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THE EFFECT OF HYDROCORTISONE ON CHOLESTEROL METABOLISM OF CULTURED HUMAN SKIN FIBROBLASTS *

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Hydrocortisone in physiologic concentrations resulted in a reduction in sterol synthesis by cultured normal human skin fibroblasts. These changes were observed when [^{14}C]acetate, [^{14}C]octanoic acid and $^{3}H_2O$ were used as precursors. However, the incorporation of [^{3}H]mevalonic acid lactone into digitonin-precipitable sterols was not affected by hydrocortisone, suggesting that hydrocortisone inhibits sterol synthesis at a site prior to the formation of mevalonic acid. In contrast, the activity of hydroxymethylglutaryl-CoA reductase was stimulated several-fold by the hormone. Thus, the inhibitory effect of hydrocortisone on the cholesterol synthetic pathway may be on hydroxymethylglutaryl-CoA synthase.

Introduction

Cultured human skin fibroblasts as well as other types of mammalian cells can acquire exogenous cholesterol by means of cell surface receptors that specifically bind low density lipoprotein (LDL) [1]. Once bound to its receptor, LDL is internalized by adsorptive endocytosis and delivered to lysosomes [2], where its protein and cholesteryl ester components are hydrolyzed. The delivery of cholesterol to the cell by the LDL pathway suppresses cholesterol synthesis by inhibiting the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (mevalonate : $NADP^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34) [2]. The conversion of hydromethylglutaryl-CoA to mevalonic acid catalyzed by this enzyme is recognized as the rate-limiting step in the biosynthesis of cholesterol [3], although other regulatory sites have been described recently [4].

When cultured cells are deprived of cholesterol by exposing them to a medium containing lipoprotein-deficient serum or to serum-free medium, hydroxymethylglutaryl-CoA reductase and sterol synthesis are stimulated and LDL receptor activity increases [5]. Conversely, exposure to LDL or cholesterol in the incubation medium results in suppression of receptor activity and in inhibition of cholesterol synthesis.

Recently we have investigated the effect of hydrocortisone on the cellular LDL pathway in cultured human fibroblasts [6]. Physiological concentrations of hydrocortisone do not affect the binding of ^{125}I-labeled LDL to its cell surface receptor, but decrease LDL uptake and degradation. These results apparently are due to an inhibition of LDL internalization by hydrocortisone. Thus, in the presence of hydrocortisone, the cells bind LDL normally, but since less LDL is internalized, less is degraded.

Since hydrocortisone influences the cellular LDL pathway in normal cultured human fibroblasts, it appeared likely that it also would affect cellular cholesterol synthesis and homeostasis.

In established cell lines, such as HeLa S3G cells, glucocorticoids have been shown to decrease cholesterol synthesis [7]. Despite reduced cholesterol synthesis, the activity of hydroxymethylglutaryl-CoA

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Abbreviation: LDL, low-density lipoprotein(s).

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reductase surprisingly was increased in a variety of established cell lines by dexamethasone treatment [8]. On the other hand, an earlier enzyme in the cholesterol biosynthetic pathway, i.e. hydroxymethylglutaryl-CoA synthase, has been reported to be suppressed by dexamethasone in HeLa S3G cells [9]. Therefore, it has been suggested that hydroxymethylglutaryl-CoA synthase controls the rate of cholesterogenesis when cholesterol synthesis is partially suppressed by glucocorticoids [9].

Since regulation of metabolic processes in established cells lines frequently differs from those in normal diploid cell strains, the present study was designed to evaluate the effects of glucocorticoids on cellular cholesterol synthesis and homeostasis in normal cultured human skin fibroblasts.

Experimental procedures

Materials. D,L-[2-^{3}H]Mevalonic acid lactone (745 mCi/mmol) was obtained from Amersham/Searle Corp. (Arlington Heights, IL). [2-^{14}C]Acetate (3.5 mCi/mmol), D,L-[*glutaryl*-3-^{14}C]hydroxy-3-methylglutaryl coenzyme A (58.6 mCi/mmol), [1-^{14}C]-octanoic acid (25 mCi/mmol), ^3H_2O (1 mCi/g) and Aquasol were obtained from New England Nuclear, Boston, MA. Sigma Chemical Co., St. Louis, MO, supplied the hydrocortisone and mevalonolactone. Dulbecco-Vogt medium and trypsin were purchased from Grand Island Biological Co., disposable plastic tissue culture flasks, Petri dishes and filters from Corning Glassworks, and disposable pipettes from Falcon Plastics.

Cell culture. Skin fibroblasts were cultured from punch biopsy specimens taken from the anterior aspects of thighs of healthy volunteers. Cells were grown and transferred after trypsinization as previously described [10] using modified Dulbecco-Vogt medium containing 10% fetal calf serum.

All experiments were performed after 2–10 transfers. 7–10 days prior to each experiment, $1 \cdot 10^5$ cells in 4 ml of medium were plated into 60-mm diameter plastic petri dishes and grown to confluence, by which time cell numbers approximated $(3–6) \cdot 10^5$ cells/dish. Cells were grown and experiments performed during incubation in an atmosphere of humidified 95% air/5% CO_2 at 37°C.

Hydrocortisone was dissolved in 50% ethanol and added to dishes in 10-μl aliquots to yield the final concentration desired. Control dishes received 10 μl of 50% ethanol.

Determination of cholesterol synthesis by the incorporation of radioactively labeled precursors into digitonin-precipitable sterols. The effect of hydrocortisone on sterol synthesis was evaluated by pulse-labeling the cell layer with the following radioactively labeled precursors in serum-free medium: [2-^{14}C]-acetate, ^3H_2O, [^{14}C]octanoic acid and D,L-[2-^{3}H]-mevalonic acid lactone. Hydrocortisone was added to the serum-free medium at a final concentration of 15 ng/ml ($4.1 \cdot 10^{-8}$ M), a physiological concentration of unbound hormone in human serum. After incubation of triplicate dishes for the times indicated, the dishes were removed from the incubator and the medium discarded. The cells were then washed and harvested by scraping the dishes with a teflon policeman. Extraction of lipids was performed according to the method of Stein et al. [11]. After the aqueous and the organic phases were separated, the pellet was dried and redissolved in 0.1 M NaOH, and its protein content was determined by the method of Lowry et al. [12]. The organic phase was dried under air and saponified with 1 M ethanol/KOH at 80–85°C for 1 h. For the ^3H_2O incorporation into digitonin-precipitable sterols, the organic phase was washed three times. After saponification and the addition of ethanol/water, the sterols were extracted with hexane and, after drying, were precipitated overnight in 5% digitonin in 50% ethanol, acetone diethyl ether and one drop of 10% acetic acid. The precipitate was washed with acetone diethyl ether, then with diethyl ether and after drying, was dissolved in glacial acetic acid. An aliquot was transferred in a glass counting vial, Aquasol was added, and radioactivity was determined in a scintillation counter.

Hydroxymethylglutaryl-CoA reductase. Hydroxymethylglutaryl-CoA reductase was determined by the method of Brown et al. [5]. Briefly, the cell pellets were ruptured by rapid freezing and thawing. The disrupted cells then were incubated for 10 min at 37°C with 0.15 ml of buffer containing 50 mM K_2HPO_4 (pH 7.4), 5 mM dithiothreitol, 5 mM EDTA, 0.2 M KCl and 0.25% Kyro-EOB (Proctor and Gamble, Cincinnati, OH). After centrifugation for 5 min at 5000 rev./min at room temperature, the supernatants (100 μl) containing 50–100 μg protein were incubated for

2 h at 37°C in a total volume of 0.2 ml, containing 0.1 M potassium phosphate (pH 7.5), 20 mM glucose 6-phosphate, 5 mM NADP, 0.7 units of glucose-6-phosphate dehydrogenase, 5 mM dithiothreitol and $2.5 \cdot 10^{-5}$ mM and 50 μM D,L-3-[*glutaryl*-3-^{14}C]-hydroxy-3-methylglutaryl-CoA. After 2 h the reaction was stopped by adding 50 μl of 3 M HCl. 60 mM [^{3}H]mevalonolactone was added as carrier. After an additional 30 min at 37°C, the [^{14}C]mevalonate formed was isolated by thin-layer chromatography and counted [5]. Values are expressed as pmol/mg protein. Hydroxymethylglutaryl-CoA reductase activity was assayed from triplicate dishes and differences were evaluated using Student's *t*-test. Active and inactive forms of the enzyme were assayed by the method of Brown et al. [13].

Efflux of cholesterol from cells. The rate of efflux of cellular cholesterol was estimated by determining the increase in sterol mass that occurred in the medium during the incubations. Cells were exposed to medium with or without hydrocortisone for 48 h. The medium was then removed for determination of its sterol mass. Stigmasterol was added to the medium as an internal standard, the sterols were extracted from the medium using chloroform/methanol (2 : 1), and were quantitated by gas chromatography.

Determination of cellular cholesterol. Extraction of cellular sterols was performed by a modification of the method of Stein et al. [1]. After the experimental incubations, dishes were chilled on ice, washed as indicated, and frozen. The frozen cells were scraped from the dishes with a teflon policeman into 1.0 ml ice-cold methanol/water (2 : 1). The dishes were washed once with the same solvents and then twice more with 1.0 ml 100% methanol. The washes were combined with 3.0 ml chloroform and the sterol was extracted at 4°C overnight. The one-phase extract was centrifuged and the pellet was dried and dissolved in 0.1 M NaOH for protein determinations. The extract was then dried and resuspended in hexane. Cholesterol was determined by the enzymatic method of Heider et al. [14].

Results

Sterol synthesis after exposure to hydrocortisone was determined by measuring the incorporation of [^{14}C]acetate and [^{3}H]mevalonic acid lactone into digitonin-precipitable non-saponifiable lipids. As expected, deprivation of the cells of exogenous cholesterol (by switching to serum-free medium) resulted in a time-related increase in sterol synthesis from [^{14}C]acetate, but by as early as 6 h after the medium change it was evident that sterol synthesis was stimulated to a smaller extent in the cells treated with physiological concentrations of hydrocortisone than in control cells not exposed to the hormone (Fig. 1). Under the same experimental conditions, the incorporation of [^{3}H]mevalonic acid lactone was not significantly affected by hydrocortisone nor by deprivation of a source of exogenous cholesterol in the medium (Fig. 1).

The effect of hydrocortisone in inhibiting sterol synthesis is not only time but also dose-dependent. Preincubation of the cells for 24 h in serum-free medium with increasing concentrations of hydrocortisone resulted in a dose-dependent decrease in cholesterol synthesis (Fig. 2). At a concentration of $4.1 \cdot 10^{-8}$ M, the physiological concentration of unbound hormone in human plasma, a 35% reduction in sterol synthesis was achieved. Maximal inhibition of sterol synthesis was achieved with $4.1 \cdot 10^{-7}$ M hydrocortisone.

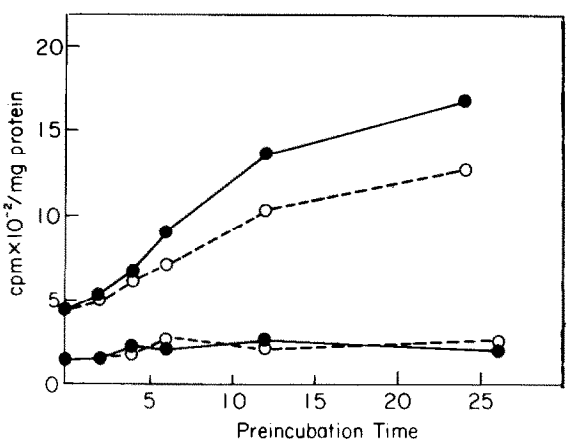

Fig. 1. Time course of hydrocortisone effect on sterol synthesis. On day 9 of cell growth each cell monolayer was exposed to $4.1 \cdot 10^{-8}$ M hydrocortisone (○) or 10 μl ethanolic solution in serum free medium (●). 2 h prior to the indicated time points the cells were pulse-labeled with 3.5 μCi/ml [^{14}C]acetate (upper curves) or 5 μCi/ml of [^{3}H]mevalonic acid lactone (lower curves) for measurement of their incorporation into digitonin-precipitable sterols.

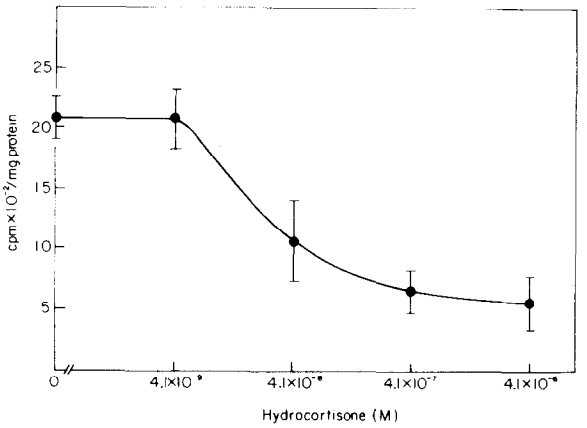

Fig. 2. Effect of hydrocortisone concentrations on sterol synthesis. On day 8 of cell growth, each cell monolayer was exposed to the indicated concentrations of hydrocortisone in serum-free medium. At the same time 5 μCi [^{14}C]octanoic acid/dish was added. After 24 h incubation the cells were extracted and the incorporation at the radioactive label into digitonin-precipitable sterol measured.

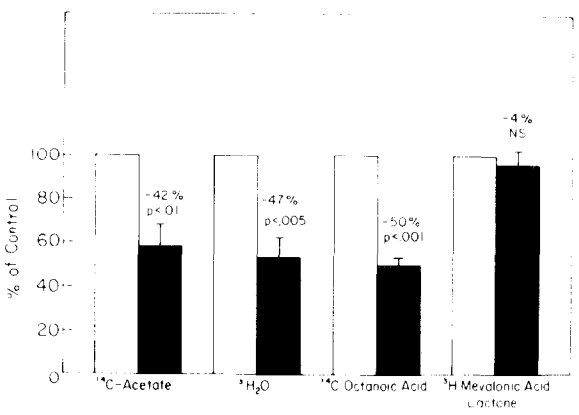

Fig. 3. Effect of hydrocortisone on sterol synthesis using different radioactively labeled precursors. On day 8 of cell growth each cell layer received $4.1 \cdot 10^{-8}$ M hydrocortisone (hatched bars) or ethanolic solution in serum-free medium (open bars). At the same time 3 μCi/dish [^{14}C]acetate, 1.5 mCi/dish ^3H_2O or 5 μCi/dish [^{14}C]octanoic acid was added. After 24 h the incorporation of the radioactive label into digitonin-precipitable sterols was measured as described under Experimental procedure.

To exclude the possibility that these changes were due to an effect of hydrocortisone on acetate pool size, hydrocortisone-treated and control cells were incubated for 24 h with either [^{14}C]acetate, [^{14}C]-octanoic acid or ^3H_2O. Regardless of which precursor was used, cholesterol synthesis was decreased a similar extent (Fig. 3). Thus, the effect of hydrocortisone on cholesterol synthesis cannot be explained by an influence of the hormone on acetate pool size. Further, when [^{3}H]mevalonic acid lactone was used as precursor, no difference between hydrocortisone-treated cells and controls could be detected (Fig. 3). These findings suggest that hydrocortisone affects cholesterol synthesis pathway at a step prior to that of mevalonic acid formation. A general toxic effect of hydrocortisone on macromolecular synthesis seems unlikely, since the addition of the hormone to the medium had no effect on either protein or RNA synthesis, or on total protein content or cell number [6].

To determine whether the effect of hydrocorti-

TABLE I

EFFECT OF HYDROCORTISONE ON CHOLESTEROL CONTENT OF INCUBATION MEDIUM AND CELL LAYERS (SERUM-FREE MEDIUM)

On day 10 of cell growth each cell layer was exposed to $4.1 \cdot 10^{-8}$ M hydrocortisone or ethanol in either serum-free medium (A) or medium containing 5% lipoprotein-deficient medium (LDS, B). After 48 h incubation the cells and the medium were extracted and their cholesterol content was determined.

	Hydrocortisone concentrations (M)	Total cell cholesterol (μg/mg cell protein)	Cell cholesterol esters (μg/mg cell protein)	Medium total cholesterol (μg/ml medium)
A. Serum-free medium	0	55.7 ± 2.3	3.3 ± 0.6	0.98 ± 0.23
	$4.1 \cdot 10^{-8}$	57.9 ± 3.3	3.8 ± 0.5	0.79 ± 0.18
B. 5% LDS	0	46.8 ± 4.1	2.4 ± 0.5	1.72 ± 0.31
	$4.1 \cdot 10^{-8}$	51.3 ± 3.9	3.2 ± 0.4	1.60 ± 0.28

sone on sterol synthesis resulted from inhibition of hydroxymethylglutaryl-CoA reductase (the rate-limiting enzyme in this process), the activity of this enzyme was assayed. Surprisingly, incubation of cells with $4.1 \cdot 10^{-8}$ M hydrocortisone for 24 h resulted in an increased activity of hydroxymethylglutaryl-CoA reductase, from 117 ± 10 to 276 ± 51 pmol/min per mg protein ($P < 0.001$). Additional experiments showed that both active and inactive forms of the enzyme were stimulated equally by hydrocortisone. Addition of the same concentration of hormone to the assay system in the absence of cells was without effect on enzyme activity measurements (data not shown).

Evidence that cholesterol efflux was not affected by hydrocortisone was obtained by measurement of the increase in cholesterol mass in the medium with time; no difference between hydrocortisone-treated cells and controls could be demonstrated (Table I).

Since cholesterol synthesis was decreased by hydrocortisone without a change in cholesterol efflux, the effect of the hormone on cellular cholesterol content was measured. There was no difference in either total cellular cholesterol or cholesterol ester content between cells incubated in the presence of hydrocortisone or in its absence (Table I).

Discussion

These results indicate that sterol synthesis is partially inhibited by physiological concentrations of hydrocortisone in a time- and dose-dependent manner in cultured human fibroblasts. The rate-limiting step of this biosynthesis pathway in this cell type is generally recognized to be the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid catalyzed by the enzyme hydroxymethylglutaryl-CoA reductase [3,13]. Whether or not this reduction step is always rate-controlling has been questioned recently [4,8,9]. As shown in this study, the activity of this enzyme in cultured human fibroblasts is actually stimulated by hydrocortisone, whereas sterol synthesis is inhibited. Since hydrocortisone did not affect enzyme activity in a cell-free system, the mode of action is not likely to be due to a competitive inhibitory mechanism on this enzyme, as has recently been shown to occur with compactin [15]. Nor is the hormone acting by increasing cholesterol efflux, since neither the unesterified cholesterol content of the cells nor the medium was influenced by the hormone during incubation either in serum-free medium or in a medium supplemented with lipoprotein-deficient serum. Finally, under the same experimental conditions, no increase in protein synthesis, which could have been an explanation for an increase in enzyme synthesis, has been seen [6]. The hydrocortisone-induced increase in hydroxymethylglutaryl-CoA reductase activity observed in our experiments is in contrast to observations made by Brown and Goldstein [2], who found no effect of high dexamethasone concentrations on the activity of this enzyme. However, in those studies cells were incubated in lipoprotein-deficient serum, which might have contained an excess of steroid hormone-binding globulins.

It is likely that the rate-limiting step at which hydrocortisone influences cholesterol biosynthesis is one prior to the formation of hydroxymethylglutaryl-CoA, possibly hydroxymethylglutaryl-CoA synthase. This enzyme, which catalyzes the formation of hydroxymethylgluratyl-CoA, has been shown to be decreased by hydrocortisone in established cell lines [9]. Thus, hydroxymethylglutaryl-CoA synthase could become rate-limiting under certain circumstances, resulting in a decreased availability of hydroxymethylglutaryl-CoA, the substrate for hydroxymethylglutaryl-CoA reductase. Decreased mevalonate and/or cholesterol formation might then lead to induction of hydroxymethylglutaryl-CoA reductase due to reduced feedback inhibition with a resultant increase in the activity of this enzyme.

A dissociation of cholesterol synthesis from hydroxymethylglutaryl-CoA reductase activity has been observed when established cell lines, some of neoplastic origin, were treated with dexamethasone [7,8].

A similar situation appears to apply in the present study using cultured diploid cells. Recently it has been reported that hydroxymethylglutaryl-CoA reductase exists in active and inactive forms [13]. Theoretically, it is possible that hydrocortisone increases the inactive form of the enzyme, i.e. a form that cannot catalyze the conversion of hydroxymethylglutaryl-CoA to mevalonic acid by cultured cells, but that might be measured during our enzyme assay. This possibility was excluded by the finding of an increase in both the active and inactive forms of the

enzyme, the active form predominating in cultured fibroblasts. Further studies are needed to delineate the precise mechanism by which hydroxymethylglutaryl-CoA reductase is activated in hydrocortisone-treated cells.

Thus, these results show that hydrocortisone decreases cholesterol synthesis in cultured human fibroblasts, while it increases the activity of hydroxymethylglutaryl-CoA reductase. These finding confirm that measurement of this enzyme cannot be taken as the sole index of cholesterol synthesis in cultured fibroblasts under all experimental conditions. Coupled with the previous finding that hydrocortisone impairs internalization of LDL, it appears that hydrocortisone limits the availability of both endogenous and exogenous cholesterol. This effect of hydrocortisone may play a role in inhibiting the replication of extrahepatic cells as exemplified by fibroblasts [16].

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