Vol. 107, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1982 Pages 217-224

EFFECT OF Ca^{2+} BLOCKING AGENTS ON THE METABOLISM OF LOW DENSITY LIPOPROTEINS IN HUMAN SKIN FIBROBLASTS

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Received May 21, 1982

The effect of Ca^{2+} entry blockers (prenylamine, fendiline, verapamil, diltiazem and nifedipine) and calmodulin inhibitors (trifluoperazine and dibucaine) on the biosynthesis of cholesterol and on the metabolism of low density lipoproteins (LDL) was studied in human skin fibroblasts in culture. Addition of prenylamine, fendiline, trifluoperazine or dibucaine (5-10 μM) to the cells incubated with lipoprotein-deficient serum decreased the LDL-mediated inhibition of the incorporation of [1-^{14}C] acetate into sterols by greater than 75%. LDL-mediated inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase was also decreased by these drugs. Verapamil and diltiazem were also effective at 50-100 μM concentrations. The drugs did not prevent the inhibition of sterol synthesis by 25-hydroxycholesterol, suggesting that they affect LDL metabolism and not HMG-CoA reductase itself. The drugs decreased the cellular degradation of LDL and enhanced the accumulation of LDL in the cells.

INTRODUCTION

Drugs which inhibit the influx of Ca^{2+} ("calcium antagonists") are widely used in the treatment of coronary artery disease (1). The underlying cause of this disease is atherosclerosis. The characteristic feature of the atherosclerotic process is the accumulation of lipids, connective tissue elements and Ca^{2+}. Recent studies in monkeys and rabbits have shown that agents which decrease Ca^{2+} deposition in tissues inhibit the formation of atherosclerotic lesions. Kramsch et al. (2) reported that treatment of cholesterol-fed monkeys with diphosphonates (anticalcifying agents) or $LaCl_3$ (a Ca^{2+} antagonist) markedly reduced plaque formation without decreasing plasma cholesterol levels. The amount of arterial collagen, Ca^{2+}, cholesterol and elastin also decreased in the drug-treated animals (3). Similar effects were obtained with cholesterol-fed rabbits treated with nifedipine (4) and verapamil (5), drugs which

Abbreviations used: LDL, low density lipoproteins; LPDS, lipoprotein-deficient serum; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase.

0006-291X/82/130217-08$01.00/0

inhibit Ca^{2+} movement into cells. The mechanism by which these compounds exert their anti-atherogenic effect is not known. A role for Ca^{2+} is implicated in most of the atherogenic processes such as increased endothelial permeability, migration of cells into the intima, secretion of collagen, and binding of LDL to receptors (2,6). In this communication results on the effect of Ca^{2+} entry blockers on LDL metabolism and sterol synthesis in human skin fibroblasts are presented.

MATERIALS AND METHODS

[1-^{14}C] Sodium acetate (51 Ci/mol) and carrier-free [^{125}I] sodium iodide were obtained from New England Nuclear Corporation. Tissue culture medium and chemicals were obtained from Grand Island Biological Co. Fetal calf serum was purchased from KC Biologicals. All other chemicals used were of analytical grade.

Drugs: The drugs used were obtained from the following sources: Verapamil and D-600 - Knoll Pharmaceuticals, Whippany, NJ; prenylamine and fendiline - Choinin Pharmaceuticals, Budapest, Hungary; diltiazem - Marion Research Laboratories, Kansas City, MO; trifluoperazine - Smith, Kline and French, Philadelphia, PA; nifedipine - Pfizer Pharmaceuticals, New York, NY; dibucaine - 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 cultured in monolayers as described earlier (7).

Human LDL (d 1.02-1.05 g/ml) and lipoprotein deficient serum (LPDS) were prepared by ultracentrifugation in KBr solutions as described previously (8). Iodination of LDL with [^{125}I] sodium iodide was carried out according to the method of Bilheimer et al. (9). Other experimental methods are described in the legends of the respective tables.

Presentation of Results: The values reported are arithmetic means of duplicate or triplicate determinations. The variation between the duplicates was less than 15%.

RESULTS

The effects of Ca^{2+} entry blockers on the incorporation of [1-^{14}C] acetate into sterols are shown in Table 1 and Figure 1. When fibroblasts were incubated in a medium containing LPDS, the rate of sterol synthesis from acetate was maximal and it was inhibited to the extent of 80-90% by the addition of LDL. Inhibition of sterol synthesis by LDL was prevented when the cells were incubated with the Ca^{2+} blocking agents. Addition of these compounds in the absence of LDL caused very little or no increase in the incorporation of acetate into sterols (data not shown). Prenylamine (10 μM) enhanced the rate of acetate incorporation into nonsaponifiable lipids relative to that which occurred when the cells were incubated in LPDS. However, the amount of

TABLE 1
EFFECT OF Ca^{2+} ENTRY BLOCKERS ON THE INCORPORATION OF [1-^{14}C] ACETATE INTO STEROLS

Additions	Concentration (μM)	Acetate incorporation: Nonsaponifiable Lipids (dpm/mg protein x 10^{-3})	Acetate incorporation: Cholesterol (dpm/mg protein x 10^{-3})
Experiment 1			
None (LPDS)		313	199
LDL (5 μg/ml)		51	16
+ prenylamine	10	440	113
+ fendiline	10	395	214
+ verapamil	10	76	31
	25	213	124
	50	363	158
+ diltiazem	25	107	53
	50	181	77
	100	276	134
+ nifedipine	10	41	16
	100	53	15
Experiment 2			
None		198	160
LDL		67	10
+ dibucaine	10	148	106
+ trifluoperazine	5	182	107
+ D-600	50	132	98
	100	318	223

Fibroblasts from confluent monolayeres were trypsinized to dissociate them from the plates according to the method of Nambudiri et al. (7). They were seeded on 35 mm culture dishes (5×10^4 cells/dish) with 2 ml of Eagles' minimum essential medium containing antibiotics and 10% fetal bovine serum (7). The cells were grown for 5 days. The medium was replaced on alternate days. On the 5th day the medium was replaced with fresh medium containing 10% lipoprotein deficient serum and incubated for 24 h. At the end of this incubation period, the drugs were added to the culture medium to give the appropriate final concentrations. Four hours later, LDL was added and the incubation was continued for 20 h. Then [1-^{14}C] acetate was added (5 μCi/dish) and incubated for 4 h. After the incubation, the medium was removed and the cells were washed three times with 0.9% NaCl. The cells were then digested with 0.1 N NaOH and saponified with 0.5 N NaOH in ethanol for 20 min in a boiling-water bath. Non-saponifiable lipids were extracted with hexane, and cholesterol was separated by thin-layer chromatography on silica gel G using acetone:petroleum ether (10:90, v/v) as the developing solvent.

radioactivity associated with cholesterol was lower than that obtained for the LPDS control, indicating that this compound, in addition to blocking the inhibitory effect of LDL, inhibited cholesterol synthesis at a step beyond the formation of squalene. Analysis of the nonsaponifiable lipids by thin layer chromatography revealed that there was an accumulation of radioactivity in methyl sterols (data not shown). Fendiline, like prenylamine, prevented the inhibition of sterol synthesis by LDL; however, it did not inhibit cholesterol synthesis. Verapamil had very little effect at 10 μM, but at higher concentrations caused an increase in the incorporation of acetate into non-saponifiable lipids. Verapamil also inhibited cholesterol biosynthesis at a post-

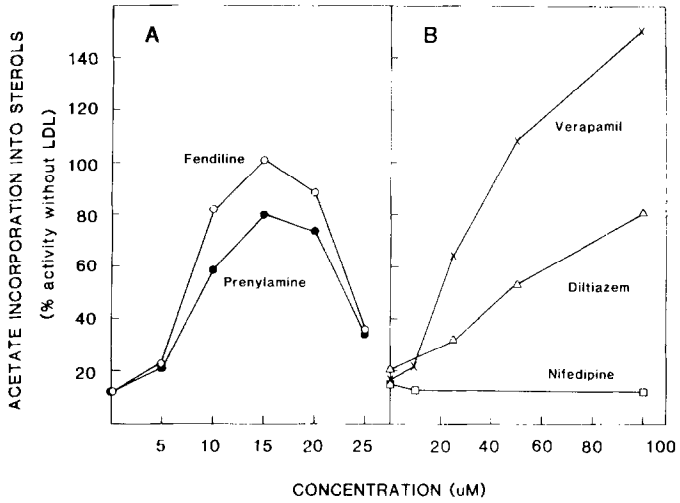

Figure 1 Effect of prenylamine, fendiline, verapamil and diltiazem on the incorporation of [^{14}C] acetate into sterols. The experimental details are as described in the legend for Table 1. [^{14}C] Acetate incorporation in cells incubated with LDL (5 μg protein/ml) and the drugs was determined and the values are represented as percent of radioactivity incorporation obtained in the absence of LDL.

squalene stage, but radioactivity did not accumulate in methyl sterols. D-600, a methoxy derivative of verapamil, also prevented suppression by LDL of cholesterol synthesis at 50 and 100 μM concentrations. Diltiazem had a similar effect but was less effective than verapamil. Nifedipine did not show any effect at 10 μM or 100 μM concentrations. Trifluoperazine, a calmodulin inhibitor, and dibucaine, a local anesthetic, were also very effective at low concentrations in blocking the inhibition of sterol synthesis by LDL.

Since it is well known that LDL inhibits sterol biosynthesis mainly by affecting HMG-CoA reductase (10), the effect of Ca^{2+} blocking agents on this enzyme activity was tested. The results shown in Table 2 indicate that the drugs prevented down-regulation of HMG-CoA reductase activity by LDL in the cells. Relative to incubation with LDL alone, HMG-CoA reductase activity was elevated by 4- to 5-fold when fibroblasts were incubated with LDL plus prenylamine (10 μM) or diltiazem (100 μM), and elevated 11- and 16-fold, respectively, when verapamil (50 μM) or trifluoperazine (10 μM) was added.

TABLE 2
EFFECT OF Ca^{2+} ENTRY BLOCKERS
ON HMG-CoA REDUCTASE ACTIVITY

Additions	Concentration (μM)	HMG-CoA Reductase Activity (pmol/min/mg protein)
None (LPDS)		106
LDL (5 μg/ml)		9
+ prenylamine	10	39
+ verapamil	50	95
+ diltiazem	100	48
+ trifluoperazine	10	138

Fibroblasts were grown in 60 mm culture dishes (after seeding with 1.5×10^5 cells/dish) by the method described in the legend to Table 1. On the fifth day, the medium was replaced with fresh medium containing 10% LPDS. The drugs were added on the sixth day and 4 h later LDL was added. After 20 h of further incubation the medium was removed and the cells were washed with 0.9% NaCl. HMG-CoA reductase activity was determined by measuring the rate of conversion of 3-hydroxy-3-methyl[3-^{14}C] glutaryl CoA to mevalonate in detergent-solubilized cell-free extracts according to the method of Brown et al. (13) with modifications described by Shapiro et al. (14).

The drug-elicited increased HGM-CoA reductase activity in the presence of LDL can be caused by inhibition of the transport of LDL into the cells or by direct stimulation of the synthesis or the activity of the enzyme. To test these possibilities, the effect of Ca^{2+} blocking agents was studied in cells in which cholesterol synthesis was inhibited by 25-hydroxycholesterol. The data presented in Table 3 show that 10

TABLE 3
EFFECT OF Ca^{2+} ENTRY BLOCKERS ON STEROL
BIOSYNTHESIS IN CELLS TREATED WITH 25-HYDROXYCHOLESTEROL

Additions	[1-^{14}C] Acetate incorporation into nonsaponifiable lipids (dpm/mg protein x 10^{-3})
None	68.5
LDL (5 μg/ml)	6.4
+ Fendiline (10 μM)	48.6
25-hydroxycholesterol (1.25 μM)	29.2
+ Fendiline (10 μM)	30.4
25-hydroxycholesterol (12.5 μM)	6.5
+ Fendiline (10 μM)	9.1

The experimental details are as described in the legend to Table 1.

TABLE 4
EFFECT OF Ca^{2+} ENTRY BLOCKING AGENTS
ON THE CATABOLISM OF LDL

Additions	Bound	Internalized (ng LDL/mg protein)	Degraded
None	30	710	5320
Fendiline (10 μM)	31	1530	2460
Diltiazem (100 μM)	52	2620	3470
Verapamil (50 μM)	48	2790	2550
Trifluoperazine (5 μM)	52	1150	440

The cells were grown according to the method described in the legend to Table 1. After 24 h incubation with medium containing 10% LPDS, [^{125}I] LDL (350 dpm/ng protein) was added to the medium at the concentration of 2.5 μg/ml and incubated for 8 h. At the end of this period, the binding, internalization and degradation of the added [^{125}I] LDL were assessed according to the method of Goldstein et al. (6).

μM fendiline enhanced the rate of acetate incorporation into nonsaponifiable lipids when the cells were incubated with LDL but not with 25-hydroxycholesterol. This result suggests that the Ca^{2+} entry blockers interfere with the cellular metabolism of LDL and not with the mechanism of regulation HMG-CoA reductase activity.

To determine the effect of Ca^{2+} blocking agents on LDL metabolism, the uptake and degradation of [^{125}I] LDL by fibroblasts were investigated and the results are presented in Table 4. The three Ca^{2+} antagonists and the calmodulin inhibitor trifluoperazine increased the intracellular level of LDL. The degradation of LDL was inhibited by all four compounds tested, trifluoperazine being the most potent.

DISCUSSION

The data presented in this report indicate that the Ca^{2+} blocking agents affect the metabolism of LDL and in turn the regulation of cholesterol biosynthesis in human skin fibroblasts. Specifically, the drugs inhibited the degradation of the internalized LDL, and as a result, blocked the delivery of free cholesterol to the feedback regulatory mechanism of cholesterol synthesis. Among the Ca^{2+} blockers tested, prenylamine and fendiline were the most potent. Verapamil and diltiazem were effective at relatively high concentrations. However, nifedipine, one of the most powerful Ca^{2+} antagonists and found to be effective in the prevention of diet-induced lipid deposition in rabbits (4), did not have any effect in the fibroblast system. This

may be due to a tissue specificity of this compound. Trifluoperazine and the local anesthetic dibucaine were also effective at 5 and 10 μM concentrations, respectively. Van Berkel et al. (11) have shown that 25 μM trifluoperazine inhibited the degradation of acetylated-LDL by non-parenchymal liver cells. Local anesthetics of this class have been known to have the characteristics of Ca^{2+} antagonists as well as calmodulin inhibitors (12). In addition to preventing down-regulation of HMG-CoA reductase by LDL, prenylamine and verapamil also inhibited cholesterol synthesis at a post-squalene step.

The mechanism by which these drugs inhibited the LDL-mediated inhibition of cholesterol synthesis is speculative. Receptor binding of LDL has a specific requirement for Ca^{2+} (6). Coated vesicles, which are responsible for receptor-mediated endocytotic processes (15) have been found to be associated with calmodulin (16). However, the drugs did not inhibit the internalization of LDL. There was, however, an accumulation of intracellular [^{125}I] LDL suggesting that the drugs inhibited LDL degradation after the lipoprotein had been internalized. The mechanism by which these drugs inhibited LDL degradation requires further study. However, the results of this study suggest that they may have affected the delivery of an endocytotic vesicle containing LDL to the lysosome.

Acknowledgements

The authors gratefully acknowledge the assistance of Ms. Rose Alden for preparing the manuscript for publication and of Ms. Gwen Kraft for preparing the figures. Special thanks go to Dr. Arnold Schwartz for his many helpful discussions during the course of this work and to Dr. Ronald Brown, Marion Laboratories, Inc. for partial support of this research. J.A.K.H. is an Established Investigator of the American Heart Association.

REFERENCES

1. Vanhoutte, P.M. (1981) Fed. Proc. 40, 2882-2887.
2. Kramsch, D.M., Aspen, A.J. and Apstein, C.S. (1980) J. Clin. Invest. 65, 967-981.
3. Kramsch, D.M., Aspen, A.J. and Rozler, L.J. (1981) Science 213, 1511-1512.
4. Henry, P.D. and Bentley, K.I. (1981) J. Clin. Invest. 68, 1366-1369.
5. Rouleau, J.L., Parmley, W.W., Stevens, J., Wikman-Coffelt, J., Sievers, R., Mahley, R. and Havel, R.J. (1982) Am. J. Cardiol. 49, 889.

6. Goldstein, J.L., Brown, M.S. and Anderson, R.G.W. (1977) International Cell Biology, pp. 639-647, Rockefeller University Press, New York.
7. Nambudiri, A.M.D., Ranganathan, S. and Rudney, H. (1980) J. Biol. Chem. 255, 5894-5899.
8. Redding, C.M. and Steinberg, D. (1960) J. Clin. Invest. 39, 1560-1569.
9. Bilheimer, D., Eisenberg, S. and Levy, R.I. (1972) Biochim. Biophys. Acta 260, 212-217.
10. Brown, M.S., Dana, D.E. and Goldstein, J.L. (1973) Proc. Natl. Acad. Sci. USA 70, 2162-2166.
11. Van Berkel, T.J.C., Nagelkerke, J.F. and Kruijk, J.K. (1981) FEBS Lett. 132, 61-66.
12. Volpi, M., Sha'afi, R.I., Epstein, P.M., Andrenyak, D.M. and Feinstein, M.B. (1981) Proc. Natl. Acad. Sci. USA 78, 795-799.
13. Brown, M.S., Dana, S.E. and Goldstein, J.L. (1974) J. Biol. Chem. 249, 789-796.
14. Shapiro, D.J., Nordstrom, J.L. Mitschelen, J.J., Rodwell, V.W. and Schimke, R.T. (1974) Biochim. Biophys. Acta 370, 369-377.
15. Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1979) Nature (London) 279, 679-685.
16. Linden, C.D., Dedman, J.R., Chafoulias, J.G., Means, A.R. and Roth, T.F. (1981) Proc. Natl. Acad. Sci. USA 78, 308-312.