Comparative study of CYP2B1/2 induction and the transport of bilirubin and taurocholate in rat hepatocyte-mono- and hepatocyte-Kupffer cell co-cultures

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ABSTRACT

Introduction: Hepatocyte-Kupffer cell (KC) co-cultures represent a promising approach for in vitro modeling of complex interactions between parenchymal and non-parenchymal cells in the liver, responsible for drug-induced liver injury (DILI). In this study we aimed to compare hepatocyte monocultures with hepatocyte-KC co-cultures regarding some basic liver functions associated with the chemical defense system. These pathways involve transporters and enzymes the function of which is highly sensitive towards hepatotoxic events.

Methods: CYP2B1/2 induction and the biliary and sinusoidal elimination of bilirubin (B) and taurocholate (TC) were studied in rat hepatocyte sandwich cultures compared with rat hepatocyte-KC sandwich co-cultures of 1:0, 6:1, 2:1 and 1:1 cell combinations representing the physiologic and pathologic conditions of the liver.

Results: KCs decreased phenobarbital inducibility of CYP2B1/2 in a cell ratio dependent manner and activation of KCs by lipopolisacharide (LPS) amplified this effect. Similarly, KCs decreased the transport of B and its glucuronides (BG) in both sinusoidal and canalicular directions resulting in its intracellular accumulation. In contrast, the uptake and the efflux of TC were greater in the co-cultures than in the hepatocyte monocultures. Immuno-labelling of sodium-dependent taurocholate transporter (Ntcp) revealed increased expression of the transporter in the presence of KCs.

Discussion: Here we presented that KCs have a direct impact on some hepatocyte functions suggesting that the co-culture model may be more suitable for drug related hepatotoxicity studies than hepatocyte monocultures.

Keywords: CYP2B1/2, hepatocyte, Kupffer cell, sandwich co-culture method, transporters.

Abbreviations: B, bilirubin; Bsep, bile salt export pump; CYP, cytochrome P-450; $GdCl_3$, gadolinium(III) chloride; H/KC, hepatocyte-Kupffer-cell co-culture; HBSS, Hanks' Balanced Salt

Solution; HPLC, high-performance liquid chromatography; KC, Kupffer-cell; LPS, lipopolisacharide; Mrp, multidrug resistance-associated protein; Ntcp, sodium-dependent taurocholate transporter; PB, phenobarbital; PBS, phosphate-buffered saline; PTX, pentoxyphylline; TC, taurocholate

1. INTRODUCTION

Drug-induced liver injury (DILI) is one of the most common adverse event causing drug non-approvals and drug withdrawals (Lee, 2003). DILI is a function of non-parenchymal and parenchymal cell interactions that cannot be adequately predicted by studies in hepatocyte monocultures since hepatocytes alone may not properly represent *in vivo* functionality especially when examining drug related hepatotoxicity (LeCluyse, Witek, Andersen, & Powers, 2012; Soldatow, Lecluyse, Griffith, Rusyn, 2013). However, validating the interaction for using co-cultures of non-parenchymal and parenchymal cells as an in vitro predictive method for DILI will require examination of the interactions relative to normal hepatic functioning. Previously we demonstrated that sandwich culture of human and rat hepatocytes is a useful model for hepatotoxicity studies. The findings supported that cholestatic effect of some drugs results mainly from inhibition of function of hepatocellular transport systems which normally mediate hepatic uptake and biliary excretion of bile salts and various non-bile salt organic anions (e.g. bilirubin). Our experimental setup allowed studying the basolateral and canalicular efflux of compounds (e.g. taurocholate, bilirubin) simultaneously, thus comparing drug-induced changes in their vectorial transport (Jemnitz et al., 2010: Jemnitz et al., 2012, Szabó et al., 2014).

Here we completed the sandwich culture of rat hepatocytes with Kupffer cells (KCs), of three KC densities in order to represent the physiologic and pathologic conditions of the liver (Nguyen et al., 2015). KCs, the resident macrophages of the liver, play an important role in the normal hepatic physiology and homeostasis as well as participate in the acute and chronic responses to toxic drugs or toxicants. KCs exert these effects by excreting intercellularly acting regulatory factors. Pro-

inflammatory cytokines produced in response to various infectious and non-infectious stimuli are potent inducers of intrahepatic cholestasis (inflammation-induced cholestasis, Trauner et al., 1999). The release of such modulators can also be induced *in vitro* by the activation of the KCs (e.g. by lipopolisacharide, LPS) leading to altered metabolic and detoxifying capacity of hepatocytes (Laskin, Sunil, Gardner, & Laskin, 2011). LPS induced cholestasis in rodents is basically attributed to the downregulation of the bile salt transporters, the basolateral sodium-dependent taurocholate transporter (Ntcp) and the canalicular bile salt export pump (Bsep) (Klaassen, & Aleksunes, 2010). Impairment of hepatic drug disposition during inflammation is mainly caused by downregulation of gene expression of members of the chemical defense system, such as drug metabolizing enzymes and transporters (Milosevic, Schawalder, & Maier, 1999; Nakamura et al., 1999; Aoki et al., 2008; Kosters and Karpen, 2010; Sekine et al., 2010; Nguyen et al., 2015).

The aim of our study was to extend our present knowledge concerning the influence of KCs on some basic functions of hepatocytes in a rat hepatocyte-KC co-culture model by testing two hypotheses. a., The addition of, and/or, increasing the ratio of Kupffer cells to hepatocytes, alters pathways involved in the chemical defense system, such as Phase I metabolism and important drug transports. b., This Kupffer cell dependent effect on Phase I and transporter processes is increased by LPS activation of Kupffer cells. We hope that our results contribute to the development of reliable, predictive *in vitro* cellular systems, which will be readily applied to identify hepatotoxic drugs, and which would improve the safety of new therapies.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals, including bilirubin, taurocholate, 7-pentoxyresorufin, dicoumarol, bovine serum albumin, type IV collagenase, dexamethasone, Percoll, insulin, LPS from Escherichia coli 055: B5, Williams' Medium E and all cell culture reagents were purchased from Sigma–Aldrich (Steinheim, Germany). ^{3}H-taurocholic acid (10 Ci/mmol) was obtained from American Radiolabeled

Chemicals, Inc. (St. Louis, MO, USA). Merck (Darmstadt, Germany) was the manufacturer of dimethyl sulfoxide, acetonitrile, methanol and HEPES used in the experiments. Triton X-100 was purchased from Reanal Ltd. (Budapest, Hungary). Matrigel was obtained from Corning (Bedford, MA, USA). 6-well and 24-well sterile cell culture plates were obtained from Greiner Bio-One GmbH (Frickenhausen, Germany).

2.2. Animals

Male Wistar rats (200-250 g) were purchased from Charles River, Hungary. Studies were approved by the Institutional Animal Care and Use Committee and the Animal Health and Animal Welfare Directorate, National Food Chain Safety Office, Budapest, Hungary (Permit Number: 22.1/2728/3/2011).

2.3. Isolation of primary rat hepatocytes and Kupffer cells

Primary rat hepatocytes and KCs were isolated from male Wistar rats by a two-step collagenase perfusion technique as described (Seglen, 1976). For separation of hepatocytes from the non-parenchymal cells, the cell suspension obtained after collagenase perfusion (approx. 200 ml) was centrifuged at 100 x g, 2 min, 4 ^{0}C. The supernatant enriched in non-parenchymal cells was used for the KC isolation. The cell pellet containing the hepatocytes was subjected to a 45 % Percoll centrifugation to remove non-viable cells; cell viability (> 90%) was determined by the trypan blue exclusion test. Hepatocytes were plated on collagen coated 24-, or 6-well plates at a density of 2.0 x 10^5 cell/cm^2 in Williams' Medium E supplemented with 10 % fetal calf serum and 100 nM insulin.

KCs were isolated as described (Gopalakrishnan & Harris, 2011) with slight modifications. In brief, the supernatant from the initial centrifugation step was centrifuged at 50 x g, 2 min, 4 ^{0}C, (pellet discarded) and then at 350 x g, 10 min, 4 ^{0}C to sediment the non-parenchymal fraction. The cell pellet was re-suspended in Williams' Medium E and the suspension was layered on a density cushion of 25 % / 50% Percoll gradient and centrifuged at 900 x g, 20 min, 4 ^{0}C. The cells floating at the boundary of the two Percoll layers (mostly KCs and sinusoidal endothelial cells) were collected,

washed and were seeded on collagen coated 24-, or 6-well plates at a density of 2.0 x 10^5 KC/cm^2 (for hepatocyte-KC co-cultures of 1:1 ratio), 1.0 x 10^5 KC/cm^2 (for hepatocyte-KC co-cultures of 2:1 ratio), or 0.33 x 10^5 KC/cm^2 (for hepatocyte-KC co-cultures of 6:1 ratio), respectively. KCs were separated from the other non-parenchymal cell types by their ability to adhere on cell culture plate within a short period of time. After incubation for 20 min, at 37 ^{0}C in a humidified incubator at 5 % CO_2, non-adherent cells were removed by gentle shaking and medium change. The attached cells were identified by immunostaining with CD163 (AbD Serotec, MCA342A647), a macrophage specific antigen (data not shown). In case of co-cultures, hepatocytes were seeded on top of the KC layer at a density of 2.0 x 10^5 cell/cm^2. Twenty-four h after seeding cultures were overlaid with Matrigel to achieve a sandwich configuration. The cells were cultured in Williams' Medium E supplemented with 10 % fetal calf serum and 100 nM insulin for at least 24 h before starting any treatment for assessing transporter or enzyme activities.

2.4. CYP2B1/2 induction studies

Hepatocyte mono- and hepatocyte-KC co-cultures were treated with 0.2 mM phenobarbital (PB) from 24 h after seeding for 3 days to induce CYP 2B1/2 enzyme expression. The enzyme activity was measured by 7-pentoxyresorufin dealkylation (Burke, Thompson, Elcombe, Halpert, Haaparanta, & Mayer, 1985; Lubet et al., 1985) at 96 h after seeding. Cells were incubated with 5 µM 7-pentoxyresorufin and 10 µM dicoumarol at 37°C and the resorufin production was monitored at 550/589 nm in a SpectraMax M5 microplate reader (Molecular Devices LLC) for 30 minutes. The enzyme activities were expressed as V_0 values derived from the linear segment of resorufin formation.

In order to activate the Kupffer cells, the mono- and co-cultures were treated with 1µg/ml LPS from 48h after plating for 48h. Control cultures were untreated. KC inhibitors, dexamethasone (100 nM), gadolinium(III) chloride ($GdCl_3$, 0.2 mM) and pentoxyphylline (PTX, 0.25 mM) were added at 1 h after seeding for 72h, control cells were treated with the vehicle. $GdCl_3$ and PTX at the

concentrations applied did not change the viability of hepatocytes (data not shown). Assays were run using four wells as one set. All experiments were carried out with mono- and co-cultures from three independent cell preparations.

2.5. Bilirubin transport experiments

Hepatocyte mono- and hepatocyte-KC co-cultures were treated with LPS as described above. Bilirubin-glucuronide efflux experiments were performed at 72 h of culturing as described (Lengyel, Veres, Szabó, Vereczkey, & Jemnitz, 2005). Briefly, cells were incubated with 10 μM B for 5 min in standard HBSS at 37°C in a humidified atmosphere of 95% air, 5% CO_2, then the cells were washed with ice-cold standard HBBS or HBSS without Ca^{2+}/Mg^{2+}, containing 1 mM EGTA (calcium-free HBSS). Thereafter, the cells were incubated with standard or calcium-free HBSS, the efflux lasted for 10 min. After removing the efflux medium the cells were lysed by a 10-minute sonication in an acetonitrile/water solution [30% (v/v)] followed by centrifugation. The amounts of B and B conjugates in the efflux medium and in the cell lysates were analyzed by HPLC (high-performance liquid chromatography). Data were normalized for the protein content analyzed in wells where the cells were solubilized by Triton X-100, (Pierce BCA Protein Kit, Thermo Scientific). In standard HBSS, the bile canalicular networks maintain their integrity, whereas in calcium-free HBSS the tight junctions are disrupted and the content of the canaliculi gets into the efflux medium (Liu et al., 1999). The biliary transport of B and B conjugates was determined by subtracting the amount of B and its conjugates in standard efflux medium from that in calcium-free efflux medium and was expressed as nmol/mg protein. Assays were run using three wells as one set. All experiments were carried out with cultures from three independent cell preparations.

2.6. HPLC measurement of bilirubin and conjugated bilirubin

The HPLC method for analysing bilirubin and conjugated bilirubin was performed as described previously (Lengyel, Veres, Szabó, Vereczkey, & Jemnitz, 2005) with modifications. In brief, samples were centrifuged at 13000 rpm for 10 min, and 200 μl of supernatant was analysed on

a Merck/Hitachi HPLC system. Bilirubin and bilirubin conjugates were resolved on a Chromolith Performance RP 18e (4.6 × 100 mm, Merck Darmstadt, Germany) reversed-phase chromatographic column. Elution was achieved at 3 ml/min flow rate using a binary gradient of solvent A (75% 0.01 M sodium phosphate buffer, pH 3.2 and 25% acetonitrile, containing 200 µl/l triethylamine) and solvent B (acetonitrile) and a total run time of 10 min. After 1 min of isocratic elution with solvent A, the gradient started reaching 10% of eluent B in 5.2 min. Following the separation of the conjugates, the column was rinsed using a gradient reaching 100% eluent B at 7.5 min, and then the column equilibration time was 2.5 min. The UV absorbance was monitored at a wavelength of 450 nm. The amount of bilirubin and bilirubin conjugates were quantified using calibration curves prepared with bilirubin as standard.

2.7. TC transport experiments

TC efflux experiments were performed similarly to that with B as substrate. ^{3}H TC was applied at 1 µM. The uptake period was 1 min and the efflux lasted for 10 min. The treatment of the cells was the same as described above. Following the efflux period the cells were lysed with 0.5 % Triton X-100 in PBS. The amount of TC in the efflux medium and the cell lysates were determined by liquid scintillation. Assays were run using three wells as one set. All experiments were carried out with cultures from three independent cell preparations.

2.8. Immunostaining of Ntcp transporter and tight junction protein ZO-1

At 72 h after plating for immunofluorescence staining of Na^{+}-taurocholate co-transporting polypeptide (Ntcp) and TJ component ZO-1 the cells were fixed and permeabilized with 4% paraformaldehyde in PBS for 15 min, then with ice cold methanol for an additional 15 min. The cells were labeled with primary antibodies against Ntcp (K4, obtained from Bruno Stieger) 1:250 and ZO-1 (Millipore, MABT11) 1:200 for overnight, following a 1 h blocking with Dulbecco's modified PBS (DPBS) containing 2 mg/ml BSA, 1 % fish gelatin, 0.1 % Triton X-100, and 5 % goat serum (pH 7.2). Goat anti-Rabbit IgG H&L Alexa Fluor® 594 (Thermo Fisher Scientific, A-11012) 1:250

and Goat anti-Rat IgG H&L Alexa Fluor® 488 (Thermo Fisher Scientific A-11006) 1:250 secondary antibodies were used. The cell nuclei were stained with 1 μM DAPI in DPBS for 10 min. The blue, green and red fluorescence of stained samples was studied by a Zeiss LSM 710 confocal laser scanning microscope using a Plan-Apochromat 20 × (numeric aperture 0.8) dry objective (Zeiss) at 405, 488 and 543 nm excitations, respectively. For visualization the publicly available software ImageJ 1.46q was used (NIH, USA).

2.9. Statistics

Data are reported as mean ± standard deviation (SD). Triplicate experiments of three cell isolations were run with at least three parallel wells for each set of condition. Statistically significant differences were evaluated using Student's t-test (a value of $p < 0.05$ was considered significant).

3. RESULTS

In order to evaluate the effect of KCs on some essential liver functions, all experiments were performed in hepatocyte mono- or co-cultures with KCs at three macrophage ratios to simulate different states of the liver. The 6:1; hepatocyte:KC (H/KC 6:1) ratio corresponds to a near physiologic, while the 2:1 (H/KC 2:1) and 1:1 ratio (H/KC 1:1) represent pathological, inflamed states of the organ. Macrophages were activated by LPS treatment, which is known to promote macrophage differentiation to a "classical" or M1 phenotype (Wan et al., 2014).

3.1 Effect of KCs on the inducibility of CYP2B1/2 enzyme activity

The basal CYP2B1/2 activity of hepatocytes was very low both in mono- and H/KC 2:1 co-cultures, 0.77 ± 0.37; 0.67 ± 0.14 pmol/10^6 cell/min, respectively. PB treatment resulted in an almost 20-fold increase of enzyme activity in hepatocyte monocultures, and the rate of induction highly depended on the number of KCs in the co-cultures (Fig 1). The presence of macrophages at a physiologic level did not alter the inducibility of CYP2B1/2. Increasing the ratio of macrophages decreased the level of induction. The Kupffer cells alone as added in pathological proportions, were sufficient to retard PB-related CYP2B induction. Compared to H/KC 6:1 co-cultures CYP2B1/2

induction was 58% lower in H/KC 2:1, and a 94% decline was measured in case of H/KC 1:1. In order to model pathologic situation, hepatocyte mono- and co-cultures were treated with LPS along with PB treatment. In hepatocyte monocultures LPS did not exert significant influence on CYP2B1/2 activity, while a 78 % decrease could be detected even in the H/KC 6:1 co-cultures. In H/KC 2:1 and 1:1 co-cultures LPS treatment completely impeded the induction of CYP2B1/2. The effect of the macrophages on CYP2B1/2 inductibility was proved by inactivating the KCs by a synthetic steroid, dexamethasone (Fig 2), $GdCl_3$ and PTX (Fig 3). Liver macrophage cytotoxic/inflammatory activity is blocked with hydrocortisone or synthetic steroids (Laskin, Sunil, Gardner,& Laskin, 2011) and $GdCl_3$ and PTX are also known inactivators of the KC (Laskin, Sunil, Gardner,& Laskin, 2011; Tukov, Luyendyk, Ganey, & Roth, 2007). H/KC 2:1 co-cultures were treated with 1 and 10 μg/ml LPS that totally precluded the induction of CYP2B1/2 (Fig 2A). Pretreatment of the co-cultures with 0.1 μM dexamethasone from 1 hour after seeding abolished the effect of LPS treatment; the rate of PROD induction after dexamethasone addition in the presence of either 1.0 or 10 μg/ml LPS was identical to the control (Fig 2B). The difference between PROD activity measured in the untreated control cultures containing or lacking dexamethasone is due to higher CYP expression in the presence of dexamethasone (data not shown). Hepatocyte mono- and H/KC 6:1 co-cultures were treated with 1 μg/ml LPS in the presence and absence of 200 μM $GdCl_3$ and 250 μM PTX, respectively. In hepatocyte monoculture neither compounds altered the rate of induction significantly. In H/KC 6:1 co-cultures both $GdCl_3$ and PTX hindered the impact of LPS, decreasing the inhibition of CYP2B1/2 induction from 78% to 6 % and 36 %, respectively (Fig 3).

3.2. Effect of Kupffer cells on bilirubin (B) and bilirubin glucuronide disposition

B is cleared from the body as bilirubin glucuronide via the hepatocytes. After conjugation in the hepatocytes, B is eliminated mainly in the forms of mono- and di-glucuronides by Mrp2 into the bile canaliculi or back into the sinusoidal space by Mrp3, respectively. Our study aimed to evaluate changes in the elimination profile of B and bilirubin glucuronides in the presence of KCs. KCs did

not influence the uptake of B (1.54 ± 0.39 nmol/ 10^6 cells/ 5 min) and similarly, formation of bilirubin glucuronides in the co-cultures did not vary significantly from that in the monocultures. The amount of bilirubin glucuronides found in the various culture systems calculated as a percent of B uptake are as follows: H/KC 2:1 70.9 ± 6.6; H/KC 6:1 71.4 ± 7.8; hepatocyte monoculture 75.9 ± 4.5; H/KC 2:1 in the presence of LPS 75.3 ± 6.4. In contrast to B uptake and conjugation, the elimination profile changed significantly. Macrophages caused significant and cell density dependent efflux transport impairment towards both the sinusoidal and the canalicular directions, which resulted in the intracellular accumulation of B and its conjugates (Fig 4). Sinusoidal efflux decreased from 27.6 % measured in monocultures to 22.9% in H/KC 6:1 and to 13.1% in H/KC 2:1 co-cultures (Fig 4 A). The biliary efflux was 38 % of the uptake in hepatocytes, which decreased to 28 % and 25 % in H/KC 6:1 and 2:1 co-cultures, respectively (Fig 4 B). Along with the decrease of the efflux transport in H/KC 6:1 and 2:1 co-cultures, the intracellular accumulation of B and bilirubin glucuronides increased to 140 % and 177 % of that in the monoculture, respectively (Fig 4 C). LPS treatment did not alter the B elimination profile in hepatocyte monocultures significantly (Fig 4). On the contrary, both Mrp3 and Mrp2 mediated transport were severely obstructed in the co-cultures, causing a significant decrease in the sinusoidal (H/KC 6:1, 42%; H/KC 2:1, 28% of that in the monoculture, Fig 4 A) and canalicular (H/KC 6:1, 34%; H/KC 2:1, 31 % of that in the monoculture, Fig 4 B) elimination. The impaired transport resulted in a marked B and bilirubin glucuronide accumulation inside the cells, doubling their intracellular concentration (Fig 4 C).

3.3 Effect of Kupffer-cells on the uptake and efflux of taurocholate

The uptake and efflux balance of bile salts is essential for bile secretion and to avoid liver damage, e.g. cholestasis. TC was used as a substrate for studying the effect of macrophages on the function of pivotal uptake (Ntcp) and efflux (Bsep) transporters involved in bile acid transport. As shown in Fig 5A, macrophages induced significant and cell ratio dependent elevation of TC uptake. In H/KC 6:1 co-cultures the uptake of TC slightly but significantly increased by 27% compared to

that in hepatocyte monocultures, and further increased in H/KC 2:1 co-cultures to more than two-fold of that in hepatocyte cultures. This effect was even more pronounced at 96 h of culturing, when TC uptake increased by 3.5-fold in H/KC 2:1 co-cultures compared to that in the monocultures.

It is widely accepted that Ntcp expression both in mRNA and protein level is highly decreased by macrophages in pathologic livers (Sturm et al., 2005). Immunofluorescence staining was used to study the expression and localization of Ntcp as a function of macrophage density in order to explain this discrepancy. As illustrated in Fig 5B, the expression of Ntcp is increased in H/KC co-cultures compared to hepatocyte monoculture. In addition the transporter showed pronounced membrane localization. To demonstrate the proper sinusoidal localization of Ntcp a canalicular protein (ZO-1 tight junction associated protein) was also stained and it showed no co-localization with Ntcp. The differences observed in TC uptake of mono- and co-cultures considerably altered the efflux transport of TC as well (Fig 6A). The elevated intracellular TC concentration after 1 min of uptake (2.89 ± 0.51 μM and 6.12 ± 1.40 μM in H mono- , and H/KC 2:1 co-cultures, respectively) was accompanied by increase of the efflux rate in both sinusoidal and biliary direction at the same extent. The sinusoidal and biliary efflux normalized for the intracellular concentration did not change, (Fig 6B). Treatment of the H/KC 6:1 co-cultures with 1μg/ml LPS slightly but not significantly decreased TC transport into both the canalicular and sinusoidal compartments, which together increased the intracellular accumulation of TC significantly by 2.5-fold.

4. DISCUSSION

In this work, we characterized some liver functions involved in the chemical defense system (Sarkadi et al., 2006) in a hepatocyte/KC sandwich co-culture model of rat for investigating heterotypic cell–cell interactions between parenchymal and non-parenchymal cells. Hepatocyte monocultures represent a reliable but simple model for testing; however, a number of papers

demonstrated that hepatocytes do not reflect the complex response of the liver to drugs or to other toxic challenges. Interactions between parenchymal and non-parenchymal cell types are involved in processes leading to liver injury (Chen, Xue, & Sun, 2013; Kostadinova et al., 2013). The present experimental setup comprises the study of a Phase I metabolic process, CYP2B1/2 induction, and of Phase 0, II and III processes by studying the hepatic elimination of B and TC. These pathways involve transporters and enzymes the activity and expression of which are highly sensitive towards hepatotoxic events. Our results demonstrate that most of the processes studied were highly influenced by KC in a cell density dependent manner supporting the importance of KC - hepatocyte interaction in the capacity of the defense system towards toxicants especially in pathologic liver. We compared the co-culture model with standard hepatocytes both grown in sandwich configuration. The 3D cell culture allows formation of in vivo-like hepatocyte architecture, better metabolic activity and prolonged functional lifetime, compared to the conventional monolayer most frequently used for toxicity screening (Dunn, Tompkins, & Yarmush, 1991; LeCluyse, 2001; De Bruyn et al., 2013). Moreover, during 3 days of culturing, hepatocytes regain their polarity; a biliary network is formed between hepatocytes that is essential for proper translocation and function of canalicular and sinusoidal transporters (LeCluyse, Fix, Audus, & Hochman, 2000; Jemnitz et al., 2015).

Comparing hepatocyte monocultures to co-cultures with tissue macrophages a significant decrease was detected in the inducibility of CYP2B1/2 in case of elevated KC density, while at physiological level KCs did not affect the rate of induction. An extreme high KC level totally precluded CYP2B1/2 induction by PB (Fig 1). The activation of KCs by LPS markedly decreased the CYP2B1/2 activity even at physiological hepatocyte:KC ratio, and that was more pronounced at higher KC density. These results show that elevated KC density, which is not necessarily due to infection, may influence the metabolic capacity of the liver. In line with previous studies, by selective blocking of the macrophages with dexamethasone (Wei et al., 2011), PTX (Tukov, Luyendyk, Ganey, & Roth, 2007) or $GdCl_3$ (Milosevic, Schawalder, & Maier, 1999) the decrease of

CYP2B induction was reduced or completely prevented (Figs 2, 3), thus demonstrating that the drop detected in co-cultures was connected to the KCs. It has been demonstrated that in hepatocyte monocultures, the influence of KC-derived mediators, TNFα, IL 1β, IL 6 on metabolic enzyme expression not always represents hepatocyte - KC interactions that underline the advantage of co-culture models. TNFα is known to downregulate CYP2B enzyme expression in vivo and in the presence of KC, but no effect was found in human hepatocyte mono-cultures in vitro (Sanada, Sekimoto, Kamoshita, & Degawa, 2011; Milosevic, Schawalder, & Maier, 1999; Nguyen et al., 2015) despite the fact that in vivo the decrease of CYP2B mRNA, protein and activity was linked to the amount of TNFα.

The activity of transporters involved in drug and endogenous compound elimination, such as Mrp2, Mrp3 and Bsep were also highly influenced by KC. Beside the notable effects on CYP2B1/2 inducibility, KC decreased the transport of B and its conjugates as well (Fig 4A). Hepatocyte monocultures transported nearly the same amount of B to the bile canaliculi and the sinusoidal space via Mrp2 and Mrp3, respectively. The presence of KC reduced the transport in both directions with the concomitant intracellular accumulation of B. Treating the cells with LPS the decline of B transport markedly increased even in co-cultures of physiologic KC density (Fig 4B). Regarding biliary transport of B and its conjugates our results are in line with previous data, showing that LPS caused a dramatic reduction of bile flow in rodents due to repressed mRNA expression of hepatic efflux transporters (Elferink et al., 2004; Klaassen, & Aleksunes, 2010). Sekine et al. (2010) demonstrated that oxidative stress caused the obstruction of hepatobiliary transport with the internalization of the key transporter Mrp2. The sinusoidal transport of B also decreased in our study, in contrast to reports describing marked Mrp3 mRNA increase in endotoxin-induced inflammation rodent models (Cherrington, Slitt, Li, & Klaassen, 2004), but in line with other reports showing significant Mrp3 mRNA decline in IL 6 and LPS treated mice (Sievert et al., 2004). However, the role of KCs in this process is not fully understood. In a recent study, in spite of that $GdCl_3$

pretreatment of mice fully impeded the elevation of TNFα excretion induced by perfluorodecanoic acid treatment, the depletion of macrophages led to minor changes in Mrp4 expression, and Mrp3 mRNA was unaffected suggesting that KC activation had no influence on Mrp3 levels (Maher et al., 2008).

In spite of the commonly accepted fact that Ntcp is highly downregulated by LPS treatment that can be prevented by KC depletion (Klaassen, & Aleksunes, 2010; Sturm et al., 2005) we observed a significant, and KC density dependent increase in TC uptake (Fig 5A). Immunostaining experiments revealed some increase in the amount of the transporter is behind these findings (Fig 5B). Previously, in line with other papers we demonstrated that Ntcp activity dramatically decreases during culturing even in sandwich configuration of rat hepatocytes (Rippin, Hagenbuch, Meier, & Stieger, 2001; Jemnitz et al., 2012). We presume that the less than 10 % Ntcp activity preserved by the cells at 72 h in culture did not decrease further by KCs not even after LPS activation. We have also demonstrated that intracellular concentration of compounds highly influences their hepatobiliary elimination (Jemnitz et al., 2015) that is in line with the present data concerning TC efflux. On the other hand, according to the present results we cannot exclude that the expression and/or the activity of Bsep changed, contributing to the elevated TC efflux. The slight and not significant decrease of both sinusoidal and biliary efflux of TC by LPS resulted in its significant intracellular accumulation (Fig 6B).

In this study, we presented that density and LPS activation of KC have a direct impact on the function of members of drug elimination pathways. This hepatocyte/KC co-culture model can be readily used for drug interaction studies modeling pathologic liver or when KC are involved by using nano-sized particles or macromolecules as drugs or drug components.

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Figure captions

Figure 1. Effect of different hepatocyte:KC ratios and macrophage activation on the inducibility of CYP2B1/2.

Hepatocyte mono- and hepatocyte-KC 6:1, 2:1 and 1:1 co-cultures were treated with 200 μM PB from 24 h after seeding for 72 h and the CYP2B1/2 activity was measured by PROD at 96 h of culturing. KCs were activated by adding 1μg/ml LPS from 48h after seeding for 48 h, along with PB treatment. H: Hepatocyte monoculture. Values are expressed as PROD activities [pmol/10^6cells/min] (A), and as relative PROD activities, LPS/control [%] (B). Assays were run using four wells as one set. All experiments were carried out with hepatocytes and KCs from at least three independent preparations. *, $p < 0.05$ for co-cultures versus mono-cultures.

Figure 2. The influence of dexamethasone on the KC related decrease of CYP2B1/2 inducibility. Hepatocyte mono- and hepatocyte-KC 2:1 co-cultures were treated with 200 μM PB for 72 h and the CYP2B1/2 activity was measured by PROD at 96 h of culturing. Cells were cultured in the presence of 0.1 μM dexamethasone (B), or the vehicle as control (A). KCs were activated by adding 1μg/ml or 10 μg/ml LPS from 48h after seeding for 48 h, along with PB treatment. Values are expressed as

mean ± SD, assays were run using four wells as one set. All experiments were carried out with hepatocytes and KCs from at least three independent preparations.

Figure 3. The influence of $GdCl_3$ and PTX on the KC related decrease of CYP2B1/2 inducibility. Hepatocyte mono- and hepatocyte-KC 6:1 co-cultures were treated with 200 μM PB for 72 h and the CYP2B1/2 activity was measured by PROD at 96 h of culturing. Cells were treated with 1 μg/ml LPS in the presence and absence of 200 μM $GdCl_3$ and 250 μM PTX, respectively, or the vehicle as control, from 1 h after seeding for 96 h. Values are expressed as mean ± SD, assays were run using four wells as one set. All experiments were carried out with hepatocytes and KCs from at least three independent preparations. *, $p < 0.05$ for $GdCl_3$ and PTX treated versus not treated cultures; #, $p < 0.05$ for co-culture versus hepatocyte mono-culture.

Figure 4. Effect of KCs and LPS on bilirubin and bilirubin-glucuronides disposition.
At 72 h of culturing in the absence or the presence of 1 μg/ml LPS, hepatocyte mono- and hepatocyte-KC 6:1, 2:1 co-cultures were incubated with 10 μM B, then (B + BG) sinusoidal (A), and canalicular (B) elimination was assayed. Intracellular accumulation is shown in C. Values are expressed as mean ± SD, assays were run using three wells as one set. All experiments were carried out with hepatocytes and KCs from at least three independent preparations. *, $p < 0.05$ for co-cultures versus mono-cultures.

Figure 5. Effect of KCs on the uptake of taurocholate.
At 72 h and 96 h of culturing hepatocyte mono- and hepatocyte-KC 6:1, 2:1 co-cultures were incubated with 1 μM TC, then canalicular and sinusoidal elimination was tested. TC uptake (A) was expressed as mean ± SD, assays were run using three wells as one set. All experiments were carried out with hepatocytes and KCs from at least three independent preparations. *, $p < 0.05$ for hepatocyte-KC 6:1 as well as 2:1co-cultures versus hepatocyte mono-cultures and #, $p < 0.05$ hepatocyte-KC 6:1 versus hepatocyte-KC 2:1 co-cultures. The expression and localization of Ntcp and ZO-1 at 72 h of culturing (B) was determined by immunofluorescence.

Figure 6. Effect of KCs and LPS on the elimination of taurocholate.

At 72 h of culturing, hepatocyte mono- and hepatocyte-KC 6:1, 2:1 co-cultures were incubated with 1 μM TC, then the canalicular and sinusoidal elimination was tested. The amount of TC excreted into the medium or into the canalicular networks, and that remained in the hepatocytes are shown in panel A, while the same data expressed in proportion of the uptake rate are shown in panel B. Values are expressed as mean ± SD, assays were run using three wells as one set. All experiments were carried out with hepatocytes and KCs from at least three independent preparations. *, $p < 0.05$ for co-cultures versus mono-culture; #, for LPS treated versus not treated hepatocyte-KC 6:1 co-cultures.

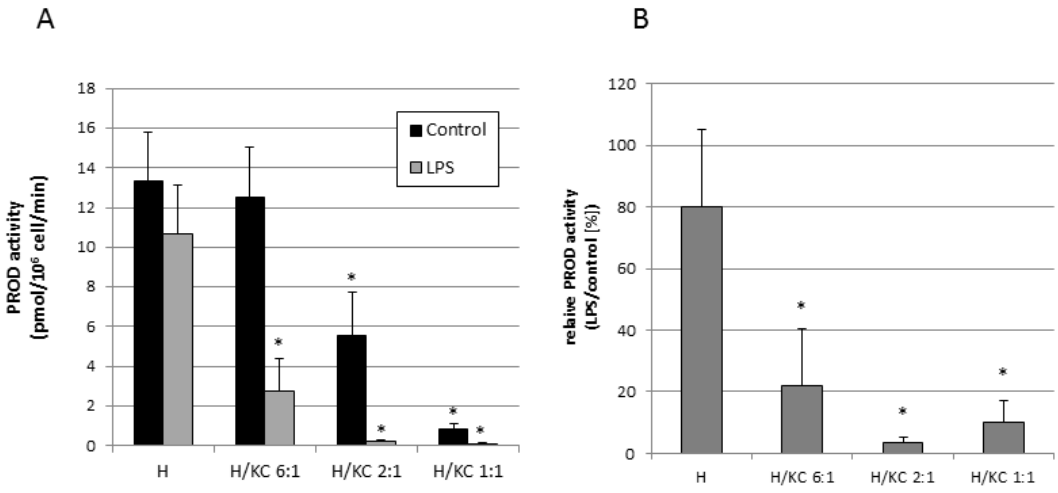

Figure 1

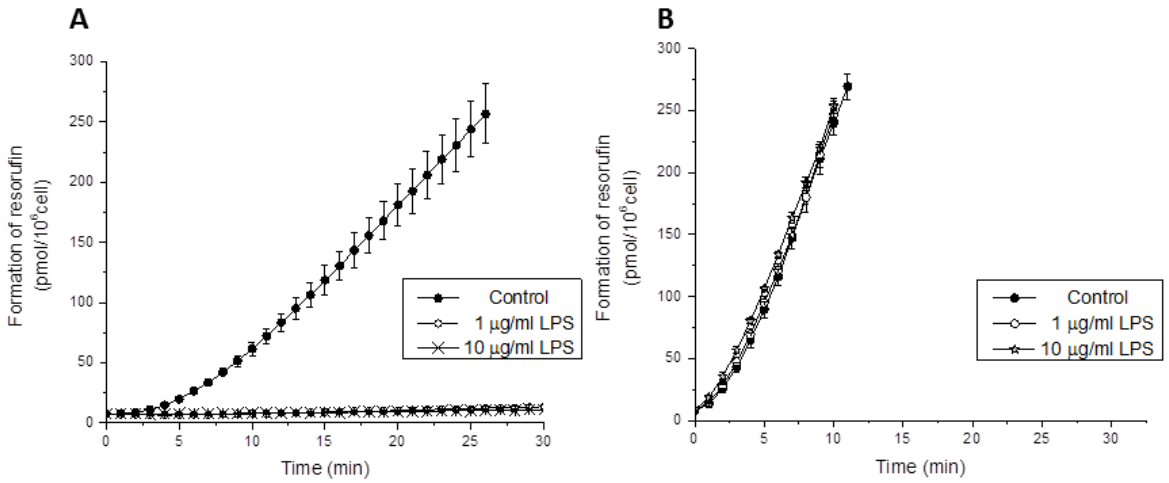

Figure 2

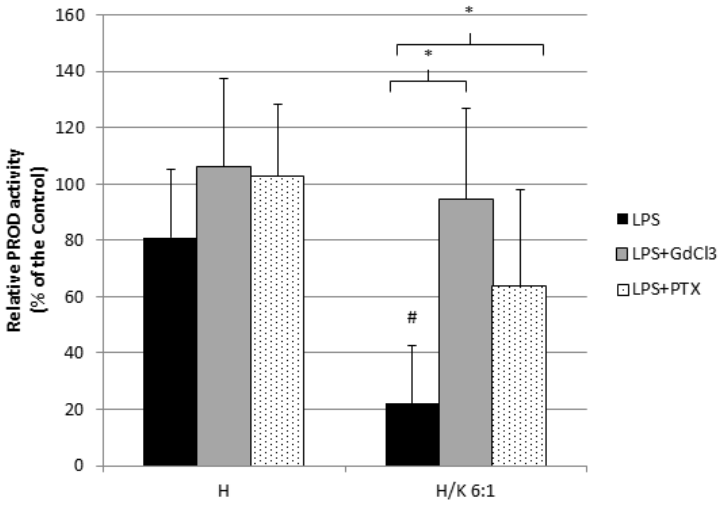

Figure 3

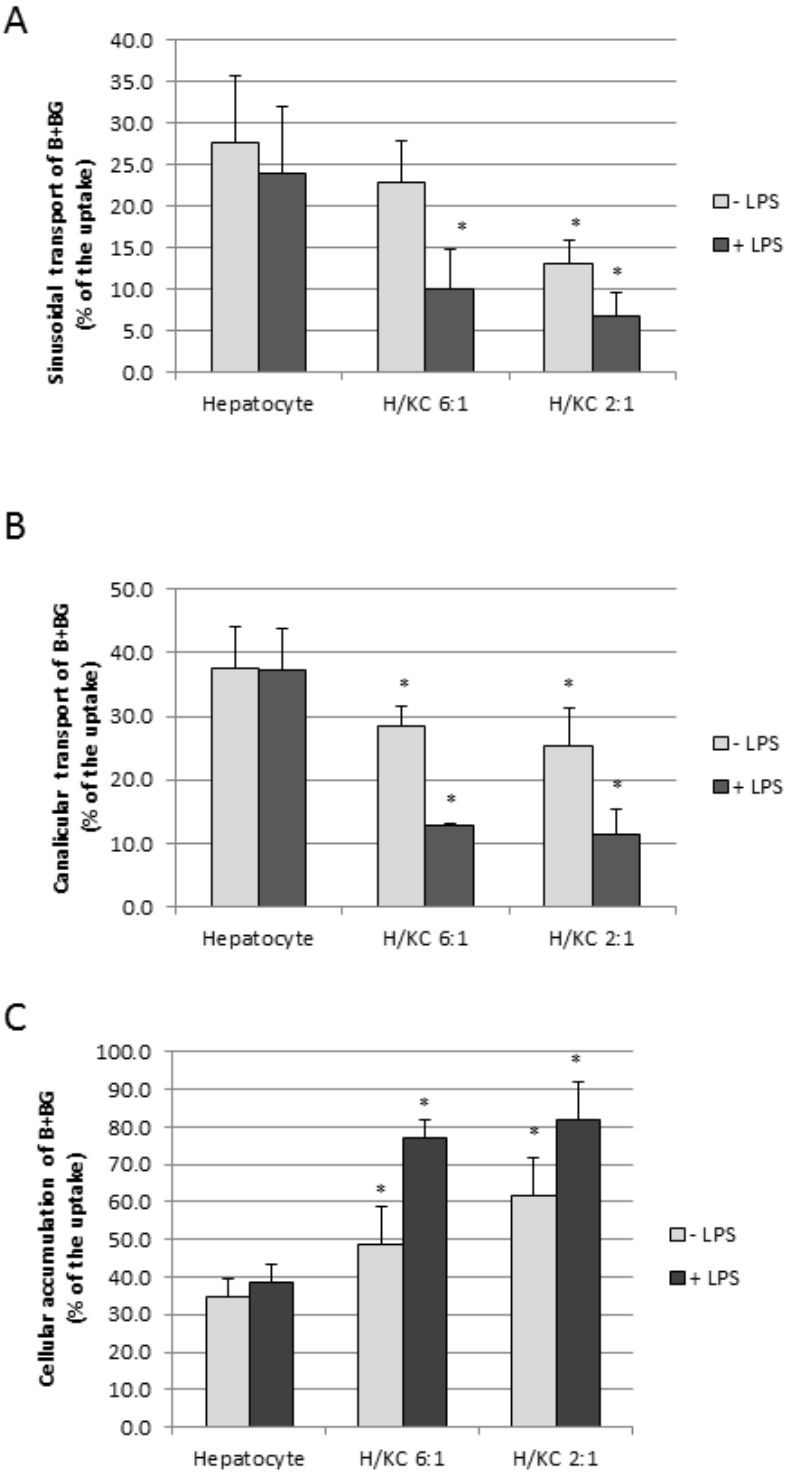

Figure 4

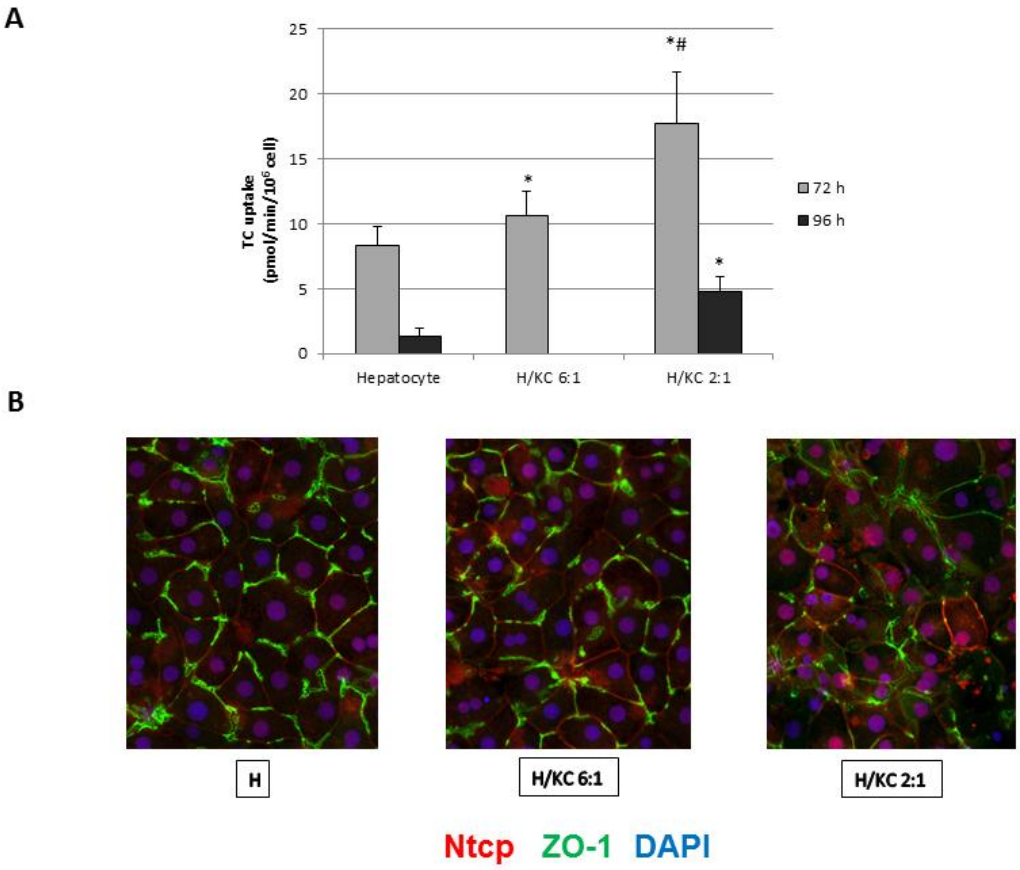

Figure 5

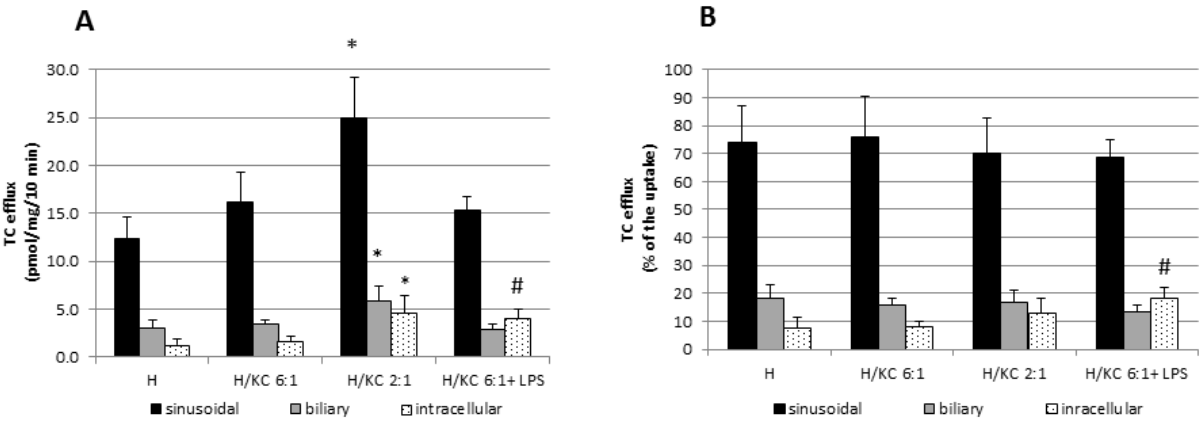

Figure 6