J. Inher. Metab. Dis. 12 (1989) 257–266

Deficit of Uridine Diphosphate Galactose in Galactosaemia

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Summary: The levels of uridine diphosphate galactose (UDPGal) and uridine diphosphate glucose (UDPGlc) have been determined in liver autopsy samples, erythrocytes and cultured skin fibroblasts from galactosaemic patients and compared to non-galactosaemic controls. In patients with undetectable erythrocyte galactose-1-phosphate uridyltransferase (transferase) activity, the levels of UDPGal were substantially lower than in controls. In patients with detectable transferase activity, even though in less than 1% of normal values, both UDPGal and UDPGlc levels were in the normal range. Incubation of erythrocytes from both galactosaemic patients and normal individuals with 10 mmol/L uridine increased UDPGal and UDPGlc levels several-fold, both in the presence or absence of galactose in the incubation medium. We hypothesize that a deficit of UDPGal is responsible for the late onset clinical manifestations in galactosaemia which include ovarian failure, speech defect and neurological abnormalities. We suggest that uridine administration may be of therapeutic value in raising the intracellular concentrations of UDPGal. We conclude that the transferase reaction, however small in activity, is essential for optimal UDPGal formation.

Galactosaemia (McKusick 23040) is an autosomal recessive disorder of galactose metabolism due to a deficiency of the enzyme galactose-1-phosphate uridyltransferase (transferase). Affected patients usually present in the neonatal period with jaundice, hepatomegaly, vomiting, lethargy and susceptibility to infections with Gram-negative organisms (Donnell *et al.*, 1967). The mortality rate is high in the untreated patient. With prompt dietary treatment patients survive and most exhibit normal physical development. However, recent reports have indicated that some patients, despite treatment from birth, have an unfavourable outcome with regards to speech development (Waisbren *et al.*, 1983), neurological status (Lo *et al.*, 1984) and ovarian function (Kaufman *et al.*, 1981). There is no clear explanation as to why treatment does not prevent these complications.

It is believed that accumulation of intracellular galactose-1-phosphate (Gal-1-P)

MS received 5.1.89 Accepted 10.3.89

is responsible for the clinical manifestations, but the mechanism for this is unclear. Gal-1-P inhibits uridine diphosphate glucose (UDPGlc) pyrophosphorylase *in vitro* (Oliver, 1961). This enzyme, in the presence of uridine triphosphate, converts glucose-1-phosphate to UDPGlc and inorganic pyrophosphate. If a similar inhibition takes place *in vivo*, concentrations of UDPGlc and UDPGal should be decreased. The ratio of these two nucleotide sugars could also be affected by the UDPgalactose-4-epimerase activity which converts UDPGlc to UDPGal and by other reactions which utilize these nucleotide sugars as substrates for the formation of glycogen, glycolipids and glycoproteins. To examine whether UDPGlc and UDPGal concentrations are altered in galactosaemia and whether these may play a role in the pathogenesis of the disease, these nucleotide sugars were measured in various tissues including liver, erythrocytes and cultured skin fibroblasts from galactosaemic patients and normal controls. Preliminary results have been presented (Ng *et al.*, 1987a, b).

MATERIALS AND METHODS

All chemical reagents such as NAD, UDPGlc, UDPGal and coupling enzymes UDPGlc dehydrogenase and UDPGal-4-epimerase were supplied by Sigma Chemical Co., St. Louis, MO, USA. Alkaline phosphatase and galactose dehydrogenase were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Inc., USA. Galactose-1-phosphate, disodium salt, D-[^{14}C(U)] was supplied by Dupont NEN Research Products, Wilmington, DE, USA.

Patient samples: The liver samples obtained at autopsy from six galactosaemic patients and six non-galactosaemic controls had been stored for up to 14 years at −80°C. Whole blood samples were obtained by venepuncture and collected in sodium heparinized tubes from galactosaemic patients and normal controls of varying ages. Skin cultured fibroblasts were derived from six patients with classical galactosaemia and six galactosaemic variants and four normal controls.

Cultured skin fibroblasts: The skin fibroblasts were grown and maintained on a 90% minimal essential medium (MEM), 10% (v/v) fetal calf serum, 50 μg/ml of gentamycin sulphate. Cells were grown in 75 mm^2 flasks and harvested when confluency was reached (Oizumi *et al.*, 1986).

Preparation of cellular extracts

Liver tissue: After thawing, tissues were blotted with Kimwipes three times. Approximately 200 mg were weighed out and homogenized in 1.0 ml of 0.1 mol/L glycine buffer, pH 8.7 in a VirTis tube. The homogenate was boiled for 5 min and then cooled in an ice bath. Subsequently, the homogenate was centrifuged at 12 000 × *g* for 10 min. The clear supernate was assayed for UDPGal and UDPGlc.

Erythrocytes: 0.5 ml of washed RBC was mixed with 1 ml of water in a 12 × 75 mm plastic tube. The mixture was boiled for 10 min, cooled in an ice bath, and then

1 ml of 0.1 mol/L glycine buffer, pH 8.7, was added, mixed and centrifuged at $3000 \times g$ for 15 min. If the supernate was not clear, it was centrifuged once more. The supernate was used for UDPGal and UDPGlc determinations.

Cultured skin fibroblasts: Cells from two or three 75 mm^2 flasks were removed by trypsinization and combined from the same patient. The cell suspensions were centrifuged and washed three times with 10 ml of saline. The cells were finally mixed with 1.0 ml of 0.1 mol/L glycine buffer, pH 8.7, and then homogenized in a VirTis tube. An aliquot was taken for protein determination (Lowry *et al.*, 1951) with bovine serum albumin as the standard. The remaining homogenate was boiled for 5 min, and after centrifugation at $12000 \times g$ the supernate was used for UDPGlc and UDPGal determinations.

Analytical biochemistry

Determination of UDPGlc and UDPGal: UDPGlc and UDPGal were determined by a fluorometric method by modifying the procedure of Fujimura *et al.* (1983). The incubation cuvettes contained the following components at final concentrations: 0.82 mol/L glycine buffer, pH 8.7, 2.75 mmol/L cysteine, 2.2 mmol/L NAD, 7.4 mmol/L $MgCl_2$, 0.005 U UDPGlc dehydrogenase and 0.025 U UDPGal-4-epimerase and 100 µl supernate from tissue or haemolysate preparation in a total volume of 1.35 ml. UDPGal and UDPGlc standards at final concentration of 1.3 µmol/L were run at the same time for each experiment. The recovery ranged from 95 to 105%. The concentration in liver was expressed as µmoles/100 g wet weight, in erythrocytes as µmoles/100 g haemoglobin and in cultured cells as µmoles/100 g protein.

Galactose-1-phosphate determination: Erythrocyte galactose-1-phosphate was determined fluorometrically by enzyme coupling using galactose dehydrogenase and alkaline phosphatase by modification of the method described by Gitzelmann (1969a).

Incubation studies: The effects of uridine and galactose in erythrocytes on UDPGal, UDPGlc and Gal-1-P levels were studied. 4 ml of whole blood were incubated in Krebs-Ringer bicarbonate buffer, pH 7.4, with 5.5 mmol/L glucose in 25 ml Ehrlenmyer flasks at time intervals of 0, 1 and 2 h. In addition, galactose (5.5 mmol/L and/or uridine (10 mmol/L) were included. At the end of incubation, mixtures were centrifuged. Buffy coats were removed. Aliquots were taken for haemoglobin determination. The remaining portions were extracted as described above.

Micro galactose-1-phosphate uridyltransferase assay: A sensitive radioactive assay was carried out according to the procedure described by Ng *et al.* (1978) to detect minute transferase activity in erythrocytes.

RESULTS

Table 1 summarizes the results of uridine nucleotide sugar measurements in liver tissue, erythrocytes and cultured skin fibroblasts of galactosaemic patients and controls. Both UDPGlc and UDPGal values were lower in the liver tissue of galactosaemic patients than in tissue from non-galactosaemic individuals. However, the decrease was more pronounced for UDPGal, which was approximately 17% of the mean control value.

Table 1 UDP sugar concentration in liver, erythrocytes and cultured skin fibroblasts from classical galactosaemic patients and non-galactosaemic controls. Classical galactosaemia is defined as patients in whom there is no demonstrable transferase activity

Tissue	*UDPGlc*	*UDPGal*
Liver (μmol/100 g wet weight)		
Controls ($n = 6$)	37.4 ± 8.2	14.2 ± 4.3
Galactosaemic ($n = 6$)	29.1 ± 8.0	2.4 ± 1.4
p	<0.01	<0.001
Erythrocytes (μmol/100 g haemoglobin)		
Controls ($n = 14$)	38.1 ± 7.1	11.2 ± 2.5
Galactosaemic ($n = 40$)	37.5 ± 9.6	3.9 ± 1.6
p	>0.1	<0.001
Cultured skin fibroblasts (μmol/100 g protein)		
Controls ($n = 4$)	194.5 ± 19.5	76.5 ± 25.6
Galactosaemic ($n = 6$)	184.8 ± 41.0	27.8 ± 4.7
p	>0.1	<0.001

In contrast to the findings in the autopsy liver tissue, the levels of UDPGlc in erythrocytes of 40 living galactosaemic patients without detectable erythrocyte transferase activity (classical) was about the same as that found for normal individuals of comparable ages. However, UDPGal values in galactosaemic patients were about 35% of the mean found for normals. These galactosaemic patients had been on lactose-restricted diets since diagnosis. Similar findings of normal levels of UDPGlc and decreased levels of UDPGal were observed in cultured skin fibroblasts from galactosaemic patients.

Among the patients who had been diagnosed as having galactosaemia either by clinical presentation or through newborn screening, we have found some who exhibited a small amount of erythrocyte transferase activity (0.2–2%) as determined by a sensitive radioisotopic method. It is of interest that erythrocytes and cultured skin fibroblasts from these patients showed normal levels of UDPGal and UDPGlc (Figure 1). The question has arisen as to whether these individuals represent one or more variants of the transferase defect.

In order to examine whether uridine could influence the levels of uridine nucleotide sugars, erythrocytes from normal and galactosaemic individuals were incubated for 1 and 2 h in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5.5 mmol/L glucose with or without uridine (10 mmol/L) and in the presence or absence of galactose (5.5 mmol/L). The results are shown in Table 2.

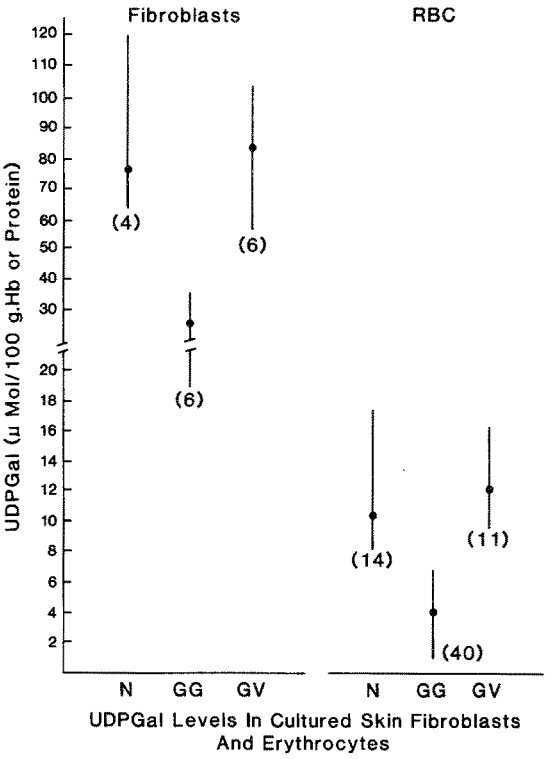

Figure 1 UDPGal levels in cultured skin fibroblasts and erythrocytes. N = normal; GG = classical galactosaemia; GV = galactosaemia variants

In cells from two normal individuals, Gal-1-P was detected only in the presence of galactose in the incubated medium, while the levels of UDPGlc and UDPGal remained about the same. When uridine was added to the incubation medium, both UDPGlc and UDPGal increased in amount. In actual quantities, more UDPGlc was formed than UDPGal. When uridine and galactose were added at the same time, both UDPGlc and UDPGal increased, but substantially more UDPGal was formed, undoubtedly due to the transferase reaction. It was also noted that more galactose-1-phosphate was present in the presence of uridine. This may be due either to enhancement of galactokinase activity or to inhibition of the transferase reaction.

In intact cells from three classical galactosaemic patients, Gal-1-P was present and concentrations of UDPGal were decreased. When galactose was added, Gal-1-P increased 7–10-fold, while the levels of UDPGlc and UDPGal remained essentially unchanged. In the presence of uridine alone, the Gal-1-P level remained the same, but UDPGlc and UDPGal increased to the same extent as in the normal control cells. In the presence of both uridine and galactose, Gal-1-P rose markedly, UDPGal increased, while the change in UDPGlc was about the same as that seen when uridine alone was used. In these cases, there were no differences in Gal-1-P concentrations in the presence or absence of uridine.

Table 2 Effects of uridine and galactose on levels of UDPGlc and UDPGal in human erythrocytes[a]

Samples	*Incubation time* (h)	*Without uridine*						*With uridine*					
		Glucose			*Glucose+galactose*			*Glucose*			*Glucose+galactose*		
		UDPGlc	*UDPGal*	*Gal-1-P*	*UDPGlc*	*UDPGal*	*Gal-1-P*	*UDPGlc*	*UDPGal*	*Gal-1-P*	*UDPGlc*	*UDPGal*	*Gal-1-P*
Normal controls													
1	0	38.2	10.6	0	38.2	10.6	0	38.2	10.6	0	38.2	10.6	0
	1	34.7	8.8	0	31.8	12.4	22.6	53.1	17.7	0	55.9	30.1	40.7
	2	36.3	11.3	0	32.7	11.1	17.0	62.8	17.7	0	51.3	49.5	20.4
2	0	47.0	15.4	0	47.0	15.4	0	47.0	15.4	0	47.0	15.4	0
	1	52.0	15.0	0	48.0	14.0	28.0	71.3	20.0	0	92.0	42.0	48.0
	2	43.5	14.0	0	47.4	14.0	29.0	72.0	20.5	0	95.0	47.0	59.0
Galactosaemic patients													
1	0	26.9	2.8	15.7	26.9	2.8	15.7	26.9	2.8	15.7	26.9	2.8	15.7
	1	26.5	1.8	12.3	23.0	4.4	118.4	38.9	16.3	13.0	42.4	23.2	133.4
	2	22.0	4.6	11.2	27.0	5.7	109.2	55.3	33.6	11.0	70.7	38.9	128.3
2	0	25.3	5.0	21.4	25.3	5.0	21.4	25.3	5.0	21.4	25.3	5.0	21.4
	1	34.7	8.0	—	45.8	6.4	176.6	47.0	12.0	21.4	68.1	17.0	163.0
	2	42.4	6.7	21.5	49.2	5.8	172.2	99.0	23.2	19.2	74.3	32.4	169.5
3	0	41.0	5.6	18.9	41.0	5.6	18.9	41.0	5.6	18.9	41.0	5.6	18.9
	1	41.0	7.6	18.9	42.0	7.6	141.6	60.0	14.0	19.3	60.0	16.0	145.3
	2	44.0	8.0	19.7	44.0	7.8	185.0	73.0	17.0	19.0	83.0	28.0	193.0

[a]Concentrations of UDPGlc, UDPGal and Gal-1-P are expressed in μmol/100 g haemoglobin.
Incubations were carried out in Krebs-Ringer bicarbonate buffer glucose, pH 7.4, [glucose] = 5.5 mmol/L, [galactose] = 5.5 mmol/L, and [uridine] = 10 mmol/L

DISCUSSION

Our findings of deficit in UDPGal in erythrocytes of classical galactosaemic patients are in agreement with the observations by Shin *et al.* (1985) using a different analytical approach. However, their normal values are three to seven times higher (Bohles *et al.*, 1986). They did not report the levels of UDPGlc. Additionally, we found that decreased levels occurred in cultured skin fibroblasts as well as in liver samples.

There are several mechanisms which may contribute to the decrease in UDPGal in tissues of patients with classical galactosaemia:

(1) lower UDPGal-4-epimerase activity,
(2) lower UDPGlc pyrophosphorylase activity,
(3) complete absence of transferase activity.

Even on lactose-free diet, there are always small amounts of Gal-1-P (2–5 mg/100 ml) present in erythrocytes of classical galactosaemic patients. It is possible that this level may inhibit the epimerase activity, resulting in decreased levels of UDPGal. We have found that the level of Gal-1-P at 0.1 mmol/L (2–3 mg/100 ml) does not produce any inhibition of the epimerase reaction *in vitro*. Even at concentration of 2.3 mmol/L (50 mg/100 ml), a level often found in untreated patients, the epimerase activity was only decreased by 20%. Our finding is in agreement with the study on bovine mammary and liver UDPGal-4-epimerase (Green *et al.*, 1977). It, therefore, does not appear that inhibition of epimerase by residual Gal-1-P explains the reduced intracellular UDPGal concentration.

It is possible that the decreased level in UDPGal may be due to inhibition of UDPGlc pyrophosphorylase by residual Gal-1-P. The fact that UDPGlc levels in erythrocytes are about the same in normal and affected patients suggests that UDPGlc pyrophosphorylase is not affected.

The third possibility is that a complete block in the transferase reaction results in decreased levels of UDPGal and that the reactions of UDPGlc pyrophosphorylase and UDPGal-4-epimerase are insufficient to generate enough UDPGal from glucose sources to maintain normal levels.

The fact that patients with low, but detectable transferase activity (galactosaemia variants) have normal levels of UDPGal is consistent with the third of these proposed hypotheses. We have reported that three adult females, who are considered to be galactosaemia variants on the basis of the presence of detectable transferase activity, have normal levels of UDPGal. These individuals have not developed, thus far, evidence of ovarian insufficiency (Kaufman *et al.*, 1988). It suggests that the transferase reaction, even at low levels of activity, is important for UDPGal formation and for clinical expression of the disorder.

We found that erythrocytes from six patients with galactokinase deficiency and cultured skin fibroblasts from a patient with generalized epimerase deficiency, grown in galactose-free medium, had normal levels of UDPGal (Xu *et al.*, unpublished). Among these patients, the enzyme deficiencies were demonstrated to be incomplete; about 5% of normal activity was detected. Thus, when the galactose pathway is not completely blocked, adequate UDPGal formation is achieved.

The finding in cells of galactosaemic patients that uridine increased both UDPGlc and UDPGal, but had little effect on the Gal-1-P levels, suggests that the erythrocyte Gal-1-P found in galactosaemic patients may not be derived from UDPGal as proposed by Gitzelmann *et al.* (1969b).

The results of our *in vivo* galactose tolerance tests performed with a dose of 35 g per m^2 on three normal adults and three galactosaemic patients are consistent with the *in vitro* findings (Kaufman *et al.*, 1989). The baseline levels of UDPGal did not change while Gal-1-P levels continued to increase during the 4-h tests. These observations again suggest that there is no relationship between changes in levels of Gal-1-P and the concentrations of UDPGal in the erythrocytes.

The fact that uridine increases UDPGal in the absence of transferase activity suggests another mechanism may be operative, possibly through UDPGal pyrophosphorylase reaction.

$$\text{UTP} + \text{Gal-1-P} = \text{UDPGal and PPi}$$

With radioactive Gal-1-P as the substrate and UTP as the co-substrate, a very small amount of activity (0.2–0.5 μmoles/h per g haemoglobin) was found in haemolysates of normal controls and all galactosaemic patients. It is possible that the intracellular level of UTP may be limiting. UDPGal pyrophosphorylase activity probably resides with UDPGlc pyrophosphorylase (Knop and Hansen, 1970). One cannot ignore the possibility of the participation of both UDPGlc pyrophoshorylase and epimerase in producing the higher concentrations of UDPGlc and UDPGal observed in both normal and galactosaemic patients under the experimental conditions.

We have proposed that decreased levels of UDPGal may have a role in the pathogenesis of galactosaemia. To support this hypothesis, decreased levels of gangliosides and glycoproteins were reported in the brain and liver tissues of an adult who died of galactosaemia (Haberland *et al.*, 1971). UDPGal and UDPGlc are precursors to their formation.

Recently, we reported that the activities of four different enzymes, related to galactose metabolism, are very high in human ovarian tissue in comparison to testes and erythrocytes (Xu *et al.*, 1987). Uridine nucleotide sugar levels are also much higher. It is possible that the ovarian failure may also be related to a deficit of UDPGal, resulting in decreased synthesis of membrane glycoproteins necessary for normal development and maintenance of the integrity of the ovary.

Our findings that both UDPGlc and UDPGal levels increase with the addition of uridine suggests an approach in the management of galactosaemia to prevent late-onset clinical manifestations. Uridine has been used for the treatment of orotic aciduria without any clinical side-effects (Becroft *et al.*, 1969). The beneficial effect of orotic acid, a related pyrimidine, was reported for galactosaemic patients more than 30 years ago (Tada *et al.*, 1962). However, the explanation was based on speculation that orotic acid stimulated galactose metabolism rather than change in nucleotide sugar levels. Segal *et al.* (1966) showed that orotic acid did not stimulate galactose oxidation from both *in vitro* and *in vivo* studies. Our study showed that uridine did not affect the concentration of galactose-1-phosphate. Evaluation of uridine in the treatment of galactosaemia requires careful controlled long-term studies.

ACKNOWLEDGEMENTS

This study has been supported in part by the Mary Duque Emeritus Endowment Fund 2578488, Children's Hospital of Los Angeles. The authors thank Mrs Lee Mollison for the preparation of the manuscript and Drs Julian Williams and Thomas Roe for their reviews. Dr Neil Buist, Dr Harvey Levy and Dr Imie Sardarwalla kindly sent blood samples from patients affected with galactokinase deficiency and cultured skin fibroblasts from a patient with generalized UDPGal-4-epimerase deficiency.

Yan-Kang Xu from Sun-Yat Sen University of Medical Sciences, Guangzhou, China is a Research Fellow in the Division of Medical Genetics at Children's Hospital of Los Angeles.

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