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EFFECT OF CALCIUM-CHANNEL-BLOCKING DRUGS ON LYSOSOMAL FUNCTION IN HUMAN SKIN FIBROBLASTS

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Abstract—Recent reports suggest that certain Ca^{2+}-channel-blocking drugs reduce the severity of atherosclerosis in cholesterol-fed animals. To determine whether the suppression of atherogenesis is related to altered lipoprotein metabolism, we have assessed the effects of these drugs on the catabolism of plasma low density lipoproteins (LDL) by human skin fibroblasts. The Ca^{2+}-channel-blocking drugs verapamil and diltiazem inhibit the lysosomal degradation of LDL by these cells; degradation of epidermal growth factor was also inhibited by the same drugs, suggesting a general effect of these drugs on lysosomal function. In contrast, nifedipine did not affect the degradation of LDL or epidermal growth factor. None of the drugs affected phospholipid or protein synthesis. Entry of LDL into the lysosomes also was not affected. [^{3}H]Diltiazem, which inhibited LDL degradation, accumulated in the lysosome-rich fraction, whereas [^{3}H]nimodipine, a drug structurally and functionally similar to nifedipine, did not accumulate. We suggest that the inhibitory effect of some of the Ca^{2+}-channel-blocking drugs on lysosomal function is due to their basic nature, causing them to accumulate in lysosomes, thereby increasing intralysosomal pH.

A number of drugs that inhibit the influx of Ca^{2+} into cells or affect the release and binding of intracellular Ca^{2+} are used in the treatment of ischemic heart disease [see Refs. 1 and 2, for review]. Recent reports suggest that some of these drugs may also be effective in preventing the development of atherosclerosis in cholesterol-fed animals [3–7]. Kramsch *et al.* [3, 4] reported that treatment of cholesterol-fed monkeys with inhibitors of arterial calcium deposition (diphosphonates) or a Ca^{2+} antagonist (lanthanum) markedly decreased the formation of fibrous plaques without decreasing plasma cholesterol levels. A reduction in atherogenesis was also obtained in cholesterol-fed rabbits treated with the Ca^{2+}-channel-blocking drugs nifedipine, verapamil and diltiazem [5–7].

To determine if Ca^{2+}-channel-blocking drugs interfere with the metabolism of plasma low density lipoproteins (LDL), a class of lipoproteins which correlates positively with coronary heart disease [8], we have studied the effects of these drugs on the binding, internalization and degradation of LDL in human skin fibroblasts. In an earlier report we showed that some of the Ca^{2+}-channel-blocking drugs inhibited the degradation of LDL and thereby prevented the LDL-mediated suppression of cholesterol synthesis [9]. The results of the present study extend these earlier findings and show that the inhibition of LDL degradation by these drugs is not a specific phenomenon but is due to a general inhibition of lysosomal function.

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MATERIALS AND METHODS

Chemicals. [^{125}I]Sodium iodide (carrier-free), [U-^{14}C]leucine (340 Ci/mole), [^{125}I]epidermal growth factor (200 μCi/μg) and [^{3}H]nimodipine (150 Ci/mmole) were obtained from the New England Nuclear Corp., Boston, MA. Carrier-free [^{32}P]-phosphoric acid was obtained from ICN Chemicals and Radioisotopes, Irvine, CA. [U-^{14}C]Sucrose (555 Ci/mole) was purchased from Amersham Radiochemicals, Arlington Heights, IL. [^{3}H]Diltiazem (112 Ci/mmole) was obtained from the Tanabe Pharmaceutical Co., Osaka, Japan. Eagle's Minimum Essential Medium, trypsin, fetal bovine serum and all chemicals for tissue culture medium were obtained from the Grand Island Biological Co., Grand Island, NY.

Drugs. The drugs used were obtained from the following sources: verapamil, Knoll Pharmaceuticals, Whippany, NJ; fendiline, Choinin Pharmaceuticals, Budapest, Hungary; diltiazem, Marion Research Laboratories, Kansas City, MO; nifedipine, Pfizer Pharmaceuticals, New York, NY; and chloroquine, Sigma Chemical Co., St. Louis, MO.

Cells. Human skin fibroblasts, GM 0043, were obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ; the cells were originally derived from a normal individual. The cells were grown in monolayers as described earlier [10], except that fetal bovine serum was used instead of newborn calf serum.

Lipoproteins. Human LDL (d = 1.02 to 1.05 g/ml) were isolated by ultracentrifugation in KBr solutions as described previously [11]. Iodination of LDL using iodine monochloride was carried out according to

the method of Bilheimer *et al.* [12]. The specific activity of iodinated LDL was 1000 dpm/ng protein. [^{14}C]Sucrose-labeled LDL were prepared from [U-^{14}C]sucrose and LDL using the method of Pittman *et al.* [13] as modified by Bigelow *et al.* [14]. The specific activity was 10,000 dpm/μg protein.

Subcellular fractionation. Cell monolayers were washed with Dulbecco's phosphate-buffered saline three times, and the cells were scraped with a rubber policeman and suspended in 0.25 M sucrose solution adjusted to pH 7.0. The cells were homogenized in a Potter–Elvehjem type homogenizer, with a motor-driven Teflon pestle, until most of the cells were disrupted as judged under the microscope. Generally, 5 ml of the sucrose medium was used to suspend the cells from a 100 mm culture dish (1–2 mg protein). The homogenate was centrifuged at 600 *g* for 10 min in a Beckman J21B centrifuge to sediment the cell debris and nuclear fraction. The supernatant fraction was then centrifuged at 8000 *g* for 10 min to isolate the lysosome-rich fraction. The pellet was suspended in a small volume and used for the assay of lysosomal enzyme activity and for the determination of radioactivity.

Assay of lysosomal enzymes. β-Glucosidase and *N*-acetyl galactosaminidase were determined fluorometrically using 4-methyl umbelliferyl derivatives of glucose and *N*-acetyl galactosamine, respectively, as substrates. The assay mixture contained 0.2 mM substrate, 50 mM acetate buffer (pH 5.0), 0.1% Triton X-100 and 5–10 μg of lysosomal protein in a total volume of 0.2 ml. After incubation for 30 min at 37°, the reaction was terminated by the addition of 3 ml of 50 mM glycine–NaOH solution (pH 10.4). The product formed with both substrates was determined by measuring the fluorescence at 460 nm with excitation at 360 nm [15].

Determination of cholesterol. Total lipids were extracted directly from the monolayers using 6 ml of hexane–isopropanol (3:2, v/v) [16]. After evaporating the solvent, cholesterol and cholesteryl esters were separated by thin-layer chromatography on silica gel using heptane–diethyl ether–acetic acid (90:10:1, by vol.) as the developing solvent [17]. The bands containing cholesterol and cholesteryl esters were scraped out, and the compounds were eluted with chloroform–methanol (1:1, v/v). The sample containing cholesteryl esters was saponified in ethanolic NaOH, and cholesterol was extracted into hexane. Free cholesterol and cholesteryl ester (after hydrolysis to free cholesterol by saponification) were determined by gas chromatography using stigmasterol as internal standard. A Perkin–Elmer model 900 Gas Chromatograph with flame ionization detector was used. The sterols were fractionated at 250° in a 1.8 m long (2 mm i.d.) silanized glass column packed with 3% OV-17 on 100/120 mesh Gaschrom Q. Nitrogen was used as carrier gas with a flow rate of 40 ml/min. The injector and detector temperatures were 300° and 310° respectively.

RESULTS

Since our previous study [9] showed that fendiline, verapamil, and diltiazem inhibit LDL degradation in human skin fibroblasts, it was of interest to assess the general effects of these Ca^{2+}-channel-blockers on the biosynthesis of phospholipids and proteins. As shown in Table 1, the Ca^{2+}-channel-blocking drugs at the concentrations tested had negligible effects on the incorporation of [^{14}C]leucine into protein. Diltiazem caused a 30% inhibition of phospholipid synthesis. However, it seems unlikely that this inhibition was related to the inhibition of LDL degradation since fendiline and verapamil were more

Table 1. Effect of Ca^{2+}-channel-blocking drugs on phospholipid and protein synthesis in human skin fibroblasts*

Additions	[^{32}P]Phosphate incorporated into phospholipids (cpm/mg cell protein × 10^{-3})	[U-^{14}C]leucine incorporated into protein (cpm/mg cell protein × 10^{-3})
None	745	202
Fendiline (15 μM)	870	223
Verapamil (50 μM)	705	214
Diltiazem (100 μM)	500	216

*Human skin fibroblasts were seeded in 35 mm culture dishes (4–5 × 10^4 cells/dish) and grown for 5 days as described in Materials and Methods. The cells were incubated with the indicated drugs for 24 hr and then pulsed with [^{32}P]phosphoric acid (20 μCi/dish) for 6 hr or with [U-^{14}C]leucine (0.5 μCi/dish) for 4 hr. After pulse labeling, the incorporation into phospholipids was determined as follows. The cells were washed four times with 0.9% NaCl and then solubilized with 0.05% sodium dodecyl sulfate; total lipids were extracted with chloroform–methanol (2:1) [18], and the radioactivity was determined. To determine leucine incorporation into protein, the cells were washed with 0.9% NaCl and lysed with 0.1 M NaOH. Trichloroacetic acid was added to a final concentration of 10% and, after 2 hr at room temperature, the precipitates were collected by centrifugation, washed three times with 5% trichloroacetic acid, and radioactivity in the precipitate was determined. The results represent mean values of duplicate determinations. The variations between duplicate values were less than 15%.

Table 2. Effect of verapamil on the uptake of [^{14}C]sucrose-labeled LDL by fibroblasts*

	[^{14}C]Sucrose-labeled LDL (cpm)				
	In cells		In lysosome-rich fraction		
Additions	Total	Per mg protein	Total	Per mg protein	% in Lysosomes
None	7,070	8,850	2,070	27,700	29.2
Verapamil (50 μM)	10,800	15,950	3,000	38,420	27.7

*Human skin fibroblasts were grown in 100 mm culture dishes for 4 days and then incubated in a medium containing lipoprotein-deficient serum for 10 hr. Verapamil (50 μM) was added and the incubation was continued for 24 hr. [^{14}C]Sucrose-labeled LDL (10 μg/ml) was then added and, after 14 hr of incubation, the cells were washed and radioactivity was determined in the total cell homogenates and the isolated lysosome-rich fraction. The results represent mean value of duplicate determinations. The variation between the duplicate values was less than 15%.

effective than diltiazem in inhibiting LDL degradation [9] and yet were ineffective in inhibiting phospholipid synthesis.

Recent studies by Altstiel and Branton [19] suggest that Ca^{2+} at micromolar concentration is involved in the fusion of endocytic vesicles and lysosomes. This finding raises the possibility that the Ca^{2+}-channel-blockers may inhibit the delivery of internalized LDL to the lysosome. To assess this possibility, we used [U-^{14}C]sucrose covalently linked to LDL as described by Pittman *et al.* [13]. The advantage of this radiolabeled compound is that when sucrose-labeled LDL enters the lysosome and is subsequently degraded, the [^{14}C]sucrose is trapped in the lysosome since the lysosomal membrane is not permeable to sucrose. The results shown in Table 2 indicate that about 30% of [^{14}C]sucrose-labeled LDL that was taken up by the cells was present in the isolated lysosome-rich fraction either in the presence or in the absence of verapamil, suggesting that the drug did not inhibit the delivery of LDL to the lysosome.

The Ca^{2+}-channel-blocking drugs did not appear to have a direct effect on the lysosomal enzyme-mediated degradation of LDL. As shown in Table 3, fendiline, verapamil and diltiazem, at the concentrations indicated, had negligible effects on the *in vitro* degradation of [^{125}I]LDL at pH 4.1, which is optimum for lysosomal enzyme activities.

To determine if the Ca^{2+}-channel-blocking drugs have a general effect on lysosomal function, their effects on the degradation of epidermal growth factor, a process known to occur in lysosomes [21], was assessed. Chloroquine was also included in this experiment since it is well known that this compound accumulates in lysosomes and inhibits enzyme activities by altering pH [22]. The data in Table 4 show that chloroquine and verapamil at the concentrations tested inhibited the degradation of epidermal growth factor by 58% and 42% respectively. On the other hand, nifedipine had only a limited effect, which is consistent with our previous finding [9] on its lack of effect on LDL degradation.

If verapamil and diltiazem have a general effect on lysosomal function, as does chloroquine, then in the presence of LDL there should be an accumulation of cellular cholesteryl esters. The data presented in Table 5 indicate that there was a 10- to 20-fold increase in cellular cholesteryl esters in the presence of chloroquine, verapamil and diltiazem. There was also a net increase in unesterified cholesterol. However, nifedipine had no effect on the cholesterol content of the cells.

Since the Ca^{2+}-channel-blocking drugs that are effective in inhibiting lysosomal functions are weak bases, it is possible that they accumulate in the lysosomes and raise the pH, as occurs with chloroquine. To test this possibility, we determined the uptake of [^{3}H]diltiazem and its distribution in the cells. The results presented in Table 6 show that the amount of radioactivity was the highest in the fraction in which the lysosomal enzyme activity was maximum, suggesting that the drug was accumulating in the lysosomes; about 30% of the drug taken up by the cells was present in the fraction rich in lysosomes. When the cellular uptake and distribution of [^{3}H]-nimodipine, a drug structurally similar to nifedipine, were determined, only 7% of the [^{3}H]nimodipine was localized in the lysosome-rich fraction (Table 6);

Table 3. Effect of Ca^{2+}-channel-blocking drugs on LDL degradation *in vitro**

Addition	Drug conc (μM)	[^{125}I]LDL degraded (ng/hr/mg cell protein
None		31.2
Fendiline	10	35.0
	100	38.6
Verapamil	50	32.1
	500	30.3
Diltiazem	100	26.1
	1000	25.7

*Human skin fibroblasts were grown in 100 mm dishes for 6 days. The cells were washed with phosphate-buffered saline and scraped into 2 ml of the buffer; the cell suspension was sonicated for 10–15 sec. The sonicated preparation was used to determine the rate of degradation of [^{125}I]LDL in the presence of the various drugs. The incubation mixture contained 1 μg of [^{125}I]LDL, 50 mM sodium acetate buffer (pH 4.1), 1 mM EDTA, 5 mM dithiothreitol and 120 μg of sonicated cell protein in a total volume of 0.5 ml. The incubation was carried out at 37° for 2 hr. The degradation of [^{125}I]LDL was determined according to the procedure described by Goldstein *et al.* [20].

Table 4. Effect of chloroquine and Ca^{2+}-channel-blocking drugs on the degradation of [^{125}I]epidermal growth factor (EGF) by fibroblasts*

Additions	[^{125}I]EGF cpm in cells	[^{125}I] cpm released into the medium at 4 hr	EGF % degraded
None	14,900	16,000	100
Chloroquine (10 μM)	22,700	9,500	42
Verapamil (50 μM)	21,700	12,500	58
Nifedipine (100 μM)	12,800	11,400	89

*Human skin fibroblasts were grown in 35 mm plates for 4 days and then incubated with various drugs for 24 hr. [^{125}I]Epidermal growth factor was added (0.2 μCi/ml) and incubated for 2 hr. At the end of this incubation, the cells were washed and the radioactivity in the cells was determined. In a duplicate set of dishes, the medium containing [^{125}I]epidermal growth factor was removed; the cells were rinsed four times with the medium and incubated for 4 hr at 37° in a medium containing the drugs. At the end of this period, the radioactivity released into the medium was determined; radioactivity in the medium represents degraded [^{125}I]epidermal growth factor [21]. The values represent means of duplicate experiments. The variation between the duplicate values was less than 15%.

85% of the drug was present in the soluble fraction of the cell.

DISCUSSION

Our earlier study [9] showed that some of the Ca^{2+}-channel-blocking drugs inhibit the degradation of LDL in human skin fibroblasts and, therefore, prevent the LDL-mediated suppression of choles terol synthesis. In the present report, we attempted to understand the mechanism of action of these drugs. These compounds do not appear to affect the general metabolism of cells because they do not inhibit phospholipid and protein synthesis. In addition, the drugs do not inhibit LDL degradation by cell homogenates.

Alstiel and Branton [19] have shown that calcium is necessary for the fusion of endocytic vesicles with lysosomes. One could surmise that the drugs which block the entry of calcium into cells could decrease the calcium levels in the cell to a level low enough to inhibit the process of delivery of the endocytic vesicles to the lysosome. However, this possibility seems unlikely since verapamil, a drug known to reduce the influx of calcium in cardiac and smooth muscle cells [1, 2], did not affect the entry of LDL into lysosomes.

Of all the Ca^{2+}-channel-blocking drugs tested, as reported in our previous paper [9], prenylamine and fendiline were the most effective in causing inhibition of LDL degradation; they are the least effective as Ca^{2+}-channel-blockers [23]. Dibucaine, a local anesthetic, and trifluoperazine, a calmodulin inhibitor, were also very effective at low concentrations. Nifedipine, an effective Ca^{2+}-channel-blocker, surprisingly did not affect LDL degradation even at high concentrations. We also found that *l*-diltiazem, a stereoisomer of diltiazem, was as effective as *d*-diltiazem in causing inhibition of LDL degradation (data not shown), whereas the *l*-isomer is known to be inactive as a Ca^{2+}-channel-blocker [24].

Examination of the chemical structure of all the compounds used in these experiments shows that they are all weak bases, except nifedipine. It is well

Table 5. Effects of chloroquine, varapamil, diltiazem, and nifedipine on the cellular content of unesterified cholesterol and cholesteryl ester in human skin fibroblasts*

Additions	Free cholesterol	Esterified cholesterol (μg/mg protein)	Total cholesterol
None	17.1	0.8	17.9
Chloroquine (25 μM)	20.6	14.9	35.5
Verapamil (50 μM)	30.5	7.2	37.7
Diltiazem (100 μM)	22.3	10.2	32.5
Nifedipine (100 μM)	16.7	0.7	17.4

*Human skin fibroblasts were grown in 100 mm dishes for 6 days. The drugs were then added and, after 2 days, the cells were washed with phosphate-buffered saline, and the lipids were extracted directly from the monolayer using 6 ml of hexane–isopropanol (3:2, v/v). Unesterified cholesterol and cholesteryl ester were separated by thin-layer chromatography, and lipid mass was determined by gas chromatography as described in Materials and Methods.

Table 6. Intracellular distribution of ^3H-labeled diltiazem and nimodipine*

Expt.	Drug used	Fraction	Radioactivity (cpm) Total	Radioactivity (cpm) per mg protein	Lysosomal enzyme activity (nmoles/hr/mg protein)
1	Diltiazem	Homogenate	27,000	19,780	14
		600 *g* pellet	4,210 (15%)	13,580	20
		8,000 *g* pellet	6,850 (25%)	57,080	52
		30,000 *g* pellet	4,490 (16%)	32,070	30
		30,000 *g* supt.	8,130 (29%)	10,040	<1
2	Diltiazem	Homogenate	13,000	15,390	80
		600 *g* pellet	3,120 (24%)	13,420	135
		8,000 *g* pellet	4,840 (37%)	37,810	243
		30,000 *g* pellet	1,010 (7.8%)	22,950	92
		30,000 *g* supt.	3,300 (25%)	7,050	4
3	Nimodipine	Homogenate	56,330	27,600	80
		600 *g* pellet	630 (1%)	2,610	32
		8,000 *g* pellet	4,090 (7.3%)	16,540	276
		30,000 *g* pellet	2,560 (4.5%)	18,700	127
		30,000 *g* supt.	48,110 (85%)	29,520	9

*Human skin fibroblasts grown in 100 mm plates for 6 days were used for the experiments. In Experiment 1, the cells were incubated with [^3H]diltiazem (1.2 μCi/dish, 10 μM) for 24 hr, and the activity of the lysosomal enzyme, β-glucosidase, was determined. In Experiment 2, the drug was incubated for 40 hr and the activity of the lysosomal enzyme, *N*-acetyl-galactosaminidase, was determined. In Experiment 3, the cells were incubated with [^3H]nimodipine (5 μCi/dish, 0.3 μM) for 24 hr and *N*-acetyl-galactosaminidase was measured as lysosomal marker. Other experimental details are as described in Materials and Methods. Two dishes were pooled for each experiment.

known that weak bases accumulate in lysosomes and increase the intralysosomal pH [25]. This increase in pH occurs because the free unprotonated base diffuses rapidly across the lysosomal membrane and is then trapped inside the lysosome after protonation. Such compounds are known as lysosomotropic agents, chloroquine being one of the better-known examples. Lysosomal enzymes have pH optima in the range of 3–5, and when the basic compounds accumulate in the lysosomes, the pH increases, causing decreased enzyme activities. To examine whether the drugs affect other lysosomal enzymes, we determined their effects on the degradation of epidermal growth factor. The results obtained indicate that the effects of the drugs on the degradation of epidermal growth factor were closely comparable to those on the degradation of LDL. In both cases, among the compounds tested, nifedipine was ineffective, verapamil was most effective, and diltiazem showed some effect. When we studied the uptake and distribution of [^3H]diltiazem in the fibroblasts, it was found that it accumulates in the fraction rich in lysosomes. Our results also show that the drugs cause accumulation of cholesteryl ester in the cells, presumably due to the accumulation of LDL from the medium and also decreased cholesterol esterase activity.

Taken together, these results support the hypothesis that the effects of these drugs on LDL degradation are due to the lysosomotropic property of the Ca^{2+}-channel-blockers and may not be associated with calcium entry into the cells. However, these findings do not explain how some of the Ca^{2+}-channel-blocking drugs decrease the development of atherosclerosis in cholesterol-fed animals. It is possible that these drugs interfere with the metabolism of native or modified LDL in macrophages or arterial smooth muscle cells, which are of major importance in the formation of atherosclerotic lesions. Experiments are currently in progress to test this possibility.

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