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KINASE/PHOSPHATASE REGULATION OF CYP7A1

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1. ABSTRACT

Bile acid synthesis powerfully influences cholesterol homeostasis by providing an avenue for cholesterol disposal and by producing signaling molecules. Bile acids are multifaceted signals, regulating gene expression both by acting as a ligand for the nuclear hormone receptor, farnesoid X-receptor (FXR), and by activating cellular kinases. Though the exact identities and sequence of the signaling events are under investigation, there is mounting evidence for the involvement of c-Jun N-terminal kinase (JNK) and extracellular-regulated kinase (ERK) 1/2 pathway. The rate of bile acid synthesis is controlled by the activity of the enzyme, cholesterol 7alpha-hydroxylase, encoded on the CYP7A1 gene. Cholesterol 7alpha-hydroxylase activity and transcription of CYP7A1 gene promoter have been reported to be affected by protein kinases and phosphatases. Cellular protein kinases may provide the mechanisms for coordinate regulation of cholesterol transport, synthesis and breakdown to bile acids. Investigations into the interrelationships between various kinases/phosphatases and nuclear hormone receptors will clarify the roles that these pathways play in bile acid gene regulation and coordinate regulation of lipid metabolism, as well as in the connection of lipid metabolism with disease onset and progression of several human diseases.

2. INTRODUCTION

Because of the importance of cholesterol 7 alpha-hydroxylase in cholesterol homeostasis, the regulation of the activity of this enzyme is the subject of intense research. Transcription of the gene for cholesterol 7 alpha-hydroxylase, CYP7A1, is specific to the liver and expression of the CYP7A1 message is tightly controlled spatially (1) and developmentally (2). The proximal promoter of CYP7A1 gene contains regulatory domains conferring the activation by the glucocorticoid, dexamethasome, and cAMP; and suppression by bile acids, phorbol esters, and insulin (3-5). Several lines of evidence show transcription of CYP7A1 is feedback repressed by bile acids (5-15). The sequence that is sufficient and required for bile acid repression of transcription (bile acid responsive element; BARE) (3, 16, 17) is an enhancer characterized by an HNF4-response element and its auxiliary factor binding sites, including alpha-fetoprotein transcription factor (FTF/CPF), chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) and basic transcription element binding protein (BTEB) (16, 18-20). The bile acid receptor (FXR) does not bind to the BARE, nor has an FXR/RXR response element been found in the CYP7A1 promoter. Moreover, all promoters with FXR/RXR elements described to date have been stimulated by cell exposure to bile acids. The bile acid repression of

CYP7A1 without FXR/RXR binding to the CYP7A1 promoter is likely to be the consequence of changes in transcription factor activity arising from changes in transcription factor protein levels and modification by phosphorylation. Bile acids cause these changes by stimulating transcription of FXR/RXR target genes and by activating kinase/phosphatase pathways, resulting in sweeping changes in bile acid synthesis, transport and excretion.

3. BILE ACID SYNTHESIS

Levels of cholesterol in the body are determined by the activity of three metabolic processes: uptake of dietary cholesterol and transport; *de novo* synthesis; and the conversion cholesterol to other steroid compounds, such as bile acids and hormones. Cholesterol from the diet is absorbed by the intestine and transported in the blood combined with proteins and other lipids. Cholesterol is moved throughout the body by a highly integrated system of acyltransferases, transporters, lipoproteins and specific receptors. The rate of *de novo* synthesis of cholesterol is controlled by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (E.C 1.1.1.34/88), the enzyme that converts 3-hydroxy-3-methyl-glutaryl CoA to mevalonate. The major metabolic fate of cholesterol is conversion to bile acids in the liver, a process controlled by the activity of the enzyme, cholesterol 7alpha-hydroxylase.

3.1. Role of cholesterol 7alpha-hydroxylase in bile acid synthesis

Cholesterol 7alpha-hydroxylase (EC 1.14.13.17) catalyzes the rate-limiting step in the neutral or classical pathway which converts cholesterol to bile acids (21). Cholesterol 7alpha-hydroxylase is 504 amino acids long and believed to be anchored to the endoplasmic reticulum via the N-terminal 23 amino acids. This cytochrome P450 enzyme, also known as cholesterol 7alpha-monooxygenase, hydroxylates cholesterol in the following reaction: cholesterol + NADPH + H^+ + O_2 produces 7alpha-hydroxycholesterol + $NADP^+$ + H_2O. 7alpha-cholesterol is converted to 7alpha-hydroxy-4-cholesten-3-one by 3beta-hydroxy-delta^5-C_{27}-steroid dehydrogenase/isomerase. The 7alpha-hydroxy-4-cholesten-3-one can follow one of two branches of the pathway, leading to either cholic acid or chenodeoxycholic acid. Cholic acid is produced if 7alpha-hydroxy-4-cholesten-3-one is hydroxylated by the microsomal enzyme, sterol 12alpha-hydroxylase, encoded on the CYP8B1 gene. For chenodeoxycholic acid synthesis, 7alpha-hydroxy-4-cholesten-3-one is converted to 5beta-cholestane-3alpha,7alpha-diol in two steps. Bile acid synthesis continues in the mitochondria with the hydroxylation of the side chain of both the 5beta-cholestane-3alpha,7alpha-diol and 5beta-cholestane-3alpha,7alpha,12alpha-diol. This reaction is catalyzed by sterol 27-hydroxylase (CYP27A1; E.C.1.14.13.60). The hydroxyl group at C27 is further oxidized to COOH, through which the choestanoyl group is linked to coenzyme A for transport into the peroxisome. The side chain is shortened by three carbons in a process similar to beta-oxidation of branch-chained fatty acids in the peroxisome. The resulting bile acid is conjugated with taurine or glycine, with the release of coenzyme A. Variations on the basic process and the action of intestinal bacteria produce the dozens of species of bile acids found in the bile (22, 23).

3.2. Acidic or Alternative bile acid biosynthetic pathway

Though normal bile production and cholesterol homeostasis require an active cholesterol 7alpha-hydroxylase, the enzyme can be bypassed by the metabolites following the acidic or alternative bile acid biosynthetic pathway (24). In this physiologically important process, the side chain of cholesterol is hydroxylated first, in contrast to the neutral pathway in which the committed step is the hydroxylation of carbon 7 of the sterol nucleus. The 7alpha-hydroxylation of the oxysterols results from the action of oxysterol 7alpha-hydroxylase (CYP7B1), which is expressed in a range of tissues (25). In addition to the CYP7B1 gene product, 24-hydroxycholesterol can be 7alpha-hydroxylated by the gene product of CYP39A1 (26). Individuals with a homozygous deletion mutation in the CYP7A1 gene have sharply reduced bile production and elevated serum lipids that are non-responsive to HMG-CoA reductase inhibitors. People with this frameshift mutation, that deletes the active site of cholesterol 7alpha-hydroxylase, have increased metabolite flow through the alternative bile acid pathway, providing the precursors for residual bile acid production (27).

The liver is the only tissue that produces bile acids, however, the oxysterols are produced in extra-hepatic tissues as part of normal and necessary metabolic processes. The major cholesterol oxidation products in the human circulation are 7alpha-hydroxycholesterol, 27-hydroxycholesterol, 24-hydroxycholesterol, and 4beta-hydroxycholesterol. These oxysterols are formed from cholesterol by specific cytochrome P450 enzymes. The levels of 7alpha-hydroycholesterol mirror the activity of hepatic cholesterol 7alpha-hydroxylase. 27-hydroxycholesterol is formed by sterol 27-hydroxylase (CYP27A1), a mitochondrial enzyme expressed in a number of tissues (28, 29). The microsomal sterol 24-hydroxylase (Cyp46A1) produces 24-hydroxycholesterol. The expression of CYP46A1 in neurologic tissue suggests a role in the mobilization of cholesterol in the central nervous system cholesterol pool across the blood/brain barrier (30). 4beta-hydroxycholesterol results from the action of CYP3A4, with levels elevated in patients treated with ursodeoxycholic acid or anti-epilepsy drugs (31).

The role of the acidic/alternative pathway is likely to be an essential cholesterol mobilization mechanism and not primarily for producing bile acids, though the point remains controversial. This hypothesis is supported by the insensitivity of CYP27A1 to bile acid feedback (32-34), patients with a defect in CYP27A1 accumulate cholesterol neurological and connective tissue (cerebrotendinous xanthomatosis) (35), as well as the observed phenotype of mice with Cyp7B1 disrupted. These mice show normal bile acid metabolism (36). Oxysterols activate the liver X receptor (LXR) (37), a nuclear hormone receptor influencing the activity of a number of genes important for lipid homeostasis. Mice

lacking a functional CYP27A1 gene were viable but had serious defects in sterol metabolism, with marked increases in bile acid and cholesterol excretion, as well as hypertriglyceridemia (38).

4. REGULATION

4.1. Nuclear hormone receptors

Nuclear hormone receptors are a diverse class of zinc-binding transcription factors that are essential for transcriptional regulation. Their transcription modulating effects are mediated through the interactions with auxiliary proteins called corepressors and coactivators (39). The activity of these factors can be controlled by protein-to-promoter ratios, DNA-binding affinity, and competition for DNA or protein interaction sites. These properties are modulated by multiple, but factor-specific, mechanisms. Factor availability is controlled by regulating the factor's transcription, intracellular localization or protein stability. Allosteric changes can be induced by binding specific small molecules (ligands) or by phosphorylation. The orphan receptors are nuclear receptors that have no identified ligand, but have been found to have primary functions in establishing, maintaining and modulating gene expression, especially in the context of hormone signaling (40).

4.1.1. Hepatocyte nuclear factor 4 alpha (HNF4)

HNF4alpha is a nuclear hormone receptor found in the liver, intestine, kidney and pancreatic islet that plays a large role in hepatoma differentiation and maintenance (41). In addition to CYP7A1 and CYP8B, many liver genes have been found to be regulated by HNF4, including glucose transport and glycolysis genes (42, 43). Though usually considered an orphan receptor, fatty acyl-CoA thioesters were found to act as ligands (44). Additionally, the transcriptional activation activity has been found to be also regulated by serine, threonine and tyrosine phosphorylation. Phospho-tyrosine is required for binding and alters intracellular localization. Activation of protein kinase A (PKA) by cAMP results in the phosphorylation of the A-box that reduces DNA binding of HNF4. Phospho-serine/threonine (45) at some locations enhances DNA binding, but AMPK phosphorylation results in the degradation of HNF4. HNF4 has been found to interact with a number of transcription factors and accessory proteins, including COUP-TFs, steroid receptor coactivator glucocorticoid receptor-interacting protein (GRIP)-1, CREB-binding protein (CBP) and the closely related p300 (46). CBP interacts with the amino acids 119-375 of HNF4 (46), an almost identical region (amino acids 128-374) was shown to be essential for the interactions required for the glucocorticoid response of the hepatic phosphoenolpyruvate carboxykinase (PEPCK) gene (47). The coactivators SRC-1 and GRIP1 associate with the AF-2 domain of HNF4, and p300and SRC-1 can synergistically potentiate HNF4 transcriptional activation activity (48). Emphasizing the importance of coactivators in human health, the two HNF4 mutations R154X and E276Q impair interaction of CBP, result in the adult onset diabetic phenotype, Maturity-Onset Diabetes of the Young Type 1 (MODY1)(49).

MODYs are genetic disorders that occur due to specific mutations in one of at least five genes. The mutations have been found in both coding and non-coding sequences. MODY1 is associated with defects in the HNF4 alpha gene (50). The E276Q substitution mutation interferes with the interaction of HNF4 with COUP-TFII (51) and p300 (49), which impairs insulin production in pancreatic beta-cells, likely due to the secondary reduction in HNF1alpha and other HNF4-target genes. The E276Q mutation is of interest in light of synergistic activation of HNF4 and COUP-TFII detected on the BAREII (18) and would be predicted to result in reduced CYP7A1 transcription. MODY2 was found to be a mutation in the gene for the glycolytic enzyme, glucokinase (52). MODY3 is the result of the genetic defect that results in the loss of function of the TCF1 gene, encoding hepatocyte nuclear factor-1alpha (50). Mice lacking a functional HNF1alpha gene (Tcf1-/-), have multiple defects, including non-insulin dependent diabetes and hypercholestrolemia presented with increased bile acid synthesis. The complex phenotype of Tcf1-/- mice can be explained in part by the reduced expression of the FXR gene, Nr1h4, an HNF1alpha target gene. The reduction of FXR reduces bile acid up-regulation of transporters. The reduced expression of liver basolateral membrane transporters and ileal bile acid transporters reduce liver, ileal and kidney uptake of bile acids (53).

4.1.2. Alpha-fetoprotein transcription factor (FTF)

A member of the fushi tarazu factor 1 (FTZ-F1) family of orphan nuclear receptors was found to bind to the BARE adjacent to HNF4 in both the CYP7A1 and CYP8B1 gene promoters (54). These factors, that bind as monomers to the DNA sequence 5'-TCAAGGTCA-3', have been grouped into two families: liver receptor homologous protein-1 (LRH-1)/FTF and steroidogenic factor 1 (SF-1)/Ad4BP (55). The homologs of FTF (NR5A2) vary according to species and tissue distribution. LRH (rat and mouse) and FTF (rat and human) are 138 nt longer than cholesterol 7 alpha-hydroxylase promoter factor (CPF) (human) (20) due to a splicing variation. FTF can be detected in the liver, intestine, ovary and pancreas; whereas SF-1 (NR5A1) is found in the adrenal gland, hypothalamus, pituitary and steroidogenic tissues but not found in the liver (56). The FTF has roles in steroidogenesis, liver growth, endocrine development and differentiation; with FTF elements found in the promoters for SF-1, SHP (57), hepatitis B virus (HBV) (58), HNF3beta (59, 60), CYP7A1 (20) and CYP8B1 (54, 61). SHP (small/short heterodimer partner) is not only a target gene for FTF, but SHP and FTF have been found to interact (62) and transcription of both SHP and FTF genes are increased by bile acid-activated FXR/RXR (63, 64). Significantly, because of the protein's relatedness to FTF, the activity of SF-1 has been shown to be influenced by PKA. FTF has been reported to interact, and thus mediate, the differential effects of SREBP-1 and –2 on the CYP8B promoter, and its response to cholesterol levels (65). FTF is believed to be important in the FXR/RXR mediated repression of gene transcription and during inflammation by its interaction or competition with other factors such as SHP (62, 66-71).

4.1.3. Farnesoid X Receptor (FXR)

Bile acids activate FXR, nuclear hormone receptor of the NR1 family and obligate heterodimeric partner of the retinoid X receptor. FXR is expressed in the

liver, intestine, kidney and adrenal cortex (72, 73). FXR is activated by the bile acids deoxycholate (DCA) and chenodeoxycholate (CDCA). The beta-hydroxy-bile acid, ursodeoxycholate (UDCA), inhibits DCA and CDCA activation of FXR (74-77). However, FXR is activated by a wide range of biomolecules, so the role of FXR is potentially very broad. FXR can be activated by the androgen catabolites, farnesol, juvenile hormone III and the sterol bronchodilatory drug forskolin (77-79). FXR has been implicated as a regulator in a variety of cellular processes, including many aspects of phospholipid and sterol metabolism.

FXR is essential for normal bile acid, cholesterol and lipid metabolism. Mice with the FXR gene disrupted displayed elevated serum bile acid, cholesterol, and triglycerides, increased hepatic cholesterol and triglycerides. The ileal BA binding protein (iBABP) and transport proteins were not induced, interfering with the normal active uptake of BA from the intestine, resulting in reduced bile acid pools (80). Cholesterol 7 alpha-hydroxylase was not repressed by bile acids in these FXR null mice, but had reduced bile acid pools due to decreased expression of the major hepatic canalicular bile acid transport protein. The lack of iBABP induction in FXR null mice was consistent with the identification of an FXR activation element in the iBABP promoter (81). An FXR-response element has been found in the promoter for the phospholipid transfer protein (PLTP) and hepatic PLTP gene transcription was induced in mice fed bile acids (82). PLTP is essential in the transfer of very low density lipoprotein (VLDL) phospholipids into high density lipoprotein (HDL). Loss of regulation of PLTP gene expression in the FXR-null mice may be the cause of the proatherogenic serum lipoprotein profile in these mice. Apolipoproteins have been found to be regulated by FXR (83). Clearly, FXR plays a large role in determining the balance of cholesterol and lipid levels, and bile acid synthesis and transport. However, FXR/RXR does not bind to the BAREII and, in every case, bile acid-activated FXR/RXR has stimulated transcription of promoters to which it binds.

4.1.4. Coactivators of transcription

The transcriptional activation activity of nuclear hormone receptors acts through protein/protein interactions. The coactivators and repressors are important proteins for modulating the activity. An increase in the expression of negative factor can result in the suppression of transcriptional activation. Indirect mechanisms for the repression of CYP7A1 by bile acid-activated FXR/RXR involving the up regulation of SHP and FTF/LRH have been proposed (63). Other studies indicate that SHP is not absolutely required (84) (85). Kerr,*et al*, 2002, (84) concluded that there were two SHP-independent regulatory pathways and one SHP dependent pathway based on studies with mice without a functional SHP gene. FTF/LRH figure prominently as a factor interacting with SHP(66, 86). Moreover, there is evidence that c-Jun N-terminal kinase (JNK) is one of the SHP-independent pathways in SHP knock-out mice (87).

The interaction of the DNA-binding receptors with these accessory proteins can me modified by phosphorylation of either the hormone receptor or the coactivator (88). In spite low expression levels in the liver (89), the transcriptional coactivator, peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1alpha), may be important in the regulation of CYP7A1(90). This factor is one of the common control elements of both carbohydrate and lipid metabolism, and may be an attractive target of therapies directed at correcting the detrimental energy usage and hypercholesteremia observed in non-insulin dependent diabetes. The protein stability of PGC-1 is increased by phosphorylation by AMPK (91). The message RNA for PGC-1alpha gene has been shown to increase in rat muscle following exercise and treatment with AICAR (92). PGC-1 is important for training adaptations of the mitochondria (89). Transcription of the CYP7A1 gene was activated by infecting HepG2 cells with a recombinant adenovirus expressing PGC-1alpha (90).

4.2. Signal transduction pathways

Gupta, *et al.*, 2001, showed that treatment of rat primary hepatocytes with taurocholate (TCA) results in a rapid and robust activation of the c-Jun N-terminal kinase (JNK) pathway, and that deoxylcholic acid (DCA) activates the Raf-1/MEK/ERK signaling cascade in primary rat hepatocytes primarily via an EGFR/Ras-dependent mechanism (93). Bile acids can activate protein kinase C *in vitro* (Huang, *et al*., 1994), treatment of HepG2 cells with bile acids results in the translocation of PKC and PKC inhibitors reduce the bile acid repression of transcription from the CYP7A promoter (Stravitz, *et al.*, 1995). Phorbol esters, which activate PKC, repress CYP7A1 transcription, and this effect maps to the same loci as the repression by bile acids and insulin (94). Moreover, the response of CYP7A1 to tumor necrosis factor alpha maps to BAREII, specifically HNF4 (95). That same study showed that expression of a dominant negative form of the stress-activated protein kinase kinase blocked repression with the bile acid CDCA in HepG2 cells. The structure-activity relationships of the different bile acids relative to FXR activation are reflected in the finding that expression of FXR target genes is unaltered in mice lacking a functional Cyp8B1 gene, the lack of which, as previously mentioned, prevents the synthesis of cholate. Synthesis of the FXR-ligands, DCA and CDCA, continues. However, there was a loss of bile acid feedback of cholesterol 7alpha-hydroxylase (CYP7A1), that was corrected with cholate feeding (96). This is consistent with a model of cholate activating JNK, resulting in a down-regulation of CYP7A1 (97). This study is compelling evidence for multiple bile acid response pathways (87). Reconciling the contribution of the kinase activation to the role of the nuclear hormone receptor, FXR, is an important long term goal of ongoing investigations, as well as determining the targets of the pathways that transmute the phosphorylation signal into changes in synthesis rates. There are several potential targets, with recent reports of RXR (98), vitamin D receptor(99) and SF-1 (100) being added to the growing list of transcription and accessory factors known to be phosphorylated in response to extracellular signals.

4.2.1. c-Jun N-terminal kinases (JNK)

Expressed as several isoforms, c-Jun N-terminal kinases (JNK) are activated by oxidative stress, and are also known as stress activated protein kinases (SAPK). JNK is

responsible for the phosphorylation c-Jun and the subsequent activation of AP-1 (Jun/Fos). Oxidative stress is followed by JNK activation, cytochrome c release form the mitochondria, caspase activation and apoptosis (101). JNK is of medical importance because of the suspected interference with hormone treatments, such as the anti-inflammatory glucocorticoid drugs used to treat asthma. The ligand- bound glucocorticoid receptor (GR) translocates to the nucleus but cannot attenuate inflammatory gene expression in patients with higher than normal JNK activation (102). Bile acids activate JNK, which is followed by repression of CYP7A1 gene expression (97, 103). Diferuloylmethane (curcumin found in the spice turmeric), an inhibitor of JNK, increases cholesterol 7alpha-hydroxylase activity and bile acid synthesis (104).

4.2.2. Extracellular-regulated kinase (ERK) 1/2 pathway

Another well-characterized mitogen-activated protein kinase (MAPK) pathway is the ERK. ERK 1/2 pathway is implicated in regulation of sterol metabolism by phosphorylating SF-1 (100), limiting JNK (105), regulation of steroidogenic acute regulatory (StAR) protein (106) and in the activation of the ERK 1/2 pathway by bile acids (107). Using primary rat hepatocytes, (107) show that 100 microM DCA activated the ERK 1/2 and AKT pathways in a mitochondria dependent process.

4.2.3. AMP-activated protein kinase (AMPK)

The long-known regulation of HMG-CoA reductase activity by phosphorylation (108, 109) is mediated by HMG-CoA reductase kinase (AMPK) (110-112) and whose action is reversed by the activity of HMG CoA reductase phosphatase (113, 114). AMPK down-regulates HMG-Co A reductase enzyme activity. One of the cell's energy sensors, AMPK shuts down anabolic processes and activates catabolic pathways when the kinase is activated by AMP and activators of AMPK improve utilization of glucose (115). This results in the conservation of ATP and is part of the conditioning response of muscle. Activators of AMPK, biguanide, metformin, and 5-amino-imidazole-4-carboxamide-1 beta D-riboside (AICAR) improve utilization of glucose (115). In addition to carbohydrate metabolism, AMPK profoundly influences lipid metabolism by a variety of mechanisms that have been implicated in bile acid metabolism. AMPK increases glucose transport into muscle through ERK pathway components, JNK, atypical protein kinase Cs (aPKCs) by activating proline-rich tyrosine kinase-2 (PYK2), and phospholipase D (PLD) (116). Interestingly, atypical protein kinase C (117), JNK and Raf-1/MEK/ERK signaling cascade (93) have been shown to be activated by bile acids. AMPK down regulates HMG-Co A reductase by covalently modifing the enzyme. AICAR, a AMPK activator, mimics the effect of insulin on PEPCK (118). Zucker rats infused with AICAR had improved serum lipid profiles. HNF4, one factor found to bind the BAREII has been reported to be degraded following activation of AMPK (119) as well as after treatment with bile acids (54). The reduction in HNF4alpha following AICAR treatment diminished mRNA levels for HNF-1alpha, GLUT2, L-type pyruvate kinase, aldolase B, apolipoprotein (apo)-B, and apoCIII (119). Additionally, AMPK has been found to influence transcription by modifing the action of CBP. Phosphorylation of CBP by activated AMPK, results in rendering the coactivator unable to interact with some nuclear hormone receptors while not changing the ability to respond to factors of other gene families. The activation of AMPK down regulates SREBP (120). SREBP-1, which is associated with regulation of fatty acid synthesis, is repressed by AMPK (high AMP levels) (120).

5. MORE THAN SEQUENTIAL PATHWAYS

Bile acids have been reported to act as agonists and antagonists for nuclear hormone receptors, signal transduction pathway activators and even a sex attractant in lampreys (121). The final response from bile acid exposure will be the result of interaction and opposition of the various pathways. Complicating interpretation of bile acid regulation of CYP7A1studies with animal models, are the observations that bile acid transport can be altered, and thus indirectly, but profoundly, influencing the activity of cholesterol 7 alpha-hydroxylase. Primary monolayer cultures of rat hepatocytes treated with 10 microM progesterone depleted cellular bile acids due to increased bile acid output. This effect was followed by a sustained increase in cholesterol 7 alpha hydroxylase activity (122). Clinical studies have indicated a decrease of bile acid recirculation upon treatment with metformin (123).

5.1. Cross-talk of signaling pathways

Activation of one pathway affects the state of components of other pathways, often referred to as cross-talk. Using human adrenocortical cells, the activity of SF-1 was found to be regulated by MAPK phosphatase-1 (MKP-1) and ERK1/2. In this study, expression of the human CYP17 promoter responded to dibutyryl-cAMP (Bt(2)cAMP) via the following events: the signal binding its receptor was followed by an increase in intracellular cAMP, which activated PKA, which phosphorylated the nuclear dual-specificity phosphatase, MKP-1, and increased its gene transcription. Additionally, knocking down MKP-1 reduced the human CYP17 promoter stimulation by cAMP, while inhibiting ERK1/2, increased human CYP17. This suggests that MKP-1 and ERK1/2 work in opposition (124). Bile acids activate the insulin receptor (125). Extrapolation between studies is complicated by the fact that the responses are often tissue-specific (98).

5.2. Physiologic verses apoptotic bile acid response

The role of JNK in apoptosis (101, 126, 127) would seemingly preclude the pathway's involvement in modulation of bile acid genes in the normal liver in the absence of some apparatus to prevent hepatocyte death. Mechanisms to prevent apoptosis would include dosage effects and survival signals that block progression to apoptosis. For instance, the normal hepatocyte is adapted to bile acid levels of the normal liver while cholestatic liver disease results from bile acid-induced apoptosis. This is consistent with what is observed in tissue culture: physiological doses of BA result in down regulation of

CYP7A1, while sustained high-dose (>100 micoM) results in apoptosis. Apoptosis was markedly reduced in rat hepatocytes and in rats by pre-exposure to trimethylglycine (betaine), a nutrient that reduces homocysteine levels. The anti-oxidant betaine prevented cytochrome c release without stopping the JNK, Erk, and p38 mitogen-activated protein kinase (MAPK) activation or protein kinase B (PKB) dephosphorylation (128). Considerable evidence exists indicating JNK-activation is proximal to the mitochondrial events preceding apoptosis, and sustained JNK-activation is the difference between apoptosis induced by taurolithocholic acid (TLCA) and the protective properties of tauroursodeocycholate (TUDCA) (129). UDCA was not protective against apoptosis of hepatocytes if the cells were treated with inhibitors of mitogen-activated protein kinase (MAPK) and phosphatidyl inositol 3-kinase (PI3K) signaling (130). A study with rat hepatocytes showed that DCA-activation of JNK was not prevented by blocking ROS (103), consistent with the JNK activation preceding the mitochondrial events. This would permit JNK to influence transcription without necessarily involving the mitochondria, followed by apoptosis. Protein kinase C inhibitors block TLCA-induced apoptosis, also (129). Moreover, there have been described non-apoptosis inducing signaling pathways that are JNK-dependent. Fibroblast growth factor-19 (FGF-19) is a target gene of FXR/RXR. FGF-19 strongly suppresses expression of CYP7A1 via JNK in primary cultures of human hepatocytes and mouse liver (131). JNK was found to by cytoprotective against nitric oxide induced apoptosis in cardiac myocytes (132).

6. TARGETS

Less studied than the upstream events are the identities of the ultimate targets of kinase/phosphatase action; the factors that transmute the signal into enzyme activity levels. Because of the cross-talk and concomitant, but abortive, activation of parallel pathways discussed above, identification of the proximal targets that transmute the signal into alterations in gene expression is essential. An additional reason for identifying the downstream targets is to validate assumptions made using information derived from models based on different species and tissues. In addition to the previously mentioned transcription factors and accessory factors, potential targets for the kinase/phosphatase cascades include transcription factors and covalent modification of enzymes.

6.1. Transcription factors

The response of the CYP7A1 promoter to number of drugs affecting the status of kinase/phosphatase pathways map to the BAREII, as previously mentioned and reviewed (23). Phosphorylation has been found to regulate the DNA-binding characteristics, interactions with co-activators/repressors, and protein stability of transcription factors. The ability to interact with its cognate binding element and coactivators of one of the factors that binds the BAREII, HNF4, is controlled by phosphorylation events (45, 133-136). RXR, which binds at several locations of the CYP7A1 promoter, is phosphorylated by JNK. This covalent modification results in reduced expression of RXR target genes (137). Because of the need for RXR as a heterodimeric partner for a number of nuclear hormone receptors including FXR, any process that affects RXR will change the response to the signals that rely on these factors. RXR-phosphorylation may underlie differing specificities and mode of actions of certain RXR ligands (138). Results from a series of experiments with linker-scanning mutations through the BAREII suggested that the activity of a factor that required the sequence adjacent to the HNF4 element was a potential target for covalent modification (Author, unpublished). This promoter sequence is recognized by FTF, which suggests FTF is a potential target analogous to the phosphorylation of the related protein, SF-1 (100, 124).

6.2. Enzyme phosphorylation

The activity of cholesterol 7 alpha-hydroxylase has been postulated to be controlled by the phosphorylation state of the enzyme (139-143). The differences between the extent of repression of transcriptional activity and enzyme activity suggest that CYP7A1 is subject to post-transcriptional regulation (8, 14, 144-152). Pandak, *et al.*, 1994, (153) found partially nephrectomized rats showed an increase in CYP7A1 enzyme activity with no increase in mRNA levels. Moreover, nuclear run-on assays determined that there was a 44% decrease in transcriptional activity of CYP7A1, in spite of the increase in activity (154). They conclude that the cholesterol 7alpha-hydroxylase activity may be regulated on the enzyme level, comparing it to the long-known regulation of HMG-CoA reductase activity by phosphorylation (108, 109). This kinase is generally identified with AMP-activated protein kinase (AMPK) (110-112) and whose action is reversed by the activity of HMG CoA reductase phosphatase (113, 114).

Cholesterol 7alpha-hydroxylase is a substrate for cAMP-dependent protein kinase *in vitro* that is reversed by treatment with alkaline phosphatase, resulting in a stimulation of activity (155). This finding was disputed by (143). The most direct evidence was found by Nguyen, *et al.*, 1996 (156), when they showed that the activity of *in vitro* expressed and purified human cholesterol alpha-hydroxylase could be modulated four-fold by cAMP-dependent protein kinase from rabbit muscle. Activation by protein kinase could be reversed with phosphatases and the activity was protected with the phosphatase inhibitor, sodium fluoride. Several protein kinases were effective, including cAMP-independent protein kinase and an uncharacterized kinase of *E. coli* origin. They demonstrated that the protein could be labeled with 32-phosphorous. Li, Wang and Chiang, 1990 (145), describe two putative Ca2+/calmodulin-activated protein kinase II phosphorylation sites as well as three glycosylation sequences. 5'AMP activates both AMPK and Ca2+/calmodulin-activated protein kinase I cascades (157). The phosphorylation state of cholesterol 7alpha-hydroxylase *in vivo* may be dependent on the physiological state of the hepatocyte. Cholesterol 7alpha-hydroxylase partially purified from diabetic rats had a lower specific activity than that isolated from normal rats and the activity from the diabetic rats could be increased by the addition of

sodium fluoride to the enzyme from the diabetic rats only (158). This result would be consistent with the presence of a phosphatase in the diabetic rat liver inactivating the enzyme.

7. PERSPECTIVES: IMPLICATIONS FOR HUMAN DISEASES

Recent advances in the study of liver gene control have increased the understanding of the molecular events that lead to the observed pathology of several human disorders. The oxysterols, are potent signaling molecules that regulate cell proliferation, apoptosis, brain function and the immune system (159). The anti-apoptotic effect of UDCA has been used to reduce neurological damage following stroke (160) and slow the progression of Huntington's disease (161). Hepatic inflammation is accompanied by reduced bile flow, which has been attributed to reduced expression of the Na+/taurocholate co-transporting polypeptide (Ntcp), resulting from IL-1 beta inhibition of RXR activity, mediated through JNK (137).

Alterations in bile transport are associated with secondary symptoms of seemingly unrelated conditions. The association of control of bile acid synthesis with diabetes stems from the early connection of diarrhea in diabetes patients with increased bile acid excretion rates (162-165). Patients with diabetes mellitus and streptozotocin-diabetic rats show larger bile acid pools and bile acid excretion rates compared to normal individuals (166, 167). Bile flow, pool size and excretion rates are interdependent parameters; reflecting rates of synthesis, export, reabsorption in the intestine and hepatocyte. The increased rate of bile synthesis in diabetes mellitus has been attributed, in part, to the de-repression of the CYP7A1 and liver CYP27A1 gene transcription by the absence of insulin (168).

The profile with Type 2 diabetes is quite different than insulin-dependent Type 1 diabetes, reflecting fundamental differences between the lack of insulin verses the loss of insulin response. The resistance of target tissues to insulin is the result of abnormal signaling. Premature death from heart disease, stroke and diabetes are major health issues in developed countries, with life-style induced non-insulin dependent diabetes (NIDDM or Type 2) rates increasing. Centers for Disease Control and Prevention (CDC) reported the proportion of people with diabetes increased from 4.9% in 1991 to 7.3% in 2000 (169), with 95% of all diabetics having Type 2. CDC and the Agency for Health Care Research and Quality found that women with diabetes were twice as likely to have cardiovascular disease (CVD) and four times as likely to require hospitalization as compared to non-diabetic women (170). The poorly-controlled hyperglycemia, obesity, hypertension, and dyslipidemia that characterize the pathophysiology of Type 2 diabetes (171), indicate the existence of inter-connections between carbohydrate and lipid metabolisms. When cholesterol and lipoprotein metabolism were studied in mildly hypercholesterolemic nonobese men with NIDDM, cholesterol synthesis was found to be significantly higher in NIDDM subjects than in the control subjects, whereas cholesterol absorption efficiency was lower than controls (172). The significance of the changes in signaling pathways found in NIDDM hepatocytes may be better understood with information provided by studies into bile acid signal transduction.

A large and growing body of literature supports the view that cellular kinases and phosphatases regulate substrate flow through and between the constituent pathways of cholesterol homeostasis. Bile acids are an important part of the complex, multi-component system that governs lipid and carbohydrate metabolisms. The control of the production of bile acids includes specific nuclear hormone receptors and signal transduction pathways interacting with elaborate compensatory mechanisms. Perturbation of the processes result in disease, but understanding of the interconnections will lead to new therapies and enable better anticipation of deleterious side-effects.

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