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Regulation of cholesterol biosynthesis in HeLa S3G cells by serum lipoproteins: Dexamethasone-mediated interference with suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase

(enzyme regulation/glucocorticoids/cholesterogenesis)

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ABSTRACT **Derepression of the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [mevalonate:NADP$^+$ oxidoreductase (CoA-acylating); EC 1.1.1.34] was elicited by the removal of serum from the growth medium of HeLa S3G cells with a concomitant expected increase in cellular sterol biosynthesis; if dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione) was present in the serumless medium, there was an augmentation of HMG-CoA reductase activity but a suppression of sterol biosynthesis. When human serum, human low density lipoprotein, or calf serum was present in the medium, there was a reduction of both the enzyme activity and sterol biosynthesis, but the presence of dexamethasone resulted in an increase in HMG-CoA reductase activity as compared to the controls containing human serum, low density lipoprotein, or calf serum alone. In contrast, either low density lipoprotein or whole serum supplementation eliminated the differences in acetate incorporation into sterols between glucocorticoid-treated and untreated cells. Human high density lipoproteins had little effect on the enzyme activity and abolished the difference in sterol biosynthesis only at relatively high concentrations. Addition of low density lipoproteins to cells after preincubation in serumless medium elicited the same rate of decay of HMG-CoA reductase ($t_{1/2}$ 3.8–4.2 hr) regardless of the presence of glucocorticoids in the medium, but there was an exaggerated lag before the onset of suppresion in the hormone-treated cells. If free cholesterol was present in the medium, the dexamethasone augmentation of HMG-CoA reductase was maintained, but the addition of either 7-ketocholesterol or 25-hydroxycholesterol abolished the difference between glucocorticoid-treated and control cells. These observations suggest that, under certain physiological conditions, HMG-CoA reductase activity no longer accurately reflects cellular sterol biosynthesis.**

Cultured mammalian cells regulate endogenous sterol biosynthesis depending on the cholesterol content of the medium (for reviews, see refs. 1 and 2). The rate-limiting step of this biosynthesis pathway is generally recognized to be the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonic acid, catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [mevalonate: NADP$^+$ oxidoreductase (CoA-acylating); EC 1.1.1.34] (3–5). Whether, in fact, this reduction is the rate-controlling step has been questioned recently (6–8), and it now appears that the control of sterol biosynthesis is more complex than previously believed.

The role of steroid hormones in the regulation of cholesterol biosynthesis is not clear, although it has been suggested (9) that a high serum estradiol/testosterone ratio may be associated with both high serum cholesterol and the atherosclerotic process. Studies involving glucocorticoid effects on cholesterogenesis *in vivo* have been in conflict (10–12), perhaps due to the superimposition of a diurnal cycle for both the pathway and hormonal fluctuations. The use of hormone-responsive cells in culture permits the isolation of a great many of these variables and is particularly valuable from two standpoints: in the study of glucocorticoid regulatory effects on cholesterol biosynthesis and the more general hormone-mediated induction of enzyme activity.

The present paper describes the response of HeLa S3G cells to serum and its lipoprotein components and the superimposition of glucocorticoid effects upon that response.

MATERIALS AND METHODS

Cells. HeLa S3G cells (HeLa$_{65}$), a strain that has a glucocorticoid-inducible alkaline phosphatase (13), were cultivated as described (13). In these studies, cells (1.3–1.5 × 10^6/60-mm petri dish) were grown for 24 hr in 5 ml of minimal essential medium supplemented with 2% calf serum. The medium was then replaced with 5 ml of fresh medium supplemented as indicated and incubated for the additional indicated times at 37° in a 5% CO_2/95% air atmosphere. Dexamethasone (9α-fluoro-11β, 17α, 21-trihydroxy-16α-methyl-1, 4-pregnadiene-3,20-dione) was added in 5 μl of ethanol, with controls receiving an equivalent amount of ethanol.

HMG-CoA Reductase Assay. The method of enzyme assay was identical to that previously reported (8), and is essentially a combination of the methods of Brown *et al.* (14), Dietschy and Brown (15), Avigan *et al.* (16), and Goldfarb and Pitot (17). In all cases, 50 μg of whole cell homogenate protein was assayed for 60 min at 37° (the homogenate was prepared with 60 strokes of a glass Dounce B homogenizer in a homogenization medium consisting of 1 mM potassium phosphate, pH 7.5/0.32 M sucrose/1 mM $MgCl_2$). The reaction was linear with respect to homogenate protein and time of incubation throughout the conditions used in these studies.

Sterol Synthesis. The rate of sterol synthesis was determined by pulse labeling cells for 1 hr with 1 μCi of [^{3}H]acetate per ml (New England Nuclear, 300 mCi/mmol) prior to harvesting at the indicated times after medium replacement. After the 1-hr pulse, cells were washed three times with 5 ml of 10 mM sodium acetate in 0.85% sodium chloride (pH 7) and then harvested in the same buffer by scraping the dishes with rubber policemen.

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoproteins; HDL, high density lipoproteins; LPDS, lipoprotein depleted serum; dexamethasone, 9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione.

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Cells were pelleted at 2000 × *g* and resuspended in 0.85% NaCl, representative portions were taken from this suspension for protein determination (18), and the cells were pelleted again. The resultant pellets were dissolved in 0.5 ml of 50% KOH for 30 min at 37°; then 2.5 ml of ethanol was added and the lipids were saponified for 2 hr at 60°. Nonsaponifiable lipids were extracted overnight into 4 ml of hexane and their radioactivity was determined in a Packard Tricarb scintillation spectrometer in a toluene-based cocktail containing 0.6% 2,5-diphenyloxazole and 0.02% 1,4-bis[2(5-phenyloxazolyl)]benzene.

Lipoproteins. Human lipoproteins were isolated from the pooled sera obtained from 12 healthy volunteers by sequential flotation as described (19); KBr was used for density adjustment. Each fraction was analyzed by agarose electrophoresis in barbital buffer and visualized by Fat Red 7B staining (20). The lipoproteins migrated as homogeneous bands, with lipoprotein-depleted serum (LPDS) showing no lipoprotein bands even when heavily loaded gels were used. The total cholesterol content of the lipoprotein fractions was determined (21) in a Varian 2100 gas/liquid chromatograph with coprostanol as an internal standard (22). All fractions were sterilized by filtration through 0.22 μm Millipore filters before addition to the cell cultures, and were used within 6 weeks after isolation. All calf sera were obtained from K. C. Biologicals, Inc. and delipidization was carried out by the method of Cham and Knowles (23). Cholesterol (Sigma CH-S grade) was purified by four recrystallizations from methanol followed by thin-layer chromatography (eluent, benzene/ethyl acetate, 7:3 vol/vol) on Silica Gel G followed by four further recrystallizations from methanol and used immediately after it was dried and dissolved in ethanol. 7-Ketocholesterol and 25-hydroxycholesterol were obtained from Steraloids and used without further purification; they appeared homogeneous by gas/liquid chromatography.

RESULTS

Previous observations (8, 24) on the effects dexamethasone exerts on sterol biosynthesis in HeLa cells and on the rate-limiting enzyme of the pathway are summarized in Fig. 1*A* and *B*. It is quite evident that the effects of opposite, i.e., the glucocorticoid stimulates HMG-CoA reductase activity while it suppresses sterol biosynthesis. The HMG-CoA reductase increase is observed in terms of either specific activity or total activity (Fig. 1*A*, numbers in parentheses) and the steroid augmentation is not apparent until 12–14 hr of exposure. The inhibition of sterol biosynthesis becomes evident almost immediately with short pulses of [^{3}H]acetate (Fig. 1*B*) and is also apparent when [^{14}C]acetate is used in the pulse or [^{3}H]acetate or ^3H_2O are used as continuous labels, although not when [^{3}H]mevalonic acid is used as the sterol precursor (24). In addition, continuous labeling with [^{14}C]octanoate or [^{14}C]glucose showed similar inhibitions (unpublished results). However, pulse labeling with glucose or tritiated water is impractical because of the high levels of radioactivity required for such studies. Further evidence for a reduction in the cellular cholesterol level was obtained from direct measurements of total cholesterol, which indicate less cellular sterol in dexamethasone-treated cells than in the controls (8).

All of these observations were made of necessity on cells that had been deprived of serum and so represent the derepression of sterol biosynthesis and the role glucocorticoids play during that process. The critical question we asked during the present studies was whether dexamethasone could exert some effect on sterol biosynthesis when the pathway was repressed by serum or its components. Fig. 1*C* demonstrates the time course of the dexamethasone-elicited induction of HMG-CoA reductase in the presence of delipidized and normal calf sera. It is evident that the induction occurs even when there is enough serum to repress the controls and that the induction occurs at the same

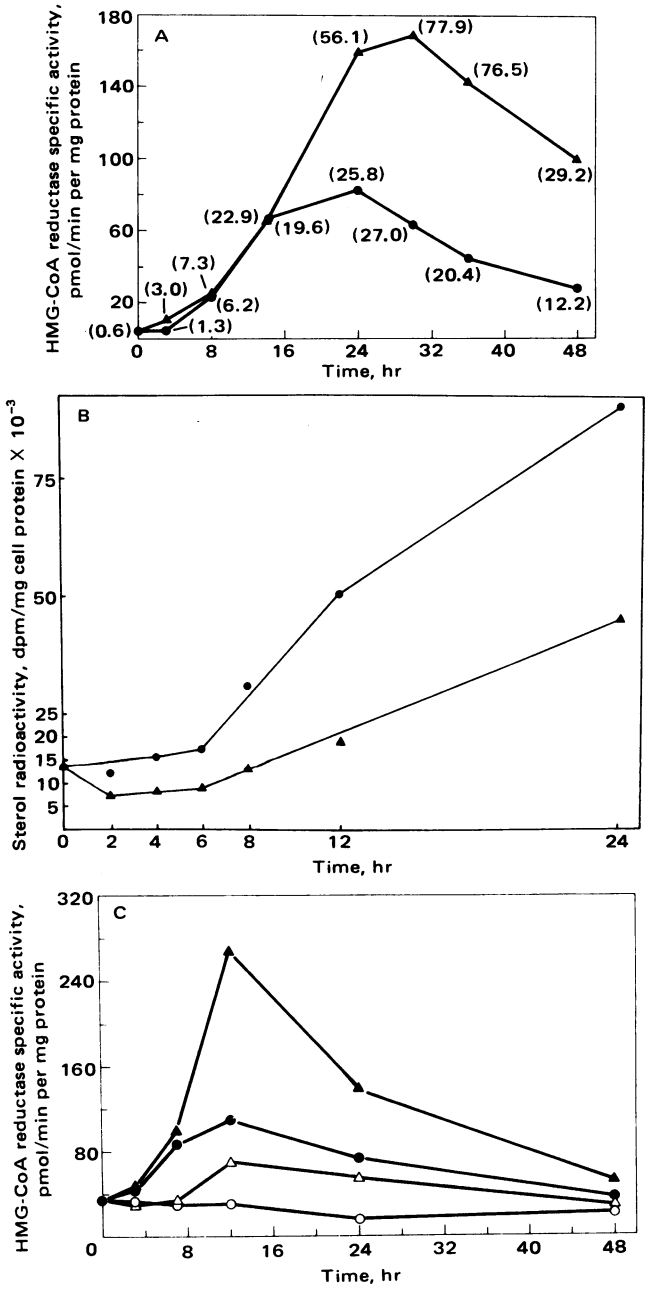

FIG. 1. Effect of growth of HeLa S3G cells in the presence of dexamethasone on HMG-CoA reductase activity and the rate of sterol biosynthesis in the absence of serum. (*A* and *B*) Cells were grown for 24 hr in minimal Eagle's medium supplemented with 2% calf serum. The medium was then replaced with unsupplemented minimal medium and either ethanol or dexamethasone (final concentration, 1 μM) was added. In *A* the activity of HMG-CoA reductase is shown (values in parentheses are the total activities); in *B* the rate of sterol biosynthesis is shown. Points represent the means of either three or four cultures with standard deviations less than 10%. ●, Control; ▲, 1 μM dexamethasone. (*C*) Cells were grown for 24 hr in minimal Eagle's medium supplemented with 2% calf serum. The medium was then replaced with minimal medium supplemented with either 5% calf serum or delipidized (23) serum (protein content equal to 5% serum), with dexamethasone or ethanol. Points represent the means of triplicate cultures with standard deviations less than 10%. ○, Control, 5% serum; △, 1 μM dexamethasone, 5% serum; ●, control, 5% delipidized serum; ▲, 1 μM dexamethasone, 5% delipidized serum.

time after hormone addition in either serum or delipidized serum. There appears to be very little difference in the induction in the presence of delipidized serum compared to that in the absence of serum (Fig. 1*A*) except for the earlier attainment of maximum activity. Fig. 2 demonstrates that low density lipoprotein (LDL) is the active regulatory component in serum that can modulate both sterol biosynthesis and HMG-CoA reductase activity. LDL was effective in suppressing both the pathway and the rate-controlling enzyme at low concentrations of cholesterol, whereas the same amount of cholesterol in the form of high density lipoprotein (HDL) had only a negligible effect at low concentrations and much less effect than LDL at higher concentrations (it is possible that even this effect was due to a small amount of contaminating LDL although that was undetected). It is generally assumed that the regulatory site of action of LDL is at a post-transcriptional level and that the mode of action is a decreased rate of HMG-CoA reductase synthesis (25). Since glucocorticoids had been shown to induce HMG-CoA reductase activity above the control level obtained in either the presence or absence of serum and since LDL exhibits characteristic effects when added to these cells, it was desirable to determine the effect dexamethasone exerted on the LDL-mediated suppression of both HMG-CoA reductase activity and sterol biosynthesis.

Table 1 displays the composite results of several experiments in which either serum or its lipoprotein components were added to cells that were growing in the presence of glucocorticoids compared to those obtained from control cells. It is evident that in the absence of any lipoprotein, dexamethasone elicited approximately a 2-fold increase in HMG-CoA reductase activity although there was a concomitant 40% inhibition of the rate of sterol biosynthesis. In both sets of cultures there were rapid concurrent decreases in both the rate of sterol synthesis and HMG-CoA reductase activity when increasing concentrations of either serum or LDL were included in the growth medium. However, in the presence of dexamethasone, the activity of HMG-CoA reductase was always greater regardless of the amount of cholesterol (from whatever source) in the medium.

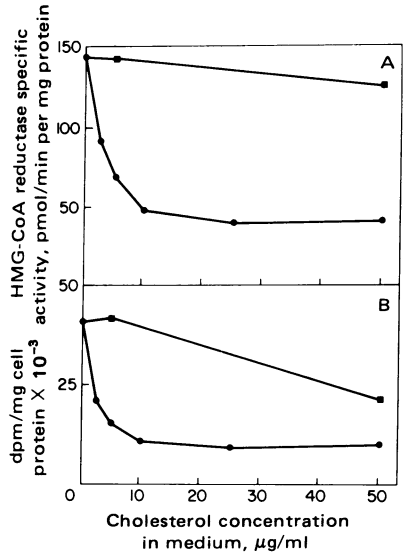

FIG. 2. Effect of growth in the presence of various concentrations of lipoproteins on HMG-CoA reductase activity (*A*) and sterol biosynthesis (*B*) in control cultures. (*A* and *B*) Cells were grown for 24 hr in minimal essential medium supplemented with 2% calf serum. The medium was replaced with unsupplemented minimal medium plus the indicated concentration of lipoprotein cholesterol and incubation was continued for 18 additional hr. All points represent the means of triplicate cultures and the experiment was repeated with essentially identical results. Standard deviations were less than 10%; controls had 2% LPDS added. ●, LDL; ■, HDL.

This was apparent not only with human serum, but also when calf serum was added to the medium. Conversely, the difference in sterol biosynthesis between control and dexamethasone-treated cells was abolished when even small amounts of human serum, calf serum, or LDL were present in the medium. The addition of LPDS to cultures (2%, an amount of protein equivalent to adding 30 μg of whole serum cholesterol per ml) had little effect on HMG-CoA reductase activity relative to the

Table 1. Response of HeLa S3G cells to serum lipoproteins in the presence and absence of dexamethasone (Dex)

Additions	Cholesterol, μg/ml	HMG-CoA reductase specific activity, % of control		Relative rates of incorporation of [^{3}H]acetate into nonsaponifiable lipids	
		−Dex	+Dex	−Dex	+Dex
None	—	100	177.0	100	60.0
Human serum	3	42.8	112.4	42.0	45.0
	7.5	14.5	24.0	28.2	34.7
	30	6.1	11.0	22.7	24.7
LDL	2.5	40.0	79.3	39.0	32.7
	10	12.5	26.8	21.5	19.7
	25	8.2	24.2	15.8	16.0
HDL	5	85.4	178.0	81.1	39.9
	50	76.2	135.0	30.1	40.1
LPDS	*	86.0	190.0	77.9	121.3
Calf serum	4	116.0	207.0	—	—
	17	68.5	127.3	54.8	66.3
	45	24.8	48.8	11.7	11.7
Delipidized calf serum	0.5†	109.9	221.5	120.0	—

Cells were grown as described in the legend to Fig. 2. At the time of medium supplementation, dexamethasone was added to half of the cells and ethanol to the other half, and the indicated serum fractions were added. After 23 hr the cells were pulse labeled and cultures were harvested for both sterol biosynthesis and HMG-CoA reductase determinations. All numbers represent the mean of three or four cultures and all experiments have been repeated two to four times. Standard deviations of all measurements were less than 15%.

* Same as 2% human serum by protein (equivalent to 25 μg of cholesterol per ml).

† Same as 5% calf serum by protein (equivalent to 45 μg of cholesterol per ml).

controls with no serum, but caused a reversal of the dexamethasone-mediated inhibition of the rate of acetate incorporation into cellular sterols; the reason for this reversal is not clear at present. When HDL was present in the medium at 5 μg of cholesterol per ml, the glucocorticoid-elicited differences were maintained with respect to both HMG-CoA reductase activity and the rate of sterol biosynthesis. The rate of acetate incorporation into saponifiable lipids was reduced in the presence of any of the lipid-containing serum fractions (serum, LDL, HDL, or LPDS) and LDL was not as effective in reducing incorporation into saponifiable as into nonsaponifiable lipids, regardless of the presence or absence of dexamethasone (unpublished results).

In order to determine whether the higher level of HMG-CoA reductase activity, which was present in dexamethasone-treated cells even in the presence of large amounts of LDL, was due to an altered response to the suppressive effects of the lipoprotein, we performed studies in which the kinetics of suppression by LDL were determined. Fig. 3A indicates that when HMG-CoA reductase activity was derepressed by serum removal, the addition of LDL caused a rapid decrease in the catalytic activity ($t_{1/2}$ of 3.8–4.2 hr, similar to those reported in refs. 25 and 26) of the enzyme from both control and dexamethasone-treated cultures. However, as is also apparent from Fig. 3A, growth in the presence of glucocorticoid caused a lag of 3 hr in the responsiveness of the cell to LDL-mediated HMG-CoA reductase suppression. This was evident only when the HMG-CoA reductase activity was increasing (in these experiments the peak of activity for both control and dexamethasone-treated cells was 18 hr after serum removal; in all other ways the curve was identical to Fig. 1A). As the results in Fig. 3B indicate, there was no apparent lag before the onset of suppression when LDL was added to the medium during the latter part of the curve, although the decay kinetics were still first order and the $t_{1/2}$ for decay was still 4 hr. Addition of dexamethasone at any time between 0 and 6 hr resulted in an increased peak level of HMG-CoA reductase activity, but if the steroid was added after the peak of activity, there was no apparent induction (unpublished observations).

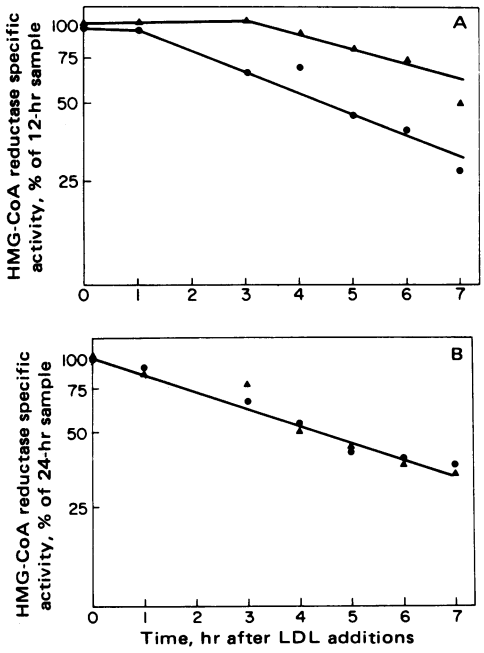

FIG. 3. Decrease in HMG-CoA reductase activity after addition of LDL. Cells were grown as described in the legend to Fig. 2 except that at the time of medium replacement dexamethasone (final concentration, 1 μM) was added to half the cultures and ethanol to the other half. Subsequently, the cells were incubated an additional 12 (*A*) or 24 (*B*) hr, at which times LDL was added to a final concentration of 10 μg of cholesterol per ml of medium. Triplicate samples were taken at the times indicated and assayed for HMG-CoA reductase activity. The data are expressed as the percentage of the initial value obtained at the time of LDL addition and were: 12 hr control, 183.0 pmol/min per mg of protein; 12 hr dexamethasone, 206.4; 24 hr control, 59.6; and 24 hr dexamethasone, 166.6. Values obtained at 18 hr were: control, 171.8; dexamethasone, 319.3. Each point represents the mean of three cultures; the experiments have been reproduced and standard deviations were less than 10%. ●, Control; ▲, dexamethasone.

Table 2 demonstrates that this dexamethasone-induced augmentation of HMG-CoA reductase was also evident in the presence of free cholesterol but not when the cells were grown with either 7-ketocholesterol or 25-hydroxycholesterol, oxygenated sterols which are much more inhibitory than cholesterol (4), a result which these data support. Our results also suggest that it is cholesterol itself rather than a metabolite that suppresses HMG-CoA reductase in these cells and that cholesterol present as serum or LDL is more effective than the free sterol in regulating the enzyme activity.

DISCUSSION

The results of these studies suggest that endogenous HeLa cell cholesterogenesis is regulated by serum lipoproteins in a manner analogous to that described for various other systems (14, 25, 27–30). In addition, when this regulation was examined in the presence of glucocorticoids, although sterol synthesis was modulated according to the cholesterol source and content of the medium, there was some interference which was manifested as a reduced level of suppression of HMG-CoA reductase. Thus, in the presence of serum or its lipoproteins, the apparent dissociation of the rate-limiting enzyme of the pathway was, perhaps, more significant than previously reported (8), providing additional evidence that under at least some physiological conditions, an alternate regulatory site of cholesterogenesis was at a different point than HMG-CoA reductase.

The mechanism by which glucocorticoids act to specifically

Table 2. Effect of cholesterol and oxygenated sterols on HMG-CoA reductase activity in HeLa S3G cells

Supplement		HMG-CoA reductase specific activity	
Compound	Conc.*	Control	1 μM dexamethasone
None		114.3 ± 10.5	207.2 ± 8.3†
Cholesterol	5	81.4 ± 11.0	165.7 ± 10.1†
	10	50.0 ± 7.4	77.9 ± 1.6†
	25	25.0 ± 4.6	43.0 ± 4.7†
7-Ketocholesterol	1	34.6 ± 4.0	43.5 ± 2.9
	5	16.1 ± 3.6	20.2 ± 0.7
	10	15.2 ± 5.3	23.4 ± 1.9
25-Hydroxycholesterol	1	16.5 ± 3.1	16.2 ± 5.2
	5	13.2 ± 4.0	18.0 ± 2.4
	10	15.4 ± 1.0	13.2 ± 0.8

Cells were grown as described in the legend to Fig. 2. At the time of medium supplementation dexamethasone, cholesterol, 7-ketocholesterol, and 25-hydroxycholesterol were added as indicated. Values represent means for triplicate cultures ± SD.

* μg/ml of medium.

† Significantly different from controls at $P < 0.01$.

elevate HMG-CoA reductase in these cells is still in question. However, it is clear that, by whatever means, either in the presence or absence of serum lipoproteins, there was greater enzyme activity in dexamethasone-treated cells than in controls in all cases, even though the difference in the rate of sterol biosynthesis seen in the absence of serum was abolished. This induction by hormone was specific for glucocorticoids, was dependent on both the time and concentration of steroid supplementation (unpublished observations), and could be eliminated by 24 hr of growth in the presence of cycloheximide or actinomycin D (8). Although it is possible that the dexamethasone effect in serum could have been caused by a decrease in either the number of LDL receptors or the efficiency of lipoprotein processing (31), this seems unlikely since the rate of sterol synthesis was rapidly suppressed to the same basal level in both control and treated cells. It is also unlikely that the hormone is acting by increasing cholesterol efflux, thereby effectively derepressing HMG-CoA reductase, since when mevalonate was used as the cholesterol precursor there was no longer a difference in the biosynthetic rate between treated and control cultures (8). Also, when cellular sterols were prelabeled for 48 hr in delipidized serum and then sterol radioactivity was followed for 24 hr there was no apparent difference in label turnover between steroid-treated and control cells (unpublished data). The indication that the half-life of decay of the enzyme activity was identical in both controls and in cultures exposed to hormone suggests that dexamethasone does not act by decreasing the degradative rate of HMG-CoA reductase. It is possible that the glucocorticoids allow the mRNA either to remain on polysomes for a longer period of time or to have an extended turnover time, as might be suggested by the extended lag phase evident in Fig. 3A. It is interesting that this lag only occurred in the time period during which the addition of dexamethasone was effective in eliciting an induction of enzyme activity.

The addition of lipoproteins did not affect cell growth within this relatively short time of exposure. This was verified by measuring total cell protein per culture, which remained unchanged. It is possible that there are more subtle alterations in cell populations (e.g., effects on the relative distribution of cells in different phases of the cell cycle).

The evaluation of the regulation of cholesterol biosynthesis in cultured cells has proved to be valuable (e.g., refs. 4, 5, 8, 25, and 27–30) since these systems offer the opportunity to isolate and control some of the many variables affecting this pathway. However, they can also lead to an oversimplification of the mechanisms of control which are probably acting in conjunction *in vivo*. As more and more variables are examined, the system of regulation that has been so elegantly described by Brown and Goldstein (31) might have to be modified and/or expanded to include the interplay of several hormonal and, possibly, other physiological factors.

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