Cholesterol-metabolizing cytochromes P450

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ABSTRACT

By catalyzing the first steps in different pathways of cholesterol degradation, cytochromes P450 (P450s or CYPs) 7A1, 27A1, 11A1, and 46A1 play key roles in cholesterol homeostasis. CYP7A1 is a microsomal liver-specific enzyme that converts cholesterol to 7α-hydroxycholesterol. CYP27A1 is a ubiquitously expressed mitochondrial P450 that metabolizes cholesterol to 27-hydroxycholesterol. CYP11A1 also resides in mitochondria but is expressed mainly in steroidogenic tissues where it catalyses the conversion of cholesterol to pregnenolone. Finally, CYP46A1 is a brain-selective microsomal monoxygenase producing 24S-hydroxycholesterol from cholesterol. Catalytic efficiencies of cholesterol-metabolizing P450s vary significantly and likely reflect physiological requirements of different organs for the rate of cholesterol turnover. P450s 7A1, 27A1, 11A1, and 46A1 represent a unique system for elucidation of how different enzymes have adapted to fit their specific roles in cholesterol elimination. Studies of cholesterol-metabolizing P450s suggest that their activities could be modulated post-translationally and that they should also be considered as targets for regulation of cholesterol homeostasis.
In mammals, excess cholesterol (5-cholesten-3β-ol) is removed mainly through conversion to bile acids, and only a small portion is utilized for production of steroid hormones (Turley and Dietschy, 1982). Enzymes called cytochromes P450 (P450s or CYPs) initiate all quantitatively significant pathways of cholesterol degradation. The P450 proteins contain a single heme group and have a characteristic absorption at 450 nm when reduced and form a complex with CO (for recent reviews on P450s see refs. (Nebert and Russell, 2002; Meunier et al., 2004; Aguiar et al., 2005; Coon, 2005; Denisov et al., 2005; Guengerich, 2005; Johnson and Stout, 2005). P450s from different families share low sequence identity (<40%), nevertheless they have a similar overall structural fold and generally carry out monoxygenation reactions (Graham and Peterson, 1999). This review describes only those P450s that act directly on cholesterol (Fig. 1), information about P450s that hydroxylate cholesterol derivatives can be found in other reviews (Russell, 2003; Payne and Hales, 2004). Furthermore, the major focus will be on human enzymes because some interspecies differences exist both in maintenance of cholesterol homeostasis and in the regulation of the activity of cholesterol-metabolizing P450s.

**Physiological and medical significance of cholesterol-metabolizing P450s**

**CYP7A1**

There are several metabolic routes that lead to the formation of bile acids and only one pathway to produce steroid hormones. In the liver, the main organ for cholesterol degradation, bile acid biosynthesis is initiated and controlled by a liver-specific enzyme CYP7A1 that converts cholesterol to 7α-hydroxycholesterol (Myant and Mitropoulos, 1977). This reaction represents the first and rate-limiting step in the classical or neutral bile acid biosynthetic pathway, which dominates under normal physiological conditions. About 400-600 mg of cholesterol are eliminated through this pathway on a daily basis by the human liver (Sabine,
Humans lacking cholesterol 7α-hydroxylase activity as a result of the mutation in the \textit{CYP7A1} gene have significant elevation of total and LDL-cholesterol levels, substantial accumulation of cholesterol in the liver and a markedly decreased rate of bile acid excretion (Pullinger et al., 2002). Increased plasma LDL-cholesterol levels are also observed in some subjects carrying nucleotide substitutions in the \textit{CYP7A1} promoter region and presumably having reduced cholesterol 7α-hydroxylase activity (Wang et al., 1998). In two population studies a frequent A-204C polymorphism (~38-46% of the population carries the -204C allele) was found to have significant or modest effect on LDL cholesterol levels (Wang et al., 1998; Couture et al., 1999), whereas in three studies the effect was inconsistent or non significant (Hegele et al., 2001; Kovar et al., 2004; Abrahamsson et al., 2005). So different results in the five studies are explained by differences in diet and in genetic background of the population. In addition to a medical significance, it is possible that the A-204C genotype has a pharmacological relevance because it was shown to be associated with a poor response to a cholesterol-lowering drug atorvastatin and influence the response of plasma lipids to increased intake of dietary cholesterol and cafestol (Hofman et al., 2004; Kajinami et al., 2005).

\textbf{CYP27A1}

Analysis of the \textit{CYP7A1} gene knockout mice and then subsequent characterization of the human subjects carrying the CYP7A1 null mutation revealed that when the classical bile acid biosynthetic pathway is suppressed, an alternative or acidic pathway of bile acid biosynthesis is up-regulated (Ishibashi et al., 1996; Schwarz et al., 1996; Pullinger et al., 2002). Under normal conditions, this pathway is initiated in extrahepatic tissues and accounts for a daily elimination of 18-20 mg of cholesterol (Duane and Javitt, 1999). The alternative pathway complements the HDL-mediated reverse cholesterol transport to the liver and is initiated by a ubiquitously
expressed CYP27A1 that converts cholesterol to 27-hydroxycholesterol (Anderson et al., 1972; Lund et al., 1996). CYP27A1 is a polyfunctional enzyme and in addition to hydroxylation of cholesterol in extrahepatic tissues, it also oxygenates bile acid intermediates in the liver and vitamin D₃ in the kidney (Wikvall, 1984; Masumoto et al., 1988; Okuda et al., 1988). The CYP27A1 products 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid are the ligands for the nuclear receptors LXRα and LXRβ that activate the transcription of several genes involved in lipid metabolism (Song and Liao, 2000; Fu et al., 2001). However, the significance of CYP27A1 in transcriptional regulation of cholesterol homeostasis via generation of biologically active oxysterols is currently unclear (Fu et al., 2001; Hall et al., 2001; Bjorkhem, 2002; Javitt, 2002; Meir et al., 2002). Deficiency of CYP27A1 activity as a result of genetic mutations causes a disease called cerebrotendinous xanthomatosis (CTX), which is characterized by abnormal deposition of cholesterol and cholestanol (5α-saturated analog of cholesterol) in multiple tissues (Cali et al., 1991; Bjorkhem et al., 1995). Phenotypic manifestations and clinical progression of CTX are variable and may include bilateral cataracts, premature atherosclerosis, tendon xanthomas, neurological and neuropsychiatric abnormalities, and osteoporosis (Bjorkhem et al., 1995). CTX is believed to be a rare disease. It has been reported in more than 200 people worldwide (Moghadasian, 2004). Most recent studies suggest that CTX is underdiagnosed and the prevalence of CTX is about 3-5 per 100,000 people (Lorincz et al., 2005). Over 40 different mutations in CYP27A1 have been described (Verrips et al., 2000; Lee et al., 2001). Of them, about a half are deletion/insertion, splice site and nonsense mutations (result in premature stop codon) that are likely abolish the enzyme expression and consequently the activity. The other half constitute missense mutations that result in amino acid substitution. No genotype-phenotype correlations have been established thus far, possibly because CYP27A1 is involved in several
metabolic pathways, contribution of each of which to the phenotype is poorly understood at the present time (Verrips et al., 2000). Studies in this laboratory (presented in the next section) may provide some insight into mechanisms underlying phenotypic heterogeneity associated with CTX. CTX is a treatable disease. Oral supplementation with chenodeoxycholic acid halts CTX progression and leads to significant neurological recovery (Beringer et al., 1984).

**CYP11A1**

In steroidogenic tissues (adrenal glands, ovaries, testis, placenta, and brain) cholesterol is converted to pregnenolone. This reaction represents the first step in the overall steroid hormone biosynthesis and is catalyzed by CYP11A1 (Stone and Hechter, 1954). About 40-50 mg of cholesterol is utilized every day to produce steroid hormones (Sabine, 1977; Turley and Dietschy, 1982). The conversion of cholesterol to pregnenolone was long thought to be the rate-limiting step in overall steroidogenesis. However, now is clear that the critical step is cholesterol delivery to the inner mitochondrial membrane where CYP11A1 resides (Stocco, 2000). Intramitochondrial cholesterol transport regulates cholesterol availability to CYP11A1 and consequently the enzyme activity and is mediated by the steroidogenic acute regulatory (StAR) protein (Stocco, 2001). StAR is expressed in many steroidogenic tissues but not all: it is not found in the placenta. Therefore in this organ the rate-limiting step in steroid synthesis is controlled by CYP11A1 (Tuckey, 2005). Mutations that completely abolish the CYP11A1 activity are believed to be incompatible with human term gestation because this enzyme is needed for placental biosynthesis of progesterone, an essential hormone required to maintain pregnancy (Miller, 1998). Recently, a patient homozygous for a nucleotide deletion leading to a premature stop codon was described indicating that in rare cases fetuses with a complete CYP11A1 deficiency may be viable (Hiort et al., 2005). The child was born prematurely, had a
complete sex reversal and congenital lipoid adrenal hyperplasia, a severe disorder of steroidogenesis in which cholesterol accumulates within the steroidogenic tissues and the synthesis of all adrenal and gonadal steroids is impaired. The adrenal crisis was treated with the hormone replacement therapy, the child has survived, and was 2 years old at the time of the paper writing. Different partially inactivating CYP11A1 mutations are also described in two patients which were born after normal pregnancies (Tajima et al., 2001; Katsumata et al., 2002). One of the patients developed symptoms of adrenal insufficiency at the age of 7 months and the other at the age of 4 years. In genetic male attenuation of CYP11A1 activity caused pseudohermaphroditism and in genetic female lack of secondary sexual characteristics. A substitution with unknown effect on enzyme activity, V179I, was found in a hyperandrogenic hirsute woman (Calvo et al., 2001). Because this was a conservative replacement, the authors do not believe that it plays a major role in the pathogenesis of hirsutism and hyperandrogenism.

**CYP46A1**

Cholesterol elimination from the human brain begins with 24S-hydroxylation catalyzed by CYP46A1 (Lund et al., 1999). In contrast to cholesterol, 24S-hydroxycholesterol can cross the blood-brain barrier, enter the circulation and then be delivered to the liver for further degradation. The brain does not appear to contribute significantly to the whole-body cholesterol balance: only 6-7 mg of cholesterol are 24S-hydroxylated every day in the brain (Heverin et al., 2004). Significance of CYP46A1 may not be limited to involvement in cholesterol degradation. 24S-Hydroxycholesterol is a potent activator of the LXR receptors (Janowski et al., 1999), therefore CYP46A1 may play a regulatory role by producing a biologically active product. It is possible that CYP46A1 may also be involved in subsequent metabolism of 24S-hydroxycholesterol because *in vitro* it was found to convert 24S-hydroxycholesterol to 24,25-
and 24,27-dihydroxycholesterols with 24S-hydroxycholesterol being a much better substrate for CYP46A1 than cholesterol (Mast et al., 2003). Furthermore, *in vitro* studies indicate that CYP46A1 has a broad substrate specificity and metabolizes a number of structurally diverse compounds including different cholesterol derivatives and drugs (Mast et al., 2003). Accordingly, CYP46A1 may participate in metabolism of neurosteroids and drugs that are targeted to the central nervous system. Accumulating evidence suggests that cholesterol is a risk factor for Alzheimer’s disease, (Burns and K., 2002; Puglielli et al., 2003; Raffai and Weisgraber, 2003), therefore CYP46A1 deficiency was proposed to play a role in the pathogenesis of this neurological disorder. Several single nucleotide polymorphisms have been identified in the introns of the *CYP46A1* gene, and two of them were studied. The results are controversial, and as of the end of 2005, six published papers suggest that there is a link between the polymorphisms and Alzheimer’s disease (Kolsch et al., 2002; Papassotiropoulos et al., 2003; Borroni et al., 2004; Combarros et al., 2004; Johansson et al., 2004; Wang et al., 2004; Papassotiropoulos et al., 2005), whereas five papers do not support this association (Desai et al., 2002; Chalmers et al., 2004; Ingelsson et al., 2004; Kabbara et al., 2004; Juhasz et al., 2005). Thus, medical significance of CYP46A1 remains unclear.

**CYP3A4**

Review of cholesterol-metabolizing P450s will not be complete without mentioning CYP3A4. This enzyme is the most abundant P450 in the human liver and has a dominating role in drug metabolism (Guengerich, 2005). Recently, CYP3A4 was found to convert cholesterol to 4β-hydroxycholesterol with the average rate of 0.3 mg/day in two healthy volunteers (Bodin et al., 2002). This slow rate suggests that cholesterol elimination by CYP3A4 is of a very minor quantitative importance. However, since 4β-hydroxycholesterol is as potent as an activator of the
LXRα receptor as 24S-hydroxycholesterol (Janowski et al., 1996), the major contribution of CYP3A4 to cholesterol homeostasis could be through transcriptional regulation. Furthermore, CYP3A4 activity in humans varies up to 40-fold (Shimada et al., 1994; Westlind et al., 1999), and many drugs induce the enzyme resulting in a 10-20-fold increase in plasma concentrations of 4β-hydroxycholesterol (Bodin et al., 2001). Therefore, in different people and under certain conditions the rate of 4β-hydroxycholesterol production could be significantly higher than that reported.

**Cholesterol-metabolizing P450s as a unique system for investigation**

P450s 7A1, 27A1, 11A1 and 46A1 share <25% sequence identity, nevertheless they bind cholesterol with nanomolar to low micromolar affinity (Table 1). The four P450s convert cholesterol to different products with CYPs 11A1, 27A1, and 46A1 hydroxylating cholesterol on the side chain and CYP7A1 on the steroid nucleus (Fig. 1). Catalytic efficiencies of cholesterol-metabolizing P450s vary and likely reflect physiological requirements of different organs for the rate of cholesterol turnover. Two of the P450s (7A1 and 46A1) reside in endoplasmic reticulum (ER), whereas the other two (27A1 and 11A1) in the inner mitochondrial membrane. Similarities and differences make cholesterol-metabolizing P450s a unique system for comparative studies to address several important questions:

1) How do enzymes that share such low sequence identity recruit and bind the very same substrate cholesterol?

2) What factors determine catalytic efficiency of cholesterol hydroxylation by P450s?

3) How does organ and subcellular location affect cholesterol binding and catalysis?

Knowledge of how key enzymes in cholesterol degradation function will provide insight into maintenance of cholesterol homeostasis and will help us to understand whether it is possible to
stimulate the rate of cholesterol degradation via post-translational modulation of activity of CYPs 7A1, 27A1 and 46A1. Current cholesterol lowering strategies act at the level of cholesterol synthesis (statins) and cholesterol absorption (ezetimibe) (Sudhop et al., 2005; Wolozin et al., 2005). Additional strategy that is being considered is aimed at transcriptional regulation via nuclear receptors (Repa and Mangelsdorf, 2000; Makishima, 2005; Michael et al., 2005). Undoubtedly, enzymes initiating bile acid biosynthesis should also be investigated as potential targets for modulation of cholesterol degradation. The following is currently known about regulation of activity of these P450s.

In humans, activity of CYP7A1 is negatively regulated by bile acids but not by cholesterol accumulation (Repa and Mangelsdorf, 2000; Chiang, 2003; Russell, 2003). Some drugs (fibrates), hormones (thyroid, steroid, pituitary and insulin) and nutritional factors (glucose and high fat diet) as well as diurnal rhythm and such physiological conditions as obesity and age also have effects on CYP7A1 activity (Chiang, 1998). An important mechanism of regulation of CYP7A1 activity is believed to take place at the level of gene transcription because changes in enzyme activity were found to parallel those in mRNA levels. A number of reports, however, indicate that control of the CYP7A1 activity could also include post-transcriptional events such as cholesterol availability, cytosolic factors, disulfide bonds in the enzyme structure and phosphorylation (Chiang, 1998). The mRNA changes do not always correlate with changes in enzyme activity (Pandak et al., 1994; Cheema et al., 1997; Li et al., 2004), also suggesting post-transcriptional regulation. Finally, it is not clear at present why CYP7A1 activity varies over a 5-10-fold range among healthy individuals (Nicolau et al., 1974; Oda et al., 1990; Reihner et al., 1990). The mechanism of regulation of CYP27A1 is just beginning to be elucidated and virtually nothing is known about the regulation of CYP46A1. Human CYP27A1 mRNA and enzyme
activity is down regulated by bile acids, however, the responses are less prominent than those of CYP7A1 (Chen and Chiang, 2003; Ellis et al., 2003). Two reports in the literature suggest post-transcriptional regulation of CYP27A1 activity, one showing a large discrepancy in the magnitude of increase in the CYP27A1 mRNA and protein levels versus enzyme activity (Hall et al., 2001), and the other significantly reduced enzyme activity when mRNA levels remained normal (Matsuzaki et al., 2002). With CYP11A1, there is acute and chronic regulation of the enzyme activity. Acute regulation occurs at the level of cholesterol availability, while chronic regulation takes place at transcriptional level and involves pituitary trophic hormones acting via the cAMP-dependent pathway (Sewer and Waterman, 2003).

Thus, activity of one of four cholesterol-metabolizing P450s is established to be regulated post-translational, and there is a possibility, based on the literature data, that there may be post-translationally regulation of activity of the other two enzymes (CYPs 7A1 and 27A1). Keeping this information in mind as well as questions outlined in the beginning of this section, the following research is being undertaken.

**Structure/function studies of cholesterol-metabolizing P450s**

Characterization of cholesterol-metabolizing P450s is mainly carried out using purified recombinant enzymes that are produced by *E. coli* when cells are transformed with the expression plasmid containing the P450 cDNA (Wada et al., 1991; Karam and Chiang, 1994; Pikuleva et al., 1997; Mast et al., 2003).

**Active Site**

The active site of CYPs 7A1 and 27A1 was investigated using substrate analogs in combination with homology modeling and site-directed mutagenesis (Mast et al., 2005; Mast et al., 2006). In the case of CYP7A1, 41 mutants, encompassing 26 amino acid residues, were
generated and characterized. Based on the homology model and phenotype of the mutant P450s, seven active site residues were identified (V280, W283, A284, N288, A358, L360, and L485) and suggested to determine the enzyme specificity for cholesterol (Fig. 2). Studies of CYP7A1 indicate that there is a tight complementarity fit between cholesterol and the enzyme active site, and a “goodness of fit” seems to be the feature that contributes in part to strict substrate specificity and high catalytic efficiency of this P450 (Mast et al., 2005).

CYP27A1 has a broader substrate specificity than CYP7A1 accommodating substrates with significantly different three-dimensional structures. For example, cholesterol, the enzyme substrate in the alternative bile acid biosynthetic pathway, is a flat molecule and has only one hydroxyl group in the steroid nucleus, the 3β-hydroxyl. In contrast, 5β-cholestan-3α,7α,12α-triol, the CYP27A1 substrate in the classical bile acid biosynthetic pathway, has a bend at the A/B ring junction, the 3-hydroxyl in the α-position instead of the β- and the two extra hydroxyl groups in the steroid nucleus. Computer models suggest that cholesterol and 5β-cholestan-3α,7α,12α-triol bind in different orientations relative to heme and occupy different regions in the active site (Fig. 3). As a result, there is a set of residues in the substrate-binding pocket that interacts with only one substrate, and a set of residues interacting with both substrates (Mast et al., 2006). Because the latter (W100, H103, T110, E298, M301, A302, V367, I481, V482) are in contact with different segments of cholesterol and 5β-cholestan-3α,7α,12α-triol, their mutations should have differential effects on binding and metabolism of the two substrates. This notion was, in general, confirmed by the mutagenesis data showing that the properties of CYP27A1 are altered in a substrate-dependent manner. Studies of the CYP27A1 active site may provide insight into why clinical symptoms are so diverse in CTX. Of 14 amino acid residues, whose replacement underlies CTX, 5 are very conserved in the P450 superfamily (R94, R362,
R372, G439, and R441, human CYP27A1 numbering) and are known to be involved in heme-binding and protein folding (Peterson and Graham, 1998). Mutation of these residues (a total of 11 missense mutations) most likely produce non-functional enzyme and lead to a complete loss of the CYP27A1 activity. Prediction of the effect of the other known 9 missense mutations (R104W, G112E, A183P, K226R, T306M, D321G, P351L, P368R and R446C) is not so straightforward. It is possible that these missense mutations lead to different disease manifestations because activity toward one substrate is disrupted whereas activity toward another substrate is partially preserved. Thus far, only one enzyme activity is usually measured when characterizing a CTX patient. Our data suggest that several substrates should be tested to begin to understand mechanisms underlying phenotypic heterogeneity of CTX.

CYP46A1 has been cloned only in 1999 (Lund et al., 1999), and efficient E. coli expression system and purification procedure to produce large quantities of the recombinant enzyme were developed in 2003 (Mast et al., 2003; Mast et al., 2004). Probing the enzyme active site with different substrate analogs showed that CYP46A1 can hydroxylate steroids that differ in the length of the steroid side chain, position of the double bound, and substitutions in the steroid nucleus and the side chain. Also, CYP46A1 was found to metabolize xenobiotics, carrying out dextromethorphan O- and N-demethylations, diclofenac 4’-hydroxylation, and phenacetin O-deethylation. (Mast et al., 2003). Thus, the CYP46A1 active site seems to be even larger than that of CYP27A1.

**Putative substrate access channel**

In some of the structurally determined P450s, the active site is isolated from the protein surface, but in most there is one or more clear channels between the buried active site and the protein exterior. On the basis of alignment with structurally characterized P450 102A1, a
putative region that forms part of the substrate-access channel in CYPs 27A1 and 11A1 was
identified and mutations were introduced in this region (Pikuleva et al., 2001). Wild type and
mutant P450s were compared with respect to catalytic activity, product pattern, substrate
binding, formation of hydrogen peroxide and interaction with redox partner. Results indicate that
the mutated residues are important for delivery of the correctly oriented substrate to the P450
active site. The I211K and F215K mutations, for example, affected the regioselectivity of
CYP27A1-dependent hydroxylation reactions and conferred the P450 capacity to cleave one of
the terminal methyl groups of 5β-cholestan-3α,7α,12α-triol during the catalytic cycle. Studies
of CYP11A1 indicate that F202 has functions similar to those of its counterpart in P450 27A1
(F215). Thus, CYPs 27A1 and 11A1 seem to have the substrate-access channel that provides an
additional mechanism to control regioselectivity of hydroxylation in mitochondrial P450s
(Pikuleva, 2004).

Association with the membrane

Studies of the microsomal enzyme CYP2C5 suggest that both mitochondrial (e.g. 27A1) and
microsomal (e.g. 7A1) P450s have a similar, mono-facial mode of association with the
membrane (von Wachenfeldt and Johnson, 1995; Williams et al., 2000). In microsomal P450s, a
large hydrophilic domain is believed to be anchored to the lipid bilayer through the N-terminal
segment and the adjacent surface of the protein formed by non-contiguous portions of the
polypeptide chain. Mitochondrial P450s do not contain the N-terminal membrane anchor, and yet
their membrane-binding motif is likely to be similar to that of microsomal P450s and includes
one or more hydrophobic regions that are partially inserted in the membrane (Williams et al.,
2000). A loop between the putative helices F and G and adjacent segments of these helices were
proposed to be one of the regions involved in membrane-binding in eukaryotic P450s (Graham-
Lorence et al., 1995; Graham and Peterson, 1999). Computer modeling and quantification of subcellular distribution of the F-G loop mutants in *E. coli* were used to assess membrane topology of CYPs 7A1, 27A1, and 11A1 (Nakayama et al., 2001; Murtazina et al., 2002; Pikuleva, 2004). The validity of *E. coli* as a model system was justified by the fact that, despite differences in the phospholipid (PL) content of bacterial and eukaryotic membranes, cholesterol-metabolizing P450s stay associated with the membrane fraction when expressed in *E. coli* and are catalytically active when *E. coli* membranes containing these enzymes are used for reconstitution of the enzyme activity. Replacements of the amino acid residues in the putative F-G loop caused alteration of subcellular distribution of the three cholesterol-metabolizing P450s strongly suggesting the F-G loop is indeed a membrane interacting area in CYPs 7A1, 27A1 and 11A1. Membrane binding of CYP11A1 was also studied by a different approach. Cysteine mutagenesis within the F-G loop was followed by fluorescent labeling of the mutated residues and then measurements of the changes in their fluorescence upon association of the mutant P450 with PL vesicles. The data obtained also support the notion that association of CYP11A1 with the membrane is mediated, at least in part, by the F-G loop (Headlam et al., 2003). Effect of the altered membrane-protein interactions on kinetic parameters for cholesterol hydroxylation of the F-G loop mutants was investigated in a reconstituted system containing exogenous NADPH, cholesterol, redox partner(s) and the P450-containing *E. coli* membranes (Nakayama et al., 2001; Murtazina et al., 2002). In CYP7A1, several substitutions increased *K*<sub>m</sub> but not *K*<sub>d</sub>, up to 12-fold, whereas in CYP27A1 there was no significant change of *K*<sub>m</sub>. The *k*<sub>cat</sub> values varied significantly in both P450s and ranged from undetectable activity to a 2-3-fold increased activity. An increase in *k*<sub>cat</sub> in the CYP7A1 mutants was not enough to compensate for the increased *K*<sub>m</sub>, therefore an overall effect was a decrease in catalytic efficiency of cholesterol hydroxylation (the
In the CYP27A1 mutants, $K_m$ values were not significantly affected, and mutants with increased $k_{cat}$ values had increased catalytic efficiencies. The K226R mutation that underlines CTX was reproduced during the course of this work (Murtazina et al., 2002). According to the computer model, K226 is located on the surface of the molecule in the putative F-G loop (Fig. 4). The K226R replacement weakened the CYP27A1 interaction with the membrane, insignificantly increased the $K_m$ and resulted in a 5-fold reduction of the $k_{cat}$. Properties of the K226R mutant indicate that a complete disruption of the CYP27A1 activity is not required for CTX to develop, even a 5-fold decrease of catalytic efficiency of cholesterol hydroxylation is sufficient to cause the disease. Overall, kinetic studies clearly demonstrated that the way in which CYPs 7A1 and 27A1 interact with the membrane influences catalytic efficiency of cholesterol hydroxylation and the effect is not always negative since in some of the mutants cholesterol hydroxylation became more efficient. Establishing that membrane-protein interactions contribute to efficiency of cholesterol hydroxylation raises a possibility that activity of cholesterol-metabolizing P450s could be modulated post-translationally through the membrane by altering its lipid composition.

**A possible strategy to modulate activity of cholesterol-metabolizing P450s**

Lipid composition of biological membranes is very sensitive to dietary fat because upon digestion fatty acids that constitute the fat are delivered to virtually every cell in the body and become incorporated into membrane phospholipids (Tahin et al., 1981). It is long known that effect of dietary fat on total plasma cholesterol levels depends on the predominant type of the fatty acids it contains (Kris-Etherton and Yu, 1997). Saturated fatty acids (SFAs), present in large amounts in butter and lard, are hypercholesterolemic. Monosaturated fatty acids (MUFAs), found in olive and rapeseed oils, exert a neutral effect or are mildly hypocholesterolemic.
Sources rich in n-6 polyunsaturated fatty acids (PUFAs) like corn, safflower and sunflower seed oils, elicit the most potent hypocholesterolemic effect when substituted for saturated fat in the diet. Finally, the n-3 PUFAs, present in high amounts in fish (salmon, sardine, menhaden, and cod) and fish oils, as well as in flaxseed, canola and soybean oils and some nuts (walnuts), do not reduce total cholesterol at low doses but have a hypocholesterolemic effect at high doses. Of all known dietary factors, n-3 PUFAs are considered to be the most protective against death from cardiovascular disease (Harris et al., 2003). The suggested cardiovascular benefits of n-3 PUFAs include decreased risks of arrhythmias and thrombosis, lower blood pressure and triacylglyceride levels, improved endothelial function, reduced inflammatory responses and decreased rate of growth of the atherosclerotic plaque (Kris-Etherton et al., 2003). Biochemical mechanisms whereby n-3 PUFAs exert their effects involve eicosanoid production, cell signaling, regulation of gene expression and alteration of the PL acyl chain composition (Jump, 2002; Lapillonne et al., 2003; Benatti et al., 2004). The latter affects membrane viscosity, permeability, amount and distribution of cholesterol in the membrane and enhances activity of a number of membrane proteins (Stubbs and Smith, 1984; Holmes and Kummerow, 1985; Kurushima et al., 1995; Bravo et al., 1998; Demaison and Moreau, 2002; Jump, 2002; Ohvo-Rekila et al., 2002; Lapillonne et al., 2003; Stulnig, 2003; Morise et al., 2004). There are three reports in the literature on the effect of dietary fat on CYP7A1. In the first study, rats were on diets supplemented with palm oil (rich in SFAs), olive oil (rich in MUFA) and corn oil (rich in n-6 PUFAs). Olive and corn oil diets increased cholesterol 7-hydroxylase activity in the liver 3-4-fold, while palm oil feeding had no effect. The mRNA expression for CYP7A1 was increased only in the olive oil diet group, suggesting that MUFAs operate at the gene level, whereas the n-6 PUFAs exert their effects at a post-transcriptional level (Bravo et al., 1998). A similar 3-fold increase of CYP7A1 activity was
also observed in hamsters fed with oleic acid; however, mRNA levels were not measured in this study (Kurushima et al., 1995). Finally, in the third investigation, hamsters were either on a “linseed” diet rich in PUFA conjugated linoleic acid or on a “butter” diet rich in SFAs. Cholesterol 7-hydroxylase activity in the liver of animals on the “linseed” diet was found to be 30-50% higher than that in the animals on the “butter” diet (Morise et al., 2004). With respect to CYP27A1, \textit{in vitro} studies suggest that phospholipids differentially control the rates of cholesterol and 5\(\beta\)-cholestan-3\(\alpha\),7\(\alpha\),12\(\alpha\)-triol hydroxylation and therefore they have potential to regulate the amount of cholesterol metabolized through the classical and alternative pathways of bile acid biosynthesis (Murtazina et al., 2004). Maintenance of cholesterol homeostasis is complex, involves many enzymes and proteins and is controlled at several levels. A comprehensive investigation is required to assess how varying ratios between different fatty acids, especially between the n-6/n-3 PUFAs, simultaneously affect plasma lipid profile and activity and expression of the key enzymes and proteins in the pathways of cholesterol synthesis and degradation. It is very likely that through these studies an \textit{optimal} composition of the dietary fat will be found which \textit{maximally} stimulates cholesterol degradation and has a strong overall hypocholesterolemic effect. If established that cholesterol balance could be regulated via post-translational modulation of activity of cholesterol-metabolizing P450s, a strong impetus will be given to studies of other classes of fatty acids. Conjugated linoleic acid could be one of such fatty acids because it has been reported to have numerous beneficial effects on human health including cardiovascular disease (Belury, 2002).

\textbf{Conclusions}

There is a clear need to identify additional therapeutic strategies to lower plasma cholesterol levels. Structure and function studies of cholesterol-metabolizing P450s provide insight into the
factors that control catalytic efficiency of these important metabolic enzymes and suggest that their activity could be modulated post-translationally. Further research is required to assess the potential of cholesterol-metabolizing P450s as targets for cholesterol lowering.
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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. The four major cytochrome P450 enzymes that initiate cholesterol degradation in different tissues.

Fig. 2. The model of the CYP7A1 active site. Cholesterol is in light grey and heme is in black. N288 is suggested to play a key role in the P450-cholesterol contacts by hydrogen bonding to the steroid 3β-hydroxyl, while V280 and A284 (not shown) are beside and W283 is above the steroid nucleus orienting the cholesterol molecule. L360, A358, and L485 appear to define the size of the active site over the heme pyrrole ring A, thus limiting the orientation and size of the substrate at the steroid A ring and directing cholesterol hydroxylation to the 7α-position.

Fig. 3. Distinct binding of cholesterol (in dark grey) and 5β-cholestane-3α,7α,12α-triol (in light grey) in the CYP27A1 active site. Oxygen atoms in the two steroids are shown in black to indicate that cholesterol and 5β-cholestane-3α,7α,12α-triol bind in flipped orientations relative to each other so that the α-hydroxyl groups in 5β-cholestane-3α,7α,12α-triol and the 3β-hydroxyl group in cholesterol point in the same direction. Heme is also in black.

Fig. 4. The model of CYP27A1 showing the putative F-G loop (dark grey) and K226 (black). The horizontal line indicates the putative interface between the matrix and inner mitochondrial membrane.
Table 1. Cholesterol-metabolizing P450s

<table>
<thead>
<tr>
<th>Property</th>
<th>CYP7A1</th>
<th>CYP27A1</th>
<th>CYP11A1</th>
<th>CYP46A1</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence identity</td>
<td></td>
<td></td>
<td>&lt;25%</td>
<td></td>
<td>(Chung et al., 1986; Cali and Russell, 1991; Cohen et al., 1992; Lund et al., 1998)</td>
</tr>
<tr>
<td>Tissue distribution</td>
<td>Liver-specific</td>
<td>Many tissues</td>
<td>Steroidogenic tissues</td>
<td>Brain-selective</td>
<td>(Chung et al., 1986; Cali and Russell, 1991; Cohen et al., 1992; Lund et al., 1999)</td>
</tr>
<tr>
<td>Subcellular distribution</td>
<td>ER</td>
<td>Mitochondria</td>
<td>Mitochondria</td>
<td>ER</td>
<td>(Simpson and Boyd, 1967; Mitton et al., 1971; Wikvall, 1984; Lund et al., 1999)</td>
</tr>
<tr>
<td>Amount of metabolized cholesterol, mg/day</td>
<td>400-600</td>
<td>18-20</td>
<td>40-50</td>
<td>6-7</td>
<td>(Sabine, 1977; Turley and Dietschy, 1982; Duane and Javitt, 1999; Heverin et al., 2004)</td>
</tr>
<tr>
<td>$K_d$, $\mu$M$^1$</td>
<td>0.050$^2$</td>
<td>0.026</td>
<td>0.3</td>
<td>0.3$^2$</td>
<td>(Pikuleva et al., 2001; Mast et al., 2006)</td>
</tr>
<tr>
<td>$K_m$, $\mu$M$^3$</td>
<td>13.3$^3$</td>
<td>7.0$^3$</td>
<td>3.4$^2,3$</td>
<td>5.3$^{2,4}$</td>
<td>(Nakayama et al., 2001; Murtazina et al., 2002)</td>
</tr>
<tr>
<td>$k_{cat}$, min$^{-1}$</td>
<td>378</td>
<td>15.8</td>
<td>13.8</td>
<td>0.34</td>
<td></td>
</tr>
</tbody>
</table>

$^1$Apparent binding constants ($K_d$) of cholesterol-metabolizing P450s strongly depend on the assay conditions, specifically on whether detergent and at what concentration is present in the assay buffer. Detergent is required to maintain solubility of full-length microsomal CYPs 7A1 and 46A1 but can be omitted when truncated forms of these enzymes, lacking the hydrophobic membrane anchor, are used. Full-length mitochondrial CYPs 27A1 and 11A1 are more soluble than full-length microsomal CYPs 7A1 and 46A1 and do not precipitate in the absence of detergent. The $K_d$ values in this row represent cholesterol binding properties of truncated microsomal P450s and full-length mitochondrial enzymes in detergent-free buffer. In separate experiments when detergent was present, we compared how truncation affects the affinity of P450 for cholesterol and found no significant differences in the $K_d$ values between the full-length and truncated CYPs 7A1 and 46A1. Another factor that influences the $K_d$ for cholesterol is the amount of the E. coli PLs that are co-purified with the recombinant P450. The PL content of CYPs 7A1, 27A1 and 46A1 was <2 $\mu$g/ nmol P450 and that of CYP11A1 is unknown. It is possible that the much higher $K_d$ of CYP11A1 is due to the increased PL content.

$^2$Unpublished data from the author’s laboratory

$^3$Kinetic parameters were measured in a reconstituted system consisting of P450-containing E. coli membranes (full-length enzymes), exogenous NADPH, cholesterol and the redox partner(s).
Determined for a purified truncated form because CYP46A1 is a very slow enzyme and its kinetic parameters can not be reliably measured when high amounts of the P450-containing *E. coli* membranes are used in the enzyme assay.
Figure 1.

The diagram illustrates the metabolic pathways of cholesterol. Cholesterol is converted to 27-hydroxycholesterol by CYP27A1 in many tissues. 27-Hydroxycholesterol is then converted to 24S-hydroxycholesterol by CYP46A1 in the brain. Cholesterol is further converted to 7α-hydroxycholesterol by CYP7A1 in the liver.
Figure 2.
Figure 3.
Figure 4.