Evidence for the stimulation of dolichol and mannoside synthesis by glucocorticoids in HeLa cells

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The effects of addition of 1 μ M-dexamethasone on the rate of transfer of mannose from GDP-mannose into mannoside have been studied in HeLa cell cultures. Concurrent with an increase in incorporation of mannose into glycoproteins, the incorporation of mannose from GDP-mannose *in vitro* into mannoside and dolichol-linked oligosaccharides was increased after dexamethasone treatment. Stimulation of mannoside synthesis showed a correlation with the 11 β ,17 α ,21-trihydroxy structure of C₂₁ steroids. Dexamethasone treatment also resulted in an increased incorporation of acetate into dolichol and dolichyl phosphate. The results suggest that the effect of dexamethasone on the cell-surface glycoprotein accumulation is related to increased sugar-linked dolichol synthesis.

According to the current concept of the biosynthesis of N-linked glycoproteins, a lipid intermediate is formed before the glycosylation of protein takes place. In eukaryotic cells this lipid intermediate has been well characterized as dolichyl phosphate saccharide (Parodi & Leloir, 1979). The glycosylation can be regulated by the cells through various mechanisms, one of which is the cellular concentration of dolichyl phosphate (Lucas & Levin, 1977; Harford *et al.*, 1977). Workers from several laboratories have examined the regulation of dolichol biosynthesis and its possible link with that of cholesterol (Mills & Adamany, 1978; James & Kandutsch, 1979, 1980; Harford *et al.*, 1979). In smooth-muscle cells, 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34) appears to be rate-limiting for both of these pathways and therefore co-ordinately controlled (Mills & Adamany, 1978). Findings in other systems suggest that although there is a common link between the two pathways, there could be a marginal degree of regulatory independence (James & Kandutsch, 1979, 1980; Keller *et al.*, 1979).

We have shown previously that the synthesis of cholesterol in HeLa cells is decreased when glucocorticoid hormones are added to the growth medium (Melnikovych *et al.*, 1976). However, hydroxy-methylglutaryl-CoA reductase showed no parallel decrease. Instead, there was an augmentation of the activity of this enzyme (Cavenee & Melnikovych,

1977; Ramachandran *et al.*, 1980a). Significantly, it is in these cells that glucocorticoid treatment causes an increase in cell-surface glycopeptides (Sinha & Melnikovych, 1973). Accumulation of glycoprotein (fibronectin) has been observed also in SV40-transformed human fibroblasts treated with dexamethasone (Furcht *et al.*, 1979). One plausible explanation for these changes in cell-surface composition may be that glucocorticoids cause an increased flux of mevalonate for dolichol synthesis, facilitating increased formation of prenol-linked saccharides, resulting in an increased glycoprotein synthesis. With the object of examining this possibility, we have studied the effect of dexamethasone on the synthesis of dolichol, dolichyl phosphate and prenol-linked mannose. The results suggest that dexamethasone, although suppressing cholesterol synthesis, stimulates the syntheses of these intermediates.

Experimental

Materials and methods

ICN Pharmaceuticals (Irvine, CA, U.S.A.) provided [2-¹⁴C]acetic acid (sp. radioactivity 55 Ci/mol). Other radiolabelled compounds, including GDP-[U-¹⁴C]mannose (sp. radioactivity 192 Ci/mol), [U-¹⁴C]mannose (sp. radioactivity 216.5 Ci/mol), [4,5-³H]leucine (sp. radioactivity 31.9 Ci/mmol) and [5,6-³H]fucose (sp. radioactivity 60.5 Ci/

mmol) were obtained from New England Nuclear Corporation. Sigma Chemical Co. (St. Louis, MO, U.S.A.) supplied dolichol, dolichyl phosphate mannose and dexamethasone. All other chemicals were analytical grade. HeLa S3G cells have been maintained in this laboratory for the past 11 years.

The method of cultivation of HeLa cells was as described previously (Ramachandran *et al.*, 1978). The culture medium was Eagle's minimal essential medium (Gibco) with 100 μ g each of penicillin and streptomycin/ml supplemented with 10% calf serum (K. C. Biologicals, Lenexa, KS, U.S.A.). The cultures were split 6–8-fold twice weekly using trypsin to detach cells from the surface.

For the experiments, cells were seeded in either 60 mm dishes (1–2 million cells/5 ml of medium) or in flasks (12–15 million cells/100 ml of medium) containing MEM supplemented with 2% calf serum. The cells were grown for 18 h. The medium was then replaced by serum-free medium. Glucocorticoids (1 μ M) were added at this time as ethanolic solutions. Control cultures received ethanol not exceeding 0.1%. The cells were used for the experiments 24 h after such additions.

Incorporation of sugars and amino acids into trichloroacetic acid-precipitable fraction

In these experiments, radioactive precursors at indicated concentrations were added to the cultures at the time of dexamethasone addition. After 24 h of incubation, the cells were scraped and transferred to tubes along with the medium. Bovine serum albumin (25 mg) was added to each tube. The tubes were chilled on ice and a cold solution of trichloroacetic acid was added to a final concentration of 25%. The tubes were allowed to stand on ice for 30 min. The precipitated protein was collected by centrifugation at 45000 *g* for 15 min and was washed four times with 5 ml portions of diethyl ether. The dried pellet was dissolved in 0.5 ml of formic acid and the radioactivity was measured using Aquasol (New England Nuclear) as the scintillation fluid.

Synthesis of mannosyl lipid in vitro

Cells harvested after 24 h of dexamethasone treatment were homogenized in 50 mM-Tris/HCl (pH 7.1) and the homogenate was used directly as the enzyme source. Transfer of [14 C]mannose from GDP-[14 C]mannose into glycolipid was assayed by the method described by Waechter & Harford (1977). The standard incubation mixture consisted of 50 mM-Tris/HCl (pH 7.1), 10 mM-MnCl₂, 2.5 mM-AMP, 16 μ g of dolichyl phosphate, 0.04% Triton X-100, 3 μ M-GDP-[14 C]mannose (273 c.p.m./pmol) and 500 μ g of protein in a total volume of 0.2 ml. Before addition, the dolichyl monophosphate was dispersed by sonication in 0.16% Triton X-100. Unless otherwise stated, the reaction mixtures were

incubated for 3 min at 37°C. The reaction was terminated by the addition of 4 ml of chloroform/methanol (2:1, v/v). The extraction mixture was allowed to stand at room temperature for 10 min and was then centrifuged at 600 *g* for 10 min. The supernatant solution was saved and the pellet was washed twice with 2 ml volumes of chloroform/methanol (2:1, v/v). The lipid extracts were pooled and washed with 0.2 vol. of 0.9% NaCl. The aqueous phase was discarded and the organic phase was again washed with 0.9% NaCl/methanol (1:1, v/v). The lower phase containing the [14 C]mannose-labelled lipids was transferred to scintillation vials, dried under a stream of air and counted in 10 ml of Econofluor (New England Nuclear). The mannose-linked lipid was identified on paper chromatograms using a solvent system consisting of isobutyric acid/NH₃/water (57:4:39, by vol.) as described by Waechter & Harford (1977).

Synthesis of dolichol-linked oligosaccharides and glycoproteins

Synthesis of dolichyl pyrophosphate oligosaccharides was measured in cell homogenates by a method basically similar to that described above. The assay system was scaled up 4-fold and the incubation was continued for 30 min. At the end of incubation, the tubes were cooled to 4°C and 5 vol. of chloroform/methanol (2:1, v/v) was added and kept at room temperature for 30 min. Three phases separated out. The insoluble material present at the interphase was washed three times with 4 vol. of butanol and three times with water. The residue was then extracted twice with 2 vol. of chloroform/methanol/water (4:4:1, by vol.). After the extraction, the residue, which contained glycoprotein, was dissolved in 0.5 ml of NCS (Amersham Corporation) and the radioactivity was measured. The extract was dried under N₂, redissolved in chloroform/methanol/water (10:10:3, by vol.) and applied to a DEAE-cellulose (acetate) column equilibrated with the same solvent. The bound lipids were eluted by sequential application of chloroform/methanol/water (10:10:3, by vol.) and chloroform/methanol/aq. ammonium acetate with increasing concentrations of ammonium acetate (1, 5, 10, 50 and 100 mM) (Herscovics & Bugge, 1980). The dolichyl phosphate oligosaccharides were eluted with 50 and 100 mM-ammonium acetate and the radioactivity was determined. The heterogeneity on DEAE-cellulose is probably due to the differences in carbohydrate composition.

Synthesis of dolichol and dolichyl phosphate

After 22 h of growth in the presence of dexamethasone, the cells were trypsinized and harvested. The cells from each flask (25–40 mg of protein) were collected by centrifugation and re-

suspended in 5 ml of MEM and transferred to 25 ml spinner flasks (Bellco Glass Co.). To each flask 10 μ Ci of [2- 14 C]acetate was added and the flasks were incubated at 37°C with stirring at very low speed for 2 h. At the end of incubation the cells were pelleted by centrifugation and treated with 5 ml of chloroform/methanol (2:1, v/v). Dolichol and dolichyl phosphate were isolated on a DEAE-cellulose column followed by high-pressure liquid chromatography by the method described by Keller *et al.* (1979). The high-pressure-liquid-chromatographic separation was carried out on a column of μ Porasil (Waters) at room temperature. Under our experimental conditions dolichol showed an absorption maximum of 215 nm.

Results

Results shown in Table 1 indicate that the incorporation of [14 C]mannose into trichloroacetic acid-precipitable protein was increased after dexamethasone treatment. Since we have combined the cells and the medium, this result represents the incorporation of the precursors into cellular and cell-originated protein. When [3 H]fucose was used as the precursor, there was no statistically significant

difference in the rate of incorporation between the two groups. Although we noticed a small decrease in the total protein content in dexamethasone-treated cultures, the incorporation of [3 H]leucine into protein showed no significant difference.

Based on these results and those reported previously (Sinha & Melnykovich, 1973), further investigation was carried out to examine whether or not glucocorticoid treatment affects the process of glycosylation of protein and its possible link with the steroid-induced changes in cholesterol biosynthesis (Ramachandran *et al.*, 1978; Johnston *et al.*, 1979). Since the formation of a dolichol-linked intermediate is known to occur before glycosylation of protein takes place, we measured the formation of this lipid-linked sugar using GDP-[14 C]mannose as the substrate in a cell-free system as shown.

The initial rate of formation of mannosylated protein increased with increasing protein concentration (Fig. 1a). Even in the absence of exogenous dolichol, a small amount of mannosylated protein was synthesized by the cell homogenates from GDP-mannose and the homogenates of dexamethasone-treated cells incorporated more label into the mannosylated fraction although this effect appeared evident only in the presence of relatively high concentrations of cell

Table 1. *Effect of dexamethasone on the incorporation of [3 H]fucose, [14 C]mannose and [3 H]leucine into trichloroacetic acid-precipitable fraction*

HeLa S3G cells were seeded in 60 mm petri dishes containing 5 ml of MEM supplemented with 2% calf serum. The cells were incubated at 37°C in an atmosphere of CO₂/air (1:19, v/v). After 18 h, the medium was replaced by serumless medium $\pm$ 1 μ M-dexamethasone (Dex). At this time, 5 μ Ci of [3 H]fucose (1 mM), 5 μ Ci of [3 H]leucine (1 mM) or 1 μ Ci of [14 C]mannose (1 mM) was added to the culture. The cells were incubated for an additional 24 h. The cellular and cell-originated proteins were then precipitated by trichloroacetic acid as described in the text. The values given are means $\pm$ S.D. for at least four cultures.

Precursor	10 ⁻³ $\times$ Radioactivity (d.p.m./culture)		10 ⁻³ $\times$ Radioactivity (d.p.m./mg of cell protein)	
	Control	Dex-treated	Control	Dex-treated
[5,6- 3 H]Fucose	29.7 $\pm$ 5.9	31.9 $\pm$ 7.5	60.0 $\pm$ 12.0	71.5 $\pm$ 16.7
[U- 14 C]Mannose	5.9 $\pm$ 1.1	8.8 $\pm$ 0.5	11.9 $\pm$ 2.1	19.8 $\pm$ 1.2
[4,5- 3 H]Leucine	77.8 $\pm$ 3.9	59.3 $\pm$ 9.0	157.3 $\pm$ 7.9	132.7 $\pm$ 20.2

Table 2. *Effects of C₂₁ steroids on mannosylated protein synthesis in HeLa S3G cells*

The experimental details and assay conditions are given in the text. Cells from at least two flasks were pooled to obtain enough enzyme. All steroids (1 μ M) were added as ethanolic solutions with the controls receiving an equal volume of ethanol. Values given are means $\pm$ S.D. for three separate assays.

Steroids (1 μ M)	10 ⁻³ $\times$ [14 C]-labelled mannosylated protein produced	
	(c.p.m./mg of protein)	Activity (%)
None	72.0 $\pm$ 1.8	100
Dexamethasone	97.9 $\pm$ 2.7*	136
Prednisolone	91.2 $\pm$ 2.8*	127
Cortisol	81.4 $\pm$ 0.8*	113
Cortisone	69.5 $\pm$ 6.8	97
Deoxycorticosterone	66.7 $\pm$ 5.6	94
Progesterone	67.0 $\pm$ 2.8	93

* Statistically different from control ($P < 0.01$).

Fig. 1. Rate of mannoside synthesis by HeLa cell homogenates

(a) The assay mixture contained various amounts of HeLa cell homogenate protein, 40 mM-Tris/HCl, pH 7.1, 10 mM-MnCl₂, 2.5 mM-AMP, 0.04% Triton X-100 (or 16 μg of dolichyl phosphate) and 3 μM-GDP-[U-¹⁴C]mannose (273 c.p.m./pmol), all in a final volume of 0.2 ml. Incubations were carried out for 3 min at 37°C. (b) Incubations were for the periods of time indicated; 0.5 mg of protein was used. The method of extraction of mannoside is described in the text. Dolichyl phosphate was dispersed by ultrasonication in 0.16% Triton X-100 and added to tubes. Δ and ▲, no dolichyl phosphate added; ○ and ●, dolichyl phosphate added. Open symbols indicate controls and closed symbols indicate homogenates from dexamethasone-treated cells. Each value represents the mean for three separate determinations. In the absence of dolichyl phosphate, the difference was significant ($P < 0.05$) only for a protein concentration of above 500 μg (Fig. 1a).

protein. Addition of exogenous dolichyl phosphate augmented the mannoside synthesis and the dexamethasone treatment caused a further increase. In all further studies 500 μg of protein was used.

Fig. 1(b) shows the reaction rate as a function of time of incubation. The rate of mannoside formation was much higher when dolichyl phosphate was

Table 3. Effect of dexamethasone (Dex) on the incorporation of acetate into dolichol, dolichyl phosphate and cholesterol

HeLa S3G cells were harvested by trypsinization 24 h after the dexamethasone treatment as described in the legend to Table 1. The cells were pelleted and resuspended in 5 ml of MEM and transferred to a spinner flask. [2-¹⁴C]Acetate (10 μCi) was added to each flask and the cells were incubated for 2 h at 37°C. After 2 h the cells were pelleted and treated with chloroform/methanol (2:1, v/v). Extraction and determination of dolichol, dolichyl phosphate and cholesterol were carried out by the method described by Keller *et al.* (1979).

Product	10 ⁻² × Acetate incorporated (c.p.m./mg of protein)	
	Control	Dex-treated
Dolichol	16.4 ± 2.33	22.68 ± 2.92
Dolichyl phosphate	4.79 ± 0.59	6.75 ± 0.87
Cholesterol	2616 ± 131	1570 ± 126

added. The reaction rate increased rapidly and levelled off within 3 min of incubation and proceeded at a slow rate. Similar rapid and slow reactions for the mannoside synthesis have been shown in rat liver microsomes (Kato *et al.*, 1980). Dexamethasone-treated cells showed a higher rate of mannoside synthesis both in the presence and absence of exogenously added dolichyl phosphate.

Chromatographic separation on silica gel indicated that the mannoside had an R_F corresponding to that of dolichyl phosphate mannose (Harford *et al.*, 1979). It was also observed that the mannoside synthesis increased with the addition of increasing amounts of exogenous dolichyl phosphate to the assay mixture, dexamethasone-treated cells showing higher rates of synthesis (results not shown).

To determine if there was any relation between glucocorticoid activity and the stimulation of mannoside synthesis, several steroids at 1 μM concentration were added to the cultures. As shown in Table 2, the stimulatory effect correlated with glucocorticoid potency, i.e. dexamethasone, prednisolone and cortisol stimulated mannoside synthesis, whereas cortisone, progesterone and deoxycorticosterone were inactive. Similar specificity has been observed previously in these cells with regard to changes in hydroxymethylglutaryl-CoA reductase and hydroxymethylglutaryl-CoA synthase (Cavenee & Melnykovych, 1977; Ramachandran *et al.*, 1978).

Since intracellular concentration of dolichyl phosphate may regulate the synthesis of mannoside (Lucas & Levin, 1977; Harford *et al.*, 1977), it was interesting to examine the synthesis of this intermediate in glucocorticoid-treated cells. This was

Table 4. *Effect of dexamethasone on the synthesis of [^{14}C]mannosylated oligosaccharide lipid and glycoproteins*
 The reaction mixture consisting of the cofactors given in the legend to Fig. 1 was scaled up 4-fold and incubated with 2mg of homogenate protein for 30min at 37°C. At the end of incubation, oligosaccharide lipids and the glycoprotein fractions were separated by the method of Herscovics & Bugge (1980). The values given are means $\pm$ S.D. for three experiments. Abbreviations used: dol. phos., dolichyl phosphate; Dex, dexamethasone.

Treatment	Radioactivity incorporated (c.p.m./mg of protein)			
	^{14}C -labelled oligosaccharide-lipid		Glycoprotein	
	-dol. phos.	+dol. phos.	-dol. phos.	+dol. phos.
Control	594 $\pm$ 84	1046 $\pm$ 85	4210 $\pm$ 152	5639 $\pm$ 179
Dex-treated	955 $\pm$ 85	2173 $\pm$ 200	4489 $\pm$ 875	9105 $\pm$ 402

particularly important since glucocorticoids induce the activity of hydroxymethylglutaryl-CoA reductase (Ramachandran *et al.*, 1980a) and this enzyme is implicated in the regulation of dolichol synthesis (Mills & Adamany, 1978). As shown in Table 3, the synthesis of dolichol from acetate was higher in dexamethasone-treated cells than in the controls ($P < 0.05$). A similar increase in the synthesis of dolichyl phosphate was observed also in dexamethasone-treated cells. The radioactivity from acetate incorporated into these two fractions was very low when compared with that incorporated into cholesterol (Table 3) and fatty acids (results not shown); therefore, a very high level of radioactivity had to be used to obtain measurable radioactivity in these fractions. Consistent with our previous report (Melnikovych *et al.*, 1976) synthesis of cholesterol was decreased in dexamethasone-treated cells.

In the reaction sequence for the glycosylation of proteins, dolichyl phosphate mannose synthesis is followed by the formation of dolichyl phosphate-linked oligosaccharides. We examined the incorporation of [^{14}C]mannose from GDP-[^{14}C]mannose into dolichyl phosphate-linked oligosaccharides and into glycoproteins. A marked increase in the radioactivity incorporated into oligosaccharide-lipid and glycoproteins was observed as a result of dexamethasone treatment (Table 4). It was also observed that in the presence of exogenously added dolichyl phosphate, the formation of these constituents was faster (Table 4).

Discussion

The results presented here show that: (a) dexamethasone increased glycoprotein synthesis in the HeLa cells; (b) syntheses of dolichol, dolichyl phosphate and dolichol-linked mannose are enhanced as a result of dexamethasone treatment and (c) the effect of dexamethasone on mannolipid synthesis is associated with glucocorticoid potency.

The present finding helps to explain how glucocorticoids bring about changes in lipid metabolism and glycoprotein synthesis. Increased incorporation

of mannose into trichloroacetic acid-precipitable fraction by the whole cells is consistent with our previous finding that these cells accumulate surface sialopeptides when grown in the presence of glucocorticoids (Sinha & Melnikovych, 1973). Further experiments using cell homogenates (Table 4) suggest that this increase in mannose incorporation should be due to increased mannosylation of the protein. While this work was in progress, Baumann *et al.* (1980) showed that in HTC cells dexamethasone causes increased synthesis of certain classes of secretory glycoproteins.

The significance of glucocorticoid-induced increase in glycosylation is not clearly understood. One possibility is indicated by the findings of Furcht *et al.* (1979), who have shown that dexamethasone increases fibronectin accumulation in SV40-transformed cells. Perhaps the mechanism by which dexamethasone changes the morphology of the transformed cells closer to normal cells is by increasing the surface fibronectin, which is a glycoprotein involved among other things in intercellular adhesion and attachment of cells to a growth surface (Hughes, 1975).

During increased demand for glycoprotein synthesis, cells can increase the glycosylation capacity by several possible mechanisms. These include (a) increased formation and recycling by several dolichols, (b) increase in the concentration of dolichyl phosphate and (c) increasing the number of nascent chains of proteins available for glycosylation. Evidence for these possibilities has been accumulating in the literature (Lucas & Levin, 1977; Harford *et al.*, 1977; Keller *et al.*, 1979). Stimulation by dexamethasone of the synthesis of dolichol-linked mannose, oligosaccharide lipids as well as dolichols is consistent with these mechanisms.

Since intracellular dolichyl phosphate concentration can control the mannosyltransferase activity (Harford & Waechter, 1980), an increase in the synthesis of this glycosyl-carrier lipid and concurrent decrease in cholesterol biosynthesis in dexamethasone-treated cells are noteworthy. A similar correlation was noticed by Hemming's group

in the liver on inclusion of cholesterol in the diet (Coolbear & Hemming, 1979; Tavares *et al.*, 1979). On the contrary, James & Kandutsch (1980) recently showed that both cholesterol and dolichol syntheses are decreased by dietary cholesterol. However, these authors did not find any correlation between decreases in dolichol synthesis and manose incorporation into liver and plasma glycoproteins. On the other hand, inhibitors of cholesterol synthesis inhibited dolichol synthesis as well as decreasing the synthesis of dolichol-dependent glycoproteins in smooth-muscle cells (Mills & Adamany, 1978). Since the cell's capacity to synthesize cholesterol far exceeds that to synthesize dolichol (Table 1; James & Kandutsch, 1979, 1980; Keller *et al.*, 1979), it seems unlikely that hydroxymethylglutaryl-CoA reductase would be the common rate-limiting step for both biosynthetic pathways. The alternative possibility might be the presence of separate cellular pools of mevalonate for cholesterol and dolichols. However, no report is hitherto available on the separate pools of mevalonate, although hydroxymethylglutaryl-CoA reductase is known to be located in both mitochondria and microsomes (Preiss & Lehoux, 1979). In dexamethasone-treated cells, hydroxymethylglutaryl-CoA reductase activity remains at an induced level, whereas hydroxymethylglutaryl-CoA synthase is inhibited (Ramachandran *et al.*, 1978, 1980b). Since inhibition at the hydroxymethylglutaryl-CoA synthase step decreases the C_2 flux towards mevalonate to a great extent, this will significantly decrease cholesterol synthesis. Still mevalonate synthesis takes place solely based on the availability of hydroxymethylglutaryl-CoA. This would sufficiently meet the increased demand for dolichyl phosphate, favoured by augmented hydroxymethylglutaryl-CoA reductase activity.

If such a mechanism is operative in the dexamethasone-treated cells, this might explain the increased synthetic rate of dolichyl phosphate in the cells. However, the parallel increase in mannosyl synthesis need not necessarily be exclusively due to the increase in dolichyl phosphate. Since the response of mannosyltransferase(s) activity to exogenous dolichyl phosphate was greater in the homogenates from dexamethasone-treated cells, the increase in dolichyl phosphate may not be of major significance in the augmentation of glycosylation process. It is possible that dexamethasone affects mannosyltransferase(s) in an independent manner not involving the increase in the concentration of cellular dolichol. Clearly, more work is needed to establish the mode of action of dexamethasone in the modulation of mannosyl transfer.

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