Diplomarbeit

Effects of cholestasis, bile acids and FXR on the adrenal glands

eingereicht von

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zur Erlangung des akademischen Grades

Doktor der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt im

Labor für Experimentelle und Molekulare Hepatologie

Klinische Abteilung für Gastroenterologie und Hepatologie

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Graz, am 11.05.2015

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Acknowledgement

First of all, I would like to express my gratitude to my supervisor Gernot Zollner for his brilliant guidance throughout the process of finishing my thesis and all the opportunities he provided me during this time. His instructions were worth its weight in gold.

For widely opening the doors to his laboratory and allowing me to enter the world of science cordial thanks to Professor Peter Fickert.

Of course, many thanks to Martin Wagner for his precious advice.

I also highly appreciate the help of all the former and current lab and group members Kati, Caro, Didi, Judith, Dagmar, Silvia, Andrea, Elisabeth, Franziska and Lei. They have supported me exemplarily in the past and I am sure they will still have words of advice for me in the future.

Last but not least thanks to my friends and family for their support.

Thank you all so much.

Abstract (German)

Hintergrund: Cholestatische Lebererkrankungen gehen mit abnorm hohen Konzentrationen von im Blut zirkulierenden Gallensäuren einher. Ältere Publikationen zeigen, dass Cholestase Remission in Patienten mit rheumatoider Arthritis induziert. Bei Patienten mit alkoholischer Hepatitis und Ikterus wurde ein Hypercortisolismus beobachtet. Verschiedene Tiermodelle zeigen eine veränderte Nebennierenrinden- (NNR) Funktion bei Cholestase, aber vieles wurde bislang noch nicht im Detail untersucht. Ziel dieser Arbeit ist es, den Einfluss von Cholestase und Gallensäuren auf die NNR-Funktion zu untersuchen. Hierzu beobachteten wir die NNR-Funktion in gallengangsligierten und mit Gallensäuren gefütterten Mäusen um deren Effekte herauszufinden und die Rolle des Gallensäurerezeptors FXR zu charakterisieren.

Methoden: Mäuse wurden für 3 Wochen (w) einer Gallengangsligatur (CBDL) unterzogen bzw. mit einer mit Chenodeoxycholsäure (CDCA)-angereicherten Diät gefüttert. Eine 7-tägige CBDL wurde bei FXR Wildtyp (WT) und Knockoutmäusen (KO) durchgeführt. Corticosteron (das Maus Homolog zum humanen Cortisol) wurde mittels ELISA im Serum gemessen. Die Geneexpression der im Corticosteronmetabolismus involvierten Enzyme in Nebenniere (NN) und Leber und die des Cholesterinstoffwechsels in der NN wurde mittels qPCR bestimmt. Der Lipidgehalt der NNR wurde mittels Ölrotfärbung visualisiert.

Ergebnisse: Die Corticosteronspiegel waren nach CDCA Fütterung und 3w CBDL etwa verdoppelt. CBDL und CDCA erhöhten in der NN die mRNA Expression der Enzyme SR-BI (Cholesterinaufnahme), HMG-CoA Reduktase und Lanosterol-14a-Demethylase (Cyp51) (Neusynthese) und CBDL verminderte die Transkription von Abca1 (Cholesterinabtransport). Die mRNA der Corticosteron-synthetisierenden Enzyme blieb unverändert. In der Leber war der Corticosteronabbau vermindert (3α-Hydroxysteroiddehydrogenase, 5β-Reduktase mRNA). Der Verlust von FXR hatte weder Einfluss auf die Corticosteronspiegel noch auf die Expression der untersuchten Gene. CBDL führte zum FXR unabhängigen Verlust von Lipiden in der NNR.

Schlussfolgerungen: Cholestase und Gallensäuren beeinflussen die NNR-Funktion deutlich. Dies ist durch die gesteigerte Synthese und Aufnahme von Cholesterin, der Ausgangssubstanz für die Steroidbiosynthese, in der Nebenniere und durch den verminderten Corticosteronabbau in der Leber zu erklären. FXR spielt hierbei keine Rolle.

Abstract (English)

Introduction: Cholestasis is a condition with abnormal high bile acid levels. There is evidence that cholestasis induces remission in patients with rheumatoid arthritis. Furthermore, patients with alcoholic hepatitis, who are jaundiced have increased cortisol levels. Also, in animal experiments cholestasis alters adrenocortical function. The link between cholestasis and adrenocortical function has not been investigated in detail so far. Therefore, we hypothesized that systemic accumulation of bile acids during cholestasis affects adrenocortical function and glucocorticoid output. We assessed adrenocortical function in mice with obstructive cholestasis and in response to bile acid feeding to determine the role of bile acids and tested the impact of the major bile acid receptor FXR in mice with genetic depletion of FXR.

Methods: Mice either underwent common bile duct ligation (CBDL) for 3 weeks (w) or were fed a chenodeoxycholic acid (CDCA)-enriched diet for 4 days (d). The role of FXR was assessed in 7d CBDL FXR wildtype (WT) and knockout (KO) mice. We assessed serum levels of corticosterone (rodent homologue to human cortisol), determined mRNA expression levels of enzymes involved in adrenal cholesterol metabolism, steroid synthesis, and hepatic steroid breakdown and visualized adrenal lipid content with oil red O staining.

Results: 3w CBDL and 4d CDCA-feeding resulted in two fold elevated serum corticosterone levels. SR-BI (adrenal cholesterol uptake), HMG-CoA reductase and lanosterol-14α-demethylase (Cyp51) (cholesterol *de novo* synthesis) mRNA levels were increased in adrenal glands after CBDL and CDCA feeding while Abca1 mRNA (cholesterol export) was decreased after CBDL. Enzymes mediating adrenal corticosterone synthesis remained unchanged after CBDL and CDCA feeding. Hepatic corticosterone breakdown (5β-reductase, 3α-hydroxysteroid dehydrogenase mRNA) was reduced in both treatment groups. Genetic loss of FXR had no impact on serum corticosterone levels or mRNA expression levels in 7d BDL mice. We observed FXR independent loss of neutral adrenal lipids.

Discussion: Obstructive cholestasis and bile acid feeding lead to hypercorticosteronemia in mice via increased adrenal cholesterol uptake and

biosynthesis (as substrate for adrenal steroid synthesis) as well as decreased hepatic corticosterone breakdown. These alterations are independent of FXR.

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Abbreviations

Acat1	acyl-coenzyme A:cholesterol acyltransferase 1
Acc	acetyl-CoA carboxylase
ACTH	adrenocorticotropic hormone
AI	adrenal insufficiency
ALT	alanine transaminase
ANIT	α-naphtylisothiocyanate
AP	alkaline phosphatase
AST	aspartate transaminase
AVP	arginine vasopressine
BA	bile acid
CA	cholic acid
cAMP	3´-5´-cyclic adenosine monophosphate
CBDL	common bile duct ligation
CDCA	chenodeoxycholic acid
CE	cholesteryl esters
CEH	cholesteryl ester hydrolase
CIRCI	critical illness-related corticosteroid insufficiency
CRH	corticotropin releasing hormone
Cyp11a1	cholesterol side chain cleavage enzyme
Cyp11b1	11β hydroxylase
Cyp21a1	21 hydroxylase
DCA	deoxycholic acid
DHEA	dehydroepiandrosterone
ER	endoplasmic reticulum
Fas	fatty acid synthase
FC	free cholesterol
FXR	farnesoid X receptor
FXRE	farnesoid X receptor responsible element

GC	glucocorticoid
GRE	glucocorticoid response elements
HDL	high-density lipoprotein
HE	haematoxylin and eosin
HPA	hypothalamic-pituitary-adrenal
HSD/Hsd	hydroxysteroiddehydrogenase
Hsd3b1	3β hydroxysteroid dehydrogenase type 1
Hsl	hormone-sensitive lipase
KO	knockout
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LpX	lipoprotein X
MC2R	melanocortin type 2 receptor
Nceh1	neutral cholesterol ester hydrolase 1
PBC	primary biliary cirrhosis
PBS	phosphor-buffered-saline
PSC	primary sclerosing cholangitis
PKA	protein kinase A
PKC	protein kinase C
POMC	proopiomelanocortin
PVN	paraventricular nucleus of the hypothalamus
qPCR	real time quantitative polymerase chain reaction
RAI	relative adrenal insufficiency
RXR	retinoid x receptor
SR-BI	scavenger receptor class B member 1
SD	standard deviation
TC	total cholesterol
TNF	tumor necrosis factor
VLDL	very low-density lipoprotein
WT	wild-type

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1 Introduction

1.1 Clinical background

1.1.1 Jaundice induces hypercortisolism

As early as 1897 observations began to emerge in the literature regarding ameliorating effects of severe jaundice on the autoimmune disorder rheumatoid arthritis. During the early 20th century the American physician Philip Hench started to review the existing literature concerning this issue and to pedantically write down his own observations. According to him, only severe jaundice induced by cholestatic liver diseases but not haemolytic anaemia was able to instantly stop disease progression. Hench also found that not only cholestasis but also other conditions like pregnancy, starvation, surgery or even anaesthesia without surgery were able to prevent progression of rheumatoid arthritis for a short period of time. His research finally lead to the discovery of the biological effects of cortisol and its application in the treatment of rheumatoid arthritis for which he was awarded with the Nobel Prize together with Kendall and Reichstein in 1950. The exact mechanisms by which cholestatic diseases alleviate the destructive joint disorder remain unknown but an induced hypercortisolism could be the common denominator (1–3).

Alcoholic liver disease could lead to cortisol excess and a condition called “pseudo-Cushing´s syndrome”. Burra et al. characterised the endocrine status in patients with fatty livers due to alcohol ingestion, alcoholic hepatitis and alcoholic cirrhosis. All three groups were comparable regarding the amount of alcohol ingestion. Patients with alcoholic hepatitis and cirrhosis had elevated cortisol concentrations in the blood compared to the ones only with fatty liver changes (at least when normalised for the impaired synthesis capacity of the livers of the cirrhotics). They also found a positive correlation between cortisol and bilirubin. Taken together these data suggest that in alcoholic liver injury alcohol itself is not the cause for the increased circulatory cortisol (4).

In a more recent study Zietz et al. showed that patients suffering from short-term obstructive cholestasis due to tumours have significantly elevated basal cortisol levels. In patients where obstruction in bile flow was not caused by tumours the

concentration of cortisol was not significantly elevated and elevation of cholestatic parameters was less pronounced. Interestingly, the cortisol / adrenocorticotropic hormone (ACTH) ratio was higher in patients with obstructive cholestasis suggesting ACTH-independent stimulation of the adrenal glands. It has been speculated that circulatory cytokines could significantly contribute to this finding (5).

Taken together these studies indicate an altered glucocorticoid homeostasis in jaundiced patients with cholestatic liver diseases. Whether retention of cholephiles like bilirubin or bile acids could play a role is still unknown. Assuming that these cholephiles alter adrenal function, one might also expect similar alterations in various other cholestatic liver diseases rendering this concept a more general one.

Cholestatic liver diseases are characterized by either an attenuation of bile flow or the defective transport of cholephiles into the bile which results in systemic accumulation of bilirubin, bile acids and inflammatory cytokines. These diseases can be divided into hereditary and acquired ones.

The hereditary cholestatic liver diseases are extremely rare and include the progressive familiar intrahepatic cholestasis (PFIC) syndromes. Depending on which transporter is disrupted, they can be divided into three subgroups. Cystic fibrosis which is caused by a defective chlorid channel, manifests also with cholestasis.

The acquired forms of cholestasis include obstructive cholestasis, inflammation induced cholestasis, drug induced cholestasis, intrahepatic cholestasis of pregnancy, and the complex forms primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC).

Obstructive cholestasis is caused by obliteration or compression of the large bile ducts by gallstones, tumours or inflammation. Inflammation induced cholestasis is caused by alterations in activities for distinct bile acid transporters in response to cytokines. Intrahepatic cholestasis of pregnancy is induced by hormones together with minor hereditary modifications of bile acid transporters. A genetic predisposition might be obligate. In most cases drug induced cholestasis is elicited by idiosyncratic effects of the administered substances. The causes of PBC and PSC are

multifactorial and the exact pathogeneses still remains to be determined [reviewed in (6)].

Chronic cholestatic liver diseases lead to severe damage of the livers (7). Systemic accumulation of bile acids during cholestasis affects various organ systems (e.g. the kidneys (8) or the cardiovascular system (9)) via the activation of their respective receptors.

1.1.2 Relative adrenal insufficiency

In critically ill patients but also in patients with liver cirrhosis the opposite of hypercortisolism called adrenal insufficiency (AI) is a common disorder. The patients show features of AI like hypotension, decreased responsiveness to vasopressors or higher mortality. Regarding these circumstances, the term "hepato-adrenal syndrom" has been coined to describe adrenal insufficiency as extrahepatic manifestation of liver cirrhosis. Thus, a deeper understanding of pathophysiological mechanisms regarding the disruption of glucocorticoid homeostasis in liver diseases is necessary, so that we are able to adequately asses the seriousness of AI or potential hypercortisolism and then give the patient the individual therapy he needs [reviewed in (10,11)].

Several pathophysiological models for development of AI exist. First, insufficient glucocorticoid (GC) production due to lack of circulatory cholesterol - mainly HDL cholesterol - results in either low basal plasma cortisol levels or a low stress response. To be more precise, the cortisol concentrations could be at least to low regarding the severity of the underlying illness, which explains the synonym relative adrenal insufficiency (RAI). Second, the intensive proinflammatory status of the patients plus their higher receptiveness to bacterial infections may result in AI. Increased levels of circulating cytokines (i.e. interleukins and tumor necrosis factor (TNF) α) may alter the function of the whole hypothalamic-pituitary-adrenal (HPA) axis (12). Third, hemodynamic and circulatory changes as a consequence of the reduced patient´s general health condition like attenuated adrenal blood flow, haemorrhages or necroses could cause impairment of the adrenals´ power to synthesize GC [reviewed in (10,11)] (13).

Despite the poor knowledge of its pathophysiology, the main problem until now is the absence of accurate diagnostic criteria of the hepato-adrenal syndrome. The lack of a consensus on diagnosis is the reason for the rather huge difference of the prevalence of AI found in literature ranging from 7% to 87% [reviewed in (10,11)].

A more recent paper gives us the hint that probably the appearance of AI is overestimated due to time biases in measurement of plasma cortisol as well as the fraction of cortisol measured. In this study, plasma free cortisol of cirrhotic patients and healthy controls was assessed for a 24-hour-period directly to avoid errors that can occur because of altered concentrations of glucocorticoid-binding proteins because of impaired liver function. The main finding was a delayed 24-hour cortisol rhythm that resulted in significant differences if measured at 08:00. Nonetheless, the lower plasma free cortisol level was only the result of the delay. In consequence, the assessing of AI may be very complex. Further investigations concerning this issue are necessary, as well as a confirmation of the results with a greater number of participants would be recommendable in order to confirm the findings (14).

In critical illnesses such as severe sepsis, a phenomenon called critical illness-related corticosteroid insufficiency (CIRCI) can be observed. Recently, Boonen et al. showed elevated basal levels of cortisol indicating that only the stress response is severely impaired. Therefore, it might be better to speak of relative adrenal insufficiency (RAI). The group claims that the reason for the found hypercortisolemia is an impaired metabolism of glucocorticoids. In this work a positive correlation between bile acids (BA) versus the level of plasma cortisol was shown. Thus bile acids could take part in the origin of RAI in liver diseases (15).

Most of the studies use the total cortisol response as the main parameter to assess RAI. That might not be adequate due to higher baseline cortisol levels as well as lower levels of corticosteroid binding globulin (CBG) and albumin, the proteins transporting cortisol in the blood (16), hence necessarily lowering total cortisol concentrations, whereas the free and active cortisol fraction remains unchanged. Thus, RAI could be overestimated (10,15).

Fatigue is another common disorder in patients with liver diseases. Swain and coworkers assumed that this may be caused by defective central corticotropin

releasing hormone (CRH) release in the entire brain. CRH fibres are widely spread in our brain and are the upstream regulatory unit controlling the adrenal glands. So there may be a connection between liver diseases, defective CRH metabolism and adrenal insufficiency (13,17).

Taken together, whether the lack of consensus on the diagnostic criteria leads to a misinterpretation of the glucocorticoid status in patients with liver cirrhosis is unknown. Due to impaired CBG and albumin synthesis in the liver, the prevalence of AI could be overestimated. What factor causes the alterations is also unknown but BA might be involved.

1.2 Bile acids act via their nuclear receptor FXR

Bile acids accumulate during cholestatic liver diseases. Bile acids are amphipathic molecules derived from cholesterol and produced in hepatocytes. They fulfil many physiological important purposes. Bile acids are a crucial detergents in the bile and hence necessary for maintaining bile flow, bile solubility and cholesterol elimination. They also contribute to cholesterol clearance simply by their synthesis. Other functions are, for instance, enabling the resorption of hydrophobic molecules such as vitamins or lipids in the intestine. As close relatives of endocrine steroid hormones bile acids also take part in complex signalling activities via distinct bile acid receptors (18,19).

Mice have a more hydrophilic bile acid pool compared to humans. In human blood, the most abundant bile acids are the primary bile acids (synthesised in the liver from cholesterol), chenodeoxycholic acid (CDCA) and cholic acid (CA) and the secondary bile acid (primary BA are metabolised by bacteria in the gut and then absorbed by enterocytes) deoxycholic acid (DCA). The unconjugated forms of these free bile acids together with their corresponding glycine conjugates account for approximately three quarters of circulatory bile acids (20). However, mice show negligible concentrations of CDCA but elevated levels of α/β/ω muricholic acids (MCA) compared to humans. In contrast to humans, the taurine-conjugated bile acids overrule the glycin-conjugated ones in rodents (21–23).

The central BA receptor, farnesoid X receptor (FXR, NR1H4), is a nuclear transcription factor and was first cloned in 1995. Before that it was called an “orphan” nuclear receptor with unknown ligand binding activity. The study by Forman et al. revealed supraphysiological concentrations of farnesol and its derivatives as natural ligands. FXR acts as a heterodimer together with the retinoic X Receptor (RXR) (24). In 1999, three works revealed that bile acids modulate the activity of FXR but already at physiological concentrations (25–27).

FXR is highest expressed in liver, intestine and kidney, but immunohistochemical tissue analysis also showed robust FXR expression in the adrenal glands of mice (28), which has also been reported preciously (24). In particular, zona reticularis shows numerous FXR-stained nuclei, whereas they are more fainted in zona

fasciculata and not detectable in zona glomerulosa and the adrenal medulla (28). The results were confirmed by in vivo imaging studies. Despite the low baseline activity of FXR, it was possible to gain a more intense signal from the murine adrenal glands after GW4064 administration, a potent synthetic FXR agonist (29). Nevertheless, the function of this important nuclear BA receptor in the adrenal glands still remains unknown.

1.3 Hypothalamic-pituitary-adrenal axis

Stress is a conquering condition caused by an internal and external stressor, which threatens our body´s homeostasis. To save the equilibrium, we have two systems working synergistically and complementarily: on the one hand, there is our autonomic nerve system. On the other hand, there is a complex neuroendocrine system, the hypothalamic-pituitary-adrenal (HPA) axis. This axis consists of three morphologically distinct units. Two are located in the central nervous system of the vertebrates whereas the last one, the adrenal gland, is located in the abdomen.

1.3.1 Anatomy and physiology of the HPA axis

The topmost level of the HPA axis is located in the hypothalamus. It is a subdivision of a small amount of cells arranged symmetrically beside the third ventricle. This area is called the parvocellular division of the paraventricular nucleus of the hypothalamus. These cells get many afferent signals from overall the brain indicating their central role in stress adaption. Their main product is a 41 amino acid-long peptide, which is called corticotropin releasing hormone (CRH). It is secreted in an ultradiurnal pulsatile pattern. The cells project to the median eminence where they release their product into the pituitary portal vessel system, which penetrates the anterior pituitary.

CRH stimulates the corticotropic cells in the anterior lobe of the pituitary gland through the CRH receptor 1. Activation of CRH receptor stimulates the expression of proopiomelanocortin (POMC), a long peptide which acts as precursor for the adrenocorticotropic hormone (ACTH). Besides CRH, the parvocellular cells of the PVN also synthesize arginine vasopressine (AVP). This nine amino acids-containing hormone potentiates the effects of CRH on the specific cells of the anterior pituitary. It connects with the V1b receptor on the cell surface and acts through the protein kinase C (PKC) pathway.

ACTH is secreted into the systemic circulation by pituitary corticotropic cells in a pulsatile fashion like CRH. It reaches the adrenal glands and binds to the melanocortin type 2 receptor (MC2R) which is mainly expressed on cells of zona fasciculata. By inducing the cAMP pathway it causes a fortified excretion of

glucocorticoids. This class of steroid hormones – in humans mainly cortisol and in rodents corticosterone – exerts its action by binding to the glucocorticoid receptor (GR), another nuclear transcription factor.

The inactive form of the GR is retained in the cytosol of the cells. After binding its ligand it forms homodimers and translocates into the nucleus where it binds to glucocorticoid response elements (GRE) on the DNA. The glucocorticoids exert a feedback inhibition to the hypothalamus and the pituitary gland and inhibit the production and secretion of CRH, AVP and ACTH. By this mechanism they regulate their own homeostasis via a negative feedback loop [reviewed in (30–33)].

1.3.2 Adrenal glands

The adrenal glands are paired organs which are symmetrically located in the abdomen of vertebrates. They are located at the upper poles of the kidneys. The adrenal glands can be divided in two totally different parts, what embryogenesis, regulation and function is concerned. The inner mass is called the adrenal medulla. It derives from ectodermal neural crest and is functionally as well as morphologically part of the autonomic nerve system. The outer layer, also known as the adrenal cortex, emerges from the mesoderm and is a component of the endocrine system. The cortex consists of three distinct zones: the exterior section right beneath the capsule is termed zona glomerulosa. Its main product is the principal mineralocorticoid aldosterone. The middle zone is the zona fasciculata, where most of the glucocorticoids are synthesized. The inner layer is the zona reticularis, where androgens (i.e. dehydroepiandrosterone (DHEA) and androstendione) are produced. In contrast to humans, mice lack a functionally or morphologically distinct zona reticularis (34). This may be due to the lack of the two major enzymes required for sex hormone synthesis named Cyp17 – restricted in rodents to gonads and placenta (35) – and Sult2a1 (36).

The mouse adrenal glands weigh about 2.5 mg to 7.5 mg, the female ones being heavier. Most of the volume consists of the zona fasciculata. However, a special feature of the mouse´s adrenals is the so called X-zone. It is the innermost zone in the rodent adrenal cortex. Its function remains to be determined (34).

1.3.3 Circadian glucocorticoid rhythm

The glucocorticoid secretion follows a diurnal rhythm. In humans peak concentrations are reached in the morning while in the night hours the synthesis is regulated down to a minimum (33). Rodents are nocturnal animals. Thus they have their peak corticosterone concentrations at around 20:00 (37–40).

1.4 Corticosterone synthesis in mice

The substrate for adrenal glucocorticoid synthesis is cholesterol. The first critical step required for production of corticosterone is supply of adequate intracellular cholesterol. Four pathways play pivotal roles in maintaining murine adrenal cholesterol homeostasis: [1] uptake of circulatory cholesterol, [2] de novo synthesis, [3] formation and hydrolysis of intracellular cholesteryl ester droplets and [4] efflux of unesterified cholesterol to avoid toxic accumulation (summarised in ***Figure 1***).

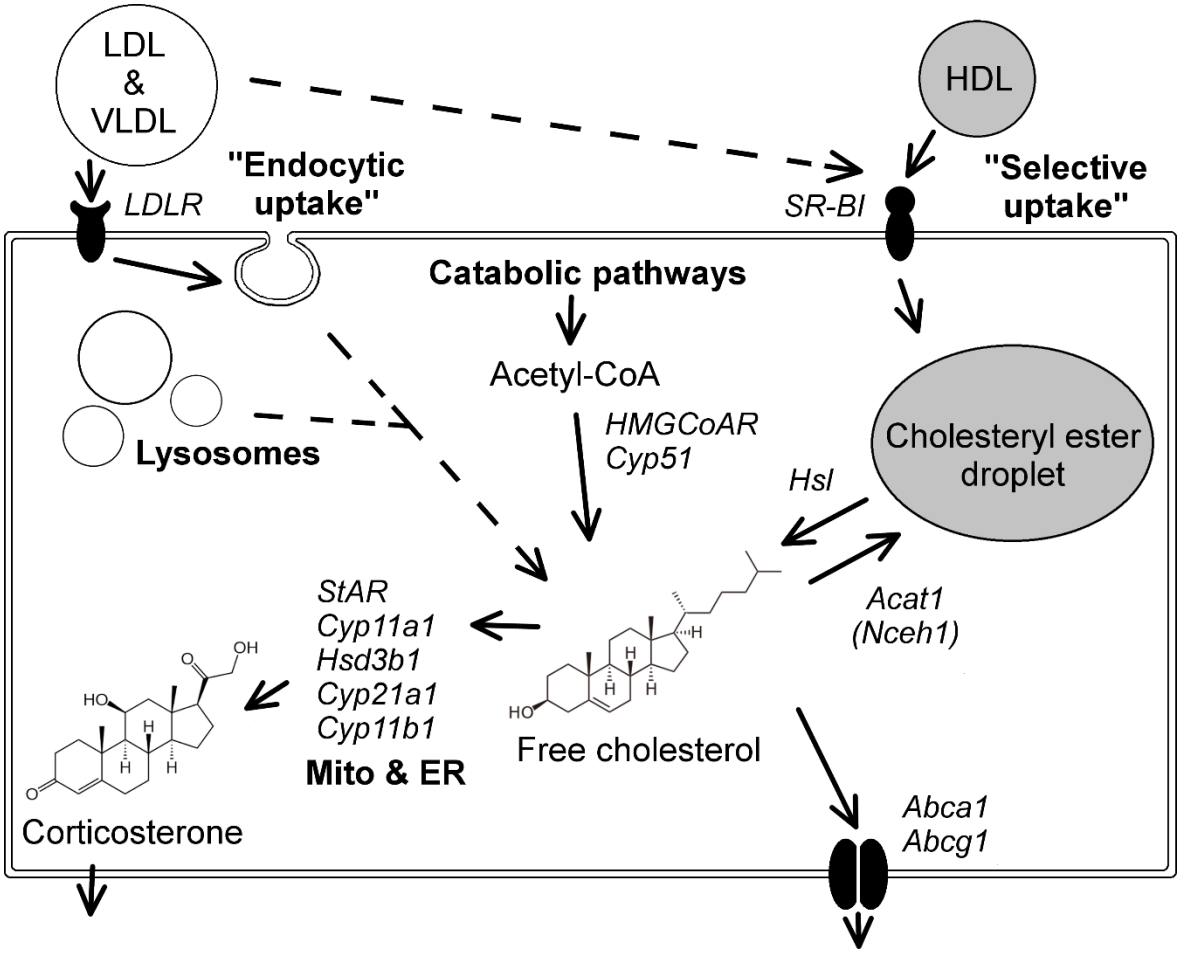

Figure 1 Adrenal cholesterol utilisation (modified after *(41)*)

Dashed lines indicate quantitatively minor pathways. Enzymes are in italic. Mito, mitochondrion; ER, endoplasmic reticulum; LDLR, LDL receptor

Cells of the adrenal cortex have two pathways for the uptake of cholesterol from the blood stream [reviewed in (41)]. On the one hand, the cells internalize low-density lipoprotein (LDL) via the action of the LDL receptor, which is subsequently degraded in lysosomes. This pathway only plays a minor role since loss of LDL receptor has no major effects and the LDL receptor is not able to compensate when the second

pathway is impaired (42,43). On the other hand adrenal cells selectively absorb the circulatory cholesteryl esters (CE) in a scavenger receptor class B member 1 (SR-BI)-dependent manner. The main substrate for SR-BI is high-density lipoprotein (HDL) (44,45). In addition SR-BI is also a receptor for apolipoprotein B and apolipoprotein E containing lipoproteins (i.e. LDL, VLDL) (42,46,47) and most of the cholesteryl esters derived from LDL are taken up via this SR-BI-mediated pathway (48). Uptake of cholesteryl esters via SR-BI might be the most important factor for adrenal cholesterol homeostasis and further glucocorticoid production. Mouse adrenals are not able to compensate the loss of SR-B1 leading to a diminished corticosterone production (43). Furthermore, loss of circulatory cholesteryl esters leads to impaired adrenal function (49). Lowering circulatory cholesterol content in rats leads to nearly 50-fold induction of adrenal de novo cholesterol synthesis, which, however, cannot compensate for the reduced uptake leading to deprivation of the stored cholesterol in the adrenal glands (44). Taken together, SR-BI mediated uptake of HDL linked cholesteryl esters is critical for sufficient acute steroidogenesis.

Cholesterol is stored in esterified form in the adrenal glands. The enzyme required for conversion of cholesterol and free fatty acyl-coenzyme A to cholesteryl esters in adrenal glands is acyl-coenzyme A:cholesterol acyltransferase 1 (Acat1) (50,51). It accounts for nearly the whole amount of adrenal cholesterol esterification activity (52,53). A similar enzyme, Acat2, is highly expressed in the liver and intestines of mice but not in their adrenal glands (54).

Hormone-sensitive lipase (Hsl) is the major neutral cholesteryl ester hydrolase (CEH) in the adrenal cortex. It catalyses cleavage of cholesteryl esters. Other hydrolases such as neutral cholesterol ester hydrolase 1 (Nceh1) have a negligible role compared to Hsl because they account for less than 10% of CEH-activity and are not able to compensate for the loss of Hsl (55). Hsl not only empties stored cholesteryl ester depots on demand but also enables the usage of cholesterol taken up via SR-BI (48). Experiments using knockout (KO) mice underline the pivotal role of Hsl in adrenal function. In vitro determination of the corticosterone output of primary adrenocortical cells reveals insufficient production both under basal as well as under stimulated conditions (48).

Cholesterol is synthesized de novo from acetyl-CoA. HMG-CoA reductase, a membrane embedded enzyme in the ER which catalyses the reaction of HMG-CoA to mevalonic acid, is considered the rate limiting step of cholesterol de novo synthesis. Another downstream acting enzyme, lanosterol 14α demethylase, indirectly affects the activity of HMG-CoA reductase via its own activity and can therefore also be considered a key regulatory control point (56).

Cholesterol export is mediated by Abca1 and Abcg1, two transmembrane proteins, protecting the cells against potentially toxic accumulation of free cholesterol (57–60).

The first direct and rate-limiting step of steroid hormone synthesis is the transport of cholesterol into the mitochondria by the steroidogenic acute regulatory protein (StAR). StAR is a 30kDa protein derived from a 37kDa precursor in the cytoplasm. It is located at the outer mitochondrial membrane where it enables the transport of free cholesterol to the inner mitochondrial membrane so the next step of steroidogenesis can occur (61–63). In the mitochondria cholesterol is transformed into pregnenolone by the cholesterol side chain cleavage enzyme (Cyp11a1, P450scc). 3β hydroxysteroid dehydrogenase type 1 (Hsd3b1), the mouse orthologue of human HSD3B2, catalyses the further reaction of pregnenolone to progesterone in both the endoplasmic reticulum (ER) and the mitochondria. Progesterone diffuses into the ER that contains 21 hydroxylase (Cyp21a1). Hydroxylation at C21 generates 11-deoxycorticosterone, which travels back to the inner mitochondrial membrane. In the zona fasciculata the last step of steroidogenesis is the formation of corticosterone by the action of 21β hydroxylase (Cyp11b1). In zona glomerulosa this reaction is followed by the synthesis of aldosterone which is catalysed by the aldosterone synthase (Cyp11b2, P450aldo). The steroid hormones are released into the systemic circulation by diffusion (35) (***Figure 2***).

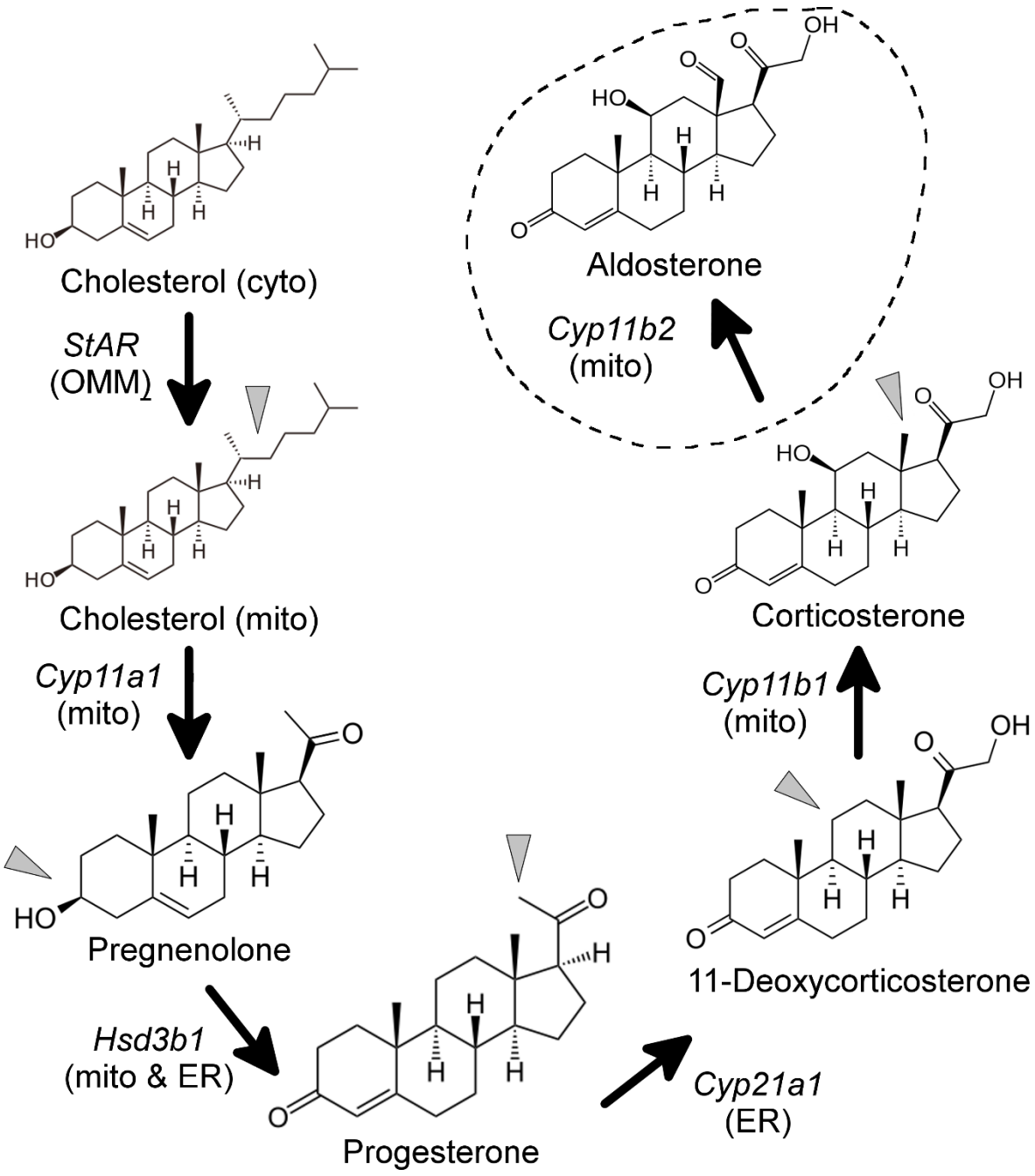

Figure 2 Biosynthesis pathway of steroid hormones in the adrenal gland

The name of the molecule is located under its structural formula. Enzymes, which catalyse the distinct reactions are in italic. Their location is in the parentheses. Their site of action is indicated by the grey arrow. All these steps occur in zona fasciculata besides the last reaction circled by the dashed line. This reaction only takes place in zona glomerulosa. OMM, outer mitochondrial membrane; cyto, cytosol; mito, mitochondrium; ER, endoplasmic reticulum

1.5 Corticosterone breakdown in the livers of mice

Most of the corticosterone is excreted as polar metabolites in the urine and the bile. Nevertheless, a small but not negligible fraction exits the body without previous metabolization. Approximately one-tenth of urinary corticosteroids is free corticosterone (64). The rest is converted in the liver and kidneys before excretion. Biliary excretion is the preferable route for corticosteroid metabolites in mice accounting for about two-thirds of secretion (65). Half of the metabolites retain their 3-oxo-4-ene structure of the A-ring. The alterations in these molecules include for the most part an α-reduction of the carbonyl group on C20 and 6β-hydroxylation. It is not known in detail which enzyme accounts for most of the 20α-hydroxysteroid dehydrogenase (20α-Hsd) activity in the livers of mice. Three enzymes are described in the mouse which are able to catalyse this reaction: Akr1c6, Akr1c12 and Akr1c18. Previous data suggest that Akr1c6 might be the enzyme which catalyses reduction at C20 in the livers of mice (66–68). Disruption of the molecular integrity of the A-ring involve saturation of the double bond between C4 and C5, catalysed by either 5α- or 5β-reductase with a consecutive reduction of C3 by 3α-hydroxysteroid dehydrogenase (3α-Hsd). Northern and Western blotting as well as measurement of enzyme activity revealed the murine liver as the organ with the highest expression of 5α-reductase type 1. The other isoform, type 2, is mainly present in male animals (in adrenals and epididymis) (69). However, loss of type 1 of this enzyme resulted in no significant changes in basal plasma corticosterone levels in mice (70). Indeed, 5β-reduced metabolites are the prominent epimers (64). 5β-reductase is expressed in the murine liver and the rat enzyme shows a high affinity to glucocorticoids (71). The downstream-acting enzyme 3α-Hsd is also expressed in high levels in the liver (72) [reviewed in (73) with emphasis on the human situation]. 5β-reductase and 3α-Hsd are also part of the bile acid synthesis machinery (18) (***Figure 3***).

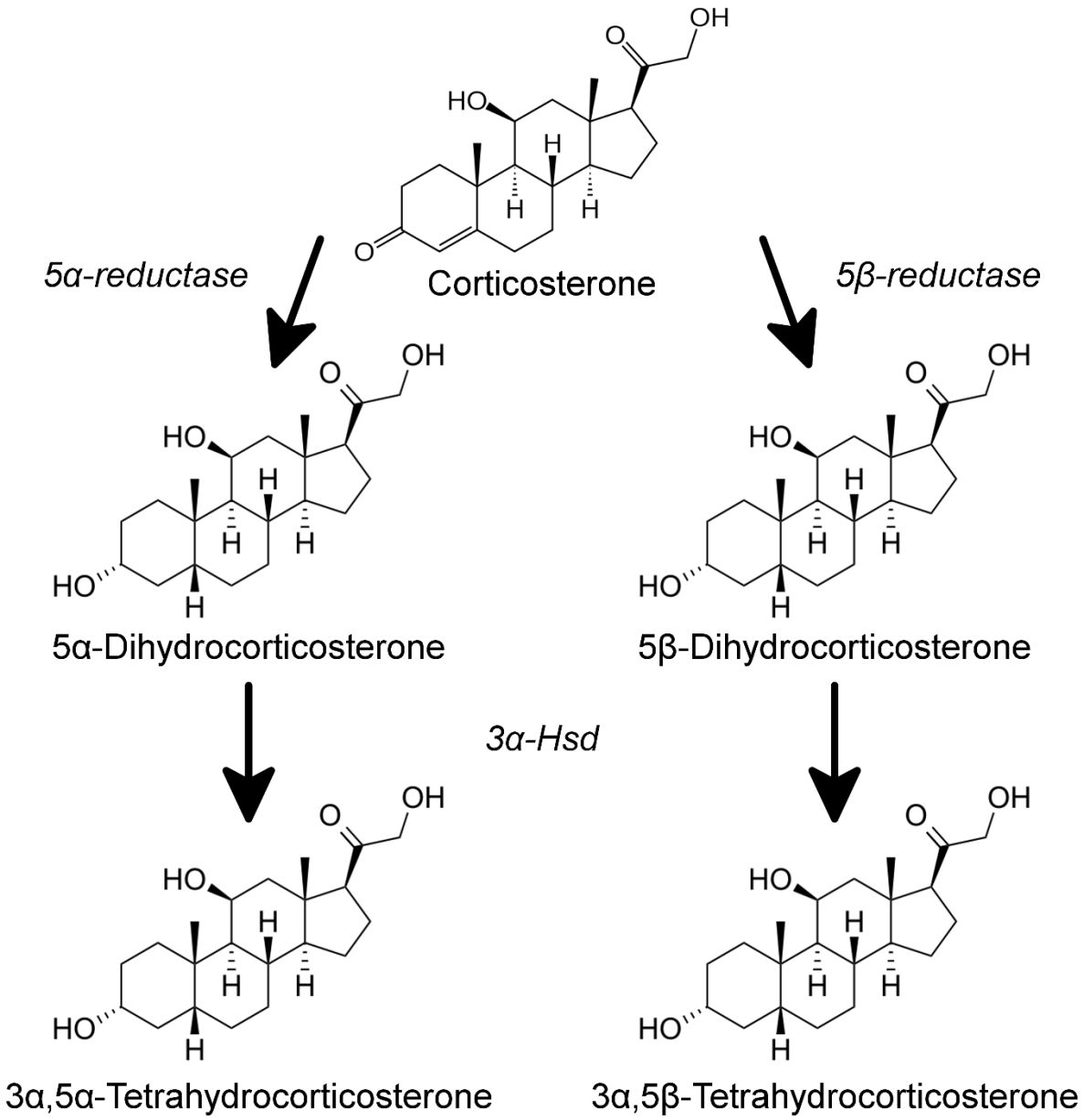

Figure 3 Corticosterone breakdown in the liver by 5α- and 5β-reductase and 3α-Hsd

Enzymes are in italic.

1.6 Modulation of adrenal steroidogenesis

1.6.1 Maintaining cholesterol homeostasis

Cholesterol is the substrate for glucocorticoid synthesis. The regulation of the enzymes involved in lipid metabolism is complex, involving alterations on both transcriptional and post transcriptional levels.

The main transcription factor maintaining intracellular lipid homeostasis is sterol regulatory binding protein (Srebp). There are three different isoforms: Srebp1a, Srebp1c, which are generated through the use of different promoters as well as alternative splicing and Srebp2. Srebp1c regulates mainly genes which express enzymes for triglyceride and fatty acid metabolism whereas Srebp2 is responsible for cholesterol biosynthesis. Srebp1a acts on both systems. Srebp´s form complexes with Srebp cleavage activating protein (Scap). Both are membrane embedded proteins. Additionally, Scap contains a sterol sensing domain measuring the content of free cholesterol in the cells. During depletion of the intracellular cholesterol pool Scap binds Srebp and escorts it from the endoplasmic reticulum (ER) to the Golgi apparatus where Srebp is cleaved by two proteases into its active transcription factor. This active form moves into the nucleus and activates the transcription of enzymes for cholesterol and fatty acid synthesis by binding to its response site. Insulin induced gene 1 (Insig1) is also one of Srebps target genes. Insig acts as its counterpart mediating negative feedback. There are also three different isoforms of Insig which are regulated differently but fulfil the same roles: Insig1, Insig2a, the liver specific form (74) and Insig2b. The Insig1 protein is degradated rapidly but strongly induced by insulin whereas Insig2 is constitutively expressed providing a functional reserve. But when free cholesterol accumulates Insig1 binds to the Srebp/Scap complex due to conformational changes of Scap because of cholesterol binding to the sterol sensing domain leading to stabilizing of the Insig1 protein and retaining the Srebp/Scap/Insig complex in the ER keeping it from activating cleavage in the Golgi. Insig itself is activated by binding oxysterols which accumulate, when free cholesterol accumulates. Insig also binds HMGCoA reductase which leads to ubiquitination and augmented degradation of the enzyme enhanced by binding of lanosterol (a metabolite of the cholesterol de-novo synthesis pathway) to a specific domain of HMGCoA reductase [reviewed in (75,76)].

1.6.2 ACTH is the master regulator of the adrenal glands

The major regulator of adrenal function and steroidogenesis is ACTH. This hormone derives from the pituitary gland and is necessary for normal adrenal steroidogenesis and adrenal cholesterol homeostasis as pointed out in experiments using mice lacking ACTH (77). ACTH acts mainly as a stimulation of the adenylyl cyclase via its binding to the G protein coupled transmembrane receptor MC2R. This results in increase of intracellular cAMP levels and leads to activation of the PKA. PKA alters the activity of various enzymes as well as of many of transcription factors by phosphorylating specific sites of the proteins (78). Downstream of ACTH and except the cAMP/PKA pathway are many other second messenger mechanisms regulating steroidogenesis including growth factors, macrophage-derived factors, steroidogenic-inducing protein, chloride ions and calcium messenger systems. However, they play a minor role compared with cAMP dependent mechanisms (79).

Chronic exposure of the adrenal glands to ACTH is required even to sustain basal steroidogenesis because of the short half time of circulatory ACTH (80).

ACTH is able to trigger corticosterone excretion of the adrenals to the maximum within thirty minutes. As already shown in the 1960s, inhibition of protein biosynthesis with the use of the potent substances puromycin and cycloheximide efficiently inhibits rapid steroidogenic response. Actinomycin D, an inhibitor of mRNA synthesis, has no effect concerning the acute stress response. Further experiments pointed out that the acute corticosterone response depends on protein synthesis of a distinct and labile enzyme prior to pregnenolone (80). The cycloheximide-sensitive factor and rate-limiting enzyme was identified to be StAR, the transporter for cholesterol into the mitochondria where further enzymes for steroidogenesis are located (61,62,79). However, intracellular StAR mRNA and protein levels increase not before one hour after ACTH administration in rats. This suggests that posttranslational modifications like enhanced cleaving of the precursor and phosphorylation of StAR may be the mechanism by ACTH that rapidly increases serum corticosterone concentrations. Indeed, phosphorylation at serine 194 of the murine protein induced by activation of the cAMP/PKA pathway approximately doubles its activity (81). In vitro experiments in Leydig cells suggest

mutual assistance of PKA and PKC for sufficient StAR phosphorylation and activity (82).

After the acute response and during the first few hours, mainly transcriptional and translational changes occur to maintain efficient steroidogenesis. Already in 1986 a group showed that ACTH administration increased transcription of Cyp11a1, Cyp21a1 and Cyp11b1 in primary bovine adrenocortical cells (83). Furthermore, microarray analyses revealed that ACTH increases the mRNA concentrations of the enzymes involved in cholesterol metabolism and steroid hormone biosynthesis including Star, Cyp11a1, Hsd3b1, Ldlr, SR-BI, HMGCoA reductase, Acat1 and Insig1, all of them mainly via pathways using PKA and PKC in Y1 mouse adrenal tumour cells (84). Another group found out that StAR mRNA levels began to rise after half an hour after ACTH administration in rats in vivo with a delayed increase in StAR protein, not obvious before one hour after ACTH treatment. Western blots revealed no major changes for the other four steroidogenic enzymes (85). Also SR-BI translation seems to be regulated by ACTH (77). Early functional analysis revealed that adrenal glands of rats treated with ACTH over four days showed increased cholesteryl ester content due to enhanced uptake of HDL (44). Accordingly, loss of ACTH production in mice resulted in depletion of adrenal CE content. Furthermore, ACTH-deficient mice showed decreased mRNA levels of StAR, increased levels of HMGCoA reductase and Ldlr and no change in levels of Acat1, Abca1, Hsl, SR-BI, Cyp11a1 and Cyp11b. Intracellular protein concentrations of StAR, SR-BI, Cyp11a1 and Cyp21a1 were reduced (77).

Long term stress adaption mediated by ACTH mainly appears by elevating the functional reserve of the adrenals by hypertrophy but not hyperplasia (77).

1.6.3 Potential role of FXR and bile acids on adrenal function

FXR is a central regulator of lipid homeostasis. This happens either through direct transcriptional effects involving other transcription factors or by indirect increase of intracellular cholesterol levels by suppressing bile acid synthesis in liver. This nuclear receptor is capable of altering the circulatory as well as the intracellular lipid composition. FXR might therefore play an important role in maintaining normal adrenal function because steroidogenesis is dependent on adequate supply of free cholesterol from the blood.

Activation of FXR might result in significant lowering of circulatory lipids and vice versa. FXR knockout (KO) mice show up to two fold elevated levels of total cholesterol (TC), cholesteryl esters (CE), free cholesterol (FC) and triglycerides in plasma as well as slight lipid accumulation in the liver, particularly in the form of triglycerides. These changes resulted in significant increase of the lipoprotein fractions high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) in the circulation (86,87). This could affect cholesterol supply for adrenal steroidogenesis.

This disruption of the normal circulatory cholesterol composition may be due to alterations of central enzymes in the liver. Loss of FXR leads to diminished transcription of lecithin-cholesterol acyltransferase and hepatic lipase in the liver which both are responsible for processing circulatory cholesterol and triglycerides. Furthermore, livers of FXR KO mice show attenuated mRNA levels of SR-BI, for cholesterol uptake, HMG-CoA reductase, for cholesterol synthesis and Hsl, for cholesterol hydrolysis. No significant change was found for Abca1. Additionally, besides the mRNA concentrations of SR-BI, also its protein concentrations were diminished and both mRNA and protein were inducible by cholic acid (CA)-feeding in WT mice (87). In contrast to the FXR KO mouse, stimulation of FXR activity with the potent synthetic agonist GW4064 in mice resulted in no significant change in hepatic mRNA expression for HMGCoA reductase but in decreased protein levels (88). The findings that Insig2a, the liver specific isoform, is up-regulated after FXR stimulation whereas lanosterol 14α-demethylase (Cyp51) transcription is down-regulated might explain this phenomenon. The diminished activity of Cyp51 results in an accumulation of lanosterol which, in cooperation with Insig2a, enhances the

degradation of HMGCoA reductase by proteasomes. This results in an overall halving of hepatic cholesterol de novo synthesis (88). That may explain the inhibitory effects of orally administered taurine-conjugated bile acids on HMG-CoA reductase in the livers of rats determined by in vitro assays (89). But feeding mice with CA did not bring significant changes of mRNA concentrations for HMG-CoA reductase and Abca1 in the liver (90). In contrast, hepatic transcription of HMG-CoA reductase was suppressed after CA feeding of mice. However, this decrease was absent in Shp KO mice, indicating a FXR/Shp dependent pathway (91). Nevertheless, treatment of rats with the hydrophobic bile acids CA and deoxycholic acid (DCA) resulted in diminished hepatic activity of HMG-CoA reductase whereas feeding of the more hydrophilic bile acid UDCA had no effect (92). The partially conflicting result may have occured due to the complex regulation of HMG-CoA reductase involving transcriptional alterations by the FXR/Shp/LRH-1 pathway as well as cholesterol/Srebp pathway and post-translation changes in enzyme activity (93).

However, studies using mice overexpressing Shp in their liver showed that the bile acid and FXR mediated alterations are multimodal. Shp transgene showed decreased levels of serum cholesterol but no changes in circulatory triglyceride levels could be observed. In fact, their livers accumulated fat. This may be due to indirect induction of Srebp-1c and PPARγ resulting in increased fatty acid synthesis. Further results include no change of HMG-CoA reductase mRNA levels but a direct decrease of Cyp51 mRNA. Further indirect effects include increase of Abca1 and diminished SR-B1 transcription (94).

Loss of FXR in the intestines resulted in increased absorption of dietary cholesterol as measured by using a dual isotope plasma ratio method (87). In line with this finding, mice that overexpressed Shp in their livers showed diminished absorption of ingested cholesterol determined by decreased serum concentration of β-sitosterol (94). This could partly be explained by an increased and diminished bile flow, respectively (87,94). In the ileum of FXR KO mice no change of mRNA levels for HMGCoA reductase were found but increased levels of Abca1 transcription (87).

Taken together, these data show effects of BA and FXR on regulating intracellular as well as circulatory cholesterol concentrations. Altered blood lipid content due to molecular alterations in the liver and the intestine might affect adequate cholesterol

supply for efficient steroidogenesis in the adrenal glands. If similar molecular alterations directly occur in the adrenal glands remains to be determined.

1.6.4 Potential role of cholestasis on adrenal function

Cholestasis (CBDL and ANIT) in rodent (rats and mice) animal models is associated with a high increase of lipids in the circulation. The disruption of the lipid composition in the blood is characterized by the appearance of abnormal lipoprotein X (LpX) and significant increases of TC, phospholipid and triglyceride concentrations. The raise of TC levels is mainly due to elevated FC but only minor changes regarding CE are observed (95–98). Cholestasis also leads to rearrangement of cholesterol in the lipoprotein fractions of rats and mice. CBDL and ANIT-administration in mice resulted in diminished HDL particles containing cholesterol but an increase of cholesterol in the VLDL and LDL fractions (95,98). However, performing CBDL in rats did not alter HDL cholesterol levels but LDL and VLDL cholesterol increased (96). These effects may be the result of a reduced activity of LCAT (95,98).

In the livers of CBDL rats increased concentrations of total and free cholesterol were found whereas CE decreased (97). However, Kamisako et al. found no differences in hepatic cholesterol content in their bile-duct-ligated rats (96). CBDL in mice resulted in increased hepatic TC and FC (95). The alterations in cholesterol homeostasis may be mainly due to increased hepatic cholesterol synthesis and HMGCoA reductase activity (95,97,98). HMGCoA reductase mRNA content in the livers of CBDL animals showed either no change (95) or increased levels (also in the intestine) (96). Other molecular alterations regarding cholesterol homeostasis which are reported during cholestatic liver diseases include: diminished hepatic protein levels of SR-BI and Abca1 in mice (95). In accordance with previous findings diminished mRNA levels of SR-BI in the livers of rats but no change of SR-BI mRNA in their intestines after CBDL (96).

We speculate that alterations in circulatory cholesterol content could alter adrenal steroidogenesis. The effects on the adrenal glands remain to be investigated in detail. In addition, if similar molecular alterations like in the rodent liver occur in their adrenal glands during obstructive cholestasis remains to be elucidated.

1.7 Effects of experimental cholestasis, bile acids and the FXR activators on the HPA axis

Several groups have investigated the effects of bile acids and/or cholestasis on adrenal gland function. Since these studies are crucial for understanding of our hypothesis, the results will be briefly summarized and discussed in this chapter.

Obstructive cholestasis induces functional und morphological alterations in adrenal glands of female rats. Cholestatic animals showed increased urinary excretion of 17-hydroxycorticosteroid as well as pronounced lipid accumulation in adrenal cortex cells (99).

Swain and co-workers postulated a central impairment of the HPA axis after CBDL caused by diminished CRH release from the hypothalamus. The reduction of hypothalamic hormone secretion could only be seen during stress. Thus, only the stress response evident as reduced CRH secretion was considerable impaired while the function of the HPA axis was maintained during basal conditions after CBDL. Consequently, also pituitary gland and adrenal glands, presented with a diminished reaction to a stressor. Moreover, after intraperitoneal ovine CRH administration, the truncal blood concentration of ACTH was lower in CBDL animals compared to sham ones. In line with these findings were the lower stress plasma corticosterone levels (100,101).

These data were confirmed by another study investigating CBDL- and ANIT-induced cholestasis. Gene expression analysis with qPCR and immunoblots revealed diminished synthesis of CRH in the hypothalamus, ACTH in the pituitary gland and steroidogenic enzymes Hsd3b and Cyp11b1 in the adrenal glands. These findings were in line with lower baseline corticosterone levels as well as with a decrease in the CRH immunofluorescence signal in the hypothalamus (102).

The altered GC concentrations during liver diseases may be due to altered metabolisation of corticosterone in the liver. A reduced activity of the GC degrading enzyme 5β-reductase in the liver and decreased mRNA levels of the steroidogenic enzyme Cyp11b1 after CDCA administration in the adrenals were observed as well as lower urinary output of corticosterone-metabolites. Lower plasma corticosterone

concentrations only appeared while combining CDCA intake with a fat free diet. After CBDL transcription of 5β-reductase, 3α-Hsd and Cyp11b1 decreased but no change in plasma corticosterone concentrations was found. In addition to the reduction of 5β-reductase mRNA, the group was able to show a diminished enzyme activity, so they are the first ones mentioned in this thesis who are able to directly contribute their results to BA (103).

Since bile acids are the main signalling molecules in cholestasis another study evaluated the effects of bile acid signalling via their receptor FXR on the adrenal glands. FXR KO mice and their wild type littermates were treated with GW4064, a specific FXR agonist. Surprisingly, no changes of mRNA levels of the FXR target genes Ostα, Ostβ and Shp were detected in adrenal glands in vivo. In contrast, incubating excised mouse adrenal glands with GW4064 resulted in induction of the FXR target genes, indicating that GW4064 might not be able to penetrate the organs in sufficient concentrations in vivo. To provoke cholestasis ANIT was used. Quantitative PCR revealed induction of Ostα, Ostβ and Shp but no change in Cyp11a1 and Hsd3b2 mRNA content in the ANIT-treated mice (104).

Experiments comparing the corticosterone concentration of FXR KO mice with their wild type littermates revealed no difference neither at basal levels nor after overnight fasting (i.e. a metabolic stressor). Thus, they claim that FXR does not alter adrenal function under physiologically conditions. However, GW4064 treatment resulted in higher plasma corticosterone levels after overnight fasting. Furthermore, enriched mRNA content of Ostβ and SR-BI (i.e. necessary for cholesterol uptake) in the adrenal glands, but lower adrenal esterified and unesterified cholesterol content were found after GW4064 treatmend. No changes were found concerning pituitary gland signalling (i.e. plasma ACTH concentration, adrenal weight and adrenal MC2R transcription) and steroidogenic enzymes mRNA expression (i.e. Cyp11a1, Cyp11b1, Cyp21a1, Hsd3b2). Higher expression of SR-BI which enhances cholesterol uptake from HDL and Ostβ that might facilitate corticosterone efflux (105) could contribute to a higher glucocorticoid synthesis and output rate reflected by a diminished adrenal cholesterol content, even despite unchanged mRNA levels of steroidogenic enzymes (106).

Another group used H295R adrenocortical cells, which are malignant human adrenal cells, and proved an FXR dependent increase of HSD3B2. But they did not find a FXRE in the orthologue of the mice, called Hsd3b1. This shows us that the mouse model only works with some restrictions (107).

Previous studies did not use an adequate time point to measure the corticosterone levels. Either blood samples were collected in the morning or it is not definitely defined when the blood was drawn. Therefore, results are questionable. By convention, cortisol is measured during peak concentrations in the morning in humans. Because mice as well as rats are nocturnal animals, their circadian rhythm is inverted, compared to humans. Due to this reason, the best time for measuring their glucocorticoid concentration would be in the evening, when peak levels can be achieved. If we use the maximum concentration and the correct time, differences will be meaningful.

1.8 Aims and Hypothesis

The overall aim of this thesis is to evaluate the effects of cholestasis with retention of bile acids on murine adrenal glands´ function. Furthermore we aim to determine, whether these effects are mediated through the nuclear bile acid receptor FXR.

We hypothesised that:

- cholestasis affects adrenal function in a murine model
- this is mediated by bile acids and their receptor FXR

1.9 Experimental setup

To determine the effects of obstructive cholestasis on adrenal function, we ligated the common bile duct of C57/BL6 mice for three weeks. To determine whether any seen alterations are mediated by bile acids via the action of FXR, we fed the mice for four days with CDCA, the most potent natural occurring FXR agonist. To evaluate FXR depended effects of obstructive cholestasis, we performed CBDL for one week in FXR WT and KO mice.

As readouts, we used serum corticosterone concentrations, measured by competitive ELISA and the transcription levels of distinct enzymes assessed by qPCR. The circulating corticosterone levels reflect the functional output of the adrenal glands. The mRNA levels of key steroidogenic enzymes and enzymes required for cholesterol homeostasis were assessed in adrenal tissue and enzymes for glucocorticoid degradation were measured in the liver.

2 Materials and Methods

2.1 Materials

Sodium chenodeoxycholate was obtained from Sigma-Aldrich (St. Louis, MO). qPCR reagents including MagNa Lyser Green Beads tubes were from Roche Diagnostics (Basel, Switzerland). TRIzol® Reagent, 5x Buffer, 0.1M DTT, Superscript were purchased from Invitrogen (Waltham, MA). Random hexamers, RNAse Inhibitor, SYBR® Green PCR Master Mix were purchased from Applied Biosystems (Waltham, MA). dNTP was obtained from GeneAmp. GlycoBlue™ Coprecipitant was purchased from Ambion (Foster City, CA). The mouse/rat corticosterone ELISA was obtained from BioVendor (Brno, Czech Republic). The reagents for histological analysis included: Hematoxylin solution modified according to Gill III, xylene, 30% H_2O_2, hydrochloric acid fuming, n-butyl acetate, oil red O Certistain®, Aquatex® and Entellan® new were from Merck (Darmstadt, Germany). Anti-human FXR mouse monoclonal antibody was purchased from Perseus Proteomics (Tokyo, Japan). Proteinase K S3004, antibody diluent, REAL Envision Detection System Anti-mouse-rabbit polymer, polyclonal rabbit anti-mouse immunoglobulins/HRP and AEC substrate chromogen were obtained from Dako (Glostrup, Denmark). Microscope slide: Micro Slides, x-traTM Adhesive was from Surgipath (Richmond, IL) for FXR immunohistochemistry.

2.2 Animal studies

C57/BL6 mice were obtained from the Division for Biomedical Research and were housed in cages together with up to four animals at the Center for Medical Research in Graz with a 12 hours light and 12 hours dark cycle. Animals had access to standard chow and water ad libitum.

All the experiments were approved by the "Österreichische Tierversuchskommission am BMf WF" and were therefore in conformity with the Austrian "Tierversuchsgesetz 2012".

2.2.1 Common bile duct ligation (CBDL)

The common bile duct was ligated in 13 to 33 weeks old male C57/BL6 mice for three weeks in wild-type (WT) mice ($n_{3wSham} = 4$; $n_{3wCBDL} = 6$) and over one week in WT mice and FXR KO littermates (n = 4 per group), respectively.

The animals were placed in supine position with all four limbs attached to the underground. Mice were anaesthezised with isofluran per inhalationem. In the next step, their abdomen was disinfected. Median laparotomy was performed by transecting the integument approximately from the urinary bladder to the sternum with subsequent cutting across the linea alba, avoiding to dissect the near arteries. The common bile duct presented itself after folding back the liver. Ductus choledochus as well as ductus cysticus were obliterated by tying them up with two separate sutures. After the procedure the abdominal cavity was closed by stitching up the median incision. Before putting the animals back in their cages mice were nestled in gauze. Cages were set on plate warmers until recovery of the mice.

For sham operation we followed the same protocol except the ligation of common bile duct and gall bladder.

The perioperative mortality (1 out of 26) was low and within the acceptable range of this procedure.

2.2.2 Chenodeoxycholic acid (CDCA) feeding

11 to 15 weeks old female C57/BL6 mice were fed a diet containing 1% CDCA for four days. The control group received standard chow without CDCA (n = 3 per group).

2.2.3 Harvesting

CBDL and CDCA-fed mice were harvested around 18:00 and 14:00, respectively. Animals were anaesthetised with isofluran followed by death through decapitation. Subsequently, truncal blood was obtained via the collum for further blood analysis. The abdominal cavity was opened and the liver and adrenal glands were removed. Adherent fat was removed from adrenals glands to avoid crosscontamination of surrounding white adipose tissue. Afterwards, one adrenal gland per mouse was snap-frozen for mRNA analysis and the other one was either snap-frozen or fixed with 4% formaldehyde and afterwards put into paraffin for further histological analyses. Livers were dissected into several parts. For mRNA expression analysis one section was put in MagNa Lyser Green Beads tubes with 800 µl TRIzol® Reagent. The other portions were again either snap frozen or fixed with 4% formaldehyde and laid in paraffin for analysis of the histology.

2.3 Serum biochemistry

Mouse serum was examined for the concentrations of alanine transaminase (ALT), alkaline phosphatase (AP), aspartate transaminase (AST), bilirubin, cholesterol, urea, lactate dehydrogenase (LDH), triglycerides and bile acids (BA) on a Hitachi 917 analyzer (Boehringer Mannheim, Germany). For serum corticosterone level determination we used a mouse/rat corticosterone ELISA according to the manufacturer´s protocol.

2.4 Quantitative PCR

2.4.1 Primer design

Primers for qPCR are shown in ***Table 1***. They were designed using the reference sequences of the UCSC Genome Browser. After visualising of the nucleobases the sequence was copied into the Primer3 Browser to design the forward and reverse primers. The most important features were: a primer size reaching from 19 to 26 nucleotides with an optimum of 20; an optimal melting temperature of 60 degree Celsius one degree up or down; the size range of the PCR product should reach from 50 to 150 nucleotides; maximum self and pair complementary were set at 4.00. Primers were picked with the highest quality possible but they had to lie within the coding sequence in two different exons to avoid DNA amplification. The retrieved primers were tested further using the UCSC Genome Browser to reveal cross-reactions. Additionally, the PCR products were assessed with the NCBI Blast online software, so that we were able to identify more unspecific cross-reactions. Hsd3b1 cross-reacts with most of its other isoforms, Cyp11b with Cyp11b1 and Cyp11b2 and Akr1c6 with Akr1c20 due to minimal differences in their coding sequence.

Table 1 Primer sequences

Gene	Accession Number	Forward Primer (5´->3´)	Reverse Primer (5´->3´)
Srd5a1	NM_175283.3	gtggttagtgggcatggtga	ctccacgagctccccaaaat
Akr1d1	NM_145364.2	ccagcgagggatagttgtca	agcaactccacatagcggac
Akr1c6	NM_030611.3	tggcactgtgaagagggaag	tgttccaagcagacccgtac
Akr1c14	NM_134072.1	gaagaggaagtaggccaggc	tccaagcaagatcggaccaa
StAR	NM_011485.4	ccaggaaggctggaagaagg	gtctaccaccacctccaagc
Cyp11a1	NM_019779	catggccaagatggtacagttg	ggagatggggtggagtctca
Hsd3b1	NM_008293.3	tccacactgctgctgtcatt	agatgaaggctggcacactt
Cyp21a1	NM_009995.2	tccaagagagtcgggaccat	ctttccattggcctgcaacc
Cyp11b	NM_001033229.3	ctgggacgtggtgtgttctt	cccttgctatcccatccacc
HMGCoAR	NM_008255.2	ccggcaacaacaagatctgtg	atgtacaggatggcgatgca
Cyp51	NM_020010.2	gttggggagaaagcggagaa	gagccaccttctcgttgagt
SR-BI	NM_016741.2	gagcacgttctacacgcag	ggtctgaccaagctatcaggtt
Acat1	NM_009230.3	tcctgtttgcgcctacactt	aaaacaggcagccaaacacc
Hsl	NM_010719.5	acctggatgtgcacttctgg	atgttggccagagacgacag
Abca1	NM_013454	ctcttcatgactctagcctgga	acacagacaggaagacgaacac
Abcg1	NM_009593.2	ctttgacaccatcccagcct	ccaagccgtagatggacagg
Fas	NM_007988.3	gctgtagcacacatcctaggca	tcgtgttctcgttccaggatc
Acc1	NM_133360	ggacagactgatcgcagagaaag	tggagagccccacacaca
Acc2	NM_133904	aaggcggaggcgggaaaggtat	gaacagtcccgtccgaacagtg
Srebp1	NM_011480.3	agagccctgcacttcttgac	cagtccccgtccacaaagaa
Srebp2	NM_033218.1	cggtcctccatcaacgacaa	cctcagaacgccagacttgt
Scap	NM_001103162.2; NM_001001144.3	cagccgtggtcacagtactt	agcccaataacgaccaccag
Insig1	NM_153526.5	ccatcgccttcctagctacg	ccactgtgacacctcctgag
Insig2	NM_001271531.1; NM_133748.2; NM_001271532.1; NM_178082.3	agagtggtccagtgtcatgc	cctactgacagtgcagccag
36b4	NM_007475	gcttcattgtgggagcagaca	catggtgttcttgcccatcag
Cyclo	NM_011149.2	ggagatggcacaggaggaa	gcccgtagtgcttcagctt
18SrRNA	NR_003278	gtaacccgttgaaccccatt	ccatccaatcggtagtagcg
FXR	NM_009108	cggctgtcaggatttgtgc	gttgtatggggagtacgattc

2.4.2 RNA isolation

Snap-frozen liver and adrenal tissues were homogenised in 800 µl TRIzol® Reagent. Liver tissue was disrupted with MagNa Lyser Green Beads by putting them twice in the MagNa Lyser Instrument (Roche Diagnostics, Switzerland) for 20 seconds at 6500 rotations per minute (rpm). The adrenal glands were homogenised with a plastic pestle to save every precious quantum of RNA.

To separate the RNA from DNA and proteins, 160 µl chloroform was added. After proper shaking so as to provide an appropriate mixture, the samples were centrifuged for 20 minutes at 13,000 rpm and 4 °C. This procedure resulted in three phases: the lower phase contains mainly proteins, the intermediate one DNA and the RNA is dissolved in the upper aqueous phase.

The steps for precipitation followed the transferring of the upper phase into a new tube. 500 µl isopropanol and, in the samples derived from adrenal tissue, also 1µ of GlycoBlue™ Coprecipitant were added and then again put in the centrifuge. Prior to the centrifugation, adrenal samples were stored for half an hour in a -80 °C freezer. Owing centrifugation and the glycogen, the pellet should be better visible. The fluid was discarded and 500 µl ethyl alcohol (70%) was added. The samples were centrifuged again. The last three steps were repeated one more time. The remaining alcohol was removed. The pellets were dried for approximately 10 minutes at room temperature and then redissolved in a particular amount of distilled water according to the pellets´ size (i.e. 12 µl for the adrenal samples and up to 800 µl for the liver samples). The tubes were incubated for 10 minutes at 65 °C and then put in the freezer overnight.

Prior to cDNA synthesis, the RNA concentration was determined using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Furthermore, the quality of the specimen was evaluated with the 260/280 (should ideally be around 2.0) and 260/230 ratio (should be ideally between 2.0 and 2.2).

2.4.3 cDNA synthesis

A total amount of 500 ng of adrenal RNA and 1.5 µg of hepatic RNA was pipetted into specific tubes together with 1.4 µl random hexamers and filled up to 10 µl

volume with distilled water. The samples were put in the MyCycler™ Thermal Cycler (Bio-Rad) for 15 minutes (5 minutes at 65 °C and 10 minutes at 25 °C).After that a “mastermix” consisting of 4 µl 5x Buffer, 2 µl 0.1M DTT, 0.3 µl dNTP, 0.5 µl Superscript, 0.5 µl RNAse Inhibitor and 2.7 µl distilled water was added. The residual steps of the program lasting 90 minutes at 42 °C and 15 minutes at 70 °C were finished. Samples were diluted 1:20. All samples were transferred into a new tube to create a cDNA pool. From this pool the cDNA was diluted for standard 1 1:5, for standard 2 1:10 and for standard 3 1:25.

2.4.4 qPCR run

For analysis of gene transcription, plates containing 1.75 µl diluted cDNA and 8.3 µl qPCR mastermix per well were analysed using the Light Cycler 480 (Roche Diagnostics). The qPCR mastermix consisted of 5.25 µl SYBR® Green PCR Master Mix, 2.8 µl distilled water and 0.35 µl forward and reverse primer, respectively. Samples and standards were arranged in duplicates. Plates were centrifuged, put into the Light Cycler and passed through the following cycles:

- Heating up to 50°C
- Denaturation at 95°C for 10 minutes
- Amplification and quantification by cycling 40 times at 95°C for 15 seconds and 60°C for one minute
- Melting curve analysis by continuous heating and measuring from 55°C to 95°C
- Cooling to 40°C

2.5 Histology of the adrenal glands

2.5.1 Hematoxylin and eosin staining

In paraffin wax embedded adrenal glands were sliced in sections approximately 2 µm thick. The slices were stained with hematoxylin and eosin using a standard protocol.

2.5.2 Oil Red O staining

Cryosections of adrenal glands from CBDL mice were stained with oil red O. For oil red O stock solution, oil red O Certistain® was mixed with 100 ml isopropanol at 60°C the day before usage. Oil red O dye was obtained by mixing stock solution with distilled water in the ratio 2:5 and filtering it. Cryosections were placed in 60% isopropanol for 5 minutes, stained in oil red O solution for 10 min, immersed in 60% isopropanol and subsequently rinsed in distilled water. Counterstaining was performed using Mayer´s hemalum solution for 3 - 5 minutes. Sections were mounted with Aquatex®.

2.5.3 FXR immunohistochemistry

Paraffin wax embedded adrenal slices and coated microscope slides were used for immunohistochemistry for FXR. Sections were deparaffinised and rinsed in PBS. Proteinase K stock was added (dilution in PBS 1:1000) and left on slides for 5 minutes. Sections were again rinsed in PBS, treated with 0.1% Sodium-citrate puffer in a microwave, rinsed in PBS, placed in H_2O_2 for 10 minutes, rinsed in PBS. The first antibody (FXR antibody) was diluted 1:100 in antibody diluent and sections were incubated overnight. Samples were rinsed in PBS. The second antibody was diluted 1:100 in antibody diluent and added. Sections were rinsed in PBS, incubated with polymer for 30 minutes, rinsed in PBS and incubated in AEC for 3 to 8 minutes. Counterstaining was performed using Mayer´s hemalum solution. Sections were mounted with Aquatex®.

2.6 Statistical analysis

Data were analysed using student´s t-test, Mann-Whitney rank sum test or ANOVA. Values are shown as means ± standard deviation (SD). A p-value < 0.05 was considered statistically significant.

3 Results

3.1 Serum biochemistry

CBDL mice showed significant elevated levels of ALT, AP, bilirubin, bile acids, cholesterol and triglycerides in serum compared to sham-operated animals in all experimental groups. (***Table 2***).

Table 2 Serum biochemistry of CBDL mice versus sham-operation

Values represent the mean ± SD. Significant differences are as follows:
* $p < 0.05$; ** $p < 0.001$; compared to the corresponding sham-operated mice
BILI, bilirubin; CHOL, total cholesterol; TRI, triglycerides; KO, FXR KO

	3 weeks CBDL experiment		1 week CBDL experiment			
	Sham	**CBDL**	**Sham WT**	**CBDL WT**	**Sham KO**	**CBDL KO**
ALT (U/l)	64 ± 47	268 ± 53**	34 ± 11	411 ± 68*	35 ± 6	1013 ± 103**
AP (U/l)	59 ± 4	1045 ± 221*	54 ± 4	746 ± 84**	43 ± 5	614 ± 112*
BILI (mg/dl)	0.06 ± 0.04	18.96 ± 4.80*	0.06 ± 0.02	19.81 ± 4.85*	0.06 ± 0.04	29.48 ± 3.69*
BA (µmol/l)	4 ± 4	1035 ± 329*	43 ± 17	4850 ± 1079	4 ± 9	9482 ± 2454
CHOL (mg/dl)	32 ± 5	167 ± 95*	107 ± 6	763 ± 184*	119 ± 17	1164 ± 403*
TRI (mg/dl)	107 ± 39	166 ± 28*	92 ± 6	296 ± 79*	159 ± 16	783 ± 185*

3.2 FXR is expressed in the adrenal glands

FXR mRNA in the murine adrenal glands could be detected using qPCR (data not shown). Additionally, FXR immunohistochemistry of adrenal glands obtained from female mice revealed a nuclear FXR staining pattern throughout the zona fasciculata and in the inner half of the innermost zone of the adrenal cortex (X-zone). Nuclei in zona glomerulosa and the adrenal medulla were clearly negative for FXR (***Figure 4***).

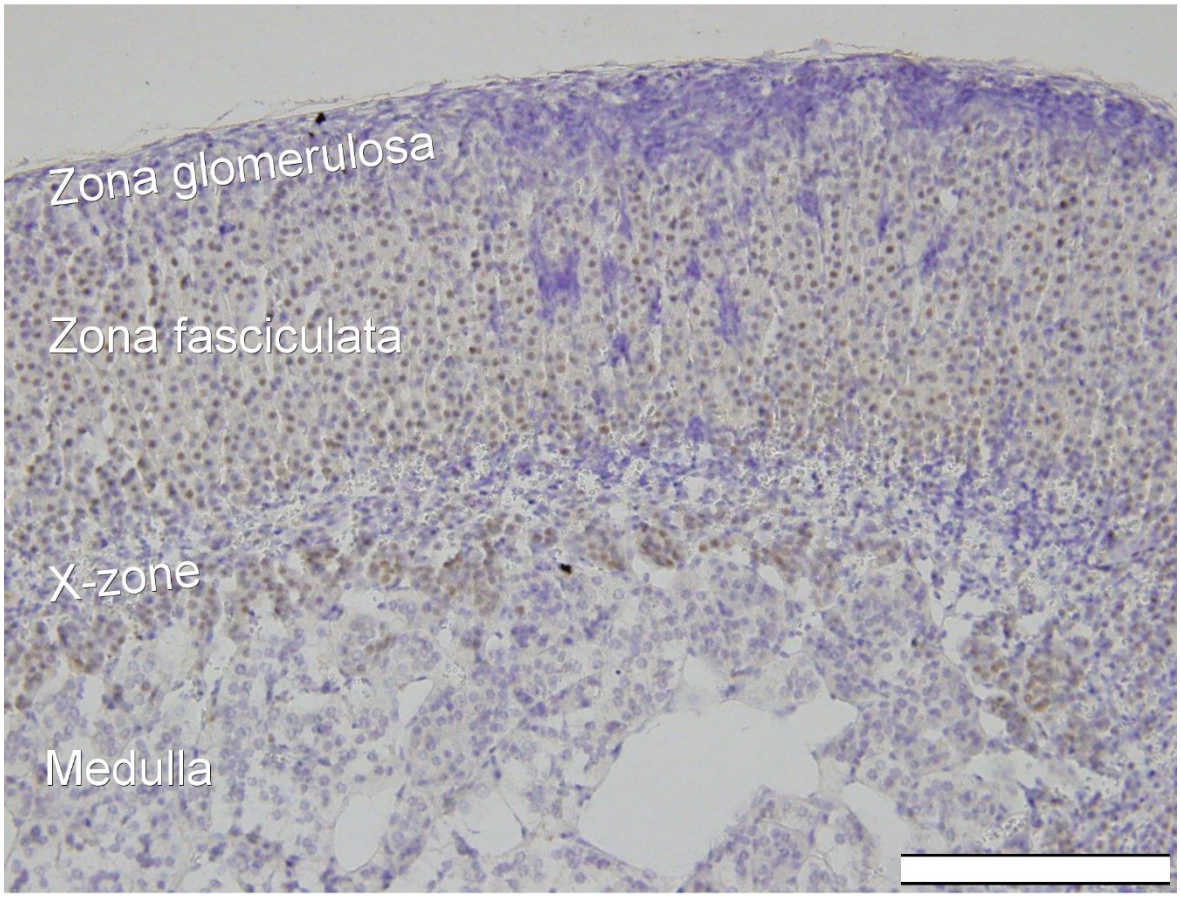

Figure 4 FXR expression pattern in adrenal glands of female mice

Adrenal FXR immunohistochemistry. Nuclear FXR positive cells show brown nuclei in contrast to the blue nuclei of FXR negative cells.
Scale bar: 200 µm

3.3 Cholestasis increases serum corticosterone levels

Three weeks CBDL led to elevated basal total serum corticosterone levels compared to sham-operated animals. However, this increase failed to reach statistical significance (192 ± 59 ng/ml in Sham vs. 484 ± 252 ng /ml in CBDL; p = 0.060) (***Figure 5A***). To test the hypothesis, that this increase in CBDL mice is mediated by bile acids, we fed CDCA for four days. Similarly, CDCA feeding increased serum corticosterone concentrations compared to chow-fed animals just failing to reach statistical significance (131 ± 22 ng/ml in Chow fed vs. 282 ± 101 ng/ml in CDCA fed; p = 0.064) (***Figure 5B***). To determine if these effects are FXR dependent we performed CBDL for one week in FXR WT and KO mice. CBDL animals showed increased levels of serum corticosterone compared to sham-operation, regardless of presence or absence of FXR. The difference reached statistical significance in WT but not in KO animals (156 ± 41 ng/ml in Sham WT vs. 373 ± 83 ng/ml in CBDL WT; p < 0.01; 223 ± 97 ng/ml in Sham KO vs 523 ± 280 ng/ml in CBDL KO; p = 0.089) (***Figure 5C***).

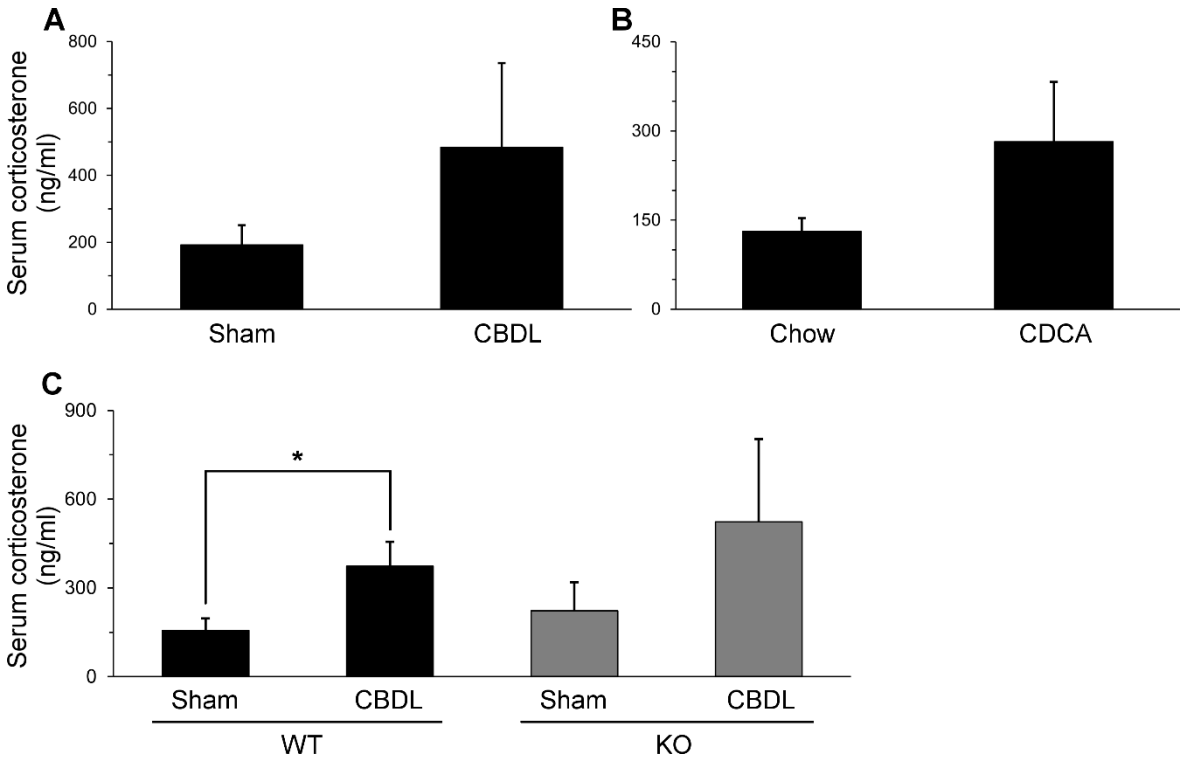

Figure 5 Total serum corticosterone concentrations

(A) Serum corticosterone from sham-operated (n = 4) versus CBDL (n = 5) mice 3 weeks after the procedure. **(B)** Serum corticosterone from Chow vs. CDCA-fed mice (n = 3) after 4 days of feeding. **(C)** Serum corticosterone from sham-operation versus CBDL in FXR WT and KO mice 1 week after the procedure.
Data represent the mean ± SD. * $p < 0.05$

3.4 Cholestasis and bile acids affect gene transcription in adrenals and liver

3.4.1 CBDL alters hepatic corticosterone breakdown

No change in transcription levels of 5α-reductase (Srd5a1) were observed whereas mRNA of 5β-reductase (Akr1d1) showed a significant increase and in contrast 3α-Hsd (Akr1c14) and 3α/17β-Hsd (Akr1c6) a significant decrease in 3 week CBDL mice compared to sham-operated animals (***Figure 6***).

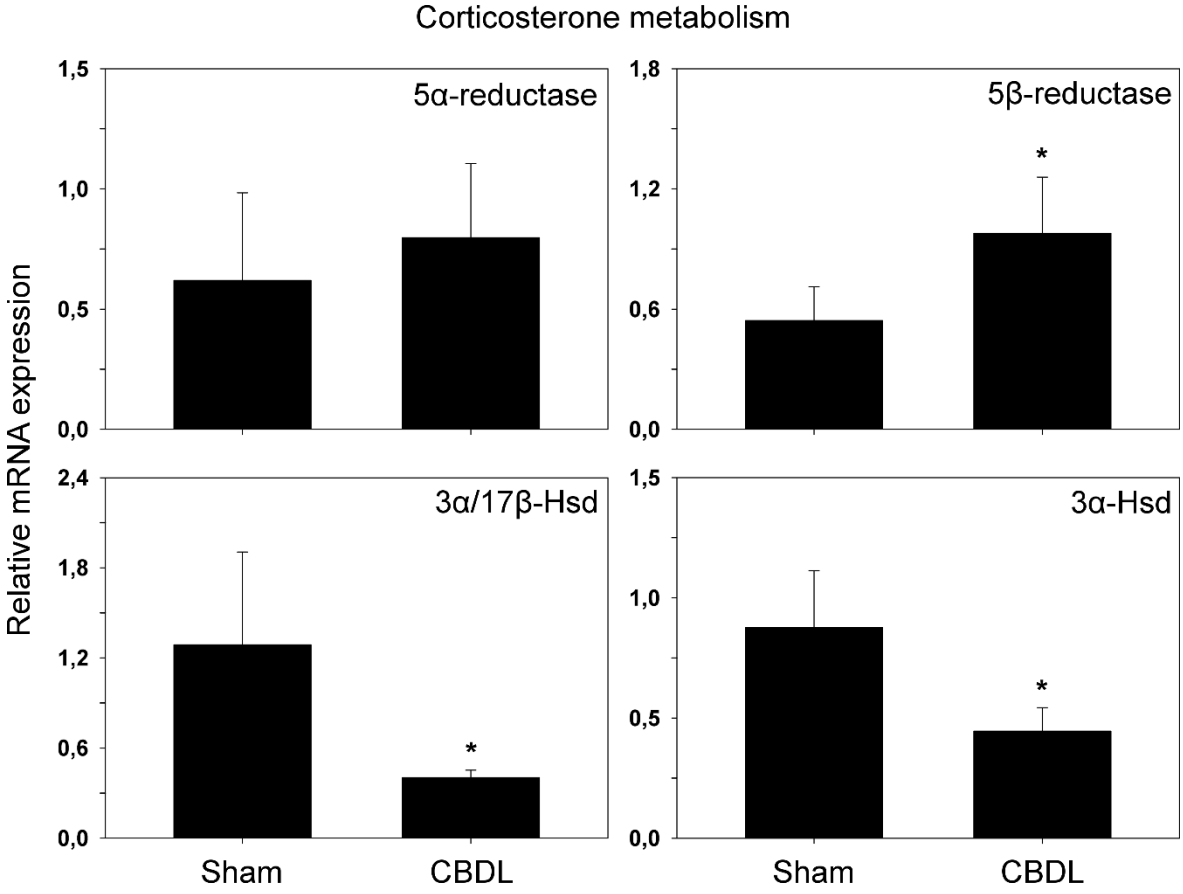

Figure 6 Regulation of genes involved in hepatic corticosterone metabolism during CBDL

Liver samples of 3 weeks sham-operated and CBDL mice were used (n = 4-5). mRNA values were normalized to 18SrRNA. Data represent the mean ± SD. * p <0.05

3.4.2 CBDL has no effect on mRNA levels of adrenal enzymes involved in corticosterone synthesis

3 week CBDL did not affect mRNA expression of enzymes for corticosterone synthesis including StAR, Cyp11a1, Hsd3b1, Cyp21a1 and Cyp11b (***Figure 7***).

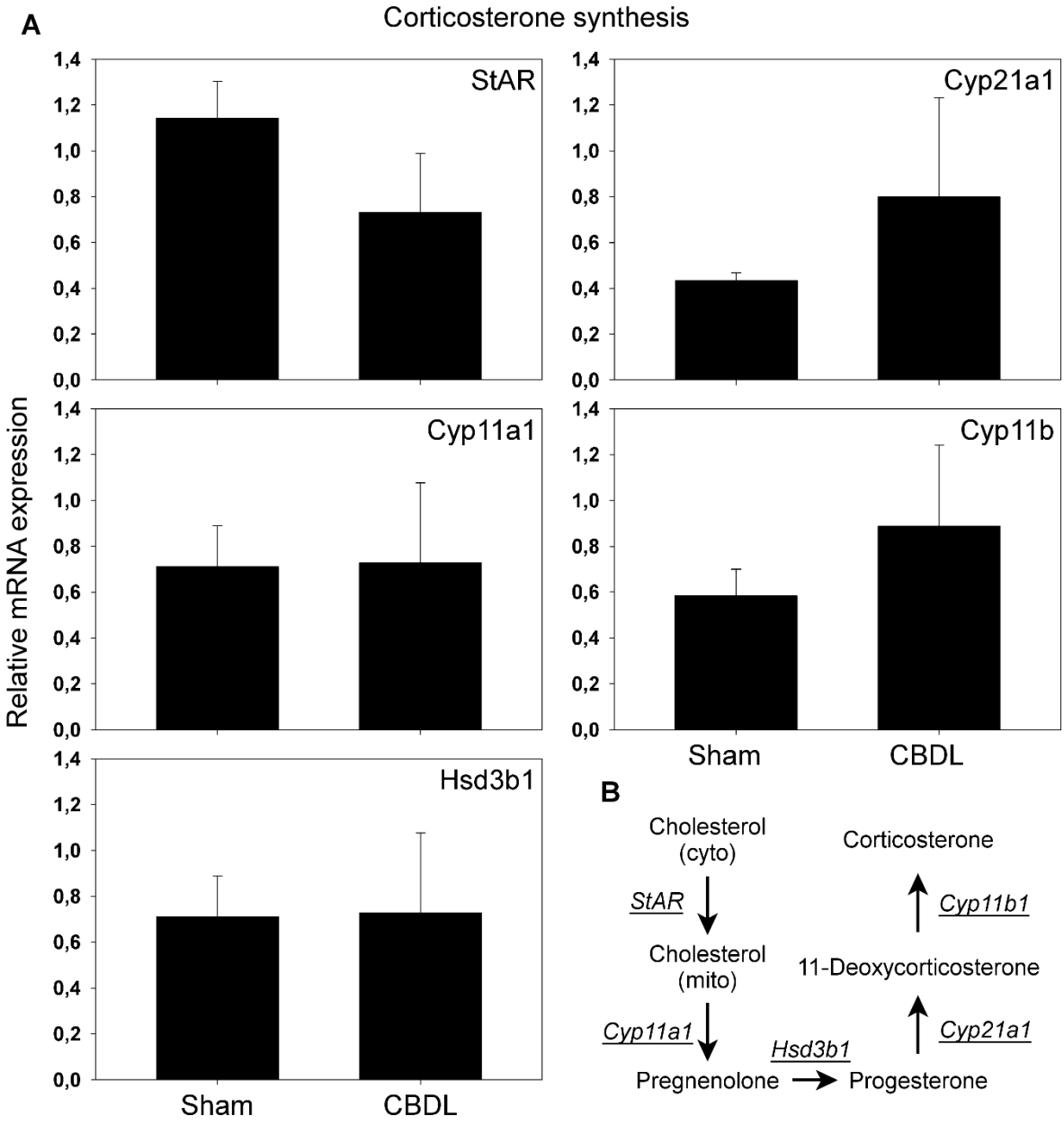

Figure 7 Regulation of genes involved in adrenal corticosterone synthesis during CBDL

(A) Adrenal glands of 3 weeks sham-operated and CBDL mice were used (n = 2-4). mRNA values were normalized to 36b4. Data represent the mean ± SD. * p <0.05 **(B)** Schematic illustration of the corticosterone biosynthesis pathway. Enzymes are underlined. cyto, cytosol; mito, mitochondrion

3.4.3 CBDL alters adrenal cholesterol homeostasis

Due to the important role of cholesterol homeostasis for adequate corticosterone synthesis, we analysed mRNA expression levels of enzymes involved in maintaining intracellular cholesterol concentrations.

In 3 week CBDL mice we found significantly elevated mRNA levels of HMG-CoA reductase and Cyp51 compared to sham-operated animals. CBDL did not change mRNA expression of Abcg1, but increased SR-BI and decreased Abca1 mRNA levels (not statistically significant) (***Figure 8***).

No change was observed in intracellular mRNA concentrations of Fas, Acc1, Acc2 (enzymes for synthesis of free fatty acids), Hsl and Acat1 (enzymes involved in cholesterol metabolism) after CBDL (***Figure 9***).

Additionally, no change was observed in mRNA expression levels of Srebp1, Srebp2 and Insig2 (regulatory proteins for enzymes involved in lipid metabolism). However, CBDL resulted in a significant increase of transcription of Insig1 and Scap (regulatory proteins for enzymes involved in lipid metabolism) compared to sham-operated animals (***Figure 10***).

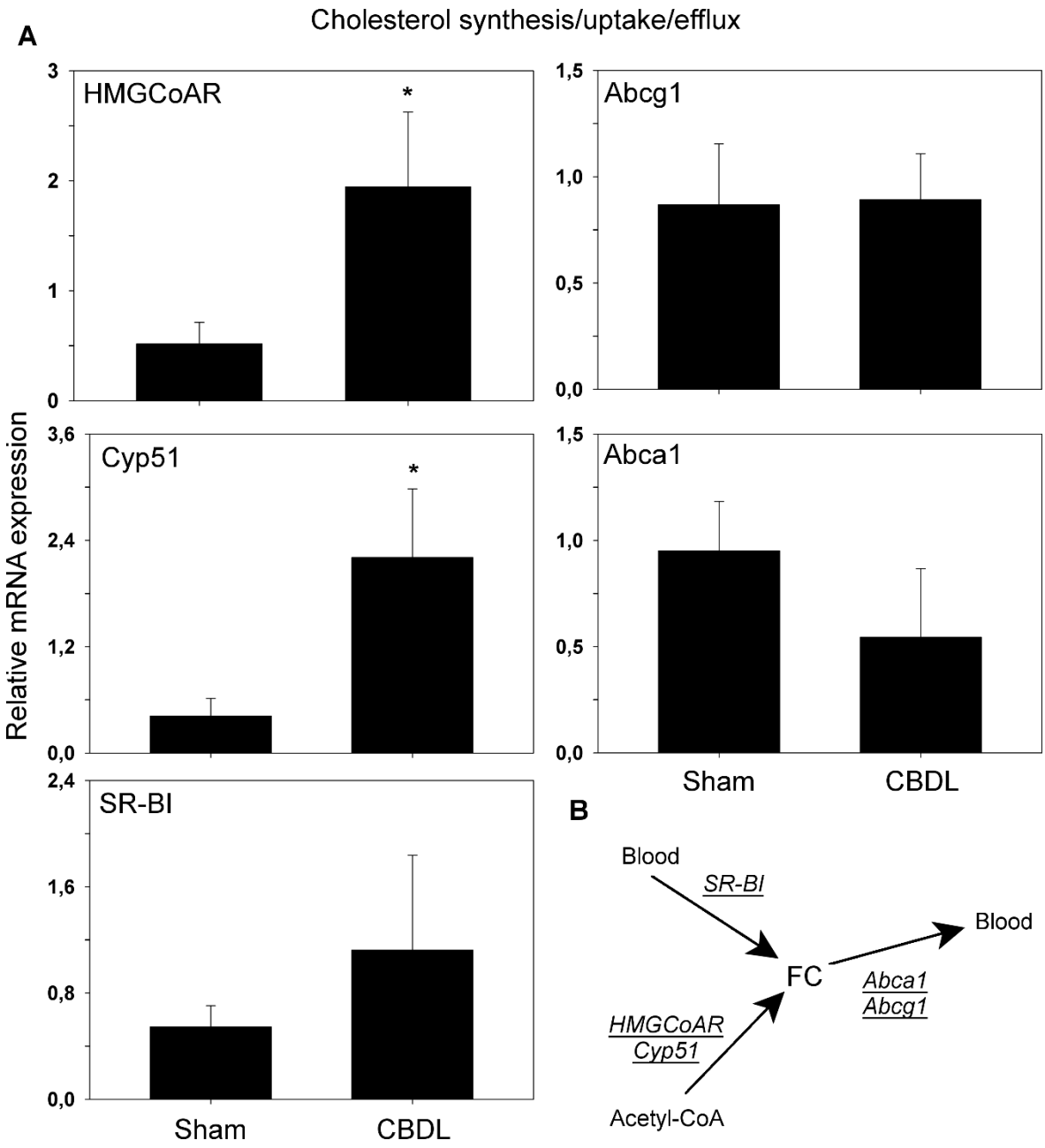

Figure 8 Regulation of genes involved in adrenal cholesterol synthesis, uptake and efflux during CBDL

(A) Adrenal glands of 3 weeks sham-operated and CBDL mice were used (n = 2-4). mRNA values were normalized to 36b4. Data represent the mean ± SD. * p <0.05 **(B)** Schematic illustration of the part the distinct enzymes play in adrenal cholesterol homeostasis. Enzymes are underlined.

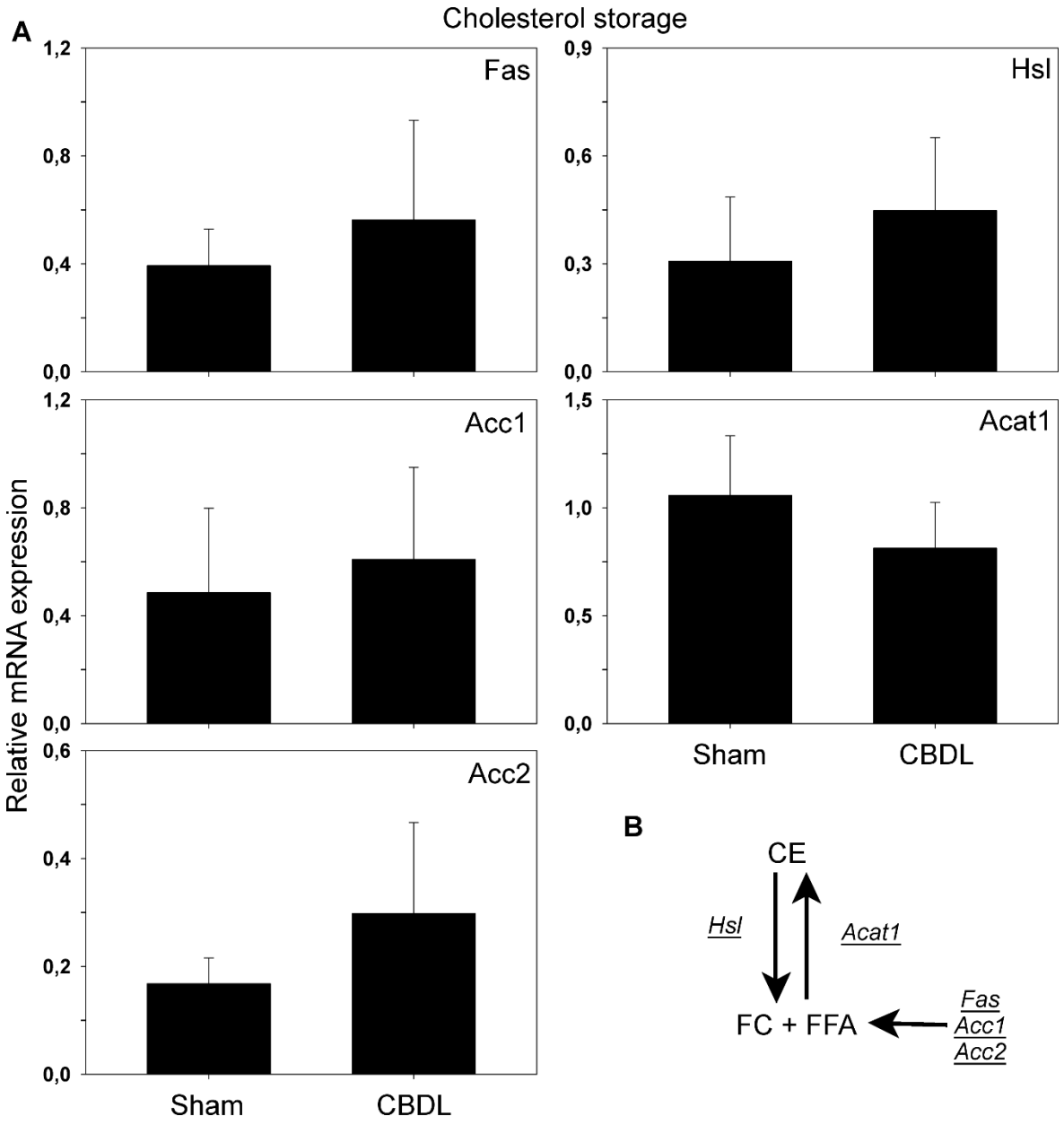

Figure 9 Regulation of genes involved in adrenal cholesterol storage during CBDL

(A) Adrenal glands of 3 weeks sham-operated and CBDL mice were used (n = 2-4). mRNA values were normalized to 36b4. Data represent the mean ± SD. * p <0.05 **(B)** Schematic illustration of the cholesteryl ester formation and hydrolysis pathway. Enzymes are underlined.

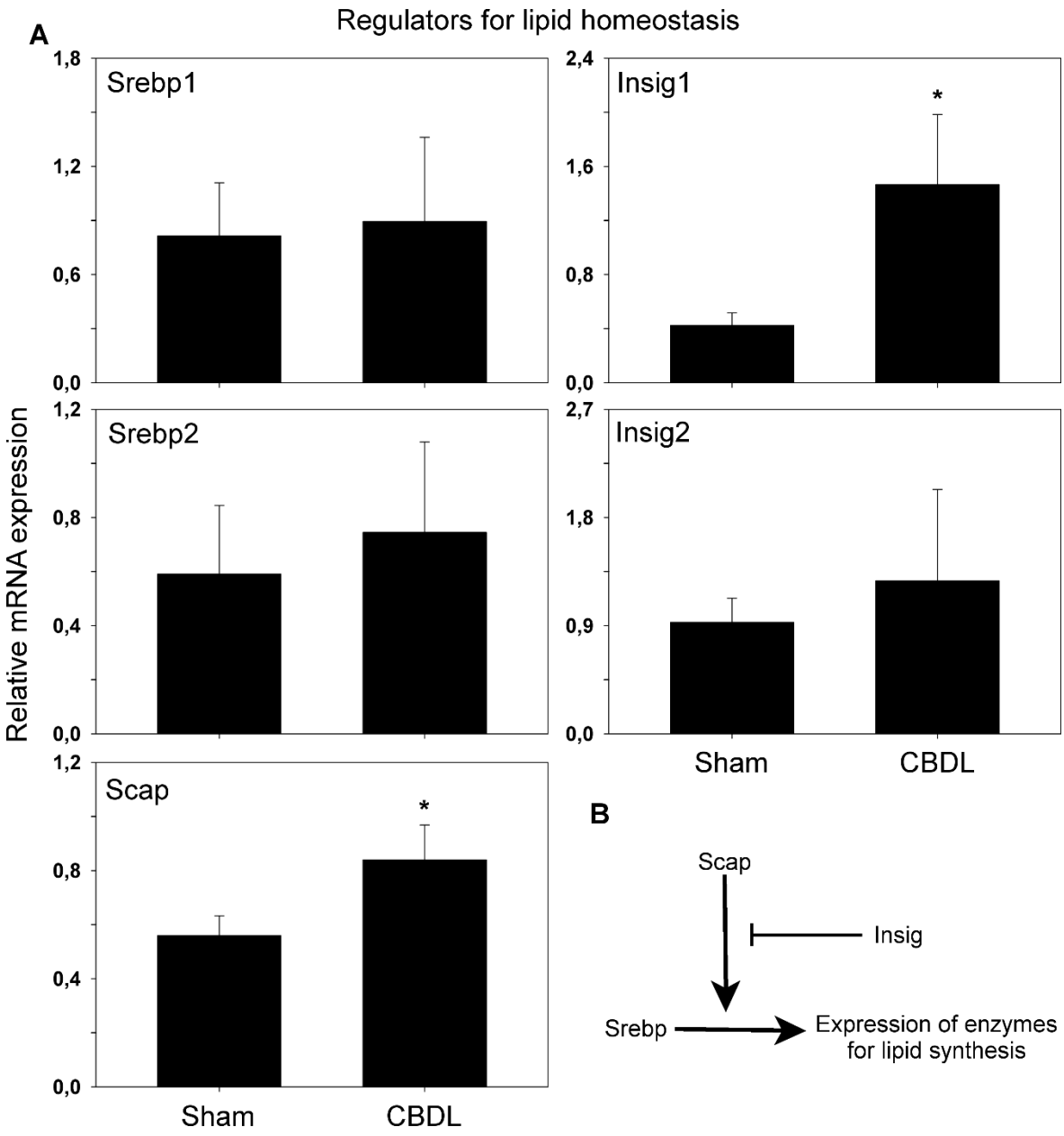

Figure 10 Regulation of genes involved in regulating adrenal lipid homeostasis during CBDL

(A) Adrenal glands of 3 weeks sham-operated and CBDL mice were used (n = 2-4). mRNA values were normalized to 36b4. Data represent the mean ± SD. * p <0.05 **(B)** Schematic illustration of the role the distinct enzymes play in regulation of lipid homeostasis.

3.4.4 CDCA feeding alters corticosterone breakdown and cholesterol homeostasis

CDCA administration resulted in increased transcription levels of HMG-CoA reductase, Cyp51, SR-BI and Abca1. However, this did not reach statistical significance. After feeding mice with CDCA no change in gene transcription was observed for StAR, Cyp11a1, Hsd3b1, Cyp21a1, Cyp11b, 5α-reductase (Srd5a1), 5β-reductase (Akr1d1), 3α-Hsd (Akr1c14). Feeding of CDCA resulted in significant decrease of hepatic mRNA concentration of 3α/17β-Hsd (Akr1c6) compared to chow fed animals (***Figure 11***).

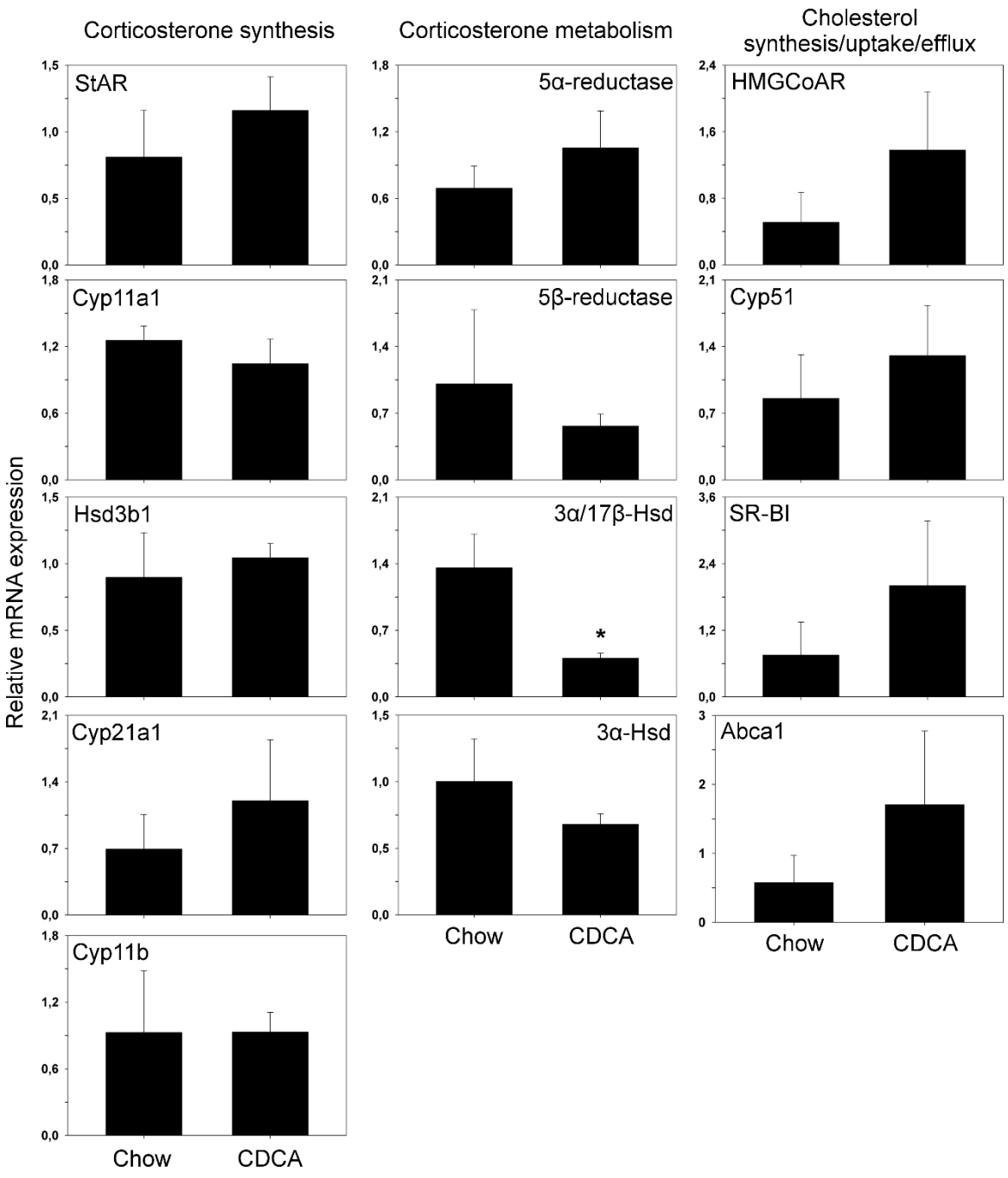

Figure 11 Regulation of genes involved in hepatic metabolism and adrenal steroidogenesis after CDCA feeding

Adrenal mRNA values were normalized to 36b4 and hepatic ("Corticosterone metabolism") mRNA values were normalized to 18SrRNA. Data represent the mean ± SD. * p <0.05

3.4.5 Effects are FXR-independent

In order to determine whether the observed changes are dependent of FXR, we performed CBDL in FXR WT and KO mice for 1 week. Performing the same analyses in mice with versus without FXR revealed no significant differences in mRNA expression levels of distinct genes between FXR WT and FXR KO mice regardless of the treatment (***Figure 12***). However, the classical FXR target genes such as Shp, Ostα and Ostβ were significantly downregulated in FXR KO mice confirming that the FXR KO animals truly have a blunted FXR signalling pathway (data not shown).

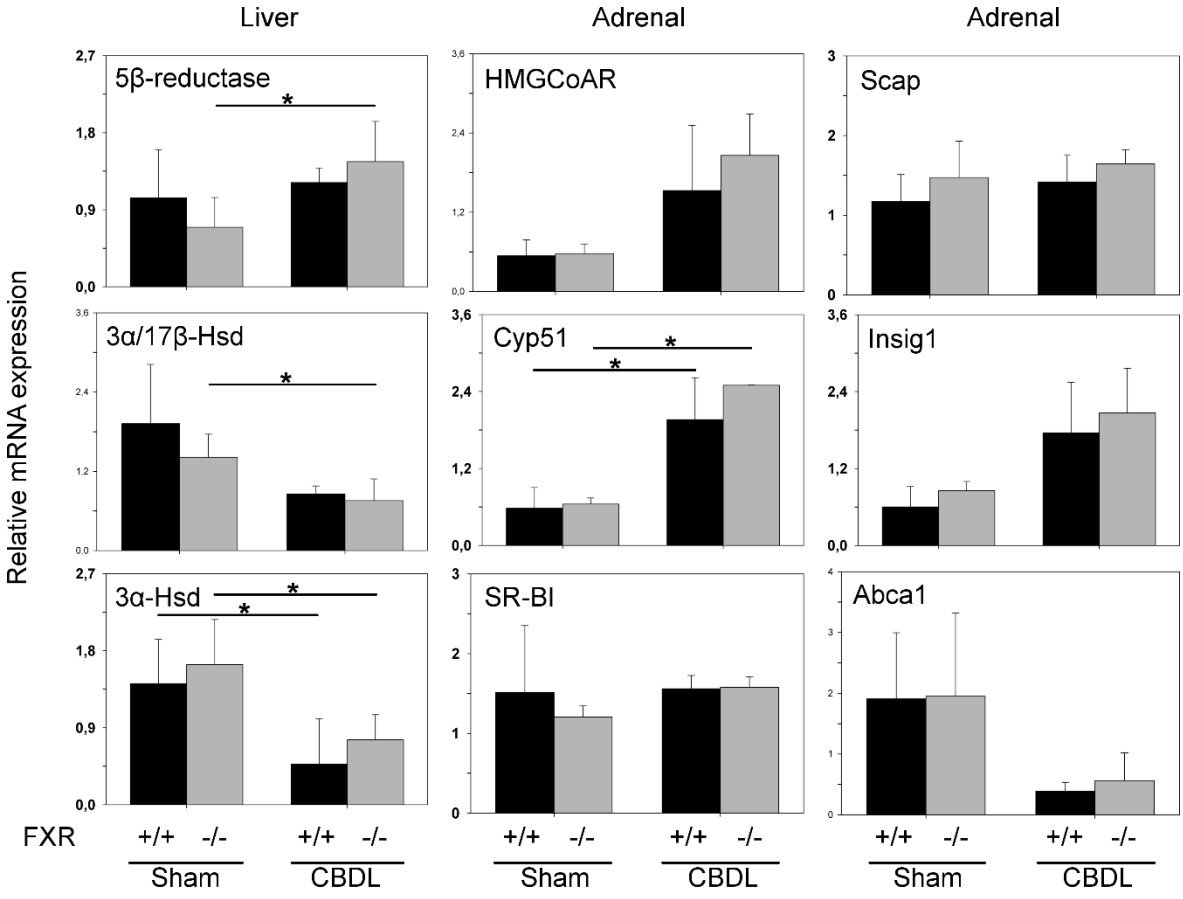

Figure 12 Regulation of genes involved in hepatic corticosterone metabolism and adrenal steroidogenesis after CBDL in FXR WT and KO mice

For the first column liver samples of 1 week sham-operated versus CBDL mice were used (n = 4) and mRNA values were normalized to 18SrRNA. For the second and third columns adrenal glands of 1 week sham-operated versus CBDL mice were used (n = 2-4) and mRNA values were normalized to 36b4. Data represent the mean ± SD. * p <0.05

3.4.6 CBDL led to loss of adrenal lipids

Oil red O staining revealed depletion of red to orange stained neutral lipid droplets in CBDL mice versus sham-operated animals. However, this effect was independent of FXR (***Figure 13***).

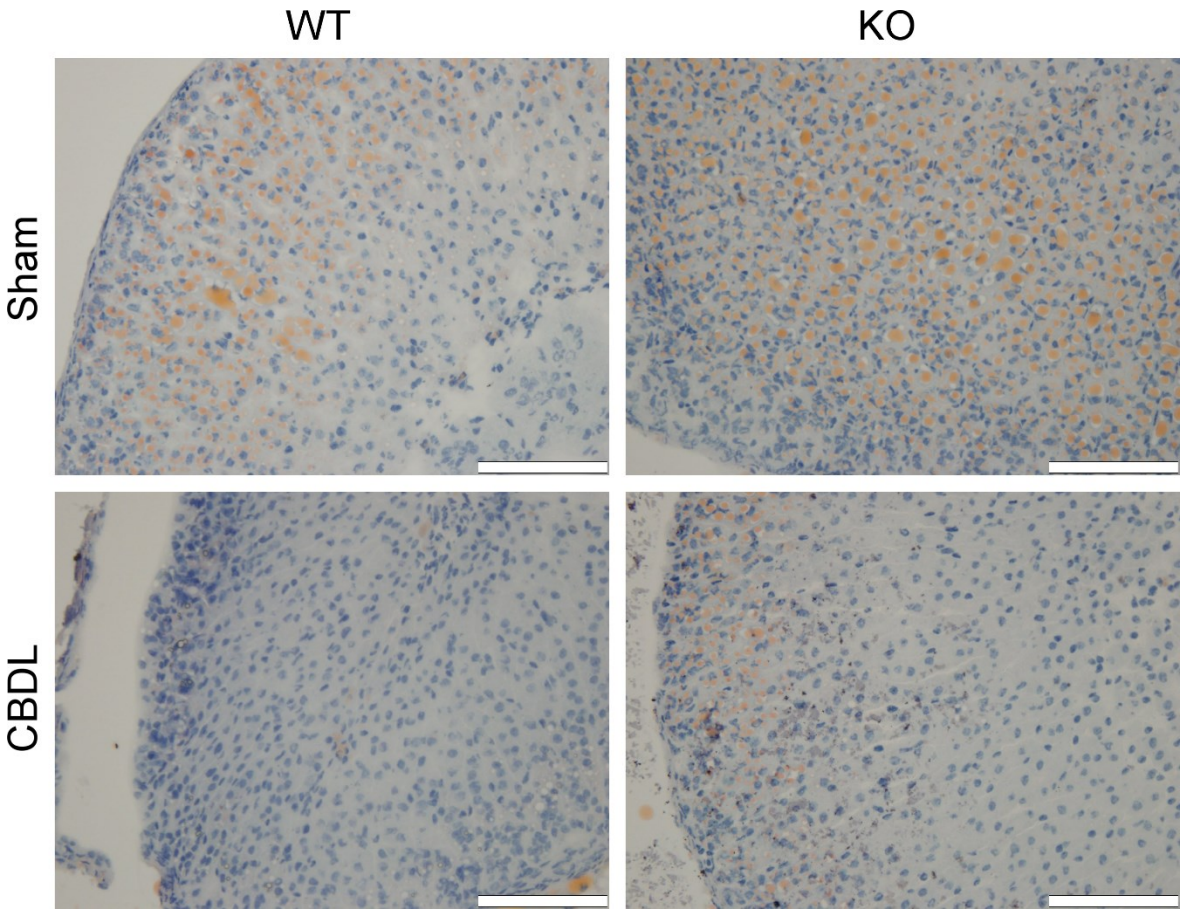

Figure 13 Oil red O staining of neutral lipids in adrenal glands

Male adrenal glands of 1 week sham-operation versus CBDL in FXR WT and KO mice were stained with oil red O. Staining resulted in orange neutral lipid droplets.
Scale bars: 100 µm

4 Discussion

This is the first work demonstrating effects of CBDL and bile acids on adrenal function and glucocorticoid homeostasis in mice.

We observed elevated corticosterone concentrations in serum of CBDL animals. CDCA feeding and the use of FXR KO mice further revealed that these effects may be BA-mediated but independent of the bile acid receptor FXR. mRNA expression data suggest, that corticosterone degradation in liver is impaired. In adrenals mRNA data suggest, significantly alter cholesterol metabolism with reduced cholesterol export, increased cholesterol uptake and de-novo synthesis. Cholesterol storage and corticosterone biosynthesis after CBDL and CDCA administration were not changed. These findings are in accordance with the elevated serum corticosterone concentrations in the mice. No differences in FXR WT versus KO animals were found. We also observed a decreased amount of neutral lipid droplets in the adrenal glands after CBDL in FXR WT and KO mice using oil red O staining. Taken it all together we conclude that obstructive cholestasis alters adrenal as well as hepatic function resulting in hypercorticosteronemia. This may possibly be via the action of BA. The bile acid receptor FXR plays only a minor role in regulating basal adrenal function during cholestasis.

The exact pathomechanisms leading to the hypercorticosteronemia in cholestatic mice remains to be determined. In general, there are two major possibilities explaining this finding: increased adrenal glucocorticoid biosynthesis and diminished hepatic breakdown and subsequent excretion. Our data suggest that both pathways are disrupted thus leading to increased corticosterone levels. A second hit might be necessary for the formation of the phenotype with elevated corticosterone levels. If only one part of the HPA axis might be disrupted the other would be able to counter-regulate. Further studies - much beyond the scope of this thesis - aiming to elucidate the relative contributions of the two mechanisms are indispensable.

Hepatic qPCR data revealed important changes at the transcriptional level of enzymes for corticosterone metabolism in the liver. In addition to the obstruction of the common bile duct and therefore logically blocking the biliary excretion of

corticosterone metabolites, the molecular breakdown seems to be impaired in cholestasis. Reduced expression of enzymes mediating corticosterone breakdown such as 3α-Hsd´s was observed in CBDL and CDCA-fed animals strongly indicating that these alterations are bile acid-induced. These changes were independent of FXR in CBDL mice. Nevertheless, we found slight but significant increased mRNA levels for 5β-reductase in CBDL mice whereas in CDCA fed mice the mRNA concentration decreased. Discrepant findings reported by McNeilly et al. showing decreased transcription of both 5β-reductase and 3α-Hsd after CBDL but not CDCA administration can be attributed to the usage of rats in this study (103). Taken together we claim bile acid-mediated but FXR independent decrease of hepatic corticosterone breakdown in mice during cholestasis.

We found no change in adrenal mRNA expression levels of corticosterone synthesizing enzymes in any of the experimental groups. This could be explained by posttranscriptional changes in protein expression, which we have not assessed so far. Especially StAR is highly regulated at the protein level as shown in in vitro experiments using Y1 cells and oxysterols (108).

However, our qPCR results point towards increased intracellular cholesterol concentrations due to increased cholesterol synthesis and uptake together with reduced export. This may lead to increased synthesis as long as the enzymes for corticosterone biosynthesis are not saturated. We observed increased mRNA expression for HMG-CoA reductase and Cyp51 for de novo cholesterol synthesis and SR-BI for cholesterol uptake in all experimental groups indicating that these effects are again bile acid-mediated but independent of FXR. The transcription of the cholesterol efflux transporter Abca1 seems to be down-regulated during obstructive cholestasis whereas bile acid feeding resulted in a not significant up-regulation. The significant elevated levels of mRNA expression for Insig1 and Scap fit to the pattern of intracellular cholesterol depletion (75,76). This may indicate increased usage of free cholesterol.

Oil red O staining revealed reduced neutral fat content in the adrenal cortex in response to CBDL. The reason for the loss of adrenal fat remains to be determined. Assuming that these lipids were cholesteryl esters there are three possible explanations:

First, there could be increased usage of intracellular cholesterol for corticosterone synthesis even despite unchanged mRNA concentrations for StAR, Cyp11a1, Hsd3b1, Cyp21a1 or Cyp11b by posttranscriptional mechanisms.

Second, the homeostasis of intracellular cholesterol storage could be disrupted towards increased hydrolysis of CE droplets at the level of Hsl and Acat1, the two major adrenal enzymes for cholesterol storage homeostasis by posttranscriptional mechanisms.

Third, a lack of substrate for CE formation could result in loss of adrenal ester droplets despite normal enzyme activity. The substrate for CE are free fatty acids and free cholesterol. Cholestatic mice show increased levels of total circulatory cholesterol as also shown in this study. However, this increase is mainly due to increased levels of FC whereas CE concentrations in the blood show no major changes but a shift into the LDL fraction. This was shown by others in rats and mice after CBDL (95–97). Because of the fact, that the HDL/SR-BI mediated uptake of CE plays the most important role in maintaining adequate intracellular cholesterol concentrations, this could explain a possible depletion. Further investigations also using BA are necessary. We also found no changes for transcription levels of Acc1, Acc2 and Fas, three enzymes for fatty acid synthesis. However, other pathways like increased β-oxidation could lead to free fatty acid depletion and therefore CE depletion.

The role of FXR in regulating adrenal function appears minor. Even after adrenal FXR activation in vivo (we observed increased mRNA expression of FXR target genes after CBDL and CDCA feeding; data not shown) we observed no differences between FXR WT and KO animals. Its potential effects seem to be overruled by compensatory pathways. However, one should not forget the findings of Hoekstra et al. showing that activation of FXR is not negligible for adrenal function during stress (106). This indicates that during the basal state FXR activity is negligible in the adrenal gland, whereas after stimulation of both the adrenals by a stressor and FXR by bile acids it significantly modulates the function this organ.

Besides FXR, other receptors regulating the function of the adrenal glands under conditions with a high bile acid load could play a major role (i.e. other BA receptors

like Tgr5, PXR or VDR). The membrane embedded bile acid receptor Tgr5 would provide an interesting target. TGR5 is expressed in the human adrenal and pituitary gland (109). This bile acid receptor acts by stimulating the cAMP/PKA pathway. Therefore, it may utilize similar signalling pathways as ACTH on the adrenal glands. The role of Tgr5 can be assessed by using Tgr5 KO mice. Previous to using the mouse model it is necessary to verify adequate Tgr5 expression in the mouse adrenal gland.

From this we conclude that obstructive cholestasis increases basal serum glucocorticoid concentrations probably via increased adrenal cholesterol synthesis and reduced hepatic degradation. These effects are bile acid-mediated but independent of the bile acid receptor FXR. We speculate that human cholestatic liver diseases are also associated with hypercortisolism, which surprisingly has not been investigated in detail so far. Deregulated glucocorticoid levels may represent a clinically important condition.

5 Outlook

Experiments in this thesis are the basis for currently on-going and future more-detailed studies investigating the molecular mechanisms underlying the presented findings. The major goal, however, is to characterize adrenal gland´s function in humans with cholestasis.

Assessing adrenal function in humans to see whether the findings in animal experiments can be extended to the human situation is the next to do. We are currently measuring the cortisol response after ACTH administration (Synacthen® test) in patients with various cholestatic disorders. Preliminary results indeed indicate elevated basal cortisol levels in human cholestatic diseases.

To elucidate the molecular mechanisms in animal experiments, analysis of protein expression is needed. We might have missed post transcriptional changes especially for StAR and the enzymes in lipid homeostasis the establishment of a Western blot will be an important step. Direct assessing of the enzyme activity might be more appropriate but also more complicated. Also, direct measurement of cholesteryl ester formation and hydrolysis displaying mainly the activities of Hsl and Acat1 could provide useful information about enzyme function. Considering that the use of radiolabeled molecules would be essential to clearly identify where the main alterations occur.

Further experiments meticulously characterizing the situation of the whole HPA axis during cholestasis and after bile acid administration are required. Tracking of glucocorticoid synthesis and metabolism using radiolabeled molecules would be an elegant approach to evaluate the function of the whole system and quantify the synthesis as well as the breakdown and excretion.

Another important point is that we need to elaborate our corticosterone detection techniques. The unbound fraction of corticosterone is widely known as the active form. Circulatory CBG which is the protein in blood for the binding and transportation of glucocorticoids may be altered during CBDL. Considering these facts only stating the total corticosterone levels may not provide enough information. Direct measurement of free corticosterone is the gold standard but its realization is

technically complex. Another approach would be the determination of the serum CBG concentration and in the next step putting it in ratio to total serum corticosterone. This value would be similar to the free cortisol index (FCI) which correlates well with free cortisol in humans (16) and pigs (110). The use of Coolen´s equation might be another possibility. But on the one hand it is adapted to the usage in humans and on the other hand it correlates worse with free cortisol in humans with liver cirrhosis than the FCI (16). These facts, however, limit its use in CBDL mice.

The non-invasive measurement of urinary corticosterone and faecal glucocorticoid metabolites directly reflect circulatory free corticosterone concentrations (111). Nevertheless, determination of faecal glucocorticoid metabolites is unsuitable in CBDL mice due to the impaired excretion through the bile. Establishing an appropriate method for collecting urine over a period of 24 hours would be an easy, non-invasive and non-stressful way to measure free corticosterone. However, to avoid biasing stress caused by housing in metabolic cages, an acclimatization period with subsequent collection of urine and determination of corticosterone levels would be required. Urinary 24-hour corticosterone concentration could be normalized to urine volume or creatinine concentration.

Due to the fact that the expression of corticosterone synthesizing enzymes is tightly regulated by pituitary ACTH excretion treating mice with dexamethasone (DEX) to suppress the upper instances of the HPA axis should be considered. This experiment can help to exclude compensatory mechanisms as a stress response during harvesting of the mice. It may be possible to visualize regulatory effects of gene transcription under basal secretory conditions. Also the measurement of corticosterone under DEX suppression would provide an important amount of information regarding the disruption of adrenal corticosterone output and metabolism.

To further determine and characterize the function of the whole HPA axis during cholestasis measuring of corticosterone after stress responses would be another important approach. Stressors include stimulation with ACTH, LPS, fasting and restrain.

By incubating adrenal cells with bile acids and determine the corticosterone concentration in the supernatant it would be possible to simply measure adrenal cells´ corticosterone output. We either could use primary adrenal cells or murine or human cell lines for the culture. This would also provide a sufficient approach to exclude the effects of the pituitary gland and hypothalamus.

A pivotal result was the decrease in the neutral lipid content of the adrenal glands. Until now we only visualized this decline in oil red O stained slices. We further need to determine the reduction of which lipid contributes to this finding. Thus, the measurement of free cholesterol and cholesteryl esters is pivotal since cholesterol is the substrate for corticosterone synthesis. Other lipids including triglycerides, phospholipids and free fatty acids, which could contribute to the shown alterations, should also be measured.

Aldosterone is another essential hormone deriving from the adrenal gland. Hence, the determination of serum aldosterone concentration would be interesting.

Besides the fact, that there is still a lot of work to do, these data already demonstrate the profound influence of obstructive cholestasis and bile acids on the adrenal glands. These findings are important since similar alterations occur in humans. Dysfunction of the adrenal glands, the pivotal organ for adaption to stress, during cholestatic diseases may lead to a higher morbidity and mortality in the patients as already observed in cirrhosis.

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