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In Vitro Induction of Bilirubin Conjugation in Primary Rat Hepatocyte Culture

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UDP-glucuronosyltransferase (UGT1A1) is a critical enzyme in the elimination of bilirubin. The aim of our study was to investigate bilirubin conjugation in primary rat hepatocyte culture and the *in vitro* inducibility of this isoenzyme by inducing compounds of different classes: dexamethasone, clofibrate, rifampicin, and methylcholanthrene. Hepatocytes exhibited a marked decline in UGT1A1 activity in the first 4 h of culturing (10% of initial activity) and the recovery took 72 h. Immunoblot analysis proved that the loss of enzyme activity was associated with the decrease of protein concentration. Marked induction was detected in the cases of dexamethasone, clofibrate, and rifampicin treatments for 96 h both in enzyme activity (178, 176, and 168%) and in UGT1A1 protein level (362, 328, and 250%). The effects of dexamethasone and clofibrate were additive (210%). Methylcholanthrene had no influence on bilirubin conjugation in our system.

Key Words: **bilirubin; UGT1A1; induction; dexamethasone; rifampicin; clofibrate.**

Bilirubin, an end metabolite of heme degradation, is produced in large quantities from normal turnover of hemoglobin and other hemoproteins. Because of its serious toxicity the control of bilirubin concentration in serum is critical (1). The major factor regulating the excretion of bilirubin is the rate of its glucuronidation in the liver. This metabolic process is catalyzed by a member of the UDP-glucuronosyltransferase family, UGT1A1 (2–5). The rate of bilirubin conjugation as a consequence of the level of UGT1A1 enzyme activity is determined by genetic and environmental factors as well (6–9). It is well documented that UGT1A1 is subject to induction by various enzyme-inducing compounds of different inducing classes. These exogenous inducers like phenobarbital, clofibrate, dexamethasone, or rifampicin act through different regulation mechanisms (most of these processes have been clarified for cytochrome P450 induction); however, most of them increase enzyme activity on the transcription level (10–14). A number of papers demonstrated the *in vivo* effect of different enzyme-inducers on bilirubin elimination and on microsomal UGT1A1 activity (14, 15). Primary rat hepatocyte culture provides a useful model to study the *in vitro* effect of inducers on UGT1A1 expression and the mechanism of induction as well. This system provides possibility to investigate the effect of different inducers in combination also. Some papers described the *in vitro* induction of UGT1A1 detected on mRNA level, but little information is available regarding protein level of UGT1A1 and enzyme activity in the intact cells following treatment with the inducers (17). The aim of our study was to investigate the effect of dexamethasone, clofibrate, rifampicin and 3-methylcholanthrene on UGT1A1 enzyme activity toward bilirubin in intact rat hepatocytes and on UGT1A1 protein level determined by Western blot analysis.

MATERIALS AND METHODS

Chemicals. Bilirubin, UDP-glucuronic acid, 3-methylcholanthrene, clofibric acid, dexamethasone, rifampicin, William's Medium E, collagenase, and saponin were purchased from Sigma Chemical Co. (St. Louis, MO); sodium dodecyl sulfate (SDS), ethyl-2-aminobenzoate, and methyl propyl ketone were from Merck (Darmstadt, Germany). Rat polyclonal antibody against UGT1A1 was a generous gift of B. Burchell (Dundee, Scotland); all other chemicals were obtained from Reanal (Budapest, Hungary).

Primary hepatocyte cell culture. Male Wistar rats (200 g) were purchased from Toxicoop Safety Toxicological Study Center (Budapest, Hungary). They were kept under standard conditions. Hepatocytes were prepared by *in situ* liver collagenase perfusion (18). Cell viability (>85%) was determined by trypan blue exclusion. Cells were seeded on collagen-coated dishes (3.0 cm) at a density of 1.3×10^6 cells per dish in William's Medium E containing 10% of fetal calf serum, 100 nM insulin, 2.5 μg/ml amphotericin B, 0.1 mg/ml gentamicin, and 30 nM Na_2SeO_3. Calf serum and amphotericin B were present for the first 24 h than omitted. Cells were maintained at 37°C in a humidified atmosphere of 95% air–5% CO_2. 4 h after plating, and every day thereafter the medium was changed to Wil-

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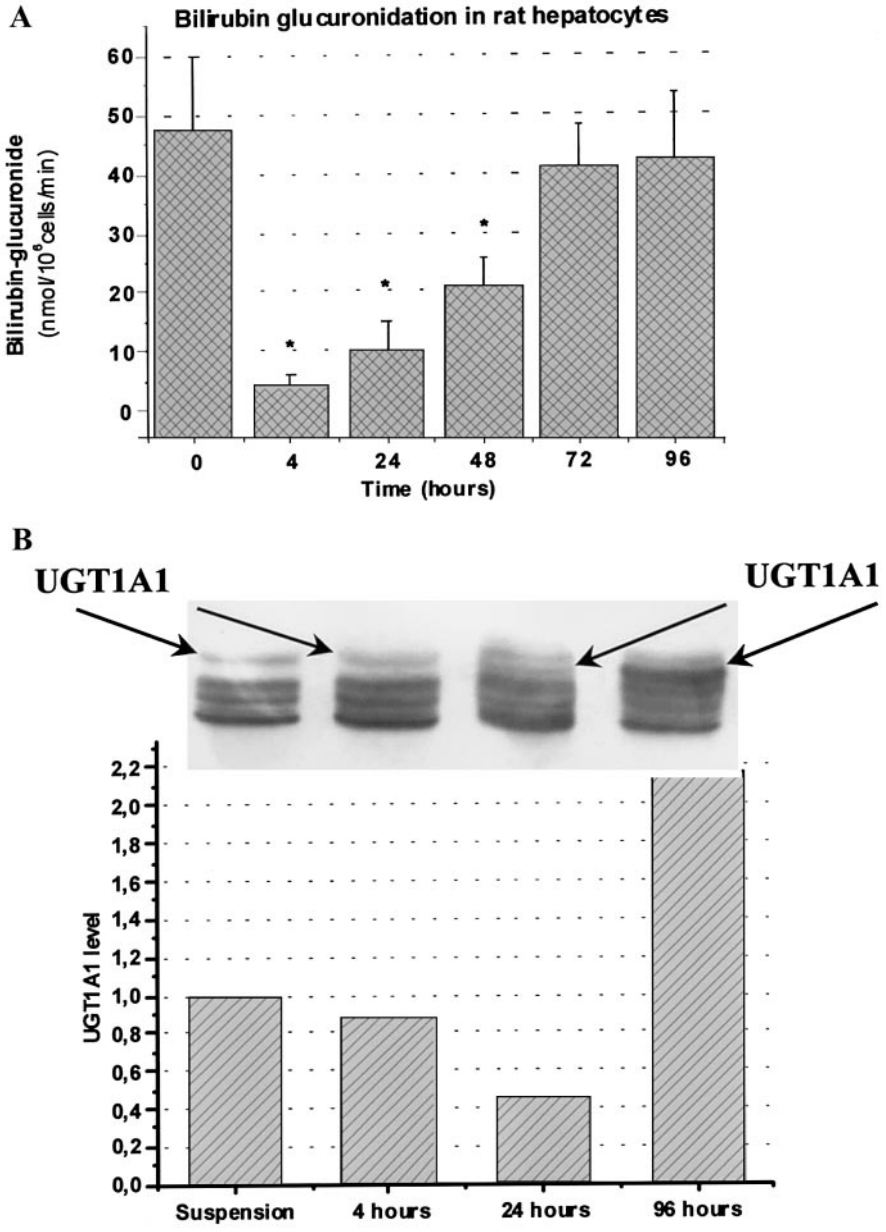

FIG. 1. Relationship between UGT1A1 enzyme activity and protein level in primary rat hepatocyte culture. (A) UGT1A1 enzyme activity toward bilirubin as a function of culturing time. Data represent means ± SD for three dishes in four experiments. *$P < 0.01$. (B) Immunoblot analysis of UGT1A1 protein in microsomes prepared from lysate of hepatocytes cultured for different time. Fifteen micrograms of protein/lane was subjected to SDS–PAGE. Relative levels of UGT1A1 were determined by UN-SCAN-IT digitizing software (Silk Scientific, Orem, U.S.A.).

liam's Medium E supplemented with 3.7 μM methylcholanthrene, 0.1, 1, 10 μM dexamethasone, 100 μM clofibrate, 50 μM rifampicin, and 10 μM dexamethasone combined with 100 μM clofibrate, respectively. Control medium contained 0.1% DMSO as it was the solvent of the inducers. The induction periods lasted for 24, 48, 72, and 96 h, respectively.

Bilirubin conjugation. After induction cells were incubated with 25 μM of bilirubin for 60 min. Bilirubin (60 mM) was dissolved in DMSO/1.0 M NaOH, 88/12 as a stock solution, diluted to 3 mM in the incubation medium containing 20 mg BSA/ml. The amount of bilirubin-glucuronide was determined by diazotation of the conjugate according the method of Burchell (19).

For preparing microsomes cells were scraped and sonicated on ice followed by centrifugation at 10,000*g* for 30 min, and the microsomes were sedimented at 100,000*g* for 60 min. Protein concentration was determined by the method of Lowry, using BSA as protein standard (20). Microsomes were stored at −70°C, and were used for Western immunoblot analysis.

Western immunoblot analysis. The microsomes were prepared for immunoblot analysis by suspension in sample loading buffer, they were heated at 100°C for 5 min, and separated by SDS–polyacrylamide gel electrophoresis (7.5%), loading 15 μg of protein into each well. After separation the proteins were transferred to nitrocellulose membranes, treated with PBS–Tween 20 (0.1%) containing 3% BSA overnight at 4°C (21). The membranes were washed in PBST before addition of primary antibodies, rat anti-liver anti-

body (RAL) (1:3000 dilution of 46.5 mg of protein/ml). Following incubation with primary antibodies, the membranes were washed, and incubated with secondary antibody, horseradish peroxidase-conjugated anti-sheep IgG (Calbiochem, San Diego, CA; dilution as recommended by the supplier). Immunoreactive proteins were visualized by chemiluminescence method (Amersham International, Arlington Heights, IL).

RESULTS AND DISCUSSION

Alterations in UGT1A1 Enzyme Activity and Protein Level during Culturing

To study the *in vitro* induction of UGT1A1 expression, we first determined the change in UGT1A1 enzyme activity as a function in a 5-day induction period due to culturing the hepatocytes. In the first 3–4 h of culturing UGT1A1 enzyme activity using bilirubin as substrate dramatically decreased to 10% of the initial enzyme activity determined in the cell suspension before seeding (Fig. 1). By 24 h of culturing the hepatocytes regained 20% of UGT1A1 activity and almost the initial activity (87%) was recovered in the 72-h culture and no further change was observed in the last 24-h period. One possible reason for the loss of enzyme activity might be the shock of cell preparation, seeding and attachment to the collagen underlay which depletes energy supplies of the cell resulting in shortage of UDP-glucuronic acid or in decrease of other energy demanding step of bilirubin-conjugation (e.g., transport). To prove this hypothesis we permeabilized the cell membrane by saponin treatment (22) or by freezing–thawing the cells. The permeability of cells was checked by trypan blue exclusion. The incubation medium for bilirubin conjugation was completed with 4 mM of UDP-glucuronic acid. Enzyme activity was not altered significantly by either treatment (Fig. 2), that contradicts the above hypothesis suggesting that neither the shortage of UDP-glucuronic acid nor the transport process plays role in the decrease of bilirubin conjugation. Another interpretation of our data is that the loss of enzyme activity is due to the degradation of UGT1A1 that may be manifested in inactivated protein and decreased protein level. Immunoblot analysis of UGT1A1 protein after different times of culturing shows, that the large decline in UGT1A1 expression in the 24-h culture is due to the loss of protein, however in the 4-h culture the protein level of UGT1A1 did not change parallel with enzyme activity (Fig. 1). This observation suggests that the degradation process has already started during the seeding period causing inactivation of the enzyme followed by protein decline. In the 96-h culture the protein concentration reached a significantly higher level than observed in the microsomes of cell suspension before seeding, and than it was expected according the enzyme activity data measured in the same culture. Ritter *et al.* reported similar data obtained in human hepatocyte culture, in a 3-day culture the protein level of UGT1A1 was similar to that of the liver before culturing (7).

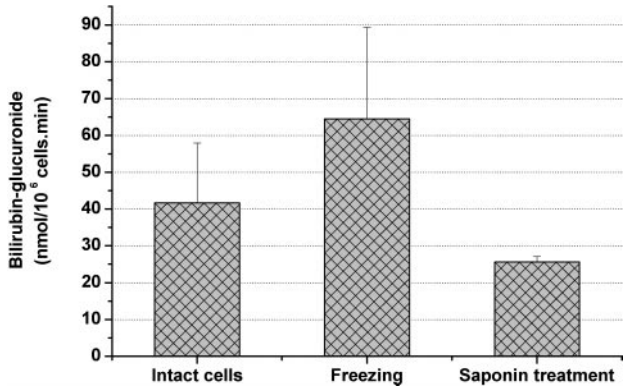

FIG. 2. Bilirubin conjugation in intact and permeabilized cells. Hepatocytes were permeabilized by freezing–thawing three times or treating with 0.005% of saponin for 5 min. Data are means ± SD of three dishes in four experiments.

Induction of UGT1A1

To the best of our knowledge this study is the first one which presents data on the *in vitro* inducibility of UGT1A1 in primary rat hepatocyte culture by inducers belonging to different classes: dexamethasone, clofibrate, rifampicin, 3-methylcholanthrene, and the combination of clofibrate with dexamethasone. All of the inducers except 3-methylcholanthrene increased UGT1A1 enzyme activity slightly even in the 48-h culture, however significant induction was measured after 96 h of induction (Fig. 3). Dexamethasone induced bilirubin conjugation in a concentration dependent manner (133, 150, and 178% by 0.1, 1, and 10 μM dexamethasone, respectively). The inductive effect of dexamethasone and clofibrate was additive in combination (210%). Clofibrate, rifampicin and 10 μM of dexamethasone exerted similar rate of induction (176, 168, and 178%, respectively). Methylcholanthrene had no effect on UGT1A1 enzyme activity. The immunoblot analysis of UGT1A1 protein amount of control and inducer-treated hepatocytes supports the enzyme activity data, the protein level of induced hepatocytes was elevated to 328, 250, and 362% by clofibrate, rifampicin, and 10 μM dexamethasone, respectively (Fig. 3). For significant rate of induction hepatocytes needed 96 h of treatment with the different inducers. Previous studies reported species and strain differences in responsiveness to methylcholanthrene treatment and the involvement of aryl hydrocarbon (Ah) receptor in the regulation process arose in the case of human hepatocytes (7, 23). Li *et al.* (17) reported, that bilirubin can induce mRNA of UGT1A1 in rat hepatocyte culture probably acting as an endogenous ligand of the Ah receptor (24, 25). In our experiment methylcholanthrene, another well described ligand of the Ah re-

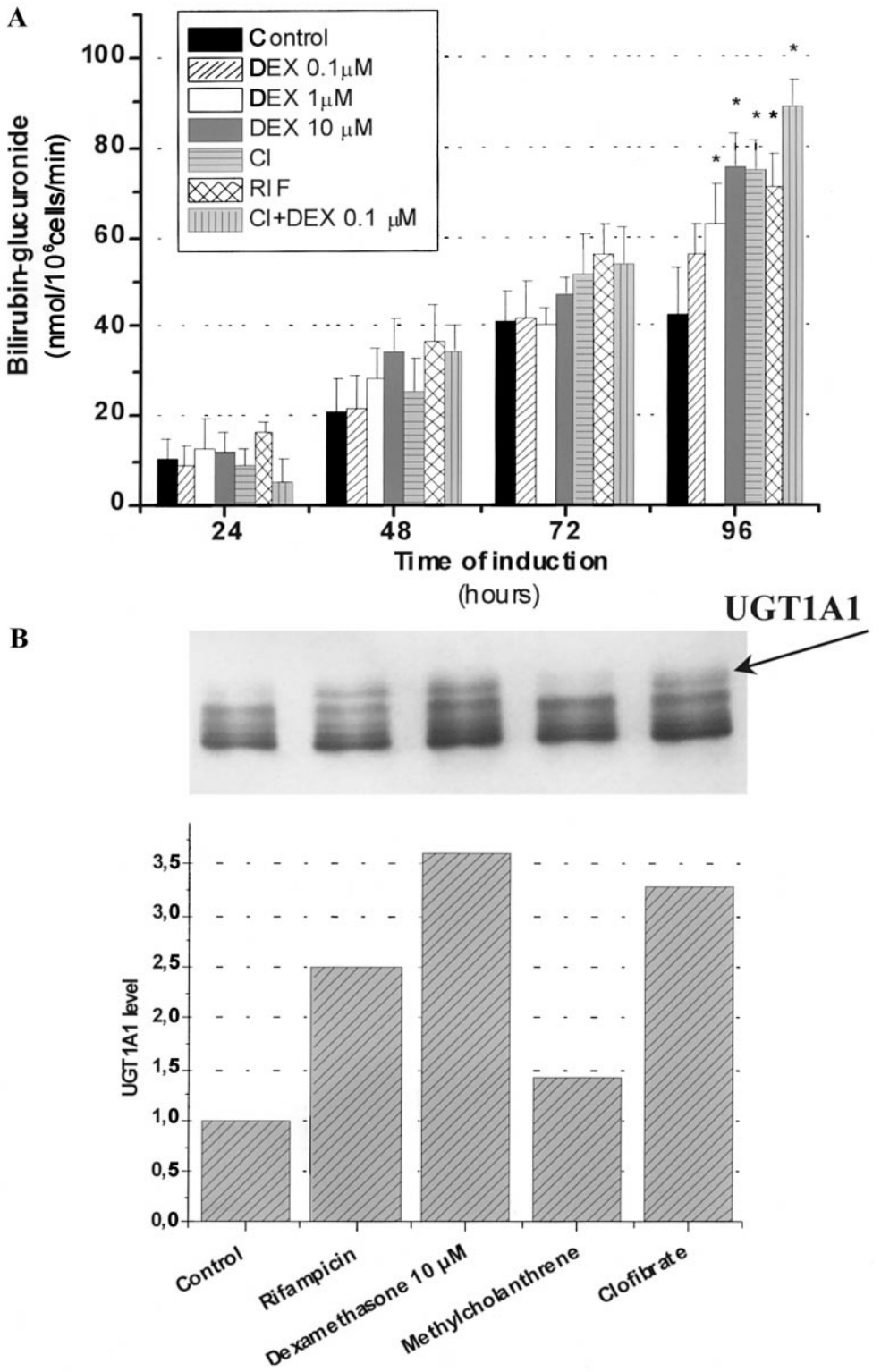

FIG. 3. *In vitro* induction of UGT1A1 expression by dexamethasone, clofibrate, rifampicin, and 3-methylcholanthrene. (A) Enzyme activity of UGT1A1 toward bilirubin after treating the cultures with the inducers for 24, 48, 72, and 96 h. Data represent means ± SD for three dishes in three experiments. $*P < 0.01$. (B) Immunoblot analysis of UGT1A1 protein in microsomes prepared from lysate of the induced hepatocytes (96 h). Fifteen micrograms of protein/lane was subjected to SDS–PAGE. Relative levels of UGT1A1 were determined by UN-SCAN-IT digitizing software (Silk Scientific).

ceptor had no influence on UGT1A1 expression either on protein level or enzyme activity measured by bilirubin conjugation (Fig. 3).

In conclusion, the results obtained demonstrate that primary rat hepatocyte culture loose most of UGT1A1 activity in the first 24 h of culturing, and the total recovery takes about 72 h. Studying the *in vitro* effect of inducing agents of different classes (dexamethasone, clofibrate, and rifampicin) seems feasible in this model system; the elevated UGT1A1 expression was detected not only by measuring enhanced enzyme activity toward bilirubin but also on protein level seen by immunoblot analysis.

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