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Influence of culture system and medium enrichment on sulfotransferase and sulfatase expression in male rat hepatocyte cultures

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Abstract

The expression of sulfotransferase and steroid sulfatase was studied in rat liver using the most promising culture models of hepatocytes, including monolayer culture with a pyruvate (30 mM) enriched medium, co-culture with rat epithelial cells from primitive biliary origin and collagengel sandwich culture. In the latter, addition of dexamethasone (1 μM) to the medium was examined. Phenol sulfotransferase enzymes (SULT1) were studied by measuring activities towards 4-methylphenol and estradiol, hydroxysteroid sulfotransferase (SULT2A) activity was determined towards dehydroepiandrosterone (DHEA). Microsomal steroid sulfatase activity was measured towards estrone sulfate. Western blot analysis was carried out using polyclonal antibodies raised against rat phenol sulfotransferase SULT1A1 (ASTIV), estrogen sulfotransferase SULT1E1 (EST) and hydroxysteroid sulfotransferase (HST). SULT2A activity towards DHEA was maintained at a high level during the whole culture time. In the co-culture it even reached the level of freshly isolated cells. Addition of pyruvate had no positive effect on the activity measured in monolayer cultures. High SULT1A1 activity towards 4-methylphenol was found in the co-culture system. In the monolayer culture, the activity initially decreased with 35% but was then kept at a constant level, while in the sandwich culture low activities were measured. For dexamethasone, an inducing effect on the various SULT activities could not be detected. Independently of the culture model used, the SULT1E1 activity towards estradiol decreased to 20% and 5% of the initial activity after four and seven days of culture, respectively. Microsomal steroid sulfatase activity was best maintained in collagengel sandwich cultures. During the first four days in culture it retained 73% of the initial activity, afterwards it decreased to 40% of the activity found in freshly isolated hepatocytes, irrespective of the culture conditions. High expectations exist for collagengel sandwich cultures, however, in our study the results were rather disappointing. Monolayer is a suitable culture model for short-term purposes. For long-term *in vitro* biotransformation studies, co-culture is preferred but is rather complex.

Keywords: Sulfotransferase; Sulfatase; Hepatocyte cultures; Dexamethasone; Pyruvate

1. Introduction

SULTs (EC 2.8.2) are a family of phase II biotransformation enzymes catalysing the transfer of a sulfuryl group from PAPS to an electrophile acceptor molecule, generally making the molecule more water-soluble. SULTs play an important role in xenobiotic detoxification, carcinogen activation, prodrug processing, cellular signalling pathways and the regulation of intra-tissue active androgen and estrogen levels [1–4]. Two main subfamilies, the phenol SULTs (SULT1) and the hydroxysteroid SULTs (SULT2) are well characterised. The estrogen SULTs are members of SULT1, although these isoenzymes are distinct members of the SULT family based on their selectivity and high affinity for estrogens. Sex-specific distribution of SULT isoenzymes in rat is described, SULT1 is designated as the male-specific family, while SULT2 is female-predominant [5].

Steroid sulfatase or arylsulfatase C (EC 3.1.6.2) is a microsomal enzyme catalysing the desulfation of sulfated steroids and other compounds. Sulfoconjugates of biomolecules not only serve as water-soluble excretion products,

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Abbreviations: CYP, cytochrome P450; DEX, dexamethasone; DHEA, dehydroepiandrosterone; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; GST, glutathione S-transferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; RLEC, rat liver epithelial cells; SULT, sulfotransferase.

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but also as an inactive transport form in the circulation. In their target tissue, they are taken up and converted to the active, parent molecule explaining the key role of estrogen sulfatase in regulating estrogen production [6,7].

Different culture models for hepatocytes exist. The best known ones are simple monolayer culture, co-culture with rat epithelial cells of primitive biliary origin and collagen gel sandwich configuration culture. The expression of sulfotransferase and sulfatase activities in hepatocyte cultures has not received the same attention as given to other biotransformation enzymes. In monolayer culture, hepatocytes remain viable in a non-proliferating state during a limited number of days and express several differentiated functions. However, a sharp decline in the CYP content (90% in the first 48 hours) and changes in CYP oxidative reactions have been noticed [8]. Various attempts, in particular enrichment of the culture medium, have been made to improve the maintenance of different functions [9–11]. As such, pyruvate-supplemented (30 mM) monolayer cultures remain morphologically intact for at least 7 days and during this period they maintain specific liver functions such as secretion of albumin, expression of glucokinase, CYP 2C11 mRNA [12] and GST activities [13,14]. However, Wirachwong and Fry [15] could not demonstrate any beneficial effect of pyruvate addition to the culture medium on CYP-dependent enzyme activities (7-ethoxy-coumarin *O*-deethylase, 7-ethoxy-resorufin *O*-deethylase and 7-pentoxyresorufin *O*-dependentylase). The co-culture system is based on the establishment of cell-cell contact between hepatocytes and RLEC originating from the hepatic stem cell compartment located in the terminal ductules connecting the canals of Hering with bile canaliculi [16–18]. The hepatocytes retain better their differentiated state, for example the expression and inducibility of CYP-dependent monooxygenases and phase II enzymes such as GST are better maintained for a longer time in comparison with simple monolayer cultures [9,19,20]. Sandwiching hepatocytes between two layers of hydrated collagen type I closely mimics the *in vivo* situation by re-establishing the polarity of the cells and has positive effects on the long-term maintenance and expression of biotransformation enzymes and on the secretion of liver-specific proteins [21–26]. DEX has often been added to the culture medium in concentration ranges between 0.1 and 1.0 μM to enhance cell survival and better maintenance of adult hepatic functions, including albumin secretion and expression of phase I and phase II biotransformation enzymes [11,27]. It has been suggested to be an inducer of SULT activity, however its effect on SULT activity is controversial [27,28]. Treatment of rats with pharmacological doses of dexamethasone resulted in increased expression of SULT1 and SULT2 enzymes at both the mRNA and activity levels [29]. Comparable results were obtained in rat hepatocyte cultures supplemented with 0.1 μM dexamethasone [30]. Liu & Klaassen [28], however, revealed that the SULT expression in rat hepatocyte cultures was lost at the mRNA level, but showed that the hepatocytes remained inducible. In contradiction, McMillan and co-workers [27] reported an accelerated decrease in SULT activities towards 1-naphthol and estrone by the addition of 1 μM DEX.

The aim of this work was to identify a culture model for rat hepatocytes, which maintains the expression of the SULTs and steroid sulfatase at a reasonable level in comparison with that of freshly isolated hepatocytes.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA, fraction V), bovine insulin, crude collagenase type I, DEX, glucagon, kanamycin monosulfate, L-glutamine, sodium pyruvate, rat albumin (fraction V), streptomycin sulfate, DHEA, estradiol, estrone sulfate, barium acetate, barium hydroxide and zinc sulfate were purchased from Sigma Chemical Company. Hydrocortisone sodiumsuccinate came from Upjohn and benzylpenicillin from Continental Pharma. Amphotericin B and sodium ampicillin were from Bristol-Meyers Squibb. DMEM without L-glutamine and without phenol red were specially prepared by B.I. BioWhittacker; FBS, Minimum Essential Medium (MEM) without phenol red and Medium 199 (M199) without phenol red were from Gibco BRL. Peroxidase conjugated rabbit anti-rat albumin was obtained from Organon Technika. [1,2,6,7-^{3}H(N)]-DHEA (115 Ci/mmol), [2,4,6,7-^{3}H(N)]-estradiol (72 Ci/mmol), PAP^{35}S (1.5–2.54 Ci/mmol), [6,7-^{3}H(N)]-estrone sulfate were purchased from Du Pont/New England Nuclear. Scintillation fluids, Emulsifier Safe and Opti Fluor O, were from Canberra Packard. All other compounds were readily available products.

2.2. Isolation and culture of adult rat hepatocytes

Hepatocytes were isolated, as previously described [31], from male outbred OFA (Orcins France stem A) Sprague Dawley rats, 200–250 g, Iffa-Credo, Belgium) with free access to food and water. Cell integrity was measured by trypan blue exclusion (87.6% $\pm$ 2.9, N = 4). Collagen gel sandwich cultures were prepared according to Beken *et al.* [32]; DMEM was used as culture medium, supplemented with 10% (v/v) FBS, 0.5 U/mL bovine insulin, 0.007 μg/mL glucagon, 7.5 μg/ml hydrocortisone sodiumsuccinate and antibiotics (7.3 IU/mL benzylpenicillin, 50 μg/mL kanamycin monosulfate, 10 μg/mL sodium ampicillin and 50 μg/mL streptomycin sulfate), and 0.25 μg/mL amphotericin B. After 4 hr, the medium was replaced by serum-free DMEM medium (described above) supplemented with 0.05% (v/v) dimethylsulfoxide (DMSO) or 1 μM DEX in 0.05% (v/v) DMSO according to the different culture conditions. Simple monolayer cultures were prepared as described [33], with the modification that the same DMEM medium was used as for the sandwich cultures. When appropriate, 30 mM pyruvate was added. Co-cultures were set

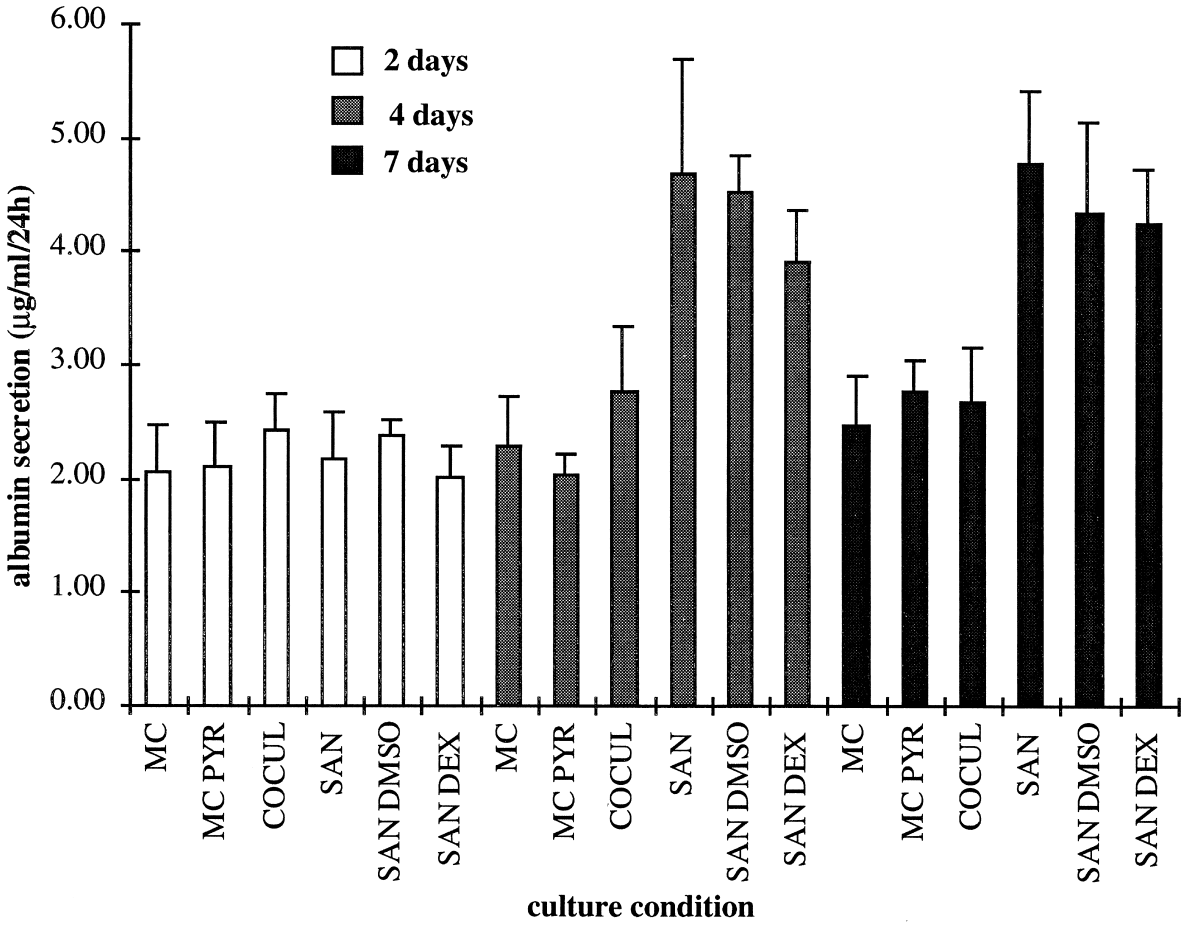

Fig. 1. Albumin secretion (μg/mL/24 hr) into the culture medium of rat hepatocytes in different models after 2, 4, and 7 days of culture. The hepatocytes were cultured in a simple monolayer culture (MC), a co-culture with RLEC (COCUL) or a collagen gel sandwich configuration (SAN). In addition, the effects of 30 mM pyruvate (MC PYR), 0.05% (v/v) DMSO (SAN DMSO) or 1 μM DEX in 0.05% (v/v) DMSO (SAN DEX) were investigated. The culture medium was renewed every 24 hr. The results are expressed as mean $\pm$ SD (N = 4 independent experiments).

up as described [33]; the culture medium was a mixture of 75% (w/w) MEM and 25% (w/w) M199 supplemented with 10% (v/v) FBS, 1 mg/mL BSA, 0,278 U/mL bovine insulin, antibiotics and L-glutamine in a final concentration of 0.244 mg/ml. The medium was changed daily by serum-free medium additionally supplemented with 2.5 μg/mL hydrocortisone hemisuccinate and 0.25 μg/mL amphotericin B. Hepatocyte cultures were kept at 37° in an atmosphere of 95% air and 5% CO_2 with a relative humidity of 100%.

2.3. Albumin secretion

Medium samples were analysed for their albumin content by an enzyme-linked immunosorbent assay (ELISA) according to Dunn *et al.* [22].

2.4. RLEC in co-cultures

When co-culturing hepatocytes with RLEC [33], approximately 6×10^5 RLEC were added per ml culture medium. For each culture experiment, the percentage of RLEC present in the co-cultures was determined as follows. After 3 days of culture, the hepatocytes were detached from the epithelial cells by using a collagenase solution (91 units/mL in PBS) at 37° and the remaining epithelial cells were scraped off. The protein content was measured using a BioRad Protein Assay kit (BioRad, Belgium) with BSA as a standard. By making the ratio of the protein content of the epithelial cells in co-culture versus the total protein content (hepatocytes and epithelial cells), the percentage of RLEC (40.2% $\pm$ 8.3, N = 4) was determined. This value was taken into account when the amount of protein originating from the hepatocytes was calculated.

2.5. Enzyme activities

Before the preparation of cytosolic fractions, rat hepatocytes cultured in a sandwich configuration were adopted to a collagenase digestion as described previously [34]. Cells from monolayer and co-cultures were harvested by scraping. Cytosol and microsomes were prepared by differential centrifugation in 10 mM Tris/HCl, pH 7.4, containing 250

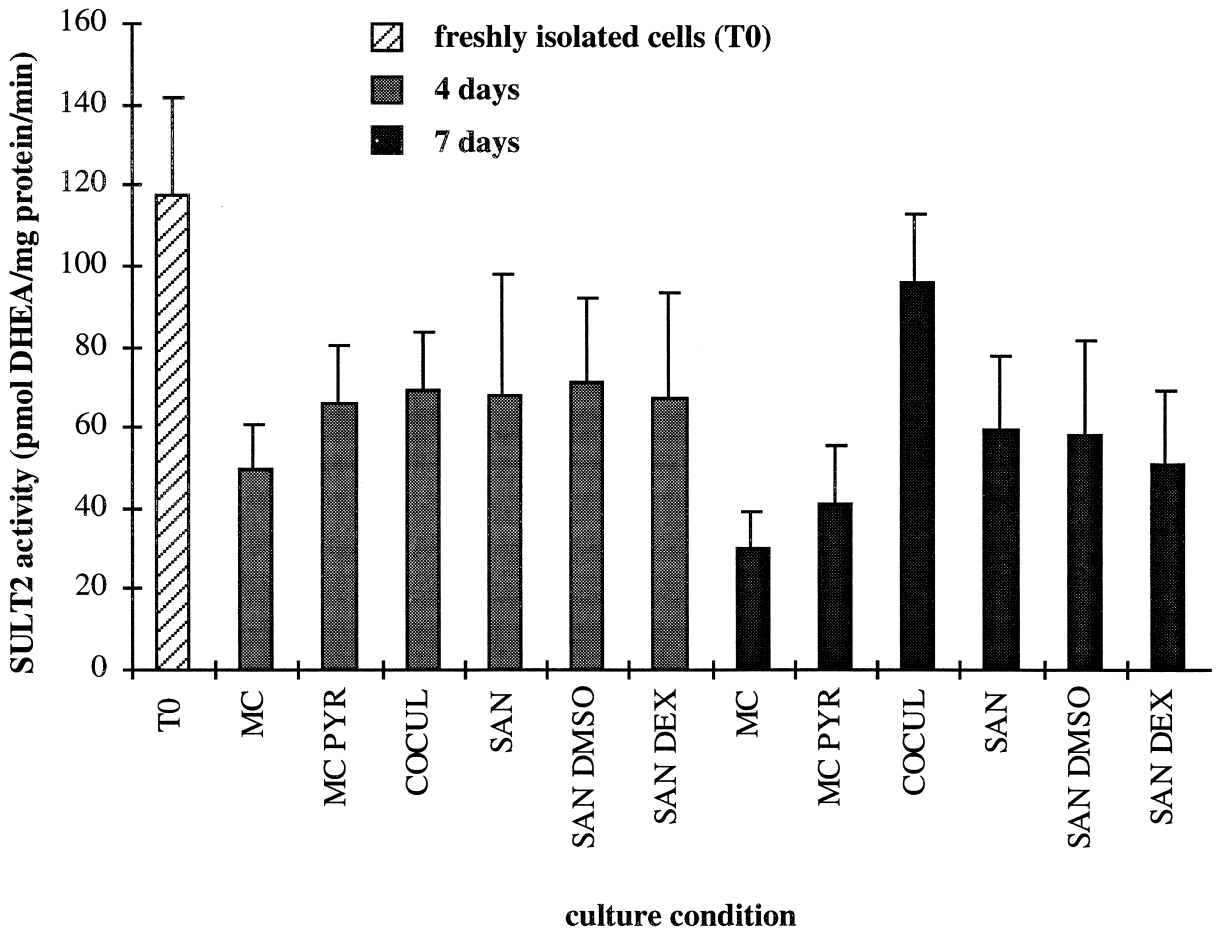

Fig. 2. SULT2A activity towards DHEA of freshly isolated rat hepatocytes and rat hepatocytes after 4 and 7 days under different culture conditions. The hepatocytes were cultured in a simple monolayer culture (MC), a co-culture with RLEC (COCUL) or a collagen gel sandwich configuration (SAN). Additionally the influence of 30 mM pyruvate (MC PYR), 0.05% (v/v) DMSO (SAN DMSO) or 1 μM DEX in 0.05% (v/v) DMSO (SAN DEX) were studied. The results are expressed as mean ± SD (N = 4 independent experiments).

mM sucrose. The microsomal pellets were resuspended in the same buffer and cytosol and microsomes were stored at −70° until use. Cytosolic and microsomal protein contents were measured using a Bio-Rad Protein Assay kit.

The assay conditions for the SULT enzyme activities, described in the literature, have been optimised in our laboratory with respect to substrate and PAPS concentrations, incubation times and protein contents. All assays were performed in duplicate.

The SULT2A enzyme activity assay with DHEA was performed as described in Sharp *et al*. [35] with minor modifications: incubations contained cytosolic protein (50 μg), [^{3}H]DHEA (10 μM, 0.1 μCi), PAPS (50 μM) and buffer (0.1 M Tris/HCl, 20 mM $MgCl_2$, pH 7.5). After incubation for 45 min at 37°, reactions were stopped by adding 3 mL of chloroform. 250 μL 0.25 M Tris/HCl (pH 8.7) were added. After shaking, phases were separated by centrifugation at 3000 *g* for 3 min. An aliquot of the aqueous layer was mixed with scintillation fluid (Emulsifier Safe) and the radioactivity was determined by liquid scintillation spectrometry. Blank incubations contained no PAPS.

The estradiol SULT1E1 assay is a modification of the method described by Borthwick *et al*. [36]: incubations contained cytosolic protein (15 μg), [^{3}H]estradiol (0.05 μM, 0.1 μCi), PAPS (50 μM) and buffer (60 mM potassium phosphate; 0.7 mM $MgCl_2$, pH 6.0). After incubation for 30 min at 37°, reactions were stopped by adding 3 mL water-saturated dichloroethane, and 300 μL of water. From here on, the same protocol as described for DHEA was followed.

SULT1A1 activity was measured with 4-methylphenol by the method of Foldes and Meek [37] with the following modifications. Incubations contained cytosolic protein (15 μg) in buffer (10 mM potassium phosphate, pH 7.4), PAP^{35}S (10 μM), 4-methylphenol (50 μM). Blanks contained no 4-methylphenol. After incubation for 20 min at 37°, the reaction was terminated by adding 200 μL barium acetate (0.1 M), 200 μL of barium hydroxide (saturated), 200 μL of zinc sulfate (0.1 M). We determined that 4-methylphenol was selective for SULT1A1, and was not a good substrate for SULT1C1 (not shown).

For estrone sulfatase activity determination, the incubation mixture contained microsomal protein (10 μg), estrone

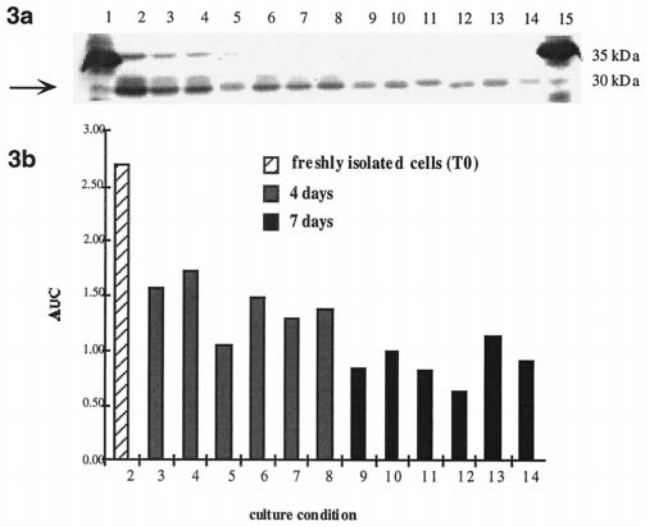

Fig. 3. Western blot analysis of DHEA SULT2A protein in rat hepatocyte cytosol (3a) and densitometric analysis of expression levels (3b). Protein samples were subjected to SDS-PAGE, and following separation were electrophoretically transferred to nitrocellulose and immunostained as described in Materials and Methods. Lane 1, purified human DHEA SULT2A1 protein. Lane 2, cytosol (20 μg cytosolic protein) from freshly isolated rat hepatocytes. Lanes 3–8, cytosol (20 μg cytosolic protein) of rat hepatocytes after 4 days of culture, respectively from monolayer culture (3), monolayer culture enriched with 30 mM pyruvate (4), co-culture (5), collagen gel sandwich culture (6), collagen gel sandwich culture with 0.05% (v/v) DMSO (7) and collagen gel sandwich culture with 1 μM DEX in 0.05% (v/v) DMSO (8). Lanes 8–14 the same culture condition but after 7 days of culture. Lane 15, purified human DHEA SULT2A1 protein. The human 2A1 protein migrates at a higher Mr than the rat orthologs.

sulfate (8 μM) and buffer (0.2 M Tris/maleate, pH 6.9). Blank incubation did not contain microsomal protein. The reaction was carried out at 37° for 10 min and was terminated by addition of 200 μL of ice-cold water and 2 mL of ethyl acetate. At this stage, microsomal protein was added to the blanks. After extraction and centrifugation at 3000 *g* for 3 min, an aliquot of the ethylacetate layer was mixed with scintillation fluid (Opti Fluor O) and the radioactivity determined by liquid scintillation spectrometry.

2.6. Western blotting

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and immunochemical localisation were performed as described previously [38]. Blots were probed with polyclonal antibodies raised against rat liver SULTs: SULT1A1 (ASTIV) antibodies, estrogen sulfotransferase (EST) and hydroxysteroid sulfotransferase SULT2A (HST) antibodies were all raised in rabbits. They were purified to homogeneity and well characterised to recognise in Western blot analysis the antigens against which they were raised [35,36,39]. There is some cross-reactivity of anti-SULT1E1 antibody with SULT1A1 (Fig. 5), and also some cross-reactivity of anti-SULT1A1 with SULT1E1 (Fig. 7). Densitometric quantification was performed and areas under the peaks (AUC) were measured (Enhanced Laser Densitometer and software Gelscan XL 2.1).

2.7. Statistical analysis

The results were analysed using an ANOVA test ($P < 0.05$ is considered to be significant), followed by Dunnett's post-hoc test.

3. Results

3.1. Albumin secretion

Albumin secretion into the medium (Fig. 1) was measured here as an indicator of differentiated liver function. It was maintained at a constant level over the course of 7 days in the simple monolayer culture and in the co-culture. Enrichment of the medium with 30 mM pyruvate, however, had no positive effect. In the sandwich cultures, albumin secretion was significantly ($P < 0.05$) higher compared to the values in the monolayer culture and co-culture. No significant effect of DMSO or DEX was observed in the sandwich cultures.

3.2. Enzyme activity and expression of SULT and sulfatase

3.2.1. SULT2A activity towards DHEA

After 4 days of culture, SULT2A activities towards DHEA diminished (around 55%) significantly ($P < 0.001$) compared to the activity of freshly isolated cells (Fig. 2). Addition of 30 mM pyruvate to the culture medium had no significant effect on the activity measured in the monolayer cultures. There was also no difference in activity when DMSO or DEX were added to the sandwich cultures. After 7 days, the SULT2A activity towards DHEA in the co-culture recovered to a level comparable to that of freshly isolated hepatocytes. However, the activities measured in simple monolayer cultures and sandwich cultures after 7 days, stayed at the same level of the values measured after 4 days. Addition of DEX or DMSO to the sandwich culture medium, had no effect. When these activity results are compared with those obtained at the protein level (Fig. 3), some discrepancies are seen. Although the amount of immunoreactive proteins after 4 days of culture, is also around 55% of the amount of freshly isolated hepatocytes, the amount in co-culture is slightly lower compared to the other culture systems. After 7 days of culture, a lower amount was seen in all the culture systems used. The discrepancy between protein and enzyme activity may be explained by the activity of other members of the SULT2A family towards DHEA which may not be detected by this antibody.

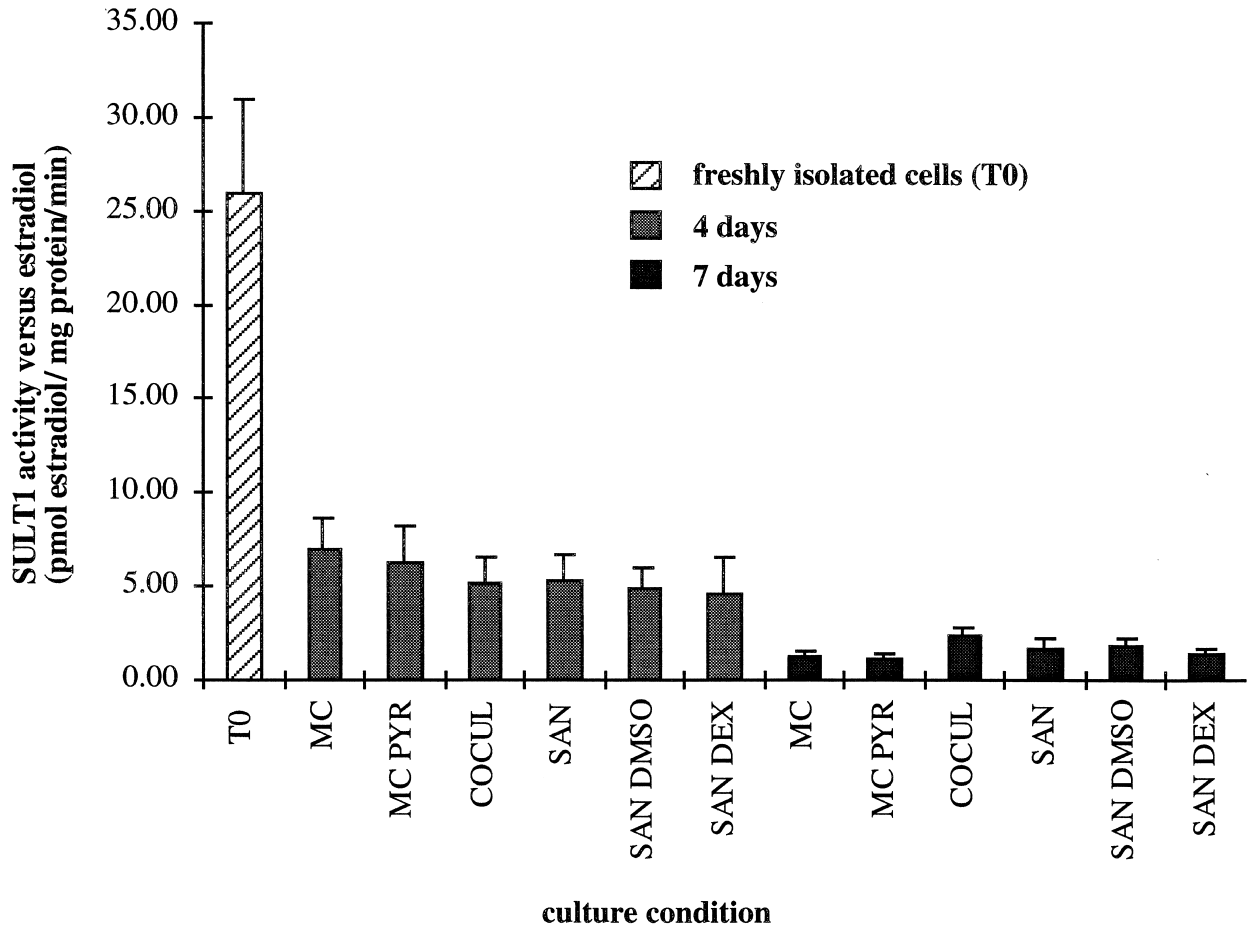

Fig. 4. SULT1E1 activity towards estradiol in freshly isolated rat hepatocytes and rat hepatocytes after 4 and 7 days under different culture conditions. The hepatocytes were cultured in a simple monolayer culture (MC), a co-culture with RLEC (COCUL) or a collagen gel sandwich configuration (SAN). Additionally the influence of 30 mM pyruvate (MC PYR), 0.05% (v/v) DMSO (SAN DMSO) or 1 μM DEX in 0.05% (v/v) DMSO (SAN DEX) were studied. The results are expressed as mean ± SD (N = 4 independent experiments).

3.2.2. SULT1E1 activity towards estradiol

After 4 days in culture, the SULT1E1 activities towards estradiol measured in the different culture models studied were only 20% of the activity observed in freshly isolated hepatocytes ($P < 0.0001$) (Fig. 4). Addition of 30 mM pyruvate to the simple monolayer cultures had no effect. DEX did not induce SULT1E1. No statistical significant differences could be observed between 4 and 7 days of culture. In western blots of the same cells, very low SULT1E1 protein contents were seen, although these were slightly higher for simple monolayer cultures and co-cultures than for sandwich cultures, both after 4 and 7 days of culture (Fig. 5).

3.2.3. SULT1A1 activity towards 4-methylphenol

With the exception of co-cultures, SULT1A1 activity towards 4-methylphenol after 4 days of culture, was statistically lower ($P < 0.0001$) than those measured in freshly isolated cells (Fig. 6). When comparing the activities in the different culture systems, it is clear that, after 4 and 7 days, in co-culture the activities are kept at a level comparable to that of freshly isolated hepatocytes. After 4 days in monolayer cultures the SULT1A1 activities, although statistically lower ($P < 0.05$) than in co-cultures, are significantly higher ($P < 0.01$) than those measured in sandwich cultures. In the latter case, SULT1A1 activities towards 4-methylphenol fell to 20% of the activity measured in freshly isolated cells and stayed at that low level during the rest of the culture time. No significant difference, could be observed when pyruvate, DMSO and DEX were added to the monolayer cultures or the sandwich cultures, respectively. After 7 days, addition of pyruvate had a negative effect on the SULT1A1 activity versus 4-methylphenol. DMSO and DEX did not have any effect on the activities measured (Fig. 6). The results of the western blots supported the data obtained from the enzyme activity measurements. Here also, the results obtained in the sandwich cultures were rather poor (Fig. 7).

3.2.4. Sulfatase activity

Compared to freshly isolated rat hepatocytes, the steroid sulfatase activity significantly ($P < 0.0001$) decreased as

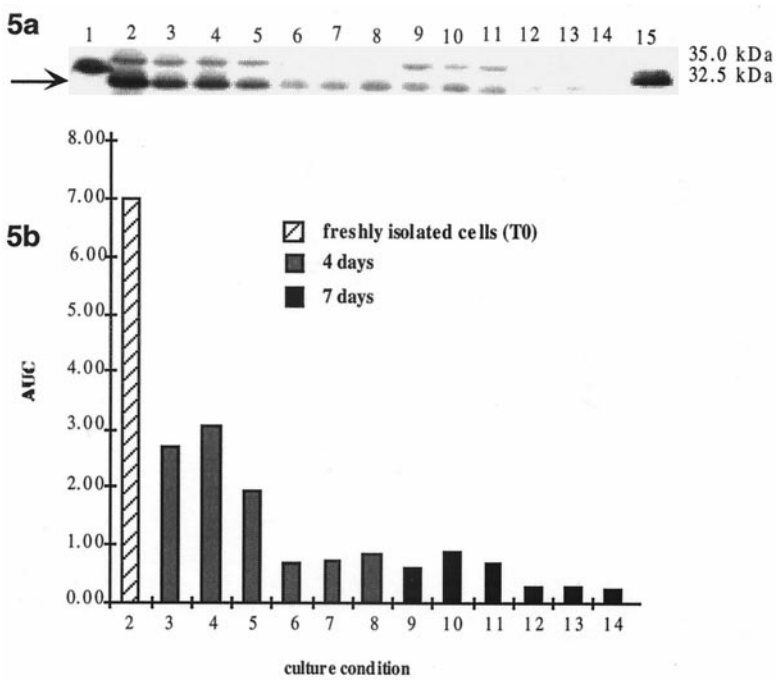

Fig. 5. Western blot analysis of estrogen SULT1E1 protein expression in rat hepatocyte cytosol (5a) and densitometric analysis of expression levels (5b). Lane 1, purified human SULT1E1 protein (200 ng). Lane 2, cytosol (20 μg cytosolic protein) from freshly isolated rat hepatocytes. Lanes 3–8, cytosol (20 μg cytosolic protein) of rat hepatocytes after 4 days of culture, respectively from monolayer culture (3), monolayer culture enriched with 30 mM pyruvate (4), co-culture (5), collagen gel sandwich culture (6), collagen gel sandwich culture with 0.05% (v/v) DMSO (7) and collagen gel sandwich culture with 1 μM DEX in 0.05% (v/v) DMSO (8). Lanes 8–14 the same culture condition but after 7 days of culture. Lane 15, purified human SULT1E1 protein (200 ng). The human SULT1E1 migrates with a larger Mr than rat SULT1E1. The second immunoreactive protein seen in some samples at 35 kDa is due to some cross-reactivity of the antibody with SULT1A1.

a function of culture time in all models tested (Fig. 8). However, it was somewhat better preserved in collagen sandwich cultures (73%) compared to monolayer cultures (59%) and co-cultures (54%). Addition of either pyruvate, DMSO or DEX did not affect the activities measured at days 4 and 7 in monolayer and sandwich cultures, respectively.

For the different substrates used, no activity could be measured in the RLEC used in the co-cultures.

4. Discussion

Long-term cultures of hepatocytes are important in pharmacotoxicologic studies, in particular when long-term effects such as induction and interaction with endogenous and exogenous molecules are involved [40]. In these models, activities of phase II biotransformation enzymes were reported to be better preserved than those of phase I enzymes [10,11]. However, the enzymes studied were principally GST and uridine diphosphateglucuronosyltransferase (UGT). SULTs have not received the same attention, although they are important phase II biotransformation enzymes involved in detoxification and activation of many drugs and exogenous compounds. Our knowledge on sulfatases in hepatocyte cultures is even more limited.

Three key factors, affecting the long-term maintenance of liver-specific functions in culture have been defined as being cell-cell contact, extracellular matrix environment and medium composition [9,11]. From our results (enzyme activities and enzyme protein level) it seems that cell-cell interaction is an important factor in maintaining SULT2A (activity towards DHEA) and SULT1A1 (activity towards 4-methylphenol) expression. On the contrary, for SULT1E1 (activity towards estradiol) expression levels were equally low in the three culture models. Since each of these is based on one of the three key factors mentioned, none of these factors seems to be of basic importance for the regulation and maintenance of the estrogen SULT expression. Pyruvate addition to the medium had no effect either and dexamethasone was not inducing. Thus for the moment an explanation why estrogen SULT expression cannot be preserved in male rat hepatocyte cultures is not available. Further experiments with sex hormones and female hepatocytes are underway and perhaps they will provide us with more information on the regulation of its expression in hepatocyte cultures.

Pyruvate has been reported to be an antioxidant having beneficial effects on albumin secretion, CYP-dependent activity and GST-expression in cultured hepatocytes [12,13]. Enrichment of the culture medium with pyruvate (30 mM) had, however, no positive effect on SULT and sulfatase activities.

During the first 4 days in simple monolayer cultures, high enzyme activities towards the different substrates were measured, corresponding with a high level of immunoresponding protein. In this simple culture condition, hepatocytes normally undergo dedifferentiation and morphological changes, accompanied with a decrease of biochemical function [11]. In our lab, Williams' medium E was formally used for culturing hepatocytes in a simple monolayer culture. After 7 days in culture, the hepatocytes have lost their normal morphology and all GST isoenzyme activities are decreased [13]. Watts and co-workers [41] studied the plating efficiency, CYP content, rate of urea synthesis and intracellular concentration of GSH of rat and sheep hepatocytes cultured in four different culture media (Modified Earle's Medium, Williams' E Medium, Medium 199 and Chee's Medium). They found that Williams' Medium E, which is widely used for culturing rat hepatocytes, performed rather badly and that Chee's Medium was one of the best culture media for rat hepatocytes. In our simple monolayer culture, DMEM was used (already containing 1 mM pyruvate) in order to work under the same culture conditions as in the sandwich cultures. We measured high activities in hepatocytes under these culture conditions, which may be explained by the different medium composition.

Only for the co-culture was the medium described by Guillouzo and co-workers [9], who developed this culture model, used to provide optimal conditions for the RLEC. Earlier work from Utesch and Oesch [42] suggested that several cell lines can express GST and UGT activity, we tested whether RLEC expressed some SULT and sulfatase activities. In preliminary experiments, SULT1, SULT2 and sulfatase activity could not be detected in pure RLEC cultures. The enzyme activities and SULT protein expression found in co-cultures were therefore only due to the hepatocytes and not to the RLEC.

It was hoped that the addition of relatively high concentration of DEX (1 μM) to the sandwich culture medium could induce SULT activity. This was, however, not the case. Our results are in line with those found by McMillan and co-workers [27], but are in contrast with those of Liu *et al*. [43]. In their cultures, hepatocytes lost their constitutive expression of SULTs but remained inducible even with a small concentration of DEX (0.1 μM). Recently, rather disappointing results have also been reported by LeCluyse *et al*. with respect to the effect of DEX on GSTP1 expression [44].

The collagen gel sandwich configuration culture is the most recent one of the sophisticated organospecific culture models available for hepatocytes [21,23–26,45,46]. It has been shown to maintain *in vivo* cellular polarity and synthesis of *in vivo* amounts of albumin, transferrin, urea and bile salts for at least 6 weeks [22]. The *in vitro* metabolism of urapidil (an antihypertensive drug) by human and rat hepatocytes in a collagen gel sandwich configuration corresponds to the pattern found *in vivo* [47]. SULT activity, however, has not often been studied. In our study, the enzyme activities in sandwich cultures are significantly changed in comparison with the values observed in the

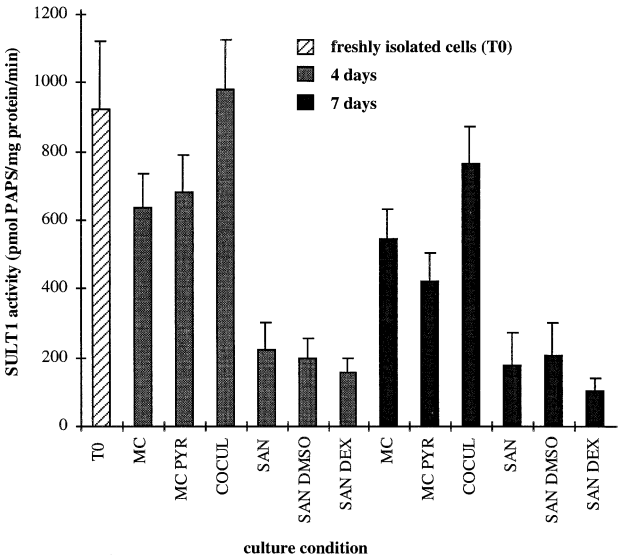

Fig. 6. SULT1A1 activity towards 4-methylphenol in freshly isolated rat hepatocytes and rat hepatocytes after 4 and 7 days under different culture conditions. The hepatocytes were cultured in a simple monolayer culture (MC), a co-culture with RLEC (COCUL) or a collagen gel sandwich configuration (SAN). Additionally the influence of 30 mM pyruvate (MC PYR), 0.05% (v/v) DMSO (SAN DMSO) or 1 μM DEX in 0.05% (v/v) DMSO (SAN DEX) were studied. The results are expressed as mean ± SD (N = 4 independent experiments).

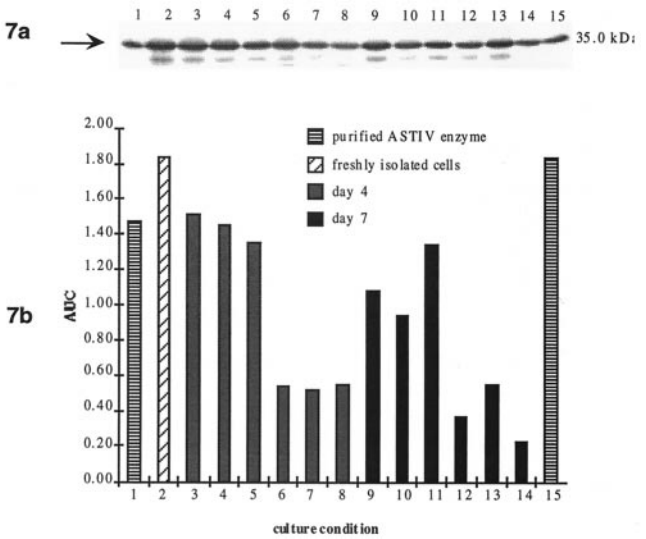

Fig. 7. Western blot analysis SULT1A1 protein expression in rat hepatocyte cytosol (7a) and densitometric analysis of expression levels (7b). Lane 1, purified rat SULT1A1 protein (0.6 μg). Lane 2, cytosol (20 μg cytosolic protein) from freshly isolated rat hepatocytes. Lanes 3–8, cytosol (20 μg cytosolic protein) of rat hepatocytes after 4 days of culture, respectively from monolayer culture (3), monolayer culture enriched with 30 mM pyruvate (4), co-culture (5), collagen gel sandwich culture (6), collagen gel sandwich culture with 0.05% (v/v) DMSO (7) and collagen gel sandwich culture with 1 μM DEX in 0.05% (v/v) DMSO (8). Lanes 8–14 the same culture condition but after 7 days of culture. Lane 15, purified rat SULT1A1 protein (1.2 μg).

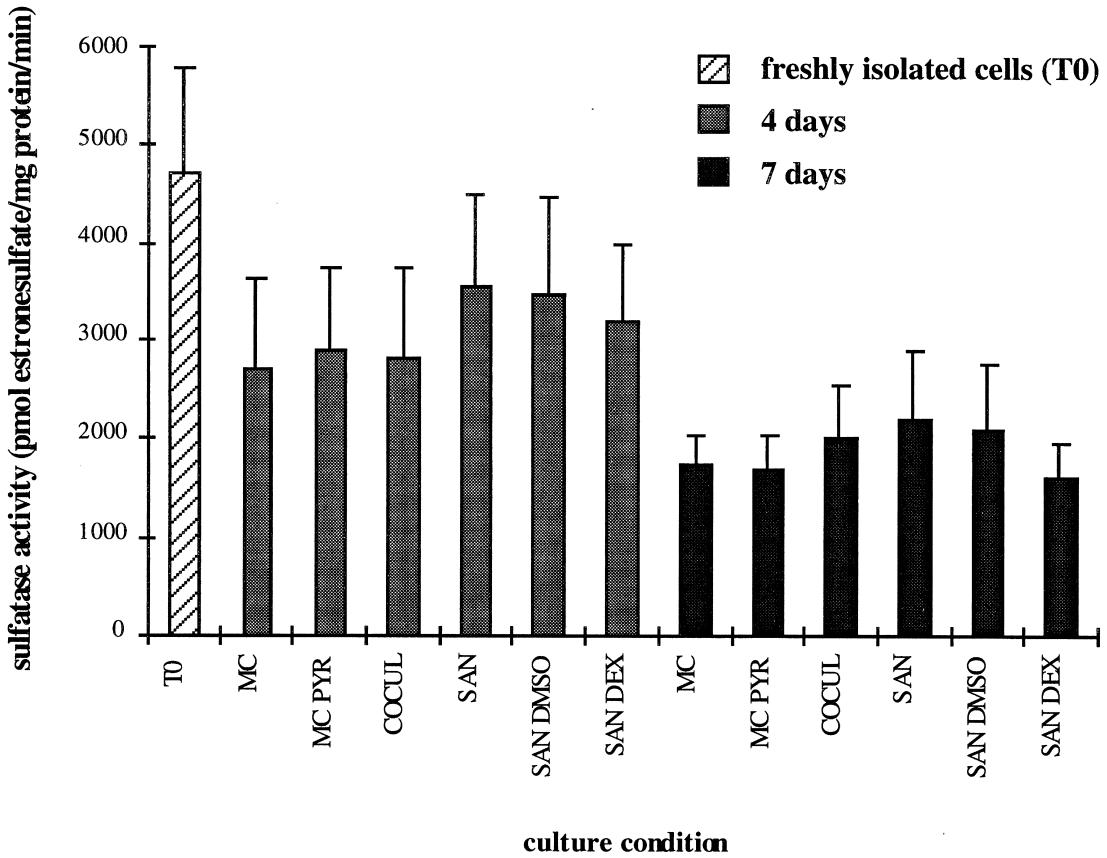

Fig. 8. Steroid sulfatase activity towards estrone sulfate in freshly isolated rat hepatocytes and rat hepatocytes after 4 and 7 days under different culture conditions. The hepatocytes were cultured in a simple monolayer culture (MC), a co-culture with RLEC (COCUL) or a collagen gel sandwich configuration (SAN). Additionally the influence of 30 mM pyruvate (MC PYR), 0.05% (v/v) DMSO (SAN DMSO) or 1 μM DEX in 0.05% (v/v) DMSO (SAN DEX) were studied. The results are expressed as mean ± SD (N = 4 independent experiments).

freshly isolated cells: a lower activity towards 4-methylphenol was measured compared to the activity in co-culture and monolayer culture; with DHEA and estradiol as substrates, the activities were similar to those found in co-culture. Liu and co-workers [43] found that the expression of all the sulfotransferases measured declined rapidly to less than 20% of the initial values, regardless of the matrix or media conditions. These results indicate that, in particular for the SULTs, the collagen gel sandwich culture is not a promising culture system, as earlier described for other xenobiotic biotransformation pathways [24–26,43,45,47,48]. Human hepatocytes are known to be more stable than their rodent counterparts [49]. Since sulfotransferase expression is poorly maintained in rat hepatocyte cultures, human hepatocytes may offer a more interesting model to study the long-term maintenance of sulfotransferase expression at elevated levels.

In conclusion, in this study co-culture was a useful culture system for rat hepatocytes, preserving a part of the SULT enzyme and sulfatase activities over an extended period of time. The complex character of the system, in particular isolating and culturing the RLEC, however, is a disadvantage of this culture system. Simple monolayer culture, which has the advantage to be an easy to apply system, is a decent culture system for short-term *in vitro* studies. In this study, collagen gel sandwich culture was not a suitable model for the study of sulfotransferase and sulfatase expression.

Further investigation will focus on the influence of hormonal addition to the culture medium on the SULT expression in co-culture of hepatocytes.

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