Endotoxin-induced reduction in biliary indocyanine green excretion rate in a chronically catheterized rat model

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Beno, David W. A., Michael R. Uthing, Masakatsu Goto, Yong Chen, Vanida A. JiymAPA-Serna, and Robert E. Kimura. Endotoxin-induced reduction in biliary indocyanine green excretion rate in a chronically catheterized rat model. Am J Physiol Gastrointest Liver Physiol 280: G858–G865, 2001.—Using a nonstressed chronically catheterized rat model in which the common bile duct was cannulated, we studied endotoxin-induced alterations in hepatic function by measuring changes in the maximal steady-state biliary excretion rate of the anionic dye indocyanine green (ICG). Biliary excretion of ICG was calculated from direct measurements of biliary ICG concentrations and the bile flow rate during a continuous vascular infusion of ICG. Despite significant elevations in mean peak serum tumor necrosis factor-α (TNF-α) concentrations (90.9 ± 16.2 ng/ml), there was no effect on mean rates of bile flow or biliary ICG clearance after administration of 100 μg/kg endotoxin at 6 or 24 h. Significant differences from mean baseline rates of bile flow and biliary ICG excretion did occur after administration of 1,000 μg/kg endotoxin (mean peak TNF-α 129.6 ± 24.4 ng/ml). Furthermore, when rats were treated with up to 16 μg/kg of recombinant TNF-α, there was no change in mean rates of bile flow or ICG biliary clearance compared with baseline values. These data suggest that the complex regulation of biliary excretion is not mediated solely by TNF-α.

TUMOR NECROSIS FACTOR-α (TNF-α) is believed to be the primary mediator of endotoxin-induced hepatocellular dysfunction. This dysfunction results in decreased bile formation, a condition known as cholestasis. In our previous studies (1) with a chronically vascularity catheterized rat model, we demonstrated that the endotoxin-induced TNF-α response is dramatically attenuated by the surgical and nonsurgical stress associated with experimental protocols. However, investigators who have proposed the mediator role for TNF-α conducted endotoxin-induced hepatic dysfunction studies with animal models in which experimental stress was not controlled (5, 29, 31). We hypothesized that the experimental models of these investigators may have induced a stress response in animals, thereby attenuating the TNF-α response and underestimating the extent of endotoxin-induced liver injury.

To test this hypothesis, we modified our previously reported (3, 27) vascularly catheterized rat model to include a chronic bile catheter. This model features several advantages for studying endotoxin-induced hepatic dysfunction. First, our experiments generate an unattenuated endotoxin-induced TNF-α response because they are performed without the adverse effects of protocol-related stress (1). Second, normal biliary physiology is maintained because bile flow from the liver to the intestine is unimpeded and, therefore, enterohepatic circulation of bile acids remains intact, permitting serial measurements of bile flow (4, 20). Finally, our model minimizes interanimal variability because serial experiments can be performed on the same animal (1, 27).

A series of preliminary studies established the accuracy of our measurements of rates of biliary indocyanine green (ICG) excretion and bile flow under physiological research conditions. We selected these indices because previous research suggested that alterations in ICG clearance from the blood and excretion into the bile are early sensitive indicators of sepsis- and endotoxin-induced hepatic dysfunction (26, 30). The purpose of this study was to determine the effect of endotoxin and TNF-α on hepatic function in vivo as measured by alterations in rates of biliary ICG excretion and bile flow using the described model.

MATERIALS AND METHODS

Reagents

Endotoxin (Escherichia coli 0127:B8; Sigma Chemical, St. Louis, MO) was prepared in sterile saline. ICG was obtained from Akorn Industries (Decatur, IL). Murine recombinant (r)TNF-α was obtained from Genentech (San Francisco, CA).

Animals

A total of 72 adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 325–350 g...
served as subjects in this study. Rats were housed singly in standard cages and were fed chow and water ad libitum. The environment was temperature and humidity controlled, with lights on and off at 0630 and 1630, respectively. The Institutional Animal Care and Use Committee of Rush University approved all procedures.

Operative Procedures

Operative procedures were performed as previously described (1). Briefly, the animals were anesthetized with 60 mg/kg ketamine and 5 mg/kg xylazine intramuscularly. Catheters were placed in the aorta, inferior vena cava (IVC), and duodenum under sterile conditions. A bile catheter was made by inserting the 0.5-cm tip of a 24-gauge Insyte catheter (Becton Dickinson Vascular Access, Sandy, UT) into a 3-cm segment of Silastic tubing. A 0.5-cm segment of PE-60 tubing (Clay-Adams, Parsippany, NJ) was inserted into the other end of the Silastic tubing, and the entire catheter was placed over a 24-gauge Insyte needle. After placement of the IVC and aortic catheters, the tip of the 24-gauge Insyte needle was introduced in a retrograde fashion into the common bile duct ~2 cm distal to the liver. The bile catheter was then advanced over the Insyte needle 0.25 cm into the bile duct, the Insyte needle was removed, and the catheter was secured with cyanoacrylate glue. The distal PE-60 tubing was inserted into 3.5-in. intermittent infusion set tubing (no. 4871; Abbott Laboratories, North Chicago, IL) that had been passed from a cervical incision into the abdominal cavity as previously described for the aortic, IVC, and duodenal catheters (27). During this procedure, the bile duct was not ligated and bile flow was not obstructed. The abdominal cavity was closed with 4-0 silk suture. The infusion sets exiting the cervical incision were sutured securely to the back of the rat with 2-0 silk suture and were glued postoperatively with silicon to form a single unit. To maintain patency, all catheters were flushed daily.

Measurement of Maximum Biliary ICG Excretion

Bile was obtained through the bile catheter by inserting a 23-gauge infusion set needle (Abbott Laboratories), from which all but 0.5 in. of tubing had been removed, with ~24 in. of saline-filled PE-60 tubing attached. A sterile 1.5-ml sampling tube was attached to the tubing end, and bile was withdrawn by suspending the sampling tube below the cage using negative pressure. Bile was collected into tared sampling tubes at 5-min intervals over a 90-min period, during which ICG was infused into the IVC at 50 μg/min. ICG (1 ml/h) was delivered by a Razel Scientific (Stamford, CT) infusion pump with a 3-ml Becton Dickinson (Franklin Lakes, NJ) syringe.

Bile flow rate was calculated using the net sampling tube weight and assuming a bile density of 1 g/ml. A total of 10 μl of bile from each sample tube was aliquoted to determine ICG concentration. The remainder of the bile was infused into the duodenal catheter to maintain enterohepatic circulation of bile acids. Biliary ICG concentrations were measured spectrophotometrically at a wavelength of 805 nm. Biliary ICG excretion was calculated for each 5-min interval by multiplying the bile flow rate by the biliary ICG concentration.

Experimental Design

Experiments were performed at least 4 days postoperatively because our previous work (1) showed that the effects of surgical stress on the endotoxin-induced cytokine response are no longer present at this time. At this time, the animals had achieved at least 95% of their preoperative weight.

Effect of endotoxin on hepatic function. Rats were assigned to receive one of three doses of endotoxin infused into the IVC over 30 s: 0 (control, n = 7), 100 (n = 9), or 1,000 (n = 8) μg/kg. Rates of biliary ICG excretion and bile flow were measured at baseline (time 0) and then 6 and 24 h after endotoxin administration. Aortic blood samples (0.2 ml) were collected at 0 and 90 min for determination of corticosterone and TNF-α concentrations, respectively. After each blood draw, the animals were transfused with an equal volume of blood obtained immediately before transfusion from other untreated chronically catheterized rats that were previously designated as donors. Samples were aliquoted and stored at -80°C until analyzed. TNF-α was measured by ELISA (Genzyme, Cambridge, MA). Corticosterone was measured by RIA (ICN Biomedicals, Costa Mesa, CA). For a subgroup of eight rats, we performed these biliary measurements at four additional time points: 2, 4, 48, and 72 h after endotoxin infusion (0 Control, n = 2), 100 (n = 3), or 1,000 (n = 3) μg/kg.

Effect of rTNF-α on hepatic function. In a separate group of animals, we tested the speculation of previous researchers that TNF-α is the primary mediator of the endotoxin-induced inhibition of bile flow rate. For these experiments, we measured rates of biliary ICG excretion and bile flow after bolus infusion of 8 (n = 5) or 16 (n = 4) μg/kg rTNF-α. These rTNF-α doses were chosen to simulate (125 ng/ml) or to double (250 ng/ml) peak serum TNF-α concentrations observed in this study after infusion of 1,000 μg/kg endotoxin (1). TNF-α was not detectable in the serum after 120 min (half-life of 15.6 min; r² = 0.96) after infusion of 8 μg/kg rTNF-α. Rates of biliary ICG excretion and bile flow were measured immediately before rTNF-α administration (baseline) and again at 4.5 and 22.5 h after rTNF-α administration. Because endotoxin-induced peak TNF-α was observed at 1.5 h after endotoxin infusion, we measured hepatic function at 4.5 and 22.5 h after rTNF-α administration. These measures simulated the timing of endotoxin-induced TNF-α effects on hepatic functions at 6 and 24 h. Aortic blood samples (0.2 ml) were obtained immediately before and 1 min after rTNF-α administration for determination of corticosterone and TNF-α concentrations, respectively. After each blood draw, the animals were transfused with an equal volume of blood obtained immediately before transfusion from other untreated chronically catheterized rats that were previously designated as donors.

Preliminary Studies

Determination of maximal biliary ICG excretion rate... To establish a sensitive and reliable measure of hepatic dysfunction, we determined the maximal biliary ICG excretion rate under physiological conditions. This measure, which detected even minor changes in biliary ICG excretion, was critical to our subsequent primary studies. We measured the blood concentrations and biliary excretion rates of ICG during a constant infusion of ICG at 10, 20, 50, 100, and 200 μg/min for 90 min in eight rats that served as their own controls for the different doses on separate days. No residual ICG from our subsequent primary studies. We measured the blood concentrations and biliary excretion rates of ICG during a constant infusion of ICG at 10, 20, 50, 100, and 200 μg/min for 90 min in eight rats that served as their own controls for the different doses on separate days. No residual ICG from the previous day’s infusion was observed in the bile for any animal on any study day. Biliary ICG excretion was measured as described in Measurement of Maximum Biliary ICG Excretion. Aortic blood samples (0.25 ml) were collected every 10 min and analyzed for ICG concentration. After each blood draw, the animals were transfused with an equal volume of blood obtained immediately before transfusion from other untreated chronically catheterized rats that were previously designated as donors. The data revealed that maximum biliary ICG excretion rate occurred after ICG infusion rates of ≥50 μg/min (Fig. 1A). After an initial 30-min equilibration.
ICG is collected with a dose-dependent excretion effect. After bolus ICG infusion, demonstrating that 87–97% of the infused ICG was excreted into the bile. Since ICG is excreted exclusively into the bile, we determined the accuracy of our calculation for bile flow rate by bypassing the bile catheter, being secreted into the intestine. Because ICG is excreted exclusively into the bile, we determined the accuracy of our calculation for bile flow rate by comparing the amount of ICG infused into the IVC with that excreted into the bile.

We studied eight rats that served as their own controls for infusions of three doses of ICG (500, 750, and 1,000 μg) on three separate days. No residual ICG from the previous day’s infusion was observed in the bile of any animal for any study day. For all ICG doses, a 1-ml bolus was infused and ICG concentration in the bile was measured at 5-min intervals for 2.5 h after infusion. After a 180-min period of steady-state mean biliary ICG excretion between 30 and 90 min of ICG infusion compared with infusion of ICG at 50 μg/min (Fig. 1B). The bile flow rate was not affected by any of the tested ICG concentrations. The data from this preliminary study indicated that the maximal rate of ICG uptake and excretion from the liver into the bile occurred after ICG infusion of 50 μg/min, so this infusion rate was incorporated into the methodology for our primary study.

Accuracy of bile flow rate calculation. This set of experiments was designed to confirm that during bile sampling all bile in the common bile duct was collected and that no bile bypassed the bile catheter, being secreted into the intestine. Because ICG is excreted exclusively into the bile, we determined the accuracy of our calculation for bile flow rate by comparing the amount of ICG infused into the IVC with that excreted into the bile.

Effect of chronic biliary catheterization on hepatic pathology and bile flow. In 10 rats, we performed serial liver function measurements and histological examination of the liver and bile ducts at scheduled time points over a 28-day period after bile duct catheterization. No hepatic or biliary histological abnormalities were noted, and mean values for liver function enzymes including alanine aminotransferase, alkaline phosphatase, γ-glutamyltranspeptidase, and albumin were unchanged during this time (data not shown).

To ensure that measures of bile flow remained constant over the time that experiments were performed, we measured bile flow rate daily for 10 rats from 3 to 14 days after surgery. For this set of experiments, bile flow rate was measured at 5-min intervals for a total of 30 min. Our data revealed that mean bile flow rates were constant over this time period (Fig. 3). Of the original 10 rats in this set of experiments, we continued to measure the bile flow rate of 3 rats for an additional 10 days. Mean bile flow rate for these rats was constant for 24 days after surgery (data not included in Fig. 3).

Effect of endogenous bile replacement on bile flow. In this set of preliminary studies, we tested the hypothesis that previous investigators’ reports of reduced bile flow rates resulted from continuous bile sampling that depleted bile acid. Because our model permits replacement of collected bile and maintains enterohepatic bile circulation, we compared mean bile flow rate with and without the replacement of bile through a duodenal catheter (Fig. 4). Chronically catheterized rats were assigned randomly to control (without bile replacement; n = 5) or experimental (with bile replacement; n = 5) groups. For both groups of rats, bile flow rate was measured at 5-min intervals for 4 h and at 15- to 60-min intervals for an additional 4 h between 4 and 7 days after surgery. Data revealed a significantly lower mean bile flow rate for the control group (Fig. 4), beginning at 35 min after bile sampling had begun (P < 0.05). In contrast, bile flow rate remained constant for experimental animals, suggesting that replacement of bile into the duodenum permits serial bile collection (up to 4 h) without significantly altering bile flow rate.
**Statistical Analysis**

All results are expressed as means ± SE. For kinetic studies, differences were compared by one-way or two-way repeated-measures ANOVA with Bonferroni post hoc correction.

**RESULTS**

**Effect of Endotoxin and rTNF-α on Bile Flow Rate**

For rats who received only endotoxin, mean bile flow rates at 6 and 24 h were unchanged from baseline values after infusion of 100 µg/kg endotoxin (Fig. 5A). However, infusion of 1,000 µg/kg endotoxin resulted in a reduction in mean bile flow rate of 42% (P < 0.001) at 6 h and 25% (NS) at 24 h compared with baseline values. Peak serum TNF-α concentrations were 90.9 ± 16.2 and 129.6 ± 24.4 ng/ml after infusion of 100 and 1,000 µg/kg endotoxin, respectively. No differences in mean bile flow rate at 6 and 24 h were noted in the control group compared with baseline values (Fig. 5A). The mean baseline corticosterone concentration for all rats reported throughout this study was 46.4 ± 14.8 ng/ml (range 34–110 ng/ml).

Mean serum TNF-α concentrations 1 min after rTNF-α administration were 122.8 ± 15.6 and 252.6 ± 28.5 ng/ml for the 8 and 16 µg/kg doses, respectively. Although greatly decreased, mean serum TNF-α concentrations were significantly elevated for up to 90 or 120 min after rTNF-α administration of 8 and 16 µg/kg, respectively (data not shown). No TNF-α was detected in the serum after 120 min. Mean bile flow rates at 6 and 24 h were unchanged from baseline values after rTNF-α administration (Fig. 5B).

**Effect of Endotoxin and rTNF-α on Biliary ICG Clearance**

Infusion of 1,000 µg/kg endotoxin resulted in a reduction in mean biliary ICG clearance of 50% (P < 0.001) at 6 h and 29% (P < 0.01) at 24 h compared with baseline values (Fig. 6A). Mean biliary ICG clearance rates at 6 and 24 h were unchanged from baseline values after infusion of 100 µg/kg (Fig. 6A). No differences in biliary ICG clearance rate at 6 and 24 h were noted in the control group compared with baseline values (Fig. 6A). Mean biliary ICG clearance rates at 6 and 24 h were unchanged from baseline values after rTNF-α administration of 8 and 16 µg/kg (Fig. 6B).

**DISCUSSION**

Our data demonstrate a dose- and time-dependent decrease in the rates of bile flow and biliary excretion of ICG after endotoxin infusion and suggest that TNF-α is not the singular mediator of this response. Whereas our endotoxin dose of 1,000 µg/kg resulted in significant reduction from baseline values for rates of biliary ICG excretion at 6 and 24 h and of bile flow at 6 h after infusion, no changes from baseline values were noted for 100 µg/kg endotoxin or for the controls. The 1,000 and 100 µg/kg doses of endotoxin resulted in peak serum TNF-α concentrations of 129.6 ± 24.2 and 90.9 ± 16.2 ng/ml, respectively.

Because 100 µg/kg endotoxin caused a significant and sustained increase in TNF-α without affecting the rates of biliary ICG excretion or bile flow, we attempted to isolate the effects of TNF-α by infusing two doses of rTNF-α that achieved either the same peak serum concentration of TNF-α or twice the peak serum concentration. This series of experiments, designed to examine the singular role of TNF-α in mediating endotoxin-induced hepatic dysfunction, revealed no change from baseline rates for biliary ICG excretion or bile flow after rTNF-α administration at either 8 or 16 µg/kg in the absence of endotoxin infusion. This finding strongly suggests that TNF-α is not the singular mediator of endotoxin-induced hepatic dysfunction be-
cause these rTNF-α doses yielded higher mean serum TNF-α concentrations than those achieved with 1,000 μg/kg endotoxin infusion (Table 1).

Our findings are inconsistent with other studies, conducted with both humans and rats, in which TNF-α has been implicated as the major mediator of cholestasis during endotoxemia. In a series of human studies, investigators demonstrated that immunotherapy with rTNF-α induces cholestasis in humans (8). Using a rat model, Whiting et al. (31) demonstrated that administration of anti-TNF-α antibody before endotoxin infusion provided protection from endotoxin-induced bile acid accumulation. In separate research with rats, the administration of extremely high concentrations of parenteral rTNF-α (0.2–3.6 mg/kg) induced severe sepsislike responses including altered cardiac output, severe hepatic dysfunction, and death (23). To minimize these toxic effects, Wang et al. (29) administered lower exogenous rTNF-α doses (0.05 and 0.25 mg/kg) to rats over 30 min. Although these lower rTNF-α doses did not alter the hepatic microcirculation, ICG clearance was reduced after the 0.25 mg/kg dose but was unaffected with the 0.05 mg/kg dose (29). These lines of evidence led researchers to conclude that elevated TNF-α concentrations play a pivotal role in hepatocellular dysfunction (17, 21, 25, 29).

In contrast, the results of our research do not support the conclusion that TNF-α is the singular mediator of reduced ICG excretion. A major difference between our methodologies and those of other researchers is that others infused very high rTNF-α doses for 30 min to achieve measurable differences in hepatic ICG clearance, whereas we administered a single bolus infusion of rTNF-α (29). However, the bolus infusion of rTNF-α did result in significantly elevated concentrations of TNF-α for up to 120 min, approximating the length of significantly elevated concentrations of TNF-α after endotoxin challenge (1). To further control for the possibility that a bolus infusion may not accurately reflect the more sustained endotoxin-induced TNF-α response, we infused endotoxin doses of 100 μg/kg to achieve and sustain elevated TNF-α concentrations comparable to those in response to 1,000 μg/kg and still found no alteration from mean baseline rates of biliary ICG excretion or bile flow. Most importantly, the extremely elevated rTNF-α concentrations that reduced ICG clearance in other studies cannot be achieved in acute endotoxemic models, precluding generalizability of those findings to physiological models of endotoxin-induced hepatic dysfunction.

Our findings are consistent with two bodies of work in which researchers tried to attenuate the endotoxin-induced TNF-α response to minimize resulting hepatic dysfunction. Lund et al. (13) incorporated an in situ perfusion method and compared mean biliary ICG clearance for two groups of rats that were infused with 1,000 μg/kg endotoxin. One group of rats was pretreated with the TNF-α inhibitor pentoxifylline, and the other group was not. No significant difference between the groups was noted, suggesting that TNF-α does not mediate endotoxin-induced hepatic dysfunction. A more specific comparison between the results of Lund et al. and our findings is impossible because that does not measure TNF-α concentrations.

In separate work, Nakamura et al. (18) compared changes in mRNA of the canalicular transporter multidrug resistance protein 2 (mrp2) for two groups of rats after endotoxin treatment (18). One group received pretreatment with anti-TNF-α antibody, whereas the other did not. The finding of Nakamura et al. of no significant differences between the groups for mRNA of mrp2 was consistent with our conclusion that TNF-α does not regulate hepatic dysfunction. Neither our results nor those of Lund et al. (13) and Nakamura et al. (18) preclude the possibility that some mixture of endotoxin-induced inflammatory cytokines serve as mediators for changes in ICG excretion. However, in combination, these studies suggest that TNF-α is not the singular mediator of this response.
Several investigators have identified mechanisms that may regulate bile flow rate and biliary transport after endotoxin infusion, including bile transporters at both the basolateral and canalicular membranes (11, 26, 28). Although our studies have not included these transporters, an understanding of their function is central to our protocol for examining rates of bile flow and ICG excretion at 6 and 24 h after endotoxin infusion. Organic anion transporting protein 1 (oatp1) is thought to be the primary transporter of ICG across the basolateral membrane, whereas cytoplasmic multispecific organic anion transporter (cMOAT), also known as mrp2, is thought to be the primary ICG canalicul transporter (12, 26, 28). These processes may be mediated by changes in mRNA or protein as well as by the intracellular compartmentalization of the transporters. Whether oatp1 and mrp2 are the exclusive transporters for ICG at their respective membrane sites is unknown.

The basolateral and canalicular transporters of ICG are differentially affected by endotoxin infusion (2). Although the mRNA of the basolateral transporter oatp1 is unaffected by endotoxin treatment by 18 h after endotoxin infusion, oatp1 protein is significantly lower than baseline concentrations at this time (13). In contrast, the mRNA of the canalicular transporter mrp2 is downregulated after endotoxin treatment, with investigators reporting significantly reduced mRNA concentrations at 12–18 h (16, 21, 26) or at 3–6 h after endotoxin infusion (18). Vos et al. (28) found that mrp2 mRNA was completely eliminated between 6 h—the earliest time point examined in their study—and 48 h after endotoxin infusion. Separate work revealed that the mean concentration of mrp2 transporter protein was significantly lower than baseline concentrations at 12 and 24 h after endotoxin administration (18, 28). Still another mechanism of regulating mrp2 function may be the redistribution of mrp2 transporter from the canalicular membrane to an intracellular subapical compartment during rapid downregulation within 3 h after endotoxin infusion (11, 26).

This series of studies addressing transporters revealed reduction from baseline concentrations in transporters as soon as 3 h after endotoxin infusion. In most experiments, the peak decrease in transporter activity occurred at 18–24 h after infusion. On the basis of this combination of studies, we anticipated that our experiments would demonstrate greater reductions in the rates of biliary ICG secretion and bile flow at 24 h than at 6 h after endotoxin infusion. However, this was not the case. Although our data revealed significant reductions at 24 h, the greatest decrease from baseline for rates of biliary ICG clearance and bile flow occurred 6 h after endotoxin infusion.

Our finding of an endotoxin dose-dependent effect on mean peak TNF-α is consistent with that of other researchers who have focused on both TNF-α and other potential mediators of ICG excretion. However, in our study, mean peak TNF-α concentrations were elevated and sustained for both endotoxin doses. We previously showed (1) a significant dose-dependent increase over baseline in mean peak TNF-α serum concentrations after endotoxin doses ranging from 10 ng/kg to 2,000 μg/kg using our nonstressed rat model. However, even in our model system, the dose of endotoxin (1,000 μg/kg) necessary to cause hepatic dysfunction is pharmacological rather than pathophysiological and greatly exceeds the endotoxin concentration observed in portal blood during sepsis (31).

Other investigators have also reported an endotoxin dose-dependent effect on hepatic dysfunction (18, 21). In studies using in situ liver perfusion or hepatocyte isolation models, parenteral injection of endotoxin at doses ≤100 μg/kg failed to induce hepatic dysfunction (21). Similarly, in a separate study, Nakamura et al. (18) found a dose-dependent decrease in mrp2 mRNA after intraperitoneal injection of endotoxin at doses equivalent to those used in our study. In the experiments of Nakamura et al. (18) endotoxin at 1,000 μg/kg reduced mrp2 mRNA to 45% of baseline values at 12 h after injection, whereas 100 μg/kg of endotoxin reduced mrp2 mRNA to just 71% of baseline values. Although consistent with our data, Nakamura et al. noted reductions in mrp2 mRNA and bile flow rate at 12 h after intraperitoneal injection of endotoxin, whereas we noted the greatest reduction from baseline in rates of biliary ICG clearance and bile flow at 6 h after parenteral endotoxin infusion. Even with these methodological differences, the findings of Nakamura et al. of an endotoxin dose-dependent effect on mRNA concentrations provide a potential explanation for the dose-dependent alterations in rates of biliary ICG clearance and bile flow that we observed.

The modification of our chronically catheterized rat model to include a bile catheter permitted direct measurement of ICG biliary excretion, which served as an indicator of hepatic dysfunction. This methodological adaptation eliminates the need to make assumptions about biliary ICG excretion based on decreased blood uptake, which is characteristic of other methodologies (19, 24, 29). The findings of our preliminary studies addressing the determination of maximum biliary ICG excretion rate indicate that steady-state ICG blood concentrations are not obtainable and therefore cannot accurately substitute for direct measures of biliary ICG excretion.

Other investigators have reported findings that are consistent with our results and underscore the lack of accuracy in using measures of hepatic blood uptake to estimate ICG biliary excretion. This related body of work suggests that the half-life of blood ICG concentration is dose dependent, as evidenced by the nonlinear kinetics for ICG uptake from bolus infusions (6, 19, 13). Therefore, hepatic uptake is a saturable process and uptake curves must be calculated as mono-, bi-, or triexponential, depending on the infused concentration (15, 24). Consistent with this line of evidence, our data reveal that higher concentrations of infused ICG resulted in significantly higher mean blood ICG concentrations, whereas the biliary ICG excretion rate remained unchanged. Thus the use of high concentrations of infused ICG to quantify hepatic blood clearance...
does not accurately reflect the biliary ICG excretion rate.

Our model system has several advantages for studying endotoxin-induced alterations in biliary ICG clearance. Our previous studies revealed that surgical stress dramatically attenuates the endotoxin-induced TNF-α response. We speculate that the lack of protocol-related stress in our model permits earlier detection of kinetic differences in the intracellular regulation and functional kinetics of ICG. These speculations are consistent with previous work that suggests that surgical stress elevates glucocorticoids, which in turn attenuates endotoxin-induced hepatic dysfunction. For example, analysis of the rat mrp2 gene promoter revealed multiple glucocorticoid response elements, which may protect hepatic function during stress (10). In studies with rats, exogenous dexamethasone inhibited the endotoxin-induced translocation of mrp2, the reduction of mRNA, and the excretion of anionic dye, whereas the bile flow rate remained unaffected (11).

Our chronically catheterized rat model controls for these effects, as evidenced by the finding that our control animals demonstrated constant biliary ICG excretion and bile flow rate over extended periods. In contrast to other methodologies, our model did not interrupt normal enterohepatic circulation of bile, because we reinfused collected bile into a duodenal catheter that was inserted 2 cm distal to the sphincter of Oddi, which is proximal to the terminal ileum, the site of bile acid reabsorption. This procedural adaptation maintained baseline bile flow rates in our experimental animals for up to 8 h of serial bile collection without the use of exogenous bile acids.

Although previous models of chronic biliary collection have addressed these issues, none to our knowledge has been adapted to study endotoxin-induced hepatic dysfunction. However, many features of these models are common to our studies. Investigators using chronic bile catheter models have emphasized that physiological collection of bile requires a nonstressed, unrestrained rat in the absence of anesthesia and the maintenance of enterohepatic circulation of bile acids (4, 7, 9, 20, 22). Unfortunately, achievement of these controls has resulted in a complex collection system in many of these models (4, 7, 9, 20, 22).

Although these models have incorporated physiological collection of bile, in situ perfusion methods have examined hepatic function under nonphysiological conditions that are inherent in the experimental protocols. Limitations of in situ perfusion methods include the induction of surgical stress to isolate the liver, the absence of endogenous blood flow during the perfusion, thereby necessitating nonphysiological regulation of infusion pressure, and the nonphysiological regulation of perfusate content such as red blood cells for tissue oxygenation and bile acids to simulate enterohepatic bile acid circulation. Each of these limiting factors may affect hepatic function, which raises the likelihood that procedures inherent in the perfusion methodology affect the outcome of the experiments. For example, the perfusion methodology significantly alters the concentrations of bile transporter proteins compared with those in nonperfused livers (13). In some perfusion studies, bile flow rate could be measured for only 36 min or less because of bile acid depletion or other protocol limitations (11, 21, 31). Finally, McKindley et al. (14) concluded in a recent study that there was no correlation between the endotoxin-induced hepatic dysfunction reported from in vivo studies and endotoxin-induced hepatic dysfunction studied under in situ conditions. As such, this group of experiments has limited generalizability to physiological models. In contrast, our model system allows sequential determination of biliary ICG excretion from individual animals, eliminating intra-animal variability.

A final advantage of our model system is its ability to detect even minor alterations in hepatic function. Our preliminary studies revealed that our model induces maximum ICG transport without significant accumulation of ICG in the blood, as evidenced by the maintenance of steady-state ICG secretion into bile. Our preliminary data revealed that the continuous infusion of ICG at 50 μg/min produced the maximum ICG excretion rate. We chose to infuse ICG at a constant rate because this method maintains steady-state biliary ICG excretion over the entire 90-min period used in this study. In contrast, bolus or sequential bolus infusions do not maintain a steady-state biliary excretion of ICG. Indeed, in our studies, 30 min of ICG infusion is required to obtain steady-state biliary ICG excretion. Therefore, we collected our data from each animal at 12 individual time points during steady-state biliary ICG excretion.

In summary, this study demonstrates that endotoxin administration causes hepatic dysfunction in vivo as measured by a decrease from baseline in the rates of ICG excretion and bile flow. Consistent with other investigators, we noted hepatocellular dysfunction at the high endotoxin dose of 1,000 μg/kg. Because the endotoxin-induced TNF-α response is unattenuated in our nonstressed experimental model, we had hypothesized that endotoxin-induced hepatic dysfunction would occur at lower doses of endotoxin than previously described. In contrast, we found that hepatic dysfunction did not occur either after endotoxin infusion at ≤100 μg/kg, doses that induced and sustained high TNF-α concentrations, or after administration of TNF-α in the absence of endotoxin. These data suggest that TNF-α is not the predominant mediator of endotoxin-induced hepatic dysfunction in vivo but do not eliminate the possibility that other components of the inflammatory response may mediate this activity.

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