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Endogenous glucocorticoids exacerbate cholestasis-associated liver injury and hypercholesterolemia in mice

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ABSTRACT

Cholestatic liver disease is characterized by a disruption of bile flow, bile acid toxicity, liver injury, and hypercholesterolemia. Relatively high secretion of glucocorticoids by the adrenals has been observed under cholestatic conditions. Here we investigated a contribution of the rise in endogenous glucocorticoids to initial stage cholestasis pathology. Adrenalectomized or sham-operated control C57BL/6 mice were given an oral dose of alpha-naphthylisothiocyanate to induce cholestasis. Adrenalectomy effectively lowered plasma corticosterone levels (18 ± 5 ng/ml vs 472 ± 58 ng/ml; $P < 0.001$) and disrupted the metabolic and anti-inflammatory glucocorticoid function. Adrenal removal did not exacerbate the cholestasis extent. In contrast, the cholestasis-associated liver injury was markedly lower in adrenalectomized mice as compared to controls as evidenced by a 84%–93% decrease in liver necrosis and plasma alanine aminotransferase and bile acid levels ($P < 0.001$ for all). Gene expression analysis on livers from adrenalectomized mice suggested the absence of bile acid toxicity-associated farnesoid X receptor signaling in the context of a 44% ($P < 0.01$) and 82% ($P < 0.001$) reduction in sodium/bile acid cotransporter member 1 transcript level as compared to respectively control and non-diseased mice. Adrenalectomy reduced the expression of the cholesterol synthesis gene HMG-CoA reductase by 70% ($P < 0.05$), which translated into a 73% lower plasma total cholesterol level ($P < 0.05$). Treatment of C57BL/6 mice with the glucocorticoid receptor antagonist RU-486 recapitulated the protective effect of adrenalectomy on indices of liver injury and hypercholesterolemia.
In conclusion, we have shown that endogenous glucocorticoids exacerbate the liver injury and hypercholesterolemia associated with acute cholestasis in mice.

1. Introduction

The liver is a key regulator of plasma cholesterol levels by removing cholesterol from lipoproteins in the blood circulation (Glomset, 1980 and Brown and Goldstein, 1986). A proper functioning of the liver is essential to overcome hypercholesterolemia and its related co-morbidities such as the development of atherosclerotic lesions and cardiovascular disease. As such, a genetic defect in for instance the low-density lipoprotein receptor (LDLR) predisposes human subjects to hypercholesterolemia and cardiovascular disease (Hovingh et al., 2013).

The presence of relatively high intracellular (free) cholesterol levels can induce oxidative stress, leading to toxicity and potentially cell death. Although the liver does contribute significantly to the regulation of plasma cholesterol levels, hepatocytes thus first and foremost aim to keep intracellular cholesterol levels within a healthy range. The majority of cholesterol acquired from lipoproteins is therefore excreted via the bile either (1) directly via the combined action of the ATP-binding

Abbreviations: ABCB4, ATP-binding cassette transporter B4; ADX, adrenalectomy; ALT/GPT, alanine transaminase/glutamic pyruvic transaminase; ANIT, alpha-naphthylisothiocyanate; APOA4, apolipoprotein A4; BSEP/ABCB11, bile salt efflux pump; CYP7A1, cholesterol 7alpha-hydroxylase; FXR/BAR, farnesoid X receptor/bile acid receptor; HDL, high-density lipoprotein; HMGCR, HMG-CoA reductase; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; NTCP1/SLC10A1, sodium/bile acid cotransporter member 1; PEPCK, phosphoenolpyruvate carboxykinase; SHP, small heterodimer partner; TAT, tyrosine aminotransferase; TDO2, tryptophan 2,3-dioxygenase; VLDL, very-low-density lipoprotein.

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cassette transporter B4 (ABCB4; MDR2) and the ABCG5/ABCG8 heterodimer or (2) after transformation into bile acids that can be excreted by the bile salt efflux pump (BSEP; ABCB11).

When bile flow is disrupted, toxic amounts of bile acids accumulate within the liver leading to the development of cholestasis (Hirschfield, 2013). Mutations in biliary epithelial transporters ABCB11, ABCB4, and ATP8B1, and mechanical blockage (gallstones) are the most common underlying causes of cholestasis (Srivastava, 2014 and McIntyre et al., 1975). Cholestatic liver disease patients generally suffer from jaundice as a result of a concomitant rise in plasma bile acid levels (Srivastava, 2014). In support of the notion that biliary cholesterol/bile acid excretion by the liver is essential to maintain normal plasma cholesterol levels, hypercholesterolemia is a common finding in cholestatic human subjects (McIntyre et al., 1975).

Interestingly, several lines of evidence have suggested that the initial induction of cholestasis is associated with a concomitant stimulation of adrenal glucocorticoid synthesis. More specifically, Zumoff et al. (1971) have shown that cortisol production rates are >50% higher in patients with biliary obstruction. In addition, studies by Zietz et al. (2001) have revealed a shift to adrenal cortisol production at the expense of adrenal androgen production in cholestatic liver disease patients. Moreover, bile duct-resected rats exhibited a marked increase in basal plasma levels of the primary glucocorticoid corticosterone as compared to sham-operated rats (Swain and Le, 1998). It should, however, be noted that in more advanced stages of the disease, i.e. in cirrhotic patients, hypothalamus-pituitary-adrenal-axis activity and adrenal glucocorticoid secretion appears to be diminished instead of increased (Jang et al., 2014 and Fede et al., 2014).

Glucocorticoids, via the action of their cognate nuclear receptor, are able to stimulate the expression of metabolic genes (Vegiopoulos and Herzig, 2007), while suppressing pro-inflammatory gene networks (Hayashi et al., 2004). A chronic increase in plasma glucocorticoid levels (hypercortisolemia) therefore translates into a high risk for metabolic disturbances including hypercholesterolemia and hypertriglyceridemia in humans (Arnaldi et al., 2010). In contrast, a relatively low adrenal glucocorticoid response rather predisposes human subjects to inflammation-related morbidities (Soni et al., 1995 and Manglik et al., 2003). Based upon the aforementioned findings, it can be hypothesized that an increase in glucocorticoid levels in acute cholestatic liver disease patients may contribute to the cholestasis-associated hypercholesterolemia and/or serve as a protective response to cope with the enhanced (hepatic) inflammatory status.

To provide in vivo proof for a causal role of glucocorticoids in initial stage cholestasis pathology, we determined the impact of removal of the endogenous glucocorticoid function on cholestasis outcome in an experimental mouse model of acute cholestatic liver disease.

2. Methods

2.1. Mice

Twelve to fifteen week old C57BL/6 were bred in house. Mice were given a standard low-fat chow diet ad libitum. Experiments were performed in a temperature and light cycle (12-hour light/12-hour dark) controlled room at the Gorlaeus Laboratories of the Leiden Academic Centre for Drug Research in accordance with the National Laws. All experimental protocols were approved by the ethics committee for Animal Experiments of Leiden University.

2.2. Adrenalectomy, RU-486 treatment, and induction of cholestasis

Bilateral adrenalectomy or a control operation in which adrenals were touched but otherwise left intact was performed under isoflurane inhalation anesthesia through a dorsal midline skin incision and lateral retroperitoneal incisions. After closure of skin wounds using a Michel suture clip, mice were left separated from each other overnight for efficient wound healing. Subsequently, 4 similarly operated mice were housed per cage for two weeks to fully recover from the operation. To compensate for the loss in adrenal function, a part of the bottom of each cage surface was heated by a heating mattress and all mice were given 0.9% NaCl and normal water ad libitum. After recovery, adrenalectomized and adrenal-intact control mice were single housed, fasted overnight, and subsequently given an oral dose of alpha-naphthylisothiocyanate (ANIT; 50 mg/kg) dissolved in 200 μl olive oil at 9:00 AM to induce cholestasis (Xu et al., 2004 and Faiola et al., 2010). After two hours, mice were re-supplied with the regular chow diet.

In a separate experiment, single housed C57BL/6 mice were injected twice daily subcutaneously with RU-486 (mifepristone; 30 mg/kg) to pharmacologically block glucocorticoid receptor function, with one dose given in the morning and the other dose given in the afternoon (Asagami et al., 2011). To prevent drug interaction effects, administration of RU-486 was started two hours after the induction of cholestasis. Mice received a total of 4 doses of the compound. Control mice received a similar injection regimen with the solvent only (100 μl 100% ethanol).

Forty-eight hours after ANIT exposure, mice were bled via tail bleeding, anesthetized by a mixture of xylazine (70 mg/kg), ketamine (350 mg/kg), and atropine (1.8 mg/kg), and euthanized by bleeding via the retro orbital plexus. Subsequently, mice were subjected to whole body perfusion with PBS (with the pressure of 100 mm Hg) via a cannula in the left-ventricular apex, and organs were harvested, weighted, and stored at −20 °C or fixed in formalin.

2.3. Histology

Cryosections (8 μM) of formalin-fixed liver specimens embedded in Sakura O.C.T. Compound™ were prepared on a Leica CM3050-S cryostat. Cryosections were routinely stained with Masson's Trichrome. Quantification of necrotic areas in liver sections was performed blinded by computer aided morphometric analysis. Images were obtained with a Leica image analysis system, consisting of a Leica DMRE microscope coupled to a camera and Leica Qwin Imaging software.

2.4. Plasma measurements

Blood samples for corticosterone analysis were drawn via tail chop between 9:00 and 10:00 AM (2 to 3 h in the light period). Levels of corticosterone in plasma were determined using a 125I radio immuno assay (RIA) according to the manufacturer's specifications (MP Biomedials). All other measurements in plasma were performed in blood collected through retro orbital bleeding at sacrifice. Bilirubin and Alanine Transaminase/Glutamic Pyruvic Transaminase (ALT/GPT) were measured via the Reflotron Plus Blood analyzer (Roche Diagnostics). Reflotron GPT/ALT and Reflotron Bilirubin sticks were used according to the manufacturer's specifications (Roche Diagnostics). Plasma concentrations of free and total cholesterol and the distribution of cholesterol over the different lipoprotein fractions obtained by fast performance liquid chromatography (Superose 6 column 3.2 × 30 mm; SMART-system; Pharmacia) were determined using enzymatic colorimetric assays (Roche Diagnostics).

2.5. Analysis of gene expression by real-time quantitative PCR

Total RNA from livers of adrenalectomized and adrenal-intact control mice was isolated using phenol-chloroform extraction and reverse transcribed using RevertAid™ reverse transcriptase. Gene expression analysis was performed using real-time SYBR Green technology (Eurogentec). Primer sequences can be found in Table 1. The housekeeping genes 36B4 and beta-actin were used for normalization to exclude that changes in the relative expression were caused by variations in the expression of the separate housekeeping genes. Where necessary for appropriate interpretation of the gene expression