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THE EFFECTS OF TWO ACYLCOENZYME A: CHOLESTEROL ACYLTRANSFERASE (ACAT) INHIBITORS, CYCLANDELATE AND A NON-HYDROLYSABLE ETHER ANALOGUE, BENZYL3,3,5-TRIMETHYLCYCLOHEXANOL ON LOW DENSITY LIPOPROTEIN METABOLISM IN MACROPHAGES AND HEPATOCYTES

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Abstract—Cyclandelate (3,3,5,-trimethylcyclohexanylmandelate) caused a dose-dependent decrease in the metabolism of radioiodinated low density lipoprotein [^{125}I-LDL] by J774 mouse macrophages. This was probably an indirect effect due to the inhibition of cholesterol esterification by the cells rather than a direct one on the interaction of LDL with its receptor, since no inhibition was seen in cells which had been cholesterol-depleted by prior incubation with lipoprotein-depleted serum for 48 hr. Cyclandelate also inhibited immediately *de novo* synthesis of cholesterol from [1-^{14}C]acetate in J774 cells, suggesting a direct action of the drug on an enzyme of the cholesterol biosynthetic pathway. The drug was an efficient inhibitor of hamster and rat intestinal acylcoenzyme A: cholesterol acyltransferase (ACAT) activity *in vitro* with an IC_{50} of 20 μM. Addition of cyclandelate to the diet of meal-fed rats caused a marked inhibition of the rate of appearance of dietary [4-^{14}C]cholesterol in the plasma. A nonhydrolysable ether analogue of cyclandelate, benzyl3,3,5-trimethylcyclohexanol, was prepared to compare hepatic and extrahepatic actions of the two molecules. The analogue inhibited cholesterol esterification in J774 cells, transformed human macrophages U937 and human umbilical vein endothelial cells with an IC_{50} of 20 μM and had effects similar to those of cyclandelate on ^{125}I-LDL metabolism in J774 cells. Differences between the analogue and cyclandelate were seen in hepatocytes and hepatic microsomal fractions, where preincubation with the analogue inhibited cholesterol esterification in both systems while cyclandelate had no inhibitory action in either. Consequently, preincubation of rat hepatocytes with benzyl3,3,5-trimethylcyclohexanol for 17 hr caused a marked decrease in the binding of ^{125}I-LDL to the cells, whereas binding to cells preincubated with cyclandelate was the same as to control cells.

Key words: cholesterol absorption; synthesis; LDL receptor; atheroma

Atherosclerosis is a disease of the vasculature and arises from a complex chronic process involving focal accumulation of lipid, leucocytes, smooth muscle cells and extracellular matrix in the intima of large arteries. Key elements in the formation of the atherosclerotic plaque include recruitment and adhesion of monocytes to the endothelium, migration of monocytes to the subendothelium and transformation of monocytes into macrophages with the subsequent generation of foam cells [1]. This last step is characterized by the intracellular accumulation of large amounts of cholesterylester [2]. Lipoprotein-borne cholesterylester is hydrolysed in the cell and undergoes reesterification under the action of ACAT‖. The activity of this enzyme appears to be regulated by substrate supply and covalent modification via an ATP-dependent process [3–6]. Interest in ACAT inhibitors derives from the possibility that their action in the intestine might decrease absorption of dietary cholesterol while action in extrahepatic tissues might promote reverse cholesterol transport [7]. We have reported previously on the actions of trimethylcyclohexanylmandelate (cyclandelate) on cholesterol esterification in cells [8] and ACAT activity in microsomal fractions [9]. This paper describes the effects of inhibition of ACAT activity by cyclandelate on lipoprotein metabolism in the mouse transformed

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‖ Abbreviations ACAT, acylcoenzyme A:cholesterol acyltransferase; cyclandelate; 3,3,5-trimethylcyclohexanylmandelate; ATP, adenosine triphosphate; TCA, trichloroacetic acid; α-MEM, Dulbecco's minimal essential medium, α-modification; FCS, foetal calf serum; LPDS, lipoprotein depleted serum; HMGCoA, 3-hydroxy-3-methylglutarylcoenzyme A; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

macrophage, J774, and compares its action in rat hepatocytes with that of a non-hydrolysable analogue.

MATERIALS AND METHODS

[9,10-^{3}H]Oleate (370 MBq/mmol), [1-^{14}C]acetate (2.2 GBq/mmol) and [4-^{14}C]cholesterol (2.2 GBq/mmol) were purchased from Amersham International plc (Little Chalfont, U.K.). [4-^{14}C]-Cholesteryl oleate was synthesized chemically [10]. [9, 10-^{3}H]Oleate–BSA complex was made essentially as described by Van Harken *et al.* [11] using 100.64 Bq [9, 10^{3}H]oleic acid and 50 μmol non-radioactive oleic acid saponified with 200 μmol KOH to yield a final oleate concentration of 10 mM of specific activity 12 dpm/pmol with a protein concentration of 60 mg/mL. All tissue culture media and sera were purchased from Imperial Labs (Europe) Ltd (Salisbury, U.K.). Cyclandelate was from Gist Brocades nv. (Delft, Holland). Benzyl 3,3,5-trimethylcyclohexanol was synthesized by the Williamson method of ether synthesis [12] using benzyl bromide (4.23 g; 24.5 mmol) and trimethylcyclohexanol (3.87 g; 24.6 mmol).

Rat liver microsomal fractions were prepared as described previously [13] and stored in aliquots in assay buffer at −20° until required. Protein was measured by the microbiuret assay [14]. Microsomal ACAT was assayed essentially by the method of Lichtenstein and Brecher [15] as described by Heffron *et al.* [8]. Primary cultures of rat hepatocytes were prepared fresh as described by Salter *et al.* [16].

The isolation and radioiodination of LDL. Human LDL was isolated from the plasma of normal volunteers in the density range 1.019–1.063 g/mL by sequential flotation according to the method described by Goldstein *et al.* [17]. The purity of each preparation of LDL was analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate which detected only apo B_{100}.

The radioiodination of LDL was performed as described by Salter *et al.* [16]. Preparations of LDL were obtained with a specific radioactivity in the range 50–175 cpm/ng protein; TCA-soluble radioactivity was usually less than 2% of the total, and less than 5% of the radioactivity could be extracted into chloroform/methanol (1:2, v/v).

LPDS was prepared from the plasma remaining after the isolation of the LDL fraction. The density of the plasma was raised from 1.063 g/mL to 1.215 g/mL by adding the correct amount of solid KBr. LPDS was then produced according to the method of Goldstein *et al.* [17]. The cholesterol content was typically less than 5% of the level found in whole serum. The concentrations of lipoproteins used in these experiments are given in terms of their protein content.

Assays. Three aspects of the metabolism of LDL were studied; the cell-surface binding and uptake of ^{125}I-LDL and the re-esterification of the LDL-derived cholesterol. Furthermore, these studies were concerned with the metabolism of all the LDL that is bound and subsequently internalized by the cell, i.e. both specifically and non-specifically. Non-specific binding was always less than 5% of the total.

Total cell-surface binding of ^{125}I-LDL. Prior to assay, cultures were washed with 2 mL of ice-cold α-MEM containing 10 mM Hepes, pH 7.1 (medium A) and then pre-cooled at 4°, for 30 min, in 2 mL of medium A containing 10% (v/v) human LPDS (5 mg protein/mL). The cell-surface binding of ^{125}I-LDL was then assayed at 4°. Radioiodinated LDL was added directly to the cultures to the indicated concentration and the incubation maintained for a further 2 hr to allow binding to reach equilibrium. Unbound ^{125}I-LDL was removed with three rapid washes in 2 mL of 50 mM Tris–HCl, pH 7.4, containing 150 mM NaCl and BSA (2 mg/mL), followed by two 10 min incubations, at 4°, in 2 mL of fresh buffer. The cell monolayers were then washed a further three times with 2 mL of 50 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, and then solubilized in 1 mL 0.1 M NaOH, for 1 hr at room temperature. Aliquots of the cell extract were assayed for cell-associated radioactivity (750 μL) or total cell protein (50 μL).

Total uptake of ^{125}I-LDL. Cultures were washed three times with 2 mL of pre-warmed (37°) medium B, α-MEM containing $NaHCO_3$ (2 g/L) and 4 mM Hepes, pH 8.1, prior to transfer to 2 mL of pre-warmed medium B containing 10% (v/v) LPDS (5 mg protein/mL) and the indicated concentration of ^{125}I-LDL. After the stated time interval the proteolytic degradation of ^{125}I-LDL was determined from the amount of TCA-soluble radioactivity released into the culture medium, using the methods of Goldstein *et al.* [17]. No-cell blanks were also included to correct for the presence of TCA-soluble radioactivity that was not produced by cellular proteolysis. The amount of cell-surface bound and internalized ^{125}I-LDL was obtained from the cell-associated radioactivity, determined as described above.

Cholesterol esterification by cells in culture. The culture of J774 cells, human transformed macrophages U937 and human umbilical vein endothelial cells and the assay of cholesterol esterification were as described previously [9].

Cell protein. Protein was measured by the microbiuret method [14] using bovine serum albumin as a standard.

Effect of cyclandelate on the appearance of dietary [^{14}C]cholesterol in the plasma of rats. Twenty-four adult male Wistar rats were trained to meal feed over a period of 6 days on a diet of normal chow. After an initial overnight fast they were allowed access to food for a period of 2 hr. After the meal, food was removed until the following day. The length of the meal was progressively reduced to 1 hr. After a week of meal feeding the animals were transferred to a diet of normal rat chow supplemented with 10% lard (w/w) and 1% cholesterol (w/w) with (12 animals) or without (12 animals) 0.05% (w/w) cyclandelate. After a further week of feeding this diet a test meal was fed in which the cholesterol content was reduced to 0.012% (w/w) and [4-^{14}C]-cholesterol was added to a specific activity of 1.111 MBq/mg cholesterol. Each animal was given a meal of 9.5 g of diet which was 95% of the average

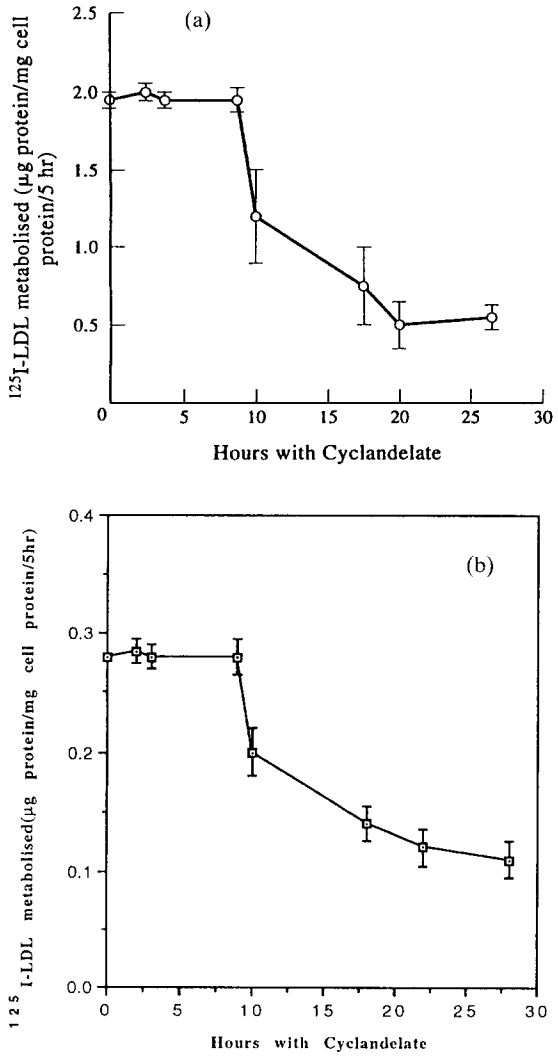

Fig. 1. Inhibition of ^{125}I-LDL metabolism by cyclandelate in J774 cells. Cells were grown in medium containing FCS (10%, v/v) and at confluence were incubated with cyclandelate (75 μM) for 0–24 hr. The medium was replaced with fresh α-MEM containing LPDS (5%, 2.5 mg protein/mL) and ^{125}I-LDL (10 μg/mL) and the degraded (a) and cell-associated (b) LDL were measured as described in Materials and Methods. Results represent mean ± SD of two separate experiments.

consumed on the previous day. All animals ate all of the food provided, implying that each consumed approximately 1 mg (0.111 MBq) of cholesterol. Three rats from each group were anaesthetized and bled by cardiac puncture at 2, 4, 8 and 20 hr after consumption of the meal. The appearance of [^{14}C]-cholesterol in the plasma was measured by scintillation counting.

RESULTS AND DISCUSSION

Previous experiments have demonstrated the inhibition of cholesterol esterification by cyclandelate in a variety of cultured cells including human umbilical vein endothelial cells and the transformed mouse macrophage line J774 [9]. Such an inhibition will increase the intracellular concentration of free cholesterol which in turn should feedback inhibit *de novo* synthesis of cholesterol at the level of HMGCoA reductase and eventually the LDL receptor [18]. The effect of addition of cyclandelate, at a concentration (75 μM) in excess of the IC_{50} (20 μM) for ACAT, on LDL metabolism in J774 cells, is shown in Fig. 1. No inhibition of binding or degradation was seen until 8 hr of incubation with the drug, after which time both parameters were markedly decreased. The decreased rate of degradation mirrored the fall in cell-associated LDL, suggesting that the fall in degradation was due to decreased binding and internalization. The lack of an immediate effect on LDL metabolism implies that cyclandelate did not prevent directly the interaction of LDL to its receptor. It also suggests that the drug acts indirectly by a reduction in LDL receptor number and that the delay in response probably reflects the half-life of the LDL receptor (11 hr). This was confirmed in studies with ^{125}I-LDL where binding to cells which had been incubated with cyclandelate for 17 hr was reduced by 30% compared to control cells (33.0 ± 4 vs 46.5 ± 4 ng LDL protein/mg cell protein: cyclandelate-treated vs control cells). The inhibition of endocytosis was related to the concentration of cyclandelate in the incubation medium (Fig. 2). In these experiments, cells were incubated with drug for 17 hr prior to addition of ^{125}I-LDL and metabolism of the drug followed over a 5 hr period. The concentration of cyclandelate required to produce a reduction in cell-associated LDL was 25 μM, similar to the IC_{50} for ACAT activity, and degradation followed a similar pattern. Although the intracellular concentration of cholesterol has not been measured in these experiments it is likely that an increase in this parameter is the cause of the inhibition, as suggested by the results shown in Fig. 3. Cells were incubated in LPDS for 0, 24 and 48 hr to reduce their intracellular free cholesterol content prior to addition of cyclandelate for 17 hr and measurement of LDL endocytosis. The effect of cyclandelate was most marked at zero time and became less so with the length of preincubation time in LPDS, such that cyclandelate caused no inhibition of endocytosis after 48 hr in LPDS. Presumably at this stage LDL receptors are fully upregulated due to a very low intracellular concentration of free cholesterol, and insufficient free cholesterol is produced in response to ACAT inhibition during the time of the experiment (5 hr) to have any effect on receptor number.

A further unexpected action of cyclandelate is the inhibition of cholesterol synthesis from acetate in J774 cells. Cholesterol synthesis from [1-^{14}C]acetate was increased 7-fold in cells which had been incubated for 24 hr in medium containing LPDS compared with cells cultured in FCS-containing medium (data not shown). Addition of cyclandelate (75 μM) to cells incubated with LPDS caused a marked inhibition (40%) of cholesterol synthesis within 1 hr (Fig. 4). Similar results were obtained

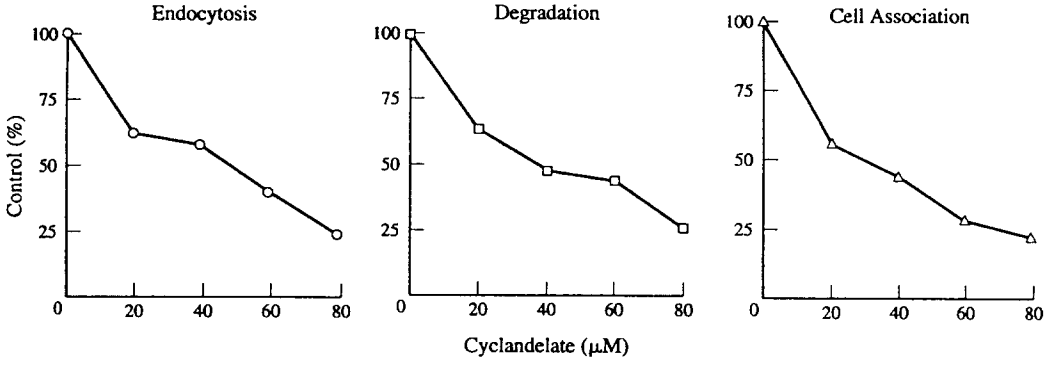

Fig. 2. The effect of the concentration of cyclandelate on ^{125}I-LDL metabolism in J774 cells. Cells were grown in α-MEM containing FCS (10%) and incubated at confluence for 17 hr with increasing concentrations of cyclandelate. The medium was replaced with fresh MEM containing LPDS (5%, 2.5 mg/mL) and ^{125}I-LDL (10 μg/mL). Metabolism of LDL was measured as described in Materials and Methods. Results are expressed as a percentage of the controls (cell-associated 0.4 ng LDL protein/mg cell protein; degraded 2.05 ng LDL protein/mg cell protein) and represent the mean of two separate experiments.

with a broken cell preparation (results not shown). As shown previously, cholesterol esterification was also inhibited immediately. It is not known whether the inhibition of cholesterol synthesis is due to a direct action of cyclandelate on an enzyme of the cholesterol synthetic pathway or an indirect one through feedback inhibition by a raised intracellular cholesterol concentration resulting from the ACAT inhibition. The rapid effect on synthesis would suggest the former but the drug shows no similarity in structure to previously described inhibitors of the cholesterol synthetic pathway, and we have not seen any direct action on HMGCoA reductase *in vitro*.

The inhibition of ACAT in extrahepatic tissues may be of value therapeutically. Cholesterol is stored intracellularly as cholesterylester and the free cholesterol pool is small. The stored ester undergoes continual hydrolysis by esterases and resynthesis by ACAT [4]. Interruption of this cycle by an ACAT inhibitor would increase the free cholesterol pool size and possibly promote reverse cholesterol transport since it is free cholesterol that is accepted by HDL [19]. Such an inhibition would also decrease *de novo* synthesis of cholesterol and down-regulate LDL receptors, leading to decreased accumulation of LDL-derived cholesterol by the cell. The activity of cellular cholesterylester hydrolases has not been measured in this study. The rationale for the use of many of the ACAT inhibitors so far described in the literature is that they will have an action in the intestine and prevent the esterification of cholesterol essential for chylomicron formation and hence the secretion of dietary lipid into the lymphatic system. The end result of inhibition of intestinal ACAT would thus lead to a decrease in the appearance of dietary cholesterol in the circulation [21, 22]. Since the life-time of the enterocyte is of the order of 3 days, the cholesterol which is absorbed into the enterocyte is lost as the cell sloughs off. The highest ACAT activity in the intestine occurs in the jejunum, the site of greatest cholesterol absorption. The average amount of cholesterol absorbed by humans may be up to 500 mg/day and prevention of this entering the body may be expected to cause appreciable lowering of plasma total cholesterol. A number of structurally unrelated compounds has been synthesized and shown to have inhibitory activity towards intestinal ACAT (see Ref. 7 for a review) *in vitro* and *in vivo*, and this inhibition can be correlated with inhibition of absorption of dietary cholesterol in rabbits [20] and rats [21]. Absorption of dietary cholesterol in some species, including humans, may be governed by cholesterol esterase activity, either pancreatic or intestinal [22]. Such activity might explain why ACAT inhibitors such as Sandoz 58-035, which appear to act solely on the intestine *in vivo*, have little effect on plasma cholesterol levels.

A decreased absorption of dietary fatty acids will also result since they appear in the circulation initially as components of triacylglycerols of chylomicrons. Cyclandelate is an efficient inhibitor of ACAT activity in rat and hamster intestinal microsomal fractions with an IC_{50} of approximately 20 μM (results not shown), similar to that for inhibition of cholesterol esterification in a number of different cell types [9]. Inclusion of cyclandelate into the diet of rats which had been trained to meal feed caused a marked decreased in the rate of appearance of dietary [^{14}C]cholesterol in the plasma. In this experiment the drug was included in the diet for 1 week before the addition of the radiolabelled sterol. Radioactivity was detected in plasma within 2 hr in the control animals, increasing to a plateau after about 10 hr (Fig. 5). A similar profile was seen in cyclandelate-dosed animals but the total radioactivity measured was only approximately 50% that of the control animals at all time points. This is consistent with an inhibition of intestinal cholesterol esterification *in vivo* and further evidence for extrahepatic actions of the drug.

The consequences of inhibition of hepatic ACAT

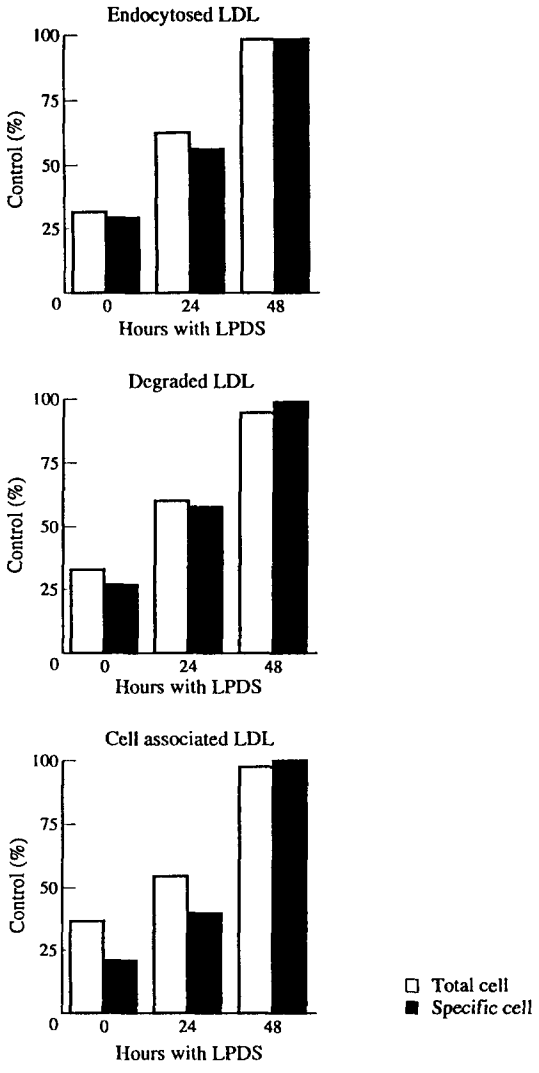

Fig. 3. The failure of cyclandelate to inhibit ^{125}I-LDL metabolism in cholesterol-depleted J774 cells. Cells were grown to confluence in α-MEM containing FCS (10%). The medium was replaced with α-MEM containing LPDS (10%, 5 mg protein/mL) for up to 48 hr. Cyclandelate (75 μM) was added to the cells 17 hr prior to addition of ^{125}I-LDL (10 μg/mL) and measurement of LDL metabolism over a period of 5 hr as described in Materials and Methods. Results are expressed as a percentage of the control values; 2021 ± 58 ng LDL degraded/mg cell protein, 537 ± 33 ng LDL degraded/cell associated/mg cell protein. Values represent the means of duplicate determinations which varied by less than 5%.

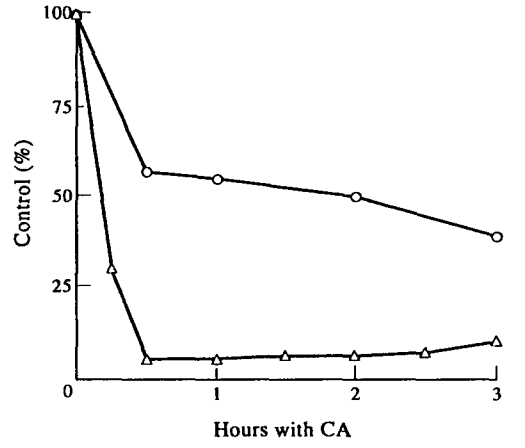

Fig. 4. Inhibition of cholesterol synthesis and esterification by cyclandelate in J774 cells. Cells at confluence were incubated in α-MEM containing LPDS (10%, 5 mg protein/mL) for 24 hr prior to the addition of cyclandelate (100 μM) and [1-^{14}C]acetate (1 mM, 10 mCi). The rates of cholesterol synthesis (○) and esterification (△) were measured over a period of 1 hr as described in Materials and Methods. Results are expressed as a percentage of control activities and represent the mean ± SD of three separate determinations. The control rate of cholesterol synthesis was 4 pmol/hr/mg protein and of esterification was 2 nmol/hr/mg protein.

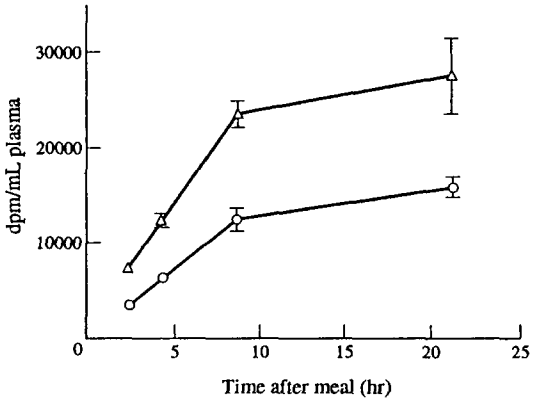

Fig. 5. The effect of cyclandelate on the appearance of dietary [4-^{14}C]cholesterol in the plasma of rats. Meal-fed rats were each given a meal containing [4-^{14}C]cholesterol (0.111 MBq) and groups of three animals were bled by cardiac puncture at 2, 4, 8 and 20 hr after consumption of the meal. The appearance of radioactivity in the plasma was measured by scintillation counting. Control (△); cyclandelate (○).

in vivo, however, are less clear. The liver plays a central role in cholesterol homeostasis with inputs from endogenous synthesis and lipoproteins (chylomicron remnants, IDL and LDL). Free cholesterol derived from any of these sources is either re-esterified for storage and eventual secretion as a component of VLDL, or secreted into bile as free cholesterol or bile acid after metabolism. Inhibition of hepatic ACAT may thus reduce plasma lipids by reducing the secretion of VLDL; the precursor of LDL and ACAT activity has been shown to be required for the secretion of apoB-containing lipoproteins in both HepG2 cells [23] and perfused monkey liver [24]. However, such an inhibition would lead to a rise in the concentration of free cholesterol and a consequent down-regulation of

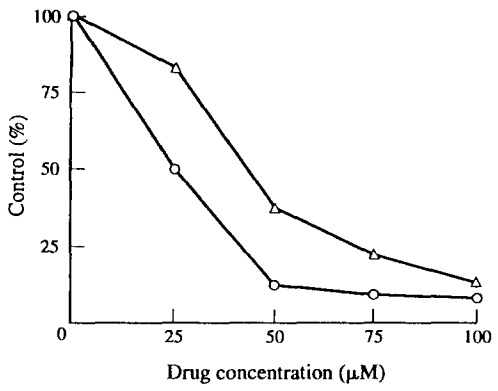

Fig. 6. Inhibition of cholesterol esterification in J774 cells by cyclandelate (○) and benzyl3,3,5-trimethylcyclohexanol (△). Cells at confluence were incubated for 1 hr with increasing concentrations of the drugs prior to measurement of cholesterol esterification as described in Materials and Methods. Results are expressed as a percentage of control activity (1630 ± 102 pmol/hr/mg cell protein). Values represent the mean of duplicate determinations which varied by less than 10%.

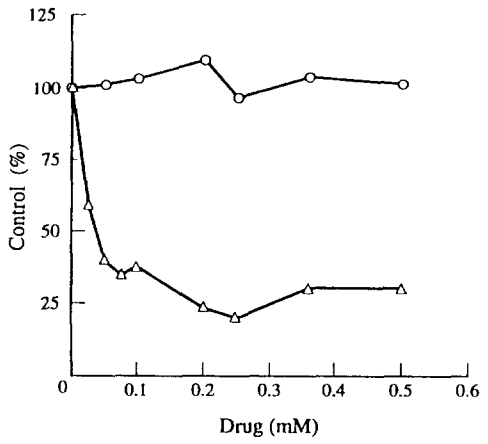

Fig. 8. The effects of benzyl3,3,5-trimethylcyclohexanol (△) and cyclandelate (○) on cholesterol esterification in rat hepatocytes. Confluent cells were incubated with increasing concentrations of the drugs for 1 hr prior to the addition of [9,10-^{3}H]oleate (300 mM). Cholesterylester synthesis was measured after 1 hr, and results are expressed as a percentage of the control value (1998 ± 705 pmol/hr/mg cell protein. Values represent the mean of triplicate determinations which varied by less than 10%.

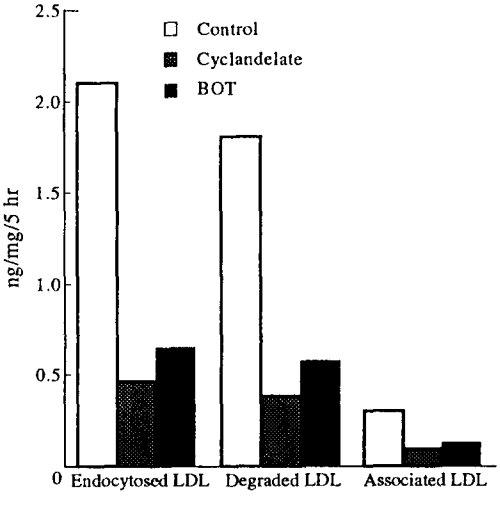

Fig. 7. Comparison of the effects of benzyl 3,3,5-trimethylcyclohexanol and cyclandelate on ^{125}I-LDL metabolism by J774 cells. Cells at confluence were incubated in α-MEM containing LPDS (10%, 5 mg protein/mL) and cyclandelate or benzyl3,3,5-trimethylcyclohexanol (75 μM) for 17 hr prior to addition of ^{125}I-LDL (10 μg/mL). Metabolism of LDL was measured as described in Materials and Methods. Results represent the mean of triplicate determinations which varied by less than 5%.

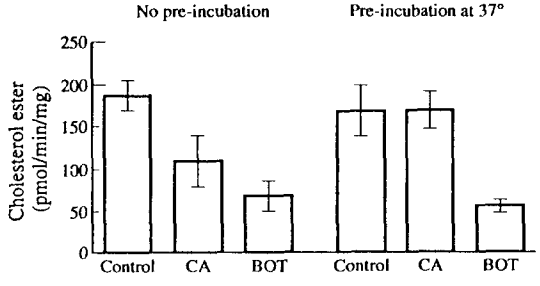

Fig. 9. The effect of preincubation of cyclandelate and benzyl3,3,5-trimethylcyclohexanol with rat liver microsomes on their ability to inhibit microsomal ACAT. Rat liver microsomes were incubated with each drug (100 μM) for 15 min at 37° prior to assay of ACAT activity as described in Materials and Methods. Results represent the mean of two separate determinations which varied by less than 5%.

hepatic LDL receptors, thereby reducing the rate of clearance of plasma LDL. Since the liver, via the bile, represents the major route of elimination of cholesterol from the body this may be an undesired effect. A further complication might be an increased lithogenicity of bile as the liver tries to reduce the intracellular free cholesterol concentration, and it is perhaps relevant that patients with gallstones appear to have a reduced hepatic ACAT activity [25]. A non-hydrolysable ether analogue of cyclandelate, benzyl3,3,5-trimethylcyclohexanol (BOT), was prepared to compare its actions with cyclandelate. The ether inhibited immediately cholesterol esterification on addition to J774 cells, transformed human macrophages U937 and human umbilical vein endothelial cells with an IC_{50} of similar magnitude to cyclandelate (Fig. 6), and had similar inhibitory effects on LDL metabolism after 17 hr incubation with J774 cells (Fig. 7). This similarity was not apparent in hepatocytes when the drugs were added to the cells 1 hr prior to measurement of cholesterol esterification. Benzyl3,3,5-trimethylcyclohexanol

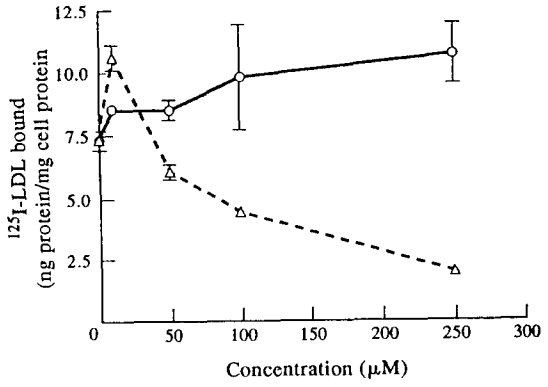

Fig. 10. The effects of cyclandelate (○) and benzyl3,3,5-trimethylcyclohexanol (△) on ^{125}I-LDL binding to rat hepatocytes. Freshly prepared rat hepatocytes were incubated in the presence of either cyclandelate (75 μM) or benzyl3,3,5-trimethylcyclohexanol (75 μM) for 17 hr, after which the medium was replaced with fresh medium containing LPDS (5%, 2 mg protein/mL) and ^{125}I-LDL (10 μg/mL). Specific ^{125}I-LDL binding to the cells was measured as described in Materials and Methods. Results represent the mean ± SD of triplicate determinations from three separate experiments.

inhibited esterification with an IC_{50} of about 20 μM as in other cells, while cyclandelate which was hydrolysed in hepatocytes was not inhibitory (Fig. 8). Similar actions were seen with microsomes isolated from rat liver and assayed directly for ACAT. When assayed immediately after addition of the drugs (100 μM), cyclandelate inhibited ACAT activity by 40% and BOT by 65%. However, when the drugs were added 15 min before the assay no inhibition was seen with cyclandelate while BOT still inhibited by 70%, again indicating that BOT was resistant to hepatic hydrolysis (Fig. 9). This difference in the hepatic actions of the two compounds was further demonstrated in LDL binding to hepatocytes after incubation with the drugs for 17 hr. There was a dose-dependent decrease in the binding of LDL to cells which had been exposed to the ether analogue such that binding in the presence of 75 μM BOT was only 30% that of control cells, implying a decreased LDL receptor number as a result of inhibition of ACAT (Fig. 10). No decrease in LDL binding was seen in cells which had been incubated with cyclandelate, even at high concentrations (250 μM), again demonstrating the differential hepatic and extrahepatic actions of the drug.

Thus cyclandelate appears to be a useful drug for the *in vivo* study of an ACAT inhibitor. Its lack of action in the liver makes it particularly attractive for the study of actions in peripheral tissues where deposition of cholesterol occurs during atherogenesis. Direct actions of the drug on arterial tissue were suggested in experiments using rabbits fed a high cholesterol diet where cyclandelate reduced the extent and severity of aortic atheroma when the cholesterol-fed animals were returned to a regression diet [26]. The drug has been used for a number of years in man for the treatment of cerebrovascular disease and peripheral vascular disorders but despite anecdotal reports of apparent improved peripheral circulation in some patients treated with cyclandelate (G. D. Bell, personal communication), no systematic study of the possible promotion of regression of atheroma by the drug in humans has been made. A major problem might be the rapid and substantial first pass effect of the drug which leads to its hydrolysis to inactive products. For it to be effective, the concentration of the drug in the peripheral blood must reach 20 μM, which might be achieved with the large doses of the drug (1–1.5 g/day) recommended.

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