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STUDIES ON STEROL 27-HYDROXYLASE (CYP27A1)

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Dedicated to Niklas,
the man of my dreams, in real life

ABSTRACT

Human sterol 27-hydroxylase (CYP27A1) has been studied in the present thesis with focus on substrate specificity and possible role of the enzyme for prevention of accumulation of cholesterol and cholestanol in tissues. Attempts have been made to evaluate a metabolic pathway to bile acids in human primary hepatocytes that starts with a 27-hydroxylation of cholesterol catalyzed by CYP27A1. The first Swedish case of sterol 27-hydroxylase deficiency (Cerebrotendinous xanthomatosis, CTX) is described and the mutation in the CYP27A1 gene is defined.

Substrate specificity
Polar substrates were found to be 27-hydroxylated more efficiently than unpolar substrates by recombinant CYP27A1 or by cells overexpressing CYP27A1. A precursor to cholesterol, 7-dehydrocholesterol, was found to be a less efficient substrate for CYP27A1. Cholestanol, a 5α-saturated analogue to cholesterol, was found to be 27-hydroxylated by CYP27A1 at about the same rate as cholesterol. Cholestenone, a precursor to cholestanol, was found to be a highly efficient substrate for CYP27A1. The findings may be part of the explanation for the accumulation of cholestanol in patients with a sterol 27-hydroxylase deficiency (CTX). Cholestanol was also found to leave cultured cells at a slower rate than cholesterol, giving a partial explanation for its selective accumulation in CTX.

Possible role in cholesterol accumulation
Cholesterolosis is a condition with accumulation of cholesterol esters in the mucosa of the gallbladder. CYP27A1 mRNA, CYP27A1 protein and CYP27A1 enzyme activity were found to be present in both normal gall bladder mucosa and in cholesterolosis in similar amounts. The esterifying enzyme ACAT was found to be upregulated in cholesterolosis, probably secondarily to the high cholesterol levels. The conclusion of the study is that CYP27A1 does not seem to be a pathogenic factor in cholesterolosis. Levels of esterified 27OH-cholesterol, the product of CYP27A1, were markedly increased in cholesterolosis, probably as a secondary effect of the increased esterification. Human tendons were found to contain CYP27A1 and the ratio between 27OH-cholesterol and cholesterol was found the be increased, suggesting that a similar mechanism is operating in tendons as in cholesterolosis. The findings are of interest in relation to the preferential accumulation of cholesterol and cholestanol in tendons of patients with CTX.

Role of 27-hydroxylation of cholesterol as the first step in bile acid formation in human hepatocytes
Labelled 27OH-cholesterol was found to be converted into cholic acid and chenodeoxycholic acid considerably less efficiently than the corresponding conversion of labelled 7αOH-cholesterol. The latter oxysterol is formed in the rate-limiting sterp in the classical pathway from cholesterol into bile acids. The preferential product of the pathway starting with 27OH-cholesterol was found to be chenodeoxycholic acid. Due to the variable degree of conversion in different experiments, no firm conclusions could be drawn with respect to the relative role of the pathway starting with a 27-hydroxylation.

First Swedish case with sterol 27-hydroxylase deficiency
A cholestatic infant boy who died from organ failure was found to have a nonsence mutation in exon 7 (G1234T, or E408stop) in the CYP27A1 gene, a mutation not previously described. Both parents, who were first cousins, were found to be heterozygous for the mutation. Together with an infection with cytomegalovirus, the lack of CYP27A1 may have contributed to the death of this patient.
The results of the studies emphasize the importance of CYP27A1 and are consistent with the suggested role of the enzyme in preventing accumulation of cholesterol in various tissues.

(Photograph on the cover: cholesterol crystal found in culture medium from primary human tenocytes and macrophages, isolated from the xanthoma of a patient with familial hypercholesterolemia. Photo by the author.)

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I
Norlin M, von Bahr S, Björkhem I, Wikvall K.
On the substrate specificity of human CYP27A1: implications for bile acid and cholestanol formation
J Lipid Res. 2003 Aug;44(8):1515-22.

II
Björkhem I, Starck L, Andersson U, Lütjohann D, von Bahr S, Pikuleva I, Babiker A, Diczfalusy U.
Oxysterols in the circulation of patients with the Smith-Lemli-Opitz syndrome: abnormal levels of 24S- and 27-hydroxycholesterol
J Lipid Res. 2001 Mar;42(3):366-71.

III
von Bahr S, Movin T, Papadogiannakis N, Pikuleva I, Rönnow P, Diczfalusy U, Björkhem I.
Mechanism of accumulation of cholesterol and cholestanol in tendons and the role of sterol 27-hydroxylase (CYP27A1)
Arterioscler Thromb Vasc Biol. 2002 Jul 1;22(7):1129-35.

IV
Strömsten A*, von Bahr S*, Bringman S, Saeki M, Sahlin S, Björkhem I, Einarsson C. (*=equal contributors)
Studies on the mechanism of accumulation of cholesterol in the gallbladder mucosa. Evidence that sterol 27-hydroxylase is not a pathogenetic factor
J Hepatol. 2004 Jan;40(1):8-13.

V
von Bahr S, Björkhem I, van't Hooft F, Alvelius G, Nemeth A, Sjövall J, Fischler B.
Mutation in the sterol 27-hydroxylase gene associated with fatal cholestasis in infancy
Manuscript

VI
von Bahr S, Ellis E, Andersson U, Diczfalusy U, Kylander C, Einarsson C, Björkhem I.
Studies on the acidic pathway for formation of bile acids in human primary hepatocytes
Manuscript

TABLE OF CONTENTS

Abstract 4

List of papers 5

Abbreviations 8

Introduction 9
Cytochrome P450-enzymes 9
Sterol 27-hydroxylase (CYP27A1) 9
Gene structure 10
Regulation of CYP27A1 activity 11
Protein structure 12
Substrate specificity of CYP27A1 13
Bile acid synthesis 13
Neutral pathway –step by step 16
Acidic pathway –step by step 17
Role of the acidic pathway in humans 17
Role of CYP27A1 in bile acid synthesis 18
Lipoprotein-mediated reverse cholesterol transport compared to the CYP27A1-mediated mechanism 20
Possible regulatory role of CYP27A1 products 20
Cerebrotendinous xanthomatosis –metabolic consequences of CYP27A1 deficiency 21
Does CYP27A1 prevent accumulation of cholesterol in tissues? 25
Conditions with increased levels of 27OH-cholesterol in the circulation 25

Aims of the study 27

Comments on methodology 28
Studies on human material 28
Incubations with recombinant human CYP27A1 28
Protein and sterol determination in human tendons 28
Incubations with primary human alveolar macrophages 29
CYP27A1 activity measurements in human gallbladder mucosa 29
DNA-sequencing 29
Incubations with primary human hepatocytes 29
Gas chromatography – mass spectrometry 30
Electrospray – mass spectrometry 30

Results and discussion **31**
Paper I 31
Paper II 31
Paper III 32
Paper IV 33
Paper V 34
Paper VI 35

General summary **36**
Substrate specificity 36
Possible role in cholesterol accumulation 36
First Swedish case of sterol 27-hydroxylase deficiency 37
Role of 27-hydroxylation of cholesterol as the first step in bile acid formation in human hepatocytes 37

Acknowledgements **38**

References **40**

Papers I-VI

ABBREVIATIONS

ABCA1	ATP-binding cassette transporter, subfamily A, member 1
BARE	bile acid responsive element
C_{27}	steroid molecule with 27 carbons
CDCA	chenodeoxycholic acid
cholestenone	3-oxo-Δ^4-cholestenone or cholest-4-en-3-one
CMV	cytomegalovirus
CoA	coenzyme A
CTX	cerebrotendinous xanthomatosis
CYP27A1	sterol 27-hydroxylase
CYP7A1	cholesterol 7α-hydroxylase
CYP7B1	oxysterol 7α-hydroxylase
CYP8B1	sterol 12α-hydroxylase
ES-MS	electrospray mass spectrometry
GC-MS	gas chromatography-mass spectrometry
HDL	high density lipoprotein
HMG-CoA reductase	hydroxymethylglutaryl coenzyme A reductase
HNF4α	hepatocyte nuclear factor 4α
HPLC	high performance liquid chromatography
kD	kilodalton
LXR	liver x receptor
NADPH	β-nicotinamide adenine di-nucleotide phosphate, reduced form
-OH	-hydroxy
SLOS	Smith-Lemli-Opitz syndrome
Sp1	an important transcription factor
StAR	steroidogenic acute regulatory protein
THCA	trihydroxycoprostanoic acid

INTRODUCTION

Cytochrome P450-enzymes

Cytochrome P450-enzymes or CYP450s are involved in the biosynthesis of several important molecules like steroids and prostaglandines. In addition, they are also responsible for the metabolism of foreign compounds like drugs. This family of enzymes are membrane-bound heme-containing mono-oxygenases and they are found in all species from bacteria to mammals. The P stands for pigment and 450 is the absorption maximum (nm) of the protein when saturated with carbon monooxide. The hydroxylation catalyzed by P450-enzymes is a mixed-function oxidation, where molecular oxygen is utilized and one of the oxygen atoms goes to the substrate, the other to form a water molecule.

Sterol 27-hydroxylase (CYP27A1)

Sterol 27-hydroxylase (CYP27A1) is a cytochrome P450 enzyme present in the inner membranes of mitochondria in most or all cell types in mammals (Andersson *et al* 1992). It has been suggested to be an anti-atherogenic enzyme, since it may protect cells from accumulation of excess cholesterol, and the lack of it causes severe cholesterol accumulation in different tissues in humans. It has an important role in the biosynthesis of bile acids. CYP27A1 catalyses the stereospecific hydroxylation of cholesterol at position 27, forming the more polar substance 27OH-cholesterol. Two subsequent hydroxylations by the same enzyme at the same site produce cholestenoic acid with a short-lived aldehyde intermediate. CYP27A1 is also active towards several non-cholesterol C_{27}-sterols, including bioactivation (25- as well as 1α-hydroxylase activity) of vitamin D (Araya *et al* 2003, Sawada *et al* 2001). From a quantitative point of view, the most important substrates for the enzyme are 7α-hydroxylated intermediates in bile acid synthesis.

In general, the enzymatic activity appears to increase with the polarity of the C_{27}-steroid. As a mitochondrial P450, the electron transfer components needed for the activity are: adrenodoxin (ADX), adrenodoxin reductase (ADR) and NADPH. In contrast, the microsomal cytochrome p450s use NADPH cytochrome p450 reductase instead of adrenodoxin and adrenodoxin reductase.

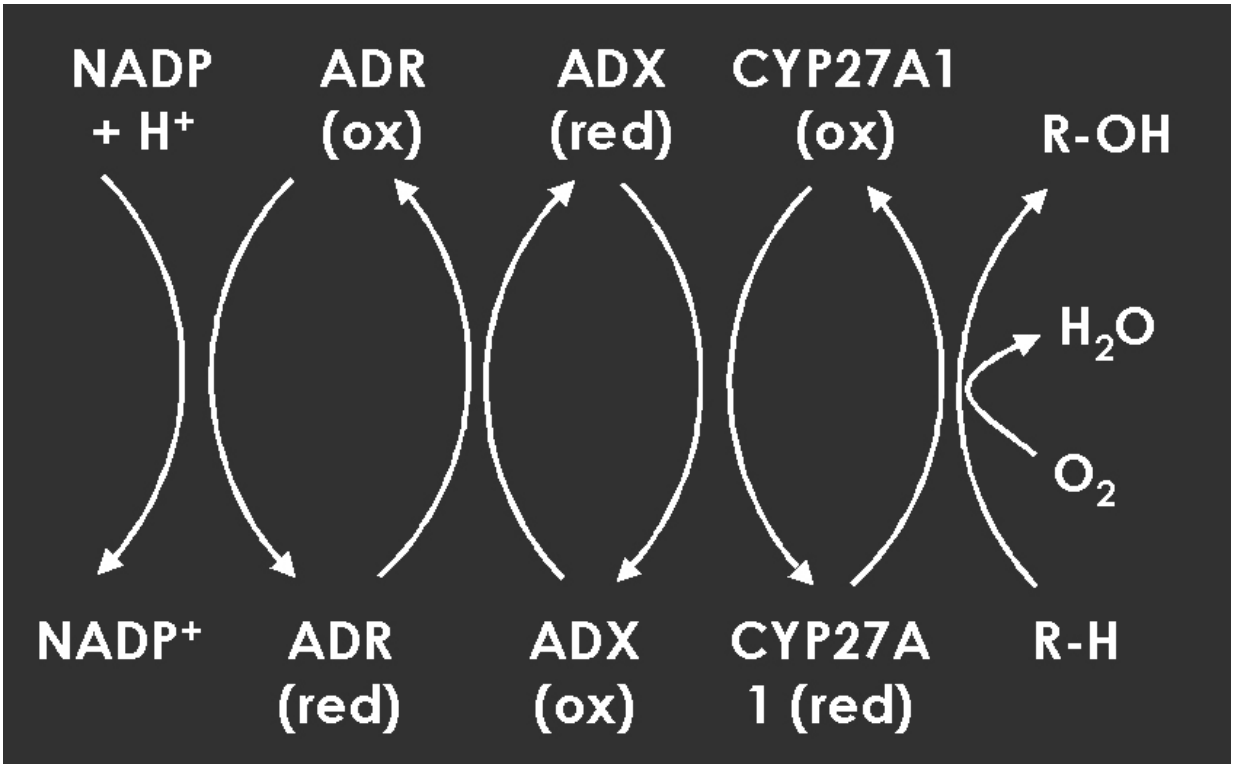

Figure 1. *Reaction mechanism of CYP27A1.*

Gene structure

The CYP27A1 gene is located on chromosome 2 in humans, and consists of nine exons and eight introns (Andersson *et al* 1992). It is shown schematically in figure 2. The first exon (containing the mitochondrial targeting signal) is the largest (667 bp), followed by a very large intron 1 (27 139 bp). Exon 2 is 192 bb, followed by intron 2 which is 2 454 bp. Exons 3-5 (with sizes 200, 198 and 173 bp) lie close together (intron sizes 130, 174, 924 bp). The same applies for exons 6-9 (with sizes 197, 79, 213 and 120 bp and intron sizes 192, 86, 153 bp). The promoter contains no discernable TATA-box, and has several transcription initiation sites (Segev *et al* 2001, Garuti *et al* 2002).

The amount of exons and introns vary between different CYP450s. CYP8B1, for instance, has no introns at all, whereas CYP46A1 has 14 introns. In addition to having regulatory elements in the promoter, it has been hypothesized that up to 30% of all genes contain regulatory regions within intron 1. The relative chances for this may increase when intron 1 is very large, as it is in CYP27A1. However, the properties of intron 1 in CYP27A1 remain to be revealed.

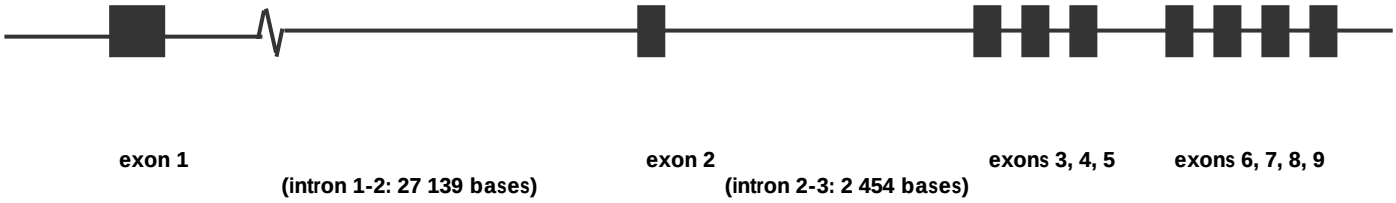

Figure 2. *Overview of the human CYP27A1 gene.*

Regulation of CYP27A1 activity

The activity of an enzyme can be regulated in several ways. The gene can be regulated transcriptionally, so that the amount of the enzyme manufactured is changed when the conditions in the cell are changed. It can be regulated through mRNA-stability, so that each blueprint is used several times or is degraded quickly. These regulations can be mediated via hormones or via the concentration of the gene product. The enzyme can also be regulated at the protein level, via activational modifications or cofactors.

The human CYP27A1 appears to have a stable activity and to be mostly regulated through substrate availability, i. e. the more cholesterol around, the more 27OH-cholesterol is made.

The promoter of human CYP27A1 gene contains several Sp1-sites, a functional HNF4α-site and a bile acid responsive element (BARE) (Chen *et al* 2003). Considerable species differences exist in the regulation of CYP27A1. In rats, the CYP27A1 gene appears to be negatively regulated by bile acids and insulin (Vlahcevic *et al* 1996, Rao *et al* 1999, Twisk 1995). According to a recent *in vivo* study by Björkhem *et al*, CYP27A1 is not significantly affected by the flux of bile acids and cholesterol at transcriptional level in humans (Björkhem *et al* 2002). When added to cultured human hepatocytes, bile acids have a slight down-regulating effect on CYP27A1 mRNA levels (Ellis *et al* 2003). This effect is however much lower than that on CYP7A1 mRNA levels. The addition of cholesterol to the culture medium of primary rat hepatocytes (Stravitz *et al* 1996) and to human extrahepatic cells (Reiss *et al* 1997, Babiker *et al* 1997) results in a significant increase in the CYP27A1 activity. At least in the latter case, this stimulation is mainly due to increased availability of substrate. CYP27A1 is upregulated by the synthetic corticosteroid dexamethasone at transcriptional level. Dexamethasone has previously been reported to increase bile acid synthesis and cholesterol 7α-hydroxylase (CYP7A1) activity (Princen *et al* 1989). Cyclosporin A inhibits the activity of CYP27A1, in particular when the substrate is unpolar (Dahlbäck-Sjöberg *et al* 1993, Princen et al 1991).

As already mentioned, substrate availability in itself seems to be the most important limiting factor *in vivo* (Pandak *et al* 2002). In rat hepatocytes, overexpression of the StAR protein, a known intracellular cholesterol transporter, leads to a 5-fold increase in bile acid biosynthesis via the CYP27A1-initiated pathway (Pandak *et al* 2000, Bauer *et al* 2000, Strauss *et al* 2003).

However, StAR mRNA or protein is not present in human liver. It is possible that another similar protein is responsible for the transport of cholesterol into mitochondria in human liver cells. MLN64, located in lysosomes and endosomes, has been suggested to be involved in intracellular trafficking of cholesterol (Zhang *et al* 2002). Other members of the StAR-family are StarD4, StarD5 and StarD6, but their roles in sterol transport remains to be elucidated.

Protein structure

The mature CYP27A1 protein consists of 531 amino acids after cleavage of the mitochondrial targeting sequence and has a molecular weight of 60 kDa. Its tertiary structure has not yet been determined. Some studies have given information about the position of the different functional domains of the enzyme (Sawada *et al* 2001).

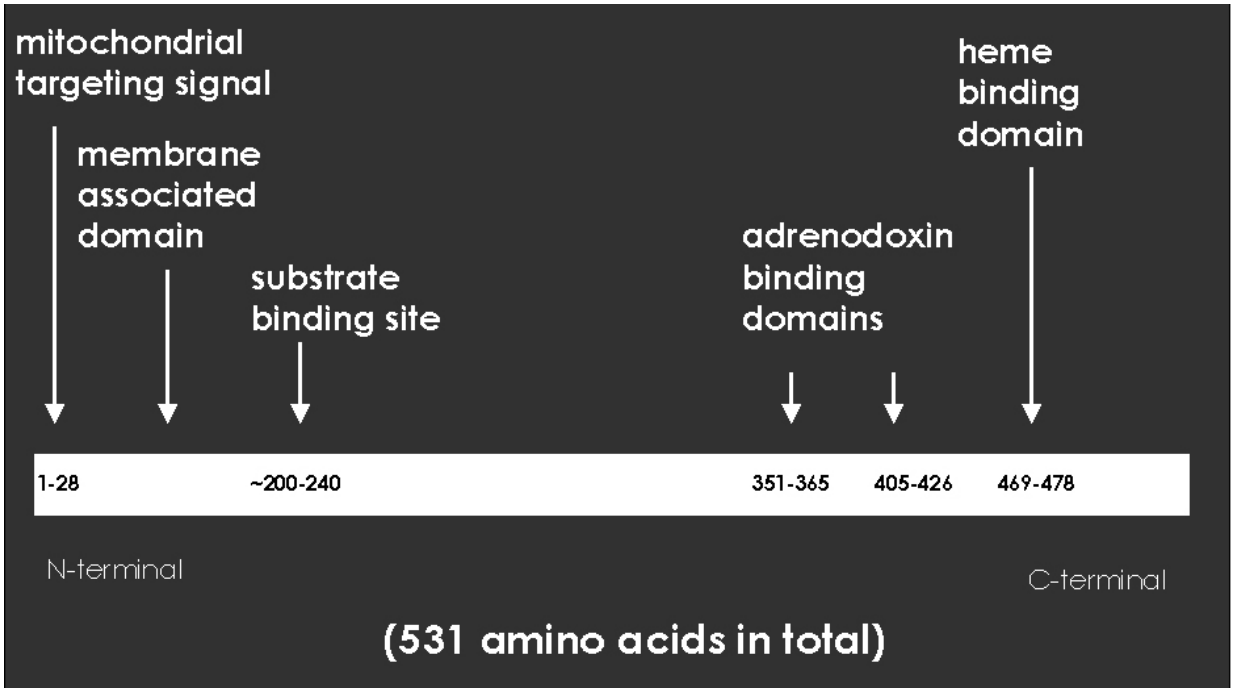

Figure 3. *Overview of the human CYP27A1 protein structure.*

Three members of the human CYP27-family have been identified to date. CYP27B1 is the major enzyme responsible for bioactivation of vitamin D, involving hydroxylation in positions 25 and 1α. These reactions can also be performed by CYP27A1. The sequence similarity between CYP27A1 and CYP27B is 53% (Sawada *et al* 2001, Araya *et al* 2003).

CYP27C1 has 43% sequence identity to CYP27A1, but it has not yet been fully characterized and its role is still unclear.

Substrate specificity of CYP27A1

CYP27A1 is considered to be a "promiscuous" enzyme with a very broad substrate specificity.

In a characterization of the mitochondrial sterol 27-hydroxylase in rat liver, Björkhem and Gustafsson reported in 1973 that 7αOH-cholesterol is 27-hydroxylated more efficiently than cholesterol itself, and with three hydroxyl groups in the C_{27}-substrate (5β-cholestane-3α, 7α, 12α-triol), the 27-hydroxylation is even faster. Thus, the activity appeared to increase with increasing polarity of the substrate. The 7α-hydroxylated intermediate in bile acid synthesis, 7α-OH-4-cholestene-3-one, was found to be a more efficient substrate than 7αOH-cholesterol.

However, as the rodent CYP27A1 differs by approximately 70% to the human enzyme, species differences may exist in substrate specificity. When the present study was initiated, there was limited information about the substrate specificity of the human enzyme. Also, it was not clear whether cholesterol precursors, cholesterol analogues, or conjugated steroids may be substrates for the enzyme.

Bile acid synthesis

Cholesterol is a lipophilic molecule, and nature has evolved sophisticated mechanisms for its transport and metabolism in the body. The most important definitive elimination of excess cholesterol from the body involves its conversion into water soluble bile acids through a series of reactions involving several different enzymes, including CYP27A1. Bile acid synthesis is important for providing a route for cholesterol excretion as well as serving as detergents for the digestion and absorption of lipids and lipid soluble vitamins from the diet.

Two main pathways for bile acid synthesis have been described, the "classical" or "neutral" and the "alternative" or "acidic".

The neutral pathway of bile acid synthesis begins with the conversion of cholesterol into 7α-OH-cholesterol through the activity of the enzyme cholesterol 7α-hydroxylase (CYP7A1). The enzymes involved in the neutral pathway of bile acid synthesis are all located in the liver. The major end product of the neutral pathway is cholic acid.

The acidic pathway starts with the hydroxylation of cholesterol at position 27 in the side chain, through the activity of the enzyme CYP27A1. Based on infusion experiments, the acidic pathway has been suggested to be responsible for about 10% of the bile acids formed in adults (Duane and Javitt,

1999). This pathway is probably more important in infants (Setchell *et al* 1998). In rats and rabbits the acidic pathway of bile acid formation seems to be more important than in humans (for a review, see Javitt *et al* 2002, Duane *et al* 2002). In rats, the acidic pathway gives rise almost exclusively to chenodeoxycholic acid, the 27-hydroxylation seems to prevent the 12α-hydroxylation and thus the formation of cholic acid. In contrast, both chenodeoxycholic acid and cholic acid seem to be formed by the acidic pathway in humans. As CYP27A1 is present in most cell types, extrahepatic 27-hydroxylation represents a quantitatively important initiation of bile acid synthesis.

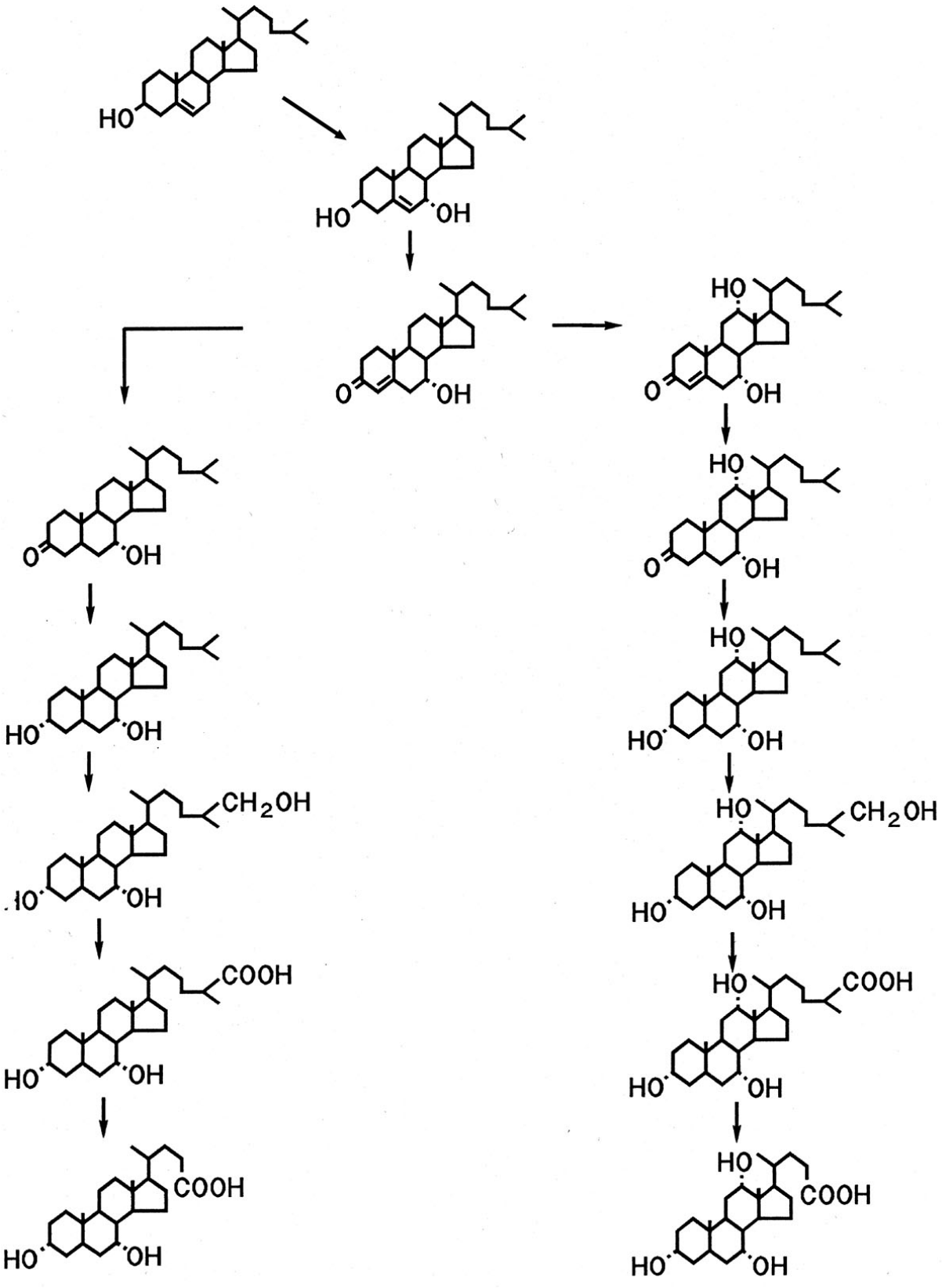

Figure 4. *Bile acid biosynthesis, the neutral pathway.*

Neutral pathway –step by step

The first step in the neutral pathway of bile acid synthesis, conversion of cholesterol to 7α-OH-cholesterol, is rate-limiting for the neutral pathway of bile acid synthesis (for a review, see Björkhem 1985, Russell *et al* 1992). CYP7A1 is subjected to a negative feed-back regulation by bile acids and is also influenced by a number of dietary and hormonal factors. Thus CYP7A1 is affecting the total cholesterol homeostasis in the body and a genetic deficiency of this enzyme leads to severe hypercholesterolemia in humans (Beigneux *et al* 2002).

The second step is the conversion of the 7αOH-cholesterol into 7αOH-4-cholestene-3-one, through the dual activity of the microsomal NAD-dependent enzyme 3β-hydroxysteroid-Δ^5-oxidoreductase/isomerase. This enzyme has two functions –it catalyses the oxidation of the 3β-OH-group as well as the isomerization of the double bond from Δ^5 to Δ^4.

At the third step, the neutral pathway diverges into two parallel routes, one leading to the formation of cholic acid and the other to the formation of chenodeoxycholic acid. In the formation of cholic acid, the conversion of 7α-OH-4-cholestene-3-one into 7α,12α-diOH-4-cholestene-3-one is catalysed by the microsomal sterol-12α-hydroxylase (CYP8B1).

The fourth step towards cholic acid formation is the conversion of 7α,12α-diOH-4-cholestene-3-one into 7α,12α-diOH-5β-cholestane-3-one by the action of the soluble NADPH-dependent cytosolic enzyme 3-oxosteroid Δ^4-steroid 5β-reductase. This enzyme does not seem to be of major regulatory importance.

The fifth step is the conversion of 7α,12α-diOH-5β-cholestane-3-one into 5β-cholestane-3α,7α,12α-triol, a reaction catalysed by the cytosolic 3α-hydroxysteroid dehydrogenase.

The sixth step in cholic acid formation is the conversion of 5β-cholestane-3α,7α,12α-triol into 5β-cholestane-3α,7α,12α,27-tetrol, catalysed by the mitochondrial sterol 27-hydroxylase (CYP27A1).

The seventh step towards cholic acid formation is also catalysed by CYP27A1, and leads to the formation of 5β-cholestane-3α,7α,12α-triOH-cholestenoic acid (trihydroxycoprostanoic acid, THCA). This step can also be catalysed by a soluble or a mitochondrial alcohol dehydrogenase and aldehyde dehydrogenase, but with lower efficiency. THCA is then CoA-activated by microsomal or peroxisomal CoA ligase and finally β-oxidised in the peroxisomes to yield the CoA-ester of cholic acid.

In the peroxisomes, the CoA of the β-oxidation product (either choloyl-CoA or chenodeoxycholoyl-CoA) is replaced with glycine or taurine, resulting in glyco/taurocholate. This reaction is performed by bile acid CoA:amino acid N-acyltransferase.

The sequence of reactions in the formation of chenodeoxycholic acid is the same or similar to that above, with the exception that there is no introduction of a 12α-hydroxyl group.

It should be pointed out that with the exception of CYP7A1, most of the enzymes in the pathway have a relatively broad substrate specificity.

Because of this, alternative sequences to those presented in figure 4 may exist in which e.g. the 27-hydroxyl group or the 12α-hydroxyl group may be introduced at an earlier or later stage.

Acidic pathway -step by step

The first step in the acidic pathway is a mitochondrial 27-hydroxylation of cholesterol by CYP27A1, yielding the product 27OH-cholesterol. This may occur in mitochondria of most cell types.

The second step in the acidic pathway may be two further hydroxylations at the same site (carbon 27) by the same enzyme (CYP27A1), giving the product cholestenoic acid and a water molecule. The intermediate aldehyde is very short-lived, and in biological situations only 27OH-cholesterol and cholestenoic acid can be isolated. Also this step may occur in any cell type with sufficient amounts of CYP27A1. If the levels of CYP27A1 are low, 27OH-cholesterol will be the only product. Cholestenoic acid and 27OH-cholesterol are able to leave the cell and enter the circulation, from where they are rapidly taken up by the liver, and participate in bile acid synthesis. Most of the cholestenoic acid present in the circulation originates from the lung, which has remarkably high levels of CYP27A1 (Babiker *et al* 1999).

The next step in the acidic pathway is the 7α-hydroxylation of cholestenoic acid or 27OH-cholesterol by the microsomal enzyme oxysterol 7α-hydroxylase (CYP7B1). There is a large amount of this enzyme in the liver, but the enzyme is also present in several extrahepatic tissues.

The remaining steps in the major acidic pathway are similar to those in the neutral pathway and lead to the formation of chenodeoxycholic acid and cholic acid, with a preference for the former.

There is little coupling between the acidic and the neutral pathway. As pointed out above, the acidic pathway does not seem to be regulated by the enterohepatic circulation of bile acids in humans. Several intermediates, 27OH-cholesterol and cholestenoic acid as well as 24S OH-cholesterol and also 7α,27-diOH-cholesterol, are formed in peripheral tissues and are taken up by the liver to enter the acidic pathway for bile acid biosynthesis.

Role of the acidic pathway in humans

The relative importance of the neutral and acidic pathway for bile acid formation in humans has not been defined with certainty. Results obtained from *in vivo* studies with intravenously administred labelled bile acid precursors are thus different from results obtained with cultured human hepatocytes. In one study, primary human hepatocytes were incubated with large quantities of the bile acid precursors 7αOH-cholesterol and 27OH-cholesterol. Both cholic acid and chenodeoxycholic acid were formed from

both precursors in similar quantities (Sauter *et al* 1996). When the present study was initiated, the relative importance for the acidic versus neutral pathway had not been studied with physiological concentrations of precursors in primary human hepatocytes.

Role of CYP27A1 in bile acid synthesis

It is evident from above that the mitochondrial CYP27A1 catalyses both the initial, rate limiting step in the acidic pathway, as well as one of the steps in the neutral pathway of bile acid synthesis. Since CYP27A1 is active in most cell types, the acidic pathway for excretion of excess cholesterol can be utilized as an alternative to the lipoprotein-mediated reverse cholesterol transport (described on page 20) (Björkhem *et al*, 1999). In this connection it is important that side-chain oxidised oxysterols are able to move across lipophilic membranes at a rate orders of magnitude faster than that of unmetabolised cholesterol (Meaney *et al* 2002). It has been estimated that about 5% of the total formation of bile acids in humans originates from 27-hydroxylated intermediates formed extrahepatically (Lund *et al* 1996).

It has been shown that most of the 27OH-cholesterol in the circulation originates from extrahepatic sources and that it is efficiently taken up by the liver (Meaney *et al* 2002, Lund *et al* 1996). In summary, CYP27A1 is an essential enzyme in bile acid synthesis and subsequent elimination of excess cholesterol from the body. Subjects born with defects in the CYP27A1 gene therefore have severe defects in their bile acid pattern and accumulate cholesterol in different tissues.

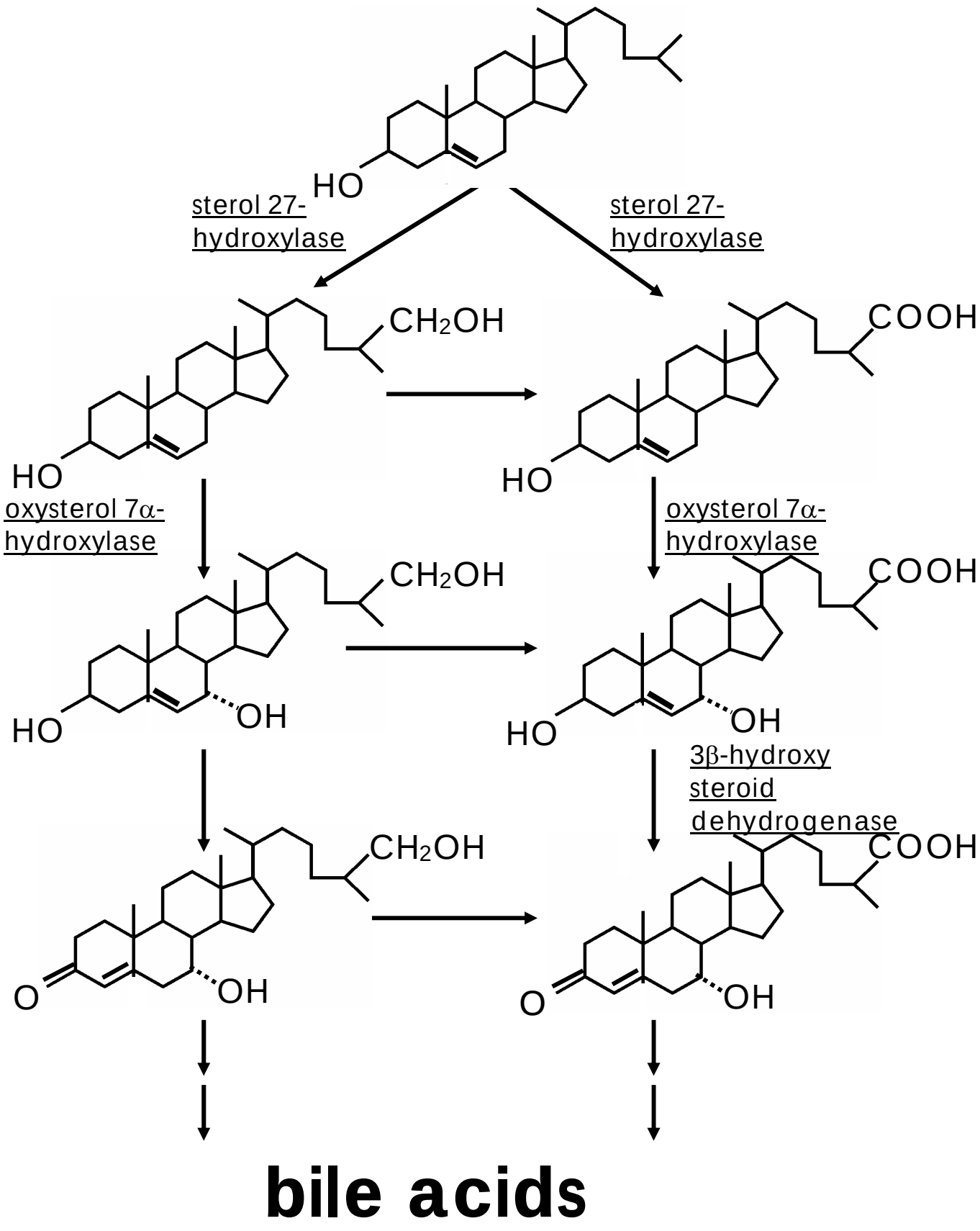

Figure 5. *The role of CYP27A1 in bile acid synthesis.*

Lipoprotein-mediated reverse cholesterol transport compared to the CYP27A1-mediated mechanism

In the intestine, dietary cholesterol is taken up after solubilization by bile acids. The cholesterol is transported in chlomicron particles and distributed to different cell types via the circulation. About 7% of the cholesterol circulates in the body bound to lipoproteins in LDL-particles. These particles interact with LDL-receptors on the cell surface and are internalized. The need for cholesterol in the cell is reflected by the amount of LDL-receptors on its surface. The uptake of LDL down-regulates the synthesis of new LDL-receptors and also of cholesterol synthesis via regulation of HMG-CoA reductase.

Excess cholesterol in cell membranes is taken up by HDL-particles and transported from extrahepatic tissues back to the liver for metabolism. This is called the "reverse cholesterol transport". In the liver, the HDL cholesterol can be converted into bile acids and excreted from the body, or it can be re-packed into LDL-particles and re-enter the circulation. LDL-cholesterol may be taken up by other tissues where it can be used (for structural purposes or hormone synthesis for example) or esterified by ACAT and stored. As a consequence, the reverse cholesterol transport may merely result in a redistribution of a large part of the existing cholesterol. Although the capacity for oxidative elimination of cholesterol from extrahepatic cells by the CYP27A1 mechanism is much lower than the capacity for lipoprotein mediated reverse cholesterol transport, the former represents a mechanism that will result in a definite removal of cholesterol from the body.

The oxidative mechanism may be of particular importance in tissues with a low degree of vascularization, such as tendons, eye lens and the central nervous system, where there is less access to the circulating lipoproteins that normally also transport cholesterol. Indeed, these specific tissues are also affected in cases of sterol 27-hydroxylase deficiency.

In the circulation, there is usually a good correlation between the levels of 27OH-cholesterol and the levels of cholesterol (Babiker, doctoral thesis 1998). This is probably mainly due to the fact that 27OH-cholesterol and cholesterol are transported in the same lipoproteins.

Possible regulatory role of CYP27A1 products

The ability of cholesterol to regulate its own homeostasis was shown by Brown and Goldstein, who were awarded the Nobel prize for this in 1985. The transcriptional regulation of several genes in cholesterol homeostasis can involve activation of so called nuclear receptors. When activated, these receptors can bind to promoter regions of specific genes and thus upregulate transcription. Several oxysterols have been suggested to be able to activate such nuclear receptors, for example side chain-oxidized oxysterols. These oxysterols seem to bind and activate the LXR (at least *in vitro*) which dimerizes upon activation and binds to specific promoter sequences and thus can

activate specific genes believed to be involved in cholesterol homeostasis. Many genes have been suggested to be activated by LXR, such as ABCA1, a cholesterol transporter molecule.

One group (Fu *et al* 2001) recently reported LXR-activation and subsequent ABCA1-upregulation by 27OH-cholesterol in cholesterol-loaded macrophages, thereby providing an alternative explanation for the ability of CYP27A1 to impact on the cholesterol efflux from cells. However, two other groups (Lehman *et al* 1997, Janowski *et al* 1996) failed to demonstrate an efficient LXR-activation by 27OH-cholesterol. It was recently shown in a preliminary study that cholesterol may interfere with the binding of oxysterols to the LXR-receptor (Meaney, doctoral thesis 2003). Since the levels of cholesterol in general are several orders of magnitude higher than those of oxysterols in most tissues, this may prevent a regulatory effect of oxysterols via LXR *in vivo*. More studies are needed before any firm conclusions can be drawn about a role of 27OH-cholesterol in ABCA1-mediated cholesterol transport.

27OH-cholesterol is an efficient inhibitor of the rate-limiting enzyme in cholesterol biosynthesis, HMG CoA-reductase *in vitro* (Hall *et al* 2001). The possibility has been discussed that the increased cholesterol synthesis that can be demonstrated in patient with CYP27A1 deficiency (c.f. below) is due to a lack of a normal down-regulation of cholesterol synthesis, due to the lack of 27OH-cholesterol (Björkhem *et al* 2001). Since the effect on cholesterol synthesis can be normalised by treatment with chenodeoxycholic acid, it is most likely that the effects on cholesterol synthesis in this disease are secondary to reduced chenodeoxycholic acid.

The finding of 27-hydroxylated and sulphatated or glucuronidated products in human circulation (Björkhem *et al* 2001) also suggests that CYP27A1 may have additional roles to play in the human metabolism, apart from the synthesis of bile acids.

Cerebrotendinous xanthomatosis and metabolic consequences of CYP27A1 deficiency

About 250-300 subjects in the world have been diagnosed with the rare inherited disease cerebrotendinous xanthomatosis (CTX) (for a review, see Björkhem *et al* 2001). Most cases have been found in Israel, Japan and Holland. Until recently, only two cases (two Norwegian sisters) were found in Scandinavia. In the summer of 2003, two new cases were found in Sweden, an infant boy of Bangladeshi origin who died at the age of 3.5 months, and an adult female of Swedish origin.

The first case of this rare disease was described in 1937 (van Bogaert 1937). Based on experiments with a liver biopsy from a CTX-patient it was concluded in 1981 that the defect is in the sterol 27-hydroxylase (Oftebro *et al* 1981). The human gene was however cloned first 10 years later (Cali, Russell 1991) and evidence was presented for a mutation in the CYP27A1 gene in CTX. Several different mutations of the CYP27A1-gene have been reported since

then, point mutations as well as deletions, insertions and splice variants (Björkhem *et al* 2001). The symptoms of CTX are dementia, ataxia, cataracts, xanthomas in tendons and brain and often premature atherosclerosis. The patients have intracellular accumulation of cholesterol, but normal cholesterol levels in the circulation. A special feature in CTX is that in addition to cholesterol, also the 5α-saturated metabolite cholestanol accumulates in the xanthomas. The xanthomas start to form after several years of life, and thus the disease can remain undiagnosed until the patient reaches puberty. It is common that infants with CTX have no symptoms of the disease. It was recently found however, that in some cases, CTX can be associated with neonatal cholestasis (Clayton *et al* 2002). With early treatment, the xanthomas and therefore the subsequent dementia, can be reduced or even prevented, so early diagnosis is of importance (Björkhem *et al* 2001, Mondelli *et al* 2001).

The production of the bile acid chenodeoxycholic acid is very low in these patients, whereas the production of cholic acid can be almost normal. Patients with CTX also excrete large amounts of 25-hydroxylated bile alcohols in bile, feces and urine. The bile alcohols accumulate due to the defective 27-hydroxylation in the bile acid synthesis (figure 6). In total, gram amounts of these bile alcohols can be excreted during a day.

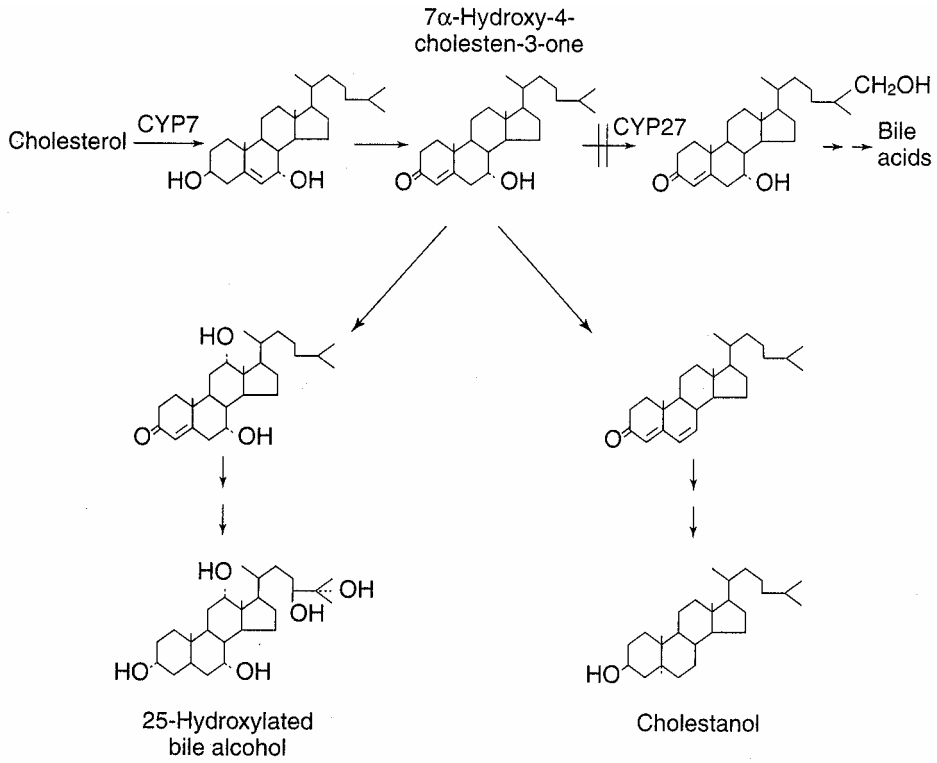

Figure 6. *Situation in CTX, illustrating the buildup of bile alcohols and cholestanol.*

The explanation for the almost normal production of cholic acid in patients with CTX is that the 25-hydroxylated bile alcohols may be converted into this bile acid by a mechanism involving cleavage of acetone from the

steroid side chain (figure 7). Since cholic acid is much less efficient as a suppressor of CYP7A1 than chenodeoxycholic acid in humans (Ellis *et al* 2003), there will be an upregulation of this enzyme, producing large amounts of 25-hydroxylated bile alcohols and cholestanol.

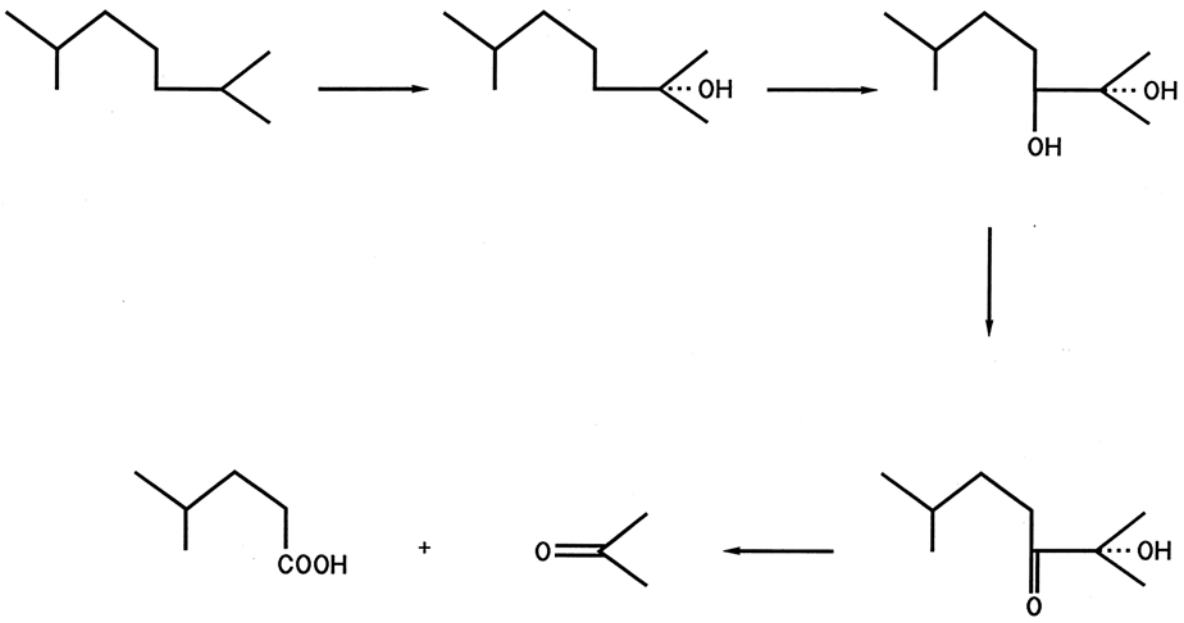

Figure 7. *Proposed mechanism for the formation of cholic acid from 25-hydroxylated bile alcohols by cleavage of acetone (Salen* et al *1979).*

The disease is generally treated by the administration of bile acids, especially chenodeoxycholic acid, which is the lacking end-product. This bile acid will, as previously mentioned, efficiently downregulate the activity of CYP7A1 and in that way reduce the amounts of 7α-OH-cholesterol and the accumulation of cholestanol and 25OH-bile alcohols formed (see figure 8). This treatment reduces the xanthomas and after some time they may even disappear totally. It is also clear that the neurological problems caused by the xanthomas in the brain are reduced by chenodeoxycholic acid treatment in CTX patients. Other bile acids, such as ursodeoxycholic acid are sometimes also used, especially if the patient is cholestatic.

Intracellular cholesterol accumulation in xanthomas also occurs in patients with high cholesterol levels in the circulation, such as in case of familial hypercholesterolemia. The reason for an intracellular cholesterol accumulation in spite of normal plasma cholesterol levels in CTX patients is likely to be the lack of the CYP27A1-mediated system for cholesterol elimination. The CTX-patients also seem to have an upregulated cholesterol synthesis, probably a secondary effect of the defective bile acid synthesis with less suppression of CYP7A1. The upregulation of CYP27A1 will cause a higher than normal

consumption of cholesterol, resulting in a compensatory increase in cholesterol synthesis (see figure 8).

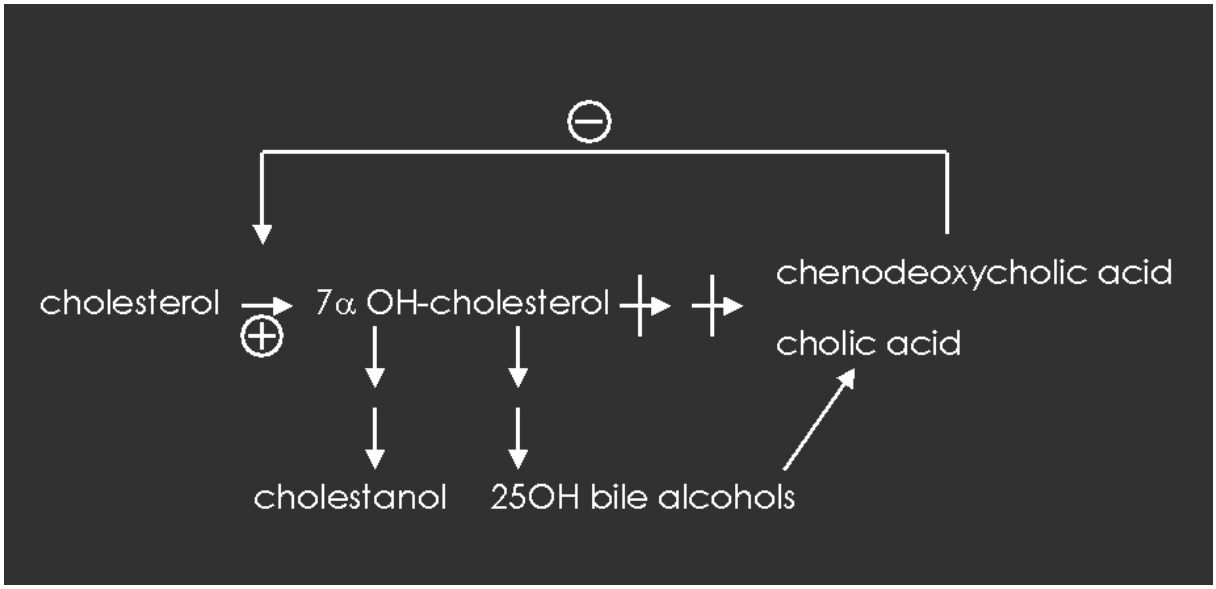

Figure 8. *In CTX, the lack of chenodeoxycholic acid means lack of downregulation of CYP7A1, leading to high levels of 7αOH-cholesterol and results in accumulation of 25-hydroxylated bile alcohols and cholestanol.*

At least part of the accumulation of cholestanol in patients with CTX has been showed to be due to accumulation of intermediates from the defective bile acid synthesis (Björkhem *et al* 2001). One such intermediate is 7αOH-4-cholesten-3-one, which normally would be metabolised by CYP27A1 in the neutral bile acid pathway. A product of accumulated 7αOH-4-cholesten-3-one is cholestanol, thus explaining the accumulation of cholestanol in CTX patients (see figure 6). Whether or not cholestanol is a substrate for CYP27A1 was not known when the present investigation was initiated.

Together with an Israeli group, our group has developed CYP27A1-knockout mice (Rosen *et al* 1998). These mice have a low bile acid production but otherwise appear to have a normal phenotype on a standard diet. They do not develop xanthomas or atherosclerosis. However, on a high-cholesterol diet they have a higher mortality than on a control diet. The reason that the CYP27A1-/- mice do not show CTX-symptoms is not clear, but it has been suggested that the 25-hydroxylation pathway is induced in these mice (Honda *et al* 2001). However, whether or not this is the explanation for the different consequences of CYP27A1 deficiency in mouse and human is not known.

In any case, it is evident that CYP27A1 is an important enzyme and that the lack of it causes severe symptoms, at least in humans.

Does CYP27A1 prevent accumulation of cholesterol in tissues?

Accumulation of cholesterol in tissues may occur in many diseases. The process of atherosclerosis involves accumulation of cholesterol in the vessel wall. In cholesterolosis, cholesterol accumulates in the gallbladder mucosa. In familial hypercholesterolemia, atherosclerosis is common, and also in particular xanthomas, accumulation of cholesterol in tendons. In CTX, as described above, cholesterol and also cholestanol accumulate in various tissues such as tendons, cns and eye lens. It is also common that CTX patients suffer from premature atherosclerosis.

The accumulation of cholesterol can have different explanations. It can be due to increased uptake or formation, and/or due to decreased metabolism or excretion.

The occurrence of xanthomas and premature atherosclerosis in patients with CTX is consistent with the possibility that CYP27A1 is an anti-atherogenic enzyme, preventing accumulation of cholesterol in various cells. This hypothesis is also consistent with the high levels of CYP27A1 enzyme found in macrophages (Babiker *et al* 1997) and the high amount of 27OH-cholesterol found in human atheromas (Björkhem *et al* 1994, Shanahan *et al* 2001). However, the relative role of CYP27A1 for prevention of accumulation of cholesterol in different tissues is not known, and systematic studies of this are lacking. In view of the preferential formation of xanthomas in tendons of CTX-patients, tendons would be of particular interest to study with respect to presence of CYP27A1 and 27OH-cholesterol. Another interesting model system for such studies may be the gallbladder wall. In this tissue there is sometimes an accumulation of cholesterol (cholesterolosis). In contrast to the accumulation of cholesterol in atheromas, this accumulation does not seem to have any pathological significance (Sahlin *et al* 1995). In the present work the hypothesis was tested that part of the difference between normal gallbladders and gallbladders affected by cholesterolosis could be due to variations in the activity of CYP27A1.

Conditions with increased levels of 27OH-cholesterol in the circulation

If CYP27A1 is an anti-atherogenic enzyme, an upregulation would be advantageous. A general upregulation of the enzyme would be expected to lead to increased levels of 27OH-cholesterol in the circulation. In our laboratory we have systematically searched for such patients and healthy subjects. Up to now three different categories of subjects have been found with high circulating levels of 27OH-cholesterol and its metabolite cholestenoic acid. One category is a minor subfraction of patients with advanced atherosclerotic disease (Amir Babiker, doctoral thesis 1998). The high levels of 27OH-cholesterol in these patients may reflect presence of an increased number of active macrophages with upregulated CYP27A1.

We have also identified one specific family of healthy members in which the mother, her son and daughter were found to have markedly and constantly elevated levels of 27OH-cholesterol. In spite of considerable efforts, we have not been able to find the mechanism behind the high levels. We have excluded the possibility that there is a mutation in the exons or proximal promoter region in the gene coding for CYP27A1 (von Bahr *et al*, unpublished observation). We have also excluded the possibility that there is a defect in the gene coding for CYP7B1, the most important enzyme involved in the metabolism of 27OH-cholesterol (Jakobsson *et al*, unpublished observation).

The third category of subjects with high levels of 27OH-cholesterol in the circulation are patients with the Smith-Lemli-Opitz syndrome (SLOS). Such patients have a defect in the cholesterol synthesis due to a functional lack of the enzyme 7-dehydrocholesterol-7-reductase, catalysing the last step in the cholesterol synthesis chain (Tint *et al* 1997). This results in the accumulation of the metabolic intermediate 7-dehydrocholesterol in tissues (this accumulation is especially profound in the brain) and markedly reduced levels of cholesterol.

The clinical symptoms of SLOS are variable, including distinctive facial appearance with microcephaly, bitemporal narrowing, ptosis, anteverted nares, cleft palate, micrognathia. There may also be cardiac, intestinal, genitourinary, skeletal and central nervous system abnormalities, often with a moderate to severe mental retardation. The different malformations may reflect the need for cholesterol during early embryonic development.

In view of the fact that substrate availability seems to be of regulatory importance for the activity of CYP27A1, at least *in vitro*, one would expect lower levels of 27OH-cholesterol in patients with SLOS. Surprisingly however, the opposite was found. The reason for this is not obvious. In principle there are two primary possibilities, increased synthesis or decreased metabolism. The possibility that CYP27A1 may act directly on the accumulating intermediate metabolite 7-dehydrocholesterol with a subsequent saturation of the Δ^7-double bond must also be considered.

AIMS OF THE STUDY

-to provide a more detailed characterization of the substrate specificity of human CYP27A1

(papers I-III)

-to investigate the role of CYP27A1 in accumulation of cholesterol and cholestanol in tendons and development of xanthomas

(paper III)

-to investigate whether CYP27A1 is of importance for the development of cholesterolosis

(paper IV)

-to characterize the first Swedish case of CYP27A1 deficiency

(paper V)

-to characterize the acidic pathway of bile acid biosynthesis in primary human hepatocytes

(paper VI)

COMMENTS ON METHODOLOGY

Studies on human material

In this study, almost all of the experiments have been made with human materials (plasma, urine, cells, enzymes or tissues). All these experiments have been approved by a local ethical committee at the Karolinska University Hospital, Huddinge (permissions n:o 365/95, 112/97, 302/99, 303/99, 78/99, 278/01, 291/03, 464/03).

Incubations with recombinant human CYP27A1

Human recombinant CYP27A1 was incubated with adrenodoxin, adrenodoxin reductase, NADPH and sterol extract under conditions as previously described (Pikuleva *et al* 1997). The substrate was dissolved in ethanol. In paper I, several substrates were evaluated, including cholesterol, cholesterol sulfate, cholesterol oleate, 7αOH-cholesterol, cholestanol, 4-cholestene-3-one. In paper I, another human recombinant enzyme, CYP7A1, was used as well as CYP27A1.

In paper II a sterol extract containing cholesterol, 7-dehydrocholesterol and 8-dehydrocholesterol in about equal amounts, was obtained from the plasma of an SLOS patient and incubated with the recombiant human CYP27A1. In addition, the system was incubated with pure cholesterol, pure 7-dehydrocholesterol and a mixture of these compounds under the same conditions.

In paper III, cholestanol and cholesterol were compared as substrates for the recombinant CYP27A1.

The incubation mixtures were extracted and analysed by GC-MS as described previously (Björkhem *et al* 1974, Dzeletovic *et al* 1995, Babiker *et al* 1999).

Protein and sterol determination in human tendons

In paper III, human achilles tendons from autopsy materials as well as from patients treated surgically for Achilles tendinosis (a tendon condition involving pain and swelling) were used. In addition to this, tendon xanthomas from a patient with familial hypercholesterolemia was obtained. The CYP27A1 levels were measured by western blotting and compared to the total protein level in each sample, measured by the Lowry method. The lipid content (free cholesterol, esterified cholesterol, cholestanol, 27OH-cholesterol) was

measured by GC-MS. The CYP27A1-localization and macrophage content was visualized by immunohistochemistry and microscopy.

Incubations with primary human alveolar macrophages

In paper III, primary human alveolar macrophages were isolated from patients undergoing broncho-alveolar lavage. These cells have the highest activity of CYP27A1 found so far and were cultured for several days and incubated with cholesterol and/or cholestanol. The culture medium was extracted and analysed by GC-MS.

CYP27A1 activity measurements in human gallbladder mucosa

Fresh gallbladder mucosa was isolated from patients with gall stones, with or without cholesterolosis. The mucosa was homogenized and incubated with the most efficient CYP27A1-substrate 5β-cholestane-3α,7α,12α-triol. The reaction mixture was extracted and analysed on GC-MS for the 27-hydroxylated product 5β-cholestane-3α,7α,12α,27-tetrol.

DNA-sequencing

DNA was isolated from the blood of an infant with CTX and from both his parents. The DNA was sequenced in order to determine the location of the mutation. DNA from control subjects was also analysed in order to detect polymorphisms of more common nature. Exons 1 and 2 of CYP27A1 were transcribed separately, including parts of untranslated regions. Exons 3-5 lie close together and were transcribed together in one pcr-reaction, as were exons 6-9, including all intron regions between the exons.

Incubations with primary human hepatocytes

Human liver tissue was obtained from patients with liver cancer undergoing reduction hepatectomy. Hepatocytes were isolated by a two-step perfusion technique, utilizing EGTA and collagenase as described in detail by Li *et al.* The hepatocytes were cultured for four to five days and then incubated with labelled 7αOH-cholesterol or 27OH-cholesterol for one to two days. The culture medium was hydrolyzed, extracted and analysed for bile acids by radio-HPLC. The endogenous bile acid content was analysed by GC.

Gas chromatography – mass spectrometry

In this study, oxysterols and other lipids from various extracts and tissues have been measured by isotope-dilution-mass spectrometry with deuterium-labelled compounds as internal standards (Björkhem *et al* 1974, Dzeletovic *et al* 1995, Babiker *et al* 1999).

Electrospray – mass spectrometry

Urine samples from a patient with CTX was analysed by ES-MS (Yang *et al* 1997). Bile acid conjugates and bile alcohols, as well as steroid hormone metabolites and other compounds are easily determined with this method.

RESULTS AND DISCUSSION

Paper I

In paper I, different substrates were incubated with CYP27A1 and the rate of conversion was compared. Polar substrates were found to be 27-hydroxylated more efficiently than unpolar. Cholesterol was thus found to be 27-hydroxylated more efficiently than cholesterol oleate, but less efficiently than cholesterol sulfate. Side chain-hydroxylated sterols like 24S-OH-cholesterol or 25-OH-cholesterol were 27-hydroxylated less efficiently than cholesterol, possibly due to steric hindrance. Steroids with a 3-oxo-Δ^4 structure were 27-hydroxylated at a much higher rate than the corresponding 3β-OH-Δ^5 steroids. A 3-oxo-Δ^4 steroid, cholestenone, was found to be 27-hydroxylated 10 times as efficiently than cholesterol. Cholestenone is a precursor to cholestanol, which is accumulated in large quantities in patients lacking CYP27A1. The possibility must be considered that there are increased levels of cholestenone in patients with CTX. In the normal case, cholestenone may be drained from the cholestanol-forming pathways by CYP27A1, probably leading to formation of bile acids.

Several oxysterols have been suggested to affect gene transcription and it is possible that 27-hydroxylation of these compounds may alter this effect and initiate their elimination from the body.

CYP27A1 has not yet been crystallized, and the possibility may be considered that there is a hydrophobic and a hydrophilic subpocket for the substrate binding. This would be in accordance with the situation in CYP3A4, another "promiscuous" enzyme with a broad substrate specificity (Shou *et al* 1994). An indication of this is the fact that cyclosporin noncompetitively inhibits the 27-hydroxylation of hydrophobic substrates but not of hydrophilic substrates (Dahlbäck-Sjöberg *et al* 1993). These possibilities remain to be investigated and will in all probability be elucidated when the complete 3D-structure of the enzyme has been solved.

Paper II

In paper II, we focused on the surprisingly high levels of 27OH-cholesterol in patients with SLOS. These patients accumulate 7-dehydrocholesterol and 8-dehydrocholesterol, but have extremely low levels of cholesterol. We investigated if these accumulating compounds were substrates for human recombinant CYP27A1.

The rate of 27-hydroxylation of these compounds by a recombinant human enzyme was found to be considerably lower than that of cholesterol, only about 20%, suggesting that a CYP27A1-activity towards these sterols is not the explanation for the high levels of 27OH-cholesterol in patients with SLOS. No significant levels of 27-hydroxylated 7-dehydrocholesterol could be found in

the circulation of patients with SLOS in our study. However, after publication of the present work, Wassif and collaborators were able to identify low levels of 27-hydroxylated metabolites of 7- and 8- dehydrocholesterol in the circulation of SLOS subjects (Wassif *et al* 2003). The ratio between 27OH-7-dehydrocholesterol and 27OH-cholesterol in their study was found to be about 0.3. In contrast to our study, Wassif and collaborators performed their experiments in the dark, in order to avoid the structural modifications that can occur with 7-dehydrocholesterol when exposed to UV-light. This may be part of the explanation for the differences in our findings.

Oxysterol 7α-hydroxylase or CYP7B1 has a key role in the metabolism of 27OH-cholesterol and cholestenoic acid in humans. A defect in CYP7B1 could be predicted to lead to accumulation of 27OH-cholesterol and cholestenoic acid. Mice with disrupted CYP7B1 also have elevated plasma-27OH-cholesterol. It is thus possible that there is a low activity of CYP7B1 in the SLOS patients. Consistent with this hypothesis we found that the levels of the CYP7B1 metabolite 7αOH-cholestenoic acid were reduced in these patients. We therefore suggest that the high levels of 27OH-cholesterol in these subjects with SLOS are due to low metabolism rather than increased synthesis.

Paper III

In this study, the hypothesis was tested that tendons and xanthomas contain CYP27A1 and that this enzyme is of importance for the elimination of cholesterol and cholestanol from this tissue. Immunohistochemistry and western blotting experiments showed that CYP27A1 is present in human tendons, both in macrophages and in tenocytes. The product 27OH-cholesterol was also found in this tissue, and the ratio between this oxysterol and cholesterol was found to be significantly higher in tendons than in the circulation.

In a pilot experiment, tenocytes and macrophages from a fresh xanthoma removed from a patient with familial hypercholesterolemia were cultured for several days. In the culture medium, cholesterol crystals precipitated (see the cover of this thesis). A net production of 27OH-cholesterol was observed when levels in the culture medium were measured before and after culture (unpublished data). The macrophages (adhesive cells) showed a higher 27-hydroxylation activity than the tenocytes.

Both recombinant human CYP27A1 and primary human alveolar macrophages with high CYP27A1-activity were able to 27-hydroxylate cholestanol into 27OH-cholestanol and cholestanoic acid, at approximately the same rate as that of cholesterol. However, cholestanol was taken up from the culture medium by the macrophages at a lower rate than cholesterol and also seemed to leave the cells at a lower rate, resulting in a selective accumulation of cholestanol compared to cholesterol. This is in accordance with the situation in CTX-patients, who can have levels of cholestanol at up to 10-20% of those of cholesterol (Björkhem *et al* 2001).

It may be speculated that the accumulation of cholestanol may decrease the efflux of cholesterol in these cells by affecting the ABCA1-transporter. If this is the case, the presence of cholestanol in the cells may initiate the formation of xanhomas in patients with CTX. This hypothesis is consistent with the observation that the levels of cholestanol decrease during the treatment with chenodeoxycholic acid in CTX-patients, in parallel with decreasing size of the xanthomas (Björkhem *et al* 2001, Mondelli *et al* 2001).

In conclusion, CYP27A1 seems to be of importance for the elimination of cholesterol and cholestanol from tendons and it seems to have a protective role in the xanthoma formation.

Paper IV

Cholesterolosis is a condition with accumulation of cholesterol in the gallbladder mucosa. It does not seem to be of pathological importance. Cholesterol-rich macrophages are found in the mucosa and, from a histological point of view, there are similarities between this condition and early atherosclerosis. The hypothesis was tested that cholesterolosis could be due to a decreased activity of CYP27A1 in these macrophages, leading to a decreased elimination of cholesterol from the tissue.

In this study, CYP27A1 activity measurements, western blotting of CYP27A1 protein and analysis of the levels of CYP27A1 mRNA from fresh human homogenised gallbladder mucosa showed that there were no significant differences between cholesterolosis and non-cholesterolosis. The levels of 27OH-cholesterol in the gallbladder mucosa were markedly elevated in cholesterolosis, probably secondary to the high cholesterol levels and thus due to increased substrate availability. Most of the cholesterol and almost all of the 27OH-cholesterol in the cholesterolosis samples were found to be esterified. The ACAT activity was found to be elevated in the cholesterolosis samples and the levels of ABCG5 mRNA, a cholesterol transporter, were also elevated. We conclude that in our group of patients, decreased CYP27A1 was not a pathogenic factor in cholesterolosis. We hypothesise that due to the high ACAT-activity, esterified 27OH-cholesterol may be trapped in the cells. Esterified 27OH-cholesterol is thus less likely removed from the tissues than the free oxysterol. The activity of ACAT is likely to increase secondarily to the increase in cholesterol content. The possibility must be considered that effects on a cholesterol transporter may be part of the explanation for the accumulation of cholesterol. The mRNA levels of ABCG5 were found to be elevated in cholesterolosis, but whether this is primary or secondary to the accumulation is not clear as of yet.

Paper V

The lack of CYP27A1 in CTX leads to decreased bile acid synthesis and to accumulation of bile alcohols. Eventually, these patients develop xanthomas with cholesterol and cholestanol in brain and tendons. This disease is very rare and in all of Scandinavia, only two cases of CTX had been diagnosed previously (two Norwegian sisters). Most often, the disease is diagnosed first after several years of accumulation of cholesterol and cholestanol, leading to xanthomas in the tendons and in the brain. The latter may lead to dementia and / or neurological problems. Treatment with chenodeoxycholic acid will reduce the xanthomas, and if the treatment is started early in life, it will prevent the xanthoma formation.

In the literature, some patients with CTX have been described who suffer from neonatal cholestasis, a condition that sometimes can be fatal. In the present work, we describe the case of a 6 week old boy with cholestasis. In spite of intensive treatment, the cholestasis in connection with an infection of CMV (cytomegalovirus) led to organ failure and the boy died at the age of 3.5 months. ES-MS analysis of urine samples showed large quantities of abnormal bile alcohols, consistent with CTX. The level of 27OH-cholesterol in the circulation was extremely low. Mutation analysis of the promoter and gene for CYP27A1 revealed a stop codon in exon 7 (DNA mutation G1234T, amino acid 408stop). This mutation has to our knowledge not been reported previously. Both parents were found to be heterozygous for the mutation.

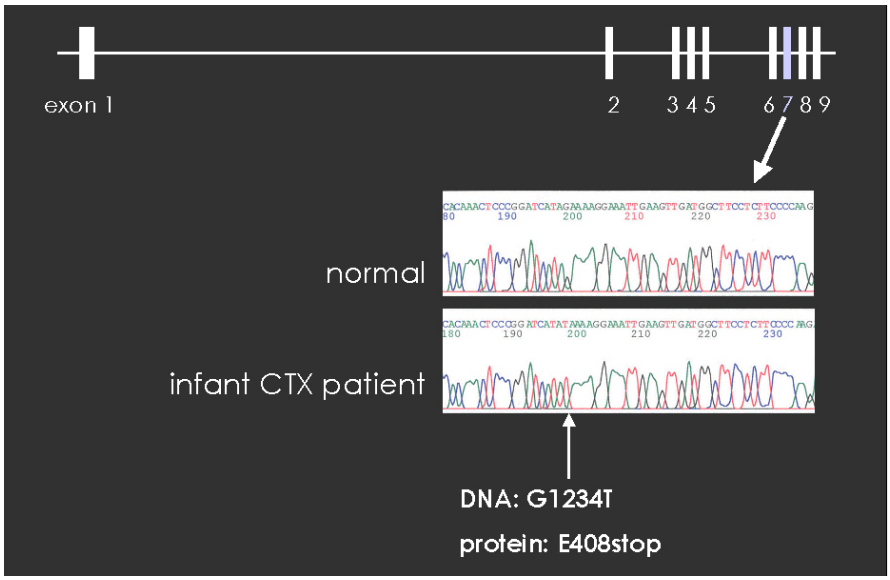

Figure 9. *Illustration of the nonsense mutation found in exon 7 at base number 1234 (amino acid number 408) in the first Swedish case of CTX.*

Three main risk factors for cholestasis could be listed in this case: low levels of CYP7A1 (which is normal in the neonatal state), CMV-infection and CTX. It Is possible that the little boy would have had better chances to survive if he had not had the CMV-infection.

It is of interest to compare the present case with the second Swedish case of CTX diagnosed a few months after the diagnosis of the first patient. This is an adult female with large xanthomas on her tendons but no xanthomas in the brain. Her levels of 7αOH-cholesterol in the circulation were found to be extremely high, whereas the 27OH-cholesterol levels were very low. She also has an abnormal pattern of 25-hydroxylated bile alcohols in the urine, consistent with the CTX-diagnosis (Florén, von Bahr, unpublished data). The mutation in the CYP27A1 gene has not yet been defined.

Paper VI

In this very preliminary study, primary human hepatocytes were incubated with labelled 7αOH-cholesterol and labelled 27OH-cholesterol and the cell culture medium was harvested after two days when the amounts of labelled cholic acid and labelled chenodeoxycholic acid were measured on radio-HPLC. The cultured hepatocytes had a relatively high production of the two common human bile acids and in general the proportions were in agreement with those in normal human bile. The labelled 7αOH-cholesterol was more efficiently converted to bile acids than the labelled 27OH-cholesterol in all of the experiments. In most of the experiments, chenodeoxycholic acid was the most important product from the precursor 27OH-cholesterol and the ratio between labelled cholic acid and labelled chenodeoxycholic acid was most often considerably higher with labelled 7αOH-cholesterol as precursor than with labelled 27OH-cholesterol. When the amount of precursor oxysterol incubated was reduced from 4 to 1 μg in 3 mL of culture medium, the results were almost the same (unpublished observation).

In the study by Sauter *et al*, no difference in the cholic acid/chenodeoxycholic acid ratio could be found between cells incubated with 7αOH-cholesterol and cells incubated with 27OH-cholesterol. However, the amounts of precursor oxysterols incubated were unphysiologically high, considerably higher than those used in our study, and may not reflect the situation *in vivo*.

In conclusion, the conversion of 27OH-cholesterol into bile acids seems to be less efficient than the corresponding conversion of 7αOH-cholesterol. Because of the great variations between the experiments, no firm conclusions can be drawn concerning the relative role of the acidic pathway.

GENERAL SUMMARY

Sterol 27-hydroxylase has been studied with respect to substrate specificity, possible role in cholesterol accumulation and role in bile acid formation in human hepatocytes. The first Swedish case of sterol 27-hydroxylase deficiency is also described.

Substrate specificity

Human CYP27A1 has a broad substrate specificity and polar substrates were found to be 27-hydroxylated more efficiently than unpolar substrates. Substrates with side-chain hydroxyl groups were 27-hydroxylated at a slower rate than those with such groups at the core of the sterol molecule, possibly due to steric hindrance.

Cholestanol, accumulating in patients with CYP27A1-deficiency (CTX) was found to be hydroxylated by CYP27A1 at about the same rate as cholesterol. Cholestenone, a precursor to cholestanol, was found to be a highly efficient substrate for CYP27A1. These findings may be part of the explanation for the accumulation of cholestanol occuring in CTX-patients.

7-dehydrocholesterol, accumulating in patients with SLOS, was found to be 27-hydroxylated by CYP27A1 at a lower rate than cholesterol.

Possible role in cholesterol accumulation

CYP27A1 enzyme is present in human tendons, as well as the product 27OH-cholesterol. The ratio of 27OH-cholesterol:cholesterol was found to be higher in tendons than in the circulation. CYP27A1 was found to be active in cultured human tenocytes and tendon macrophages. We suggest that CYP27A1 may prevent the accumulation of cholesterol and cholestanol in tendons, and play a protective role in the development of xanthomas. Cholestanol was found to enter and leave cultured human alveolar macrophages at a slower rate than cholesterol, giving a partial explanation for its selective accumulation in tendons of CTX patients.

CYP27A1 is present and active in human gallbladder mucosa. The activity and the amount of protein was found to be similar in cholesterolosis gallbladder and non-cholesterolosis gallbladder. The amounts of esterified cholesterol was elevated in cholesterolosis and the esterifying enzyme ACAT was upregulated. This upregulation is probably secondary to the high cholesterol levels. CYP27A1 does not seem to be a pathogenic factor in cholesterolosis.

First Swedish case of sterol 27-hydroxylase deficiency

The first Swedish case of CTX, a cholestatic infant boy who died from organ failure, was found to have a nonsense mutation in exon 7 (G1234T, or E408stop), not previously described. Both parents, who were first cousins, were found to be heterozygous for the mutation.

Role of 27-hydroxylation of cholesterol as the first step in bile acid formation in human hepatocytes

The acidic pathway for bile acid biosynthesis is of importance in cultured human hepatocytes, but the relative role of the acidic versus the neutral pathway could not be ascertained. Chenodeoxycholic acid appeared to be the preferred product from the acidic pathway.

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