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Forkhead box transcription factor O1 inhibits cholesterol 7 α -hydroxylase in human hepatocytes and in high fat diet-fed mice

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Abstract

The conversion of cholesterol to bile acids is the major pathway for cholesterol catabolism. Bile acids are metabolic regulators of triglycerides and glucose metabolism in the liver. This study investigated the roles of FoxO1 in the regulation of cholesterol 7 α -hydroxylase (CYP7A1) gene expression in primary human hepatocytes. Adenovirus-mediated expression of a phosphorylation defective and constitutively active form of FoxO1 (FoxO1-ADA) inhibited CYP7A1 mRNA expression and bile acid synthesis, while siRNA knockdown of FoxO1 resulted in a ~ 6-fold induction of CYP7A1 mRNA in human hepatocytes. Insulin caused rapid exclusion of FoxO1 from the nucleus and resulted in induction of CYP7A1 mRNA expression, which was blocked by FoxO1-ADA. In high fat diet-fed mice, CYP7A1 mRNA expression was repressed and inversely correlated to increased hepatic FoxO1 mRNA expression and FoxO1 nuclear retention. In conclusion, our current study provides direct evidence that FoxO1 is strong repressor of CYP7A1 gene expression and bile acid synthesis. Impaired regulation of FoxO1 may cause down-regulation of CYP7A1 gene expression and contribute to dyslipidemia in insulin resistance.

Keywords

bile acid synthesis; insulin; gene expression; nuclear receptor; metabolic diseases

1. Introduction

The conversion of cholesterol to bile acids is the predominant pathway for cholesterol catabolism. Bile acids facilitate intestinal fat and vitamin absorption and hepatic disposal of toxic metabolites and xenobiotics [1]. Approximately 95% of bile acids are reabsorbed in the intestine, transported back to the liver to inhibit bile acid synthesis. This circulation of bile acids between liver and intestine is referred to as the enterohepatic circulation of bile acids.

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Bile acids are endogenous ligands of the nuclear receptor farnesoid X receptor (FXR) [2]. FXR plays a central role in mediating bile acid inhibition of cholesterol 7 α -hydroxylase (CYP7A1), the first and rate-limiting enzyme in bile acid biosynthesis [1]. Two FXR-mediated pathways for bile acid inhibition of CYP7A1 gene transcription have been proposed. In the liver, FXR induces a negative nuclear receptor small heterodimer partner (SHP) that interacts with liver related homologue-1 (LRH-1) to repress CYP7A1 gene transcription [3]. In the intestine, FXR induces fibroblast growth factor 15 (FGF15), which activates hepatocyte FGF receptor 4 (FGFR4) signaling to repress CYP7A1 gene transcription [4]. CYP7A1 is also repressed during hepatic injury and inflammation by pro-inflammatory cytokines as an adaptive response to protect the liver from bile acid cytotoxicity [5,6]. Bile acids and FXR agonists reduce serum triglyceride levels by inhibition of lipogenesis and stimulation of triglyceride clearance, and improve hyperglycemia and insulin sensitivity in diabetic animal models [7-11].

FoxO1 belongs to the family of Forkhead box transcription factors. FoxO1 induces the gluconeogenic genes and contributes to hyperglycemia during hepatic insulin resistance [12, 13]. FoxO1 is a direct target of insulin signaling. Phosphorylation of FoxO1 by PKB/AKT promotes FoxO1 nuclear exclusion and proteasome degradation in the cytosol [14-16]. This insulin-controlled sub-cellular localization of FoxO1 provides the molecular basis of insulin action on controlling hepatic glucose and lipid metabolism [17]. FoxO1 expression and nuclear localization are increased in non-alcoholic steatohepatitis and in mouse models of diabetes and obesity [18,19]. Increased hepatic expression of FoxO1 is associated with elevated hepatic glucose production and fatty accumulation in mice [20,21]. Inhibition of hepatic FoxO1 by genetic ablation or an antisense oligonucleotide decreases hepatic glucose production and steatosis, and improves insulin sensitivity in diabetic mice [22,23].

A previous study from this laboratory shows that physiological concentrations of insulin stimulate CYP7A1 mRNA expression in primary human hepatocytes and FoxO1 inhibits CYP7A1 promoter/reporter activity [24]. In this study, we further investigated the role of FoxO1 in mediating insulin regulation of the CYP7A1 gene in primary human hepatocytes and in a mouse model of diet-induced insulin resistance. Our study suggests that FoxO1 is a key factor involved in the regulation of CYP7A1 gene transcription. Dysregulation of FoxO1 activity in insulin resistance and fatty liver diseases may lead to down-regulation of CYP7A1 gene expression and bile acid synthesis, which may contribute to impaired hepatic lipid homeostasis.

2. Materials and methods

2.1. Cell Culture

The human hepatoblastoma cell line, HepG2, was purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's modified Eagle medium and F-12 (Sigma, St. Louis, MO) supplemented with 100U/ml penicillin G/streptomycin sulfate (Mediatech, Herndon, VA) and 10% (v/v) heat-inactivated fetal bovine serum (Irvine Scientific, Santa Ana, CA). Primary human hepatocytes were isolated from human donors and were obtained through the Liver Tissue Procurement and Distribution System of the National Institutes of Health (S. Strom, University of Pittsburgh, Pittsburgh, PA). Cells were maintained in Hepatocyte Maintenance Medium (HMM) supplemented with 10⁻⁷ M of insulin and dexamethasone (Lonza, Walkersville Inc, MD). Cells were cultured in insulin-free media for 24 hr before performing all experiments.

2.2. RNA Isolation and Quantitative Real-time PCR

RNA isolation, reverse transcription reactions and real time PCR were performed as described previously [24]. All primers/probe used were TaqMan Gene Expression Assays purchased from Applied Biosystems (Foster City, CA). Amplification of Ubiquitin C (UBC) was used as an internal control. Relative mRNA expression was quantified using the comparative CT (Ct) method and expressed as $2^{-\Delta\Delta C_t}$.

2.3. Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed as described previously [6]. Antibodies against HAtag, HNF4 α and PGC-1 α (Santa Cruz Biotechnology, Santa Cruz, CA) were used to immunoprecipitate chromatin. TaqMan primers/probe were designed to detect the CYP7A1 promoter region containing a previously identified bile acid response element that binds HNF4 α (-180 to -111) and the human PEPCK gene proximal promoter containing the HNF4 and FoxO1 binding sites (-490 to -406). Quantitative real-time PCR was used to quantify the ChIP assay. The non-specific background detected in negative controls immunoprecipitated with non-immune IgG was subtracted from the precipitated CYP7A1 chromatin. The relative binding strength was expressed in arbitrary units with control set as “1”.

2.4. Recombinant Adenovirus

Adeno-EGFP was obtained from Dr. Li Wang (University of Utah, Salt Lake City, UT). Adeno-siFoxO1, Adeno-FoxO1-ADA expressing an N-terminal HA-tagged constitutively active FoxO1 and Adeno-FoxO1- Δ 256 [17] expressing an N-terminal HA-tagged truncated form of FoxO1, which lacks the C-terminal trans-activation domain and binds DNA as a dominant negative form were provided by Dr. D. Accili (Columbia University, NY). Recombinant adenoviruses were amplified in HEK293A cells and purified with Adeno-X Virus mini purification kit (BD Biosciences, San Jose, CA). Virus titer was determined by Ad-easy viral titer kit (Stratagene).

2.5. GST Pull-down Assay

GST or GST-full length human HNF4 α fusion protein was expressed in *E. coli* BL21 cells. Bacterial cell lysate containing either GST or GST-HNF4 fusion protein was then incubated with glutathione-conjugated agarose beads for 2 hr at 4 degree. Beads were then washed three times in 1 X PBS and resuspended in 1 X PBS as 50% slurry. HepG2 cells were infected with adenovirus expressing HA-tagged FoxO1-ADA or HA-tagged FoxO1- Δ 256 for 48 hr. Cells were collected by centrifugation and resuspended in 1X-GST binding buffer (1X PBS, 0.1% NP40, 0.5mM DTT, 10% Glycerol) and lysed by sonication. HepG2 cell lysates and fusion protein bound glutathione-agarose beads were then incubated at 4 degree for 2 hr. Beads were washed three times in GST wash buffer (1X PBS, 0.1% NP40, 0.5mM DTT, 100 mM KCl), and bound protein was eluted in 1% SDS lysis buffer at 95 degree and used for western blot detection of FoxO1-ADA or FoxO1- Δ 256 with an anti-HA antibody (Santa Cruz Biotechnology, CA). Ten % of whole cell lysates were used as “Input” controls.

2.6. Immunofluorescence Staining

Cells were fixed with 4% formaldehyde and permeabilized with 0.1% TritonX100. Anti-FoxO1 (Cell Signaling Technology, Danvers, MA) or anti-HA (Santa Cruz Biotechnology, CA) antibodies were used for detecting endogenous FoxO1 or exogenously expressed FoxO1-ADA, respectively. Alexa Fluor 488 conjugated secondary antibody (Molecular Probes, Carlsbad, CA) was used for detection under a confocal microscope. Non-immune IgG was used as background control.

2.7. Quantification of Total Bile Acids

Total bile acids from whole cell lysates and culture media were extracted with the Sep-Pak C18 cartridge (Walters Corp., Milford, MA) and quantified with total bile acid colorimetric assay kit (Bio-Quant, San Diego, CA) following the manufacturer's instruction.

2.8. Animal Study

Age-matched C57BL/6 male mice were fed either a standard chow diet or a high fat Western diet containing 42% fat calories (saturated fat from anhydrous milk fat) + 0.2% cholesterol (TD88137, Harlan Teklad) for a period of 20 weeks. Body weight was measured at 18 weeks of feeding. Mice were housed in a room under 12 h light and dark cycle (7 am on, 7 pm off). All mice were sacrificed around 10:00 am after over night fasting.

2.9. Analysis of plasma and hepatic lipids

Total liver cholesterol, triglyceride and free fatty acids were analyzed using lipid analysis kits (triglycerides and cholesterol, Thermo Electron Co., non-esterified fatty acids, Wako Chemicals Inc., Richmond, VA) following the manufacturer's instructions after chloroform/methanol (2:1 v/v) extraction [25]. Plasma insulin was measured using an ELISA kit (Crystal Chem, Chicago, IL). Plasma non-esterified fatty acids, triglycerides, cholesterol, glucose, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured by the Comparative Pathology Laboratory at Baylor College of Medicine.

2.10. Statistical Analysis

Results from cell-based studies were expressed as mean $\pm$ S.D. Results from animal studies are expressed as mean $\pm$ SEM. All statistical analyses were performed with student's t-test. A *p* value of <0.05 was considered as statistically significant difference between two groups.

3. Results

3.1. Adenovirus-mediated gene transfer of FoxO1 represses CYP7A1 and bile acid synthesis in human primary hepatocytes

In our previous study, we showed that physiological concentrations of insulin rapidly induced CYP7A1 mRNA expression in primary human hepatocytes and FoxO1 repressed human CYP7A1 reporter activity [24]. To directly test the effect of FoxO1 on CYP7A1 mRNA expression, we infected primary human hepatocytes with recombinant adenovirus expressing a phosphorylation defective and constitutively active form of FoxO1 (ADA) (Fig 1A, inset). Expression of FoxO1-ADA resulted in 60% inhibition of CYP7A1 mRNA expression, but had no effect on the mRNA expression of CYP27A1 mRNA expression (negative control). FoxO1-ADA significantly inhibited total bile acid synthesis by $\sim 50\%$ in primary human hepatocytes (Fig. 1B).

To test the hypothesis that FoxO1 directly interacts with HNF4 α and results in inhibiting CYP7A1 gene transcription, we performed GST pull down assays. As shown in Fig 2A, the GST-HNF4 α fusion protein interacted with both the constitutively active FoxO1-ADA, and the dominant negative FoxO1- $\Delta 256$ suggesting that HNF4 α may interact with FoxO1 through the N-terminal region that contains the DNA binding domain. Adenovirus-mediated transduction of FoxO1- $\Delta 256$ in human hepatocytes also resulted in $\sim 70\%$ repression of CYP7A1 mRNA expression (data not shown), suggesting that FoxO1 regulation of CYP7A1 is independent of its transactivating activity. Furthermore, ChIP assays showed that adenovirus-mediated transduction of FoxO1-ADA in primary human hepatocytes increased the association of FoxO1-ADA to the CYP7A1 chromatin (Fig 2B, left panel). FoxO1-ADA did not affect HNF4 α binding (Fig 2B, middle panel), but decreased its recruitment of PGC-1 α to CYP7A1

chromatin (Fig. 2B, right panel). We did ChIP assay of the human phosphoenolpyruvate carboxykinase (PEPCK) gene promoter as a control. FoxO1 and HNF4 α are known to bind PEPCK promoter and stimulate PEPCK gene transcription. As shown in Fig 2C, FoxO1-ADA did not affect the HNF4 α and PGC-1 α binding to the PEPCK gene promoter. These results suggest that FoxO1 acts as a co-repressor of HNF4 α , which blocks HNF4 α interaction with PGC-1 α and results in inhibition of CYP7A1 gene transcription. This mechanism seems to be specific for regulation of CYP7A1, but not PEPCK.

3.2. Knocking down of endogenous FoxO1 by RNA interference induces CYP7A1 mRNA expression in primary human hepatocytes

To further test the role of FoxO1 in regulating CYP7A1 gene expression, we used adenovirus-mediated transfer of FoxO1 siRNA (Ad-siFoxO1) to knock down endogenous FoxO1 expression in primary human hepatocytes. Fig. 3A shows that Ad-siFoxO1 reduced FoxO1 mRNA and protein levels by ~ 50% in primary human hepatocytes. Interestingly, such a decrease in endogenous FoxO1 expression resulted in a 6-fold increase in CYP7A1 mRNA expression (Fig. 3B). Immunostaining assays showed that FoxO1 was predominantly localized in hepatocyte nuclei (Supplemental Fig. 1). This loss-of-function study demonstrated that FoxO1 is a strong repressor of CYP7A1 gene expression.

3.3. Retention of FoxO1 in the nucleus prevented insulin induction of CYP7A1 mRNA expression

We next evaluated the role of FoxO1 in mediating insulin regulation of CYP7A1 mRNA expression in human primary hepatocytes by expressing a phosphorylation defective and constitutively active FoxO1 in human hepatocytes. Fluorescence imaging (Supplemental Fig. 1) shows that insulin treatment for 2 hr resulted in nuclear exclusion of endogenous FoxO1 in non-infected human hepatocytes (upper panel), but had no effect on exogenously expressed FoxO1-ADA that was exclusively localized in the nuclei (lower panel). To test how this FoxO1-ADA may affect insulin induction of CYP7A1 mRNA expression, hepatocytes were infected with adenovirus expressing either EGFP (negative control) or FoxO1-ADA and cultured for an additional 24 hr in insulin-free media. Cells were then treated with vehicle or insulin (10 nM) for 2 hr. Fig. 4 shows that insulin (10 nM) treatment caused a 5-fold induction of CYP7A1 mRNA in Ad-EGFP infected cells (negative control) as expected. Adenovirus-mediated transduction of FoxO1-ADA blocked insulin induction of CYP7A1 mRNA expression in primary human hepatocytes (Fig 4). These results provided the evidence that FoxO1 retained in the nuclei directly inhibited CYP7A1 gene transcription in human hepatocytes.

3.4. The CYP7A1 gene is down regulated in mice of high fat diet-induced insulin resistance

In order to gain further insights into the FoxO1 regulation of CYP7A1 *in vivo*, we fed mice a high fat Western diet for 20 weeks to study CYP7A1 expression. High-fat diet-fed mice developed fatty livers with severe hepatic accumulation of triglycerides, free fatty acids and cholesterol. A trend toward increased plasma AST and ALT levels was also noted in Western diet-fed mice, although they did not reach statistical significance. Consistent with hepatic fatty accumulation, high fat diet-fed mice showed a 3-fold increase in fasting plasma insulin levels over that of control mice, indicating impaired insulin sensitivity (Supplemental Table 1). Western diet-fed mice also showed elevated plasma cholesterol levels, but normal plasma triglycerides and free fatty acid levels (Table 1). Gene expression analysis by real-time PCR showed that high fat diet feeding caused ~ 60% decrease of CYP7A1 mRNA expression in mouse livers (Fig. 5A). Although the hepatic mRNA expression of FoxO1 was not significantly increased (Fig. 5A), high fat diet feeding resulted in a significant increase in nuclear content of FoxO1 protein (Fig. 5B). These results suggest that in hepatic steatosis and insulin resistant

conditions, both oxidative stress [26] and impaired insulin signaling [21] may cause nuclear accumulation of FoxO1, which inhibits CYP7A1 expression and bile acid synthesis

4. Discussion

Numerous studies in the past have established that dysregulation of FoxO1 contributes to both impaired hepatic glucose production and lipid metabolism. Using cell-based reporter assays, we showed previously that co-transfection of FoxO1 into HepG2 cells repressed a human CYP7A1 promoter/luciferase reporter activity, indicating that FoxO1 may regulate hepatic bile acid synthesis [24]. However, direct evidence for FoxO1 regulation of CYP7A1 expression and bile acid synthesis in human hepatocyte is still lacking. In this study, we employed adenovirus-mediated transfer of constitutively activate FoxO1 as a gain-of-function and siRNA to FoxO1 as a loss-of-function to study the role of FoxO1 on CYP7A1 gene expression in primary human hepatocytes. Furthermore, we examined the role of FoxO1 in regulation of CYP7A1 gene expression in a mouse model of high fat diet-induced insulin resistance. In this study, we demonstrated that the constitutively activate FoxO1 inhibits CYP7A1 expression and bile acid synthesis whereas knockdown of FoxO1 by RNA interference strongly stimulates CYP7A1 expression in human hepatocytes. The fact that expression of a phosphorylation defective FoxO1 completely abolished the insulin effect on the CYP7A1 gene provides direct evidence that FoxO1 is the down stream mediator of insulin action and a strong repressor of human CYP7A1 gene expression. Our data suggest that FoxO1 directly interacts with HNF4 α to reduce PGC-1 α occupancy in CYP7A1 chromatin and results in inhibiting CYP7A1 gene transcription. FoxO1 is subject to a complex regulation by cellular signaling pathways and post-translational modifications to affect FoxO1 cellular localization and activity [26-28]. It would be interesting to study how FoxO1 may regulate bile acid synthesis in response to different cellular stimuli.

Regulation of CYP7A1 gene expression in insulin resistance and hepatic dyslipidemia is currently unclear. This study also attempted to investigate the role of FoxO1 in CYP7A1 gene regulation in such disease conditions using long-term Western diet-fed mice as an *in vivo* model. This high saturated fat and high cholesterol-containing diet mimics the human "Western" diet and has been widely used in rodents for the studies of metabolic syndromes [29-32]. Consistent with these studies, we found that high fat diet feeding resulted in fatty livers and insulin resistance in these mice as reflected by excessive hepatic lipid accumulation and hyperinsulinemia (Table 1). We found that *cyp7a1* mRNA expression levels were markedly decreased in livers of high fat diet-fed mice when compared to chow-fed mice. This correlates with a significant increase in FoxO1 nuclear retention in hepatocytes, suggesting that increased FoxO1 nuclear retention may contribute to the down-regulation of the *Cyp7a1* gene as in primary human hepatocytes. These findings are also consistent with previous reports that feeding mice a diet containing high cholesterol and high saturated fat, but not polyunsaturated fat, inhibited *cyp7a1* gene expression, activity and bile acid pool [33,34]. Impaired insulin signaling may contribute to increased FoxO1 nuclear retention. How different dietary fat may regulate FoxO1 is currently not clear. Saturated fat accumulation in the liver may lead to oxidative stress and activation of JNK pathway to cause FoxO1 nuclear retention [26]. It should be noted that hepatic steatosis is usually associated with elevated hepatic production of cytokines [32]. We also found that hepatic expression of inflammatory cytokines TNF α and IL-1 β were all significantly increased (data not shown), it is likely that cytokine activation may also play a significant role in inhibition of CYP7A1 gene expression in hepatic fatty accumulation and inflammation [35].

In conclusion, this study provides direct evidence that FoxO1 is a repressor of CYP7A1 gene transcription and may be implicated in regulation of hepatic bile acid synthesis in diabetes and fatty liver diseases. Down-regulation of CYP7A1 in insulin resistance and fatty liver disease

may lead to disrupted bile acid homeostasis, which may contribute to dyslipidemia by affecting cholesterol, triglyceride and glucose metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

5. Acknowledgements

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A **B**

Figure 1. FoxO1-ADA inhibited CYP7A1 mRNA expression in primary human hepatocytes

Primary human hepatocytes prepared from seven different donor livers were infected with either adeno-EGFP or adeno-FoxO1-ADA at a MOI of 10. A. Twenty-four hr after infection mRNA expressions were measured by real-time PCR (n=7). Inset figure: FoxO1-ADA expression was detected by western blot with anti-HA antibody. B. Total bile acid amount was determined in primary human hepatocytes and culture media 48 hr after adeno-FoxO1-ADA infection (n=3). All results are expressed as mean $\pm$ S.D. The “*” indicates statistically significant, $p < 0.05$, Ad-FoxO1-ADA vs. Ad-EGFP.

Figure 2. FoxO1 interacted with HNF4 α and repressed CYP7A1 mRNA expression

A. Upper panel: a diagram of the structure of wild type FoxO1 and FoxO1-Δ256. DBD:DNA binding domain; TA: transactivating domain. Lower panel: GST pull down assay of FoxO1 and HNF4 α interaction. Total cell lysates from HepG2 cells infected with adeno-FoxO1-ADA or adeno-FoxO1-Δ256 at MOI of 10 for 24 hr were used in GST pull-down assay. Ten % of total cell lysates were used as "Input". FoxO1-ADA or FoxO1-Δ256 detected by western blot with an anti-HA antibody. B. Chromatin immunoprecipitation assay of the effect of Ad-FoxO1-ADA (Ad-ADA) on FoxO1, HNF4 and PGC-1 association with CYP7A1 and PEPCK chromatin. Primary human hepatocytes were infected with adeno-FoxO1-ADA for 24 hr at an

MOI of 10. ChIP assays were performed using antibodies against HA, HNF4 or PGC-1 α as described in Material and Methods.

Figure 3. Knocking down endogenous FoxO1 induced CYP7A1 mRNA expression in primary human hepatocytes

Primary human hepatocytes were infected with either adeno-EGFP or adeno-siFoxO1 at MOI of 10 for 48 hr. FoxO1 mRNA (A) and CYP7A1 mRNA (B) were determined by real-time PCR (n=4). FoxO1 protein was determined by western blot with an anti-FoxO1 antibody (A, inset). Results are expressed as mean $\pm$ S.D. The “*” indicates statistically significant, $p < 0.05$, Ad-siFoxO1 vs. Ad-EGFP.

Figure 4. Constitutively active FoxO1 blocked insulin induction of CYP7A1

Human hepatocytes were infected with either adeno-EGFP or adeno-FoxO1-ADA at MOI of 10 for 24 hr in insulin-free media, followed by treatment with insulin (10 nM) for 2 hrs. CYP7A1 mRNA was determined by real-time PCR (n=5). Results are expressed as mean $\pm$ S.D. The “*” indicates statistically significant, $p < 0.05$, insulin vs. vehicle. The “**” indicates statistically significant, $p < 0.05$, Ad-FoxO1-ADA vs. AdEGFP.

Figure 5. Decreased expression of *cyp7a1* mRNA in livers of western diet-fed mice

A. Quantitative real-time PCR was used to assay CYP7A1 and FoxO1 mRNA expressions in chow group (chow) or western diet group (WD) (n=3-4). Results are expressed as mean $\pm$ S.D. The “*” indicates statistically significant, $p < 0.05$, WD vs. chow. B. Immunoblot analysis of nuclear FoxO1 expression in mouse liver. Mouse livers from chow-fed and Western diet-fed (WD) mice (n =3-4) were pooled and nuclear extract was isolated for immunoblotting with antibodies against FoxO1 or Lamin B (negative control).