

β 2-adrenergic receptor-mediated *in vitro* regulation of human hepatic drug transporter expression by epinephrine

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ABSTRACT

The catecholamine epinephrine is known to repress expression of hepatic drug metabolizing enzymes such as cytochromes P-450. The present study was designed to determine whether epinephrine may also target expression of main hepatic drug transporters, that play a major role in liver detoxification and are commonly coordinately regulated with drug detoxifying enzymes. Treatment of primary human hepatocytes with 10 μM epinephrine for 24 h repressed mRNA expression of various transporters, such as the sinusoidal influx transporters NTCP, OATP1B1, OATP2B1, OAT2, OAT7 and OCT1 and the efflux transporters MRP2, MRP3 and BSEP, whereas it induced that of MDR1, but failed to alter that of BCRP. Most of these changes in transporter mRNA levels were also found in epinephrine-exposed human highly-differentiated hepatoma HepaRG cells, which additionally exhibited reduced protein expression of OATP2B1 and MRP3, increased expression of P-glycoprotein and decreased transport activity of NTCP, OATPs and OCT1. Epinephrine effects towards transporter mRNA expression in human hepatocytes were next shown to be correlated to those of the selective β 2-adrenoreceptor (ADR) agonist fenoterol, of the adenylate cyclase activator forskolin and of the cAMP analogue 8-bromo-cAMP. In addition, the non-selective β -ADR antagonist carazolol and the selective β 2-ADR antagonist ICI-118,551, unlike the α -ADR antagonist phentolamine, suppressed epinephrine-mediated repressions of transporter mRNA expression. Taken together, these data indicate that epinephrine regulates *in vitro* expression of main hepatic drug transporters in a β 2-ADR/adenylate cyclase/cAMP-dependent manner. Hepatic drug transport appears therefore as a target of the β 2-adrenergic system, which may have to deserve attention for drugs interacting with β 2-ADRs.

KEY-WORDS

Epinephrine; drug transporters; hepatocyte; β 2-adrenoreceptor; cAMP.

1. INTRODUCTION

Epinephrine is an endocrine hormone mainly produced by adrenal medulla in response to stress (Tank and Lee Wong, 2015). This catecholamine exerts various major physiological effects, concerning notably the cardiovascular system, the respiratory system and the endocrine system, through acting as a potent agonist of α and/or β adrenergic receptors (ADRs) (Strosberg, 1993). Hepatocytes, that display notable expression of α 1- and β 2-ADRs (Aggerbeck et al., 1983), are recognized as known cellular targets of epinephrine (Morgan et al., 1983). In this context, the hepatic drug detoxifying system is likely one of the various hepatic metabolic ways affected by the catecholamine. Indeed, epinephrine has been shown to modulate expression of several drug-metabolizing cytochromes P-450 (CYPs) in rat hepatocytes (Daskalopoulos et al., 2012; Iber et al., 2001). It also targets CYP levels in human hepatic cells, notably those of CYP3A4 and CYP1A2 that are reduced in both primary human hepatocytes and human high-differentiated hepatoma HepaRG cells exposed to epinephrine, which has been suggested to be linked to a β 2-adrenergic pro-inflammatory effect exerted by the catecholamine via interleukin (IL)-6 induction (Aninat et al., 2008).

Besides drug metabolizing enzymes, drug transporters are now recognized as major actors of the hepatic drug detoxification system (Funk, 2008; Lecureur et al., 2000; Li et al., 2009). They are implicated in uptake of drugs into hepatocytes at their sinusoidal pole (Fahrmayr et al., 2010) and also in biliary secretion of drugs or drug metabolites at the canalicular pole of hepatocytes (Pfeifer et al., 2014), acting thus in a coordinate manner with drug metabolizing enzymes for permitting hepatobiliary elimination of xenobiotics (Stieger and Hagenbuch, 2016). Transporter expression is also coordinately regulated with that of drug metabolizing enzymes such as CYPs (Eloranta et al., 2005; Fardel et al., 2001). Activation of drug sensing receptors like aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) has thus been demonstrated to control hepatic

expression of CYP2B6, CYP3A4 and drug transporters like P-glycoprotein, encoded by multidrug resistance gene 1 (MDR1) gene (*ABCB1*), multidrug resistance-associated protein (MRP) 2 (*ABCC2*), MRP3 (*ABCC3*), MRP4 (*ABCC4*) and breast cancer resistance protein (BCRP/*ABCG2*) (Amacher, 2016; Klaassen and Aleksunes, 2010; Kohle and Bock, 2009). Inflammatory cytokines such as IL-1 β and IL-6 also modulate in a similar way hepatic levels of CYPs and drug transporters (Fardel and Le Vee, 2009; Morgan et al., 2008; Petrovic et al., 2007). Whether epinephrine, regulating hepatic CYP expression as mentioned above (Aninat et al., 2008), may also act on that of hepatic drug transporters remains however unknown. The present study was therefore designed to analyze the effects of epinephrine on functional expression of main hepatic solute carrier (SLC) and ATP-binding cassette (ABC) transporters in human hepatocytes. Our data indicate that epinephrine treatment down-regulates expression of several sinusoidal uptake and canalicular hepatic transporters, through activation of a β 2-ADR/adenylate cyclase/cAMP pathway.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Epinephrine, fenoterol, forskolin, methoxamine, phentolamine, carazolol, ICI-118,551, N-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide (H89), probenecid and verapamil were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). 8-bromo-cAMP (8-Br-cAMP), N⁶-benzoyladenosine-cAMP (6-Bnz-cAMP) and 8-(4-chlorophenylthio)-2'-O-methyl-cAMP (8-pCPT-cAMP), used under its cell-permeable acetoxymethyl ester form, were from Tocris (Bristol, United Kingdom), whereas Gö 6983 was from Calbiochem (La Jolla, CA, USA). Recombinant human IL-6 was supplied by R&D Systems (Minneapolis, MN) and ruxolitinib by Selleck Chemicals (Houston, TX). [³H(G)] taurocholic acid (TC) (sp. act. 1.19 Ci/mmol), [6,7-³H(N)] estrone-3-sulfate (E3S) (sp. act.

57.3Ci/mmol) and [$1\text{-}^{14}\text{C}$] tetra-ethylammonium (TEA) (sp. act. 2.4 mCi/mmol) were purchased from Perkin-Elmer (Courtaboeuf, France). Mouse monoclonal antibodies against P-glycoprotein (clone C219), MRP2 (clone M2III-6), MRP3 (clone M3II-9) and BCRP (clone BXP-21) were from Alexis Biochemicals (Lausen, Switzerland), whereas that against heat shock cognate 70 protein (HCS70) was from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit polyclonal antibodies raised against organic anion transporter polypeptide (OATP) 1B1 (*SLCO1B1*) or OATP2B1 (*SLCO2B1*) have been previously described (Huber et al., 2007). All other compounds and reagents were commercial products of the highest purity available.

2.2. Cell culture

Human hepatocytes were obtained from 7 adult donors undergoing hepatic resection for tumors. The processing of biological samples was performed through the Centre de Ressources Biologiques (CRB) Santé of Rennes BB-0033-00056, conducted under French legal guidelines and fulfilled the requirements of the local institutional ethics committee. After being prepared by enzymatic dissociation of histologically-normal liver fragments (Fardel et al., 1993), hepatocytes were seeded at a density of $2 \cdot 10^5$ cells/cm² in Williams' E medium (Invitrogen, Cergy-Pontoise, France), supplemented with 10% (vol/vol) fetal calf serum (Perbio Sciences, Brébieres, France), 5 $\mu\text{g}/\text{mL}$ bovine insulin (Sigma–Aldrich), 20 IU/mL penicillin, 20 $\mu\text{g}/\text{mL}$ streptomycin, and 2 mM glutamine (Invitrogen). After 24 h, this seeding medium was discarded, and primary hepatocytes were routinely cultured in the fetal calf serum-containing Williams' E medium defined above and supplemented with $5 \cdot 10^{-5}$ M hydrocortisone hemisuccinate (SERB laboratoires, Paris, France) and 2% (vol/vol) dimethyl sulfoxide (DMSO), as previously reported (Le Vee et al., 2015). All experimental procedures complied with French laws and regulations and were approved by the National Ethics

Committee. Some human hepatocytes (from 10 donors) were also commercially obtained as 24-well-plated hepatocytes from Biopredic International (Saint Grégoire, France); after receipt, they were cultured in the same medium conditions than described above. Characteristics of hepatocyte donors are indicated in Table S1.

Human hepatoma HepaRG cells were also used in the study, owing to the limited, scarce and unpredictable availability of human hepatocytes. Indeed, differentiated HepaRG cells are now recognized as surrogates for human hepatocytes in drug metabolism and transport studies (Bachour-El Azzi et al., 2015; Lubberstedt et al., 2011; Nelson et al., 2017), even if differences between HepaRG cells and human hepatocytes for expression of some transporters such as OATP1B3 (*SLCO1B3*) exist and have likely to be kept in mind (Le Vee et al., 2013b). HepaRG cells were cultured as previously reported (Le Vee et al., 2013b). Briefly, cells were initially grown in Williams' E medium supplemented with 10% (vol/vol) fetal calf serum, 20 IU/mL penicillin, 20 µg/mL streptomycin, 5 µg/mL bovine insulin, 2 mM glutamine, and $5 \cdot 10^{-5}$ M hydrocortisone hemisuccinate for two weeks. Cells were next cultured for additional two weeks in the same medium supplemented with 2% (vol/vol) DMSO in order to promote hepatic differentiation of the cells (Gripon et al., 2002).

2.3. RNA isolation and analysis

Extraction of total mRNA from cells was performed using the TRIzol reagent (Invitrogen). RNA (10 ng) was then subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR), using the fluorescent dye SYBR Green methodology and an ABI Prism 7300 detector (Applied Biosystem, Foster City, CA, USA) (Sparfel et al., 2010). Specific ready-to-use gene primers for α -adrenergic receptor isoforms were purchased from Qiagen (Hilden, Germany). Other gene specific primers were exactly as previously described (Mayati et al., 2015) or are listed in Table S2. Amplification curves of the PCR products were

analyzed with the ABI Prism SDS software using the comparative cycle threshold method. Relative quantification of the steady-state target mRNA levels was calculated after normalization to the 18S rRNA level, used here as an internal control. Data were routinely expressed as fold factor change comparatively to control untreated cells or, alternatively, in arbitrary units relatively to 18S rRNA as previously reported (Le Vee et al., 2013b).

2.4. Western-blot analysis

Cellular protein extracts, prepared from HepaRG cells as previously described (Mayati et al., 2015), were separated on polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Gel loading and transfer were checked by staining membranes with Ponceau red. After blocking in Tris-buffered saline containing 4% bovine serum albumin, membranes were incubated overnight at 4°C with primary antibodies directed against target proteins or HCS70, used here as a control expression protein. Secondary peroxidase-conjugated monoclonal antibodies were thereafter used to detect the primary antibodies. After washing, immunolabeled proteins were finally visualized by chemiluminescence. Intensities of antibody-stained bands were measured by densitometry using ImageJ 1.40 g software (National Institute of Health, Bethesda, MA) and normalized to HCS70 content.

2.5. Transport assays

Activities of the sinusoidal transporters sodium taurocholate cotransporting polypeptide (NTCP/*SLC10A1*), OATPs and organic cation transporter 1 (OCT1/*SLC22A1*) were determined through measuring inhibitor-sensitive accumulation of reference substrates, as previously described (Jigorel et al., 2005). In brief, HepaRG cells were incubated for 5 min at 37° C with radiolabeled substrates, *i.e.*, 30 µM TEA (substrate for OCT1), 4 nM E3S (substrate for OATPs) or 40 nM TC (substrate for NTCP), in the absence or presence of

reference transporter inhibitors, *i.e.*, 50 μ M verapamil (OCT1 inhibitor) or 2 mM probenecid (OATP inhibitor), or in the presence or absence of Na^+ (for NTCP activity) (Le Vee et al., 2013b). The transport buffer used for assays consisted of 5.3 mM KCl, 1.1 mM KH_2PO_4 , 0.8 mM MgSO_4 , 1.8 mM CaCl_2 , 11 mM D-glucose, 10 mM HEPES, and 136 mM NaCl, adjusted to pH=7.4; NaCl was replaced by choline chloride for transport assays in the absence of Na^+ . Cells were then washed twice in phosphate-buffered saline and subjected to lysis in distilled water. Accumulation of radiolabeled substrates was next determined through scintillation counting of cell lysates and normalized to protein content, determined by the Bradford method (Bradford, 1976). TEA uptake in the absence of verapamil minus uptake in the presence of verapamil, E3S uptake in the absence of probenecid minus uptake in the presence of probenecid and TC accumulation in the presence of Na^+ minus accumulation in the absence of Na^+ are thought to correspond to OCT1, OATP, and NTCP activities, respectively (Jigorel et al., 2005).

2.6. Statistical analysis

Quantitative data were usually expressed as means \pm S.E.M. They were statistically analyzed using paired Student's t test, analysis of variance (ANOVA) followed by the Dunnett's post-hoc test or the nonparametric Spearman's rank correlation method. The criterion of significance was $p < 0.05$.

3. RESULTS

3.1. Responsiveness of primary human hepatocytes and HepaRG cells to epinephrine

To determine whether primary human hepatocytes and highly-differentiated human hepatoma HepaRG cells may be relevant models for analyzing the effects of epinephrine towards human hepatic drug transporter expression, we first analyzed mRNA expression of

ADR isoforms in these cells and in freshly isolated human hepatocytes. As shown in Fig. 1A, α 1a- and β 2-ADRs were the predominantly expressed isoforms in HepaRG cells and primary human hepatocytes, as well as in freshly isolated human hepatocytes; other isoforms such as α 1b-, α 1d-, α 2a-, α 2b-, α 2c- and β 1-ADR were found to be not, or only marginally, expressed in primary human hepatocytes and HepaRG cells. Because α 1- and β 2-ADRs are thought to be the main ADRs present in hepatocytes (Aggerbeck et al., 1983), these data suggested that both primary human hepatocytes and HepaRG cells may be responsive to epinephrine, at least for signaling pathways involving α 1a- and β 2-ADRs. This hypothesis was confirmed through the demonstration that 10 μ M epinephrine hugely, although transiently, induced mRNA expression of the orphan nuclear receptor NR4A1 (also known as Nur77), an early responsive gene well-known to be transiently inducible in response to adrenergic stimulation (Maxwell et al., 2005), in both cultured hepatocytes and HepaRG cells (Fig. 1B). In agreement with previous data (Aninat et al., 2008), epinephrine additionally repressed CYP3A4 mRNA levels in both primary human hepatocytes and HepaRG cells; the repression factors, *i.e.*, the ratio mRNA levels in control untreated cells versus those found in epinephrine-treated cells, were 2.5 ± 0.4 - and 8.4 ± 1.2 -fold in human hepatocytes and HepaRG cells, respectively, exposed to the catecholamine for 24 h (Fig. 1B). Inducing effect of epinephrine towards mRNA expression of the adrenergic responsive gene NR4A1 was next shown to be concentration-dependent (Fig. S1); the 10 μ M concentration, that maximally induced NR4A1 mRNA levels in HepaRG cells exposed for 2 h to the catecholamine (Fig. S1), was consequently retained for the rest of the study.

3.2. Regulation of drug transporter mRNA expression by epinephrine

SLC and ABC transporter mRNA expression was first investigated in primary human hepatocytes exposed to 10 μ M epinephrine for 2 h, 6 h and 24 h. As shown in Fig. 2A, mRNA

levels of the SLC transporters OATP1B1, OATP2B1, organic anion transporter (OAT) 2 (*SLC22A7*) and OAT7 (*SLC22A9*) were decreased as soon as a 2 h-exposure to the catecholamine, knowing that longer exposure (6 h or 24 h) was required to get maximal repression. The maximal observed repression factors were 2.1 ± 0.4 (for OATP1B1), 2.5 ± 0.6 (for OATP2B1), 4.5 ± 2.1 (for OAT2) and 5.5 ± 1.8 (for OAT7). Epinephrine similarly reduced mRNA expression of NTCP and OCT1, but only after a 6 h- and 24 h-treatment (NTCP) or a 24 h-treatment (OCT1) (Fig. 2A). OATP1B3 was also repressed, but only transiently in response to a 6 h-exposure to the catecholamine. With respect to ABC transporters (Fig. 2B), MDR1 mRNA levels in human hepatocytes were moderately induced in response to a 6 h- and 24 h-exposure to epinephrine; the induction factors, *i.e.*, the ratio mRNA level in epinephrine-treated cells versus that in control untreated cells, were 2.2 ± 0.3 (6 h-exposure) and 1.9 ± 0.3 fold (24 h-exposure). The catecholamine concomitantly repressed mRNA expression of MRP2, MRP3 and bile salt export pump (BSEP/*ABCB11*); maximal repression was reached after a 6 h-exposure for MRP2 (repression by a 3.4 ± 0.8 fold-factor) and MRP3 (repression by a 1.5 ± 0.2 fold factor) or after a 24 h-exposure for BSEP (repression by a 4.5 ± 2.8 fold factor) (Fig. 2B). By contrast, BCRP mRNA levels in primary human hepatocytes were not impaired by epinephrine, whatever the time of exposure (Fig. 2B). The epinephrine-triggered transporter mRNA regulations described above were found in each analyzed human hepatocyte population (data not shown), thus most likely discarding any major inter-individual variability with respect to the responsiveness to the catecholamine.

HepaRG cells exposed to 10 μ M epinephrine also displayed altered mRNA expression of drug transporters (Fig. S2). Like human hepatocytes, they thus exhibited reduced mRNA expression of NTCP, OCT1, OATP1B1, OATP2B1, OAT2 and OAT7 after a 24 h-exposure to epinephrine; several of these SLC transporters were also down-regulated for shorter

exposure (2 h and/or 6 h) to the catecholamine (Fig. S2A). OATP1B3 mRNA expression was by contrast induced by epinephrine, but only in response to a 24 h-treatment (Fig. S2A). The catecholamine also induced MDR1 mRNA levels in HepaRG cells, but only transiently and for short exposures (2 h and 6 h), whereas those of MRP2 were concomitantly transiently reduced (Fig. S2B). Epinephrine additionally reduced mRNA expressions of MRP3 and BSEP in HepaRG cells, whatever the exposure time, whereas BCRP mRNA levels were not altered (Fig. S2B).

To directly compare the effects of a 24 h-exposure to epinephrine on the profile of drug transporter mRNA expression in human hepatocytes and HepaRG cells, transporters were next ranked from the most repressed to the most induced by the catecholamine and the ranking obtained for human hepatocytes was compared to that for HepaRG cells using the Spearman rank correlation method. As shown in Fig. S3, epinephrine-induced changes of transporter expression in human hepatocytes were found to be significantly correlated to those observed in HepaRG cells ($\rho=0.64$, $p=0.0019$), thus indicating that response to epinephrine in terms of transporters was close in human hepatocytes and HepaRG cells.

3.3. Regulation of protein expression and activity of drug transporters by epinephrine

Owing to the scarce and limited availability of human hepatocytes, HepaRG cells were used to characterize the effects of epinephrine on protein expression and activity of drug transporters. As indicated in Fig. 3A, a 48 h-exposure to 10 μ M epinephrine slightly, but significantly, enhanced P-glycoprotein expression by a 1.3 ± 0.1 fold factor. It concomitantly reduced OATP2B1 protein expression, by a 6.9 ± 3.8 fold factor, and MRP3 protein expression, by a 2.6 ± 0.6 fold factor (Fig. 3A). OATP1B1 was also repressed, but only by a slight 1.3 ± 0.1 fold factor that failed to reach statistical significance, whereas MRP2 and BCRP protein levels were concomitantly not significantly altered by the catecholamine (Fig.

3A). Epinephrine treatment was next shown to significantly reduce NTCP activity (by a 1.6 ± 0.1 fold factor), OATP activity (by a 1.3 ± 0.2 fold factor) and OCT1 activity (by a 1.9 ± 0.1 fold factor) in HepaRG cells (Fig. 3B).

3.4. Implication of β 2-ADR/adenyl cyclase/cAMP pathway in epinephrine-mediated drug transporter regulation

To determine whether epinephrine-triggered regulations of transporter expression were related to the activation of ADRs and to identify the potential ADR isoform involved, we analyzed the influence of various non-selective and selective adrenergic antagonists, *i.e.*, the α -adrenergic blocking agent phentolamine (Faulds et al., 1991), the β -ADR antagonist carazolol (Manalan et al., 1981) and the specific β 2-ADR blocker ICI-118,551 (O'Donnell and Wanstall, 1980), on transporter mRNA expression changes caused by the catecholamine in human hepatocytes. Carazolol and ICI-118,551, unlike phentolamine, were able to fully suppress the repressing effects of epinephrine toward OATP1B1, OATP2B1, OAT2 and OAT7 mRNA expression (Fig. 4A), thus supporting an involvement of β 2-ADR. We next analyzed the effects of the specific β 2-ADR agonist fenoterol (Faulds et al., 1991) on drug transporter expression in primary human hepatocytes. As indicated in Fig. 4B, a 24 h-exposure to 10 μ M fenoterol significantly decreased mRNA expression of various transporters also repressed by epinephrine such as NTCP, OCT1, OATP1B1, OATP2B1, OAT2, OAT7, MRP2 and BSEP. Moreover, the ranking of fenoterol-induced changes in transporter mRNA expression, from the most to the less repressing effects, was found to be significantly correlated to that related to epinephrine effects ($\rho=0.72$, $p=0.0005$) (Fig. 4C), thus underlining the fact that epinephrine and fenoterol treatment most likely resulted in similar global alterations of transporter expression profile in human hepatocytes. By contrast,

treatment by the α 1-ADR agonist methoxamine failed to significantly alter mRNA expression of drug transporters in human hepatocytes (Fig. S4).

The β 2-ADR pathway classically implicates adenylate cyclase activation and subsequent generation of the second messenger cAMP as key downstream signaling events (Johnson, 1998). We therefore investigated whether forskolin, a natural labdane diterpene compound known to act as a potent activator of adenylate cyclase (Daly, 1984), may alter drug transporter expression in human hepatocytes. As shown in Fig. 5A (Left panel), exposure of human hepatocytes to 50 μ M forskolin for 24 h resulted in decreased mRNA expressions of NTCP, OCT1, OATP1B1, OATP2B1, OAT2, OAT7, MRP3 and BSEP, whereas those of MDR1, BCRP and MRP2 remained unchanged. Such forskolin-induced changes in transporter expression profile were found to be significantly correlated to those caused by epinephrine ($\rho=0.81$, $p<0.0001$) (Fig. 5A, right panel).

Adenylate cyclase-related generation of the second messenger cAMP usually results in the activation/regulation of various possible downstream signaling pathways of cAMP, such as protein kinase A (PKA) (Kleppe et al., 2011), exchange proteins activated by cAMP (EPACs) (Gloerich and Bos, 2010) or cyclic nucleotide-gated ion channels (CNGCs) (Biel, 2009; Kraus-Friedmann, 2000). Interestingly structurally-diverse cAMP analogs have been shown to differently activate PKA and EPACs, thus allowing to possibly discriminate between these two cAMP-related signaling ways (Poppe et al., 2008), with however the caution that the effects of these cAMP analogs on CNGCs remain not characterized. A 24 h-treatment by 100 μ M 8-Br-cAMP, that activates both PKA and EPACs (Poppe et al., 2008), was found to inhibit mRNA expressions of NTCP, OATP1B1, OATP2B1, OCT1, OAT2, OAT7, BCRP, MRP2 and BSEP, whereas that of MDR1 was induced and those of OATP1B3 and MRP3 not significantly impaired (Fig. 5B, left panel); such changes of transporter expression were found to be significantly correlated with those elicited by epinephrine

($\rho=0.69$, $p=0.0008$) (Fig. 5B, right panel). In the same way, a 24 h-treatment by 100 μM 6-Bnz-cAMP, that activates PKA, but not EPACs (Christensen et al., 2003), repressed mRNA expressions of NTCP, OATP1B1, OATP2B1, OCT1, OAT2, OAT7, BCRP, MRP2 and BSEP in human hepatocytes, whereas those of OATP1B3, MDR1 and MRP3 were not significantly impaired (Fig. 5C, left panel). These changes in transporter expression were significantly correlated with those triggered by epinephrine ($\rho=0.72$, $p=0.0005$) (Fig. 5C, right panel). By contrast, exposing cells to 100 μM 8-pCPT-cAMP, a cAMP analogue activating EPACs but not PKA (Christensen et al., 2003), failed to alter mRNA expression of transporters in human hepatocytes (Fig. S5). The PKA inhibitor H89 was next shown to not prevent the inhibitory effects of epinephrine towards mRNA expression of OATP1B1, OATP2B1, OAT2 and OAT7 (Fig. S6). Such repressing effects of epinephrine were similarly not suppressed by the pan-protein kinase C inhibitor Gö 6983 (Fig. S7).

3.5. Lack of IL-6 implication in epinephrine-mediated drug transporter regulation

Epinephrine has previously been shown to induce IL-6 secretion in human hepatocyte cultures, which has been hypothesized to be involved in epinephrine-mediated suppression of CYP3A4 expression (Aninat et al., 2008). Because IL-6 is additionally known to modulate hepatic drug transporter expression (Le Vee et al., 2009), we wondered whether IL-6 may be also implicated in epinephrine-mediated drug transporter regulations. The profile of mRNA transporter expression changes caused by IL-6 in human hepatocytes was however not correlated with that due to epinephrine treatment ($\rho=0.02$, $p=0.68$) (Fig. 6A). Blocking the Janus kinase (JAK)-signal transducer and activator of transcription (STAT)-related downstream signaling pathway of IL-6 (Heinrich et al., 2003) using the JAK1/2 inhibitor ruxolitinib (Quintas-Cardama et al., 2010) was fully efficient for counteracting IL-6 effects as demonstrated by the inhibition of the C-reactive protein (CRP) mRNA overexpression

triggered by the cytokine (Fig. 6B) and of IL-6-mediated repression of OAPT1B1 and OATP2B1 (data not shown); ruxolitinib however failed to prevent OATP1B1, OATP2B1, OAT2 and OAT7 mRNA repression caused by epinephrine (Fig. 6C).

4. DISCUSSION

The present study demonstrates that epinephrine can regulate *in vitro* expression of human hepatic drug transporters. Associated to the fact that this catecholamine also impairs levels of hepatic CYP expression (Aninat et al., 2008), these data therefore support the idea that epinephrine likely acts as a physiological regulator of the hepatic drug detoxifying machinery. The catecholamine may consequently be added to the growing list of effectors that coordinately regulate drug metabolizing enzymes and drug transporters (Kohle and Bock, 2009).

Most of transporters regulated by epinephrine were down-regulated at the mRNA level in human hepatocytes. This was notably the case for the sinusoidal influx transporters NTCP, OCT1, OATP1B1, OATP2B1, OAT2 and OAT7 and for the efflux transporters MRP2, MRP3 and BSEP, whereas, by contrast, MDR1 mRNA expression was induced and that of BCRP remained unchanged. Such regulations were also found in HepaRG cells, with minor variations, *i.e.*, MRP2 and MDR1 mRNA levels were only transiently down-regulated and up-regulated, respectively, in HepaRG cells. Such a similar transporter regulation in response to epinephrine occurring in both HepaRG cells and human hepatocytes fully supports the now well-established relevance of HepaRG cells as surrogates for human hepatocytes (Antherieu et al., 2012). OATP1B3 mRNA expression was however markedly differently regulated in human hepatocytes versus HepaRG cells; this influx transporter was thus transiently repressed in human hepatocytes exposed to epinephrine, whereas it was induced in HepaRG cells. A similar discrepancy for OATP1B3 regulation between

hepatocytes and HepaRG cells has already been reported in response to the protein kinase C activator phorbol myristate acetate (Mayati et al., 2015) or to all-trans retinoic acid (Le Vee et al., 2013a). This may be due to the very low constitutive expression of OATP1B3 in HepaRG cells (Le Vee et al., 2013b). Interestingly, epinephrine-mediated regulations of transporter mRNA levels in HepaRG cells were associated with parallel regulations of transporter protein levels, notably for OATP2B1, MDR1/P-glycoprotein and MRP3, whereas protein expression of BCRP remained unchanged, as for its mRNA levels. MRP2 protein expression was also not obviously altered by epinephrine, which likely reflects the fact that MRP2 mRNA repression by the catecholamine was only transient and rather slight. In parallel, epinephrine reduced activity of NTCP, OCT1 and OATP, thus indicating that the down-regulation of these transporters has functional consequences.

The putative physiological meaning of epinephrine-related hepatic drug transporter regulation remains to be established. It is nevertheless noteworthy that the catecholamine rather decreases expression of sinusoidal SLC transporters like NTCP, OAT2, OAT7, OCT1, OATP1B1 and OATP2B1 whereas those of canalicular ABC transporters, excepted that of BSEP, were better preserved, *i.e.*, both BCRP and MRP2 protein levels were unchanged whereas that of MDR1/P-glycoprotein was even induced. Taken together, transporter regulation in response to epinephrine may consequently be interpreted as a protective mechanism for hepatic cells, possibly leading to decreased intracellular accumulation of xenobiotics through reduction of their uptake and preservation or enhancement of their efflux. Such disturbance of hepatic transport may in fact be considered as a part of the physiological adaptive processes triggered by stress, that hugely stimulates secretion of epinephrine by adrenal medulla, and aimed at favoring the adrenergic “fight-or-flight” response (Tank and Lee Wong, 2015). Whether physiological activation of the adrenergic component of the

autonomic nervous system, that directly innerves the liver (Jensen et al., 2013), may *in vivo* impairs drug transporter expression remains however to be determined.

Effects of epinephrine towards hepatic drug transporters involve activation of the β 2-ADR because (i) β 2-ADR expression is notable in both cultured human hepatocytes and HepaRG cells, as in freshly isolated hepatocytes, (ii) the non-selective β -ADR antagonist carazolol and the selective β 2-ADR blocker ICI-118,551, but not the α -ADR blocker phentolamine, prevented OATP1B1, OATP2B1, OAT2 and OAT7 repression due to epinephrine, (iii) fenoterol, a selective β 2-ADR agonist, led to transporter changes similar to those caused by epinephrine, (iv) the α 1-ADR agonist methoxamine failed to significantly alter expression of drug transporters, (v) Gö 6983, a potent inhibitor of the key protein kinase C-related signaling way activated by α 1a-ADR (Zhong and Minneman, 1999), an ADR isoform highly expressed by hepatocytes and HepaRG cells, failed to inhibit OATP1B1, OATP2B1, OAT2 and OAT7 repression caused by epinephrine, and (vi) forskolin, a potent activator of the immediate downstream target of β 2-ADR, *i.e.*, adenylate cyclase, also mainly reproduced epinephrine effects towards transporters, as well as 8-Br-cAMP, used as an analogue of physiological cAMP generated by the β 2-ADR/adenylate cyclase pathway. From cAMP generation, various signaling ways have to be considered for putative implication in epinephrine-mediated alterations of transporter expression (Gloerich and Bos, 2010; Kleppe et al., 2011). That linked to EPACs can most likely be discarded because the selective EPAC activator 8-pCPT-cAMP failed to affect transporter expression, whereas the cAMP analogue 6-Bnz-cAMP, which does not interact with EPACs, induced transporter changes close to those caused by epinephrine. PKA, although known to be potently activated by 6-Bnz-cAMP (Christensen et al., 2003), may also likely not be involved in epinephrine-mediated alterations of transporter expression because the PKA inhibitor H89 did not prevent transporter expression changes such as OATP1B1, OATP2B1, OAT2 and OAT7 mRNA repression

caused by the catecholamine; the protein kinase inhibitor peptide (PKI), an endogenous inhibitor of PKA (Dalton and Dewey, 2006), similarly failed to counteract these epinephrine-mediated transporter changes (data not shown). PKA- and EPACs-independent ways may therefore be involved in epinephrine-mediated changes of transporter, as already reported for other epinephrine effects like induction of the defense factor REDD1 in macrophages (Yanagawa et al., 2014). In this context, the putative implication of CNGCs, that are expressed by hepatocytes (Kraus-Friedmann, 2000), has likely to be considered in future studies.

The inflammatory cytokine IL-6 is an additional factor that has been hypothesized to contribute to epinephrine-mediated repression of drug detoxifying proteins such as CYP3A4 (Aninat et al., 2008). Indeed, this CYP is well-known to be repressed by IL-6 (Dickmann et al., 2011) and epinephrine has previously been shown to trigger IL-6 secretion in HepaRG cell cultures, which appeared to be fully functional, as demonstrated by the concomitant secretion of CRP, a well-established target of IL-6 (Aninat et al., 2008). IL-6 is however unlikely to be involved in epinephrine-mediated changes of transporter expression because such changes were not correlated to those caused by the cytokine. Moreover, the JAK1/2 inhibitor ruxolitinib, which counteracted the effects of IL-6 such as CRP up-regulation through inhibiting the IL-6 receptor-downstream JAK/STAT pathway, failed to prevent OATP1B1, OATP2B1, OAT2 and OAT7 repression caused by epinephrine.

Putative relevance of hepatic drug transporter alterations caused by epinephrine, and beyond, by marketed drugs acting as β 2-ADR agonists like fenoterol or salbutamol, in terms of possible drug-drug interactions (DDIs), is most likely a key issue that has to be considered. In addition, modulation of transporter expression levels by physiological or pharmacological β 2-ADR stimulators could contribute to inter-individual variability in disposition of transporter substrates. It is however noteworthy that no major alteration of drug

pharmacokinetics has been associated with increased plasma concentrations of epinephrine, that occurs in response to stress, during diseases like pheochromocytoma or after the therapeutic administration of the catecholamine, notably for the treatment of anaphylaxis or cardiac arrest. In the same way, to the best of our knowledge, the use of β 2-ADR agonists has not been reported to result in possible notable DDIs involving drugs well-known to be handled by hepatic transporters like the cholesterol-lowering statins (substrates for OATPs) (Rodrigues, 2010) or the antidiabetic metformine (substrate for OCT1) (Koepsell, 2015). Such data therefore suggest that putative regulation of *in vivo* hepatic transporter expression due to epinephrine or β 2-ADR stimulants is unlikely to cause major DDI. However, this conclusion should be substantiated by complementary and more complete investigations on this issue. Along the same line, whether the clinical use of β -ADR blockers may perturb physiological epinephrine-related regulation of hepatic transporters remains to be determined. Overall, it may nevertheless be hypothesized that transporter-related DDIs are likely primarily due to full blockage of transport activity by DDI perpetrators, that directly and physically interact with drug transporters (Endres et al., 2006; Giacomini et al., 2010). The numerous reported examples of established clinical DDIs strongly argue in favor of this hypothesis (DeGorter et al., 2012; Konig et al., 2013). Xenobiotic-mediated down-regulations of transporters, that commonly result in decrease, but not full abolition of drug transporter expression (Jigorel et al., 2006; Le Vee et al., 2013a), may thus fail to trigger major DDIs, probably because of notable basal remaining transport activity. This may explain why the characterization of potential direct drug transporter inhibition, and not that of drug transporter expression regulation, is the first goal of preclinical transporter studies recommended by drug regulatory agencies during the pharmaceutical development of new molecular entities (Liu and Sahi, 2016).

In summary, epinephrine was shown to impair expression of various main drug transporters in cultured human hepatocytes, through activation of a β 2-adrenergic/adenylate cyclase/cAMP pathway. This suggests that hepatic drug transport may constitute a physiological and previously-unrecognized target of the adrenergic system, which may have to be considered for drugs interacting with β 2-ADRs.

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LEGENDS TO FIGURES

Fig. 1. Responsiveness of human hepatocytes and HepaRG cells to epinephrine.

(A) ADR mRNA expressions in freshly isolated human hepatocytes, primary human hepatocytes and in highly-differentiated hepatocarcinoma HepaRG cells were determined by RT-qPCR. Data are expressed in arbitrary units normalized to 18S RNA expression and are the means \pm SEM of values from four independent hepatocyte populations or three independent HepaRG cell cultures. (B) Primary human hepatocytes and HepaRG cells were either untreated (CTR) or exposed to 10 μ M epinephrine for 2, 6 or 24 h. NR4A1 and CYP3A4 mRNA expressions were next determined by RT-qPCR. Data are expressed as fold change comparatively to mRNA levels found in untreated cells and are the means \pm SEM of values from at least three independent hepatocyte populations or HepaRG cell cultures. *, $p < 0.05$ when compared to control untreated cells. Expression levels (in arbitrary units) of CYP3A4 mRNAs in control untreated cells are: Primary human hepatocytes, 958.9 ± 140.7 ; HepaRG cells, 596.0 ± 102.6 .

Fig. 2. Regulation of drug transporter mRNA expression by epinephrine in primary human hepatocytes.

Primary human hepatocytes were either untreated (CTR) or exposed to 10 μ M epinephrine for 2, 6 or 24 h. (A) SLC and (B) ABC drug transporter mRNA expressions were next determined by RT- qPCR. Data are expressed as fold change comparatively to mRNA levels found in control untreated cells and are the means \pm SEM of values from at least four independent hepatocyte populations. *, $p < 0.05$ when compared to untreated cells. Expression levels (in arbitrary units) of transporter mRNAs in control untreated hepatocytes are: NTCP, 55.3 ± 6.2 ; OCT1, 216.3 ± 30.7 ; OATP1B1, 27.5 ± 3.4 ; OATP1B3, 2.2 ± 0.7 ; OATP2B1, 35.7 ± 4.0 ;

OAT2, 27.9 ± 2.4 ; OAT7, 1.4 ± 0.1 ; MDR1, 12.3 ± 2.1 ; BCRP, 6.3 ± 0.3 ; MRP2, 57.5 ± 4.7 ; MRP3, 40.4 ± 2.8 ; BSEP, 5.9 ± 1.1 .

Fig. 3. Effects of epinephrine on (A) drug transporter protein expression and (B) drug transporter activity.

HepaRG cells were either untreated (CTR) or exposed to 10 μ M epinephrine for 48 h. (A) Drug transporter protein contents were determined by Western-blotting. Left panel, a representative blot is shown for the effect of epinephrine on each transporter. Right panel, for each transporter, data were quantified by densitometric analysis, normalized to HSC70 content and expressed relative to transporter expression found in control untreated cells, arbitrarily set at the value of 100% and indicated by a dotted line; they are the means \pm SEM of values from at least three independent assays. *, $p < 0.05$ when compared to untreated cells. P-gp, P-glycoprotein. (B) Drug transporter activities were determined as described in the Materials and Methods section. Data are the means \pm SEM of at least three independent assays. *, $p < 0.05$ when compared to control untreated cells.

Fig. 4. Implication of β 2-ADR in epinephrine-mediated regulation of hepatic transporter mRNA expression.

(A) Primary human hepatocytes were either untreated or exposed to 10 μ M epinephrine for 24 h, in the absence (CTR) or presence of 50 μ M carazolol (a non-selective β -ADR blocker), 50 μ M ICI-118,551 (a selective β 2-ADR blocker) or 50 μ M phentolamine (a non-selective α -ADR antagonist). Drug transporter mRNA expressions were next determined by RT-qPCR. Data are expressed as fold change comparatively to mRNA levels found in control untreated cells and are the means \pm SEM of values from at least four independent hepatocyte populations. *, $p < 0.05$ when compared to cells exposed to epinephrine alone. (B) Primary

human hepatocytes were either untreated or exposed to 10 μM fenoterol for 24 h. Drug transporter mRNA expressions were next determined by RT-qPCR. Data are expressed as fold change of mRNA expressions in fenoterol-treated cells versus mRNA levels found in untreated counterparts and are the means \pm SEM of values from three independent hepatocyte populations. *, $p < 0.05$ when compared to untreated cells; the dotted line corresponds to the absence of change of mRNA expression, *i.e.*, a value of 1 for fold-change. (C) Spearman rank correlation analysis of fenoterol versus epinephrine effects towards drug transporter mRNA expressions in primary human hepatocytes. Drug transporters were ranked according to regulation of their mRNA expression in response to a 24 h-treatment by 10 μM epinephrine (data from Fig. 2) or 10 μM fenoterol (data from Fig. 4B), from the most repressed transporter to the most induced. Correlation was next analyzed using the Spearman's rank correlation method. Spearman's rank coefficients (ρ) and p values are provided on the top of the correlation graph.

Fig. 5. Effects of the adenylate cyclase/cAMP β 2-ADR effectors on hepatic drug transporter regulation.

Primary human hepatocytes were either untreated or exposed for 24 h to (A) 50 μM forskolin, (B) 100 μM 8-Br-cAMP or (C) 100 μM 6-Bnz-cAMP. Drug transporter mRNA expressions were next determined by RT-qPCR. (A, B, C, left panels) Data are expressed as fold change in treated cells comparatively to mRNA levels found in untreated counterparts and are the means \pm SEM of values from three independent hepatocyte populations. *, $p < 0.05$ when compared to control untreated cells; the dotted lines correspond to the absence of change of mRNA expression, *i.e.*, a value of 1 for fold-change. (A, B, C, right panels) Drug transporters were ranked according to regulation of their mRNA expression in response to a 24 h-treatment by 10 μM epinephrine (data from Fig. 2), 50 μM forskolin (data from Fig. 5A, left

panel), 100 μ M 8-Br-cAMP (data from Fig. 5B, left panel) or 100 μ M 6-Bnz-cAMP (data from Fig. 5C, left panel), from the most repressed transporter to the most induced. Correlation was next analyzed using the Spearman's rank correlation method. Spearman's rank coefficients (ρ) and p values are provided on the top of the correlation graphs.

Fig. 6. Lack of involvement of IL-6 in epinephrine-triggered effects on hepatic drug transporter expression.

(A) Drug transporters were ranked according to regulation of their mRNA expression in primary human hepatocytes in response to a 24 h-treatment by 10 μ M epinephrine (data from Fig. 2) or 10 ng/mL IL-6 (data from Le Vee et al., 2009). Correlation was next analyzed using the Spearman's rank correlation method. Spearman's rank coefficients (ρ) and p values are provided on the top of the correlation graphs. (B) Primary human hepatocytes were either untreated or exposed to 10 ng/mL IL-6 for 24 h in the absence (CTR) or presence of the JAK1/2 inhibitor ruxolitinib (5 μ g/mL). CRP mRNA expression was next determined by RT-qPCR and expressed as fold change of mRNA expressions comparatively to mRNA levels in control untreated cells. Data are the means \pm SEM of values from at least three independent hepatocyte populations. *, $p < 0.05$. (C) Primary human hepatocytes were either untreated or exposed to 10 μ M epinephrine for 24 h in the absence (CTR) or presence of 5 μ g/mL ruxolitinib. Drug transporter mRNA expressions were next determined by RT-qPCR. Data are expressed as fold change of mRNA expressions comparatively to mRNA levels found in untreated cells and are the means \pm SEM of values from three independent hepatocyte populations. NS, not statistically significant.

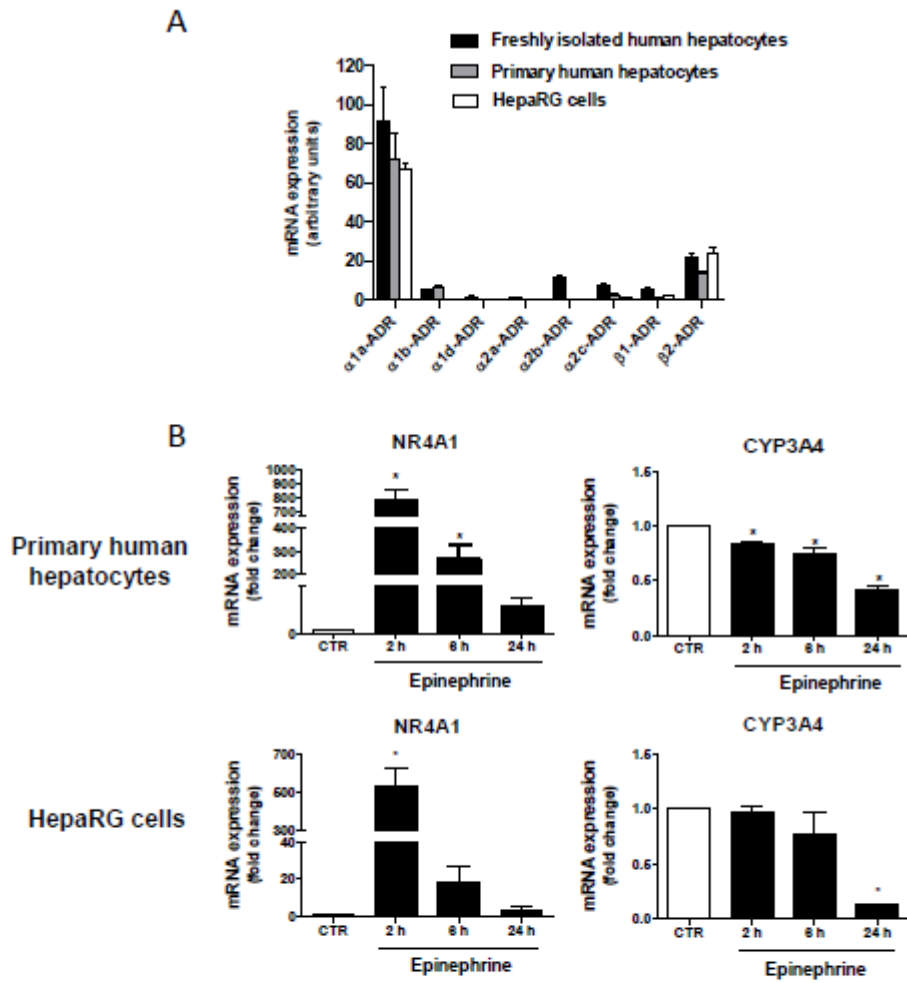


Figure 1

ACC

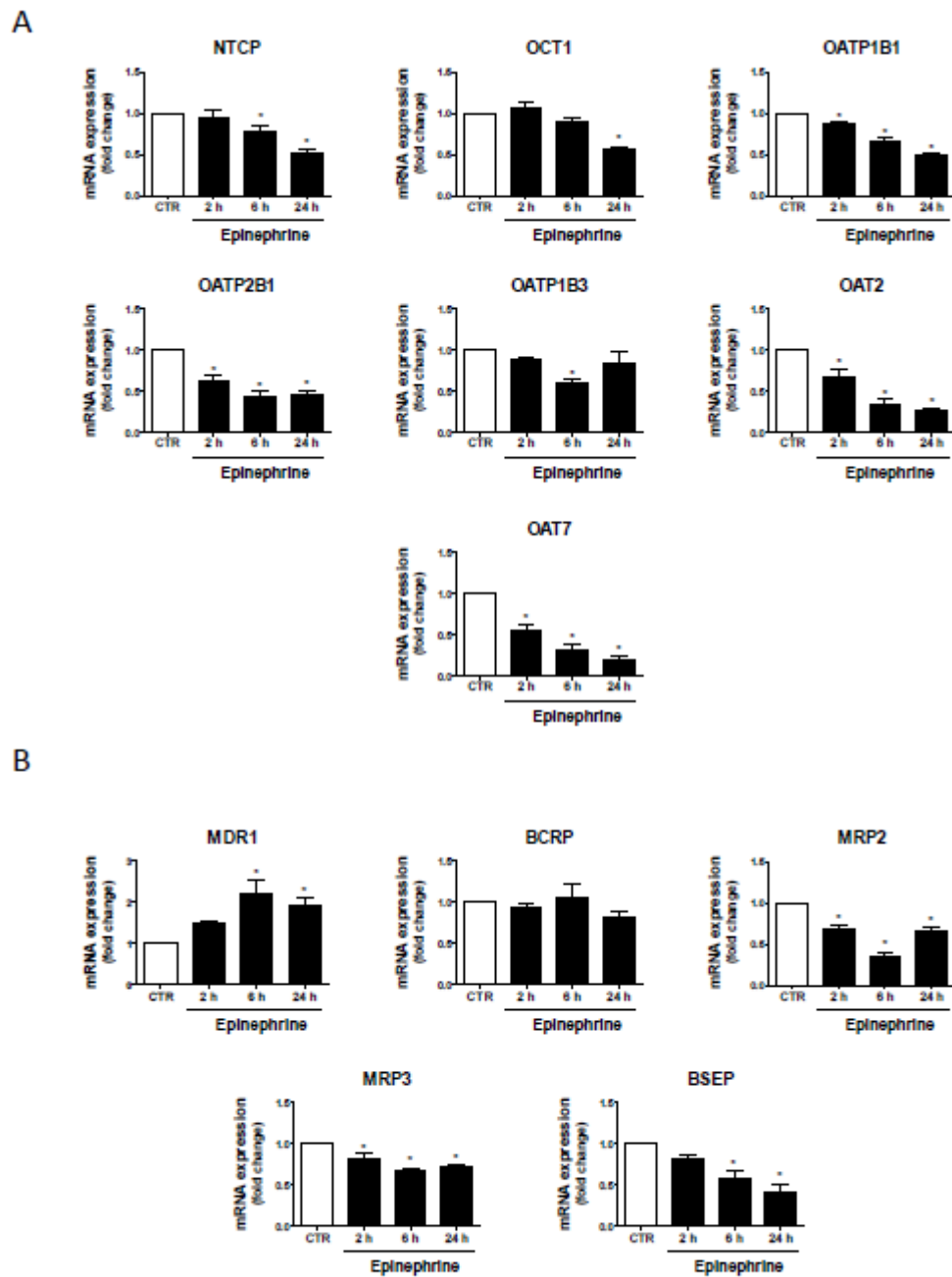


Figure 2

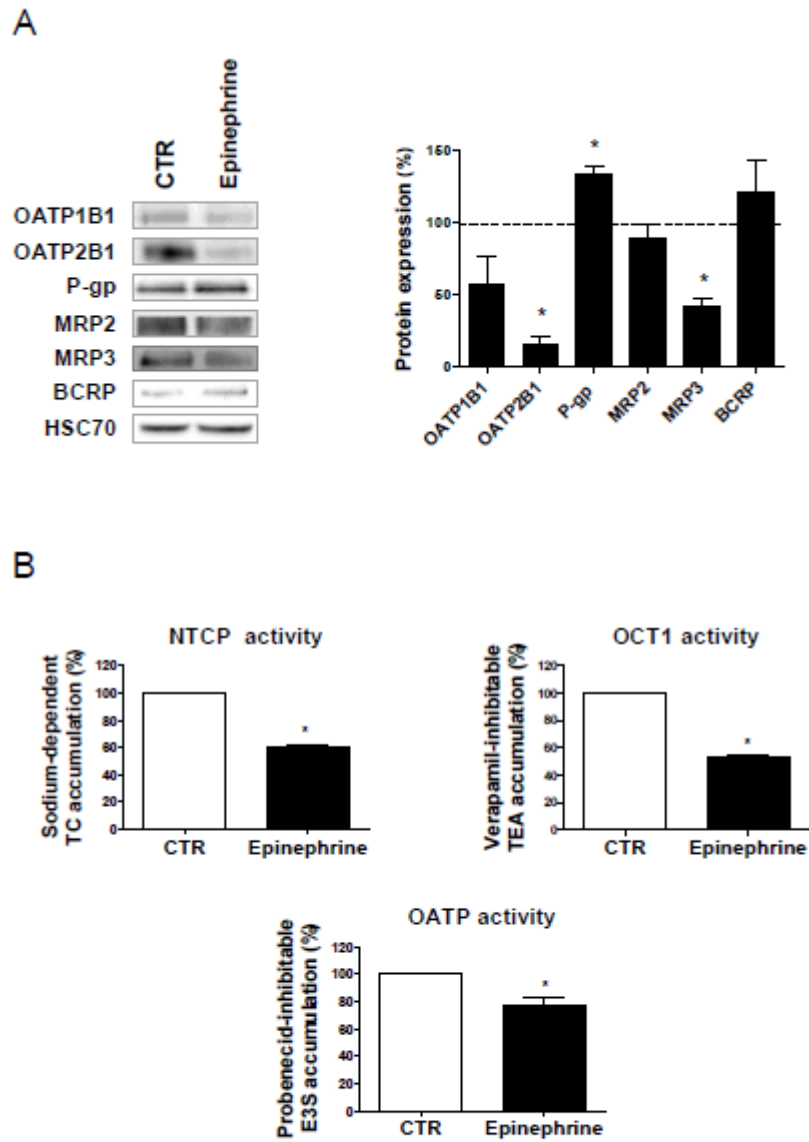


Figure 3

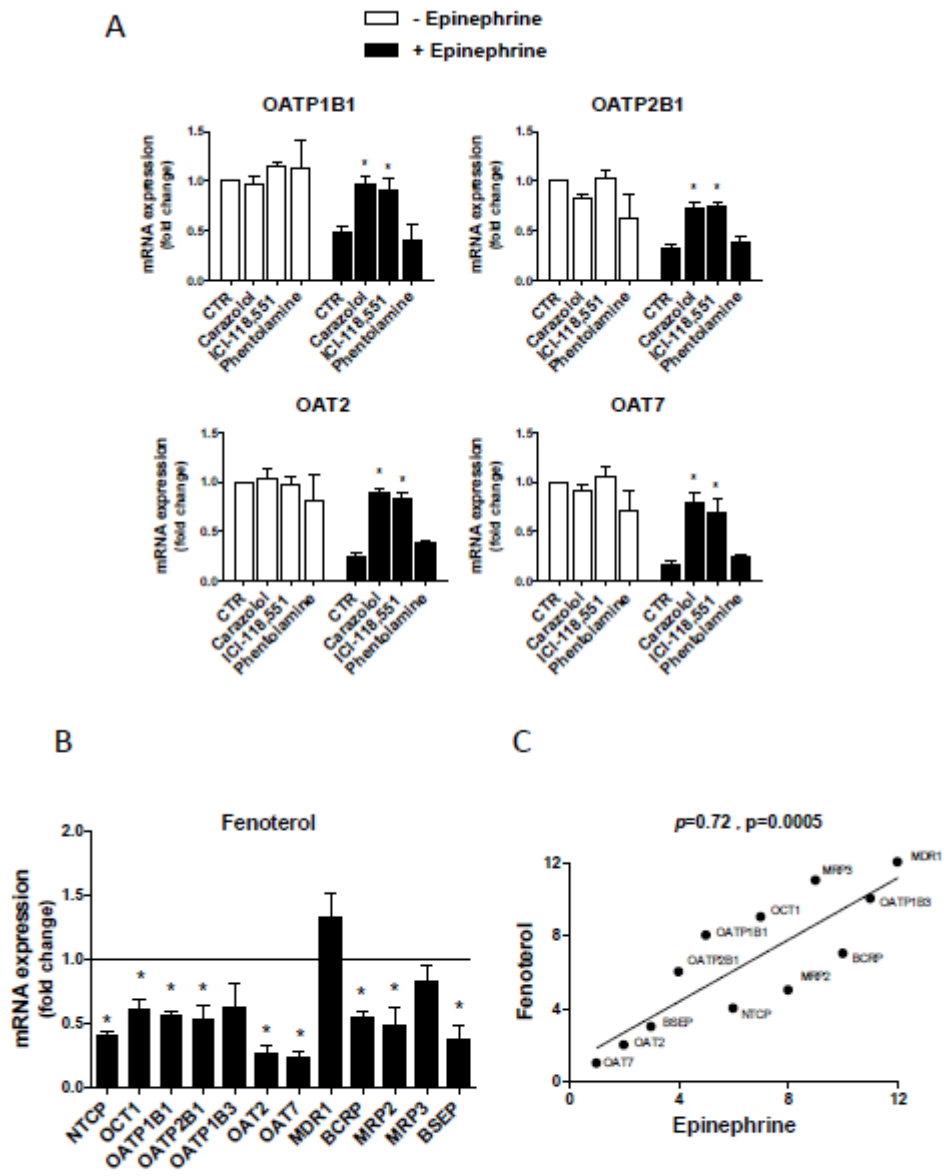


Figure 4

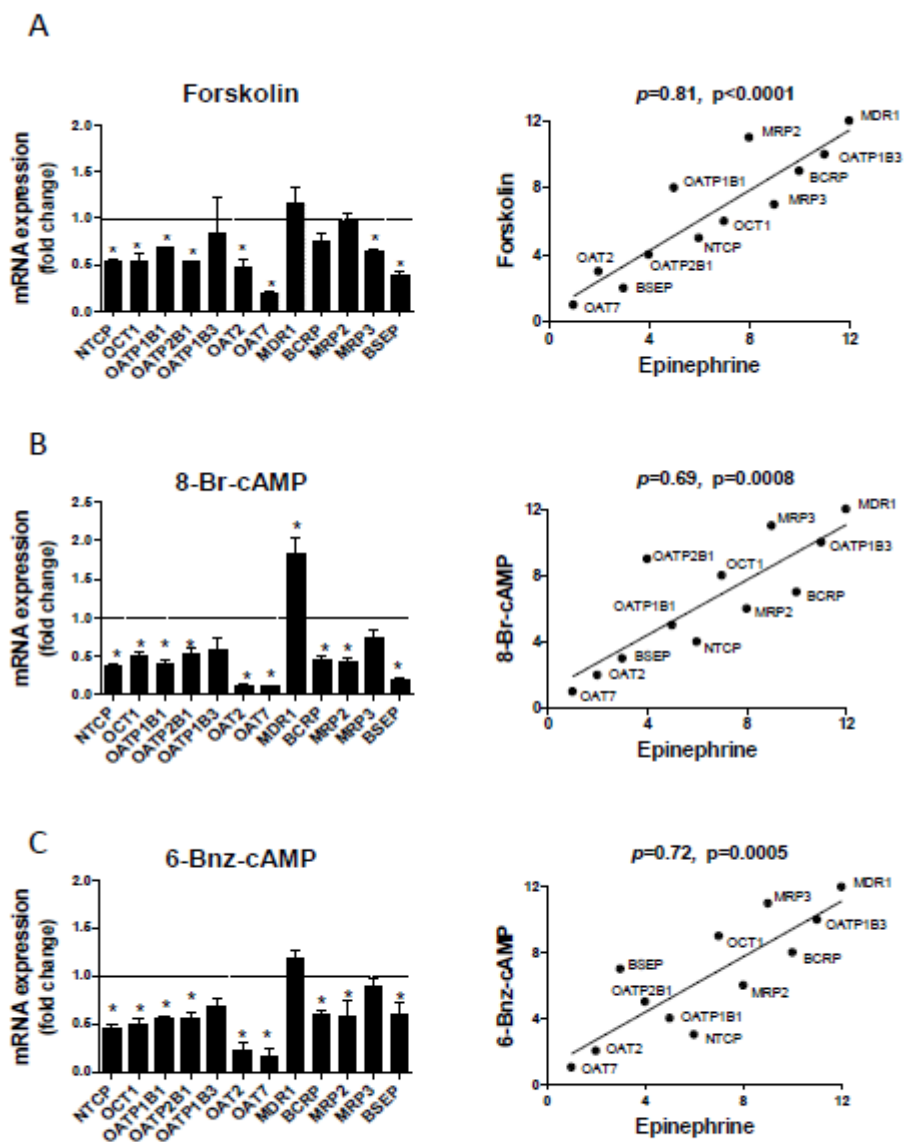


Figure 5

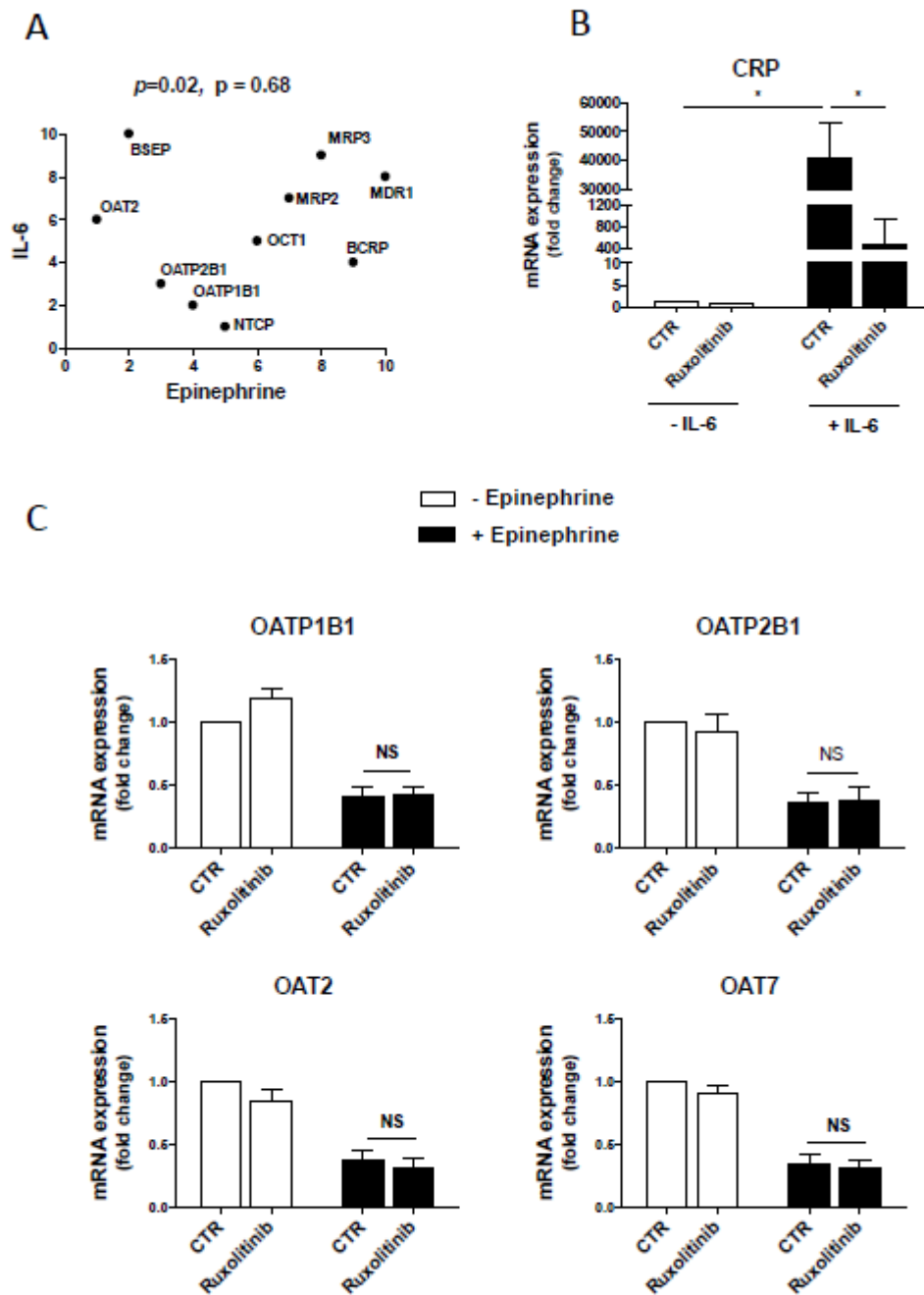
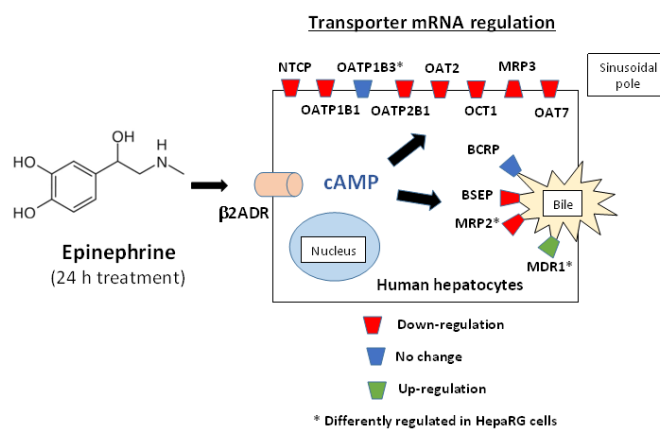


Figure 6



Graphical abstract