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Inhibition of cholesteryl ester deposition in macrophages by calcium entry blockers: an effect dissociable from calcium entry blockade

[1]Alan Daugherty, Debra L. Rateri, Gustav Schonfeld & Burton E. Sobel

Cardiovascular Division and Lipid Research Centre, Department of Medicine, Washington University School of Medicine, 660 South Euclid Avenue, Box 8086, St. Louis, Missouri 63110, U.S.A.

1 The effects of calcium entry blockers on stimulated cholesteryl [^{3}H]-oleate deposition in cultured macrophages were characterized in order to elucidate mechanisms underlying possible antiatherosclerotic effects. Stimulation of intracellular cholesteryl [^{3}H]-oleate deposition was initiated by incubation of macrophages with β-very low density lipoproteins (β-VLDL).

2 Nifedipine (Class I) markedly reduced cholesteryl [^{3}H]-oleate deposition at all concentrations tested. However, Bay K 8644, a dihydropyridine which is known to stimulate calcium entry, also reduced cholesteryl [^{3}H]-oleate deposition with a similar potency to nifedipine.

3 The effects of three Class II calcium entry blockers were evaluated: verapamil, methoxyverapamil, and diltiazem. Verapamil inhibited cholesteryl [^{3}H]-oleate deposition in a concentration-dependent manner. Similarly, methoxyverapamil reduced cholesteryl [^{3}H]-oleate deposition in a concentration-dependent manner although the reduction was not as great as that produced by verapamil. In contrast, diltiazem at any concentration tested did not inhibit cholesteryl [^{3}H]-oleate deposition.

4 Flunarizine (a Class III calcium entry blocker) produced a modest stimulation of cholesteryl [^{3}H]-oleate deposition at the lowest concentration used (10^{-7}M) but marked depression at the highest concentration (10^{-5}M).

5 The results indicate calcium entry blockers may exert protective effects on the development of atherosclerosis in animal models of diet-induced hyperlipidaemia by inhibiting intracellular cholesteryl ester deposition, but this effect may not be related to their calcium entry-blocking effects.

Introduction

Henry & Bentley (1981) first demonstrated that a calcium entry blocker (nifedipine) inhibited atherogenesis in dietary-induced hypercholesterolaemia in rabbits. Subsequently, several calcium entry blockers have been evaluated in animals with atherosclerosis induced by cholesterol supplemented diets or by genetic abnormalities, such as those in Watanabe heritable hyperlipidaemic rabbits. Antiatherosclerotic effects of calcium entry blockers have been inconsistent (Stender *et al.*, 1984; Naito *et al.*, 1984; Willis *et al.*, 1985; Sugano *et al.*, 1986), although one calcium entry blocker, verapamil, has appeared consistently to reduce atherogenesis in cholesterol-fed rabbits (Rouleau *et al.*, 1983; Ginsburg *et al.*, 1983). The antiatherosclerotic property of verapamil is evident despite a lack of change in the plasma concentrations of lipoproteins. Consequently it probably involves effects on the metabolism of cells within the arterial tissue.

Atherosclerosis with diet-induced hyperlipidaemia in rabbits is associated with the accumulation in plasma of cholesteryl ester-rich β-migrating very low density lipoproteins (β-VLDL) (Daugherty *et al.*, 1985), that enter resident macrophages of several tissues including arteries via specific β-VLDL receptors (Mahley *et al.*, 1980). With diet-induced hypercholesterolaemia in rabbits, high concentrations of β-VLDL in plasma results in continual delivery of the cholesteryl esters of β-VLDL into macrophages and converts these phagocytes to foam cells which are characteristic of early atherosclerotic lesions (Faggiotto *et al.*, 1984). Calcium entry blockers may exert their antiatherosclerotic action by prevention of cholesteryl ester loading in macrophages. The aim of the study was to evaluate this possibility directly and to characterize the relationship between effects on

[1] Author for correspondence.

macrophages and calcium entry blocking properties. This was accomplished by comparing the effects of at least one drug from each class of calcium entry blocker (Spedding, 1985) and the effects of the calcium entry stimulant Bay K 8644 (Schramm *et al.*, 1983).

Methods

Harvest of lipoproteins

β-VLDL was obtained from rabbits that had been fed a cholesterol-enriched (2% w/w) laboratory diet for 2 to 4 weeks. Blood was obtained from a marginal ear vein and collected in tubes containing EDTA (1.5 mg ml^{-1}). Plasma was subjected to ultracentrifugation at d = 1.006 g ml^{-1} (Havel *et al.*, 1985), in a Beckman L8–55 ultracentrifuge (Beckman Instruments Co., Palo Alto, CA) with a Beckman 60Ti rotor at 45,000 r.p.m. for 18 h at 10°C. The $d < 1.006$ g ml^{-1} fraction was washed by dilution in NaCl (0.15 M)/EDTA (1 mM; pH 8.2) solution and repeat ultracentrifugation under the same conditions. Isolated lipoproteins were dialyzed against NaCl (0.15 M)/EDTA (1 mM; pH 8.2) solution. Protein concentrations of lipoproteins were measured conventionally (Lowry *et al.*, 1951).

Harvest of macrophages

Macrophages were obtained by lavage of the alveolar cavity of normolipidaemic rabbits. Rabbits were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}) and exsanguinated via the dorsal aorta. A cannula was placed in the trachea and the lungs were lavaged with 50 ml of calcium and magnesium-free saline containing heparin sulphate (5 uml^{-1}; Sigma Chemical Co., St. Louis, MO). This procedure was repeated five times. Approximately 80×10^6 cells were obtained from each rabbit.

Cells were washed in Dubelcco's minimum essential medium (DME, Gibco, Grand Island, NY) containing no serum, and plated into 35 mm wells (Falcon, Oxnard, CA) at a density of $3-5 \times 10^6$ cells per well in DME medium containing newborn calf serum (15% v/v). Cells were maintained at 37°C overnight in a 5% CO_2 incubator.

Determination of cholesteryl ester deposition

Cholesteryl ester deposition in macrophages was assessed by the method of Brown *et al.* (1980). One day after harvest, the macrophages were pre-incubated at 37°C for 2 h with DME medium containing the drug under investigation. Following incubation with drug in DME medium, the medium was replaced with one containing the drug, selected concentrations of β-VLDL, and [^{3}H]-oleate (0.1 mM) complexed to bovine serum albumin (0.02 mM). The specific radioactivity of oleate incubated with cells was approximately 24 mCi mol^{-1}. Cells were incubated in this mixture for 5 h at 37°C, after which the medium was removed. The cells were washed twice with ice-cold phosphate-buffered saline. Monolayers were then incubated with hexane:isopropyl alcohol (3:2; 1 ml, containing carrier lipid; triolien and cholesteryl palmitate, both 1.5 mg 100 ml^{-1}) for 30 min at room temperature. The organic layer was transferred to a tube and the entire process was repeated. After extraction of lipids, cells were incubated with sodium hydroxide (1 ml, 1 N) overnight at 4°C. Aliquots of dissolved cells were used for determination of cellular protein by the method of Lowry *et al.* (1951).

Hexane: isopropyl alcohol extracts were evaporated to dryness by mild heating and a flow of nitrogen. Lipids were resuspended in chloroform:methanol (2:1) and blotted on thin layer chromatography plates. Plates were developed in petroleum ether:diethyl ether:glacial acetic acid (168:30:2). Cholesteryl esters were located by exposure of the plate to iodide vapours and transferred to scintillation vials containing Ready-Solv (10 ml, Beckman). Radioactivity was determined with the use of a Beckman LS-2800 liquid scintillation spectrophotometer. Quenching of samples was determined with the H number technique.

New preparations of β-VLDL were isolated for each study and consequently a control concentration-response curve was constructed for each assay. For drugs which required dissolution in ethanol, control concentration-response curves were determined in the presence of ethanol in the incubation medium.

Results are expressed as a percentage of the maximum cholesteryl ester deposition determined from the control concentration-response curve. For each concentration-response curve, the selected concentrations of β-VLDL were performed in duplicate wells. Variation of these duplicates was less than 8% of their mean value. Intra-assay variation was examined by performing three control concentration-response curves within a single batch of experiments. Maximum cholesteryl ester deposition for these three control experiments was 2.12 ± 0.03 nmol cholesteryl ester g^{-1} cell protein (mean ± s.e.mean) for a single preparation of β-VLDL assayed with macrophages which were plated from a single harvest (range = 2.00 to 2.17 nmol cholesteryl ester g^{-1} cell protein). The EC_{50} for these controls was 6.4 ± 0.5 μg protein ml^{-1}. The selected concentrations of the drug under investigation were studied in a single batch of experiments and a control concentration-response curve was constructed for each drug within a batch of experiments. Inter-assay variation in the maximum deposition of cholesteryl ester was much greater, as would be expected using different preparations of β-VLDL and macro-

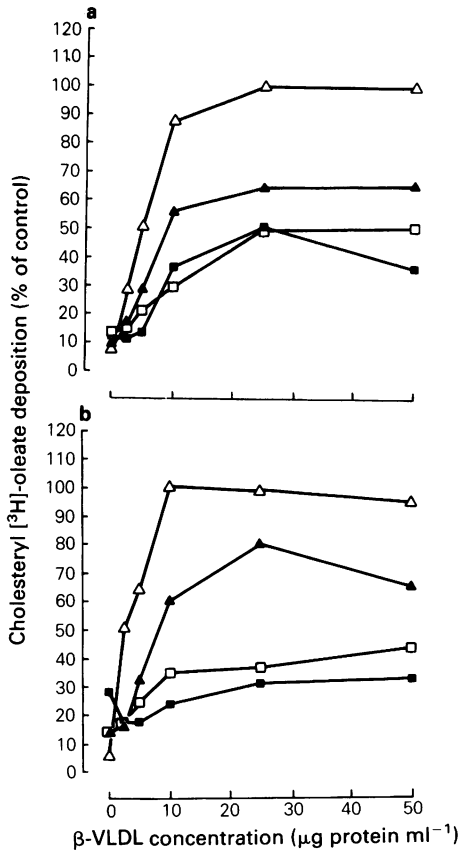

Figure 1 Effects of dihydropyridines on stimulation of cholesteryl [^{3}H]-oleate deposition in cultured macrophages by β-very low density lipoproteins (β-VLDL). Shown are incubations with lipoprotein alone (△), and in the presence of nifedipine (a) and Bay K 8644 (b) at the following concentrations: 10^{-7}M (▲), 10^{-6}M (□), and 10^{-5}M (■). Each point represents the mean of duplicate observations. The control incubations were performed in the presence of ethanol at a final concentration of 0.05% v/v in the well.

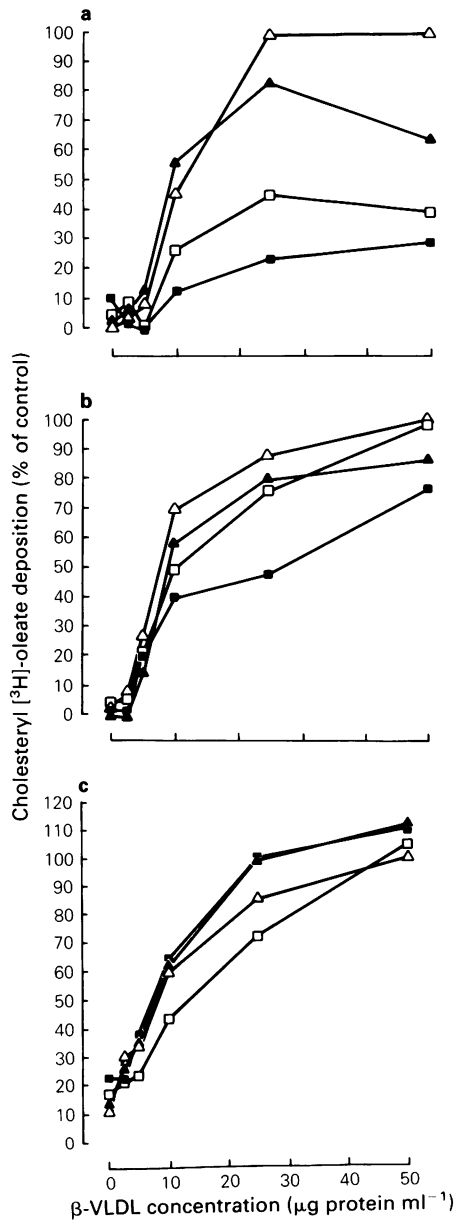

Figure 2 Effects of Class II calcium entry blockers on β-VLDL-stimulated cholesteryl [^{3}H]-oleate deposition in cultured macrophages. Shown are incubations with lipoprotein alone (△), and in the presence of verapamil (a), methoxyverapamil (b) and diltiazem (c) at the following concentrations: 10^{-7}M (▲), 10^{-6}M (□), and 10^{-5}M (■). Each point represents the mean of duplicate observations.

phages (range = 2.00 to 6.22 nmol cholesteryl ester g^{-1} cell protein). To ensure the consistency of drug-induced changes, the assay of verapamil and methoxyverapamil on β-VLDL stimulated cholesteryl ester deposition was performed in three separate batches of experiments. At each concentration of drug, the percentage reduction in cholesteryl ester deposition varied by no more than 13% of their mean value for each concentration of β–VLDL.

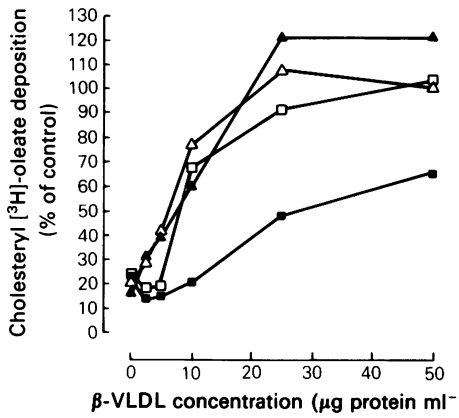

Figure 3 Effects of the Class III calcium entry blocker flunarizine on β-VLDL-stimulated cholesteryl [^{3}H]-oleate deposition in cultured macrophages. Shown are incubations with lipoprotein alone (△) and in the presence of flunarizine 10^{-7}M (▲), 10^{-6}M (□) and 10^{-5}M (■). Each point represents the mean of duplicate observations. The control incubations were performed in the presence of ethanol at a final concentration of 0.05% v/v in the well.

Drugs used

The drugs used in this study were verapamil hydrochloride (Knoll Pharmaceuticals, Whippany, NJ), methoxyverapamil hydrochloride (Sigma Chemical Co., St. Louis, MO), diltiazem (Marion Labs, Kansas City, MO), flunarizine (Janssen, Belgium), nifedipine and Bay K 8644 (Bayer, Germany). Verapamil, methoxyverapamil, and diltiazem were diluted initially in physiological saline and subsequently in DME medium. Flunarizine, nifedipine and Bay K 8644 were initially dissolved in ethanol (50% v/w) and subsequently diluted in DME. All experiments with nifedipine and Bay K 8644 were performed in a darkened room to avoid degradation of these drugs.

Results

β-VLDL stimulated the incorporation of [^{3}H]-oleate into cholesteryl [^{3}H]-oleate in rabbit alveolar macrophages in a concentration-dependent manner (Figure 1), confirming results obtained by other workers using the same lipoprotein fraction and the same cell type but derived from mouse peritoneum (Mahley *et al.*, 1980). Using this effect as a model, the ability of calcium entry blockers to inhibit cholesteryl [^{3}H]-oleate deposition was investigated.

Nifedipine (Class I) reduced cholesteryl [^{3}H]-oleate deposition in a concentration-dependent manner (Figure 1a). Bay K 8644, a dihydropyridine which, as opposed to nifedipine, stimulates calcium entry, also reduced cholesteryl [^{3}H]-oleate deposition (Figure 1b) with a potency similar to that of nifedipine.

Verapamil, methoxyverapamil and diltiazem were evaluated as examples of Class II compounds. Verapamil reduced cholesteryl [^{3}H]-oleate deposition in a concentration-dependent manner with a reduction in both the slope and peak response (Figure 2a). Methoxyverapamil also produced a reduction in slope and peak response of β-VLDL-stimulated cholesteryl ester deposition (Figure 2b), although this reduction was not as pronounced as demonstrated in the presence of verapamil. In contrast, diltiazem did not consistently reduce cholesteryl [^{3}H]-oleate deposition at any of the concentrations used (Figure 2c).

Flunarizine (a representative of the Class III of calcium entry blockers) produced a mild stimulation of cholesteryl [^{3}H]-oleate deposition at the lowest concentration used (10^{-7}M) but a high concentration (10^{-5}M) produced a marked depression of both the slope and the peak response (Figure 3).

None of the drugs investigated, at the concentrations used, caused any apparent cellular toxicity as judged by gross morphological examination and by the consistency of the cellular protein which was present in the wells at the end of the incubation period.

Discussion

Calcium entry blockers inhibit the development of the aortic atheromatous lesions that characteristically appear in aortae of rabbits fed cholesterol-enriched laboratory diets (Henry & Bentley, 1981; Rouleau *et al.*, 1983; Ginsburg *et al.*, 1983). In such animals, lipid-laden lesions appear to be produced at least in part as a consequence of β-VLDL being taken up by macrophages present in the subendothelial space. This results in deposition of cholesteryl esters via a pathway involving, first, the hydrolysis of lipoprotein associated cholesteryl esters in lysosomes; and second, the migration of the resulting unesterified cholesterol to microsomes where re-esterification with oleate is catalyzed by acyl-cholesterol acyl-transferase (ACAT). Atherosclerotic lesions contain predominantly cholesteryl oleate (Zilversmit *et al.*, 1961; Brown *et al.*, 1980). Consequently, atherogenesis proceeding via the mechanism outlined should be amenable to modification by inhibition of cholesterol esterification in macrophages.

The antiatherosclerotic action of drugs such as chlorpromazine has been attributed to inhibition of ACAT (Bell & Schaub, 1986). Indeed, selective inhibitors of ACAT have been developed for use in the

prevention of atherosclerosis (Ross *et al.*, 1984). Results obtained in the present study demonstrate that some calcium entry blockers inhibit cholesterol esterification in macrophages incubated with the potentially atherogenic lipoprotein, β-VLDL.

The mechanisms by which calcium entry blockers inhibit cholesteryl ester deposition have not been elucidated. Possibilities include (a) effects on lysosomal function that inhibit or prevent the hydrolysis of cholesteryl esters; (b) translocation of cholesterol to microsomes; and (c) direct effects on the ACAT reaction. At least in the case of verapamil, generalized inhibition of lysosomal enzymes such as chloroquine does not appear to be responsible, since lysosomal degradation in rabbit skin fibroblasts of the protein moiety of ^{125}I-labelled β-VLDL is not reduced by the drug (Daugherty, unpublished observations).

In the present study, nifedipine was effective in reducing cholesteryl ester deposition in macrophages, which contrasts with the findings of other workers (Henry, 1984). This disparity may reflect the effects of preincubation of the cells with the drugs for 2 h before their exposure to β-VLDL. Since several of the calcium entry blockers are lipophilic, it is likely that they bind to lipoproteins and are thereby excluded from the site of drug action. Although drug-lipoprotein binding was not characterized specifically in this study, it is likely to be an important consideration for elucidation of the antiatherosclerotic effects of calcium entry blockers both *in vitro* and *in vivo*.

Henry (1984) has proposed that an antiatherosclerotic effect of the calcium entry blocker, nifedipine, is attributable to the blockade of calcium entry into vascular tissue. Results of the present study demonstrate that some calcium entry blockers affect lipid metabolism by a mechanism somewhat independent of the inhibition or blockade of calcium entry. Thus a comparison of the inhibition by verapamil of incorporation of [^{3}H]-oleate into cholesteryl esters with respect to that of its methoxy derivative, a considerably more potent calcium entry blocker, (Fleckenstein, 1977), indicates a reduced inhibitory effect on β-VLDL-stimulated cholesteryl [^{3}H]-oleate accumulation compared with verapamil at all concentrations of drug evaluated. Furthermore, the other Class II drug investigated, diltiazem, failed totally to inhibit cholesteryl ester deposition. In addition, although nifedipine inhibited cholesteryl [^{3}H]-oleate deposition in a concentration-dependent manner, Bay K 8644, a dihydropyridine structurally similar to nifedipine but one that stimulates rather than blocks calcium entry, reduced cholesteryl [^{3}H]-oleate deposition with a similar efficacy to nifedipine. Accordingly, calcium entry blockers appear to inhibit cholesteryl ester accumulation by mechanisms independent of calcium entry blockade *per se* and which apparently involve direct effects of the drugs.

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