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Gut microbiota and nuclear receptors in bile acid and lipid metabolism

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

2014

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Out, C. (2014). *Gut microbiota and nuclear receptors in bile acid and lipid metabolism: bile acids, more than soaps*. [Thesis fully internal (DIV), University of Groningen]. [S.n.].

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CHAPTER 7

Prednisolone increases enterohepatic cycling of bile acids by induction of Asbt and promotes reverse cholesterol transport

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Journal of Hepatology, 2014 Mar 26.

and

Bile acids and cholestasis

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Gastroenterology, 2013 Feb;144(2):e17-8.

ABSTRACT

Background & Aim: Glucocorticoids, produced by the adrenal gland under control of the hypothalamic-pituitary-adrenal axis, exert their metabolic actions largely via activation of the glucocorticoid receptor (GR). Synthetic glucocorticoids are widely used as anti-inflammatory and immunosuppressive drugs but their application is hampered by adverse metabolic effects. Recently, it has been shown that GR may regulate several genes involved in murine bile acid (BA) and cholesterol metabolism, yet the physiological relevance hereof is controversial. The aim of this study is to provide a mechanistic basis for effects of prednisolone on BA and cholesterol homeostasis in mice.

Methods: Male BALB/c mice were treated with prednisolone (12.5 mg/kg/day) for 7 days by subcutaneous implantation of slow-release pellets, followed by extensive metabolic profiling.

Results: Sustained prednisolone treatment induced the expression of the apical sodium-dependent bile acid transporter (Asbt) in the ileum, which stimulated BA absorption. This resulted in elevated plasma BA levels and enhanced biliary BA secretion. Concomitantly, both biliary cholesterol and phospholipid secretion rates were increased. Enhanced BA reabsorption suppressed hepatic BA synthesis, as evident from hepatic gene expression, reduced plasma C4 levels and reduced fecal BA loss. Plasma HDL cholesterol levels were elevated in prednisolone-treated mice, this likely contributed to the stimulated flux of cholesterol from intraperitoneally injected macrophage foam cells into feces.

Conclusions: Sustained prednisolone treatment increases enterohepatic recycling of BA, leading to elevated plasma BA levels and reduced BA synthesis in the absence of cholestasis. Under these conditions, prednisolone promotes macrophage-derived reverse cholesterol transport.

INTRODUCTION

Glucocorticoids (GCs) are steroid hormones produced by the adrenal gland under control of the hypothalamic-pituitary-adrenal axis (HPA-axis). Related to their well-known effects on the immune response, synthetic GCs, *e.g.* prednisolone, are widely used anti-inflammatory and immunosuppressive drugs. GCs bind and activate the glucocorticoid receptor (GR) by dissociating receptor-bound heat shock proteins that maintain GR in an inactive state. Activated GR translocates to the nucleus and regulates the expression of target genes via transactivation or transrepression, by binding as a homo- or heterodimer. Besides the genes involved in the well-known anti-inflammatory function and those that influence glucose and lipid metabolism, GR also regulates several genes involved in bile acid (BA) metabolism (1, 2). Since BA act as integrators of metabolic regulation (3), modulation of BA metabolism by GR activation may underlie some of the side-effects on lipid and energy metabolism associated with chronic use of GCs. BA are synthesized from cholesterol via complex multi-enzyme pathways in the liver. The first committed step of this pathway is catalyzed by the rate-controlling enzyme CYP7A1, leading to the formation of 7 α -hydroxycholesterol. The expression of CYP7A1 is regulated by multiple signals and transcription factors, *e.g.* FXR, HNF4 α and FoxO1 (4). Glucose and insulin are major postprandial factors that induce CYP7A1, whereas bile acids down regulate the expression of CYP7A1 (4). CYP7A1 expression and activity exhibit a diurnal rhythm and, interestingly, the peak of hepatic CYP7A1 mRNA expression coincides with the peak in serum corticosterone levels in mice (5). Adrenalectomy results in a greatly decreased amplitude of CYP7A1 expression leading to decreased BA synthesis rate and BA pool size, which can be restored by cortisol administration (6). Absence of hepatic GR in mice substantially changed systemic BA metabolism, with enhanced fecal loss and a decrease in gallbladder BA content. Interestingly, mice harboring a DNA binding-defective GR mutant (GRdim) exhibit a very similar phenotype (7). These studies exemplify the important role of GCs in control of murine BA metabolism. GCs also impact on human BA metabolism. For example, short-term prednisolone treatment led to elevated plasma BA levels in patients with chronic active hepatitis (8) and, accordingly, plasma BA levels were found to be significantly elevated in patients with Cushing syndrome (9).

Recently, Lu et al. described a molecular mechanism by which GCs might interact with BA homeostasis, i.e., via blocking the transcriptional activity of the BA-activated nuclear receptor FXR (farnesoid X receptor; NR1H4) in the liver (9). However, FXR also exerts major control on BA homeostasis via the small intestine, by stimulating the production of Fgf15 in the terminal ileum. Fgf15 and the human homologue FGF19 are circulating FGFs that inhibit CYP7A1 expression upon binding FGF receptor 4 (4). Actually, the vast majority of BA is reabsorbed in the terminal ileum, and transported back to the liver via the portal circulation. BA reabsorption in the terminal ileum occurs via active transport by the apical sodium-dependent BA transporter (Asbt; Slc10a2). Two functional glucocorticoid response elements (GREs) have been identified within the promoter region of Asbt (10). Therefore, GCs may have a dual action on BA metabolism via hepatic and intestinal FXR. Yet, the complex nature of BA metabolism and its regulation prevents prediction of actual consequences on the basis of gene expression analysis only. In this study we therefore investigated the effects of synthetic GCs on BA physiology *in vivo* in mice. Changing BA metabolism can have great impact on cholesterol homeostasis as well. The impact of cholesterol and different classes of lipoproteins on the development of atherosclerosis has been investigated extensively during the past 50 years. Cholesterol is mainly eliminated from the body via the liver in the form of BA and, in general, biliary BA secretion is the driving force for biliary cholesterol secretion (11). HDL-cholesterol is the preferred substrate for BA synthesis (12), and the HDL receptor, SR-BI, is regulated in parallel with BA synthesis (13). SR-BI is a crucial factor in the pathway of reverse cholesterol transport (RCT), the transport of cholesterol from peripheral cells back to the liver for subsequent excretion into the bile (14), which is considered to be an important process to inhibit the development of atherosclerotic lesions (15). In the adrenal SR-BI supplies cholesterol required for GC synthesis (16). GCs can in turn influence the expression of several genes involved in cholesterol metabolism, *e.g.* ABCA1, SR-BI, LDL receptor (17, 18). Thus, GCs may influence cholesterol metabolism directly and indirectly via BA. However, the role of GCs in the development of atherosclerosis is not yet clearly established. Human data show contradictory influences of GCs on cardiovascular disease and cardiovascular risk, whereas animal studies suggest an atheroprotective role of GCs (19). The mechanism underlying these anti-atherosclerotic effects of GCs are incompletely understood.

In this study we show that treatment of mice with GCs is associated with increased enterohepatic cycling of BA leading to elevated plasma BA levels and choleresis, i.e., stimulation of bile formation. Prednisolone treatment also induced plasma HDL-cholesterol levels and biliary cholesterol secretion, which resulted in increased movement of cholesterol from macrophages to the feces. This may contribute to the anti-atherosclerotic effects of glucocorticoids, at least in animal models.

MATERIALS AND METHODS

Animal experiments

Twelve-week old male BALB/c mice (Charles River, France) were housed individually in a temperature- and light-controlled facility with 12 hours light-dark cycling. All mice were fed commercially available laboratory chow (RMH-B; Hope Farms, Woerden, The Netherlands) ad libitum. Slow-release prednisolone pellets (15mg; 60 day release; Innovative Research of America, Sarasota, Florida, USA) with a calculated release of 12.5 mg/kg/day were implanted subcutaneously. After 7 days gallbladder cannulation was performed to collect hepatic bile. Bile was collected during 20 minutes under Hypnorm (fentanyl/fluanisone; 1 ml/kg) and diazepam (10 mg/kg) anesthesia using a humidified incubator to maintain body temperature. Blood was obtained via heart puncture and, after sacrificing the mice, the liver and ileum were excised and snap-frozen in liquid nitrogen for gene expression and protein analysis. All experiments were approved by the Ethical Committee for Animal Experiments of the University of Groningen.

Measurements

Bile acid analysis, cDNA measurements, Western blotting and RCT studies were all performed as described before. For detailed information please go to supplemental material and methods. For the quantification of 7 α -hydroxy-4-cholesten-3-one (C4) in plasma samples, we developed a sensitive and highly specific automated on-line solid-phase extraction method coupled to high performance liquid chromatography-tandem mass spectrometry (XLC-MS/MS). Sample pretreatment consisted of addition of deuterium labelled C4 (C4-d7, Toronto Research Chemicals) as an internal standard, followed by acetonitrile protein crash. Subsequently 5 μ L plasma equivalent was injected into the automated solid-phase extraction system (Spark Holland). On-line SPE cartridges

containing C8-sorbent (Spark Holland) were used. Reversed phase chromatography was applied using an XBridge RP18-column (Waters). Mass spectrometric detection was performed in selective reaction monitoring mode, using a quadrupole tandem mass spectrometer (Quattro Premier, Waters) with positive electrospray ionization. Total run-time including on-line SPE was 10 minutes. Intra- and interassay analytical variation were <3%. Linearity in the 0–550 nmol/L calibration range was excellent ($R^2 > 0.99$). Quantification limit was 0.5 nmol/L and no interferences were detected.

RESULTS

Prednisolone treatment stimulates bile formation

Male BALB/c mice were treated with prednisolone (12.5 mg/kg/day) for 7 days by subcutaneous implantation of slow-release pellets. This induced dramatic effects on bile acid (BA) metabolism. Biliary BA secretion was more than doubled in treated mice compared to controls (Figure 1A). Since formation of bile is driven by BA secretion, bile flow was increased in mice that received prednisolone (2.0 ± 0.3 vs. 1.5 ± 0.1 $\mu\text{L}/\text{min}$; $n=8-10$; $p=0.006$). BA also drive the secretion of phospholipids and cholesterol into the bile, and correspondingly these processes were also strongly simulated (Figure 1B/C). Interestingly, prednisolone treatment significantly altered biliary BA composition: the primary BA cholic acid (CA) increased at the expense of deoxycholic acid (DCA) inducing a shift in the ratio of primary to secondary BA (Figure 1D). Secretion of β -muricholic acid (β -MCA), a murine-specific BA species derived from chenodeoxycholic acid (CDCA), increased as well after prednisolone treatment but the difference between groups was just not statistically significant (Figure 1D). Overall, the percentage of primary BA was higher in bile of prednisolone-treated mice (87.1 ± 2.4 % vs. 78.7 ± 2.3 % in controls; $n=8-10$; $p<0.001$).

Prednisolone treatment enhances bile acid reabsorption

Biliary BA mainly represent molecules that are maintained within in the enterohepatic circulation, de novo synthesized BA comprise only a minor fraction of biliary BA. In the terminal ileum, BA are efficiently reabsorbed by the apical sodium-dependent BA transporter (Asbt; Slc10a2) and transported back to the liver via the portal circulation. In line with the increased biliary BA secretion both Asbt gene expression as well as

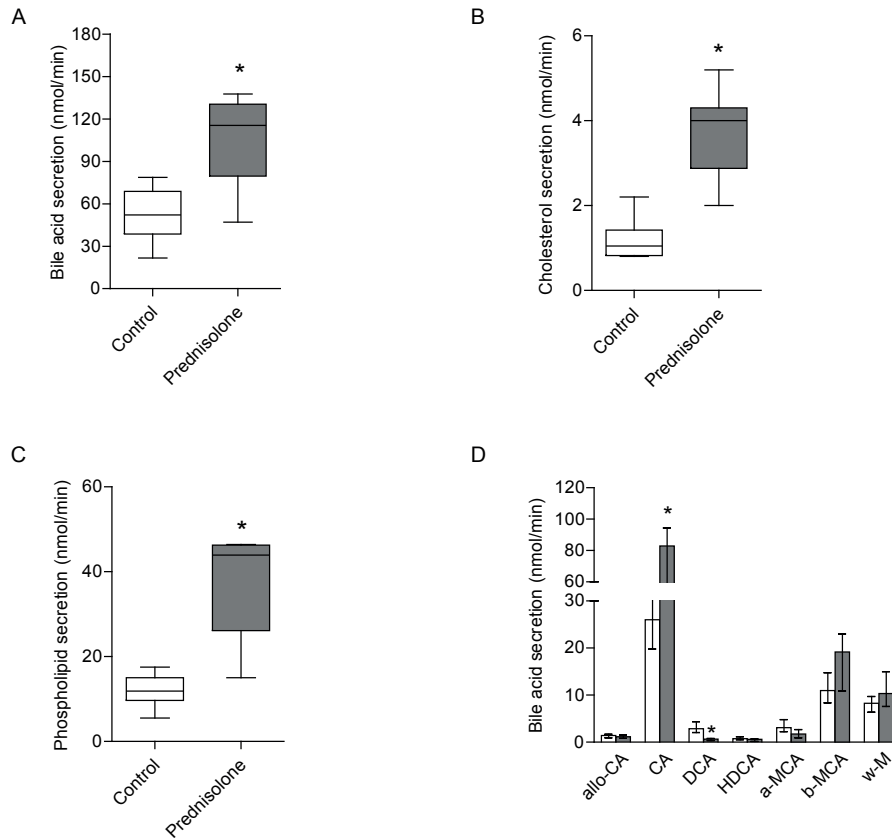


Figure 1. Prednisolone treatment promotes biliary secretion of bile acids, cholesterol and phospholipids in mice. Biliary BA (A), cholesterol (B) and phospholipid (C) secretion are increased in prednisolone-treated BALB/c mice. (D) Biliary BA composition analysis revealed increased levels of CA and decreased levels of DCA in prednisolone-treated mice. Data are presented as median \pm range, $n = 8-10/\text{group}$, * $p < 0.05$.

Asbt protein expression in ileum were clearly enhanced by prednisolone treatment (Figure 2A/B). In contrast, ileal expression of the BA-responsive genes, Fgf15, Shp, Ibabp and Osta, were all markedly decreased (Figure 2A). The high expression of Asbt resulted in decreased fecal BA excretion (Figure 2C) and an increase in plasma BA levels (Figure 2D). The rise in plasma BA was mainly accounted for by increased plasma concentrations of both conjugated and unconjugated CA (Figure 2E). Furthermore, increased concentrations of tauro- α -MCA, β -MCA, DCA and ω -MCA were present in plasma of prednisolone-treated mice compared to controls. Analysis of fecal BA composition revealed a decreased excretion of all BA species after prednisolone treatment

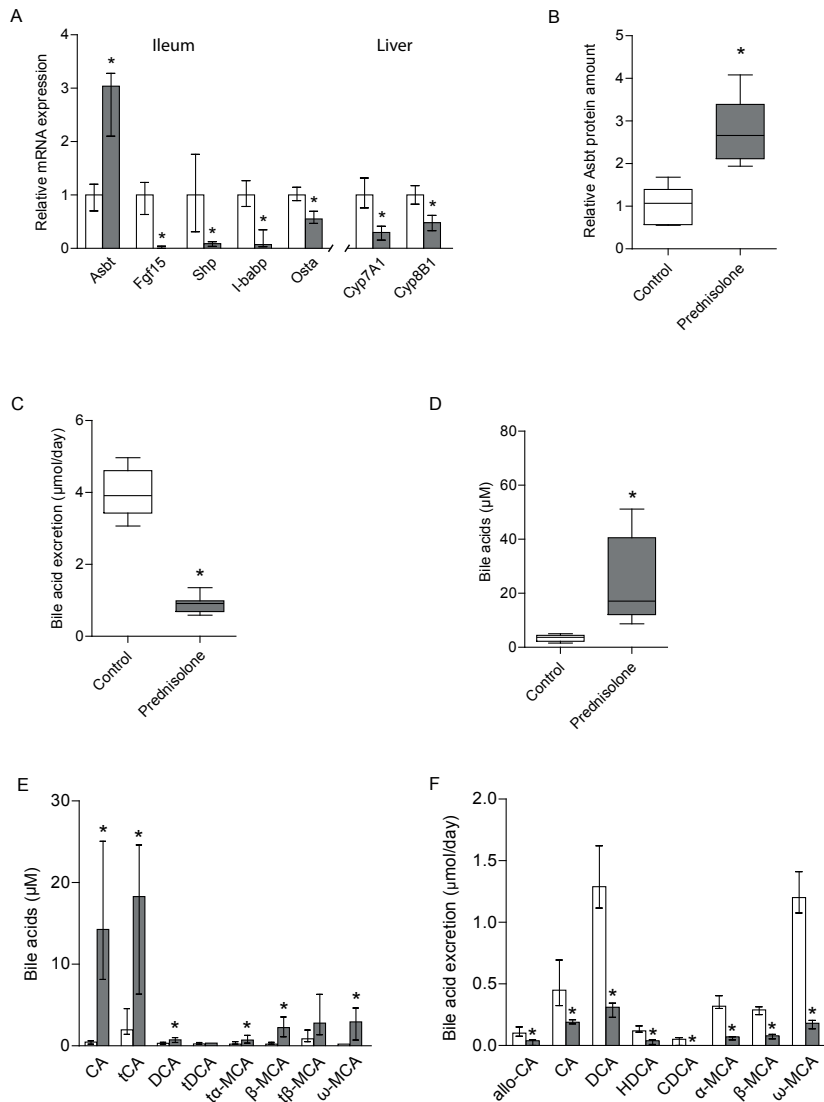


Figure 2. Prednisolone treatment increased ileal *Asbt* expression leading to enhanced reabsorption of BA and decreased BA synthesis in mice. (A) Prednisolone treatment increased the ileal expression of *Asbt*, whereas the expression of other, FXR-responsive genes was decreased. Hepatic mRNA expression of *Cyp7a1* and *Cyp8b1* is decreased upon prednisolone treatment. (B) Increased ileal *Asbt* protein expression in prednisolone-treated mice. (C) Mice treated with prednisolone showed suppressed fecal BA excretion. (D) Plasma BA levels are clearly elevated in prednisolone-treated mice compared to controls. (E) Plasma BA profile analysis revealed elevated levels of all major BA species in prednisolone-treated mice. (F) Fecal BA composition analysis revealed lower concentrations of all major BA species in prednisolone-treated mice. Data are presented as median \pm range, $n = 8-10/\text{group}$, * $p < 0.05$.

(Figure 2F). Analysis of the distribution of bile acids in the pool showed that the total amount of BA in plasma and liver was greater in prednisolone-treated mice (Table 1). The amount of BA in gallbladder bile and the small intestine showed an increasing trend (Table 1). Therefore, the total BA pool size in the enterohepatic circulation is enlarged in prednisolone-treated mice. The amount of BA in the large intestine, predetermined to be excreted, was decreased upon prednisolone treatment, consistent with decreased fecal excretion (Table 1).

Table 1. Total amount of bile acids in different compartments of the enterohepatic circulation in control and prednisolone-treated mice.

Bile acids (μmol)	Control	Prednisolone
Plasma	0.02 (0.02 – 0.02)	0.05 (0.04 – 0.07)*
Liver	0.58 (0.54 – 0.65)	0.82 (0.72 – 1.06)*
Gallbladder	1.78 (1.13 – 2.22)	2.40 (1.86 – 2.57)
Small intestine	1.54 (1.41 – 1.96)	1.90 (1.53 – 2.19)
Total	4.03 (3.38 – 4.30)	5.36 (4.27 – 5.73)*
Large intestine	0.58 (0.57 – 0.77)	0.20 (0.16 – 0.24)

* $p < 0.05$

Prednisolone treatment decreases BA synthesis

The decreased fecal excretion of BA (Figure 2C) indicates a decrease in hepatic BA synthesis, because under steady-state conditions, fecal BA loss equals hepatic de novo synthesis. Indeed, hepatic gene expression of *Cyp7a1*, the rate-controlling enzyme for BA synthesis, and *Cyp8b1*, involved in the synthesis of CA, was decreased in prednisolone-treated mice (Figure 2A). Consistent with hepatic gene expression and fecal BA excretion, plasma levels of 7 α -hydroxy-4-cholesten-3-one (C4), an intermediate in BA synthesis with a strong correlation to the enzymatic activity of hepatic CYP7A1 (20), tended to be lower (59.5 ± 20.7 vs. 38.4 ± 20.4 nmol/L; $n=8-10$; $p=0.19$) upon prednisolone treatment. Bile acid kinetics were studied using deuterium labeled cholic acid. The cholic acid pool increased from 2.7 ± 0.2 to 8.7 ± 1.9 μmol , fractional turnover decreased from 0.54 ± 0.14 to 0.35 ± 0.20 day⁻¹ and the cycling time of cholic acid was calculated to be 1.5 ± 0.1 h and 2.2 ± 0.2 h after prednisolone treatment. After prednisolone treatment the calculated cholic acid pool size was higher than chemically measured (Table 1), indicating possible overestimation because of a lack of steady state during the kinetic measurement. The hepatic expression of the nuclear receptors Fxr

and *Lrh1* and their target gene *Shp* was unchanged (Table 2). Also, expression of the hepatic BA transporters *Ntcp* and *Bsep* was unchanged (Table 2).

Table 2. Hepatic gene expression in control and prednisolone-treated mice.

Hepatic gene expression	Control	Prednisolone
<i>Fxr</i>	1.00 (0.89 – 1.07)	0.85 (0.83 – 0.93)
<i>Lrh1</i>	1.00 (0.95 – 1.03)	0.81 (0.77 – 0.86)
<i>Shp</i>	1.00 (0.79 – 1.52)	0.84 (0.67 – 1.09)
<i>Ntcp</i>	1.00 (0.94 – 1.11)	1.21 (1.11 – 1.25)
<i>Bsep</i>	1.00 (0.81 – 1.03)	1.06 (0.96 – 1.36)
<i>ApoA1</i>	1.00 (0.92 – 1.09)	1.21 (1.10 – 1.38)*
<i>Srb1</i>	1.00 (0.85 – 1.16)	1.55 (1.46 – 1.65)*
<i>Abca1</i>	1.00 (0.90 – 1.12)	1.22 (1.16 – 1.32)*

* $p < 0.05$

Effects of prednisolone treatment on cholesterol homeostasis

Changes in BA metabolism may impact on cholesterol homeostasis. Plasma cholesterol levels were significantly increased in prednisolone-treated mice (Figure 3A). FPLC analysis to determine the effect of prednisolone treatment on the distribution of cholesterol over the different lipoprotein subfractions, revealed that the excess cholesterol upon prednisolone treatment was present in HDL-sized fractions (Figure 3B). The hepatic expression of apolipoprotein A-I (ApoA1), the main apolipoprotein on HDL particles, was increased upon prednisolone treatment (Table 2), suggesting increased HDL formation as a potential underlying mechanism. The hepatic expression of scavenger receptor class B type I (*Srb1*) and ATP-binding cassette transporter *Abca1* also increased significantly (Table 2). In addition to the increases in plasma cholesterol and in biliary cholesterol secretion, fecal neutral sterol excretion was significantly higher in response to prednisolone treatment (Figure 3C).

Prednisolone treatment increases macrophage-to-feces reverse cholesterol transport

Since prednisolone treatment increased plasma HDL cholesterol levels, as well as biliary cholesterol and fecal neutral sterol excretion, we next investigated if prednisolone treatment would also have an impact on macrophage-specific reverse cholesterol transport (RCT). Therefore, *in vivo* RCT was traced in control and prednisolone-treated mice after intraperitoneal injection of primary mouse macrophages loaded with ³H-cholesterol. The appearance of tracer in plasma was significantly increased in the prednisolone-

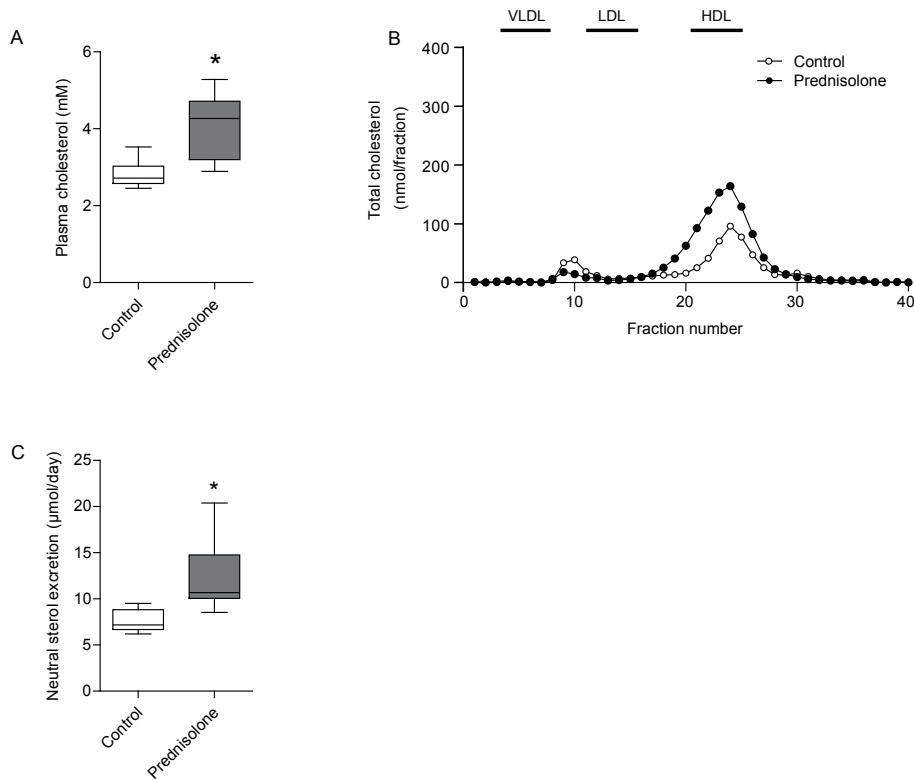


Figure 3. Prednisolone treatment alters cholesterol homeostasis in mice. (A) Plasma cholesterol levels are increased in prednisolone-treated BALB/c mice. (B) Total cholesterol levels separated by FPLC in pooled plasma samples of control (open symbols) and prednisolone-treated (closed symbols) mice. (C) Prednisolone treatment increased fecal neutral sterol excretion. Data are presented as median \pm range, $n = 8-10/\text{group}$, * $p < 0.05$.

treated group at 24h and 48h but not at 6h after macrophage injection (Figure 4A). Note that total plasma cholesterol also increased after treatment with prednisolone. The amount of macrophage-derived tracer recovered within the liver at 48h was not affected by prednisolone treatment (Figure 4B). Consistent with the higher biliary and fecal excretion of sterols, prednisolone treatment significantly enhanced the total excretion of 3H-cholesterol originating from macrophages into the feces (Figure 4C). Since tracer recovery in the fecal BA fraction remained unaltered this increase was attributable to a 3.3-fold higher excretion of 3H-cholesterol in the fecal neutral sterol fraction (Figure 4C). These results demonstrate that prednisolone treatment increases the movement of cholesterol from macrophages to the feces.

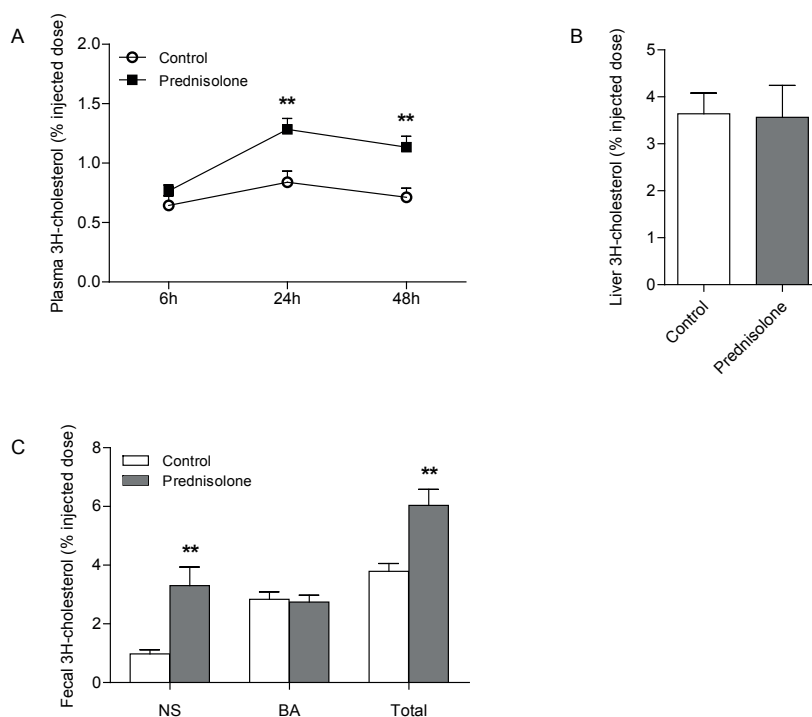


Figure 4. Prednisolone treatment increased *in vivo* macrophage-to-feces reverse cholesterol transport.

Control mice and prednisolone-treated mice received intraperitoneal injections with ^3H -cholesterol-loaded primary mouse macrophage foam cells. (A) ^3H -cholesterol tracer appearance in plasma 6, 24 and 48 h after macrophage administration. (B) ^3H -cholesterol tracer recovery within liver 48 h after macrophage injection. (C) ^3H -cholesterol tracer appearance in feces collected continuously from 0 to 48 h after macrophage administration. Data are presented as means \pm SEM, $n = 8$ -10/group. Statistically significant differences from control mice are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

The glucocorticoid receptor has more than 8000 binding sites in nuclear DNA as shown by ChIP-seq studies (21). Although it is not yet clear whether all these sites are functional, it is not surprising that activation of GR by an exogenous agonist causes a plethora of effects. It is, however, surprising that the consequences of glucocorticoid activation on BA and in particular cholesterol homeostasis have received relatively little attention. In this study we show that sustained treatment of mice with prednisolone profoundly impacts on both BA and cholesterol metabolism in mice. Treatment of BALB/c mice with

prednisolone for 7 days appeared to increase enterohepatic cycling of BA, leading to altered compartmentalization of the BA pool with high concentrations in plasma and bile. Fecal BA excretion decreased whereas biliary BA secretion was induced, pointing towards enhanced BA reabsorption in the small intestine. Asbt (Slc10a2), the main intestinal BA transporter localized in the terminal ileum, was induced upon prednisolone treatment. Previously, two functional glucocorticoid response elements (GREs) have been identified within the promoter region of Asbt (10) and GC treatment was shown to enhance taurocholic acid absorption in in situ ileal loops (22) and to restore the diminished affinity for taurocholic acid uptake, as well as expression levels of Asbt, during chronic ileitis (23). In our experiments, induced Asbt expression was associated with elevated plasma BA levels, indicating that GCs modulate BA reabsorption in vivo as well. Surprisingly, despite the increased uptake of BA by enterocytes, ileal expression of the BA-responsive genes, Fgf15, Shp, Ibabp and Osta, were all markedly decreased. It seems plausible that this is due to inhibition of the transcriptional activity of intestinal FXR by prednisolone-activated GR, as described by Lu et al. (9). Thus, both direct stimulation of Asbt by GR, and inhibition of intestinal FXR activity leading to decreased expression of SHP with derepression of LRH-1-mediated Asbt gene expression (24) and less FGF15-mediated Asbt-repression (25), likely all contribute to the high Asbt expression during GC-treatment. Increased intestinal BA reabsorption induces higher portal influx of BA to the liver exceeding the first-pass hepatic uptake capacity and hence spillover to the peripheral circulation, resulting in elevated plasma BA levels (26). In response, hepatic BA synthesis is lowered, as is evident from the decreased expression of hepatic Cyp7a1 and Cyp8b1, decreased levels of plasma C4 and decreased fecal BA excretion. Apparently, the strongly increased influx of BA into the liver overruled GR inhibition of FXR activity in the liver as well as the putative stimulating consequences on BA synthesis of reduced Fgf15 production in the ileum. These results are consistent with the previously reported increase in plasma BA levels in patients treated with prednisolone or suffering from Cushing syndrome (9), yet detailed analysis of BA metabolism in these conditions has to the best of our knowledge not been reported.

Recently, Lu et al. showed that treatment of mice with dexamethasone, another frequently used synthetic GC, resulted in elevated plasma and hepatic BA levels. On the basis hereof the authors concluded that GR activation results in cholestasis (9). Although their experiment was performed in C57Bl6 mice using dexamethasone, which is far

more potent than prednisolone, our data challenge the concept that GC treatment induces cholestasis (27). The term cholestasis is derived from the Greek *chole* (bile) and *stasis* (standing still) and is therefore physiologically defined as an impairment of bile formation. Here we show that treatment of mice with GCs is indeed associated with elevated plasma BA levels but actually induces choleresis, i.e., stimulation of bile formation, instead of cholestasis. In our opinion, increased plasma BA levels during prednisolone treatment simply reflect an increase in enterohepatic cycling of BA. Therefore, the clinical definition of cholestasis, based on assessment of plasma levels of BA, by no means always reflects the existence or the severity of the pathological event, i.e., impaired formation of bile by the liver. Prednisolone-induced hypercholanemia clearly provides another example of such a dissociation. The reported effect of GCs on BA homeostasis may have consequences for clinical practice. For example, restoration of BA absorption in patients with Crohn's disease (10), which often suffer from BA malabsorption (28-30), should be considered a beneficial effect. On the other hand, when using GCs in patients at risk for developing high BA levels (cholestasis, NASH, hepatitis), plasma BA levels should be monitored during GC treatment, or treatment may be combined with a BA sequestrant to prevent too high plasma BA levels, and pruritus (31). In other situations, such as primary biliary cirrhosis, treatment with glucocorticoids can be used – and combined with UDCA – to prevent plasma bile acid levels from skyrocketing (32, 33).

The increased enterohepatic cycling of BA upon prednisolone treatment induced a concomitant increase in biliary cholesterol secretion. Increased cholesterol mobilization may indicate stimulated reverse cholesterol transport (RCT), although earlier studies have shown that the rate of biliary cholesterol excretion does not determine RCT (34). We assessed the effect of prednisolone treatment on efflux of cholesterol from injected foam cells using the method developed by the group of Rader (35). As depicted in figure 4, prednisolone increased cholesterol flux from macrophage foam cells to the feces indicating stimulated macrophage-derived RCT. This was mainly due to increased fecal tracer recovery within the neutral sterol fraction, while tracer excretion in the BA fraction was unaltered. However, total fecal BA excretion decreased about threefold after prednisolone treatment indicating that the relative contribution of macrophage-derived cholesterol to BA synthesis actually did increase. This effect of GC treatment on cholesterol homeostasis indicates another potential beneficial effect. The increase in

HDL-cholesterol, probably attributable to increased ApoA-I expression, likely provides the underlying basis for the increase in RCT induced by prednisolone administration.

The question arises whether these results can be translated to the human situation. Increased ApoA-I synthesis upon GC treatment (36), is consistent with increased total cholesterol, HDL-C and ApoA-I levels in patients treated with prednisolone (8, 37-39). However, the role of GCs in the development of atherosclerosis is not clearly established in humans or animals. In humans, complex and inconsistent effects of GC on cardiovascular disease and cardiovascular risk have been reported (19). Primary adrenal insufficiency (Addison's disease) is associated with a higher risk for cardiovascular mortality (40), while systemic GC excess, such as in Cushing's syndrome, is also associated with a higher cardiovascular risk (41, 42). The effects of elevated GCs in animal models contrast to the human situation (43). In fat-fed rabbits, GCs and other anti-inflammatory steroids prevent or arrest atherosclerosis, despite causing hyperlipidemia (43). Atherogenic diet feeding to *Ldlr*^{-/-} mice subjected to adrenalectomy resulted in a higher susceptibility for diet-induced atherosclerosis compared to control *Ldlr*^{-/-} mice (44), whereas adrenal transplantation induced a reversal of the adrenalectomy-associated atherosclerotic lesion development in this model (44). Corticosterone treatment to APOE*3-Leiden.CETP mice fed a Western-type diet decreased macrophage content of the plaque and total atherosclerotic lesion area by -39% without lowering plasma cholesterol levels (45).

The mechanisms underlying this discrepancy in the effects of GCs on atherosclerosis are incompletely understood. GCs can negatively influence cardiovascular risk factors, interact positively with cells of the heart and vascular wall and inhibit inflammation (19). Our present data suggest that an increase in RCT contributes to the anti-atherosclerotic effects of GCs, at least in animal models. However, the various actions of GCs on the heart, vascular wall and inflammatory cells, combined with their ability to influence other cardiovascular risk factors (e.g. LDL-cholesterol, hypertension, insulin resistance and obesity), make it difficult to predict their overall effect on cardiovascular disease in vivo in the human situation (19).

In summary, we show that prednisolone treatment strongly affects sterol homeostasis in mice. BA reabsorption via induction of *Asbt* is enhanced, resulting in elevated plasma BA levels, choleresis and a decrease in fecal BA excretion. Thus, GR activation, particularly in the intestine, plays an important role in regulating BA homeostasis by controlling enterohepatic recycling of BA and bile production. Concomitantly, GC

administration increases macrophage-to-feces RCT, pointing towards potential, non-characterized, effects of this class of drugs to provide protection against development and progression of atherosclerotic cardiovascular disease.

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