Effect of dexamethasone on mannosid synthesis by hepatocytes prepared from control and inflamed rats

Mohan SARKAR and Sainen MOOKERJEA*

Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland A1B 3X9, Canada

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Hepatocytes were prepared from control and inflamed rats. Mannose incorporation into dolichol monophosphate mannose in homogenate and microsomal fraction of the hepatocytes was increased 2-fold over the controls 24 h after induction of inflammation by turpentine injection. Incubation of hepatocytes from both control and inflamed rats with 0.1–10 μM -dexamethasone produced a 1.5-fold increase of dolichol phosphate mannose formation, whereas, 100 μM -dexamethasone decreased its formation. The increase in the ratio of dolichol phosphate mannose formation in inflamed over controls was virtually eliminated when the cell homogenate assay mixtures included 30 nmol of exogenous dolichol phosphate. This supports the earlier suggestion that the increase in the enzyme activity in inflammation could be due to higher concentrations of endogenous dolichol phosphate [Coolbear & Mookerjea (1981) *J. Biol. Chem.* **256**, 4529–4535]. In contrast, the increase in the ratio of dolichol phosphate mannose formation between dexamethasone-treated and untreated hepatocytes remained unchanged when increasing concentrations of exogenous dolichol phosphate were added to the assays. This suggests that the increase in glycosylation of dolichol phosphate in dexamethasone-treated hepatocytes is probably due to the increased mannosyltransferase activity, rather than due to higher concentrations of endogenous dolichol phosphate in these cells.

The 'acute-phase reactants' are certain plasma protein components whose concentrations are significantly increased in response to inflammatory agents (Glenn *et al.*, 1968; Ashton *et al.*, 1970; Jamieson *et al.*, 1972, 1975; Koj, 1974). Almost all acute-phase reactants contain significant amounts of carbohydrate, and all are synthesized in liver parenchymatous cells. Although the mechanism of action of the inflammatory response is not fully known, it has been suggested that the initial response to inflammation is the release of an activator from the inflammatory site (Koj, 1974). The elevated concentration of the acute-phase reactants was shown to be the result of increased synthesis by liver (Maung *et al.*, 1964; Neuhaus *et al.*, 1966; Koj & McFarlane, 1968; Gordon, 1976). More recent studies have shown (Langstaff *et al.*, 1980) that inflammation invariably resulted in a marked increase in serum cortisol concentration in rats. Adrenalectomy caused a pronounced

decrease in the response of some of the acute-phase reactants to turpentine-induced inflammation, which were restored by replacement therapy with cortisol (Weimer & Benjamin, 1966; Weimer & Coggs, 1967). Other studies with hepatoma tissue-culture cells (Baumann *et al.*, 1983), perfused liver and hepatocyte suspension (John & Miller, 1969; Jeejeebhoy *et al.*, 1977) have indicated that dexamethasone or other corticosteroids promoted the synthesis of acute-phase reactants. Increased activity of several nucleotide-sugar:glycoprotein glycosyltransferases have been demonstrated in liver membranes from inflamed rats (Turchen *et al.*, 1977; Lombart *et al.*, 1980; Coolbear & Mookerjea, 1981). Most, if not all, serum glycoproteins are synthesized via dolichol-linked intermediates (Behrens, 1974; Hemming, 1974; Parodi & Leloir, 1979). Inflammation has been shown to increase the concentrations of some of these intermediates and also to increase some of the enzymic steps of dolichol-linked glycosylation (Mookerjea *et al.*, 1983). We therefore decided to study the

* To whom requests for reprints should be addressed.

effect of dexamethasone on the mechanisms involved in the formation of dolichol-dependent intermediates of glycoprotein synthesis.

Materials and methods

Reagents and chemicals

GDP-[U- 14 C]mannose (specific radioactivity 269 mCi/mmol) was purchased from New England Nuclear Corp., Lachin, Quebec, Canada. Dolichol phosphate and collagenase (lot. no. 82F-6822) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Waymouth medium MB 752/1 was from Gibco Laboratory Supplies, Grand Island, NY, U.S.A., and frozen donor horse serum was from Flow Laboratories, Melean, VA, U.S.A. The phosphate salt of dexamethasone (Hexadrol) from Organon (Canada), Toronto, Ont., Canada, was used because of its solubility in aqueous medium. All other reagents were of analytical grade.

Animals and induction of inflammation

Starved (24h) male Sprague-Dawley rats (300–350g body wt.) were divided into two groups. Inflammation was induced in one group by the subcutaneous injection of turpentine (0.5 ml/100g body wt.) in the dorsolumbar region, equal volumes being injected to either side of the midline. The period of inflammation was 24h. The control group was injected in an identical fashion with 0.9% NaCl.

Preparation of hepatocytes

Isolation of hepatocytes was carried out by slight modification of the established methods (Seglen, 1976). Starved (24h) male Sprague-Dawley rats (300–350g body wt.) were anaesthetized with diethyl ether, and for 4 min a non-circulating perfusion of the liver was performed through the portal vein with 400 ml of Ca^{2+} -free Hanks solution (Hanks & Wallace, 1949) containing 0.5 mM-EGTA at a flow rate of 40 ml/min. Then a recirculating perfusion was established for 15 min with modified Hanks solution containing 0.12% collagenase and 4 mM- Ca^{2+} . The temperature was maintained at 37°C, and the perfusion was flushed with an O_2/CO_2 (19:1) gas mixture (carbogen). The swollen liver capsule was opened with scissors, and the cells were dispersed by gentle agitation. The undispersed cells were removed by filtration on four layers of sterile gauze, and the filtrate was centrifuged at 70g for 1 min to obtain the cells. The cells were suspended in 30 ml of Krebs & Henseleit (1932) medium or with Waymouth medium MB 752/1 adjusted to pH 7.4 with Na_2CO_3 (Krul & Dolphin, 1982). Krebs-Henseleit medium was mixed with 2% (w/v) bovine serum albumin, 5 mM-glucose and amino acid mixture as

present in Waymouth medium MB 752/1 with the exception of cysteine.

Incubation of hepatocytes

A suspension of hepatocytes from one liver (30 ml, 150×10^6 cells) was incubated in two (one treated with dexamethasone and the other untreated) 250 ml Erlenmeyer flasks with Krebs-Henseleit medium or Waymouth medium MB 752/1 supplemented with 17.5% (v/v) heat-inactivated (heated at 56°C for 30 min) horse serum in a total volume of 55 ml. In all experiments the medium contained penicillin (60 i.u./ml) and streptomycin sulphate (0.05 mM). The experimental cells were incubated with different dosages of dexamethasone phosphate, whereas an equal volume of 0.9% NaCl solution was added to untreated cells.

The cell suspensions were maintained at 37°C with gentle shaking and continuous gassing with carbogen.

To estimate the number of viable cells during incubation, lactate dehydrogenase activity was monitored periodically (Höberg *et al.*, 1975). Hepatocytes prepared in our hands retained more than 80–85% of total measurable lactate dehydrogenase activity. Trypan Blue-exclusion tests (Bergmeyer & Bernt, 1974) also showed 80–85% viability of the cells throughout incubation. The hepatocytes prepared in our laboratory were found to synthesize glycoproteins and responded to the effects of colchicine and tunicamycin. On tunicamycin treatment the overall incorporation of carbohydrate into glycoprotein was found to be significantly impaired, whereas with colchicine the glycoprotein secretion was decreased, resulting in an increased retention of the intracellular glycoproteins.

Homogenization and fractions of hepatocytes

The cells were washed with 0.25 M-sucrose solution, suspended in 20 ml of 0.145 M-NaCl in 10 mM-Tris/HCl buffer, pH 7.4, and homogenized in a glass mortar with a Teflon pestle for about ten strokes at 500 rev./min. Further disruption of cells was done by sonication with the fine tip of a Branson Sonifier (model S-125; Branson Instruments, Melville, Long Island, NY, U.S.A.) at a setting of 0.5 A. Sonication was performed in a cooling bath for a total of 30 s, with 10 s for each burst at 2 min intervals. The debris and nuclei were removed by centrifugation at 480g for 10 min. The supernatants were then centrifuged at 4300g for 20 min, and the pellets were resuspended in homogenizing buffer.

For preparation of total microsomal fraction the suspension of the disrupted cells was centrifuged at 10000g for 20 min and the supernatant was centrifuged at 105000g for 60 min to give the total micro-

somal fraction. The marker-enzyme assays for fractions were done as described by de Duve *et al.* (1955), and protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Assay of mannosyltransferase

Hepatocyte homogenate or other subcellular fraction (0.3 mg of protein) was incubated with GDP-[U- 14 C]mannose (0.14 nmol) in a final volume of 80 μ l containing 6.32 mM-MnCl₂, 1.45 mM-EDTA, 18.9 mM-Tris/HCl buffer, pH 7.4, and 0.03% Triton X-100. Dolichol phosphate, when included in incubations, was first evaporated to dryness and then solubilized in the required amount of Triton X-100 by sonication in a Branson (Shelton, CT, U.S.A.) Ultrasonic Cleaner. Incubations were performed at 37°C for 2 min or as stated otherwise in the text. Dolichol phosphate mannose was extracted (Coolbear *et al.*, 1979) by stopping the incubation mixture with 2 ml of chloroform/methanol (2:1, v/v) and 2 ml of water. The chloroform phase was removed, dried under N₂ and counted for radioactivity in 12 ml of toluene/Omnifluor (New England Nuclear Corp.).

Characterization of mannolipid formed in the assay

T.l.c. was performed on silica-gel plates (Analtech, NY, U.S.A.) to characterize the dolichol phosphate mannose by the method described by Coolbear & Mookerjee (1981). The chromatogram was developed in chloroform/methanol/water (65:25:4, by vol.), and the identity of the product was established by comparing its *R_F* (0.34) with that of authentic dolichol phosphate mannose. Chromatography was carried out for 48 h at room

Fig. 1. Effect of different doses of dexamethasone on the synthesis of mannolipid by the hepatocytes

Hepatocytes isolated from control and turpentine-inflamed rats were incubated with 0.1–100 μ M-dexamethasone phosphate at 37°C for 4 h in Krebs-Henseleit medium. The enzyme activity was measured in the homogenates. The assay mixture contained 0.3 mg of homogenate protein, 6.32 mM-MnCl₂, 1.45 mM-EDTA, 18.9 mM-Tris/HCl buffer, pH 7.4, 0.03% Triton X-100 and GDP-[U- 14 C]mannose (0.14 nmol) in a final volume of 80 μ l. Other details of incubation, mannolipid extraction and measurement of radioactivity are described in the Materials and methods section. The panels are as follows: (a) and (b) are controls without or with dolichol phosphate respectively; (c) and (d) are preparations from inflamed rats without or with dolichol phosphate respectively. Dose of dexamethasone: $\circ$, none; $\bullet$, 0.1 μ M; Δ , 1 μ M; $\square$, 10 μ M; $\blacksquare$, 100 μ M. The results are from a typical one of three separate experiments.

Table 1. Effect of dexamethasone on the synthesis of mannolipid by rat hepatocytes

Hepatocytes isolated from control and inflamed rats were incubated in Krebs-Henseleit medium for 4 h either in the presence (1 μM) or in the absence of dexamethasone phosphate. The measurement of enzyme activity in homogenates and the mannolipid extraction procedures are described in the Materials and methods section. Each value represents the mean for six experiments. The values given are means ± s.d.

Treatment	Specific activity			
	Without dolichol phosphate		With dolichol phosphate	
	(pmol/mg of protein)	(% increase)	(pmol/mg of protein)	(% increase)
Saline control (0.9% NaCl)	3.52 ± 0.39		6.01 ± 0.99	
Dexamethasone	4.98 ± 0.7*	41	8.43 ± 1.3*	40
Inflamed (turpentine)	6.8 ± 0.81*		9.65 ± 0.73*	
Inflamed and dexamethasone	9.37 ± 1.14*	37	13.22 ± 1.57*	37

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

* Significantly different from control (P < 0.01).

Fig. 2. Effect of dexamethasone on the rate of mannolipid synthesis by hepatocytes up to 4 h in Krebs-Henseleit medium

Arrows indicate addition of dexamethasone phosphate at different time intervals. At these times the cells were removed and homogenized, and the homogenate was used to measure the formation of dolichol phosphate mannose. The panels are as follows: (a) and (b) are controls without or with dolichol phosphate respectively; (c) and (d) are preparations from inflamed rats without or with dolichol phosphate respectively. Dose of dexamethasone: ○, none; ●, 1 μM. Other details of cell isolation, incubation medium and enzyme assays are indicated in Fig. 1 legend. The results are from a typical one of three separate experiments.

temperature on Whatman no. 1 paper (5cm $\times$ 35cm), with the upper phase of butan-1-ol/acetic acid/water (4:1:5, by vol.) as the developing solvent. The mannoslipid was detected by cutting the chromatogram into 0.5cm sections that were then counted for radioactivity as described above. The mannoslipid appeared as a single peak 14.5cm from the origin.

Results

The formation of dolichol phosphate mannose proceeds rapidly in the homogenates prepared from isolated hepatocytes incubated for 4h in Krebs-Henseleit buffer (Figs. 1a–1d). The overall rate of mannoslipid formation in the hepatocytes from inflamed rats (Figs. 1c and 1d) is higher than in hepatocytes from control rats (Figs. 1a and 1b). In both control and inflamed hepatocytes, dexamethasone at a dose of 0.1–10 μ M increased the rate of mannoslipid formation. However, a higher dose of 100 μ M-dexamethasone decreased the mannoslipid formation in both control and inflamed-rat hepatocytes.

Results in Table 1 show the effect of 1 μ M-dexamethasone on mannoslipid formation in homogenate of the hepatocytes from control and inflamed rats. Assays were done for 2min, and the results from six different paired experiments were statistically analysed. Dexamethasone caused a significant increase (37–41%; $P < 0.01$) of mannoslipid formation in both control and inflamed-rat cells assayed with or without exogenous dolichol phosphate. Results in Table 1 also show that the mannoslipid formation is about 1.5–2-fold greater in the hepatocytes of inflamed rats than in controls. The mean rate of mannose incorporation for the six paired experiments from control hepatocytes was between 3.52 ± 0.39 (endogenous) and 6.01 ± 0.99 (with dolichol phosphate) pmol/2min per mg of protein (Table 1).

Mannosyltransferase activity in hepatocytes of control and inflamed rats was maintained at a steady level for about 4h (Figs. 2a–2d). Addition of

Fig. 3. Effect of dexamethasone on the rate of mannoslipid synthesis by hepatocytes up to 12h in Waymouth medium. Hepatocytes were incubated over a 12h period in Waymouth medium supplemented with 17.5% (v/v) heat-inactivated horse serum. Arrows indicate addition of dexamethasone phosphate and determination of formation of dolichol phosphate mannose at different time intervals. The panels and symbols are as described in Fig. 2 legend. Other details of cell isolation and enzyme assays are as indicated in Fig. 1 legend. The results are from a typical one of three separate experiments.

dexamethasone in the incubations at different intervals resulted in increased mannolipid formation in assays with (Figs. 2*b* and 2*d*) or without exogenous dolichol phosphate (Figs. 2*a* and 2*c*).

Fig. 3 shows the results of a similar study on the effect of dexamethasone on mannolipid formation by hepatocytes incubated for longer times. In these experiments, cells were incubated in Waymouth medium MB 752/1 supplemented with heat-inactivated horse serum. Hepatocytes incubated in this medium remained viable for at least 12 h. The mannosyltransferase activity assayed in presence of dolichol phosphate was markedly increased by treatment with dexamethasone (Figs. 3*b* and 3*d*). The enzyme activity of hepatocytes from control and inflamed rats remained at a constant level during 12 h of incubation, whereas treatment with dexamethasone increased the enzyme activity during 4–12 h of incubation (Figs. 3*a* and 3*c*).

As dexamethasone produced a greater increase of mannosyltransferase activity in the assays with exogenous dolichol phosphate, the effect of increasing quantities of dolichol phosphate (0–30 nmol) on mannolipid formation was studied in hepatocytes incubated for 8 h. The ratio of enzyme activity between the control and dexamethasone-treated groups as a function of increasing amounts of dolichol phosphate is shown in Fig. 4. The dexamethasone treatment of the cells, from either control or inflamed rats, maintained a much higher rate of enzyme activity in the presence of all concentrations of dolichol phosphate tested. The results in Fig. 4 also show that the increase in ratio of the enzyme activity between the hepatocytes from control and inflamed rats was decreased to near unity with increasing concentrations of dolichol phosphate in the assay.

Subcellular distribution of mannosyltransferase

The cell fractions of the hepatocytes prepared from either control or inflamed rats, treated with dexamethasone or untreated, maintained the higher values of their respective enzyme specific activities. Mannosyltransferase activity was enriched in microsomal fractions in all the experiments (Table 2). Both the specific activity and the total enzyme activity were increased in the microsomal fraction as a result of either inflammation or dexamethasone treatment. The increase of such enzyme activity in total homogenate was somewhat less than that in the microsomal fraction.

Discussion

Hepatocytes prepared from rat liver and incubated in two different media have been shown to synthesize mannolipid. Addition of dexamethasone to hepatocytes prepared from either

Fig. 4. Effect of increasing amounts of exogenous dolichol phosphate on mannosyltransferase activity

Hepatocytes prepared from control and inflamed rats were incubated in Waymouth medium for 8 h either in the presence (1 μ M) or in the absence of dexamethasone phosphate. Enzyme activity was measured in the homogenates in the presence of increasing quantities of dolichol phosphate (0–30 nmol). Results are presented as ratios of activities between inflamed rats and controls in the absence of dexamethasone (●), control cells in the presence and in the absence of dexamethasone (○) and inflamed-rat cells in the presence and in the absence of dexamethasone (□).

control or inflamed rats caused increased synthesis of dolichol phosphate mannose. The effect of dexamethasone was dose-dependent up to 10 μ M, but at a much higher dose of dexamethasone (100 μ M) the mannosyltransferase activity was inhibited in all experiments studied. The increase of enzyme activity resulting from dexamethasone treatment compared with untreated hepatocytes was evident even when the assay mixtures contained up to 30 nmol of exogenous dolichol phosphate. This suggests that the amount of dolichol phosphate as acceptor is not the limiting factor for the increased enzyme activity. In a previous study with control and inflamed rats it was shown that the increase in the ratio of mannosyltransferase activity in the microsomal fraction resulting from inflammation was eliminated by adding increasing concen-

Table 2. *Subcellular localization of mannosyltransferase*
 Experimental details are described in the Materials and methods section. Values in parentheses indicate the total activity.

Treatment	Subcellular fraction	Specific activity			
		Succinate:cytochrome <i>c</i> reductase (μmol of cytochrome <i>c</i> reduced/min per mg of protein)	NADPH:cytochrome <i>c</i> reductase (μmol of cytochrome <i>c</i> reduced/min per mg of protein)	β -Glucuronidase (μmol of glucuronic acid released/min per mg of protein)	Mannosyltransferase (pmol of product/2 min per mg of protein)
Control	Homogenate	0.028 (3.52)	0.048 (6.04)	0.13 (16.38)	2.05 (258)
	Debris (480 g pellet)	0.0015 (0.15)	0.002 (0.2)	0.05 (5.0)	0.9 (90)
	Mitochondria (4300 g pellet)	0.18 (2.61)	0.015 (0.2)	0.09 (1.2)	1.2 (90)
	Microsomes (105 g pellet)	0.006 (0.06)	0.24 (2.4)	0.06 (0.6)	4.95 (49.5)
Dexamethasone	Homogenate	0.032 (4.03)	0.052 (6.55)	0.12 (15.2)	3.1 (390)
	Debris (480 g pellet)	0.005 (0.5)	0.003 (0.3)	0.04 (4)	1.1 (110)
	Mitochondria (4300 g pellet)	0.22 (3.02)	0.01 (0.14)	0.08 (1.1)	1.5 (20)
	Microsomes (105000 g pellet)	0.007 (0.07)	0.23 (2.3)	0.06 (0.6)	7.2 (72)
Inflamed	Homogenate	0.033 (4.15)	0.054 (6.8)	0.12 (15.12)	4.01 (505)
	Debris (480 g pellet)	0.0018 (0.18)	0.0035 (0.35)	0.052 (5.2)	1.1 (110)
	Mitochondria (4300 g pellet)	0.22 (3.02)	0.02 (0.275)	0.1 (1.37)	1.6 (22)
	Microsomes (150000 g pellet)	0.008 (0.08)	0.25 (2.5)	0.07 (0.7)	9.5 (95)
Inflamed and dexamethasone	Homogenate	0.031 (3.9)	0.051 (6.42)	0.13 (16.38)	5.69 (717)
	Debris (480 g pellet)	0.0019 (0.19)	0.0033 (0.33)	0.04 (4.0)	1.2 (120)
	Mitochondria (4300 g pellet)	0.2 (2.75)	0.025 (0.34)	0.12 (1.65)	1.7 (23.5)
	Microsome (105000 g pellet)	0.008 (0.08)	0.23 (2.3)	0.06 (0.6)	13.3 (133)

trations of exogenous dolichol phosphate to the assay mixtures (Coolbear & Mookerjee, 1981). It was concluded from that study that the increase in mannosyltransferase in inflammation was due to an increase in the endogenous concentration of the dolichol phosphate in the liver and that there was no true increase of the enzyme activity. Results of our hepatocyte experiments also support the above conclusion with regard to control and inflamed conditions. In contrast, the present study, showing a lack of any effect of increased amounts of exogenous dolichol phosphate in the assay mixtures (Fig. 4), suggests that dexamethasone treatment of the hepatocytes resulted in an increase of the mannosyltransferase enzyme and that this effect of dexamethasone is not mediated by increasing the endogenous acceptor content in the cells.

A stimulatory effect of dexamethasone on the synthesis of certain classes of secretory glycoproteins has been reported by Baumann *et al.* (1983) in hepatoma tissue-culture cells and in primary hepatocyte culture. Furthermore, increased formation of dolichol-linked sugar derivatives by dexamethasone has been shown in HeLa cells (Ramachandran *et al.*, 1982). The results given in the present paper are complementary to those observations.

Results obtained by us and others on the effect of dexamethasone to stimulate glycoprotein synthesis and also to increase dolichol-mediated glycosyltransferases in hepatocytes and other cell cultures have opened an important question concerning the mechanism of glycoprotein synthesis in inflammation. There are reports that inflammation increases the concentration of cortisol in serum (Langstaff *et al.*, 1980) in addition to various other humoral factors and mediators (Glenn *et al.*, 1968). It may be suggested that the increased concentration of corticosteroids in serum would produce a direct effect on the dolichol-linked glycosyltransferases in liver. Increased glycoprotein synthesis in inflammation is therefore probably not because of inflammation as such, but due to increased concentration of corticosteroids in serum. This would constitute a new hypothesis to explain the role of inflammation in glycoprotein synthesis. Obviously, further work will be necessary to substantiate the hypothesis.

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