Research Article

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Bile acids modulate glucocorticoid metabolism and the hypothalamic–pituitary–adrenal axis in obstructive jaundice☆

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Background & Aims: Suppression of the hypothalamic–pituitary–adrenal axis occurs in cirrhosis and cholestasis and is associated with increased concentrations of bile acids. We investigated whether this was mediated through bile acids acting to impair steroid clearance by inhibiting glucocorticoid metabolism by 5β-reductase.

Methods: The effect of bile acids on glucocorticoid metabolism was studied *in vitro* in hepatic subcellular fractions and hepatoma cells, allowing quantitation of the kinetics and transcript abundance of 5β-reductase. Metabolism was subsequently examined *in vivo* in rats following dietary manipulation or bile duct ligation. Finally, glucocorticoid metabolism was assessed in humans with obstructive jaundice.

Results: In rat hepatic cytosol, chenodeoxycholic acid competitively inhibited 5β-reductase (K_i 9.19 ± 0.40 μM) and reduced its transcript abundance (in H4iiE cells) and promoter activity (reporter system, HepG2 cells).

In Wistar rats, dietary chenodeoxycholic acid (1% w/w chow) inhibited hepatic 5β-reductase activity, reduced urinary excretion of 3α,5β-tetrahydrocorticosterone and reduced adrenal weight. Conversely, a fat-free diet suppressed bile acid levels and increased hepatic 5β-reductase activity, supplementation of the fat-free diet with CDCA reduced 5β-reductase activity, and urinary 3α,5β-reduced corticosterone. Cholestasis in rats suppressed hepatic 5β-reductase activity and transcript abundance.

In eight women with obstructive jaundice, relative urinary excretion of 3α,5β-tetrahydrocortisol was significantly lower than in healthy controls.

Conclusion: These data suggest a novel role for bile acids in inhibiting hepatic glucocorticoid clearance, of sufficient magnitude to suppress hypothalamic–pituitary–adrenal axis activity. Elevated hepatic bile acids may account for adrenal insufficiency in liver disease.

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Introduction

Activation of the hypothalamic–pituitary–adrenal (HPA) axis and enhanced release of cortisol is crucial to a successful response to stress, but this homeostatic mechanism is disrupted in liver disease. In cirrhosis, impaired responsiveness of the adrenal to ACTH contributes to increased mortality with haemodynamic impairment [1,2]. Replacement with low-dose hydrocortisone significantly improves resolution of shock and survival [3]. Similarly, in cholestatic rats, secretion of corticotrophin-releasing hormone is suppressed, and adrenal responses to stress are impaired [4]. However the cause of dysregulation of the HPA axis is not understood.

If metabolism of cortisol is impaired, then negative feedback control of the HPA axis causes suppression of ACTH levels, atrophy of the adrenal gland and a reduced rate of production of cortisol, a pattern also seen in cirrhosis [5,6]. As the liver is the major site of cortisol metabolism [7], impaired clearance of cortisol in liver disease may be caused by the reduced functional liver mass or by an inhibitor of glucocorticoid metabolism.

Hepatic enzymes that inactivate glucocorticoids include 5α- and 5β-reductases and 3α-hydroxysteroid dehydrogenase (3αHSD), which convert cortisol into tetrahydrometabolites [8]. In addition, 5β-reductase and 3αHSD are involved in bile acid synthesis [9]. Bile acids are cytotoxic, so their formation and elimination are tightly regulated by the up-regulation of genes encoding proteins that induce their detoxification and/or

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Abbreviations: HPA, hypothalamic–pituitary–adrenal; ACTH, adrenocorticotropic hormone; 3αHSD, 3α-hydroxysteroid dehydrogenase; FXR, farnesoid X receptor; *Cyp7a1*, cholesterol 7α-hydroxylase; 11βHSD, 11β-hydroxysteroid dehydrogenase; BDL, bile duct ligation; THB, tetrahydrocorticosterone; DHB, dihydrocorticosterone; GCMS, gas chromatography mass spectrometry; CDCA, chenodeoxycholic acid; CA, cholic acid; DCA, deoxycholic acid; GCDCA, glyco-CDCA; K_i, inhibitor constant; TCDCA, tauro-CDCA; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; FF, fat-free; ECRP, endoscopic retrograde cholangiopancreatography; ANOVA, analysis of variance; ALT, alanine transaminase; ALP, alkaline phosphatase; *Cyp11b1*, 11β-hydroxylase; NEFA, non-esterified fatty acids; SEM, standard error of mean.

excretion, and by the suppression of genes (mainly through the farnesoid X receptor (FXR)) encoding proteins that regulate cholesterol catabolism, e.g., cholesterol 7α-hydroxylase (*CYP7A1*) [9].

Inhibition of renal 11βHSD2 by bile acids impairs glucocorticoid inactivation [10,11] and contributes to the sodium retention and potassium wasting observed in cirrhosis and cholestasis [12] as well as following bile duct ligation (BDL) [13], through illicit occupation of mineralocorticoid receptors by excess cortisol. Bile acids also inhibit hepatic 11βHSD1 [8,14–16], preventing glucocorticoid reactivation. Effects on glucocorticoid metabolism other than by 11βHSDs have not been investigated, although inhibition of 5β-reduction of aldosterone by bile acids has been demonstrated [17].

We hypothesised that bile acid accumulation in cholestasis inhibits hepatic 5β- reductase, contributing to impaired glucocorticoid clearance and attenuation of HPA axis activity. The effects of bile acids on the activity and transcription of hepatic 5β-reductase were investigated *in vitro* in liver and in hepatoma cells. The effect of bile acids on HPA activity was assessed *in vivo* in rats following dietary manipulation [18] or BDL. Glucocorticoid metabolism was also studied in humans following obstruction of the common bile duct by gallstones.

Materials and methods

Sources unless stated: solvents (Rathburn, Walkerburn, UK), cell culture reagents (Gibco BRL, Paisley, UK), molecular biology reagents (Promega, Southampton, UK), chemicals (Sigma–Aldrich, Poole, UK), radiochemicals (GE-Healthcare, Aylesbury, UK).

Effects of bile acids on enzyme kinetics in vitro

All experiments followed the guidelines of the Home Office, UK or the Danish Animal Experiments Inspectorate. Male Wistar rats (9 weeks; Harlan Olac, Bicester, UK) were sacrificed by decapitation (08:00 h) within 60 s of being disturbed.

$[^3H]_4$-Tetrahydrocorticosterone (5β-THB) (5β-reductase activity) was generated in hepatic cytosol (100 μg/ml protein), incubated 4 h with $[^3H]_4$-corticosterone (25 nM), corticosterone (975 nM, IC_{50}; 0.01–1000 μM, kinetics) and an NADPH-generating system [19]. Conversion of 5β-dihydrocorticosterone (5β-DHB; 2 μM) to 5β-THB (3αHSD activity) was measured following incubation (10 min) as above. 5β-THB was quantified by gas chromatography mass spectrometry (GCMS) [20].

Bile acids (chenodeoxycholic acid (CDCA), cholic acid (CA), deoxycholic acid (DCA), glyco-CDCA (GCDCA) or tauro-CDCA (TCDCA), (10^{-2}–10^{-9}M) were added to incubations. Inhibition of velocity (IC_{50}) was calculated relative to controls without bile acid. K_i values were calculated by a global fit model of competitive inhibition ($K_{mapp} = K_m * (1 + I)/K_i$; $Y = V_{max} * X/(K_{mapp} + X)$ using CDCA at its IC_{50}.

Effect of bile acids on transcript abundance in cultured cells

H4iiE cells (ECACC, UK), were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, UK) supplemented with foetal calf serum (10% v/v), penicillin (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) (37 °C, humidified carbon dioxide:air (5:95)). Cells were transferred to fresh media 1 h prior to the addition of bile acid (100 μM) or vehicle (ethanol, <1% v/v) and then incubated (16 h) in triplicate. Total RNA was isolated using Trizol® Reagent (Invitrogen, UK) and cDNA generated using First-Strand cDNA Synthesis Kit. Quantification of transcripts (normalised to cyclophilin A) was performed by qPCR (PRISM 7900, Applied Biosystems, UK). Primers and probes were either designed using *Primer Express* Software (5β-reductase, 3αHSD) or by Applied Biosystems (*Cyp7a1*, NM012942; cyclophilin A, NM017101).

Effect of bile acids on promoter activity of 5β-reductase

A fragment of the 5′ untranslated region of h*AKR1D1* (5β-reductase) representing the region –383 to +446 kb (relative to transcription start site) [21] was cloned from RPCI-11 HS BAC clone 386M10 (Invitrogen, UK). A construct was generated using high-fidelity PCR amplification using the following oligonucleotides, incorporating a *Kpn* site (underlined) and confirmatory sequencing performed.

5′AKR1D1 (−383), GGTACCAGTCCTGCTGCATCCAAATC;
3′AKR1D1 (+446) GGTACCTGTGGAGAACCTGACTGTAGGA.

Amplimers were inserted into the multiple cloning site of the promoter-less firefly luciferase reporter vector, pGL3-Basic, and their orientations were confirmed. Maxi DNA preparations of the luciferase construct were prepared using Qiagen maxi kits (Crawley, UK).

The human hepatoblastoma cell line, HepG2 (ATCC, Rockville, USA), was cultured in DMEM supplemented with foetal calf serum, L-glutamine and penicillin as above. Plasmid DNA (∼10 μg) was transfected [22] into cells, along with pCH110 plasmid (2 μg; β-galactosidase (β-Gal), Amersham, UK). CDCA (50 μM) or ethanol was added 24 h after transfection. Luciferase activity was assayed in cell lysates 72 h after transfection [23]. Transfection efficiency was assessed by β-Gal activity assayed using the Tropix Galacto-Light kit (Cambridge Bioscience, UK). Experiments were performed in triplicate three times utilising more than one preparation of plasmid.

Effects of bile acids in vivo in rats

Dietary manipulation of bile acids

Male Wistar rats (4–6 weeks; $n = 8$/group) were singly housed (6 days). In the first protocol, animals received standard chow ± CDCA (1% w/w, 4 weeks). In the second, they received a fat-free (FF) diet (D05052506; Research Diets Inc, USA) ± CDCA (1% w/w; D05052507) instead.

Daily production of glucocorticoids was studied in urine from animals housed in metabolic cages for 6 days, after 3 weeks on their respective diets. In the first protocol only, responses to restraint stress were studied after 2.5 weeks of the diet. Animals were acclimatised to handling (7 days) and then placed in restraint tubes (20 min, 08:00) and returned to normal cages. Blood was obtained at 0 (immediately prior to restraint) and at 20, 40, 60, and 90 min following restraint.

Bile duct ligation (BDL)

The common bile duct was ligated in male Wistar rats ($n = 6$/group; M&B Ejby, Denmark) or rats subjected to sham surgery. Rats were sacrificed by decapitation after 7 weeks, when jaundice and hepatosplenomegaly were evident and decompensated liver failure had occurred [13].

Ex vivo measurements

Enzyme activities were determined at the following substrate concentrations: 5β-reductase (25 nM, 1 μM) and 3αHSD (1 μM). Plasma and hepatic biochemistry and urinary steroids were quantified [19,20,24,25]. Bile acids were quantified using a Total Bile Acid Kit (Trinity Biotech, Ireland) [26], and liver function tests were quantified using a Modular P Analyser (Roche Diagnostics, Switzerland). Transcripts of 5β-reductase and *Cyp11b1* were quantified by qPCR [19,27] and normalised to 18S RNA or β-actin (Applied Biosystems), respectively. Transcript abundance of *Cyp7a1* was quantified by northern blot analysis [24] and normalised to U1 (M14386) [19].

Cortisol metabolism in obstructive jaundice in humans

With Local Ethical Committee approval (06/S1103/38) and written informed consent, women ($n = 8$; 48.2 ± 7.0 y) were recruited following hospitalisation with obstructive jaundice secondary to gallstone disease and studied prior to endoscopic retrograde cholangiopancreatography (ERCP). Healthy control women ($n = 5$; 40.0 ± 7.4 y) were recruited by advertisement. Subjects receiving systemic corticosteroid therapy within 3 months were excluded. Serum biochemical data were recorded, and an overnight urine collection obtained the night before ERCP and steroids was quantified by GCMS [28].

Statistical analysis

Results are mean ± SEM. *In vitro* data were analysed by ANOVA with post hoc Fisher LSD tests. *In vivo* data were analysed by unpaired Student's *t* tests or by one-way or repeated measure ANOVA. Correlations were analysed by Pearson's product moment and partial correlation analyses.

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