthesis, chylomicron remnant uptake and catabolism of low density lipoprotein (LDL) is esterified by ACAT; and the resultant cholesteryl ester is partly utilized in the synthesis of very low density lipoprotein (VLDL), which is secreted into the plasma. In the arterial wall, cholesteryl ester-loaded macrophages or foam cells are a characteristic feature of atherosclerotic lesions (2); ACAT is presumed to play a key role in foam cell formation. Although ACAT has been studied intensively, its structure and regulatory mechanism have not yet been established. Experiments with a histidyl-modifying reagent, diethyl pyrocarbonate (DEP), suggested the existence of two distinct subtypes of ACAT, DEP-resistant ACAT, typified by liver ACAT, and DEP-sensitive ACAT, typified by aortic ACAT (3). Recently, cDNA cloning of ACAT from THP-1 was achieved with the use of Chinese hamster ovary cell mutants deficient in ACAT activity (4); this should lead to an improved understanding of the molecular structure and regulation of this enzyme.

Inhibition of ACAT would be expected to reduce plasma cholesterol and to prevent the accumulation of cholesteryl ester in arterial walls. Several ACAT inhibitors, such as CL277082 (5) and CI976 (6), have been reported recently. During the course of extensive screening for ACAT inhibitors, we discovered a novel inhibitor, E5324 (7, 8). The next step was to evaluate its inhibitory activity in vitro towards ACAT from several tissues. In this paper, we describe the ACAT inhibitory profile of E5324 and the effect of the inhibitor on the cholesteryl ester synthesis and cholesterol accumulation in macrophages in vitro.

MATERIALS AND METHODS

Materials
E5324 (n-butyl-N-[2-[3-(5-ethyl-4-phenyl-1H-imidazol-1-yl)propoxy]-6-methylphenyl]urea, Fig. 1), CI976 and CL277082 were synthesized at Eisai Tsukuba Research Laboratories. [1-¹³C]Oleoyl CoA, [³H]oleic acid and [³H]-
cholesterol were purchased from Du Pont-New England Nuclear (Boston, MA, USA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (St. Louis, MO, USA). Tissue culture media and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Grand Island, NY, USA).

Lipoproteins

LDL (d = 1.020 - 1.063 g/ml) was prepared by ultracentrifugation of human plasma derived from blood collected from healthy volunteers. Acetyl LDL (AcLDL) was prepared by reaction with acetic anhydride as described by Basu et al. (9). βVLDL (d < 1.006) was prepared from the plasma of cholesterol-fed New Zealand white (NZW) rabbits by ultracentrifugation. The lipoproteins and lipoprotein-deficient serum (LPDS, d > 1.25) were dialyzed against 150 mM NaCl, 1 mM EDTA (pH 7.4) at 4°C and sterilized by filtration (0.45 μm).

Macrophage cell culture

U937 and THP-1, human monocytic leukemia cell lines, were grown in T-75 plastic flasks (Corning, NY, USA) in RPMI1640 medium supplemented with 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin (Gibco). Media were exchanged every 2 or 3 days. In order to induce cell adherence and differentiation to macrophages, U937 and THP-1 cells were suspended in RPMI1640 containing 10% FBS plus PMA and plated at a density of 1 x 10⁶/ml into 12-well culture plates (Flow Laboratories, McLean, VA, USA). The cells were incubated for 2–5 days in this medium containing PMA. Human mononuclear cells were isolated by density gradient centrifugation with ficoll-paque (Pharmacia LKB, Uppsala, Sweden) from blood collected from healthy volunteers. The mononuclear cells were washed twice with RPMI1640, and the cells were put into 12-well culture plates (3 x 10⁶ cells in 1 ml per well). The adherent cells were cultured in RPMI1640 containing 20% autologous serum, and the medium was changed twice weekly. Human monocyte-derived macrophages were used within 7–10 days.

Enzyme preparation

Microsomes were prepared from the liver and small intestine of male NZW rabbits and male Sprague-Dawley (SD) rats according to the method of Field and Mathur (10). Briefly, the intestinal mucosa and liver of NZW rabbits and SD rats were each homogenized in a buffered sucrose solution (0.1 M sucrose, 0.05 M KCl, 0.03 M EDTA and 0.04 M potassium phosphate buffer, pH 7.4) with a glass Dounce homogenizer. The homogenate was spun at 10,000 x g for 20 min at 4°C. The resulting supernatant was centrifuged at 105,000 x g for 1 hr at 4°C. The microsomal pellet thus obtained was rehomogenized in cold buffered sucrose solution and stored at −80°C until used. Aortic ACAT was prepared as follows: NZW rabbits were fed a 0.5% cholesterol and 0.5% olive oil-supplemented rabbit chow diet (ORC-4; Oriental Yeast Co., Ltd., Tokyo) for 12 weeks. The aorta was excised, and adventitial tissue grossly adhering to the aorta was removed. Then the intimal tissue was homogenized in 1 ml of 154 mM potassium phosphate buffer (pH 7.4) by the methods of Morisaki et al. (11). The homogenate was centrifuged for 5 min at 300 x g to remove cellular debris. The resulting supernatant was used as rabbit aortic ACAT. Human artery was obtained from an abdominal aneurysmectomy specimen (Tsukuba University). The artery was freed from adventitia and homogenized in a glass Dounce homogenizer. The homogenate was centrifuged at 10,000 x g for 30 min at 4°C, and the supernatant was stored at −80°C until used as human arterial ACAT. CaCo2, a human intestinal cell line, and HepG2, a human hepatoma cell line, were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. PMA-treated THP-1 was cultured in LPDS/RPMI containing 20 μg/ml of AcLDL for 6 days. These cells were removed from the culture dishes with a cell scraper and washed with 154 mM potassium phosphate buffer (pH 7.4) three times. The cells were homogenized by sonification and centrifuged at 300 x g for 5 min to remove intact cells. The resulting supernatants were used as cell-free ACAT enzyme.

Assay of cell-free ACAT activity

The ACAT activity was determined by the method described by Heider et al. (12). The reaction mixture consisted of 0.25 ml of potassium phosphate buffer (154 mM, pH 7.4) containing 2.4 mg/ml bovine serum albumin (Buffer A) and 42 μM [14C]oleoyl CoA (specific radioactivity: 15 dpm/pmol) for hepatic and intestinal ACAT. For aortic ACAT and cultured cell homogenate ACAT, the reaction mixture consisted of 0.25 ml of Buffer A containing 14 μM [14C]oleoyl CoA (specific radioactivity: 50 dpm/pmol). Test compounds were dissolved in dimethylsulfoxide at the concentration of 10 mM and diluted with
Buffer A to yield the final desired concentration. The test compound solution (30 μl) was added to the reaction mixture, which was then preincubated at 37°C for 3 min before the addition of the ACAT enzyme source (20 μl). The reaction mixture was incubated for 2 min (for liver and intestine) or 10 min (for aorta and cultured cell homogenate); then the reaction was terminated by the addition of 0.5 ml of 0.04 N HCl and 3 ml of chloroform : methanol (2:1; v/v/v). After shaking, the chloroform layer was taken to dryness under a stream of nitrogen. The extracted lipids were separated by thin layer chromatography (TLC, silica gel G plastic sheets; Merck Co., Rahway, NJ, USA) using heptane : diethyl ether : acetic acid (90:30:1; v/v/v) and were visualized with iodine vapor. The region corresponding to cholesteryl oleate was cut into pieces, which were placed in scintillation vials, and the radioactivity was determined by liquid scintillation counting. The IC₅₀ values were calculated by nonlinear least-squares fitting of the data from experiments at various concentrations to a log dose-response curve (duplicate assays were done at each concentration).

Assay of ACAT activity in cultured cells

[^3H]Oleate incorporation into cholesteryl[^3H]oleate: The media of cultured PMA-treated THP-1, PMA-treated U937 and human monocyte-derived macrophages were removed and replaced by RPMI1640 containing 5% LPDS (LPDS/RPMI), and the cells were incubated overnight. Then the media were replaced by 900 μl of LPDS/RPMI containing lipoproteins. In the study of the inhibitory effect of E5324, PMA-treated THP-1 cells were incubated in LPDS/RPMI containing 50 μg protein/ml βVLDL for 5 hr. Then, 100 μl of each concentration of E5324 solution or vehicle (LPDS/RPMI) was added to the cultured cells. After incubation with E5324 for 30 min, 20 μl of[^3H]oleate-BSA complex ([^3H]oleate: 5 mM, specific radioactivity of 17.6 dpm/pmol; BSA: 120 mg/ml) prepared by the method of St. Clair and Leight (13) was added, and the mixture was incubated for 2 hr. The lipids were extracted from the cells with hexane : 2-propanol (3:2, v/v/v) and then separated by TLC. The remaining cellular protein was dissolved in 0.1 N NaOH.

[^3H]Cholesterol incorporation into[^3H]cholesterol esters: [^3H]Cholesterol-containing liposomes were prepared by the methods of Tabas et al. (14). Briefly, 167 μCi of[^3H]cholesterol (0.9 μg) and 1.8 μg of egg phosphatidylcholine were dried under a stream of nitrogen to remove the solvent and sonicated for 10 min in 1.5 ml of Hank’s balanced salt solution (Gibco) at 4°C. Monolayers of PMA-treated THP-1 were preincubated in LPDS/RPMI (900 μl) for 16 hr. Then E5324 solution (100 μl) and[^3H]-cholesterol-containing liposomes (20 μl) were added to the cultured cells. After incubation for 16 hr, the cells were washed with Tris-HCl buffered saline (pH 7.4), and then the media were replaced by LPDS/RPMI containing 50 μg protein/ml βVLDL and the same concentration of E5324 as before washing. After further incubation for 7 hr, the lipids in the cells were extracted, and the cholesteryl esters and remaining cellular protein were determined as described above.

Cholesterol mass determination

PMA-treated THP-1 cells were incubated with AcLDL in the presence or absence of E5324 for 3 or 6 days. The cellular contents of unesterified and total cholesterol were determined after lipid extraction by the fluorescent enzymatic method of Heider and Boyett (15).

| Table 1. Inhibitory effects of E5324, CI976 and CL277082 on microsomes prepared from various tissues or cultured cell homogenate |

| Species | Tissues | E5324 | CI976 | CL277082 |
|---------|---------|-------|-------|----------|
| Rat     | Liver   | 0.11 ±0.02 (2) | 0.30 ±0.12 (2) | 2.9 ±0.3 (3) |
|         | Intestine | 0.044±0.018 (3) | 0.11 ±0.01 (3) | 0.28 (1) |
| Rabbit  | Aorta   | 0.19 ±0.03 (4) | 0.29 ±0.04 (6) | 2.9 ±0.4 (11) |
|         | Liver   | 0.16 ±0.02 (4) | 0.016 (1) | N.T. |
|         | Intestine | 0.053±0.013 (3) | 0.056±0.009 (6) | 0.16±0.02 (7) |
| Human   | Aorta   | 0.097±0.023 (3) | 0.47 ±0.03 (2) | N.T. |
|         | HepG2   | 0.068 (1) | 0.8 (1) | 3.2 ±1.4 (2) |
|         | CaCo2   | 0.089±0.021 (4) | 0.20 ±0.05 (3) | 3.2 ±0.8 (2) |
|         | THP-1   | 0.16 (1) | 0.20 (1) | N.T. |

Values are expressed as means ±S.E.M. Numbers in parentheses represent the number of experiments. The ACAT assay was done as described in Materials and Methods. N.T.: not tried.
Protein determination

Protein concentration was determined by the BCA protein assay reagent (Pierce, Rockford, IL, USA).

Statistical analyses

Values are expressed as means±S.E.M. The significance of differences between the mean values of the groups was evaluated by one-way analysis of variance and Student's t-test.

RESULTS

Cell-free ACAT activity

The results on the inhibitory activity of E5324 and reference ACAT inhibitors in the ACAT assay system using microsomes and cultured cell homogenates are shown in Table 1. E5324 shows potent ACAT inhibition with IC$_{50}$ values in the range of 0.044 to 0.19 μM. The IC$_{50}$ values of C1976 for rabbit intestine and rat liver were 0.056 and 0.29 μM, respectively, in reasonable agreement with the values (rabbit intestine: 0.073 μM and rat liver: 0.18 μM) reported by Krause et al. (16), despite some differences in the assay methods. E5324 and C1976 show approximately equal ACAT inhibitory potency. CL277082 shows tissues and species differences; the inhibitory potency is weaker against rabbit aorta and CaCo2 cells homogenate compared to rabbit intestine.

The effect of E5324 on triglyceride synthesis was estimated by measuring $[^{14}C]$oleoyl CoA incorporation into triglyceride in rat liver microsomes. E5324 showed no effect on triglyceride synthesis up to 10 μM (data not shown). E5324 also had no effect on bovine pancreatic cholesterol esterase or lecithin:cholesterol acyltransferase.
Kinetic studies on E5324 indicate that inhibition of rat intestine ACAT is competitive with respect to oleoyl CoA (Fig. 2).

Incorporation of \[^3H\]oleate into cholesteryl \[^3H\]oleate

It has been reported that U937 and THP-1, human monocytic cell lines, differentiated to adherent cells when incubated with PMA. Before examination of the inhibitory effects of E5324 on cholesteryl ester synthesis, the stimulatory effects of LDL, \(\beta\)VLDL and AcLDL on \[^3H\]oleate incorporation into cholesteryl \[^3H\]oleate in U937, THP-1 and human monocyte-derived macrophages were studied (Fig. 3). We also examined the effects of PMA treatment of U937 and THP-1 on the stimulation by lipoprotein (Fig. 3). In human monocyte-derived macrophages, AcLDL stimulated the incorporation of \[^3H\]oleate into cholesteryl \[^3H\]oleate. In U937, \(\beta\)VLDL increased the rate of incorporation of \[^3H\]oleate into cholesteryl \[^3H\]oleate, and this effect was enhanced after PMA treatment. LDL was also stimulatory, but AcLDL did not stimulate cholesteryl \[^3H\]oleate synthesis in PMA-treated U937. In contrast, AcLDL as well as \(\beta\)VLDL and LDL could stimulate cholesteryl \[^3H\]oleate synthesis in PMA-treated THP-1. Compared to U937, differentiated THP-1 cells behave more like native monocyte-derived macrophages, so that we used PMA-treated THP-1 cells as a human macrophages model. The effects of E5324 on the incorporation of \[^3H\]oleate into cholesteryl \[^3H\]oleate were evaluated in PMA-treated THP-1 (Fig. 4). E5324 inhibited the incorporation of \[^3H\]oleate into cholesteryl \[^3H\]oleate in a concentration-dependent manner with IC\(_{50}\) values of 0.44 \(\mu\)M. As shown in Fig. 4, the \[^3H\]oleate incorporation into triglycerides and phospholipids was unaffected by E5324 up to 10 \(\mu\)M in PMA-treated THP-1.

Incorporation of \[^3H\]cholesterol into \[^3H\]cholesteryl esters

Usually, the effect of ACAT inhibitor on cholesteryl ester synthesis was studied with \[^3H\]oleate as the substrate in macrophages. We next determined whether the inhibitory activity of E5324 changed when \[^3H\]cholesterol was used as the substrate. Although little is known about the delivery of cholesterol as a substrate to ACAT, it has been reported that plasma membrane cholesterol in J774 macrophages was translocated to ACAT when LDL

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**Fig. 4.** Effect of E5324 on \[^3H\]oleate incorporation into cholesteryl \[^3H\]oleate. THP-1 cells were differentiated into macrophage-like cells by treatment with PMA. PMA-treated THP-1 cells were incubated in LPDS/RPMI overnight. The media were replaced with LPDS/RPMI containing \(\beta\)VLDL (50 \(\mu\)g/ml) for 5 hr at 37°C, and then the E5324 solution and \[^3H\]oleate-BSA complex were added to the cell culture. After a 2-hr incubation, \[^3H\]oleate incorporations into triglycerides (○), phospholipids (△) and cholesteryl oleate (□) were determined. The results are given as means±S.E.M. of triplicate assays. **P<0.01 and ***P<0.001 vs control.

**Fig. 5.** Effect of lipoproteins on \[^3H\]cholesterol incorporation into \[^3H\]cholesteryl ester. PMA-treated THP-1 cells were incubated in LPDS/RPMI overnight and then labeled with \[^3H\]cholesterol phosphatidylcholine liposomes for 17 hr at 37°C. The medium was removed, and the cells were incubated in LPDS/RPMI without lipoprotein (○) or with \(\beta\)VLDL (50 \(\mu\)g/ml, □), LDL (50 \(\mu\)g/ml, △) or AcLDL (50 \(\mu\)g/ml, ■) for 7 hr at 37°C. Synthesized \[^3H\]cholesteryl esters and cellular protein were determined. The values are expressed as mean±S.E.M. of triplicate assays. *P<0.05, **P<0.01 vs without lipoprotein.
was included in the culture medium (14). We studied the effect of lipoproteins on [3H]cholesterol incorporation into [3H]cholesteryl ester in PMA-treated THP-1 prelabeled with [3H]cholesterol-containing phospholipid liposomes. As shown in Fig. 5, increased [3H]cholesteryl ester synthesis was observed upon the incubation with βVLDL and AcLDL.

The effect of E5324 on [3H]cholesterol incorporation into [3H]cholesteryl ester was evaluated under conditions of βVLDL chase. As shown in Fig. 6, E5324 inhibited [3H]cholesteryl ester synthesis with IC50 values of 0.41 μM.

Accumulation of cholesterol
To analyze the effect of E5324 on cholesterol accumulation in AcLDL-loaded THP-1 cells, the cellular contents of free and total cholesterol were measured as described in Materials and Methods. Cholesteryl ester mass was calculated by subtracting the content of unesterified cholesterol from total cholesterol. As shown in Fig. 7, PMA-treated THP-1 cells exposed to AcLDL (50 μg/ml) accumulated free cholesterol and cholesteryl ester. However, simultaneous incubation of the macrophages with AcLDL and E5324 resulted in inhibition of the accumulation of cellular total and esterified cholesterol. The increase of total cellular cholesterol in the presence of E5324 (1 and 10 μM) was predominantly due to free cholesterol, and the accumulation of free cholesterol was enhanced by E5324 in a concentration-dependent manner.

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DISCUSSION

An ACAT inhibitor with systemic bioavailability would be expected to lower plasma cholesterol levels by inhibition of ACAT in the small intestine and also to directly prevent foam cell formation within the arterial wall. Several ACAT inhibitors, such as melinamide (17), octimamide sulfate (18), SaH58-035 (19), CL277082 (5) and C1976 (20), have been evaluated for ACAT inhibitory activity, hypcholesterolemic activity and anti-atherosclerotic activity. However, CL277082 failed to demonstrate efficacy in a clinical study, probably because the dose was insufficient; in order to inhibit cholesteryl ester synthesis after cholesterol feeding, 8 times higher doses were necessary in monkeys compared to rats (21). In addition to species difference, the presence of ACAT subtypes, aortic ACAT and liver ACAT, has been identified on the basis of differential diethyl pyrocarbonate sensitivity (3). Therefore, we evaluated the effects of our novel ACAT inhibitor, E5324, and reference ACAT inhibitors on ACAT activity in microsomes or homogenates prepared from various tissues (intestine, liver and aorta) and species (rat, rabbit and human). As shown in Table 1, inhibitory potency of CL277082 is 10-times weaker against CaCo2 cells homogenate compared to rat intestine. In contrast, E5324 inhibited ACAT with IC50 values of 0.044 - 0.19 μM (Table 1). With respect to tissue selectivity, E5324 was approximately fourfold more potent in rabbit intestine than in rabbit aorta. However, in the human case, E5324 showed approximately equal inhibitory potency against intestinal (CaCo2 homogenate) and aorta ACAT. These inhibitory potencies were approximately equal against rat and rabbit intestinal ACAT.

It has been considered that foam cells of macrophage origin are abundant in all phases of the atherogenic process (22). In mouse peritoneal macrophages, cholesteryl ester accumulation can occur with AcLDL (23) and βVLDL from hypercholesterolemic animals (24). We investigated the effect of lipoproteins on ACAT in human monocyte-derived macrophages and human monocyte cell lines. In the PMA-treated U937 cell line, cholesteryl ester synthesis could not be stimulated by incubation with AcLDL, probably because expression of scavenger receptor activity is not induced by the PMA treatment, as reported by Via et al. (25). In THP-1 cells, in contrast, PMA-treatment resulted in increased rates of cholesteryl ester synthesis during incubation with AcLDL as well as βVLDL. This result confirms the reports of Hara et al. (26) and Via et al. (27). We used PMA-treated THP-1 cells as a human macrophage model because the availability of human monocyte-derived macrophages was limited. As shown in Fig. 4, E5324 inhibited the incorporation of [3H]oleate into cholesteryl [3H]oleate in a concentration-dependent manner with IC50 values of 0.44 μM. We next determined whether the inhibitory activity of E5324 changed when [3H]cholesterol was used as the substrate. In J774 macrophages incubated with LDL, the plasma membrane free cholesterol is translocated to the ACAT substrate pool (14). In rat peritoneal macrophages incubated with AcLDL, [3H]cholesterol present in the plasma membrane serves as an efficient substrate for ACAT (28). Using [3H]cholesterol-loaded THP-1, we observed stimulatory effects of AcLDL or βVLDL on [3H]-cholesterol incorporation into [3H]cholesterol ester (Fig. 5). E5324 inhibited [3H]cholesterol incorporation into [3H]cholesterol ester, and its inhibitory activity in THP-1 was essentially the same as in the experiment on [3H]oleate incorporation into cholesteryl [3H]oleate.

As shown in Table 1, E5324 inhibited cell-free ACAT prepared from THP-1 with IC50 values of 0.16 μM. A higher concentration was needed to inhibit cholesteryl ester synthesis in intact cells in comparison with cell-free ACAT inhibition. These differences might be attributed to the permeability through the cell membrane of THP-1.

When a monocyte differentiates into a macrophage, the disappearance of the LDL-receptor and concomitant appearance of the scavenger receptors, which are not feedback regulated by cellular cholesterol content, occur. It is considered that these changes in the expression of genes in lipid metabolism, which have been observed in PMA-treated THP-1 (26, 27), are important in the process of foam cell formation. Therefore, it is believed that the THP-1 cell line is a valuable model for studying the mechanism of foam cell formation (29).

When PMA-treated THP-1 cells were incubated with AcLDL instead of βVLDL, E5324 inhibited the incorporation of [3H]oleate into [3H]cholesterol oleate with IC50 values of 0.42 μM (data not shown). Therefore, we next determined the effect of E5324 on the cellular accumulation of free and esterified cholesterol in AcLDL-loaded THP-1 cells. In macrophages, AcLDL cholesterol is thought to be utilized as follows: AcLDL is endocytosed via a scavenger receptor and then delivered to lysosomes, where cholesteryl ester is hydrolyzed by acid cholesterol esterase. Part of the released cholesterol is excreted from the cells and the rest is reesterified by ACAT (30). E5324 inhibited the cholesteryl ester accumulation and concomitantly enhanced free cholesterol accumulation. It might be considered that in the presence of E5324, part of the incorporated AcLDL cholesterol is excreted from the cells so that total cholesterol accumulation is reduced. It is generally accepted that high density lipoprotein (HDL) stimulates the removal of cholesterol from the cells (31). Furthermore, Schmitz et al. have reported that the ACAT inhibitor octimamide enhances HDL receptor activity and promotes cholesterol efflux from cultured macrophages.
(32). Thus, HDL might stimulate the efflux of free cholesterol from macrophages in the presence of ACAT inhibitor in vivo. We have reported that in experimental atherosclerosis, E5324 reduced free and esterified cholesterol content in the aortic arch in pre-established hypercholesterolemic rabbits (33). Thus, E5324 may be useful for the treatment of atherosclerosis in humans.

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