Effect of adjuvant-induced systemic inflammation in rats on hepatic disposition kinetics of taurocholate

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Roberts MS, Liu X, Zou Y, Siebert GA, Chang P, Whitehouse MW, Fletcher L, Crawford DH. Effect of adjuvant-induced systemic inflammation in rats on hepatic disposition kinetics of taurocholate. Am J Physiol Gastrointest Liver Physiol 300: G130–G136, 2011. First published October 28, 2010; doi:10.1152/ajpgi.00162.2010.—It has been reported that the adjuvant-induced inflammation could affect drug metabolism in liver. Here we further investigated the effect of inflammation on drug transport in liver using taurocholate as a model drug. The hepatic disposition kinetics of [3H]taurocholate in perfused normal and adjuvant-treated rat livers were investigated by the multiple indicator dilution technique and data were analyzed by a previously reported hepatobiliary taurocholate transport model. Real-time RT-PCR was also performed to determine the mRNA expression of liver bile salt transporters in normal and diseased livers. The uptake and biliary excretion of taurocholate were impaired in the adjuvant-treated rats as shown by decreased influx rate constant k_in (0.65 ± 0.09 vs. 2.12 ± 0.30) and elimination rate constant k_out (0.09 ± 0.02 vs. 0.17 ± 0.04) compared with control rat group, whereas the efflux rate constant k_out was greatly increased (0.07 ± 0.02 vs. 0.02 ± 0.01). The changes of mRNA expression of liver bile salt transporters were investigated by the multiple indicator dilution technique and data were analyzed by a previously reported hepatobiliary taurocholate transport model. Real-time RT-PCR was also performed to determine the mRNA expression of liver bile salt transporters in normal and diseased livers. The uptake and biliary excretion of taurocholate were impaired in the adjuvant-treated rats as shown by decreased influx rate constant k_in (0.65 ± 0.09 vs. 2.12 ± 0.30) and elimination rate constant k_out (0.09 ± 0.02 vs. 0.17 ± 0.04) compared with control rat group, whereas the efflux rate constant k_out was greatly increased (0.07 ± 0.02 vs. 0.02 ± 0.01). The changes of mRNA expression of liver bile salt transporters were investigated by the multiple indicator dilution technique and data were analyzed by a previously reported hepatobiliary taurocholate transport model. Real-time RT-PCR was also performed to determine the mRNA expression of liver bile salt transporters in normal and diseased rats.

In this study, we examine the effects of systemic inflammation on hepatic drug disposition in rats. The in situ perfused rat liver preparation model was used in this work for data analysis. The resulting pharmacokinetic parameters were then related to observed changes in liver physiology induced by the systemic inflammation. We found that the hepatic extraction ratio of taurocholate was well correlated to ATP levels in the liver.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma Chemical (Castle Hill, New South Wales, Australia). [3H]taurocholate was supplied by New England Nuclear (Boston, MA).

Animals and treatment. Animal studies were carried out according to protocols approved by the University of Queensland Animal Ethics Committee. Animals were treated as previously reported (18). One group of female dark Agouti rats (150 ± 10 g) were given 0.5 mg of heat-killed and delipidated Mycobacterium tuberculosis (mixed human strains, Ministry of Agriculture, Weybridge, UK) dispersed in 0.1 ml of squalane by a single subcutaneous injection into the tail base to elicit adjuvant-induced polyarthritis (27, 38). Another group of normal control rats was treated with vehicle alone. Animals were euthanized after 15 days, when the polyarthritis was fully expressed.

Serum biochemistry measurements. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) levels were assessed and reported previously (18). In situ rat liver perfusions. The in situ perfused rat liver preparation was prepared as follows and has been described in detail elsewhere (7). Briefly, normal and adjuvant-induced systemic inflammatory rats were anesthetized by an intraperitoneal injection of xylazine 10 mg/kg and ketamine-hydrochloride 80 mg/kg. Following laparotomy, rats were heparinized (heparin sodium, 200 units) via the inferior vena cava. The bile duct was cannulated with PE-10 tubing (Clay Adams).
The portal vein was then cannulated by an intravenous catheter and the liver perfused via this cannula with 25 mM MOPS-buffered Ringer solution (pH 7.4) containing 2% bovine serum albumin and 15% (vol/vol) prewashed canine red blood cells (obtained from School of Veterinary Sciences, The University of Queensland, Brisbane, Australia) and was oxygenated via a Silastic tubing lung ventilated with oxygen. The perfusion system was noncirculating and employed a peristaltic pump. Animals were euthanized by thoracotomy after perfusion initiation and the thoracic inferior vena cava was cannulated with PE-240 tubing. Oxygen consumption, bile flow, perfusion pressure, and macroscopic appearance were used to assess liver viability.

After a 10-min stabilization period, aliquots (50 μl) of perfusate containing [3H]taurocholate (3 × 10⁶ dpm) and Evans blue dye-labeled albumin (3 mg/ml) were administered to the liver through the portal vein cannula and outlet samples were collected (1 s × 20, 4 s × 5, 10 s × 5, 30 s × 5) via a fraction collector for 4 min.

Collected samples were centrifuged, and aliquots (100 μl) of the clear supernatant were taken for scintillation counting as measurement of [3H]taurocholate using a MINIAXI beta TR-CARB 4000 series liquid scintillation counter (Packard Instruments). Aliquots of 100 μl were also removed from the outflow samples for absorption spectrophotometric measurement of the Evans Blue dye at 620 nm by using a Spectracount plate counter (Packard).

**Histopathological analyses.** Normal and adjuvant-treated rat liver samples were prepared as previously described for transmission electron microscopy and scanning electron microscopy (18).

**Biochemical analyses of hepatic components.** Intrahepatic AAG levels; hepatic micromosal protein, cytoskeleton residue, and cytochrome P-450 levels; and hepatic iron concentrations of normal and adjuvant-treated rat liver were determined as described previously (18).

**Determination of hepatic Gst level.** Glutathione S-transferase (Gst) tissue levels were assayed according to Alin et al. (2). The method was based on the reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione in a sodium phosphate buffer reaction system (pH 6.5, 30°C). Activity was measured spectrophotometrically at 340 nm (ε = 9,600 M⁻¹·cm⁻¹). Briefly, 1 ml of total reaction mixture contained 1 mM CDNB dissolved in 100% ethanol, 1 mM glutathione, and 50 μl of liver tissue homogenate in assay buffer. The reaction rate for all enzyme activity assays (μmol-min⁻¹·mg⁻¹·protein) was determined by subtracting the background activity rate. Cellular protein content was estimated by Lowry method (23).

**Determination of hepatic ATP concentration.** ATP content was measured according to Evdokimova et al. (8). Briefly, liver slices were washed twice in saline and sonicated instantly in 1 ml of 2% perchloric acid. The intracellular ATP content was measured in neutralized perchloric acid extracts using the ATP Bioluminescence Assay Kit CLS II (Boehringer-Mannheim, Mannheim, Germany). The results were expressed as nanomoles ATP per milligram protein.

**Determination of mRNA expression for bile salt transporters.** Liver tissue was snap frozen and stored at −70°C before RNA extraction. RNA was extracted from 100–150 mg liver tissue using TRIzol Reagent (Invitrogen, Mt Waverley, Victoria, Australia) as per manufacturer’s instructions. Following RNA extraction, DNA contaminations were removed by incubation with RNase-free DNase-I. First-strand cDNA was synthesized from 1 μg total RNA using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT)₁₅ as per manufacturer’s instructions. Real-time RT-PCR was then performed to quantitate the gene expression of bile transporters and the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPD) for each sample. An ABI Prism 7700 sequence detection system (Applied Biosystems, Scoresby, Victoria, Australia) was used for real-time RT-PCR experiments. Reactions were performed in a 25 μl volume with 400 nM forward primer, 400 nM reverse primer and 12.5 ng of cDNA. Nucleotides, MgCl₂, Taq polymerase, and SYBR green were included in the Quantitect SYBR Green PCR master mix (Qiagen, Clifton Hill, New South Wales, Australia). The PCR conditions included a denaturation step at 94°C for 15 min. Amplification was carried out for 40 cycles (denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s). Quantification was performed by using the standard curve method for relative quantification of gene expression (Applied Biosystems, Scoresby, Victoria, Australia). Primer sequences used for analysis of GAPD, Ntcp (Slc10a1), Bsep (Abcb11), Oatp1a1 (Slc21a1), Oatp1a4 (Slc21a5), Oatp1b2 (Slc21a10), and multidrug resistance-associated protein 3 (Mrp3, Abcc3) were as follows: GAPD forward TGG ACC ACC AAC TAC TTA GC; GAPD reverse GCC TGC TTC ACC TTC TTG ATG; Ntcp forward CAA GTC CAA AAG GCC ACA C; Ntcp reverse GCC CAC ATT GAT GAG AG A; Bsep forward GGG CAG TCC ACC CAT CTA C; Bsep reverse AGC CCA GGA TGA CAA AGA AC; Oatp1 forward TCT GCC TGC CT TTT CAT TC; Oatp1a1 reverse GTG TGC TCG TCT TCC TTC TC; Oatp2 forward TGC GGA GAT GAA GCT TAC C; Oatp1a4 reverse TCC GCC TCT GAC CTT CAT AC; Oatp1b2 reverse ATA TGT AGA CCT GAG AAG TG; Oatp1b2 reverse ATG CTT CTC AGA CCT GAG CAT AG; Mrp3 forward TCC CAC TTC TCG GAG ACA GTA ACT; Mrp3 reverse CAC TTC TCG GAG ACA GTA ACT; Mrp3 reverse CAC TTC TCG GAG ACA GTA ACT. Primers were designed by use of Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA). Results for each sample are calculated as a ratio of the GAPD concentration.

[3H]taurocholate recovery in bile. Bile was collected continuously for entire 20-min period after bolus injection by using PE-10 tubing and was quantified by weight. Aliquots (100 μl) of bile were taken for scintillation counting. The ratio of [3H]taurocholate recovery in bile (Fbile) was determined from the following equation:

\[
F_{\text{bile}} = \frac{\text{total radioactivity recovered from bile}}{\text{total radioactivity injected to liver}}
\]

**Data analysis.** A mixture of two inverse Gaussian density functions with correction for catheter effects was used to describe the outflow concentration-time profiles of Evans Blue dye-labeled albumin. A detailed description of the mathematical theory and modeling methods has been given previously (15, 16). Kinetic parameters of [3H]taurocholate were derived from a physiologically based, hepatobiliary taurocholate transport model that accounts for vascular dispersion, described in detail elsewhere (17). A schematic representation of hepatobiliary taurocholate transport is illustrated in Fig. 1. The equations for describing the pharmacokinetics of taurocholate \( \tilde{f}(s) \) in the cell and the Laplace domain function (transit time density function) \( \hat{f}(s) \) for taurocholate extraction across the liver are as follows:

\[
\tilde{f}_{\text{s}}(s) = \frac{k_{\text{out}}}{k_{\text{out}} + k_{\text{in}} + s}
\]

\[
\hat{f}(s) = \tilde{f}(s)\left[1 + k_{\text{in}}\left(1 - \tilde{f}(s)\right)\right]
\]

where \( s \) is the Laplace variable, \( \tilde{f}(s) \) is the transit time density of nonperfusing reference molecule (Evans Blue dye-labeled albumin used in this study), and \( \hat{f}(s) \) is the Laplace domain function (transit time density function) for taurocholate extraction across the liver.

Nonparametric estimates of hepatic availability (F), mean transit time (MTT), and normalized variance (CV²) were determined from the outflow concentration (C) vs. time (t) profiles for the reference from Eqs. 3-6 by using the parabolas-through-the-origin method (extrapolated to infinity) as previously described (28). In brief,
where $AUMC$ is the area under the first moment curve.

$CV^2 = \frac{\sigma^2}{MTT^2}$

Statistical analysis. All data are presented as means $\pm$ SD unless otherwise stated. Statistical analysis was performed by Student’s t-test and regression analysis where appropriate. Statistical significance was taken at the level $P < 0.05$.

RESULTS

Liver physiology and biochemistry. The experimental parameters of the perfusion studies (perfusion rate, perfusion pressure, bile flow, oxygen consumption) were comparable to those reported previously (15–17), indicating the success of experiments. The adjuvant-treated rats displayed marked alterations in liver histology with inclusions and cristae tubulization of mitochondria, irregularly shaped lysosomes, and a marked decrease in size of endothelial fenestrae. The adjuvant-treated rats also had significantly elevated serum biochemistry markers such as AST, ALT, and ALP. The adjuvant-treated group had significantly higher AAG content ($P < 0.05$) and iron levels ($P < 0.001$), but significantly lower levels of cytochrome P-450 ($P < 0.05$). These results have been reported in the previous publication (18). In addition, ATP and Gst level in the livers from adjuvant-treat animals were significantly lower ($P < 0.01$) than those levels in control group as shown in Table 1.

Table 1. Comparison of liver ATP and Gst levels between normal and adjuvant-treated rats

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>Tissue Level</th>
<th>Normal</th>
<th>Adjuvant treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP, nmol/mg protein</td>
<td>2.64 ± 0.85</td>
<td>1.30 ± 0.48*</td>
</tr>
<tr>
<td></td>
<td>Gst, µmol·min$^{-1}$·mg$^{-1}$ protein</td>
<td>20.1 ± 2.87</td>
<td>9.65 ± 1.00*</td>
</tr>
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</table>

Values are means $\pm$ SD; $n = 6$. ATP, adenosine triphosphate; Gst, glutathione S-transferase. *Significantly different from normal group ($P < 0.01$).

Fig. 2. Comparison of bile salt transporter mRNA expression level between normal and adjuvant-treated rats (means $\pm$ SD, $n = 6$).
with the normal group. Although there was \( \sim 13 \) and 7% decrease in \( \text{Ntcp} \) and \( \text{Oatp1b2 mRNA expression level} \) in adjuvant-treated rats, the changes did not reach statistical significance.

### Table 2. Comparison of nonparametric moments and \([^{3}\text{H}]\text{taurocholate} \) \( F_{\text{ bile}} \) between normal and adjuvant-treated rats

<table>
<thead>
<tr>
<th>Moment Results</th>
<th>Normal</th>
<th>Adjuvant treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E )</td>
<td>( 0.93 \pm 0.05 )</td>
<td>( 0.86 \pm 0.05^* )</td>
</tr>
<tr>
<td>MTT, s</td>
<td>( 10.3 \pm 1.23 )</td>
<td>( 14.9 \pm 2.85^\dagger )</td>
</tr>
<tr>
<td>( CV^2 )</td>
<td>( 1.18 \pm 0.31 )</td>
<td>( 0.91 \pm 0.27 )</td>
</tr>
<tr>
<td>( F_{\text{ bile}} )</td>
<td>( 0.41 \pm 0.07 )</td>
<td>( 0.31 \pm 0.05^* )</td>
</tr>
</tbody>
</table>

Values are means ± SD; \( n = 6 \); \( E \), hepatic extraction ratio; MTT, mean transit time of \([^{3}\text{H}]\text{taurocholate} \) in the liver; \( CV^2 \), normalized variance; \( F_{\text{ bile}} \), biliary recovery ratio. Significantly different from normal group (*\( P < 0.05 \), †\( P < 0.01 \)).

### Hepatic extraction ratio and biliary recovery ratio

Table 2 shows a comparison of nonparametric moments and \([^{3}\text{H}]\text{taurocholate} \) biliary recovery ratio between normal and adjuvant-treated rats. The treated group showed significantly decreased hepatic extraction ratio (\( P < 0.05 \)) and \([^{3}\text{H}]\text{taurocholate} \) biliary recovery ratio. As shown in Fig. 3, the extraction ratio was significantly correlated with ATP levels in the liver (\( P < 0.001 \)). The MTT was significantly increased in the treatment group. No significant difference in \( CV^2 \) values was found between treated animals and controls.

### Modeling and data fitting of outflow concentration fraction-time profiles

Figure 4 shows a comparison of typical measured and predicted (fitted data) outflow concentration fraction-time profiles for taurocholate in the isolated perfused normal and adjuvant-treated rat liver. Co-administered Evan blue dye-labeled albumin and \([^{3}\text{H}]\text{water} \) were used for estimation of sinusoidal and cellular volumes. Data were well fitted by a physiologically based hepatobiliary taurocholate transport model.
therefore it would be useful to understand how endogenous
mon feature in the early stage of many liver diseases and
information is important because the inflamed liver is a com-
how this process was controlled by hepatic transporters. This
inflammation on the uptake of taurocholate in hepatocytes and
DISCUSSION
Figure 4 also demonstrates that the area under the curve for
and ATP content. A poor correlation between
k
be was significantly decreased in adjuvant-treated
inflamed rat livers whereas k
out was significantly increased
in addition, the biliary recovery of taurocholate dropped ~25%
in the adjuvant-treated group.
Stepwise regression analysis showed that the hepatic ATP
level was a good predictor of E. The decreased E of tauro-
cholate is a product of a decreased k
in,k
be, permeability surface
area product, and an increased k
out. As shown in Fig. 4, a linear
relationship existed between E values and hepatic ATP content
in normal and adjuvant-treated rats and hepatic ATP levels
were reduced in adjuvant-treated rats. Although the basolateral
uptake of taurocholate is not primarily dependent on ATP,
transporter-mediated (Mrp3) efflux of taurocholate from hepa-
tocyte to sinusoid via Mrp3, and excretion from the hepatocytes into the bile via the canalicular
membrane (mediated with Bsep) (25). Using the multiple
indicator dilution (MID) technique in perfused rat livers, we
characterized these steps by their pharmacokinetic parameters:
 influx rate constant k
in, efflux rate constant k
out, and elimination
rate constant k
be. Data fitting from the MID study showed
k
be was significantly decreased in adjuvant-treated
inflamed rat livers whereas k
out was significantly increased
instead. In addition, the biliary recovery of taurocholate dropped ~25%
in the adjuvant-treated group.
Table 3 compares the kinetic parameters derived from the
hepatobiliary taurocholate transport model fitting for tauro-
cholate between normal and adjuvant-treated animals. Adju-
vant-treated animals showed significantly smaller k
in (P <
0.001) and k
be (P < 0.01) values compared with normal
animals, whereas adjuvant-treated animals had a significantly
larger k
out value (P < 0.001). As shown in Fig. 3, k
be correlated
better to liver ATP level compared with k
in and k
out.

Table 3. Comparison of kinetic parameters derived from the
hepatobiliary taurocholate transport model fitting for
[^H]taurocholate between normal and adjuvant-treated rats

<table>
<thead>
<tr>
<th>Kinetic Parameter</th>
<th>Animal Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
</tbody>
</table>
| k
in, s
−1          | 2.12 ± 0.30           | 0.65 ± 0.09*     |
| k
out, s
−1          | 0.02 ± 0.01           | 0.07 ± 0.02*     |
| k
be, s
−1          | 0.17 ± 0.04           | 0.09 ± 0.02*     |

Values are means ± SD; n = 6. k
in. Plasma-to-hepatocyte influx rate constant; k
out, hepatocyte to plasma efflux rate constant; k
be, biliary elimination rate constant. *Significantly different from normal group (P < 0.01).
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responsible for each step after adjuvant treatment may have an association with lower level of ATP, although its mechanism is not yet defined.

As shown in Fig. 4, adjuvant treatment affected taurocholate outflow fraction-time profiles: outflow profiles for the adjuvant-treated group have a larger AUC and longer tail segment compared with the control group. This is consistent with the smaller hepatic extraction ratio and larger hepatic retention (MTT) of taurocholate in diseased liver. In this study, Evans blue dye-labeled albumin was used as a surrogate marker to measure liver sinusoidal volume. The sinusoidal volume in diseased livers was found to be significantly larger than that in normal livers \( P < 0.05 \). In addition, there was a slow efflux of taurocholate for diseased livers, consistent with outflow profiles seen for blood retained in parts of the liver such as the interconnecting vessels relative to large and branch vessels (29). In this case, the retention of blood probably reflects the reported decreased perfusion in certain microvascular segments after endotoxin insult, probably partly due to adhering leukocytes impairing flow (14, 24, 34). The increased retention of taurocholate in adjuvant-treated animals is also partly due to a reduction in biliary excretion \( (k_{be}) \). The larger liver weight observed in adjuvant-treated rats would also account for an increase in MTT.

We also measured mRNA expression of hepatic transporters. The expression of transporters responsible for taurocholate uptake and bile excretion (e.g., Oatp1a1, Oatp1a4, and Bsep) was downregulated in adjuvant-treated rats, whereas the expression of Mrp3 (mainly mediates efflux of taurocholate from hepatocytes to sinusoid) was upregulated nearly twofold in inflamed rat livers. Our results are consistent with previous report that inflammation decreased expression of hepatobiliary transporters at the basolateral or canalicular membrane (9). Inflammatory cytokines including TNF-\( \alpha \), IL-1\( \beta \), and IL-6, secreted by macrophages, and Kupffer cells have been characterized as mediators that reduce bile flow and organic anion excretion (30, 39). TNF-\( \alpha \)-mediated upregulation of Mrp3 has been reported before in rat (3). Recent studies in human hepatocytes also showed that both TNF-\( \alpha \) and IL-6 upregulated expression of the sinusoidal MRP3 efflux pump at the protein level (21). Previously, the effects of inflammation on the expression and/or functionality of other transporters in the liver, such as P-glycoprotein, were also intensively investigated. The results showed that, after induction of inflammation, mRNA expression of mdr1a was constantly reduced (10, 26, 33), except in one study where it was not modified (36). Contradictory results were observed for mdr1b with either an increase (10, 36) or a deduction in mRNA expression (26). The functionality of P-glycoprotein was found to be depressed in inflammation (10, 26). Since P-glycoprotein does not play an important role in the transport of taurocholate in liver, its expression and functionality was not examined in our study. Downregulation of some other proteins such as calcium channels and \( \beta \)-adrenergic target proteins has been reported in inflammation (20, 22, 31). Different from the changes of hepatic transporters in inflammatory condition, these changes would not affect drug pharmacokinetics in vivo but lead to alteration of drug pharmacodynamics instead. Reduced response to propranolol was reported in adjuvant arthritis rats although increased plasma concentration was observed (13), which was attributed to altered \( \beta \)-adrenergic receptors function.

In the inflamed liver we would therefore predict reduced uptake and increased efflux of taurocholate, thus reducing the concentration of the molecule inside hepatocytes. This could be due to reduced ATP and therefore reduced activity of ATP-dependent transporters, coupled to lower transporter expression levels due to the downregulating effect of proinflammatory cytokines. In vivo, the reduced transporter activity might result in higher drug plasma concentration, and, therefore, altered drug response.

In conclusion, administration of adjuvant to rats and the ensuing systemic inflammation impacted on hepatic architecture, biochemistry, and solute disposition by a combination of transporter expression changes on the mRNA level and decrease in hepatic ATP content. It was shown that the hepatic extraction of taurocholate was significantly impaired, consistent with a decreased uptake of taurocholate and a reduction in biliary excretion. The changes in hepatic extraction ratio in inflamed animals might be the result of an impaired ATP-dependent canalicular transport process and an increase in basolateral efflux transporter expression. The hepatic ATP levels were found to be a good predictor for hepatic extraction ratio of taurocholate. The reduced ATP level in diseased rat livers may also reflect other changes that have affected hepatic extraction.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


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