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Cholesterol 7α-hydroxylase is phosphorylated at multiple amino acids ☆

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Abstract

The activity of cholesterol 7α-hydroxylase (gpCYP7A1), the rate limiting enzyme in bile acid synthesis, has been postulated to be regulated by phosphorylation/dephosphorylation. This study has found that several kinase activators rapidly reduce the amount of bile acid produced by the human hepatoma cell line, HepG2, and that gpCYP7A1 from HepG2 cell extracts eluted in the phosphoprotein fraction of FeIII columns. After incubating the HepG2 cells with radioactive orthophosphate, the band identified as gpCYP7Al on immunoblots was strongly labeled. Recombinant gpCYP7A was expressed as 6× HIS fusion polypeptides and subjected to kinase assays. The locations of phosphorylation were mapped further by screening synthetic peptides against AMP-activated protein kinase (AMPK), c-Jun N-terminal kinase, protein kinase A, and a panel of nine protein kinase C isoforms. AMPK, also known as 3-hydroxy-3-methylglutaryl coenzyme A reductase kinase, phosphorylated cholesterol 7α-hydroxylase, suggesting a potential mechanism of coordination of cholesterol synthesis and degradation.

Keywords: Bile acid synthesis; Cholesterol 7α-hydroxylase; Cytochrome P450; Posttranslational control; Protein kinase A; AMP-activated protein kinase; Protein kinase C

Cholesterol is transformed into bile acids at a rate determined by the activity of cholesterol 7α-hydroxylase, encoded on the *CYP7*A1 gene. The expression of this enzyme is highly regulated on multiple levels, including control of initiation of transcription and message stability. Transcription of *CYP7*A1 is repressed by bile acids, phorbol esters, and insulin and activated by activation by cAMP [1,2]. Bile acids (BA) regulate gene expression both by binding farnesoid X receptor (FXR) [3–6] and by activating cellular kinases [7–10].

Bile acids can activate protein kinase C in vitro [11], treatment of HepG2 cells with bile acids results in the translocation of protein kinase C (PKC) and PKC inhibitors reduce the bile acid repression of transcription from the *CYP7*A promoter [12]. Phorbol esters, which activate PKC, repress *CYP7*A1 transcription, and the phorbol ester response element maps to the same loci on the *CYP7*A1 promoter as the repression by bile acids, BAREII [13]. Gupta et al. [8], showed that treatment of rat primary hepatocytes with taurocholic acid results in a rapid and robust activation of the c-Jun N-terminal kinase (JNK) pathway, and that deoxycholic acid activates the Raf-1/MEK/ERK signaling cascade in primary rat hepatocytes primarily via an EGFR/Ras-dependent mechanism [14].

In addition to the rapid modulation of bile acid production, the BA-activation of kinase/phosphatase signal transduction pathways mediate programmed cell death observed following exposure to concentrations of BA higher than that found in the normal liver. Though the exact identity and order of events that result in apoptosis following exposure to high-dose BA have not yet

☆ *Abbreviations:* AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; cAMP, adenosine 3′:5′-cyclic monophosphate; *CYP7*A1, cholesterol 7α-hydroxylase gene; FTF, α-fetoprotein transcription factor; FXR, farnesoid X receptor; HNF4, hepatocyte nuclear factor 4; PKA, protein kinase A; PMA, phorbol-12-myristate-13-acetate; PKC, protein kinase C.

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been determined, activation of the JNK is involved [15,16] as well as extracellular-regulated kinase (ERK) pathway and the inactivation of protein tyrosine phosphatases [17].

Less well-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. The enzyme may be subjected to post-translational covalent modification, and it has been postulated that the activity of cholesterol 7α-hydroxylase is controlled by the phosphorylation state of the enzyme [18–22]. The differences between the steady-state levels of mRNA and enzyme activity suggest that *CYP7*A1 is subjected to posttranscriptional regulation [23]. Cholesterol 7α-hydroxylase is a substrate for cAMP-dependent protein kinase (PKA) in vitro that is reversed by treatment with alkaline phosphatase, resulting in a stimulation of activity [24]. This finding is disputed by [21] using human liver tissue samples. However, the activity of in vitro expressed and purified human cholesterol 7α-hydroxylase could be modulated fourfold by PKA from rabbit muscle [25]. Activation of enzyme activity 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 *Escherichia coli* origin. The observation that phosphorylation increases activity suggests that the enzyme activity can be modulated in a manner similar to the regulation of HMG-CoA reductase by HMG-CoA reductase kinase (AMPK) and the HMG-CoA reductase phosphatase [26]. These in vitro studies are compelling, but will not reveal other possible effects of covalent modification, such as changes in protein stability.

In the course of examining factors that coordinate bile acid synthesis with the production of cholesterol, it was observed that the total bile acid production of the human hepatoma cell line, HepG2, was altered on short treatment times. The rapid response was consistent with the regulation by phosphorylation hypothesis. If bile acid synthesis is directly regulated by phosphorylation/dephosphorylation events, then the rate limiting enzyme, cholesterol 7α-hydroxylase, would be the likely target. Therefore, recombinant protein and peptides based on the cholesterol 7α-hydroxylase amino acid sequence were tested against kinases associated with the control of bile acid and cholesterol synthesis. The results of that study are reported here.

Materials and methods

Materials

Human hepatocellular blastoma cells HepG2 (ATCC HB8065) were obtained from American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM): F12, penicillin G/streptomycin, and trypsin-EDTA were purchased from Gibco Life Technologies (Rockville, MD). Fetal calf serum was from Hyclone (Logan, UT). Cholesterol, 8-bromoadenosine cyclic monophosphate (8-Br-cAMP), bile acids, and their taurine conjugates were supplied by Sigma (St. Louis, MO). 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was obtained from Toronto Research Chemicals (North York, ON, Canada). Phorbol-12-myristate-13-acetate was purchased from Calbiochem–NovaBioChem, La Jolla, CA. Restriction enzymes and other modifying enzymes were purchased from Promega (Madison, WI). Nucleobond DNA purification kit was from Clontech (Palo Alto, CA). Proteins were determined using the Bradford assay or the BCA Protein Assay (Pierce, Rockford, IL). Double-stranded oligonucleotides were synthesized by Life Technologies (Gaithersburg, MD).

Methods

Total bile acid assay. Determination of bile acid produced by HepG2 cells was performed using the Total Bile Acids Assay Kit (BioQuant Laboratories, San Diego, CA). HepG2 cells were plated in 100 mm tissue culture plates (Sarstedt, Newton, NC) and incubated in 3% CO_2 at 37 °C for 5 days. The DMEM:F12 medium with 10% fetal calf serum from the now confluent monolayers were exchanged for the same medium without fetal calf serum or phenol red. After 4 h, the monolayers were washed 2 times with DMEM:F12, no phenol red, to remove any bile acids produced, and overlaid with 10 ml DMEM:F12, no phenol red, containing 8-bromo-cyclic AMP, AICAR, PMA or dimethyl sulfoxide (DMSO) as the vehicle alone control. After the indicated incubation times, the media were recovered and bound to a SepPak C18 column (Waters, Milford, MA) and the RNA was purified from the monolayer using the NucleoSpin kit (BD Biosciences, Palo Alto, CA) for comparison of message levels using real-time polymerase chain reaction. The SepPak C18 columns were washed with 2 column volumes of water, followed by 2 column volumes of 95% methanol. The methanol fractions were concentrated in a vacuum centrifuge and the dried pellet was resuspended in 20 μl water. Five microliters of the resulting solution was assayed in triplicate using the BioQuant Total Bile Acids Kit according to the manufacturer's directions, but modified for use with 96-well plate format. Bile acids were measured by comparing the rates of increase of thio-NADH produced by the oxidation of bile acids by of 3-α hydroxysteroid dehydrogenase in reactions containing material recovered from cell culture overlays to that of known amounts of bile acids. Increase in absorbance at 405 nm was recorded with a SpectraMax Plus 384 UV/vis plate reader and Softmax Pro software (Molecular Devices, Sunnyvale, CA). Data were analyzed using Prism3 software (GraphPad Software, San Diego, CA).

Enrichment of phosphorylated proteins from metabolically labeled HepG2 cells. HepG2 cells were grown to confluency in 100 mm dishes as described above, washed two times with pre-warmed DMEM:F12 medium, and overlaid with 5 ml DMEM:F12 containing 50 μCi [^{32}P]orthophosphate. After two hour incubation, TCDCA was added to 25 μM to two of the plates and the plates were incubated for two additional hours. Approximately 20% of the label was removed from the medium after four hours. The cells were harvested by washing the monolayer twice with cold PBS, scraping the cells into 1 ml cold PBS, and the cells were recovered by centrifugation. The cell pellets were resuspended and disrupted according to the BD Phosphoprotein Enrichment Kit instructions (BD Biosciences, Palo Alto, CA). Cells from two 100 mm dishes were resuspended in 2 ml extraction/loading buffer (BD Biosciences), frozen in liquid nitrogen, and centrifuged at 13,000*g* for 20 min. The resulting supernatant was reacted with one milliliter of the FeIII immobilized metal resin, the unbound material was collected (flowthrough) and the settled column was washed three times with 5 ml of the extraction/loading buffer. The bound proteins were eluted with 20 mM sodium phosphate, pH 8.4, 0.5 M sodium chloride, collecting 0.5 ml fractions. Proteins were determined by the