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EFFECT OF VERAPAMIL AND NIFEDIPINE ON CHOLESTERYL ESTER METABOLISM AND LOW-DENSITY LIPOPROTEIN OXIDATION IN MACROPHAGES

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Abstract—Using mouse macrophage cultures, the effects of verapamil and nifedipine on cholesteryl ester and low-density lipoprotein (LDL) metabolism were studied with special reference to the following parameters: (a) incorporation of [^{14}C]oleate into cholesteryl esters (ChE), (b) contents of total and free cholesterol (FCh), (c) liberation of [^{14}C]oleate from ChE and incorporation of [^{3}H]FCh into ChE, (d) excretion of [^{3}H]Ch from the cells, and (e) LDL oxidation. Verapamil and nifedipine (10–100 μM) were shown to decrease in a dose-dependent manner the incorporation of [^{14}C]oleate into ChE and to increase the concentration of FCh but had no appreciable effect on the concentration of total cholesterol in macrophages cultured in the presence of acetylated LDL. The drugs stimulated the liberation of [^{14}C]oleate from cellular ChE. The pharmacological concentrations (25–75 μM) of verapamil and nifedipine increased the excretion of [^{3}H]FCh from ChE of macrophages in the presence of serum and high-density lipoproteins. The same concentrations of the drugs inhibited both LDL-derived malonyldialdehyde-like products and nitroblue tetrazolium dye reduction in a dose-dependent fashion. The results obtained suggest that verapamil and nifedipine exert their macrophage-mediated antiatherosclerotic effect via reduction of LDL oxidative modification, reduction of intracellular ChE synthesis, stimulation of ChE hydrolysis and cholesterol excretion from the cells.

Key words: nifedipine; verapamil; cholesteryl ester metabolism; cholesterol excretion; LDL oxidation; macrophages

The phenilalchylamine derivative, verapamil, and dihydropyridine derivative, nifedipine, belong to the group of calcium blockers, which inhibit selectively the transmembrane calcium influx into the cell [1]. During the past few years the antiatherogenic effects of these drugs have been intensely discussed [2, 3]. Calcium antagonists have been shown to inhibit atherosclerotic lesion development and cholesterol accumulation in blood vessel walls of rabbits with no decrease in cholesterol (Ch) content in plasma LDL‡ [4–7]. Nifedipine's ability to decrease intracellular cholesterol concentration and to increase cytosolic cholesteryl ester hydrolase activity independently of its effect upon membrane calcium channels has been demonstrated in cultures of foam cells isolated from rabbit [8] and human [9] atherosclerotic vessels. The experiments with labelled precursors have demonstrated that verapamil induced cholesterol synthesis in isolated human monocytes [10] and decreased the synthesis of ChE in mouse macrophages cultured in the presence of β-very low-density lipoproteins [11]. None the less, the mechanisms underlying nifedipine and verapamil's effects inhibiting intracellular Ch and ChE accumulation remain unclear. Furthermore, no information is available on the influence of calcium blockers on oxidative modification of LDL in macrophages, although LDL oxidation has been suggested to be a key event in atherogenesis [12]. Macrophages, when exposed to Ca^{2+}-ionophores, develop a state of "respiratory burst" or "oxidative burst", associated with intense reactive oxygen species generation [13, 14]. So, it could be assumed that calcium blockers can depress the oxidative process and, hence, LDL modification.

The aim of the current study was to investigate the effects of verapamil and nifedipine on LDL oxidation, ChE synthesis and hydrolysis and Ch excretion in cultured mouse macrophages, which are a convenient tool for investigating biochemical mechanisms of foam cell formation in the early stages of atherogenesis [15].

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Oleate (56 mCi/mM) and [1,2-^{3}H]cholesterol (10 Ci/mM) were obtained from Amersham Corp. (Oakville, Ovd). Verapamil, nifedipine, cholesterol, cholesteryl oleate, nitroblue tetrazolium and bovine serum albumin were purchased from Sigma Chemical Co. (St Louis, MO,

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‡ Abbreviations: LDL, low-density lipoproteins; acetyl-LDL, acetylated low-density lipoproteins; HDL, high-density lipoproteins; Ch, cholesterol; FCh, free cholesterol; ChE, cholesteryl esters; NBT, nitroblue tetrazolium; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; FCS, fetal calf serum.

U.S.A.). The tissue culture supplies and reagents were purchased from Gibco (Mississauga, Ont. All other reagents for assays were obtained from Serva or Boehringer Mannheim (Germany).

Preparative methods. Blood from nonlipidemic donors fasted overnight was collected directly into syringes containing EDTA (1 mg/mL) and benzamidine (2 mM). Phenylmethylsulfonyl fluoride (0.5 mM in DMSO), chloramphenicol (50 μg/mL), and gentamicin (100 μg/mL) were then added to the combined plasmas. At the end of the isolation by ultracentrifugation, LDL were purified from KBr by chromatography on Sephadex G-25 and, immediately after chromatography, used in experiments of LDL oxidation.

Human LDL (density 1.019–1.063 g/mL) and HDL_3 (density 1.125–1.215 g/mL) were isolated by ultracentrifugation from the plasma of individual healthy subjects [16]. LDL was acetylated with repeated additions of acetic anhydride [17]. Low molecular weight reaction products and KBr were removed by chromotography on Sephadex G-25, followed by dialysis of acetyl-LDL and HDL_3 against 0.05 M phosphate buffer, pH 7.4, with 0.15 M NaCl (PBS). Acetyl-LDL radiolabelled with [^{3}H]-cholesterol was prepared by the absorption method, in which [^{3}H]cholesterol was incorporated into acetyl-LDL during 4 hr incubation at 37° [18]. [^{14}C]-Oleic acid was complexed with albumin by adding the oleate to PBS containing 10% BSA [17]. The specific radioactivity of oleate–albumin complexes was 4200–8600 cpm/nmol albumin. The emulsions of FCh and [^{3}H]FCh in PBS contained 10% BSA and were prepared by the method of Werb and Cohn [19]. The solutions were sterilized by filtration using a pore diameter of 0.22 μm.

Mouse macrophage monolayers. Elicited peritoneal cells were harvested from C57Bl/6J mice (all 4–8 weeks old) that were injected i.p. 5 days previously with 1.0 mL of sterile 5% glycogen solution [20, 21]. Peritoneal cavities were lavaged with ice-cold PBS. Peritoneal lavage fluid from 40 mice was pooled, and the cells were collected by centrifugation (400 *g*, 10 min) and washed once with 30 mL of Dulbecco's medium. The cells were resuspended in RPMI 1640 medium containing 20% (v/v) fetal calf serum and gentamycin (100 U/mL) at a final concentration of 2×10^6 cells/mL. The 2 mL aliquots of this cell suspensions were plated into Petri dishes 35 mm in diameter and incubated in humidified atmosphere of 5% CO_2 at 37° for 2 hr. The cell monolayers were washed three times with 4 mL of RPMI 1640 medium to remove nonadherent cells, after which the macrophage monolayers were used for experiments. Verapamil or nifedipine in ethanol solution was added to the incubation medium in a volume not exceeding 5 μL/mL of medium. Equivalent amounts of ethanol were added to the control cell cultures.

Incorporation of [^{14}C]*oleate into macrophage ChE.* The cells were incubated in RPMI 1640 medium supplemented with 0.2% BSA, 2 mM L-glutathione and 0.2 mM [^{14}C]oleate–albumin complex for 18 hr at 37° [17]. Acetyl-LDL (50 μg protein/mL) or cholesterol–albumin complex (50 μg cholesterol/mL) was added to the incubation medium. The medium was then aspirated and the cells were washed four times with 2 mL of cold PBS. The cell lipids were extracted from the macrophage monolayers *in situ* by incubating with hexane–isopropyl alcohol (3:2) for 30 min at room temperature [17]. The organic solvent was collected, each monolayer was rinsed briefly with 1 mL of solvent of the same composition, and the two organic solvents were combined and evaporated under N_2. The lipids were separated on silica gel G plates using hexane/diethyl ether/acetic acid/methanol (80:20:1:1, v/v) and visualized by exposure to iodide vapors [21]. Bands corresponding to ChE were scraped, transferred to vials containing 10 mL toluene scintillator (4 g PPO, 0.3 g POPOP in 1 L of toluene) and counted in a Mark-III liquid scintillation counter. After the lipids had been extracted, the cells in the monolayer were dissolved in 1 mL of 0.2 N NaOH and protein was determined by the procedure of Lowry *et al.* [22] with BSA as a standard. Results were expressed as nmol cholesteryl [^{14}C]oleate formed per 18 hr per mg protein.

Hydrolysis of cholesteryl [^{14}C]*oleate and esterification of* [^{3}H]*cholesterol in macrophages.* To study the effect of the drugs on synthesis and hydrolysis of ChE in macrophages, the method of Brown *et al.* was used [17]. Macrophage monolayers were cultured in RPMI 1640 medium, supplemented with 2 mg/mL of human albumin, 50 μg/mL of acetyl-LDL, and 0.2 mM [^{14}C]oleate–albumin complex at 37° for 24 hr. Afterwards, the monolayers were washed and incubated for 24 hr with the same medium in the absence of lipoprotein. On day 2 each monolayer was washed and triplicate monolayers were harvested to determine the cellular contents of Ch-[^{14}C]oleate (zero time). The remaining monolayers were incubated with 1 mL of RPMI 1640 medium containing 1 mg/mL of human albumin and [^{3}H]Ch–albumin complex (about 10^6 cpm/mL) in the presence or absence of verapamil or nifedipine. After 18 hr incubation at 37° the triplicate monolayers were washed four times with 2 mL of cold PBS and the cellular contents of Ch-[^{14}C]oleate and [^{3}H]ChE were determined by thin layer chromotography as described above, followed by double label scintillation counting according to the STD 5 program (Tracor Analytic). Data were corrected for the spill-over between isotope channels. Results were expressed as cpm of Ch-[^{14}C]oleate and [^{3}H]ChE per mg of cellular protein per 18 hr.

Excretion of [^{3}H]*Ch from macrophages.* Macrophages were incubated with RPMI 1640 medium containing 0.2% human albumin and [^{3}H]Ch conjugated with acetyl-LDL (50 μg protein/mL and 1,000,000–840,000 cpm/mL) for 18 hr. Then cells were washed twice and incubated for the next 18 hr in the absence of lipoprotein with the same culture medium. Triplicate monolayers were then washed with PBS and harvested to determine the cellular contents of free [^{3}H]Ch and esterified [^{3}H]Ch (zero time). The remaining monolayers were cultured with 1 mL of RPMI 1640 medium in the presence or absence of 20% fetal calf serum or HDL_3 (180 μg protein/mL). Verapamil or nifedipine was added to individual groups of dishes at zero time, and cell monolayers were incubated for 18 hr at 37°. Cellular

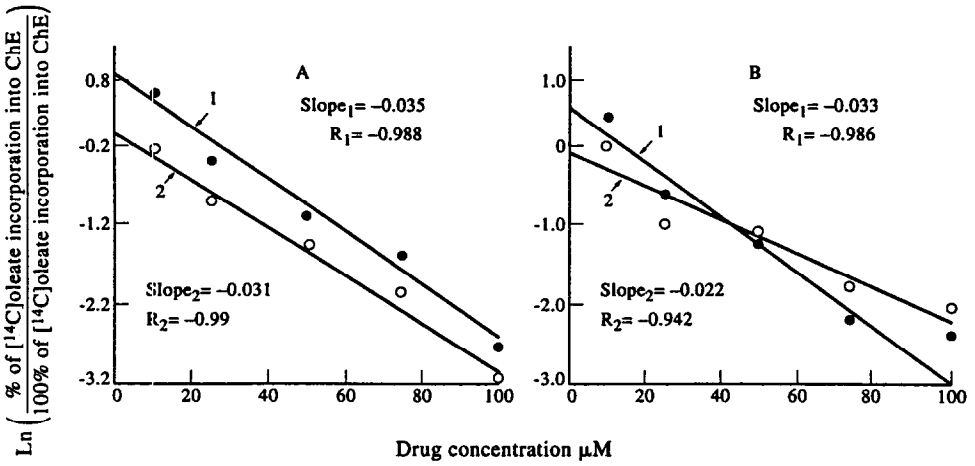


Fig. 1. Effects of verapamil (●, lines 1) and nifedipine (○, lines 2) on cholesterol esterification in cultured mouse peritoneal macrophages. Cell monolayers were incubated for 18 hr with [^{14}C]oleate and 50 μg/mL of acetyl-LDL (A) or 50 μg/mL of FCh-emulsion (B), and radioactivity in ChE was measured as described in Materials and Methods. The incorporation of [^{14}C]oleate into cellular ChE is plotted on the natural $\log(R/100 - R)$ scale vs drug concentration increase, where R is the mean % value of [^{14}C]ChE compared to control values without drug treatment. The contents of ChE formed in controls (100%) was 224 ± 18 nmol/mg/18 hr and 275 ± 23 nmol/mg/18 hr for acetyl-LDL and FCh, respectively. Mean values were obtained from groups of six cultures, each assayed in triplicate. R_1 and R_2 are the correlation coefficients for verapamil and nifedipine, respectively. P values for verapamil were <0.002 (A) and <0.001 (B), and for nifedipine were <0.005 (A) and <0.02 (B).

esterified [^{3}H]Ch and free [^{3}H]Ch were extracted and separated by thin layer chromatography as described above. FCh was extracted from the culture medium with chloroform–methanol (1:2, v/v) according to the method of Bligh and Dyer [23]. Results were expressed as nmol of [^{3}H]Ch per mg of protein per 18 hr.

Assay of LDL lipid oxidation in cultured macrophages. Macrophages were cultured in 12-well tissue culture plates at densities of 10^6 cells/well with RPMI 1640 medium (1 mL/well) containing LDL at a concentration of 250 μg cholesterol/mL. Incubations were carried out at 37° in a humidified atmosphere of 5% CO_2/95% air for 24 hr unless otherwise specified. The presence of oxidation products in culture medium was determined by the method of Schuh *et al.* [24] that detects MDA and MDA-like compounds which react with TBA. Although it is not specific for MDA this method is widely used as a reliable index of lipid oxidation. Components that react with TBA are referred to as TBARS. Briefly, 1 mL of 25% TCA was added to 400 μL samples, followed by 1 mL of 1% TBA. The samples were then vortexed and incubated for 45 min at 95° and centrifuged (1000 *g*) for 15 min. Supernatant TBARS were detected by fluorescence at 515 nm excitation and 553 nm emission using 1,1,3,3-tetramethoxypropane (Sigma) as standards (0–10 nmol). Data were expressed in terms of MDA equivalents (nmol MDA/mL of sample).

Nitroblue tetrazolium reduction test. NBT is thought to act as an unspecific electron acceptor in biochemical pathways activated during the oxidative burst [25]; therefore, the NBT test is widely used as a measure of this oxidative burst [26]. The NBT dye reduction was determined as described by Rook *et al.* [27]. Briefly, the incubation medium, consisting of Dulbecco's medium containing 1 mg/mL NBT and 5 mM glucose, was added to macrophage monolayers for 30 min at 37°, 100% humidity, 5% CO_2. After incubation the wells were washed three times with methanol and dried at 37°. The reduced NBT was extracted for 1 hr with dimethyl sulfoxide after the addition of 500 μL 2 M KOH. The contents of the wells were mixed with a pipette and the optical density was measured at 630 nm.

Cellular Ch content determination. Adherent macrophages were lipid extracted *in situ* [17]. Aliquots of the lipids extracted were used for the enzymatic fluorometric determination for unesterified and total Ch by the method adopted for culture cells [28]. The protein contents of lipoproteins were determined using the method of Lowry *et al.* [22].

Statistical analysis. Dose–response dependences were analysed with the statistical graphics system software "Statgraphics" (STSC Inc., Statistical Graphics Corp.) by regression analysis methods (logit transform or simple linear regression) so that the data were plotted on a natural log (mean value expressed as % of control /100% − mean value expressed as % of control) scale or on a natural log (mean value) scale, respectively, vs drug concentration increase. The increase or decrease of response values with increasing drug dose was considered significant if the slope was statistically different from zero ($P < 0.05$), and the correlation coefficient was calculated. Mean values were

Table 1. Effect of verapamil and nifedipine on total (TCh) and free cholesterol (FCh) contents in macrophages incubated in the presence of acetyl-LDL

Condition of incubation	Concentration of drugs (μm)	Content Ch (μg/mg protein) FCh	TCh
Nonlipid medium		24.61 ± 1.1	25.05 ± 1.19
Acetyl-LDL		33.51 ± 1.1	82.95 ± 0.64
Acetyl-LDL + verapamil	10	34.8 ± 1.65	86.02 ± 1.93
	25	39.77 ± 1.15*	85.75 ± 1.63
	50	53.54 ± 3.2*	87.4 ± 1.79
	75	55.28 ± 2.62**	85.59 ± 1.7
Acetyl-LDL + nifedipine	10	38.32 ± 1.45*	84.93 ± 1.83
	25	40.04 ± 1.53*	86.14 ± 1.66
	50	54.92 ± 1.73**	84.02 ± 1.2
	75	59.88 ± 1.59**	75.54 ± 1.96

Cultured mouse macrophages were incubated for 24 hr with or without verapamil and nifedipine in the presence of 50 μg/mL acetyl-LDL. The cellular content of free and total cholesterol was measured by enzymatic procedure as described in Materials and Methods. Results are given as the mean and SEM of six cultures, each assayed in triplicate.

* $P < 0.05$, ** $P < 0.002$ (compared to controls incubated with acetyl-LDL without drug treatment).

obtained from the groups, with sizes equal to six separate culture dishes, assayed at least in duplicate. The Student's *t*-test was used to evaluate the data shown in the table, where the dose–response dependence was not analysed.

RESULTS

[^{14}C]*Oleate incorporation into ChE and Ch content in macrophages cultured in the presence of acetyl-LDL and FCh*

The culturing of macrophages for 18 hr in lipid-deprived medium, containing 50 μg of protein/mL of acetyl-LDL or 50 μg/mL of FCh, resulted in a sharp acceleration of [^{14}C]oleate incorporation into cellular ChE (8.0 ± 0.5; 224.4 ± 8.3 and 275.3 ± 10.0 nM of esterificated oleate/mg protein in control, in the presence of acetyl-LDL and FCh, respectively). The addition of nifedipine or verapamil into the culture medium decreased [^{14}C]oleate incorporation in a dose-dependent fashion (Fig. 1). Analysis of the ChE cpm data by linear regression method (logit transform) revealed a significant reduction of ChE synthesis response with increasing dose (10–100 μM) of both nifedipine and verapamil in macrophages cultured with acetyl-LDL- (Fig. 1A) and FCh- (Fig. 1B) supplied media. The differences between nifedipine and verapamil effects were found to be nonsignificant.

At the same time, verapamil and nifedipine increased FCh contents in macrophages cultured in the presence of acetyl-LDL, whereas the addition of the drugs to acetyl-LDL-containing medium had no effect upon total Ch intracellular accumulation (Table 1). These results testify that the calcium blockers under investigation prevent ChE accumulation in macrophages.

Liberation of [^{14}C]*oleate from ChE and* [^{3}H]*FCh incorporation into ChE*

Studying the influence of calcium blockers upon ChE hydrolysis and formation we applied the method enabling simultaneous registration of both [^{14}C]-oleate liberation from stored Ch[^{14}C]oleate and [^{3}H]FCh incorporation into ChE [17]. Macrophage culturing in acetyl-LDL and [^{14}C] oleate-containing medium for 18 hr resulted in labelled oleate incorporation into ChE (1.8–2 × 10^6 cpm/mg of cell protein). After replacement of the culture medium with lipid-deprived [^{3}H]FCh-containing medium the cells were cultured for 18 hr in the presence of verapamil or nifedipine at various concentrations (from 25 to 100 μM). It was established that under these conditions nifedipine and verapamil caused the acceleration of [^{14}C]oleate liberation from Ch-[^{14}C]oleate (Fig. 2B) and the lowering of the [^{3}H]-FCh esterification rate (Fig. 2A). Along with the growth of drug concentration the gradual reductions of specific radioactivity of [^{14}C]oleate and [^{3}H]FCh in ChE were observed so that simple linear regression analysis revealed significant dose–response dependences. This reduction testified to verapamil's and nifedipine's ability to inhibit FCh esterification and to stimulate ChE hydrolysis in macrophages. It is noteworthy that nifedipine was more effective than verapamil in [^{14}C]oleate liberation from macrophage ChE ($P < 0.05$).

Excretion of [^{3}H]*Ch from macrophages, cultured in the presence of serum and* HDL_3

To investigate the effect of verapamil and nifedipine upon Ch excretion macrophages were previously cultured for 24 hr in the medium containing [^{3}H]FCh conjugated with acetyl-LDL. The culture resulted in acetyl-LDL-derived Ch incorporation into cellular ChE and FCh (319.5 and 180.4 nmol of Ch per mg of cellular protein, respectively). During the following 18 hr culture in the presence of 0.2% BSA, cells excreted only 2–3 nmol [^{3}H]Ch/mg of protein into the medium. The addition of 20% FCS or 180 μg/mL HDL_3, which are able to accept cellular Ch, caused an increase in

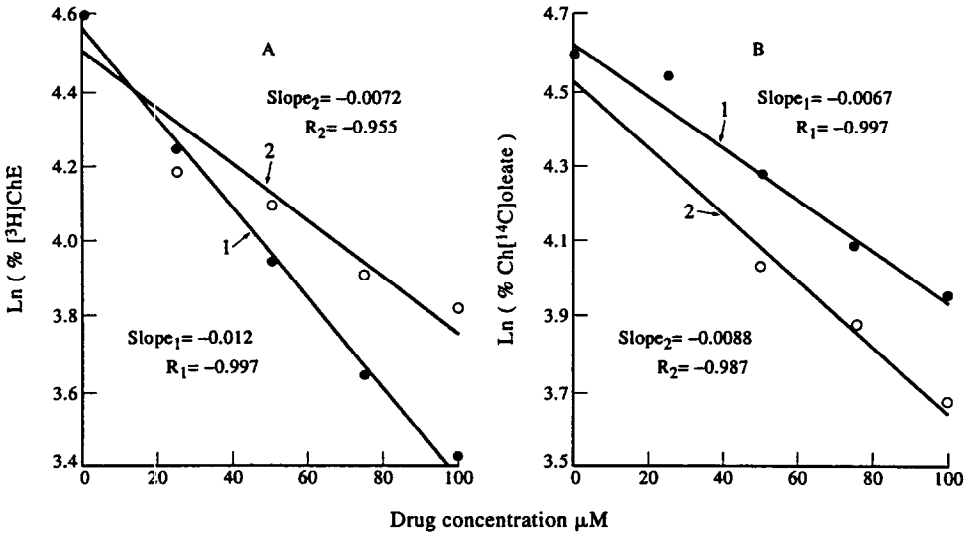


Fig. 2. Effect of verapamil (●, lines 1) and nifedipine (○, lines 2) on the incorporation of [^{3}H]Ch into ChE (A) and on hydrolysis of stored Ch-[^{14}C]oleate (B). Cultured macrophages were incubated with [^{14}C]oleate in the presence of 50 μg/mL of acetyl-LDL for preparation of stored Ch-[^{14}C]oleate as described in Materials and Methods. The monolayers were then washed and incubated for 24 hr with the same medium in the absence of acetyl-LDL. The cells were washed and incubated with or without nifedipine and verapamil for 18 hr in the presence of [^{3}H]Ch. The content of cellular Ch-[^{14}C]oleate and [^{3}H]ChE were determined by double label scintillation counting. Results are plotted as simple regression lines on the natural log (mean % value of [^{3}H]ChE cpm) (A) or on the natural log (mean % value of Ch-[^{14}C]oleate cpm) (B) scales vs drug concentration increase. Controls were the data obtained without drug treatment. The mean contents of ChE in control (100%) were $0.42 \times 10^6 \pm 0.019 \times 10^6$ and $1.2 \times 10^6 \pm 0.09 \times 10^6$ cpm/mg/18 hr for [^{3}H]ChE and Ch-[^{14}C]oleate, respectively. Mean values were obtained from groups of six cultures, each assayed in duplicate. P values for verapamil were <0.001 (A) and <0.001 (B), and for nifedipine were <0.01 (A) and <0.01 (B).

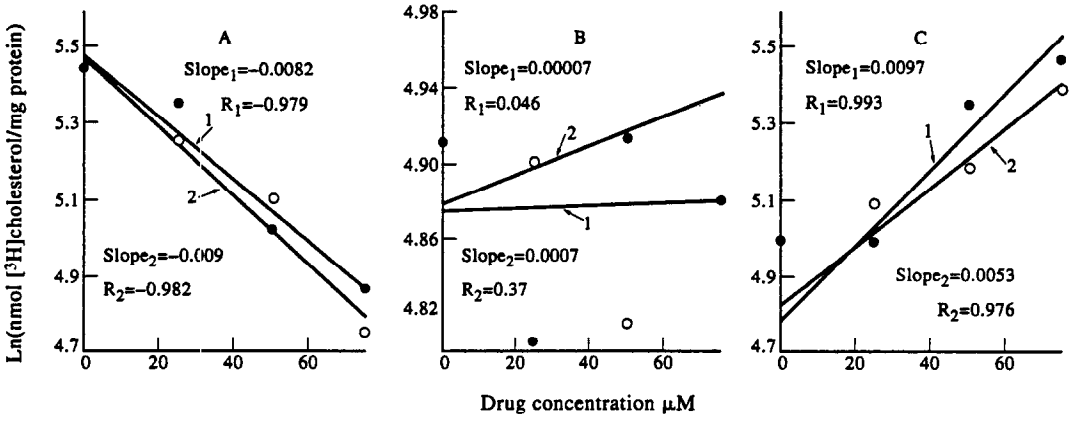


Fig. 3. Effects of verapamil (●, lines 1) and nifedipine (○, lines 2) on excretion of [^{3}H]Ch from macrophages in the presence of 20% FCS. Cultured macrophages were incubated for 24 hr with acetyl-LDL containing [^{3}H]Ch, washed, and incubated for a further 24 hr without lipoproteins, according to the standard procedure. The cells were then washed, and macrophages were incubated in the presence of 20% FCS with or without verapamil or nifedipine for 24 hr. The contents of cellular [^{3}H]Ch (A), [^{3}H]ChE (B), and excreted [^{3}H]Ch in cultured medium (C) were determined as described in Materials and Methods. Results are plotted as simple regression lines on the natural log (mean nmol values of [^{3}H]Ch/mg protein/24 hr) scale vs drug concentration increase. Mean values were obtained from groups of six cultures, each assayed in duplicate. Controls were the cultures without drug treatment. P values for verapamil were <0.05 (A), =0.95 (B), and <0.01 (C), and for nifedipine were <0.02 (A), =0.68 (B), and <0.05 (C).

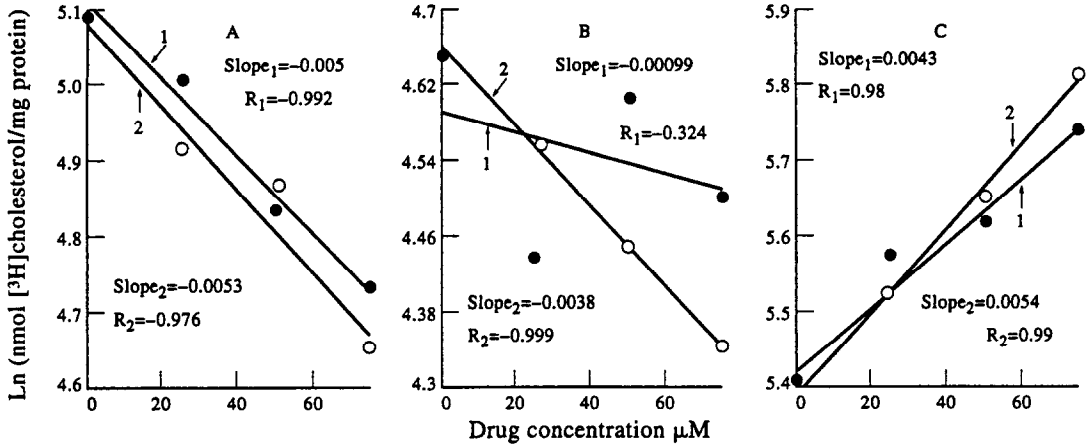


Fig. 4. Effect of verapamil (●, lines 1) and nifedipine (○, lines 2) on excretion of [^{3}H]Ch from macrophages in the presence of HDL_3. Macrophages were stored with [^{3}H]ChE for 24 hr, washed, and incubated with verapamil and nifedipine in the presence of 180 mg/ml of HDL_3, according to the standard procedure. The contents of cellular [^{3}H]Ch (A) and [^{3}H]ChE (B), and excreted [^{3}H]Ch in cultured medium (C) were determined as described in Materials and Methods. Results are plotted as simple regression lines on the natural log (mean nmol values of [^{3}H]Ch/mg protein/24 hr) scale vs drug concentration increase. Mean values were obtained from groups of six cultures, each assayed in duplicate measurements. Controls were cultures without drug treatment. P values for verapamil were <0.01 (A), =0.69 (B), <0.02 (C), and for nifedipine were <0.05 (A), <0.001 (B), and <0.001 (C).

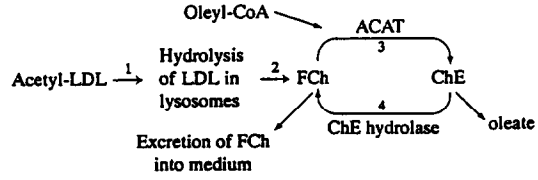
[^{3}H]Ch excretion from cells into the medium up to 40.8 and 79.2 nmol [^{3}H]Ch/mg protein, respectively. The linear regression analysis of the data has demonstrated that the addition of verapamil and nifedipine into fetal calf serum (Fig. 3) or HDL_3-containing medium (Fig. 4) caused a significant decrease of [^{3}H]Ch concentration in cellular ChE (Fig. 3A, 4A) and the increase of [^{3}H]FCh concentration in the culture medium (Figs 3C, 4C) in a dose-dependent fashion. At the same time, at concentrations from 25 to 75 μM the drugs investigated had no dose-dependent effect upon the value of [^{3}H]FCh radioactivity in macrophages cultured during 18 hr in the presence of 20% serum (Fig. 3B). In the presence of HDL_3, verapamil induced no significant changes in [^{3}H]FCh radioactivity in macrophages, whereas nifedipine decreased its level in a dose-dependent manner (Fig. 4B). The results obtained testify that under the influence of verapamil and nifedipine, [^{3}H]FCh excretion from macrophages into Ch acceptor-containing medium increases mainly because of the removal of the label from cellular ChE.

Oxidative modification of LDL and nitroblue tetrazolium dye reduction

Verapamil and nifedipine were tested for their ability to attenuate LDL oxidation and NBT reduction in cultures of elicited peritoneal macrophages. As can be seen in Fig. 5, when macrophages were incubated for 18 hr in the presence of the drugs the decrease of the level of TBARS in LDL was observed in dose-dependent manner. Nifedipine (25–100 μM) inhibited the LDL oxidation by 50–90% as compared to control TBARS level in LDL-containing medium. The effect of 75 μM nifedipine was similar to that of 5 μM BHT, a standard antioxidative agent. At the same concentrations verapamil reduced the LDL oxidation by 33–66%. Nifedipine and verapamil protection from macrophage-induced LDL oxidation was accompanied by the significant dose-dependent decrease of the NBT reduction. Concurrent dose-dependent depression of LDL oxidation and NBT reduction were markedly consistent with each other. Therefore, the drugs investigated appear to protect against macrophage LDL oxidation via the depression of the production of reactive oxygen species.

DISCUSSION

As shown earlier in a large number of experiments with rabbits [4–7], verapamil and nifedipine have a considerable protective effect against atherosclerosis development. In recent studies of macrophage-derived foam cell formation *in vitro* verapamil and nifedipine were found to reduce the rate of Ch esterification [11] and to accelerate Ch efflux from cholesterol-overloaded cells [29]. The data obtained demonstrate that nifedipine and verapamil anti-atherogenicity may be connected with their effects upon ChE metabolism. According to the two-compartment model of Brown and Goldstein [15] ChE metabolism in macrophages can be outlined in the following conventional scheme:



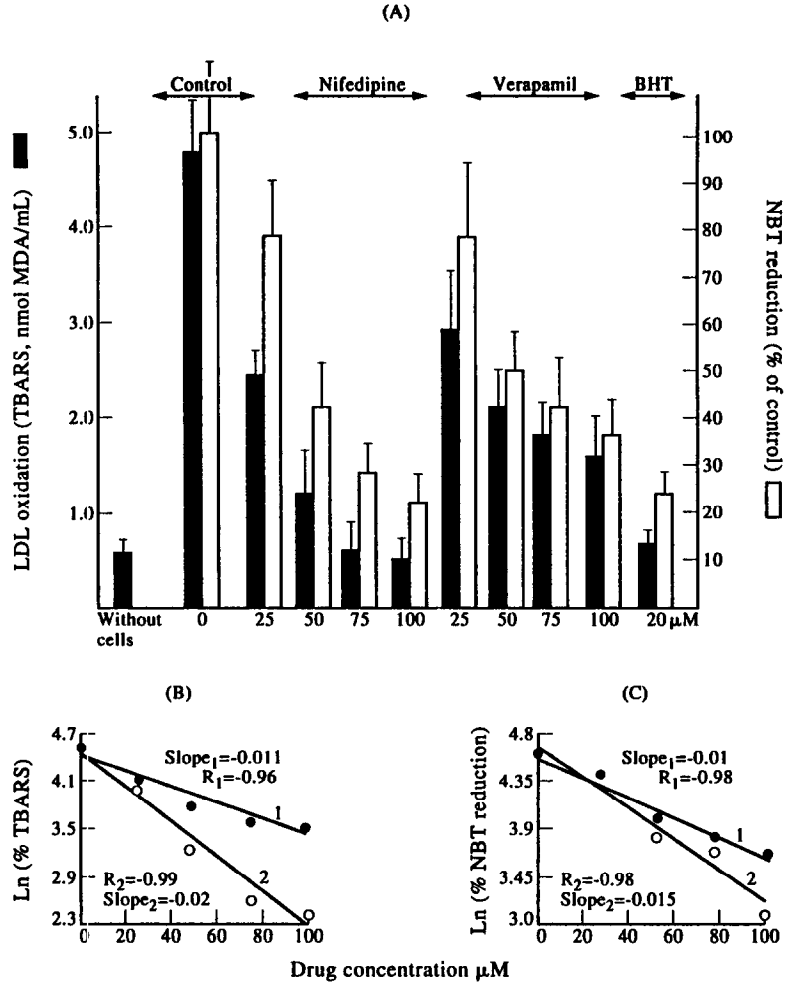


Fig. 5. Effect of verapamil and nifedipine on macrophage LDL oxidation and NBT reduction. The cultured cells were incubated with 250 μg/mL LDL and with or without verapamil, nifedipine and butylated hydroxytoluene (BHT). LDL oxidation and NBT reduction were determined as described in Materials and Methods. (A) The results of LDL oxidation (dark columns) are given as mean and SD of TBARS and expressed in nmol of MDA equivalents/mL of LDL-containing medium. The results of NBT reduction (clear columns) are given as mean and SD of extracted reducted NBT dye and expressed in percentage compared to controls without treatment. The mean optical density of the controls was 0.458 ± 0.129. Each value is the mean of six cultures, each assayed in quadruplicate. The dose–response dependences for verapamil (●, lines 1) and nifedipine (○, lines 2) are demonstrated in (B) and (C) as simple regression lines and the results are plotted on the natural log (mean nmol values of MDA expressed as % vs controls) scale (B) and on the natural log (mean values of % of reduced NBT vs controls) scale (C) vs drug concentration increase. P values for verapamil were <0.01 (B) and <0.005 (C), and for nifedipine were <0.002 (B) and <0.005 (C).

Following this scheme, receptor-mediated transfer of acetyl-LDL into macrophage lysosomes, where the hydrolysis of ChE and liberation of FCh into the cytoplasm (stages 1 and 2) take place, is associated with the sharp enhancement of acyl-CoA-cholesterol-*O*-acyltransferase (ACAT) activity. This enzyme catalyses Ch esterification (stage 3). Our results on the verapamil- and nifedipine-induced decrease of [^{14}C]oleate incorporation into ChE might enable us to assume an impairment of acetyl-LDL transfer into cells. None the less, as previously demonstrated by Yatsu *et al.* [10] verapamil does not reduce the rate of labelled acetyl-LDL degradation in monocyte-derived macrophages. This implies that acetyl-LDL uptake by cells is not impaired. Our data on constant Ch contents in macrophages, cultured in the presence of nifedipine or verapamil with acetyl-LDL, also contradict the assumption of acetyl-LDL transfer impairment. The same holds true for the data on the decrease of [^{14}C]oleate incorporation into ChE of macrophages, cultured with FCh, which accelerate FCh esterification independently of acetyl-LDL uptake. Thus, the effects of verapamil and nifedipine are most likely to be mediated through the inhibition of FCh esterification (stage 3) and ChE hydrolysis enchancement (stage 4). Indeed, we demonstrated

that verapamil and nifedipine both inhibit [^{3}H]FCh incorporation into ChE and stimulate [^{14}C]oleate liberation from ChE. Apparently, precluded ChE accumulation may in turn cause the stimulation of Ch excretion (stage 5), as we observed in macrophages cultured in the presence of serum or HDL_3. This conclusion is consistent with the opinion of Etingen and Hajjar [9, 30], who demonstrated that cytoplasmic ChE hydrolase activation suppresses excessive ChE accumulation. ChE hydrolase activation, under the action of calcium blockers, is supposed to be connected with their ability to inhibit phosphodiesterases and to increase the intracellular cAMP level [8].

Oxidative modification of LDL by blood monocyte-derived macrophages is presently considered to be of great importance for the initiation of atheroma development [12]. Macrophages are generally accepted to be precursors of foam cells, the formation of which signifies the beginning of atheroma lesion development [15]. Scavenger receptor-mediated binding and uptake of oxidized LDL is a key event of foam cell formation [12]. A mechanism of LDL oxidation by monocytes and macrophages is closely connected with their ability to generate reactive oxidants during respiratory burst [31]. Ca^{2+}-ionophores are known to induce reactive oxygen species generation by mononuclear phagocytes [13]. As this study has demonstrated, the calcium blockers verapamil and nifedipine efficiently inhibited NBT reduction in elicited macrophage cultures. At the same time, μM concentrations of the drugs inhibited the oxidation of LDL, when the latter were incubated for 18 hr with macrophage cultures. As shown earlier in the *in vitro* model of myocyte sarcolemmal membrane lipid peroxidation, nifedipine and verapamil display antioxidant activities [32]. Under this, nifedipine was a more efficient antioxidant than verapamil. In our present experiments with macrophage cultures, nifedipine was also the more efficient protector against LDL oxidation.

The mechanisms of the LDL oxidation inhibition remain unclear. It might be suggested that, together with the direct antioxidant effects demonstrated in the membrane model system [32], the calcium blockers inhibit calcium influx into macrophages and suppress LDL oxidation via the decrease in reactive oxidant species generation and/or through the inhibition of lipooxygenase, which was shown to be of significance for LDL oxidation in macrophages [33].

Our data therefore allow one to assume that the inhibition of LDL oxidation, reduction of FCh esterification rate, ChE hydrolysis stimulation and, as a consequence, the increase of Ch excretion from cells, are likely to be the mechanism of verapamil's and nifedipine's antiatherogenicity.

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