Biochemical Pharmacology, Vol. 45, No. 9, pp. 1829–1834, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

THE INHIBITION OF CHOLESTEROL ESTERIFICATION BY CYCLANDELATE IN TRANSFORMED MOUSE MACROPHAGES

FRANCESCA HEFFRON, BRUCE MIDDLETON and DAVID A. WHITE*
Department of Biochemistry, Nottingham University Medical School, Queen's Medical Centre, Clifton Boulevard, Nottingham NG7 2UH, U.K.

(*Received* 18 *December* 1992; *accepted* 29 *January* 1993)

Abstract—Cyclandelate (trimethylcyclohexanyl mandelate) inhibited cholesterol esterification in a transformed mouse macrophage cell line (J774) with a concentration of approximately 20 μM being required for half-maximal inhibition. The intact drug was required for its inhibitory action since neither of its hydrolysis products, trimethylcyclohexanol and mandelic acid, caused any inhibition even at high concentrations. The drug entered the cells very rapidly with inhibition being apparent within the shortest time possible to measure esterification (15 min after drug addition). The rate of cholesterol esterification returned to control values when drug-inhibited cells were incubated in drug-free medium indicating a rapid loss of drug from the cells. Loading of cells with cholesterol had no effect on the inhibitory action of cyclandelate, and the inhibition of esterification of cholesterol appeared to be specific, since the syntheses of phospholipid and triacylglycerol (which also involve the action of acyltransferases) were not affected by the drug. Similar inhibitions of cholesterol esterification were seen in four other cell lines, a human osteosarcoma, Chinese hamster ovary cells, a human transformed macrophage cell line (U937) and human umbilical cord vein endothelial cells, as well as in slices of pig aorta, indicating a general action in extra-hepatic tissues where the drug is not hydrolysed.

Accumulation of cholesteryl ester and calcium in the arterial wall accompanies atherogenesis and comprises, with fibrous tissue, the body of the atherosclerotic plaque. Lipid-laden macrophages are essential components of both fatty streaks and fibrous plaques while much extracellular lipid and smooth muscle-derived foam cells may also be present [1]. Many studies have shown that the monocyte-derived macrophage is involved in the initiation of the fatty streak and its subsequent development into the atherosclerotic plaque [2–6]. The major lipid in the plaque, cholesteryl ester, is synthesized intracellularly from lipoprotein-derived cholesterol and fatty acyl CoA in a reaction catalysed by acyl Co A:cholesterol acyl transferase (ACAT†). Since reverse cholesterol transport, i.e. cholesterol efflux from the cell, which would lead to regression of atheroma, involves free rather than esterified cholesterol [7], there is growing interest in inhibitors of ACAT which might increase the intracellular concentration of free cholesterol and thereby promote cholesterol efflux. A number of chemically unrelated potential inhibitors have been described (reviewed in Ref. 8). Cyclandelate, the trimethylcyclohexanyl ester of mandelic acid, is a non-toxic vasoactive drug and has been used extensively for many years in the treatment of cerebrovascular disease and peripheral vascular disorders. It is an effective inhibitor of the ACAT of microsomal preparations from humans, rats and rabbits [9]. The drug was rapidly hydrolysed by hepatic microsomal fractions to products which were non-inhibitory [10]. A direct action of the drug on arterial tissue was suggested in experiments on rabbits fed a high cholesterol diet for 10 weeks [11]. Inclusion of cyclandelate in the subsequent low cholesterol regression diet reduced the extent and severity of aortic atherosclerosis seen in animals fed the regression diet alone. In view of the rapid hydrolysis of cyclandelate by the liver and the lack of ACAT inhibitory activity of the hydrolysis products it is probable that the intact drug reaches the peripheral tissues at a low concentration, which is however sufficiently high to exert the anti-atherosclerotic effect found. The present paper describes the actions of cyclandelate on cholesterol esterification in a transformed macrophage cell line.

MATERIALS AND METHODS

Materials. [9,10-^{3}H]Oleate (370 MBq/mmol) and [4-^{14}C]cholesterol (2.2 GBq/mmol) were purchased from Amersham International (Little Chalfont, U.K.). [4-^{14}C]Cholesteryl oleate was synthesized chemically [12]. [9,10-^{3}H]Oleate–bovine serum albumin (BSA) complex was made essentially as described by Van Harken *et al.* [13] 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.).

* Corresponding author. Tel. (0602) 709371; FAX (0602) 422224.

† Abbreviations: ACAT, acyl CoA:cholesterol acyl transferase; cyclandelate, trimethylcyclohexanyl mandelate; BSA, bovine serum albumin; α-MEM, minimal essential medium α-modification; FCS, foetal calf serum; 58-035, 3-[decyldimethylsilyl]-*N*-[2-(4-methylphenyl)-1-phenylethyl]propanamide; DMSO, dimethyl sulphoxide.

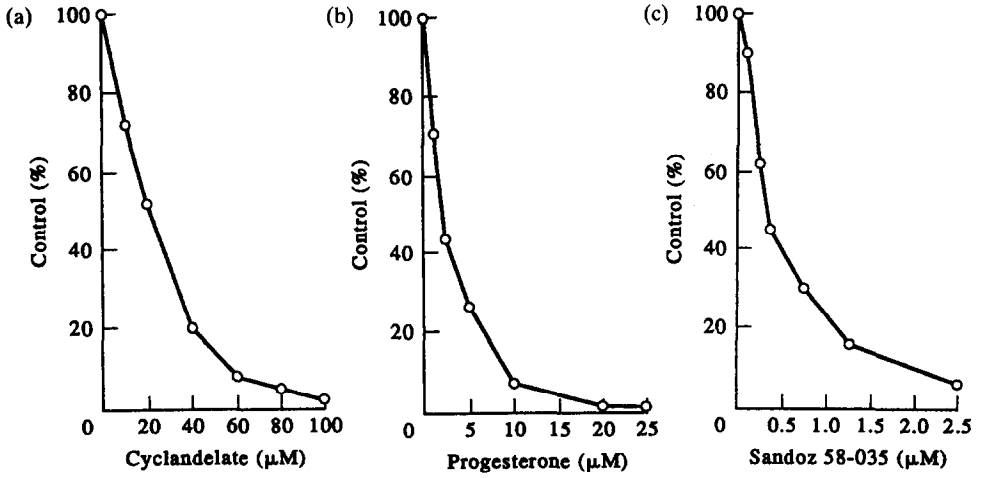

Fig. 1. The effect of increasing concentrations of (a) cyclandelate, (b) progesterone and (c) Sandoz 58-035 on cholesterol esterification in J774 cells. Cells were incubated for 1 hr in the presence of increasing concentrations of drug and the rate of esterification was determined as described in the Materials and Methods. Results are expressed as a percentage of the control value which was 1849 ± 101 pmol/hr/mg protein. Values represent the means of duplicate determinations which varied by less than 5%.

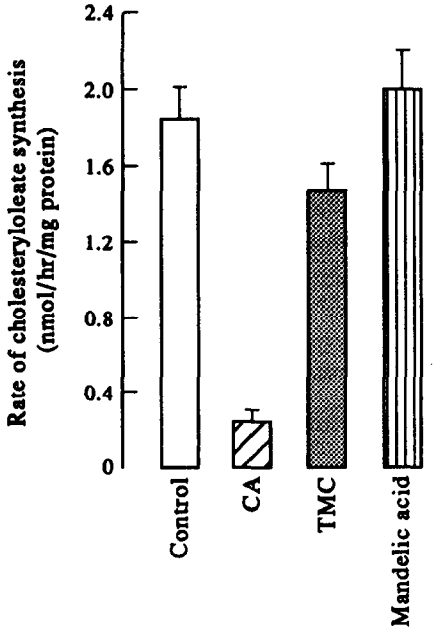

Fig. 2. The effect of cyclandelate and its hydrolysis products on cholesterol esterification in J774 cells. Cells were incubated for 1 hr in the presence of either cyclandelate, trimethylcyclohexanol or mandelic acid all at a concentration of 100 μM. Esterification was determined as described in Materials and Methods. Results represent the means ± range of two separate determinations.

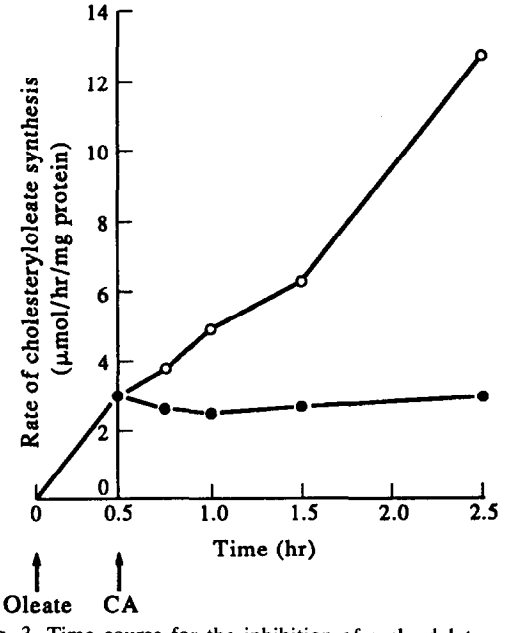

Fig. 3. Time course for the inhibition of cyclandelate on cholesterol esterification in J774 cells. Cells were incubated with 100 μM [^{3}H]oleate for 30 min, after which cyclandelate (final concentration 100 μM) was added in DMSO to the medium. The rate of cholesterol esterification at subsequent times was measured as described in Materials and Methods. Results represent the means ± range of two determinations. (●) Cyclandelate in DMSO; (○) DMSO alone.

Cyclandelate (Cyclospasmol) was from Gist Brocades NV (Delft, The Netherlands) and 58-035 {3-[decyldimethylsilyl] - *N* - [2 - (4 - methylphenyl) - 1 - phenylethyl]propanamide} was a gift from Dr H. Heider, Sandoz Research Institute (E. Hanover, NJ, U.S.A.). All other reagents were purchased from commercial suppliers.

Cell culture. J774 cells, a transformed line of mouse macrophage cells were grown in plastic tissue culture dishes in minimal essential medium α-

modification (α-MEM) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), $NaHCO_3$ (2 g/L) and 4 mM HEPES in an atmosphere of 5% CO_2. The following antibiotics were used in rotation during culture and propagation: kanomycin (100 mg/L), gentamycin (50 mg/L), penicillin (105 U/L) and streptomycin (100 mg/L).

Chinese hamster ovary cells were grown in monolayer culture in α-MEM supplemented with 10% (v/v) FCS.

Human osteosarcoma 2OS were also grown in monolayer culture in α-MEM supplemented with 10% (v/v) newborn calf serum.

U937 cells, a transformed human monocyte line, were grown in suspension culture in RPMI supplemented with 10% newborn calf serum.

Human vascular endothelial cells were isolated by the method of Van Hinsbergh *et al.* [14] from veins of umbilical cords which had been delivered less than 24 hr previously. Cells were grown in monolayer culture in α-MEM containing 20% FCS, endothelial growth factor (200 μg/mL) and heparin (5 units/mL).

Measurement of cholesterol esterification by cells in culture. [9,10-^{3}H]Oleate (10–15 dpm/pmol) complexed with defatted BSA was added at a final concentration of 100 μM to the incubation medium of cells growing in a 60 mm culture dish. Dishes were rotated gently to disperse the substrate and returned to the CO_2 incubator for the appropriate incubation time (usually 1.5 hr). The reaction was stopped by removal of the medium and cooling to 4°. The cells were rinsed with 2 × 2 mL of ice-cold buffer A (50 mM Tris–HCl pH 74 containing 150 mM NaCl and 2 mg/mL fatty acid-poor BSA) and 2 × 2 mL of the same buffer lacking the albumin. [4-^{14}C]Cholesteryl oleate (2000 dpm) was added to the cells as recovery standard and lipids were extracted twice into hexane:isopropanol (3:2 v/v; 2 mL). The lipid extracts were combined and evaporated to dryness under nitrogen. The dried lipid extracts were redissolved in chloroform (100 μL) containing unlabelled cholesteryl oleate (50 μg) and separated on silica gel-impregnated paper (SG 81) developed in hexane:diethyl ether:acetic acid (98:2:1 by vol.) Lipids were visualized by staining with iodine vapour and radioactivity on the paper was detected using a Pannax scanner. Areas on the chromatogram corresponding to cholesteryl oleate, phospholipid or triacylglycerol were cut out and assayed for radioactivity by liquid scintillation spectrometry. When the effects of cyclandelate, progesterone and 58-035 were measured they were added as a solution in dimethyl sulphoxide (DMSO) or ethanol to a final concentration of 2.5% (v/v). Control dishes received DMSO or ethanol only.

Measurement of reversibility of drug inhibition. Cell monolayers were exposed to cyclandelate, progesterone or 58-035 for 1 hr at 37°. The medium was removed and the cells washed with warm phosphate-buffered saline prior to incubation in drug-free medium containing [9,10-^{3}H]oleate (100 μM) for a further 1 hr. Cholesteryl oleate was then assayed as described above.

Cholesterol loading of cells. The cholesterol content of J774 cells was increased by incubating the cells for 24 hr in medium containing 2 mg/mL free cholesterol as described for fibroblasts by Gavigan and Knight [15]. The cholesterol was added dissolved in ethanol such that the final ethanol concentration in the medium was 0.5% (v/v). The cholesterol-supplemented medium was replaced after 24 hr with normal medium prior to assay of cholesterol esterification.

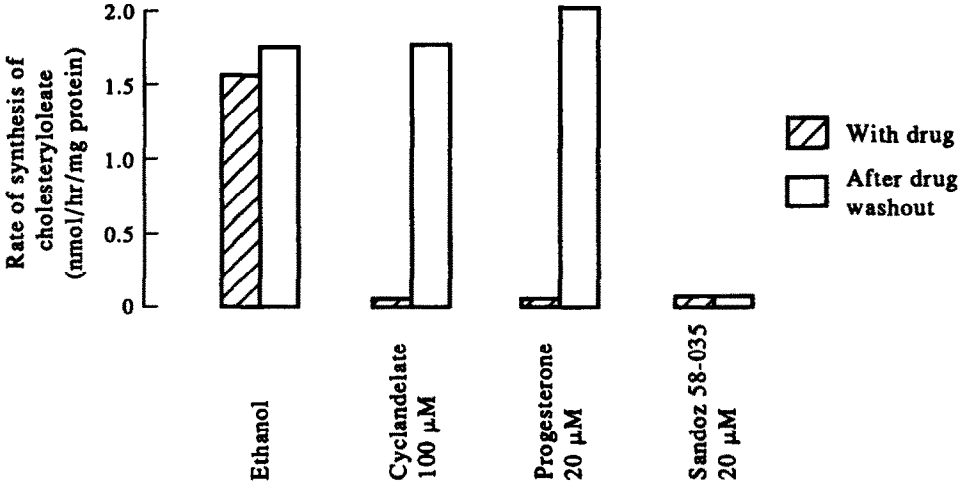

Fig. 4. The effect of drug-free medium on the inhibition of cholesterol esterification in J774 cells. Cells were incubated for 1 hr in the presence of increasing concentrations of (0–100 μM) cyclandelate, progesterone (20 μM) or Sandoz 58-035 (20 μM). They were then divided into two equal groups. The medium in one group was replaced with drug-free medium prior to the addition of [^{3}H]oleate (final concentration 100 μM) to all cells and further incubation for 1 hr. The rate of cholesterol esterification was determined as described in Materials and Methods. Values represent the means of duplicate determinations which varied by less than 5%.

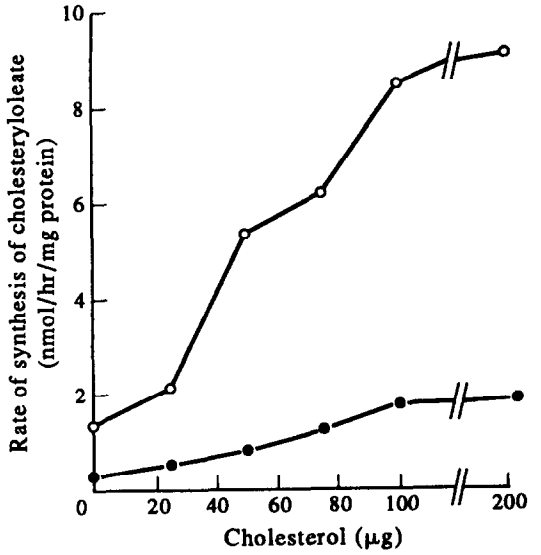

Fig. 5. The effect of increasing the intracellular cholesterol concentration on the inhibition of esterification by cyclandelate in J774 cells. Cells were preincubated for 24 hr in the presence of increasing concentrations of cholesterol added to the medium as a solution in ethanol. The rate of cholesterol esterification was then measured in the presence of DMSO (○) or cyclandelate (50 μM) added in DMSO (●) as described in Materials and Methods. Results represent the means which varied by less than 5% of two separate determinations.

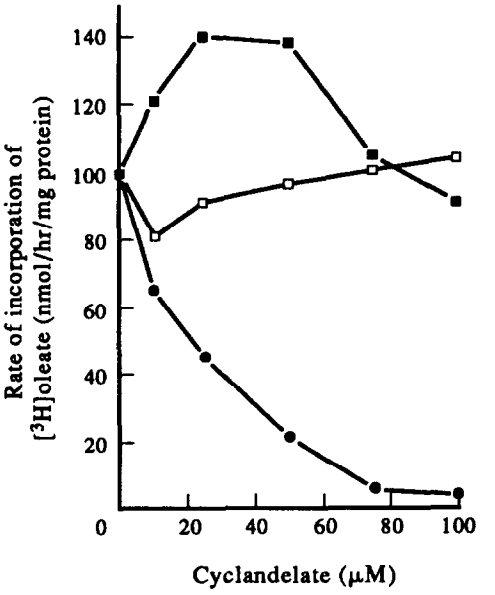

Fig. 6. The effect of cyclandelate on triglyceride (■), phospholipid (□) and cholesteryl ester (●) synthesis in J774 cells. Cells were incubated with increasing concentrations of cyclandelate prior to addition of [^{3}H]-oleate (100 μM) for a further 1.5 hr. Radiolabelled lipids were separated by TLC and assayed for radioactivity as described in Materials and Methods. Results are expressed as percentages of the control (zero drug) activities and represent the means of duplicate determinations which varied by less than 10%.

RESULTS AND DISCUSSION

Preliminary experiments established that esterification of endogenous cholesterol by radiolabelled oleate (100 μM) was linear for at least 3 hr in J774 cells. The effect of increasing concentrations of cyclandelate on this esterification is shown in Fig. 1. Greater than 90% inhibition was achieved at 100 μM cyclandelate with an IC_{50} of between 20 and 25 μM. Comparison was made with two known ACAT inhibitors, Sandoz 58-035 and progesterone, both of which inhibited cholesterol esterification more potently (IC_{50} values of approximately 0.4 and 2.5 μM, respectively) in our system. The drug was inhibitory at all points on the growth curve but was not toxic to the cells as judged by Trypan blue exclusion. The inhibition of esterification by cyclandelate was due to the intact drug since neither of the two products formed by its hydrolysis had any significant effect (Fig. 2). To determine how quickly cyclandelate exerted its effect in whole cells, the cells were incubated with 100 μM oleate for 30 min to establish a measurable esterification rate before the drug was added to the medium. The extent of esterification was then measured at various times after the addition of drug. The rapid action of cyclandelate is seen in Fig. 3. There was a linear increase in the amount of cholesteryl ester formed with time in the absence of cyclandelate. In contrast, no further increase in the amount of ester was seen in the presence of the drug even at the shortest time that allowed measurement (15 min), indicating a rapid and direct effect of the drug on its target.

Cyclandelate is not as potent an inhibitor of ACAT as progesterone or 58-035 (Fig. 1), but unlike these two drugs it is without effect in the liver. Its effect appears also to be reversible, being lost rapidly when cells were returned to a drug-free medium (Fig. 4). After incubation for 1 hr with varying concentrations of cyclandelate (0–100 μM) cells were washed and incubated in drug free medium containing [^{3}H]oleate for a further hour. The results for 100 μM cyclandelate are shown in Fig. 4, but at each concentration the control rate of incorporation of oleate into cholesteryl ester was obtained. A similar reversible inhibition was seen with progesterone and contrasts with the irreversible inhibition caused by 58-035 (Fig. 4 [16]).

Loading of J774 cells with cholesterol by incubating them overnight in medium containing increasing concentrations of free cholesterol (0–200 μg) caused a progressive increase in the rate of cholesterol esterification (Fig. 5), presumably due to an increase in the size of the pool of free cholesterol, one of the substrates for ACAT. Addition of cyclandelate (50 μM) to the culture medium after the loading period caused a consistent inhibition (>70%) of cholesterol esterification at all loading concentrations. The effect of cyclandelate was thus not due to impeded entry of one of the substrates, cholesterol,

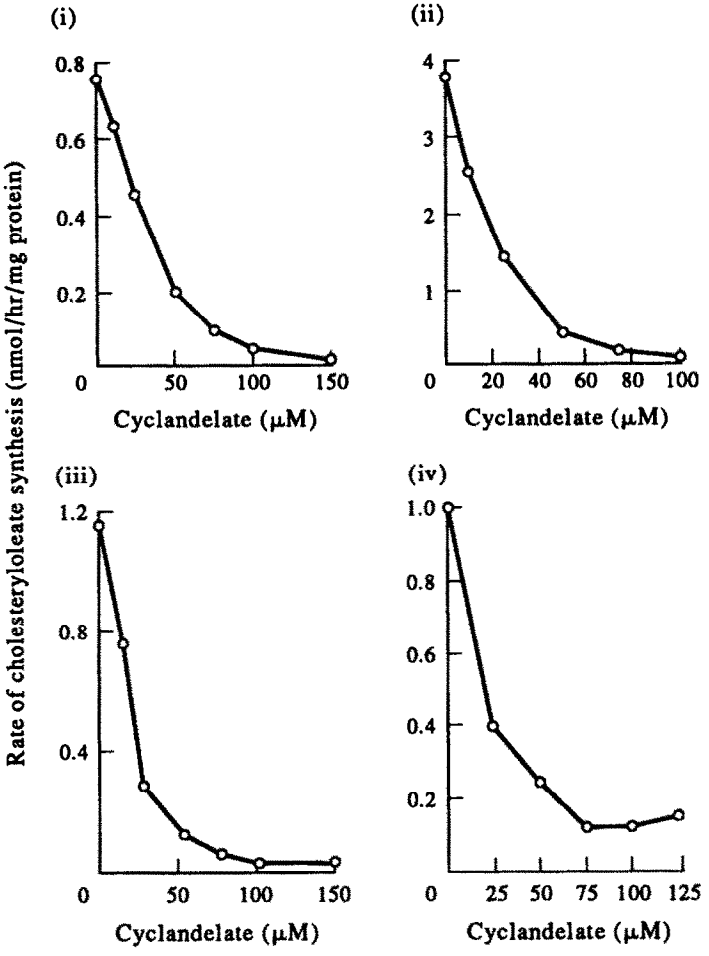

Fig. 7. The effect of cyclandelate on cholesterol esterification in osteosarcoma 2OS cells (i), Chinese hamster ovary cells (ii), human transformed macrophages U937 (iii) and human umbilical cord vein endothelial cells (iv). Cells were incubated for 1 hr with increasing concentrations of cyclandelate and the rate of cholesterol esterification measured as described in Materials and Methods. Results represent the means of duplicate determinations which varied by less than 5%.

into the cell. Neither did it appear to block oleate uptake since there was no significant effect on the incorporation of oleate into phospholipids or triacylglycerols (Fig. 6). The apparent stimulation of incorporation of radioactivity from oleate into triacylglycerols at lower concentrations of cyclandelate is unexplained and was not seen at higher concentrations of the drug. This lack of inhibition of other microsomal acyltransferases implies that the inhibition of cholesterol esterification by cyclandelate is specific.

We have shown previously [10] that cyclandelate is unlikely to have any inhibitory effect in the liver because it is rapidly hydrolysed in this tissue. However, in extra-hepatic tissues which lack the ability to hydrolyse the drug, cyclandelate inhibited cholesterol esterification to the same extent as in J774 cells. Such inhibition as a function of cyclandelate concentration is shown for four cultured cell lines: human osteosarcoma 2OS, Chinese hamster ovary cells, a transformed human monocyte U937 and human vascular endothelial cells (Fig. 7). In each cell line 50% inhibition of esterification was seen at a cyclandelate concentration of approximately 20 μM. We have also suggested from *in vivo* studies in cholesterol-fed rabbits that cyclandelate might have a direct action on arterial tissue [11]. In the current experiments, exposure of slices of pig aorta to cyclandelate for 6 hr did indeed inhibit the incorporation of [H]oleate into cholesteryl ester (results not shown). The tissue was very tough and somewhat inert metabolically with cholesterol esterification rates three orders of magnitude lower than in the five cell types studied. Higher concentrations of cyclandelate were required to produce significant inhibition, presumably due to the problems presented in penetrating active tissue.

In conclusion, cyclandelate is an effective reversible inhibitor of ACAT which is active extra-hepatically. It remains the only drug capable of inhibiting ACAT that has received significant clinical use. ACAT is responsible for intracellular

esterification of cholesterol and leads to deposition of the storage form of cholesterol as its ester. Accumulation of cholesteryl ester is a characteristic of the atherosclerotic plaque and inhibition of ACAT may be a therapeutic target in the regulation of plaque formation. Inhibition of the enzyme would lead to an increased intracellular concentration of free cholesterol and possibly promote cholesterol efflux from cells [7]. Indeed, Middleton [16] has shown that cyclandelate and progesterone both inhibit cholesterol esterification and down-regulate low density lipoprotein binding in human fibroblasts.

Although not as potent an inhibitor of ACAT as progesterone, 58-035 or CL 277,082, cyclandelate appears fortuitously to be tissue selective with no action in the liver [10]. Inhibition of hepatic ACAT might not be desirable since the consequent rise in hepatic-free cholesterol might cause down-regulation of low density lipoprotein receptors and decrease cholesterol clearance from the circulation [17]. However, inhibition of extra-hepatic ACAT might lead to cholesterol removal from these tissues, and in this respect the inhibitions reported here in macrophages and vascular endothelial cells might explain the decrease in atherosclerosis caused by dietary cyclandelate in cholesterol-fed rabbits [11].

REFERENCES

1. Haust MD, The natural history of human atherosclerotic lesions. In *Vascular Injury and Atherosclerosis* (Ed. Moore S), pp. 1–23. Marcel Dekker, New York, 1981.
2. Brown MS and Goldstein JL, Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu Rev Biochem* **52**: 223–261, 1983.
3. Gerrity RG, The role of the monocyte in atherogenesis (1). The transition of blood-borne monocytes into foam cells in fatty lesions. *Am J Pathol* **103**: 181–190, 1981.
4. Gerrity RG, The role of the monocyte in atherogenesis (2). Migration of foam cells from atherosclerotic lesions. *Am J Pathol* **103**: 191–200, 1981.
5. Rosenfeld ME, Tsukeda T, Gown AM and Ross R, Fatty streak initiation in Watanabe Heritable Hyperlipaemic and comparably hypercholesterolaemic fat-fed rabbits. *Arteriosclerosis* **7**: 9–23, 1987.
6. Rosenfeld ME, Tsukeda T, Chait A, Bierman EL, Gown AM and Ross R, Fatty streak expansion and maturation in Watanabe heritable hyperlipidaemic and comparably hypercholesterolaemic fat-fed rabbits. *Arteriosclerosis* **7**: 24–34, 1987.
7. Reichl D and Miller NE, The anatomy and physiology of reverse cholesterol transport. *Clin Sci* **70**: 221–231, 1986.
8. Sliskovic DR and White AD, Therapeutic potential of ACAT inhibitors as lipid lowering and anti-atherosclerotic agents. *Trends Pharmacol Sci* **12**: 194–199, 1991.
9. Heffron F, Middleton B and White DA, Inhibition of acyl coenzyme A:cholesterol acyl transferase by trimethylcyclohexanylmandelate (cyclandelate). *Biochem Pharmacol* **39**: 575–580, 1990.
10. White DA, Heffron F, Miciak A, Middleton B, Knights S and Knight D, Chemical synthesis of dual-radiolabelled cyclandelate and its metabolism by rat hepatocytes and mouse J774 cells. *Xenobiotica* **20**: 171–178, 1990.
11. Middleton B, Middleton A, White DA and Bell GD, Dietary cyclandelate decreases pre-established atherosclerosis in the rabbit. *Atherosclerosis* **171**: 171–178, 1984.
12. Mitropoulos KA, Balasubramaniam S, Venkatesan S and Reeves BEA, On the mechanism for the regulation of the 3-hydroxy-3-methylglutaryl coenzyme A reductase, of cholesterol 7α-hydroxylase and of ACAT by free cholesterol. *Biochim Biophys Acta* **530**: 99–111, 1978.
13. Van Harken DR, Dixon CW and Heimberg M, Hepatic lipid metabolism in experimental diabetes. *J Biol Chem* **244**: 2278–2286, 1969.
14. Van Hinsbergh V, Havakes L, Emeis J, van Corvene E and Scheffer M, Low density lipoprotein metabolism by endothelial cells from human umbilical arteries and veins. *Atherosclerosis* **3**: 547–559, 1983.
15. Gavigan S and Knight B, The effects of low density lipoprotein and cholesterol on acyl-coenzyme A: cholesterol acyltransferase activity. *Biochem J* **216**: 93–100, 1983.
16. Middleton B, Inhibition of cellular cholesterol esterification can decrease LDL receptor number in human fibroblasts. *Biochem Biophys Res Commun* **145**: 350–356, 1987.
17. Salter AM, Ekins D, Al-Seeni M, Brindley DN and Middleton B, Cholesterol esterification plays a major role in determining low-density lipoprotein receptor activity in primary monolayer cultures of rat hepatocytes. *Biochem J* **262**: 255–260, 1989.