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Evolution of the activity of UGT1A1 throughout the development and adult life in a rat

N. Bustamante, M.H. Cantarino, R.M. Arahuetes, F.J. Cubero, A. Ortiz *

Departamento de Fisiología (Fisiología Animal II), Facultad de Biología, Universidad Complutense de Madrid (UCM), 28040, Madrid, Spain

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Abstract

Biliary excretion is the main route of disposal of bilirubin and impaired excretion results in jaundice, a well recognisable symptom of liver disease. Conjugation of bilirubin in the liver is essential for its clearance. The glucuronidation of bilirubin is catalysed by the microsomal UDP-glucuronosyltransferase UGT1A1. Patients with Crigler–Najjar syndrome type 1 and Gunn rats, mutant strain of the Wistar rats, bear an autosomal recessive disorder resulting in hyperbilirubinemia. The aim of this work is to add new data about activity of UGT1A1 during the perinatal period and adult life. The results showed that activity of UGT1A1 is detectable from day 22 of the gestation. After birth, activity of UGT1A1 gradually increases and reaches the levels of adult life. Furthermore, bilirubin azopigments have been separated and characterized by thin layer chromatography. We have found that concentration of samples by evaporation and ulterior storing at −20 °C seemed to be suitable for the maintenance of samples.

Keywords: UGT1A1 activity; Glucuronidation; Monoglucuronides; Diglucuronides; Diazotization; Microsome; Azopigments; Bilirubin

Introduction

Patients with jaundice present yellow-colored skin and mucus caused by accumulation of bilirubin. The resulting toxic side effects, such as those that affect the nervous system of the newborn, can produce neonatal encephalopathy, known as kernicterus (Shapiro, 2003). This is due to the polar character of bilirubin, which passes through the blood brain barrier (Hayward et al., 1986).

Bilirubin is the degradation product of heme, the bulk of which is derived from hemoglobin of senescent erythrocytes and hepatic hemoproteins. Bilirubin is carried in the circulation by binding to plasma albumin. The endothelial lining of the hepatic sinusoids is fenestrated, and the albumin–bilirubin complex entering the liver through the portal circulation, passes through the fenestrae to reach Disse's space, where bilirubin comes into direct contact with the sinusoidal and basolateral plasma membrane domains of the hepatocyte. Bilirubin, but not albumin, passes into the hepatocyte throughout a specific uptake mechanism, implying that bilirubin must dissociate from albumin before its uptake into the hepatocyte. A fraction of the bilirubin is also derived from catabolism of hepatocellular proteins. Storage within the hepatocyte is accomplished by the binding of bilirubin to a group of cytosolic proteins, the ligandins. Binding to these proteins keeps bilirubin in solution and inhibits its efflux from the cell, thereby increasing net uptake (Cubero et al., 2001). Conjugation of bilirubin in the endoplasmic reticulum with UDP-glucuronic acid (UDPGA) is catalysed by the hepatic enzyme bilirubin UDP-glucuronosyl transferase (UGT1A1) (Mackenzie et al., 2003), forming bilirubin monoglucuronide and diglucuronide. Conjugation is obligatory for efficient transport across the bile canaliculus and disposal of bilirubin (González-Gallego and Tiribelli, 1995).

Patients with Crigler–Najjar syndrome type I (Crigler and Najjar, 1952) and homozygous Gunn rats (Gunn, 1938) show a lack of hepatic bilirubin conjugation activity due to mutations in the *UGT1A1* gene.

This work is focused on the determination of UGT1A1 activity in rat liver tissue and the study of glucuronidation activity throughout the development. Furthermore, bilirubin azopigments from different samples were evaluated by thin layer chromatography.

* Corresponding author. Fax: +34 91 3944935.
E-mail address: agustino@bio.ucm.es (A. Ortiz).

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Methods

Chemicals and apparatus

KH_2PO_4, diethyl ether (Panreac, Barcelona, España), K_2HPO_4, DTT, EDTA, ethyl-anthranilate, 2-Penthanone, Butylacetate (Boehringer Mannheim, Germany), Bradford's, Brij-56 (polyoxyethylene 10 cetyl ether), pig bile, biliary acids, biliary salts, methanol, Tris–Maleate/$MgCl_6H_2O$ (Sigma-Aldrich Chemie, Steinheim, Germany), biliverdin dimethylesther, conjugated bilirubin/sodium ditaurate bilirubin salt (Frontier Scientific, United Kingdom), chloroform (Merck, Darmstadt, Germany).

Spectrophotometer Hitachu U-2000, homogenaizer Heidolph RZR1, water-bath Bunsen, centrifuge Eppendorf 5403, ultracentrifuge Beckman XL-90, rotor 90 Ti.

Animals

Inbred Gunn and Wistar rats were bred and maintained in the Animal Facility Unit of the Facultad de Ciencias Biológicas de la Universidad Complutense de Madrid. Animals were housed in a climate-controlled (21 °C) room with a 12-h light–dark cycle and maintained on standard laboratory chow and water ad libitum.

19–22 gestational day foetuses; 1, 4 and 6 day neonatal pups were used; 30 day postnatal and adult rats weighing 250–260 g were used.

Liver isolation and the obtaining of the microsomal fraction. Caesarean section was carried out for the extraction of the foetuses. Then, both the foetuses and the neonatal pups were decapitated for extraction of the liver. Thirty-day postnatal pups and adult rats were anesthetized with ether and subjected to midline laparotomy. Livers were weighed and chopped up. Solution I, pH 7.4, 10–15% (w/v) (20 mM KH_2PO_4, 80 mM K_2HPO_4, 1 mM DTT, 1mM EDTA and 20% glycerol was used to homogenize the liver. Microsomes were obtained by the method of Heirwegh et al. (1972) and modified in our laboratory. Briefly, three centrifugations were assessed at 4 °C, 3000 rpm, for 10 min. The supernatant was then ultra centrifuged at 4 °C, 24.200 rpm, for 15 min. Then, the supernatant obtained was ultra centrifuged at 4 °C, 33.800 rpm, 45 min. Finally, the pellet was re-suspended in 300 μl of the same solution.

Protein content

The protein content of microsomes was determined by the Bradford method (1976). Microsomes were kept at −80 °C until determination of UGT1A1 activity was carried out.

Determination of UGT1A1 activity (quantitative analysis)

The determination of the activity of microsomal UGT1A1 is based on the method described by Van Roy and Heirwegh (1968), modified by Heirwegh et al. (1972), Viollon-Abadie et al. (1999) and Ortiz et al. (2003).

- Activation of microsomesTheactivation of microsomes with 0.02% Brij-56, 30 min at 37 °C, was carried out according to the method previously described by Viollon-Abadie et al. (1999).
- Diazotisation of bilirubin glucuronidesEthyl-antranilate was employed for selective diazotisation of bilirubin glucuronides as previously described Viollon-Abadie et al. (1999). Activity was expressed in nanomole conjugated bilirubin/min/mg cellular protein.

Separation of bilirubin azopigments by TLC (qualitative analysis)

The separation of bilirubin azopigments by TLC was done with the purpose of separating and qualitatively analysing conjugated and unbound bilirubin (monoglucuronides and diglucuronides, respectively). The samples used were: rat serum and bile (Wistar and Gunn), pig bile (5% in Tris–Maleate buffer), biliary acids, biliary salts, sodium ditaurate, biliverdin (0.5 mM in Tris–Maleate buffer), and liver microsomal fractions. Non-microsomal samples were also diazotised.

Pre-coated silica-gel plates were used to separate pigments. The chromatogram was developed at 18–20 °C using a mobile phase that consisted of chloroform–methanol–water (60:35:8 ml). Most standards and samples were evaporated and stocked in a dark room at room temperature (some standards and samples at −20 °C). Then, re-dilution in 2-pentanone-butylacetate (17:3, v/v) was performed. All the chromatographies were developed within a mean time of 1 h.

Table 1
Weight of animals, liver weight, UGT1A1 mean activity ± SD

	N°	Weight of animals (g) ± s.d.	Liver weight (g) ± s.d.	UGT1A1 activity ± s.d.
19 days'gestation	10	1.81 ± 0.087	0.15 ± 0.020	0 ± 0
20 days'gestation	10	2.85 ± 0.22	0.20 ± 0.023	0 ± 0
21 days'gestation	10	4.55 ± 0.31	0.28 ± 0.053	0 ± 0
22 days'gestation	8	6.01 ± 0.48	0.33 ± 0.063	0.0174 ± 0.0044
1 day	9	6.73 ± 0.54	0.30 ± 0.017	0.0490 ± 0.0089
4 days	11	8.8 ± 0.90	0.30 ± 0.028	0.0627 ± 0.0152
6 days	9	13.69 ± 2	0.37 ± 0.067	0.0912 ± 0.0201
1 month	8	72.36 ± 5.62	3.03 ± 0.19	0.175 ± 0.0199
Adults	9	257.36 ± 38.1	7.77 ± 0.99	0.275 ± 0.020

The activity is shown as nmol of conjugated bilirubin/min/mg protein.