Effects of LPS on transport of indocyanine green and alanine uptake in perfused rat liver

Mette Lund, Leslie Kang, Niels Tygstrup, Allan W. Wolkoff, and Peter Ott.

Lund, Mette, Leslie Kang, Niels Tygstrup, Allan W. Wolkoff, and Peter Ott. Effects of LPS on transport of indocyanine green and alanine uptake in perfused rat liver. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G91-G100, 1999.—Lipopolysaccharide (LPS) initiates cholestasis. Whether this process is mediated by tumor necrosis factor-α (TNF-α) and whether the cholestatic response to LPS is associated with intrahepatic accumulation of possibly toxic substances are under debate. To study these questions the hepatic uptake and biliary excretion of indocyanine green (ICG) was examined in the isolated perfused rat liver 18 h after intravenous treatment of rats with either saline, 1 mg/kg body wt LPS, or LPS and intraperitoneal pentoxifylline (POF) (n = 6 in each group). POF inhibits TNF-α release after LPS administration. LPS induced a typical acute-phase response with increased mRNA for acute-phase proteins, reduced albumin mRNA, and increased hepatic uptake of alanine. Intrinsic hepatic clearance of ICG in controls (1.01 ± 0.05 ml min⁻¹ g liver⁻¹) was similarly decreased by LPS alone (0.62 ± 0.04 ml min⁻¹ g⁻¹; P = 0.002 vs. control) or combined with POF (0.66 ± 0.06 ml min⁻¹ g⁻¹). A kinetic analysis indicated that LPS reduced both uptake and excretion processes in a balanced manner, so that intrahepatic ICG content was unaffected or even slightly reduced, as confirmed by measurement of ICG contents in the perfused livers. In livers from parallel-treated nonperfused rats, mRNA for the organic anion transporting protein-1 (Oatp1, which is likely to mediate ICG uptake) was unaffected by LPS, whereas the concentration of Oatp1 protein was reduced. Thus LPS induced an acute-phase response that included downregulation of ICG uptake by reduction of Oatp1 protein concentration, possibly at a posttranscriptional level. TNF-α appears not to be the mediator because POF did not modify these LPS effects.

Oatp1; cytokines; acute-phase response; endotoxin; tumor necrosis factor-α; lipopolysaccharide

Although jaundice in septic patients is common and associated with a poor prognosis (7), the mechanism is still under investigation. The circulating bilirubin in septic patients is predominantly in the relatively non-toxic conjugated forms (29). This suggests that hyperbilirubinemia in sepsis results from altered transport of conjugated bilirubin. Hepatic transport of conjugated bilirubin includes uptake, transcellular transport, and biliary excretion. Roelofsen et al. (35) observed that both hepatic uptake and biliary excretion of conjugated bilirubin were reduced in rats 18 h after 1 mg/kg body wt iv of lipopolysaccharide (LPS) (35). Similarly, Bolder and co-workers (2) found reduced transport of sulfobromophthalein (BSP) in both basolateral and canalicular membrane vesicles from rat livers 12 h after intraperitoneal injection of LPS at 3 mg/kg body wt. They speculated that primary reduction of canalicular transport leads to accumulation of toxic compounds in the liver and that downregulation of sinusoidal uptake may be a secondary event (2) protecting against liver damage during sepsis. This clinically important hypothesis requires further investigation.

In the present study we examined the effects of LPS administration to rats on hepatic transport of indocyanine green (ICG). ICG is removed exclusively by the liver and shares transport pathways with both unconjugated (9, 38) and conjugated bilirubin (30, 43). Because ICG is excreted into the bile in unmetabolized form, it is ideal for the study of hepatic transport phenomena. A kinetic model was used to quantify the effects of LPS on uptake, excretion, and accumulation of ICG by the perfused rat liver. The organic anion transporting protein-1 (Oatp1) is a probable mediator of ICG uptake (16). Downregulation of Oatp1 has been demonstrated after bile duct ligation (6) and after 5 days of ethinyl estradiol treatment (40). To further examine the role of Oatp1 in LPS-mediated regulation of ICG transport, we also measured the expression of Oatp1 mRNA and protein. In addition, because the changes in transport after LPS injection may be a part of the hepatic acute-phase response (20), hepatic uptake of alanine and expression of mRNA for specific acute-phase proteins were assessed.

The cholestatic responses to LPS are likely to be mediated by cytokines. Concentrations of interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) in blood are temporarily increased (3) after LPS administration, and these cytokines have all been related to the hepatic acute-phase response (20). TNF-α has been advocated as a major mediator of septic cholestasis (56), and injection of a large dose of TNF-α was followed by an altered plasma disappearance curve of ICG after bolus injection (51). For these reasons we hypothesized that TNF-α mediates the LPS-induced downregulation of organic anion transport. This hypothesis was examined in the present study by use of pentoxifylline (POF), a methylxanthine that inhibits the increase in plasma TNF-α concentrations after LPS without affecting the levels of IL-6 (3).

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MATERIALS AND METHODS

Materials

Female Wistar rats (Moellegaard Breeding Centre, L1. Skensved, Denmark; 65–70 days old; 180–220 g) were fed ad libitum and had free access to water. ICG was from Paesel and Lohr (Frankfurt am Main, Germany). LPS from Escherichia coli (0111:B4) (catalogue no. L4130) and taurocholate (no. T0750) were obtained from Sigma (St. Louis, MO). POF was kindly provided by Hoechst (Rødovre, Denmark). Pentobarbital sodium (thiomebumal sodium), heparin, and glucose were from Syneusapotekene. Human albumin was from Statens Seruminstitut (Copenhagen, Denmark). Outdated human erythrocytes were from the local blood bank. Buffer salts were from Bie and Berntsen (Roedovre, Denmark). Haemaccel was from Behringwerke (Marburg, Germany). Hypnorm [fentanyl citrate (0.315 mg/ml) and fluanisone (10 mg/ml)] was from JanssenPharma (Birkedø, Denmark). Midazolam was from Roche (Basel, Switzerland). Alanine ([L-alanine no. 1007] was from Merck (Darmstadt, Germany).

Study Design

Perfused livers. Rats were pretreated 18 h before examination of ICG transport and alanine uptake during once-through liver perfusion. There were three pretreatment groups: control, LPS alone, and LPS with POF (LPS + POF) (Table 1). After the perfusions, livers were frozen for later analysis of ICG content, mRNA for specific proteins, and concentration of Oatp1 protein.

Nonperfused livers. Rats were treated according to the same protocols as above. Eighteen hours later they were killed, and the livers were removed and frozen for later measurement of specific mRNAs and Oatp1 protein concentration.

Pretreatment

The pretreatment schedules are outlined in Table 1. The dose of LPS corresponds to 1 mg/kg body wt, which was enough to produce maximum TNF-α response (3, 50) and maximum inhibition of excretion of dinitrophenylglutathione (34) and bilirubin ditaurocholate (35) in rats. Eighteen hours was based on studies that found the maximum LPS effect on transport after 12–24 h (34, 56) and on mRNA after 16 h (10, 45), respectively. The POF dosage was from a titration study (3) in which POF reduced TNF-α release after 2 mg/kg LPS by 80% without effects on IL-6.

Table 1. Pretreatment of the 3 groups on day before liver perfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Intra peritoneal (t = 0 min)</th>
<th>Intravenous (t = 15 min)</th>
<th>Intra peritoneal (t = 135 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.3 ml 0.9%</td>
<td>0.5 ml 0.9%</td>
<td>0.3 ml 0.9%</td>
</tr>
<tr>
<td>LPS</td>
<td>NaCl</td>
<td>NaCl</td>
<td>NaCl</td>
</tr>
<tr>
<td>LPS + POF</td>
<td>0.3 ml POF (20 mg/ml)</td>
<td>0.5 ml PFS (0.5 mg/ml)</td>
<td>0.3 ml POF (20 mg/ml)</td>
</tr>
</tbody>
</table>

LPS, lipopolysaccharide; POF, pentoxifylline.

plastic bags containing citrate, phosphate, dextrose, and adrenaline (CPD-adrenaline, F-78310, Baxter) and kept in a 37°C water bath for 1 h. Following centrifugation, the buffy coat was removed and the erythrocytes were washed two to three times in Ringer lactate buffer containing 20,000 IU heparin/l and 5 g mannitol/l. The washed erythrocytes were stored at 4°C overnight in Ringer lactate. On the day of the perfusion, the suspension was centrifuged, passed through a leukocyte filter (RC-50, Pall Biomedical), and used in the preparation of the perfusates. Perfusion 0 was prepared by gently mixing 1,099 ml of packed rejuvenated human erythrocytes (hematocrit ~0.70) with 2,400 ml Krebs-Henseleit buffer containing 102 g bovine albumin, 12 ml 50% glucose, and 164 mg taurocholate. The final concentrations of albumin (560 µmol/l), glucose (12 µmol/l), and taurocholate (112 µmol/l) are close to physiological values in the rat (8). Perfusion 1 consisted of perfusate 0 to which 0.5 µmol/l ICG and 1 µmol/l alanine were added. The perfusate hemoglobin concentrations (mean ± SD, 5.20 ± 0.14 mmol/l) and hematocrits (0.22 ± 0.01 vol/vol) were not statistically significantly different among treatment groups (ANOVA).

Rat liver perfusion. On the day of the perfusion, the rats were anesthetized with 22.5 mg pentobarbital sodium intraperitoneally, and the livers were prepared for perfusion as previously described in detail (26). Livers were perfused single pass at a fixed perfusion rate, aiming at 2 ml·g ·min·1. Initially, the livers were perfused with perfusate 0 without ICG or alanine for 35 min. After this equilibration period, perfusate 1 with 0.5 µmol/l ICG and 1 mmol/l alanine was used for 36 min. Perfusion pressure, liver temperature (aim: 37°C), and perfusate pH (7.40) were kept stable during the experiment, and perfusion pressure was constantly monitored. Experiments were only accepted if the bile flow was >1 µl·g ·min·1, oxygen consumption was >2 nmol·min·1·g·1 in the experimental period, and perfusion pressure was <12 mmHg. To avoid influence from sex differences (42), only female rats were used.

From the start of perfusion with perfusate 1, samples of perfusate from the inflow and the outflow as well as from bile were collected every 4 min for a total of 36 min. After the perfusions the livers were removed, weighed, and then frozen at −18°C for further analyses of ICG content, Oatp1 protein, specific mRNA (see below), and liver dry weight. In this series, six animals were included in each treatment group.

Series of Nonperfused Livers

Thirty-six female Wistar rats were divided into three groups and treated as described above (Table 1). Four animals in the LPS + POF group died after the second POF injection. The following day the remaining rats (12 controls, 12 LPS, and 8 LPS + POF) appeared healthy. They were killed by cervical dislocation. The livers were quickly removed, frozen in liquid nitrogen, and stored at −80°C except for the shipping from Copenhagen to New York on dry ice. Liver samples from two additional animals in each treatment group were cut into slices of 0.5-cm thickness and kept in formaldehyde for examination by light microscopy.

Analyses

ICG concentrations in the perfusate, liver tissue, and bile were measured by high-performance liquid chromatography as previously described (24). Alanine concentrations in the perfusate were measured enzymatically (11). Dry weight (g/g liver) was determined as weight after freeze-drying divided by weight before freeze-drying of a 1- to 2-g sample.

mRNAs for specific proteins were measured in 200 mg of frozen liver tissue after RNA extraction using Promega kit.
ZS110, based on the thiocyanate method, modified by ethanol extraction as previously described (47). Northern blots were prepared using the wick method (4). Slot blots were prepared by adding 50 µl of 10× standard saline citrate (SSC) in each spot in a Schleicher & Schuell Minifold followed by a 5-µg RNA sample. The following cDNA probes were used: Oatp1 (12), α1-acid glycoprotein (32), β-fibrinogen (5), albumin (36), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (46). The probes were labeled using Amersham multiprime kit (RPN 1601 Z and isolated by a QiAquick nucleotide removal kit (Qiagen). Northern and slot blots were performed as previously described (47). Autoradiography was performed on an imaging plate BASIII under lead shield, and the signal was analyzed in a FUJIX bioimaging analyzer system BAS 2000 (Fuji Photo Film). Filters were washed and rehybridized with rat 18S rRNA, and readings for specific mRNAs were adjusted according to the 18S reading. Because the amount of 18S rRNA is constant, we find this way of standardization most reliable under conditions in which the pattern of mRNA changes dramatically. However, one of the housekeeping enzymes (GAPDH) was measured for comparison. 

Liver tissue was extracted with 0.1 M Na₂CO₃ as previously described (1). In brief, ~0.3 g of frozen tissue was weighed, crushed, and homogenized by 25 loose Dounce strokes in 10 ml/g of 1 mM NaH₂CO₃ containing the following protease inhibitors (Sigma): N-α-benzoyl-L-arginine methyl ester (10 mg/ml), TAME (10 mg/ml), soybean trypsin inhibitor (STI) (10 mg/ml), leupeptin (1 mg/ml), phenylmethylsulfonyl fluoride (0.2 M), EDTA (100 mM), and aprotinin (2 mg/ml). The homogenate was filtered through one layer of cheesecloth, and the volume was brought to 35 ml by addition of 0.1 M Na₂CO₃ containing protease inhibitors. The solution was rotated at 4°C for 30 min and was then centrifuged at 100,000 g for 1 h at 4°C. The supernatant was removed, and the pellet was washed twice with ice-cold PBS. The resulting pellet was resuspended in 600 µl of ice-cold PBS, divided into appropriate aliquots, and frozen at −70°C until used. Protein concentration was determined by the method of Lowry (18) using bovine albumin as standard. Following 10% SDS-PAGE of 20 µg of protein, immunoblot analysis by chemiluminescence (Renaissance kit, DuPont) was performed in duplicate using a rabbit antibody that was prepared against a 13-amino acid peptide corresponding to the derived Oatp1 sequence near its COOH terminus (1). A standard rat liver extract was included in duplicate on each immunoblot and was used as an internal standard. Intensity of immunoreactive Oatp1 was quantified after laser scanning densitometry on a Pharmacia UltraScan XL densitometer.

Calculations

The oxygen consumption (µmol·g liver⁻¹·min⁻¹) was calculated as perfusate hemoglobin concentration (µmol/ml)·(SO₂,in – SO₂,out)·F (ml·min⁻¹·g liver⁻¹), where SO₂,in and SO₂,out are the perfusate inlet and outlet hemoglobin oxygen saturations, respectively, and F is the perfusate flow per gram liver. Bile flow (µl·g liver⁻¹·min⁻¹) was measured gravimetrically, assuming a specific density of 1.00. Hepatic extraction fraction (E) of ICG or alanine was calculated as 1 – outlet concentration/inlet concentration. Intrinsic clearance (ml·min⁻¹·g liver⁻¹) was calculated as –F × (1 – Hct) × ln(1 – E), where Hct is the perfusate hematocrit (15). The intrinsic clearance is a flow-independent measure of the intrinsic ability of the liver to take up a substance from the blood (15).

Fitting Procedures

A kinetic model was fit to the data to elucidate which transport step was primarily affected by LPS. This procedure is described in APPENDIX.

Statistical Evaluation

Results are reported as means ± SE or as stated. One-way ANOVA was used for statistical evaluation. Whenever this test was significant (P < 0.05), group differences were looked for with Tukey's method. A computer program (Statgraphics 6.1, Manugistics) was used for these procedures.

RESULTS

Perfused Livers

All rats survived the pretreatment in this series (n = 6 control, 6 LPS, and 6 LPS + POF livers). For the first hours after injection of LPS or LPS + POF, rats appeared lethargic and showed piloerection. On the day of perfusion they appeared normal. The body weights were not statistically different in the three groups (control: 206 ± 5 g, LPS: 201 ± 7 g, and LPS + POF: 202 ± 5 g). Details of the physiological parameters in these groups are shown in Table 2. Compared with controls, the treatment groups had slightly higher perfusion pressures and slightly lower perfusion flow rates per gram liver (Table 2). Because postperfusion liver weights were slightly higher in the LPS-treated groups (Table 2), the total perfusion rates were similar in the three groups (data not shown). Bile flow rates and oxygen consumption did not differ (Table 2). The major observations regarding ICG transport and alanine uptake are given in Table 3.

ICG uptake and excretion. The extraction fraction of ICG was equally reduced in LPS and LPS + POF groups compared with the control group (Fig. 1A). The mean intrinsic clearance of ICG was clearly reduced by

Table 2. Physiological control parameters in perfused livers

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS</th>
<th>LPS + POF</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat weight on day of perfusion, g</td>
<td>206.67 ± 4.46</td>
<td>200.83 ± 7.05</td>
<td>202.17 ± 5.04</td>
<td>0.20</td>
</tr>
<tr>
<td>Perfusion pressure, mmHg</td>
<td>6.49 ± 0.37</td>
<td>7.61 ± 0.67</td>
<td>8.09 ± 1.66</td>
<td>0.051</td>
</tr>
<tr>
<td>Oxygen consumption, µmol·g liver⁻¹·min⁻¹</td>
<td>3.82 ± 0.10</td>
<td>3.60 ± 0.11</td>
<td>3.58 ± 0.14</td>
<td>0.32</td>
</tr>
<tr>
<td>Bile flow, µl·g liver⁻¹·min⁻¹</td>
<td>7.23 ± 0.34</td>
<td>8.58 ± 0.56</td>
<td>7.79 ± 0.24</td>
<td>0.0001</td>
</tr>
<tr>
<td>Liver dry weight, g/g</td>
<td>0.31 ± 0.007</td>
<td>0.29 ± 0.004</td>
<td>0.30 ± 0.007</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 livers/group. If P < 0.05 by 1-way ANOVA, Tukey's method was used to look for heterogeneity between control and LPS groups (*), between control and LPS + POF group (†), or between LPS and LPS + POF group (‡).
LPS with or without POF (Table 3). The biliary excretion rate of ICG was always larger in the control group than in the LPS group and the LPS + POF group (Fig. 1B). The cumulative biliary excretion of ICG was more than twice as high in the control group than it was in LPS or LPS + POF groups (Table 3, P = 0.001). The liver content of ICG after 36 min of perfusion was slightly lower in the LPS group, but this difference was not statistically significant (Table 3).

Analysis according to the kinetic model. In both treatment groups the rate constants for influx from the sinusoidal lumen into the hepatocyte (i.e., from perfusate to liver) (k_{in}) and for excretion of ICG from hepatocyte to bile (k_{ex}) were lower than in the controls (Table 3). As a test of the kinetic model, the measured liver contents of ICG at the end of the experiments were compared with those predicted from the model and the fitted parameters. Reasonable agreements were observed in all groups (Table 3).

Alanine uptake. The hepatic extraction fraction of alanine decreased more dramatically with time than that of ICG (compare Fig. 1, A and C), so intrinsic clearance was highly time dependent. Instead, we report in Table 3 the average hepatic extraction fraction, which is proportional to the area under the curves in Fig. 1C. The average hepatic extraction fraction of alanine was statistically significantly larger in the LPS group than in the control group. In contrast to ICG observations, the alanine uptake in the LPS + POF group showed only intermediate LPS effect (Fig. 1C; Table 3). This suggests that TNF-α was at least partly responsible for the upregulation of alanine uptake after LPS.

Oatp1 mRNA and protein. With values from control rats normalized to 100 ± 13.7, Oatp1 mRNA in the perfused livers (slot blot) was 83.5 ± 12.8 in LPS and 60.6 ± 4.1 in the LPS + POF group (Fig. 2, P = 0.045 by ANOVA). Further evaluation with Tukey’s test pointed out only the LPS + POF group as significantly different from the control group (Fig. 2). Oatp1 protein was reduced in the treatment groups in parallel with the intrinsic clearance of ICG (Fig. 2). This was not significant in the perfused livers, but because of the large variation there is a risk of a type-2 error. In the nonperfused livers the same differences were found, and they were statistically significant (see Nonperfused Livers).

mRNA for other proteins. As described in detail in MATERIALS AND METHODS, mRNA was normalized to 18S rRNA. mRNA for acute-phase proteins such as acid-α-glucoprotein [a type I acute-phase protein (20)] and β-fibrinogen (type II) were upregulated whereas mRNA for albumin [a “negative acute-phase protein” (20)] was downregulated. In contrast, mRNA for the housekeeping enzyme GAPDH was unaffected (Fig. 2).

Nonperfused Livers

Hepatic Oatp1 mRNA and protein are shown in Fig. 2 (n = 12 control, 12 LPS, and 8 LPS + POF livers). Changes in Oatp1 mRNA (slot blot) were of marginal significance only in the LPS + POF group. In contrast, a statistically significant reduction of Oatp1 protein was found in both LPS and LPS + POF groups compared with controls. Results for the other proteins studied were similar to results in the perfused livers (Fig. 2). Hepatic content of mRNA for Oatp1 as presented in Fig. 2 was quantified by slot blot as described in MATERIALS AND METHODS. Validity of slot blot quantification was confirmed by comparison with Northern blot in a subgroup of the nonperfused livers (n = 6 in each group). Under high-stringency conditions, two major bands of 3.3 and 4.3 kb were found (Fig. 3) as reported previously (6, 12). Because the concentration of the two bands varied linearly (P < 0.0001, r^2 = 0.91) with each other, the relative changes observed by slot blot should correctly reflect the changes of these bands. In accordance with these results, the results of the Northern blots (the 3.3-kb band) were in good agreement with results of the slot blots from the same animals (Fig. 4). The same was true with the 4.3-kb band (not shown). Light microscopy showed only minor changes in LPS livers with a light edema of the portal area and central venous endothelium with few adjacent lymphocytes. LPS + POF and control livers were histologically normal.

Table 3. Perfused livers

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS</th>
<th>LPS + POF</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrinsic clearance of ICG, ml·g liver⁻¹·min⁻¹</td>
<td>1.01 ± 0.05†</td>
<td>0.62 ± 0.04</td>
<td>0.66 ± 0.06</td>
<td>0.002</td>
</tr>
<tr>
<td>Accumulated bile excretion of ICG, nmol/g liver</td>
<td>3.40 ± 0.38†</td>
<td>1.44 ± 0.16</td>
<td>1.64 ± 0.22</td>
<td>0.001</td>
</tr>
<tr>
<td>Postperfusion ICG content, nmol/g liver</td>
<td>7.30 ± 0.69</td>
<td>5.92 ± 0.34</td>
<td>6.23 ± 0.33</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Analysis by kinetic model

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS</th>
<th>LPS + POF</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>k_{in}, min⁻¹</td>
<td>3.21 ± 0.27†</td>
<td>2.04 ± 0.22</td>
<td>2.34 ± 0.47</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>k_{ex}, min⁻¹</td>
<td>0.0049 ± 0.0048</td>
<td>0.0020 ± 0.0041</td>
<td>0.0060 ± 0.0039</td>
<td>0.30</td>
</tr>
<tr>
<td>ICG content predicted by model, nmol/g liver</td>
<td>8.27 ± 1.41</td>
<td>6.27 ± 0.86</td>
<td>5.93 ± 1.10</td>
<td></td>
</tr>
</tbody>
</table>

Mean hepatic extraction fraction of alanine | 0.138 ± 0.012* | 0.196 ± 0.015 | 0.166 ± 0.014 | 0.038   |

Values are means ± SD. For each group, n = 6. If P < 0.05 by 1-way ANOVA, Tukey’s method was used to look for heterogeneity between control and LPS groups (*) or between control and LPS + POF group (†).
DISCUSSION

In the present study LPS pretreatment decreased hepatic transport of ICG in the perfused rat liver. A kinetic analysis suggested that both the hepatic uptake and biliary excretion of ICG were reduced. LPS pretreatment reduced the concentration of Oatp1 protein but not Oatp1 mRNA. In contrast to ICG, hepatic alanine uptake was enhanced by LPS, indicating an acute-phase catabolic response, and expression of acute-phase proteins was upregulated. Addition of POF, an inhibitor of TNF-α release that is normally seen 1–3 h after LPS treatment, did not modify the LPS effects on ICG transport.

Effects of LPS

Nonspecific or specific functional changes. LPS may produce important nonspecific effects. Thus LPS reportedly induces a 5–10% loss of body weight (45) and a small increase of liver weight (31) as in the present study (Table 2). Perfusion pressures were slightly higher in LPS livers (Table 2), as also observed by others (45). These observations could support the view that nonspecific changes induced by LPS, as, for example, disturbed microcirculation (21), lead to deterioration of liver function. However, the almost normal light microscopic appearances, the similar bile flow rates and oxygen consumptions, and the increased alanine uptake after LPS render this possibility unlikely. The latter observations strongly suggest that inhibition of hepatic ICG transport by LPS was a result of a specific downregulation of this particular liver function.

Bile flow rates. Bile flow rates were unaffected by LPS treatment (Table 2) in the presence of 112 µmol/l taurocholate in the perfusate. This is in accordance with the general observation (2, 33–35) that bile acid-dependent bile flow is unaffected whereas bile acid-independent bile flow is reduced 12–24 h after LPS. The similar bile flow rates in the groups made interpretation of the data easier because the bile flow rate did not have to be included in the kinetic model.

Indications of a hepatic acute-phase response. LPS administration with or without POF resulted in increased expression of mRNA for a type I acute-phase protein (α1-acid glycoprotein) and a type II (β-fibrinogen) and reduced expression of a negative acute-phase protein (albumin). Alanine uptake increased as was also observed in septic catabolic patients (57). All together, these findings confirm that an acute-phase response was induced by LPS and further support the view that the livers were not structurally damaged by endotoxemia.

Kinetic observations. Both the uptake rate constant, kpl, and the rate constant for excretion, kbp, were reduced in the LPS and LPS + POF groups, whereas the backflux rate constant (i.e., flux from liver to perfusate), klp, did not change significantly (Table 3). The backflux rate constant was included in the compartmental model (Fig. 5) because it was the simplest way to account for the slight decline of hepatic extraction fraction of ICG with time (Fig. 1A). This particular assumption may not be uncontroversial because such backflux could not be demonstrated in intact pigs (23, 25). Thus backflux of ICG may be species dependent, or the slight decline of the hepatic extraction of ICG with time in the present study may have other causes. In one report (35) LPS pretreatment increased the permeability of the paracellular pathway, which could lead to increased backflux after LPS, but such an effect was not detectable in the present study. In summary, the LPS effect was primarily due to reduced sinusoidal uptake and biliary excretion.

Postperfusion hepatic contents of ICG. Postperfusion hepatic contents of ICG were not significantly different...
among the groups (Table 3), in agreement with the predictions based on the kinetic model (Table 3). In one study (2), transport of cholyltaurine, chenodeoxycholyltaurin, sulfolithocholyltaurine, and BSP was reduced 12 h after LPS in vesicles of both basolateral and apical hepatocyte membranes. It was speculated that because of the large sinusoidal surface some accumulation of toxic compounds could result after LPS pretreatment (2). This would also be expected from the usual assumption that the ATP-dependent canalicular transport step is rate limiting (13). In this case, downregulation of sinusoidal transporters could be a secondary phenomenon. Rather, our data indicate a balanced reduction of transport activities with no increase of intracellular ICG after LPS. A similar finding was reported in another study (35) in which LPS pretreatment reduced the liver content of unconjugated bilirubin, whereas the contents of conjugated species were unchanged during steady-state uptake of unconjugated bilirubin in the perfused rat liver. Thus we suggest that LPS induces balanced changes of transport steps that reduce transport without significant accumulation of hepatotoxic substances. This hypothesis is in accordance with the clinical observation that transaminases are generally only moderately elevated in patients with septic cholestasis.

Oatp1 mRNA and protein concentration. Sinusoidal uptake of ICG was assumed to be mediated by Oatp1, because ICG reduced BSP uptake by Oatp1-transfected oocytes to 10% of the control value (16). However, as with BSP (16, 58), the sinusoidal membrane may have more than one ICG transporter. Although Oatp1 mRNA was unchanged by LPS, the concentration of Oatp1 protein was almost halved (Fig. 2). This picture was similar in the two series (Fig. 2), although the result of the statistical evaluation was slightly different because of higher variability in the perfused series. This variability could be expected because of the smaller sample size and less-optimal storage conditions in the series of perfused livers (2°C vs. 8°C for the nonperfused livers). Our findings should be compared with previous studies of other models of cholestasis (6, 40). After bile duct ligation Oatp1 mRNA was dramatically reduced (to 20% of control on day 1 and 51% at day 3), whereas the reduction of Oatp1 protein was slower (to 90% day 1, 43% day 2) (6). These observations (6) suggest that the half-life of Oatp1 protein is 2 days. During daily administration of ethynylestradiol, mRNA for Oatp1 was reduced to 28% of control after 12 h and then

Figure 2. Main results of studies. Intrinsic clearance (Cl-int) of ICG was estimated from perfusate flow, hematocrit (Hct), and hepatic extraction ratio as explained in MATERIALS AND METHODS. In both perfused and nonperfused livers, Western blot was used to measure concentration of organic anion transporting protein-1 (Oatp1 protein). Slot blot was used to measure hepatic mRNA for Oatp1, albumin (ALB), α1-acid glycoprotein (α1AG), β-fibrinogen (β-fib), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). ns, Not significant. For treatment details, see Table 1.

Figure 3. Representative Northern blots of Oatp1 mRNA from nonperfused livers. Two bands were seen, corresponding to the 4.3- and 3.3-kb bands described by others (6, 12). L, LPS; P, LPS + POF; C, control.

Figure 4. Comparison of mRNA measurements by Northern and slot blot in 6 livers from each treatment group in nonperfused series. Treatment groups are as in Fig. 1.
further decreased to 10% at day 5. Also in this study, Oatp1 protein concentration decreased more slowly than mRNA, to 54% of control at day 1 and further to 38% at day 5. The reduction to 54% at day 1 was faster than could be predicted from the bile duct ligation studies, suggesting enhanced degradation of Oatp1 protein during ethynylestradiol administration. In our study Oatp1 protein was reduced to 50% after 18 h even though mRNA for Oatp1 was unchanged (Fig. 3). Our study cannot exclude that mRNA for Oatp1 was reduced at some time between the pretreatment and the measurement, but even then the observed downregulation of Oatp1 protein is difficult to explain without assuming that the degradation of Oatp1 protein increased after LPS. A similar mechanism has been proposed for downregulation of Ntcp protein after a larger dose (15 mg/kg) of LPS (19), but in this case downregulation of mRNA was also observed.

LPS also reduced the canalicular excretion of ICG (rate constant \( k_0 \)) (Table 3). The canalicular transport protein of ICG is not known for certain. Biliary excretion of ICG is reduced but not absent in TR protein of ICG is not known for certain. Biliary excretion of ICG is reduced within 1–2 h after a sublethal dose of LPS and are normalized within the following 2 h (3). Among other effects, cytokines alter expression of mRNA for acute-phase proteins and albumin (20) as was observed in the present study. Three reports suggested that TNF-\( \alpha \) mediated a reduction of systemic ICG clearance shortly after hemorrhage (51, 52, 54). Also, in a trial of TNF-\( \alpha \) as an anticancer drug, a high incidence of cholestatic side effects was observed (39). Based on these reports and experiments in perfused rat liver and hepatocytes (10, 56), TNF-\( \alpha \) was strongly advocated as the mediator of the cholestasis during sepsis (56) and after a LPS challenge (10, 56). To examine this hypothesis, the LPS + POF group was included.

TNF-\( \alpha \) concentrations were not measured in our study. That would have required a number of blood samples over a 1- to 2-h period after the pretreatment, and, as blood loss itself may affect ICG transport (54, 55), this could blunt the LPS effect. The dosages used did effectively reduce TNF-\( \alpha \) in similar studies (3, 17, 54). TNF-\( \alpha \) is known to upregulate hepatic uptake of alanine (19, 27, 28) and other amino acids (27, 28). In accordance with those findings, the alanine uptake was in fact less upregulated in the LPS + POF group than in the LPS group (Fig. 1C). TNF-\( \alpha \) mediates the LPS-induced increase in liver weight (31). Again, the increase in liver weight was less pronounced in the LPS + POF group than in the LPS group (Table 2). Thus, in the present study, administration of POF most likely reduced TNF-\( \alpha \).

POF did not modify the LPS effect on ICG transport (Table 3, Fig. 1, A and B). The expression of mRNA for Oatp1 was not affected by LPS, whereas LPS + POF actually reduced Oatp1 mRNA in both perfused and nonperfused livers (Fig. 2), and POF seemed to enhance the LPS-induced reduction of Oatp1 protein in the nonperfusion series (Fig. 2). As these possible effects of POF were in the opposite direction of our prediction, the data do not support the hypothesis that TNF-\( \alpha \) mediated the downregulation of ICG transport 18 h after LPS injection.

In apparent conflict with this conclusion, ICG transport was reported to be reduced 1 h after intravenous injection of \( 3 \times 10^6 \) U/kg body wt of recombinant murine TNF-\( \alpha \) (51). Important methodological differences may account for this. First, plasma concentrations of TNF-\( \alpha \) may differ from our study, and a lower dose (6 \( \times \) \( 10^5 \) U/kg body wt) had no effect on ICG transport (51). Second, the conclusions of Wang et al. (51) were based on the systemic plasma concentration decay curves of ICG after bolus injection without hepatic vein concentrations, and this method cannot separate effects of flow and of hepatocellular function. According to that analysis (51) both maximal velocity (\( V_{\text{max}} \)) and the Michaelis-Menten constant (\( K_m \)) for ICG transport were similarly reduced. In such cases the first-order intrinsic clearance (equal to \( V_{\text{max}}/K_m \)) is not changed, a fact that was overlooked (51). In addition, ICG was measured spectrophotometrically, a procedure that may bias kinetic studies because it is not as specific as HPLC (24). Third, these short-term (1 h) observations (51) may not be directly comparable to
ours (~15 h after the TNF-α peak). As has been pointed out by others (33), the observation time may be very important for the comparison of studies of the effect of LPS on transport. One to four hours after LPS injection, bile flow is reduced (both bile acid-dependent and bile acid-independent flow) (49, 56) as is transport of BSP (48, 49), dinitrophenylglutathione (34), taurocholate (56), and ICG (53). In this period there is a dramatic efflux of glutathione with a reduction of intracellular content (34). TNF-α seems important for this short-term response as bile flow and Na-dependent taurocholate transport can be protected by pretreatment with anti-TNF-α antibodies (56). Twelve to twenty-four hours later, plasma cytokines are again normal (3), and the hepatic glutathione storage is recovered (34). Bile acid-independent bile flow is still reduced, while bile acid-dependent bile flow is restored (2, 34, 35). At that time there is still a reduction of Mrp2 mRNA, protein, and function (44); Ntcp mRNA, protein, and function (2, 10, 19, 56); uptake of unconjugated bilirubin and ditauro-bilirubin (35); ATP-dependent bile acid excretion (2, 19); and Oatp1 function (Ref. 2 and present study). The importance of TNF-α for these late (18–24 h after LPS) responses has not been extensively investigated. Inasmuch as POF did not affect ICG transport in the present study, it may be that TNF-α only mediates the less specific acute effects whereas other substances mediate the later effects of LPS.

In conclusion, LPS induced a typical acute-phase change in hepatic mRNA for certain proteins, and the enhanced alanine uptake indicated a hepatic shift toward catabolic functions. LPS induced a significant reduction in both ICG uptake and excretion in a balanced way so that the hepatic ICG content was not affected. Although mRNA for the candidate transport protein for sinusoidal ICG uptake, Oatp1, was unchanged, the Oatp1 protein concentration was reduced, whether because of decreased posttranscriptional processing or accelerated degradation. The reduction of ICG biliary excretion could in part be due to reduced Mrp2 protein expression, but this protein is not the only canicular transporter for ICG. While the observed LPS effect is probably mediated by cytokines, TNF-α appears not to be involved because POF did not change the response to LPS.

APPENDIX

The model illustrated in Fig. 5 was used to analyze kinetic data. The perfusate plasma flow with a rate F carries ICG into the liver with a constant concentration, C i. The influx (nmol·g liver·1·min−1) from liver to perfusate is assumed to be proportional to C i and equal to C i · V · k pi, where V is the sinusoidal volume. The hepatocellular content of ICG at time t is L(t). The backflux of ICG from liver cell to perfusate is equal to L(t) · k b, and the rate of irreversible excretion of ICG from liver cell to bile [b(t)] is equal to L(t) · k b. Thus
dL(t) = [C i · V · k pi − L(t) · (k pi + k b)] · dt ⇒ L(t) = C i · V · k pi − L(t) · k b − L(t)

where L(t) can be substituted from Eq. 1 into Eqs. 2 or 3. The measured values for F, C i, hepatic ICG concentration [C i(t)], and biliary excretion rate [b(t)] were used in the fitting procedures. In the absence of a directly measured value, V was taken from multiple indicator dilution studies (reviewed in Ref. 22) to be 0.25 ml/g liver in all groups. A computer program (SAAM II, version 1.0, SAAM Institute, University of Washington) was used to estimate k pi, k b, and k b by fitting Eqs. 2 and 3 simultaneously to the data. Each experiment was fitted individually, and means ± SD of the rate constants in each treatment group were reported.

To reach the final formulation of Eq. 1, we used L(t = ∞) = C i · V · k pi / (k pi + k b) derived by setting dL(t = ∞)/dt = 0. The kinetic model and Eq. 1 imply that

\[ b(t) = L(t) \cdot k_b \] (2)

and

\[ C_i(t) = C_i - C_i \cdot V \cdot k_{pl} / (k_{pi} + k_b) \cdot L(t) \]

\[ F \] (3)

REFERENCES


