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EFFECTS OF DEXAMETHASONE ON THE FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE IN HeLa CELLS

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Summary

Treatment of HeLa cells for 24 h with dexamethasone resulted in lower steady-state polarization values of 1,6-diphenyl-1,3,5-hexatriene in both intact cells and dispersions of whole cell lipid extracts. Dexamethasone also reduced the polarization values in isolated membrane fractions from treated cells. These effects are similar to the effect of 25-hydroxycholesterol, a potent inhibitor of cellular sterol synthesis.

Steady-state fluorescence polarization techniques have been increasingly utilized to monitor the dynamic nature of cellular lipid membranes. Polarizations analyzed with nonpolar probes such as 1,6-diphenyl-1,3,5-hexatriene have been compared to polarizations in standard homogeneous nonpolar solvents to calculate the microviscosity of the cell membranes using the Perrin equation [1—5]. Inbar, Shinitzky and coworkers have found differences between normal and leukemic lymphocytes using this technique [3, 5]. The interpretation of steady-state polarization measurements of intact cells is complicated by recent studies which have found 1,6-diphenyl-1,3,5-hexatriene does not follow a single exponential decay [6, 7], that 1,6-diphenyl-1,3,5-hexatriene rotations are dominated by hindered torsional motions in lipid vesicles [8] and that probes may not be confined to the plasma membrane [9].

In spite of these reservations, the steady-state fluorescence polarization technique using 1,6-diphenyl-1,3,5-hexatriene can still provide valuable information concerning the dynamic properties of cellular membranes. Relative

differences in the polarization values themselves can accurately reflect changes in the physical properties or structure of the probe environment. The differences in polarizations of 1,6-diphenyl-1,3,5-hexatriene in leukemic and normal lymphocytes, found by Inbar and Shinitzky and coworkers [3, 5] for example, do represent differences in the environment of the probe but these differences should not be expressed in terms of microviscosity.

In the present study we have investigated the effects of dexamethasone and 25-hydroxycholesterol on the inhibition of cholesterol synthesis and the resultant reduction of cellular cholesterol levels. The concomitant changes in cellular membrane physical properties were determined by measuring the relative differences in the steady-state polarization of 1,6-diphenyl-1,3,5-hexatriene in both whole cells and in isolated plasma membranes.

The origin and maintenance of HeLa cells has been previously described [10]. The incorporation of [^{3}H]acetate into lipids was determined by pulse labeling cells for 1 h with 1 μCi/ml (285 mCi/mmol) prior to harvesting the cells. Total incorporation into saponifiable and nonsaponifiable lipid fraction was measured as previously described [11]. Total cholesterol was measured using the nonsaponifiable lipid extract by the method of Ishikawa et al. [12] using coprostanol as an internal standard.

To isolate membranes, cells were grown in roller bottles to near confluency in Eagles' minimal essential medium plus 10% calf serum. The medium was replaced with Eagles' minimal essential medium plus 2% calf serum for 24 h followed by unsupplemented Eagles' minimal essential medium with and without dexamethasone for another 24 h. Cells were harvested by scraping with rubber policemen. Membrane fractions were isolated by the method Thom et al. [13]. This method essentially involves hypoosmotic lysis of cells in borate buffer. The membranes are isolated by differential centrifugation followed by a sucrose single-step gradient. (Na^+-K^+)-ATPase (EC 3.6.1.8) and 5'-nucleotidase (EC 3.1.3.5) were assayed by the methods of Wallach and Kamat [14] and Porteus and Clarke [15] respectively. Alkaline phosphatase (EC 3.1.1.4) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (EC 1.1.1.34) were assayed as previously described [10, 16].

Polarization values were measured according to the method of Inbar et al. [3] using 1,6-diphenyl-1,3,5-hexatriene as the probe. Corrections were made for background and light scattering by using the appropriate sample (cells, microsomes or lipid dispersions) which was not incubated with 1,6-diphenyl-1,3,5-hexatriene. Polarization values were measured on a SLM model 8000 spectrofluorometer using a channel ratio method. The excitation and emission wavelengths used were 350 nm and 460 nm respectively.

Lipids were extracted from cells using chloroform/methanol (2:1, v/v) overnight at 4° C. The extracts were washed according to Folch et al. [17].

Previous studies using [^{14}C]octanoate and ^3H_2O [11, 18, 19] have shown that acetate incorporation into total nonsaponifiable lipids is an accurate measure of cholesterol synthesis in HeLa cells grown under the conditions used in this study. Table I shows the effects of dexamethasone and 25-hydroxycholesterol on the incorporation of [^{3}H]acetate into both saponifiable and nonsaponifiable lipid fractions. Dexamethasone inhibited the incorporation of acetate into nonsaponifiable lipids but not into saponifiable lipids.

TABLE I

EFFECT OF DEXAMETHASONE AND 25-HYDROXYCHOLESTEROL ON LIPID SYNTHESIS, CHOLESTEROL CONTENT, AND POLARIZATION VALUES IN WHOLE CELL LIPID EXTRACT AND ISOLATED MEMBRANE FRACTIONS

Cell growth and isolation of membranes are described in the experimental procedures. Incorporation of acetate into lipid fractions and the cholesterol/protein ratios represent the mean values from 4 separate cultures ± S.D. Cholesterol/protein ratios for membranes are mean values for 3 determinations of a single membrane preparation. The polarization values represent the mean of multiple measurements on duplicate samples. The measurements on a single sample varied by less than 0.005 units.

	Saponifiable lipids (dpm × 10^{-3} / mg protein)	Nonsaponifiable lipids (dpm × 10^{-3} / mg protein)	Cholesterol/protein (μg/mg)	Polarization P
Control	435 ± 10	174 ± 3	19.1 ± 0.2	0.245
Dexamethasone	518 ± 34	126 ± 7	16.4 ± 1.5	0.211
25-Hydroxycholesterol	315 ± 26	34 ± 2	11.2 ± 1.2	0.202
Control membranes	—	—	220	0.337
Dexamethasone-treated membranes	—	—	180	0.300

The inhibition by dexamethasone was not as effective as that caused by 25-hydroxycholesterol, a potent inhibitor of cholesterol synthesis in several other studies [20—22]. A slight inhibition of incorporation into saponifiable lipids also resulted from 25-hydroxycholesterol addition.

Inhibition of cholesterol synthesis resulted in cellular cholesterol/protein ratios which are significantly lower in dexamethasone- and 25-hydroxycholesterol-treated cells than in control cells. The values for cells treated with 25-hydroxycholesterol are lower than those receiving dexamethasone which reflects the greater inhibition of cholesterol synthesis by 25-hydroxycholesterol.

Since the total phospholipid content is not altered by 24 h treatment with dexamethasone (unpublished results) the polarization values should reflect changes in cholesterol/phospholipid ratio according to Inbar, Shinitzky and coworkers [1—3, 5]. It is clear from Table I that dexamethasone and 25-hydroxycholesterol both decrease the polarization of 1,6-diphenyl-1,3,5-hexatriene. In a separate experiment, we have also measured the fluorescence polarization values in intact cells from control and dexamethasone-treated cells. The mean values from 4 replicate dishes were 0.266 ± 0.008 and 0.227 ± 0.010 respectively (which is a significant difference at $P < 0.001$ by the Student's *t*-test). The higher values found in intact cells vs. cell lipid extracts suggests that nonlipid substances (e.g. proteins) can, to some extent, affect the polarization of fluorescent probes. This is similar to results of Inbar et al. [23] in lymphocytes.

The results from studies on isolated plasma membranes are also given in Table I. These results are in accordance with the whole cell data since both cholesterol/protein ratio and 1,6-diphenyl-1,3,5-hexatriene polarization are significantly lower in membranes isolated from dexamethasone-treated cells. The generally higher polarization values found in isolated membranes might be due to different growth conditions. In order to obtain reasonable quantities of membranes, cells were grown in large batches in roller bottles and nor-

mally to a higher confluency than used in dishes. Inbar et al. [24] and Nicolau et al. [25] have both found that polarization values increase as the cell density is increased. It is, therefore, probable that the polarization values were higher in isolated membranes as a result of the higher cell density. This is also similar to the results of Petitou et al. [5].

Calculations of microviscosities from polarization values have been questioned [6—8] since cell membranes are not homogenous-nonpolar solvents and probe molecules can appear in other organelles [9]. This second problem can be solved if isolated plasma membranes are also used. Since the fluorescent polarization values themselves can provide a valid measurement of the relative changes in the dynamic nature of the cell membrane brought on by specific treatments it is not necessary to calculate differences in microviscosities. The present results demonstrate that dexamethasone does alter the physical properties of cellular membranes whether measured in intact cells or isolated plasma membranes. Whether these alterations represent a general fluidizing of a homogeneous membrane or a partition of the probe into regions of altered composition can not be determined, but the fluorescent polarization technique can determine that a physical change, whatever it may be, has taken place.

Our results show that dexamethasone acts in a similar fashion to 25-hydroxycholesterol in inhibiting cholesterol synthesis. This causes a lower cholesterol/protein ratio not only in intact cells but also in isolated plasma membranes. The lower cholesterol levels cause a decrease in the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene, which is again similar to the effect of 25-hydroxycholesterol. According to Inbar and Shinitzky, cholesterol helps to maintain the rigidity of a membrane. A lower cholesterol level would allow more motion (increasing fludity), thus increasing the rotation of a fluorescent probe and therefore lowering the polarization value.

The consequences of such membrane alterations are not entirely known. Kandutsch and Chen [16] have shown that cells cease to grow if cholesterol synthesis is inhibited by 25-hydroxycholesterol. Glucocorticoids are also known to reduce cell growth which, although the mechanism is not known, may involve alterations in the physical properties of membranes. Glucocorticoids have also been shown to modify the activation of membrane-bound enzymes such as alkaline phosphatase [10] and 5′-nucleotidase [27] suggesting that inhibition of cholesterol synthesis could be the cause, acting by altering the membrane properties. Whether the actions of glucocorticoids can be generally explained on the basis of membrane effects must await further study.

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