Tailored care in resectable perihilar cholangiocarcinoma

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PART 3
Intra-operative management
CHAPTER 7

Atorvastatin protects against cholestatic liver injury but does not reduce ischemia-reperfusion damage in cholestatic rat livers

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Submitted
ABSTRACT

Background: Extrahepatic cholestasis sensitizes the liver to ischemia/reperfusion (I/R) injury, since it is associated with pre-existent sterile inflammation, microvascular perfusion defects, and impaired energy status. Statins have been shown to protect against I/R injury in normal and steatotic mouse livers. Therefore, the hepatoprotective properties of atorvastatin (ATV) were evaluated in a rat model of cholestatic I/R injury.

Methods: Male Wistar rats were subjected to 70% hepatic ischemia (30 min) 7 d after bile duct ligation (BDL). Rats were randomized to ATV treatment or vehicle-control in three test arms: (1) oral treatment with 5 mg/kg during 7 days after BDL; (2) intravenous treatment with 2.5, 5, or 7.5 mg/kg 24 h before ischemia; and (3) intravenous treatment with 5 mg/kg 30 min before ischemia. Hepatocellular damage was assessed by plasma alanine aminotransferase (ALT) and histological necrosis.

Results: I/R induced severe hepatocellular injury (~10-fold increase in ALT at 6 h after I/R and ~30% necrotic areas at 24 h after I/R). Oral ATV treatment decreased ALT levels before ischemia, but did not reduce ALT levels after I/R. Intravenous ATV treatment at 5 mg/kg 24 h before ischemia was the only regimen that reduced ALT level at 6 h after reperfusion, but not at 24 h after reperfusion. None of the tested regimens were able to reduce histological necrosis at 24 h after reperfusion.

Discussion: Pre-treatment with ATV did not protect cholestatic livers from hepatocellular damage after I/R. Clinical studies investigating the role of statins in the protection against hepatic I/R injury should not include cholestatic patients, who require (pharmacological) interventions that specifically target the cholestasis-associated hepatopathology.
INTRODUCTION

Liver surgery in patients with perihilar cholangiocarcinoma is associated with a high rate of postoperative mortality (between 5 to 18%) because the tumor causes extrahepatic cholestasis, sensitizing the liver to surgical injury.\(^1\) The effects of cholestasis include impaired liver regeneration and increased susceptibility to ischemia/reperfusion (I/R) injury. Biliary drainage can be used to reduce cholestasis preoperatively, but it is a risky intervention since complications after biliary drainage (e.g. cholangitis) may outweigh the benefit of biliary decompression.\(^2\) Therefore, pharmacological interventions targeting the effects of extrahepatic cholestasis are desired as a potentially superior alternative to biliary drainage.

Hepatic I/R injury is an inevitable side effect of surgery that results from the temporary deprivation of blood supply to the liver; a contrived procedure that is aimed at preventing excessive perioperative blood loss. However, the reinstatement of blood flow after resection may have detrimental consequences that affect the liver parenchyma and reduce liver function. Reoxygenation of the liver causes overproduction of reactive oxygen and nitrogen species (ROS and RNS, respectively) in mainly mitochondria as a result of excessive electron leakage from the respiratory chain.\(^4\) The ROS/RNS oxidize biomolecules and subsequently induce cell death programs that, in combination with the depletion of energy stores during the ischemia phase, results in mainly necrotic cell death.\(^5\) Dying and dead hepatocytes release numerous endogenous molecules that act as damage-associated molecular patterns (DAMPs), which constitute a chemotactic gradient for neutrophils and activate Kupffer cells to produce ROS and release pro-inflammatory cytokines that further contribute to neutrophil chemotaxis. The attracted neutrophils adhere to the afflicted liver microvasculature, leading to their activation and production of more ROS, altogether accounting for exacerbation of intrahepatic oxidative stress, (micro)vascular constriction, and inflammation.\(^6\)

Livers with a healthy parenchyma can effectively cope with I/R when exposed to a limited duration of ischemia. In contrast, cholestatic livers are less apt to tolerate I/R due to the pre-existent hepatopathology. Cholestasis is associated with the accumulation of hydrophobic bile acids in cell and organelle (mitochondrial) membranes, which leads to increased mitochondrial ROS/RNS production, sterile inflammation, and cell death.\(^7,9\) In addition, cholestasis is characterized by microvascular perfusion defects that result from compression of vasculature due to biliary hyperdilatation\(^10\) and from a ROS/RNS-driven shift in the balance between vasodilators (nitric oxide, prostacyclin, carbon monoxide) and vasoconstrictors (endothelin-1, thromboxane A2) towards a vasoconstrictive state.\(^11,12\) These perfusion defects, together with bile acid-mediated mitochondrial perturbations,\(^13\) lead to reduced production of ATP and other metabolically important nucleotides and hence an impaired energy status and overall metabolic dysfunction in cholestatic livers.\(^14\)

In light of this disease profile, the aim of the study was to determine whether the resilience of cholestatic rat livers to I/R could be pharmacologically augmented by the administration of statins prior to ischemia induction. Statins are normally used in the prevention of cardiovascular disease...
because of their lipid-lowering properties, but this class of drugs exhibits multiple additional effects. Among the lipid metabolism-independent effects, statins have been shown to influence bile acid homeostasis and reduce markers of liver injury in animal models of cholestasis. Although these effects remain to be confirmed in clinical studies, statins seem to reduce bile acid production and increase cholesterol transport back to plasma; both regulated of nuclear receptors. An additional effects of statin therapy is protection against I/R injury, as previously demonstrated in normal and steatotic mouse livers. In that respect, the dual hepatoprotective properties of statins may prove especially helpful against I/R injury in the context of cholestatic hepatopathology.

The lipid metabolism-independent pleiotropic effects of statins include antioxidant, vasoprotective, anti-inflammatory, and anti-thrombotic effects. The proximal steps in cholesterol metabolism include formation of farnesyl and geranyl/geranyl pyrophosphate. Inhibition of these precursors by statins prevents isoprenylation of several G-proteins; inhibition of Rac1 prevents oxidative stress through a reduction in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, and parallel inhibition of RhoA upregulates endothelial nitric oxide synthase (eNOS) production. eNOS stimulates the production and bioavailability of NO, which subsequently causes vasodilatation of the hepatic microvasculature, deters apoptosis of sinusoidal endothelial cells (SECs), and suppresses thromboxane A2 production. Accordingly, atorvastatin (ATV) ameliorates microcirculatory defects and endothelial dysfunction in normal and steatotic mouse livers. Pre-treatment with statins also suppresses hepatic protein levels of Toll-like receptor-4 (TLR-4), which is expressed by Kupffer cells, sinusoidal endothelial cells, and hepatocytes. The lower TLR-4 levels translated to reduced NF-κB activity and decreased production and release of various chemokines and cytokines that collectively drive sterile inflammation. It was therefore hypothesized that statins reduce I/R injury in cholestatic livers, as schematically outlined in Figure 1 for ATV, given that the intervention sites correspond to the molecular origins of cholestasis- and I/R-mediated liver damage.

The hypothesis was tested in a bile duct ligation (BDL)-based rat model of obstructive cholestasis, which is most representative of the patients requiring liver surgery for e.g., perihilar cholangiocarcinomas. The experiments demonstrated that oral ATV administered daily during 7 d BDL (5 mg/kg) as well as a single intravenous bolus 24 h before animal sacrifice (5 mg/kg) reduced the extent of cholestasis-induced liver damage during 7-d BDL, indicating that ATV reached the liver and conferred a hepatoprotective effect. However, systemic administration of ATV did not protect the liver against I/R damage, regardless of the dosage (up to 7.5 mg/kg) and time of administration (24 h or 30 min before ischemia induction). Further mechanistic elucidation was therefore not performed and the study was terminated prematurely. The main implications of our findings are that cholestatic patients may benefit from ATV therapy in the period between diagnosis and biliary drainage, but that ATV therapy does not constitute a viable treatment option for surgical patients with cholestasis.
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MATERIALS AND METHODS

Animals

All animal experiments were approved by the institute’s animal ethics committee (BEX102781) and animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, rev. 2011). Specific pathogen-free male Wistar rats (N = 115, Harlan Laboratories, Horst, the Netherlands) weighing between 250-270 g were acclimated for 1 wk in a temperature-controlled room with 12-h dark/light cycles and ad libitum access to water and standard chow.

Anesthesia and surgical procedures

Standard bile duct ligation (BDL) was used to induce cholestasis. Rats were subjected to 70% hepatic ischemia for 30 min at 7 d days after BDL. For both procedures, rats were anesthetized with 3-5% isoflurane (O2:air ratio of 1:1, 2 L/min, Forene, Abbott Laboratories, Queensborough, UK) and analgesic care was provided by subcutaneous administration of buprenorphine (0.03 mg/kg, Temgesic, Schering-Plough, Kenilworth, NJ). Maintenance anesthesia comprised 2-2.5% isoflurane (O2:air ratio of 1:1, 1 L/min).

Experimental setup

Rats were randomized to ATV treatment or vehicle-control in three test arms according to Figure 2. The group sizes are indicated in the figure. In the first test arm, the effects of orally and intravenously
administered ATV were investigated in the context of cholestatic liver injury. For oral administration, ATV (Pfizer, New York, NY) was dissolved in sterile 0.9% NaCl solution (B. Braun Melsungen, Melsungen, Germany) at a 1.0-mg/mL concentration. ATV (or its vehicle control) was administered per gavage once daily during 7 d after BDL at a dose of 5.0 mg/kg body weight. For intravenous administration, atorvastatin (PZ0001, Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at a 10.0-mg/mL stock concentration and diluted with NaCl to a concentration of 1.0 mg/mL, corresponding to an ATV dose of 5.0 mg/kg body weight. In the second and third test arms, the effect of a single intravenous dose of ATV administered 24 h or 30 min before ischemia induction, respectively, was investigated in terms of I/R-induced liver injury at 6 and 24 h reperfusion. ATV in DMSO (10.0 mg/mL) was diluted with NaCl to a concentration of 0.5, 1.0, or 1.5 mg/mL, corresponding to administered doses ATV of 2.5, 5.0, or 7.5 mg/kg body weight. Systemically dosed ATV was administered via the tail vein.

Blood sampling was performed via the tail vein when the animals remained in the experiment (test arm 3) or via cardiac puncture when the animals were sacrificed by exsanguination. Following sacrifice, liver specimens were harvested for histological processing.

**Assessment of liver injury, inflammation, and fibrosis**

Serum ALT and bilirubin levels were assayed in blood samples (Figure 2) by routine clinical chemistry using a Cobas 8000 modular analyzer (Roche, Basel, Switzerland). Histological sections were processed as described previously and stained with hematoxylin and eosin (H&E).

The extent of necrosis was quantified by an experienced hepatopathologist (JV) in liver biopsies collected after I/R (Figure 2). Analysis was performed in 10 random fields of view (FOVs) per liver and expressed as a percentage of the total FOV surface. Inflammation (density of polymorphonuclear leukocytes) and fibrosis were assessed according to the Ludwig scoring system. Statistical analysis was performed in GraphPad Prism (GraphPad Software, San Diego, CA). Results are presented as mean ± SEM. ATV and vehicle control-treated groups were compared using an unpaired student’s t-test with Welch’s correction. The Gaussian distribution of each data set was confirmed with a Shapiro-Wilk test (n < 8) or a D’Agostino-Pearson omnibus test (n ≥ 8). A P-value of ≤ 0.05 was considered statistically significant.

**RESULTS**

The 7-d BDL resulted in severe cholestasis, as evidenced by a mean total bilirubin of 174 ± 23 µM. Histological sections acquired after I/R exhibited septal fibrosis characteristic of Ludwig stage 3, which had developed before I/R due to the chronic nature of this process. Cholestasis was also associated with elevated plasma ALT levels (Figure 3A), reflecting hepatocellular injury that was consistent with previous reports.
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Daily oral doses of 5 mg/kg ATV during the 7-d BDL period as well as a single intravenous dose of 5 mg/kg on day 6 during the 7-d BDL period (test arm 1) reduced ALT levels by 50% and 20%, respectively (Figure 3A). These data indicate that ATV (a) reached the liver following oral as well as systemic administration and (b) conferred a hepatoprotective effect during the progression of cholestatic liver injury.

Inasmuch as cholestatic hepatopathology is amplified as a result of I/R, the pharmacodynamic efficacy of ATV was examined in a dose escalation setting at 6 h reperfusion following 30 min of ischemia. As shown in Figure 3B, i.v. administration of ATV at 5 mg/kg 24 h before the induction of ischemia resulted in significantly reduced hepatocellular damage. Consequently, this dosing regimen was more closely investigated in terms of hepatocellular damage (ALT) and histological necrosis.

Figure 2. Experimental setup consisting of 3 test arms. The first test arm entailed oral or intravenous (i.v.) administration of atorvastatin (ATV) without subjecting the animals to ischemia/reperfusion (I/R) to determine the effect of ATV on the pathology of cholestasis. This test arm also served to demonstrate that ATV is targeted to the liver following oral or i.v. administration. Test arms 2 and 3 encompassed intravenous (i.v.) administration of ATV followed by I/R. The gray segment delineates the period of bile duct ligation (BDL), the blue segment indicates the ischemic phase, and the red segment designates the reperfusion time frame. The times in every segments at which procedures were performed are indicated with black vertical lines, protracted in gray throughout the figure. The legend at the top explains the significance of the symbols, the numbers in parentheses refer to the group size.
Although ATV reduced hepatocellular damage at 6 h reperfusion, the protective effects were abrogated at 24 h reperfusion (Figure 3C and E). Given that histological necrosis is irreversible, these results demonstrated that ATV was unable to ultimately protect the liver from damage when administered systemically 24 h before I/R.

It is known that ATV is taken up by sinusoidal endothelial cells and hepatocytes, and metabolized in and excreted by the liver. Administering ATV 24 h before I/R may therefore have resulted in subtherapeutic intrahepatic ATV levels, accounting for the absence of hepatoprotection. To resolve
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this potential pharmacokinetic hurdle, ATV was administered 30 min before ischemia induction at the optimal concentration (5 mg/kg) in accordance with previous reports (24). Nevertheless, this treatment regimen also did not result in reduced liver damage at 24 h of reperfusion (Figure 3D and E). In light of these data, subsequent experiments were discontinued, as there was no compelling evidence that ATV protects the liver from I/R injury.

DISCUSSION

The anti-inflammatory, vasoregulatory, and anti-thrombotic effects of statins have been shown to protect against I/R injury in normal and steatotic mouse livers.21-23 Even short-term therapy with ATV (5 mg/kg) just 1 h before ischemia increased the bioavailability of NO and inhibited NF-κB activation that resulted in improved post-I/R outcomes.28 Based on the literature and the well-established pharmacodynamic properties of ATV in juxtaposition to cholestatic I/R pathophysiology, we hypothesized that ATV would protect cholestatic livers against I/R injury. Cholestasis is associated with pre-existing liver damage and sterile inflammation,7-9 microvascular perfusion defects,10-12 and impaired energy status.13 ATV intervenes in several of these processes. Although ATV ameliorated liver damage during the manifestation and progression of cholestasis, as has been described before for statins,17-19,35 there was no beneficial effect of ATV on I/R-induced liver damage following different dosing regimens.

Patients with perihilar cholangiocarcinoma typically present with obstructive jaundice and cholestasis upon admission, which constitute severe risk factors in liver surgery. Consequently, biliary drainage is often employed to alleviate these conditions prior to surgery. However, patients selected to undergo surgery without biliary drainage,2 may benefit from statin treatment as a preoperative intervention, based on our results and those of others17-19,35. Similarly, patients with primary biliary cirrhosis may benefit from statin treatment. One report described lower total bile acid levels,26 and several reports described a reduction in cholestasis markers in patients with primary biliary cirrhosis.36,37 It should be noted that recent studies did not reproduce these findings,38,39 so the actual clinical benefit of statins in the treatment of cholestasis remains unclear.

The exact reasons for the lacking therapeutic efficacy of ATV in cholestatic liver I/R are currently elusive. Several mechanisms may explain the failure of ATV to prevent hepatocellular damage in cholestatic livers. Microvascular perfusion defects and impaired energy status may have persisted after ATV treatment. This could have been caused by mechanical compression of the hepatic microcirculation due to biliary hyperdilatation as a result of the cholestasis, impairing intrahepatic blood flow and energy metabolism.14 Alternatively or additionally, cholestasis may have caused a pre-existent vasoconstrictive state that rendered the hepatic microcirculation unreceptive to ATV-mediated inhibition of vasoconstrictors (endothelin-1, thromboxane A2) and upregulation of NO, which would generally result in improved microcirculation, oxygen delivery, and energy metabolism. Also, the poor responsiveness to ATV treatment may have been exacerbated by the prevailing
state of oxidative stress and sterile inflammation,\textsuperscript{14} which cannot be fully resolved by TLR4 and NF-κB inhibition with ATV.\textsuperscript{22} In light of the negative results, we chose not to further investigate the mechanisms that underlie the lacking therapeutic efficacy of ATV. Instead, future research efforts should be directed at evaluating other types of pharmaceutical agents that target the multifarious pathogenic features of I/R injury in cholestatic livers.

Lastly, readers should note that this study has several limitations. First, the experimental model was associated with substantial variability in outcomes, despite the broad experience with this model in our laboratory.\textsuperscript{14,40-42} The group sizes had to be extended during the study following interim analysis to overcome the considerable standard deviations, but were still inadequate to statistically resolve minor beneficial effects of ATV. However, it is questionable whether such small improvements in outcome would justify the use of ATV as an intervention. Moreover, liver damage following I/R was extensive, probably owing to 7 days of BDL prior to ischemia induction. Although this is a widely used model for extrahepatic cholestasis, we cannot preclude that ATV does not protect the liver against I/R injury in cases of milder cholestasis.

In conclusion, pre-treatment with statins did not protect cholestatic rat livers from hepatocellular damage after I/R. Clinical studies warrant the investigation of statins for the amelioration of I/R injury in livers with otherwise healthy or steatotic parenchyma, but not with cholestasis-affected parenchyma.
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