Inhibition of protein *N*-glycosylation by 2-deoxy-2-fluoro-D-galactose

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The effects of 2-deoxy-2-fluoro-D-galactose (dGalF) on *N*- and *O*-glycosylation of proteins was studied in rat hepatocyte primary cultures and in human monocytes. In hepatocytes, dGalF at concentrations of 1 mM or higher completely inhibited *N*-glycosylation of α_1 -antitrypsin and α -acid glycoprotein, whereas 4 mM-2-deoxy-D-galactose (dGal) only slightly impaired *N*-glycosylation. In monocytes, 1 mM- or 4 mM-dGalF blocked *N*-glycosylation of α_1 -antitrypsin and of interleukin-6, while *O*-glycosylation of interleukin-6 remained unaffected. In monocytes, dGal had no effect on protein *N*-glycosylation. Addition of uridine effectively prevented the UTP deficiency induced by dGalF, but had no effect on the inhibition of protein *N*-glycosylation by dGalF. Using ^{19}F -n.m.r. spectroscopy, 2-deoxy-2-fluoro-D-galactose 1-phosphate (dGalF-1-*P*), UDP-dGalF and UDP-dGlcF could be identified as the major metabolites of dGalF in hepatocytes as well as in monocytes. In conclusion, compared with dGal, dGalF is a more efficient inhibitor of protein *N*-glycosylation. The effect is not caused by the depletion of UTP induced by dGalF, but rather by metabolites of dGalF. dGalF is metabolized not only in hepatocytes but also in peripheral blood monocytes, which can be used for *ex vivo* studies of disturbances in D-galactose metabolism.

INTRODUCTION

Replacement of the hydroxyl group at the C₂ position in D-galactose by fluorine (Adamson & Marcus, 1972; Haradahira *et al.*, 1984; Diksic & Jolly, 1986; Oberdorfer *et al.*, 1988) has enabled studies of the metabolism *in vivo* of 2-deoxy-2-fluoro-D-galactose (dGalF) by ^{19}F -n.m.r. spectroscopy and by positron emission tomography when the positron-emitting ^{18}F -labelled dGalF was used (Fukuda *et al.*, 1986; Ishiwata *et al.*, 1989; Grün *et al.*, 1990). dGalF thus offers unique advantages for studies of the D-galactose pathway by non-invasive techniques.

After administration to rodents *in vivo*, dGalF and its metabolites were found to be predominantly localized in the liver (Grün *et al.*, 1990), with accumulation of the corresponding UDP-hexose analogues (Ishiwata *et al.*, 1988; Kanazawa *et al.*, 1988; Grün *et al.*, 1990). This finding is similar to that observed after the administration of other D-galactose analogues modified at C₂, e.g. 2-deoxy-D-galactose (dGal; De Robichon Szulmajster, 1961; Smith & Keppler, 1977; Starling & Keppler, 1977), D-galactosamine (Maley *et al.*, 1968; Keppler *et al.*, 1970, 1974), and D-galactosone (Keppler *et al.*, 1982). The accumulation of UDP-2-deoxy-2-fluoro-D-hexoses (dHexF) observed after the administration of dGalF *in vivo* led to a rapid depletion of hepatic UTP, UDP-glucose and UDP-galactose (Grün *et al.*, 1990), an effect also observed with other D-galactose analogues modified at C₂.

It has recently been shown that the D-galactose analogue D-galactosamine impairs but does not completely block the *N*-glycosylation of proteins. This effect was found to occur only in hepatocytes (Gross *et al.*, 1990a). Evidence has been provided that metabolites of D-galactosamine, presumably dolichyl monophosphate glucosamine (Dol-*P*-GlcN; McDowell *et al.*, 1986), are responsible for the inhibition of protein *N*-glycosylation

(Gross *et al.*, 1990a). Based on these findings, it was of interest to investigate whether protein glycosylation can be impaired by D-galactose analogues with other substituents at C₂. We therefore examined and compared the effects of dGalF and dGal on the *N*-glycosylation of α_1 -antitrypsin and α -acid glycoprotein in rat hepatocyte primary cultures, as well as on the *N*-glycosylation of α_1 -antitrypsin in human monocytes and on the *N*- and *O*-glycosylation of interleukin-6 in human monocytes. We found that replacement of the C₂ hydroxyl group of D-galactose by fluorine results in the potent inhibition of *N*-glycosylation, and that this effect is not restricted to hepatocytes.

MATERIALS AND METHODS

Chemicals

dGalF was synthesized by F. Oberdorfer as previously described (Oberdorfer *et al.*, 1988) from D-galactal, and purified by h.p.l.c. to a purity of at least 90% (96% in the experiments presented), as determined enzymically with galactose dehydrogenase (Edwards *et al.*, 1977) and analytically by ^1H -n.m.r. and ^{19}F -n.m.r. (Grün *et al.*, 1990). Enzymic analysis was not corrected for the water content of the hygroscopic material. N.m.r. spectroscopy indicated the presence of less than 4% of the non-metabolizable 2-deoxy-2-fluoro-D-talose.

Nucleotides, nucleosides and enzymes were obtained from Boehringer-Mannheim (Mannheim, Germany) and Sigma (St. Louis, MO, U.S.A.) at the highest purity available. L-[^{35}S]Methionine (>1000 Ci/mmol) was purchased from Amersham-Buchler (Braunschweig, Germany). Protosol was from New England Nuclear (Dreieich, Germany); medium 199 was from Boehringer-Mannheim. RPMI 1640 medium was purchased from Seromed (Berlin, Germany). Ficoll-paque and Protein A-Sepharose 4B (approx. 10 mg of Protein A/g of

Abbreviations used: dGal, 2-deoxy-D-galactose; dGalF, 2-deoxy-2-fluoro-D-galactose; dGalF-1-*P*, 2-deoxy-2-fluoro-D-galactose 1-phosphate; dGlcF, 2-deoxy-2-fluoro-D-glucose; dHexF, 2-deoxy-2-fluoro-D-hexoses (dGalF and dGlcF); Dol-*P*-GlcN, dolichyl monophosphate glucosamine.

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Sephacrose 4B) were obtained from Pharmacia (Freiburg, Germany). Lipopolysaccharide from *Salmonella typhimurium* was from Sigma.

Preparation and ^{35}S -labelling of rat hepatocyte primary cultures

Hepatocytes were prepared as previously described (Katz *et al.*, 1979; Giffhorn-Katz & Katz, 1986). They were isolated from fed adult female Wistar rats (180 g) by recirculating perfusion with collagenase *in situ*. Non-parenchymal cells and debris were removed by several washings and sedimentation of the hepatocytes at 30 g for 2 min. The parenchymal cells ($3 \times 10^6/60\text{-mm}$ dish) were cultured in basal medium 199 containing glucose (5.5 mM), insulin (0.5 nM), dexamethasone (5 nM), BSA (2 g/l), penicillin (60 mg/l), streptomycin sulphate (120 mg/l) and NaHCO_3 (18 mM). Neonatal calf serum (3%) was present only during the first 4 h. Incubation was performed at 37 °C and 5% CO_2 in humidified air. For the experiments, 48-h-old hepatocyte primary cultures were used. Cells were radioactively labelled with [^{35}S]methionine (25 $\mu\text{Ci}/3 \times 10^6$ cells) in methionine-free RPMI 1640 medium without BSA and without neonatal calf serum. dGalF and dGal were added to the culture medium at various concentrations up to 4 mM. If not stated otherwise, the hepatocytes were incubated with dGalF or dGal for 1 h before the radioactive labelling, to avoid a mixture of normally processed and differently *N*-glycosylated proteins. The same concentrations of dGalF or dGal were maintained during the labelling period of 2 h. At the end of the incubation the media were separated from the cells. Then the cells of each dish were washed twice with 10 mM-Tris/HCl (pH 7.5)/0.15 M-NaCl, homogenized by stirring for 20 min in 1 ml of 25 mM-Tris/HCl (pH 7.5)/20 mM-NaCl/1% sodium deoxycholate/1% Triton X-100, and centrifuged for 15 min at 12000 g. The supernatant from this centrifugation and the culture medium were used for the immunoprecipitations.

Preparation and ^{35}S -labelling of human monocytes

For the preparation of human monocytes, buffy coats obtained from the blood of normal blood donors were used. The preparation of monocytes was carried out essentially as described by Andreessen *et al.* (1986). Mononuclear cells were separated from granulocytes and residual erythrocytes by centrifugation over Ficoll-paque. Monocytes were then separated from lymphocytes by adherence to plastic at 37 °C in RPMI 1640 medium containing 10% fetal calf serum. RPMI 1640 was supplemented with L-glutamine, antibiotics and vitamins at all stages of the monocyte preparation. The non-adherent cells (lymphocytes) were removed by repeated gentle washing with RPMI 1640 medium. The adherent cells were then incubated overnight with RPMI 1640 medium containing 5% fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO_2 . To stimulate the synthesis of α_1 -antitrypsin or interleukin-6, lipopolysaccharide was added to the culture medium at a concentration of 100 ng/ml. For the labelling of monocytes obtained after overnight incubation, methionine-free RPMI 1640 medium without fetal calf serum was used. About 3×10^6 cells/dish were incubated with 150 μCi of [^{35}S]methionine. Labelling times and the conditions for the incubation with dGalF and dGal were the same as for hepatocyte cultures.

Immunoprecipitation and electrophoresis

Immunoprecipitations were carried out essentially according to Macchecini *et al.* (1979). A 1 ml portion of medium or of the supernatant obtained from the cell homogenate was added to 5 ml of 20 mM-Tris/HCl, pH 7.5, 0.14 M-NaCl, 5 mM-EDTA and 1% Triton X-100. After addition of 10 μl of a specific antiserum against rat α_1 -antitrypsin (Gross *et al.*, 1983), rat α_1 -acid glyco-

protein (Gross *et al.*, 1984), human α_1 -antitrypsin (Gross *et al.*, 1990b) or human interleukin-6 (Gross *et al.*, 1989) and incubation at 0 °C overnight, the antigen-antibody complexes were bound to 10 mg (dry weight) of Protein A-Sepharose 4B and washed four times with the above-mentioned buffer and twice with 50 mM-sodium phosphate buffer, pH 7.5. Elution was performed by incubation with 0.1 M-Tris/HCl (pH 6.8)/5% β -mercaptoethanol/5% SDS/10% glycerol at 95 °C for 5 min. The eluted proteins were analysed by electrophoresis in SDS/polyacrylamide slab gels (King & Laemmli, 1971) and fluorography (Bonner & Laskey, 1974).

Radioactivity determinations

Trichloroacetic acid-precipitable radioactivity was determined according to Mans & Novelli (1961). To ascertain the radioactivity in individual proteins, the bands of the respective proteins were excised from SDS/polyacrylamide slab gels and incubated with 1 ml of Protosol/water (9:1, v/v) at 45 °C overnight. After addition of 0.1 ml of acetic acid, radioactivity was determined in a liquid scintillation spectrophotometer.

H.p.l.c.

For h.p.l.c. analysis, hepatocytes or monocytes were homogenized in 0.6 M- HClO_4 at 0 °C. After centrifugation at 12000 g for 15 min, the supernatant was neutralized by addition of 1 M-KOH. H.p.l.c. determinations of UDP-hexoses, UDP-dHexF, UTP, ATP and ADP were done as previously described (Grün *et al.*, 1990).

^{19}F -n.m.r. spectroscopy

Neutralized acid-soluble extracts of hepatocytes or monocytes incubated with 1 mM-dGalF for 3 h were prepared according to the same protocol as for h.p.l.c. ^1H -coupled ^{19}F -n.m.r. spectra were obtained at 470.6 MHz (11.7 T) and 28 °C with a Bruker AM-500 FT-NMR spectrometer using techniques described previously (Grün *et al.*, 1990). The hepatocyte extract (0.38 ml) was measured for approx. 10 h in a 5 mm probehead; the monocyte extract (1.5 ml) was measured for 12 h in a 10 mm probehead. At pH 7.4, dGalF and all of its metabolites can be completely resolved and identified based on chemical shifts and coupling constants (see data in [7]). The purity of the dGalF used was found to be 96%, with 2-deoxy-2-fluoro-D-talose as the major impurity; however, this was not metabolized.

RESULTS

Effects of dGalF and dGal on synthesis and *N*-glycosylation of α_1 -antitrypsin and α_1 -acid glycoprotein in rat hepatocyte primary cultures

Two molecular mass forms of α_1 -antitrypsin can be found in rat hepatocyte primary cultures: a 49 kDa intracellular form, and a 54 kDa form which is secreted into the medium. It has been shown in previous publications that rat α_1 -antitrypsin contains three N-linked oligosaccharide chains. The 49 kDa form has been identified as the high-mannose-type glycosylated intracellular precursor, and the 54 kDa form as the complex-type glycosylated mature glycoprotein (Gross *et al.*, 1982; Carlson & Stenflo, 1982). Fig. 1 shows the effects of dGalF and of dGal on the glycosylation of α_1 -antitrypsin in rat hepatocyte primary cultures. Addition of dGalF at increasing concentrations up to 4 mM resulted in the progressive disappearance of the bands normally found for the 49 kDa and 54 kDa forms of α_1 -antitrypsin (Fig. 1, lanes 1 and 2) and the appearance of bands of lower molecular mass (lanes 5–8). At 4 mM-dGalF the major band corresponded to 41 kDa (lanes 7 and 8), which is the molecular mass of the unglycosylated form of α_1 -antitrypsin

Fig. 1. Effects of dGalF and dGal on the glycosylation of α_1 -antitrypsin in rat hepatocyte primary cultures

Rat hepatocyte primary cultures were incubated without D-galactose analogue (Co; lanes 1, 2, 9 and 10), or with dGalF at 0.1 mM (lanes 3 and 4), 1 mM (lanes 5 and 6) or 4 mM (lanes 7 and 8), or with dGal at 0.1 mM (lanes 11 and 12), 1 mM (lanes 13 and 14) or 4 mM (lanes 15–18; results of two different experiments). dGalF and dGal were added to the cells 1 h prior to labelling of proteins with [35 S]methionine as described in the Materials and methods section. After a labelling period of 2 h, α_1 -antitrypsin was immunoprecipitated from cell homogenates (c) and media (m). Molecular mass standards were catalase (60 kDa), ovalbumin (43 kDa) and alcohol dehydrogenase (37 kDa).

Table 1. Net synthesis of total proteins and of α_1 -antitrypsin in rat hepatocyte primary cultures

Rat hepatocyte primary cultures were incubated without or with dGalF and dGal at final concentrations of 0.1 mM, 1 mM or 4 mM for 1 h. The cells were then labelled with [35 S]methionine (25 μ Ci/ 3×10^6 cells) in the presence of dGalF or dGal in the above-mentioned concentrations for 2 h. Total protein synthesis was measured by the determination of trichloroacetic acid-precipitable radioactivity in cells and media, and the synthesis of α_1 -antitrypsin was measured by the determination of the radioactivity in α_1 -antitrypsin immunoprecipitated from cells and media (glycosylated and not glycosylated) as described in the Materials and methods section. Results represent means $\pm$ S.D. of at least four independent experiments.

Addition	Concn. (mM)	Net synthesis (% of control)	
		Total proteins	α_1 -Antitrypsin
dGalF	0.1	88 $\pm$ 34	48 $\pm$ 7
dGalF	1	81 $\pm$ 22	34 $\pm$ 7
dGalF	4	76 $\pm$ 23	23 $\pm$ 4
dGal	0.1	109 $\pm$ 22	93 $\pm$ 18
dGal	1	101 $\pm$ 25	81 $\pm$ 3
dGal	4	100 $\pm$ 19	81 $\pm$ 19

produced in the presence of tunicamycin (Gross *et al.*, 1982). At 1 mM-dGalF, three bands were found for the cellular fraction (Fig. 1, lane 5) corresponding to protein with no, one or two oligosaccharide chains. In contrast, dGal did not impair (Fig. 1, lanes 15 and 16) or only slightly affected (Fig. 1, lanes 17 and 18) the N-glycosylation of rat α_1 -antitrypsin. Compared with dGal, dGalF always strongly inhibited N-glycosylation.

As shown in Table 1, dGalF had a minor and dGal had no inhibitory effect on total protein synthesis during the first 3 h after addition to rat hepatocyte primary cultures. However, in the presence of 1 mM- or 4 mM-dGalF the amount of newly synthesized α_1 -antitrypsin detectable after a labelling period of 2 h with [35 S]methionine decreased by a factor of 3–4. This is also visible on the gel in Fig. 1 (lanes 7 and 8). In contrast to dGalF,

Fig. 2. Effect of dGalF on the glycosylation of α_1 -acid glycoprotein in rat hepatocyte primary cultures

Rat hepatocyte primary cultures were incubated without (lanes 1 and 5) or with dGalF at 0.1 mM (lanes 2 and 6), 1 mM (lanes 3 and 7) or 4 mM (lanes 4 and 8). dGalF was added to the cells 1 h prior to protein labelling for 2 h with [35 S]methionine, after which α_1 -acid glycoprotein was immunoprecipitated from cell homogenates (c) and media (m). Molecular mass standards were BSA (68 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa).

dGal did not significantly decrease the amount of radioactively labelled newly synthesized α_1 -antitrypsin.

To address the question of whether the decreased recovery of α_1 -antitrypsin in the presence of dGalF is due to an inhibition of the *de novo* synthesis of α_1 -antitrypsin or to an increased rapid degradation of the unglycosylated protein, hepatocytes were incubated with the proteinase inhibitor Tos-Lys-CH₂Cl. When rat hepatocyte primary cultures were incubated with dGalF at 4 mM for 1 h or 3 h and then labelled with [35 S]methionine (25 μ Ci/ 3×10^6 cells) for 2 h, the amount of radioactively labelled α_1 -antitrypsin was reduced to 41 % or 38 % respectively. When the cells were incubated in addition with Tos-Lys-CH₂Cl at 1 mM 2.5 h before the labelling period, the amount of detectable α_1 -antitrypsin was only decreased to 86 % or 63 % respectively. During this extended incubation with dGalF (5 h), the effects of dGalF on N-glycosylation were the same as during the typical 3 h duration in the other experiments. These data indicate that increased degradation accounts for a large portion of the decrease in the total amount of α_1 -antitrypsin in the presence of dGalF.

As shown in Fig. 2, dGalF also inhibited the N-glycosylation of rat α_1 -acid glycoprotein, which contains six N-linked oligosaccharide chains (Joshima *et al.*, 1981) and is normally present in hepatocytes as a 39 kDa intracellular high-mannose type precursor (Fig. 2, lane 1). The secreted complex-type glycosylated form has a molecular mass of 45–50 kDa (lane 5). dGalF at 1 mM or 4 mM inhibited the N-glycosylation of α_1 -acid glycoprotein nearly completely, and led to the formation of a predominant 23 kDa form, which had the same electrophoretic mobility as unglycosylated α_1 -acid glycoprotein synthesized in the presence of tunicamycin (Ludolph *et al.*, 1989). Although the degree of inhibition of N-glycosylation was the same for α_1 -acid glycoprotein and α_1 -antitrypsin, dGalF did not lead to a significant decrease in the total amount of immunoprecipitable α_1 -acid glycoprotein comparable with that observed for α_1 -antitrypsin. This indicates that in the presence of dGalF the unglycosylated forms of different glycoproteins are degraded to different extents.

Effects of dGalF and dGal on *N*-glycosylation and *O*-glycosylation in human peripheral blood monocytes

As in hepatocytes, the synthesis of human α_1 -antitrypsin in monocytes involves a high-mannose-type glycosylated intracellular precursor (51 kDa) and a complex-type glycosylated form (56 kDa) which is secreted (Gross *et al.*, 1990b). The apparent molecular masses of these two differently glycosylated forms of human α_1 -antitrypsin as well as that of the unglycosylated form of the protein are 2 kDa higher than those of rat α_1 -antitrypsin. Fig. 3 shows that dGalF effectively prevented the *N*-glycosylation of human α_1 -antitrypsin in monocytes. In the presence of 1 mM- or 4 mM-dGalF, the glycosylated forms of human α_1 -antitrypsin could not be detected. Instead, a 43 kDa unglycosylated form of the protein (Gross *et al.*, 1990b) was found. The two bands with molecular masses of 57 kDa and 38 kDa (indicated by arrowheads in Fig. 3) are minor contaminations which are sometimes found when α_1 -antitrypsin is immunoprecipitated from human monocyte cultures. In contrast, dGal at concentrations up to 4 mM did not inhibit the *N*-glycosylation of α_1 -antitrypsin in human monocytes.

To find out whether dGalF inhibits only *N*-glycosylation or whether it can also prevent *O*-glycosylation, we studied the glycosylation of interleukin-6 in human monocytes (Fig. 4). It has been shown that interleukin-6 is both *O*- and *N*-glycosylated (Gross *et al.*, 1989). There is a predominant form with an apparent molecular mass of 24 kDa which is *O*-glycosylated, and a form with an apparent molecular mass of 27.5 kDa which is both *O*- and *N*-glycosylated. Treatment of human monocytes with 1 mM- or 4 mM-dGalF led to the disappearance of the *N*-glycosylated form of interleukin-6 (Fig. 4, lanes 6 and 8). The *O*-glycosylated 24 kDa band, however, remained unaffected. Since even minor alterations of the *O*-linked oligosaccharides of interleukin-6 (e.g. cleavage of sialic acid) lead to an increase in

Fig. 4. Effect of dGalF on the *N*- and *O*-glycosylation of interleukin-6 in human monocytes.

Human monocytes were incubated without galactose analogues (lanes 1 and 2) or with dGalF at 0.1 mM (lanes 3 and 4), 1 mM (lanes 5 and 6) or 4 mM (lanes 7 and 8). After 1 h, proteins were labelled with [35 S]methionine (150 μ Ci/ 3×10^6 cells) for 2 h. Then human interleukin-6 was immunoprecipitated from cell homogenates (c) and media (m). Molecular mass standards were glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa) and soybean trypsin inhibitor (20 kDa).

Table 2. Net synthesis of total proteins, α_1 -antitrypsin and interleukin-6 in human monocytes

Human monocytes were incubated without or with dGalF and dGal at final concentrations of 0.1 mM, 1 mM or 4 mM for 1 h. The cells were then labelled with [35 S]methionine (150 μ Ci/ 3×10^6 cells) for 2 h in the presence of dGalF or dGal at the concentrations given. The net synthesis of total protein was measured by the determination of trichloroacetic acid-precipitable radioactivity in cells and media, and the net synthesis of α_1 -antitrypsin and of interleukin-6 were measured by the determination of the radioactivity in the immunoprecipitated proteins (glycosylated and not glycosylated) from cells and media as described in the Materials and methods section. Results represent means $\pm$ S.D. of at least four independent experiments.

Addition	Concn. (mM)	Net synthesis (% of control)		
		Total proteins	α_1 -Antitrypsin	Interleukin-6
dGalF	0.1	78 $\pm$ 31	65 $\pm$ 21	108 $\pm$ 8
dGalF	1	66 $\pm$ 26	35 $\pm$ 10	100 $\pm$ 10
dGalF	4	52 $\pm$ 20	20 $\pm$ 4	63 $\pm$ 2
dGal	0.1	79 $\pm$ 21	72 $\pm$ 13	110 $\pm$ 10
dGal	1	79 $\pm$ 23	74 $\pm$ 17	92 $\pm$ 9
dGal	4	58 $\pm$ 20	70 $\pm$ 29	69 $\pm$ 6

Fig. 3. Effects of dGalF and dGal on the glycosylation of α_1 -antitrypsin in human monocytes

Human monocytes were incubated without galactose analogue (Co; lanes 1, 2, 9 and 10) or with dGalF at 0.1 mM (lanes 3 and 4), 1 mM (lanes 5 and 6) or 4 mM (lanes 7 and 8), or with dGal at 0.1 mM (lanes 11 and 12), 1 mM (lanes 13 and 14) or 4 mM (lanes 15 and 16). After 1 h, proteins were labelled with [35 S]methionine (150 μ Ci/ 3×10^6 cells) for 2 h. Thereafter α_1 -antitrypsin was immunoprecipitated from cell homogenates (c) and media (m). Molecular mass standards were the same as in Fig. 1. The two bands indicated by arrowheads are minor contaminations, which are sometimes found when α_1 -antitrypsin is immunoprecipitated from human monocyte cultures.

the electrophoretic mobility (Gross *et al.*, 1989), thus providing strong evidence that dGalF does not affect *O*-glycosylation.

As shown in Table 2, dGalF at 4 mM produced a somewhat greater inhibition of total protein synthesis in human monocytes as compared with rat hepatocytes. However, the decrease in the recovery of α_1 -antitrypsin to about 20% of control was the same for both cell types. The reduction in the detectable amount of radioactively labelled interleukin-6 in monocytes (to 63% of control) was similar to the effect on total protein synthesis. Although dGal had little effect on total protein synthesis in rat hepatocytes (see Table 1), 4 mM-dGal resulted in a moderate decrease in the detectable amounts of radioactively labelled interleukin-6 or α_1 -antitrypsin (to $\sim$ 70% of control), similar to

Table 3. Effect of dGalF and uridine on levels of UDP-dHexF and UTP in rat hepatocyte primary cultures

Rat hepatocyte primary cultures were incubated without (control) or with dGalF or simultaneously with dGalF+uridine at the concentrations indicated. After 3 h the medium was removed and the cells were carefully rinsed with 0.9% NaCl and prepared for h.p.l.c. as described in the Materials and methods section. For each analysis, 30 mg of hepatocytes (wet weight) was used.

	UDP-Hex + UDP-dHexF ($\mu\text{mol/g}$)	UTP ($\mu\text{mol/g}$)	ATP/ADP
Control	0.43*	0.80	4.3
0.1 mM-dGalF	0.31†	0.43	7.9
1 mM-dGalF	0.32†	0.07	3.4
4 mM-dGalF	0.56†	0.07	2.0
0.1 mM-dGalF + 4 mM-uridine	0.38†	1.02	4.0
1 mM-dGalF + 4 mM-uridine	1.13†	0.61	3.1
4 mM-dGalF + 4 mM-uridine	0.86†	0.49	1.8

* Control cells only contain UDP-Hex (UDP-glucose and UDP-galactose) but no UDP-dHexF.

† H.p.l.c. did not separate UDP-dHexF and UDP-Hex.

the effect on total protein synthesis (60% of control). These findings indicate that the significantly greater decrease in the net synthesis of α_1 -antitrypsin versus that of total proteins induced by dGalF in both cell types is a result of the impaired *N*-glycosylation of α_1 -antitrypsin.

Effect of dGalF on nucleotide metabolism in rat hepatocyte primary cultures

It has been shown that dGalF administered *in vivo* leads to a depletion of hepatic UTP, UDP-glucose and UDP-galactose (Grün *et al.*, 1990). To determine whether or not this uracil nucleotide depletion is responsible for the inhibition of *N*-glycosylation by dGalF, we quantified the levels of UDP-sugars, UTP and the glycosylation of α_1 -antitrypsin in dGalF-treated hepatocytes and in hepatocytes incubated with dGalF plus uridine. Table 3 shows that 1 mM- or 4 mM-dGalF led to a substantial depletion of the UTP pool in hepatocyte primary cultures (0.07 $\mu\text{mol/g}$ versus 0.80 $\mu\text{mol/g}$). The ATP/ADP ratio was reduced by a factor of two at 4 mM-dGalF. When hepatocytes were incubated with dGalF plus 4 mM-uridine, the depletion of the UTP pool was prevented, e.g. the UTP content was 0.49 $\mu\text{mol/g}$ in the presence of 4 mM-dGalF plus 4 mM-uridine, versus 0.07 $\mu\text{mol/g}$ in the presence of dGalF alone.

If UTP depletion were responsible for the inhibition of *N*-glycosylation by dGalF, it should thus be possible to prevent this effect by the addition of uridine. Immunoprecipitation of α_1 -antitrypsin during the experiment presented in Table 3, however, revealed that the addition of uridine did not prevent the inhibition of *N*-glycosylation by dGalF (results not shown). This excludes the possibility that the depletion of the UTP pool is responsible for the impaired *N*-glycosylation in the presence of dGalF.

Furthermore, the data presented in Table 3 allow the conclusion that dGalF does not impair protein glycosylation by acting as an unspecific inhibitor of energy metabolism. In the presence of 1 mM-dGalF, the ATP/ADP ratio was still within normal limits, whereas 1 mM-dGalF was sufficient to inhibit effectively the *N*-glycosylation of proteins (Figs. 1–4).

Metabolites of dGalF in hepatocytes and in monocytes

To learn more about the mechanism of action of dGalF, the metabolites of dGalF produced in hepatocytes and in monocytes

Table 4. ^{19}F -N.m.r. analysis of metabolites of dGalF in rat hepatocyte primary cultures and in human monocytes

Rat hepatocyte primary cultures and human monocytes were incubated with 1 mM-dGalF for 3 h. After removal of the media, cells were carefully rinsed with 0.9% NaCl and prepared for ^{19}F -n.m.r. spectroscopy as described in the Materials and methods section. For the n.m.r. analysis, 100 mg of hepatocytes (wet weight) and 12 mg of monocytes (wet weight) were used.

	Metabolites ($\mu\text{mol/g}$ cells)	
	Hepatocytes	Monocytes
dGalF ($\alpha + \beta$)*	0.36	3.69
dGalF-1- <i>P</i>	5.93	3.81
UDP-dGalF	0.41	0.28
UDP-dGlcF	0.62	0.29
Unidentified dHexF†	0.19	0.27

* β/α anomer ratio ~ 1.7 .

† Approx. 3% of total F; based on J_{HF} couplings this may be a talose analogue with F axial, which frequently contaminated the dGalF preparations.

were analysed by ^{19}F -n.m.r. spectroscopy (Table 4). When hepatocytes or monocytes were incubated with 1 mM-dGalF for 3 h, dGalF 1-phosphate (dGalF-1-*P*), UDP-dGalF and UDP-2-deoxy-2-fluoro-D-glucose (dGlcF) were found as the major metabolites. The uptake of dGalF was similar for both cell types, but the conversion to dGalF-1-*P* was slower in the monocytes. The levels of UDP-dGalF and UDP-dGlcF were lower in monocytes, but the ratios of their levels to that of dGalF-1-*P* were similar for both cell types. The results clearly show that monocytes can metabolize dGalF and are able to catalyse the 4'-epimerization reaction to form UDP-dGlcF.

DISCUSSION

The metabolic effects of D-galactose analogues have been found to be most pronounced in hepatocytes and hepatoma cells, since the enzymes of the D-galactose pathway exhibit high activities in these cells (Cuatrecasas & Segal, 1965; Decker & Keppler, 1974; Bauer *et al.*, 1980). Major metabolic effects include an accumulation of UDP-hexose analogues (De Robichon Szulmajster, 1961; Maley *et al.*, 1968; Keppler *et al.*, 1970, 1974, 1982; Smith & Keppler, 1977; Starling & Keppler, 1977; Ishiwata *et al.*, 1988; Kanazawa *et al.*, 1988; Grün *et al.*, 1990) and consequently a depletion of the hepatic UTP pool (Keppler *et al.*, 1974, 1985; Grün *et al.*, 1990). In addition to these effects, inhibition of protein *N*-glycosylation has been found in the presence of the D-galactose analogue D-galactosamine (Gross *et al.*, 1990a). This effect was observed in hepatocytes but not in monocytes. In contrast, dGal did not cause a marked impairment of protein *N*-glycosylation in either hepatocytes or monocytes (Figs. 1 and 3).

In contrast to the behaviour of D-galactosamine and dGal, dGalF is an effective inhibitor of the *N*-glycosylation of proteins in hepatocytes as well as in monocytes (Figs. 1–4). Thus replacement of the hydroxyl group at C₂ of D-galactose by fluorine results in enhanced *N*-glycosylation inhibitory properties in comparison with the other D-galactose analogues examined. These differences in inhibition for the three D-galactose analogues dGalF, dGal and D-galactosamine (Gross *et al.*, 1990a) suggest that this effect is not due to their common depletion of the cellular UTP pool. This was confirmed by incubations in the presence of uridine, whereby the UTP deficiency was avoided but not the inhibition of protein *N*-glycosylation by dGalF.

Of particular advantage is the ability to use ^{19}F -n.m.r. spectroscopy to study the metabolism of dGalF. Analysis of its metabolites in rat hepatocytes showed results comparable with those obtained in mouse liver after administration of similar doses and dGalF *in vivo* (Grün *et al.*, 1990). The same pattern of metabolites of dGalF, including dGalF-1-*P*, UDP-dGalF and UDP-dGlcF, was found in monocytes, albeit with a lower rate of conversion of dGalF to dGalF-1-*P*. The results of the analysis of the metabolites of dGalF in hepatocytes and in monocytes allow the following conclusions. (i) Monocytes are suited for *in vitro ex vivo* studies of D-galactose metabolism by ^{19}F -n.m.r. spectroscopy using dGalF as substrate, i.e. peripheral blood monocytes might be useful for the diagnosis of alterations in D-galactose metabolism. (ii) Metabolites of dGalF may be directly responsible for the inhibition of protein N-glycosylation in hepatocytes as well as in monocytes.

In the case of the D-galactose analogue D-galactosamine, it has been shown that its metabolite UDP-glucosamine can be used by microsomal preparations of both chick embryo cells and rat liver for the synthesis of Dol-*P*-GlcN (McDowell *et al.*, 1986). Since the synthesis of the unphysiological Dol-*P*-GlcN competes with the synthesis of the physiological dolichol-linked saccharides Dol-*P*-Glc, Dol-*P*-Man and Dol-*PP*-(GlcNAc)₂, which are necessary for the biosynthesis of N-linked oligosaccharides of all types, this effect of D-galactosamine could lead to a perturbation of protein glycosylation. In the case of dGalF, a similar mechanism leading to the formation of dolichol derivatives of metabolites of dGalF is conceivable. The fact that D-galactosamine inhibits N-glycosylation only in hepatocytes, whereas the effect of dGalF is not restricted to liver cells, indicates that the metabolites of dGalF are more potent than the metabolites of D-galactosamine as glycosylation inhibitors.

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