ELSEVIER

International Journal of Pharmaceutics 125 (1995) 1–5

international journal of pharmaceutics

Research Papers

Modulation of membrane cholesterol content in cultured LLC-PK$_1$ cells

David B. Lebo, Pardeep K. Gupta *

Department of Pharmaceutics, Philadelphia College of Pharmacy and Science, 600 South Forty-third Street, Philadelphia, PA 19104–4495, USA

Received 21 December 1994; revised 16 March 1995; accepted 20 March 1995

Abstract

This study was designed to develop a cell culture model to evaluate the effect of cell membrane fluidity on the function of drug carrier proteins. Fluidity of the cell membrane has been linked to its cholesterol content. LLC-PK$_1$ cells were grown as a monolayer and were treated with a BSA-PVP-cholesterol complex in time-dependent studies. The total cholesterol content of the cells was increased 4-fold (from 52 to 200 fmol/mg protein) as a result of this treatment. The cholesterol increase appears to level off at 2 h of treatment. Most of the incorporated cholesterol (77%) was determined to be free cholesterol, the rest being esterified. Membrane fractions of the treated cells were isolated and were determined to carry 88% of the cholesterol content. The studies indicate that it is feasible to deliver cholesterol to cultured epithelial cells and that LLC-PK$_1$ cell monolayers can be used as a model for such studies.

Keywords: Membrane fluidity; Cholesterol; LLC-PK$_1$ cell line; BSA-PVP-cholesterol complex

1. Introduction

Membrane fluidity has been implicated as one of the important factors in membrane translocation of both passive diffusion and active transport of drugs and model compounds. The fluidity is considered to be a major function regulator of cellular activities (Cooper et al., 1978; Yuli et al., 1981). It has been shown that carrier-mediated transport of glucose in human erythrocytes and mouse fibroblasts was markedly different when the rigidity of the membranes of these cells was changed (Yuli et al., 1981). The V_{max} of glucose transport in these studies increased with initial increase in the cholesterol content and decreased with further increase in cholesterol content. Other studies have shown that changes in membrane fluidity alter ion flux (Mahfouz et al., 1989a,b), serotonin binding to brain membranes (Heron et al., 1980), and transport of *p*-aminohippurate across the brush-border membrane vesicles from normal and mutant rats (Bresler et al., 1989). All of these studies were carried out in cell or vesicle suspensions.

The methods used to vary membrane fluidity involve the use of various agents that can be incorporated into the membrane. These include

* Corresponding author.

Elsevier Science B.V.
SSDI 0378-5173(95)00095-X

cholesterol and its esters (Yuli et al., 1981), subjecting the cells to magnesium-deficient media (Mahfouz et al., 1989a,b), and using certain phospholipids in the cell or membrane suspensions (Heron et al., 1980). Deficiency in magnesium has been shown to change the biosynthetic pathways of phospholipids, thereby modifying the lipid composition of the membrane (Mahfouz et al., 1989a,b). The problem with these studies is that final lipid composition and changes that occur in the membrane are not well described. Modulating the cholesterol content in the cell membranes appears to be a more consistent way of changing the membrane fluidity.

Due to the limited solubility of cholesterol, it is not possible to incorporate enough cholesterol or its esters into the media for their transport to the membranes. Several approaches have been used in an effort to improve cholesterol uptake by membranes. These include delivering cholesterol as a complex with lecithin in PBS (Cooper et al., 1978), the use of cholesterol-phospholipid mixture (Cooper et al., 1978; Pal et al., 1981), employing cholesterol-PVP complex (Shinitzky et al., 1979), and the use of cholesterol succinate-PVP complex (Heron et al., 1980). Vesicles of a mixture of phospholipids without the cholesterol have also been used to extract cholesterol from cell membranes (Heron et al., 1980). The problem with using lecithin to deliver or remove cholesterol is that lecithin itself becomes incorporated into the membrane, thus altering the membrane permeability characteristics. It has also been shown to adversely affect cell viability (Yuli et al., 1981). The PVP complex appears to work well but so far has been examined only in cell or membrane suspensions. Although referred to as PVP-cholesterol complex, it does contain 1% BSA and 2.5% PVP added to the system (Shinitzky et al., 1979). In our preliminary studies on incorporating cholesterol into cell monolayers, a system containing 3.5% PVP and 1% BSA did not deliver significant amounts of cholesterol to the cells.

In order to explore the role of membrane fluidity in drug transport, we needed a method which alters membrane fluidity in cell monolayer systems, but allows the monolayer to stay intact for use in membrane transport studies after the cholesterol treatment. The present method describes the use of BSA-PVP-cholesterol complex to alter cholesterol content in LLC-PK_1 monolayers. The LLC-PK_1 cell line was used because, being a proximal kidney cell line, it contains transport proteins for various kinds of drugs, including anionic drugs (e.g., *p*-aminohippuric acid) and cationic drugs (e.g., tetraethylammonium). The biochemistry of this cell line is well characterized and appears to be conserved in cultured systems. The goal of this study was to develop a cell culture system which can allow the study of the effect of membrane fluidity on drug transport in cell monolayers.

2. Materials and methods

2.1. Cells and media

LLC-PK_1 cells were obtained from the American Type Culture Collection, Rockville, MD. Dulbecco's modified Eagle medium (DME) with Ham's F-12 nutrient mixture mixed 50:50 was supplied by Cellgro, Herndon, VA. Fetal bovine serum (FBS) was obtained from Sigma, St. Louis, MO. Cell culture plates were purchased from Corning.

2.2. Chemicals

Horseradish peroxidase type VI A, cholesterol esterase from porcine pancreas, cholesterol oxidase, sodium taurocholate, polyethylene glycol 4000, bovine serum albumin (BSA) from fraction V, and polyvinylpyrrolidone (PVP) were obtained from Sigma, St. Louis, MO. All other chemicals were obtained from Fisher Scientific, Pittsburgh, PA.

2.3. BSA-PVP-cholesterol complex formation

Essentially fatty acid free bovine serum albumin, polyvinylpyrrolidone (MW_r 40 000), and a 50 mM glucose solution were mixed with a 10 × stock solution of Dulbecco's phosphate-buffered saline (DPBS) to obtain an isotonic solution con-

taining 3% BSA and 2% PVP in the final solution. A cholesterol stock solution of 250 mg in 10 ml chloroform was added to the BSA-PVP solution during sonication using a Branson sonifier (setting 8, 80% cycle, 30 s). The cholesterol was diluted a total of 500 to 1. The resulting dispersion was evaluated visually for the presence of a Tyndall effect and then analyzed in a Coulter N4 submicron particle size analyzer. A usable dispersion had both a Tyndall effect and had two particle sizes from the N4 of 450 and 1700 nm. This suspension was termed the BSA-PVP-cholesterol complex.

2.4. Experimental protocol

2.4.1. Cholesterol uptake studies

BSA-PVP-cholesterol complex was added to the apical side of the confluent cell layer from subcultures 205 to 220. The cells were then incubated at 37°C while being rotated at 60 rpm on an orbital stirrer. After incubation for 60, 120, and 180 min, the cells were washed three times with DPBS at 4°C. Each plate was then scraped with a cell scraper and the cells were washed and centrifuged three times with DPBS using a serological centrifuge. Each sample was then frozen and lyophilized to remove water. Isopropyl alcohol of HPLC grade (0.4 ml in each sample) was then added and the samples were sonicated with a micro tip on the Branson Sonifier (setting 4, 60% cycle). The dispersion was then centrifuged for 10 min in an Eppendorf microcentrifuge. The supernatant fluid was assayed for free (unesterified) and total cholesterol and the precipitate was dissolved in 1 N sodium hydroxide and assayed for protein using a BCA assay kit (Pierce).

2.4.2. Cholesterol assay

The method of Heider and Boyett (1978) was used with some modifications. Briefly, 0.02 ml of cholesterol extract was added to 0.4 ml of assay solution (either free or total cholesterol assay solution as listed at the end of this section). The samples were then incubated for 60 min at 37°C while shaking at 120 rpm on a rotary shaker. The reaction was terminated by adding 0.8 ml of 1 N sodium hydroxide solution. The samples were allowed to cool for 30 min, and the fluorescence was measured using a Perkin Elmer Spectrofluorometer. The excitation wavelength was 319 nm and the emission was 405 nm. Cholesterol standards were made by placing cholesterol or cholesterol hemisuccinate in isopropyl alcohol. Cholesterol was used as the standard for the free cholesterol assay and cholesterol hemisuccinate was used for the total cholesterol assay. The assay provided linear standard curves for the concentration range of 5×10^{-6}–1×10^{-4} M. (Free cholesterol assay solution: phosphate buffer pH 7.0, 0.05 M; cholesterol oxidase, 0.08 U/ml; horseradish peroxidase, 30 U/ml; *p*-hydroxyphenylacetic acid, 0.15 mg/ml. Total cholesterol assay solution: the ingredients of free cholesterol assay plus cholesterol ester hydroxylase, 0.8 U/ml; sodium taurocholate, 5 mmol; and polyethylene glycol 4000, 0.17 mmol.)

2.4.3. Membrane isolation

The method of Schmidt-Ullrich et al. (1976) was used to isolate the plasma membrane vesicles from the cell suspension. Briefly, confluent cell layers from subcultures 205 to 220 were incubated for 8 h in DME media with 5 μCi of [^{3}H]cholesterol added. BSA-PVP-cholesterol complex with 10 μCi of [^{3}H]cholesterol was added after 6 h of incubation for treated cells. After a total of 8 h of incubation, all cells were harvested by washing three times with DPBS at 4°C. The cells were then incubated for 30 min at 37°C in trypsin and EDTA solution. The cells were then washed three times at 4°C in lysing buffer containing 0.065 M sodium chloride, 0.01 M Hepes, pH 7.4, 0.002 M $MgCl_2$, and 0.075 M KCl. Samples were then sonicated for 30 s at setting 4, 80% power cycle with a microtip on a Branson sonifier. The cells were centrifuged at 4°C for 10 min in an Eppendorf microcentrifuge in lysing buffer with dextran added to make the density 1.05 g/cm^3. Radioactivity in the supernatant fluid was counted as the membrane fraction and the precipitate was counted for nonspecific binding using a scintillation counter (Wallac model 1410). The particle size of the suspended membrane vesicles/fragments, as determined by the N4, was 238 ± 15 nm for control and 267 ± 17 nm for the

treated cells (values are average from six studies with standard deviations).

3. Results and discussion

Since minor modifications were made in the procedure for assay for cholesterol, linearity of fluorescence with concentration was verified. Curves were also obtained for the various cholesterol esters and were found to be linear. The precision of the assay is excellent. Since all the determinations of cholesterol for the rest of the studies rely on this assay, it is important that the assay works well.

Fig. 1 shows the curve for incorporation of total amount of cholesterol with time using the BSA-PVP-cholesterol complex. The values were normalized to total protein content in the cells.

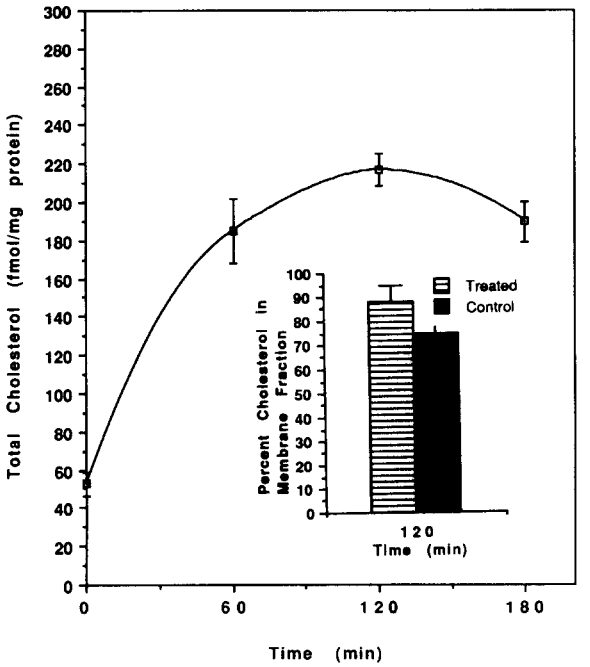

Fig. 1. Change in the total cholesterol content of LLC-PK$_1$ cells after treatment with BSA-PVP-cholesterol complex. Inset: percent cholesterol content in membrane fraction of treated and untreated cells 2 h after the treatment. Each data point in both the plots is an average of six determinations. Error bars are the standard deviations.

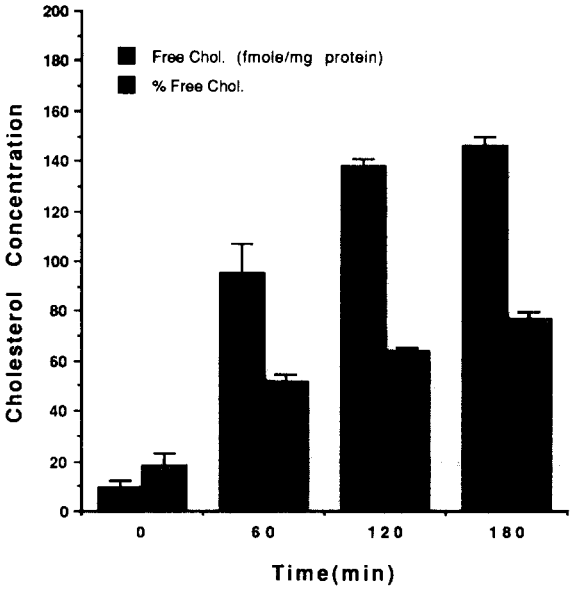

Fig. 2. Change in the free cholesterol content of LLC-PK$_1$ cells after treatment with BSA-PVP-cholesterol complex. Each data point is an average of six determinations. Error bars are the standard deviations.

The total amount of cholesterol in cells increases from about 52 fmol/mg protein before treatment to 218 fmol/mg protein after 2 h of treatment. In cells that were treated with a suspension of cholesterol in the growth media or the buffer, there was no significant change in total cholesterol content of the cells. Since our goal is to deliver cholesterol primarily to the membrane, it is important to determine the fraction of this amount that is actually in the plasma membrane fraction. The bar graph (inset in Fig. 1) shows the percent cholesterol in the membrane fraction at the end of 2 h treatment. In the treated cells, 88% of the cholesterol was found to be bound to the membrane vesicles that were isolated from the cells. This value for the untreated cells is 77%. Since the total cholesterol content of the treated cells is roughly 4-times greater than in the untreated cell, it appears that the about 90% of the delivered cholesterol becomes incorporated into the cell membrane.

Fig. 2 shows the changes in free cholesterol levels in the treated cells. The data at the zero

time point show the cholesterol content in untreated cells. This value is approx. 11 fmol/mg of protein, and corresponds to 17% of the total cholesterol content of these cells. Following treatment with BSA-PVP-cholesterol complex, the free cholesterol concentration in the cells increases to 146 fmol/mg protein. This value amounts to 77% of the total cholesterol in these cells. This indicates that the treated cells contain a much larger fraction of free cholesterol as compared with the untreated cells. Since the increase in total cholesterol in cells after 2 h treatment is about 166 fmol/mg protein, of which 153 fmol/mg protein is free, the total esterified cholesterol amounts to 54 fmol/mg protein (13 fmol/mg protein from delivered cholesterol and 41 fmol/mg protein from natural cholesterol in untreated cells). When this value is compared with the esterified amount of cholesterol of untreated cells (41 fmol/mg protein esterified, 11 fmol/mg protein free), it becomes clear that most of the cholesterol that is delivered to the cell membranes exists as free cholesterol. Our results are in agreement with the studies of Yuli et al. (1981) who studied the incorporation of cholesterol hemisuccinate as a cholesterol ester-PVP-lecithin complex. Their studies showed that the total cholesterol content in treated cells levels off at 120 min after incubation. Our studies demonstrate the same trend in time-dependent uptake studies.

In summary, BSA-PVP-cholesterol complex appears to deliver cholesterol in significant amounts to cultured LLC-PK1 cells. Most of this cholesterol is in the free form and is associated with the plasma membrane fraction of the cells. This system, therefore, can serve as a model for studying the effect of membrane fluidity on the function of membrane proteins in carrier-mediated drug transport.

References

Bresler, V.M., Balter, S.N., Jerebtsova, M.A., Isayev-Ivanov, V.V., Kazbekov, E.N., Kleiner, A.R., Orlov, Yu.N., Ostapenko, I.A., Suchodolova, A.T. and Fomichev, V.N., The influence of the lipid bilayer phase state on the *p*-aminohippurate (PAH) transport and the activity of the alkaline phosphatase in brush-border membrane vesicles from normal and mutant rats. *Biochim. Biophys. Acta*, 982 (1989) 288–294.

Cooper, R.A., Leslie, M.H. Fischkoff, S., Shinitzky, M. and Shattil, S.J., Factors influencing the lipid composition and fluidity of red cell membranes in vitro: production of red cells possessing more than two cholesterols per phospholipid. *Biochemistry*, 17 (1978) 327–331.

Heider, J.G. and Boyett, R.L., The picomole determination of free and total cholesterol in cells in culture. *J. Lipid Res.*, 19 (1978) 514–518.

Heron, D.S., Shinitzky, M., Hershkowitz, M. and Samuel, D., Lipid fluidity markedly modulates the binding of serotonin to mouse brain membranes. *Proc. Natl. Acad. Sci. USA*, 77 (1980) 7463–7467.

Mahfouz, M.M., Smith, T.L. and Kummerow, F.A., Changes in linoleic acid metabolism and cellular phospholipid fatty acid composition in LLC-PK_1 cells cultured at low magnesium concentrations, *Biochim. Biophys. Acta*, 1006 (1989a) 70–74.

Mahfouz, M.M., Smith, T.L. and Kummerow, F.A., Changes in phospholipid composition and calcium flux in LLC-PK_1 cells cultured at low magnesium concentrations, *Biochim. Biophys. Acta*, 1006 (1989b) 75–83.

Pal, R., Barenholz, Y. and Wagner, R.R., Depletion and exchange of cholesterol from the membrane of vesicular stomatitis virus by interaction with serum lipoproteins or poly(vinylpyrrolidone)complexed with bovine serum albumin. *Biochemistry*, 20 (1981) 530–539.

Schmidt-Ullrich, R., Wallach, D.F. and Davis, F.D., Membranes of normal hamster lymphocytes and lymphoid cells neoplastically transformed by simian virus 40: I. High-yield purification of plasma membrane fragments. *J. Natl. Cancer Inst.*, 57 (1976) 1107–1116.

Shinitzky, M., Skornick, Y. and Haran-Ghera, N., Effective tumor immunization induced by cells of elevated membrane-lipid microviscosity. *Proc. Natl. Acad. Sci. USA*, 76(1979) 5313–5316.

Yuli, I., Wilbrandt, W. and Shinitzky, M., Glucose transport through cell membranes of modified lipid fluidity. *Biochemistry*, 20 (1981) 4250–4256.