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Effects of Cholic Acid/CDCA and FGF-19 on the Protein Levels of the Endogenous Small Heterodimer Partner (SHP) in the Mouse Liver and HepG2 Cells

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Recent studies determined that a chronic western-style diet increased the endogenous small heterodimer partner (SHP) protein levels in mice. In experiments with cell cultures, chenodeoxy cholic acid (CDCA) treatment increased endogenous SHP protein levels and reduced the degradation rate of exogenously expressed flag-SHP levels in the human hepatoma cell line, HepG2 cells. In addition, bile acid-induced intestinal fibroblast growth factor-19 (FGF-19) increased the half-life of the exogenously expressed SHP when HepG2 cells were transfected with ad-flag-SHP. However, both the expression level and the degradation rate of the endogenous SHP in response to cholic acid and FGF-19 have not been well understood, either in mice or in cultured HepG2 cells. This study examined the effects of cholic acid treatment on the endogenous SHP protein levels in mice and the effects of FGF-19 on the degradation rate of the endogenous SHP protein in HepG2 cells. Mice fed 0.5% cholic acid in normal chow showed an increase in endogenous SHP protein levels during both 12 hr and 24 hr treatment periods as compared to control mice fed only normal chow. In cultured HepG2 cells, treatment with CDCA did not noticeably change the rate of degradation in the endogenous SHP protein from cells not treated with CDCA. Although consistent with the previous studies on the exogenous ad-flag-SHP protein, treatment with FGF-19 significantly decreased the degradation rate of the endogenous SHP protein when HepG2 cells were treated with cyclohexamide. These results suggest that both bile acids and FGF-19 increase the endogenous SHP protein levels in mouse liver and HepG2 cells.

Key words : CDCA, cholic acid, endogenous SHP, FGF-19

Introduction

Despite important physiological roles of cholesterol and bile acids, their levels must be tightly controlled in order to maintain energy homeostasis and prevent metabolic diseases [6,12,13]. An orphan receptor small heterodimer partner (SHP) has been reported to play an important role in this regulation by mediating bile acid induced repression of the cholesterol 7α hydroxylase (*CYP7A1*) gene expression in the bile acid synthetic pathway [3,11,12]. The *CYP7A1* gene encodes the first and rate-limiting enzyme in converting cholesterol to bile acids in the liver [1,8]. One of the multiple bile acid-activated signaling pathways involves farnesoid-X-receptor (FXR)-SHP nuclear receptor cascade in which the nuclear bile acid receptor FXR binds the elevated hepatic bile acids and induces transcription of the SHP gene [3-5,7,10,11,14]. The expressed SHP protein then recruits inhibitory chromatin remodeling complex to the promoter region of the *CYP7A1* gene and inhibits transcription of the gene [2,9]. The bile acid-activated FXR was also demonstrated to induce expression of fibroblast growth factor-19 (FGF-19) in the small intestine [7]. FGF-19 is secreted into the circulatory system and binds to and activates FGF membrane receptor-4 in the liver [7]. Although the downstream pathway of FGF-19 is not well understood, it was reported that secreted FGF-15 (mouse homolog of human FGF-19) inhibits transcription of the *CYP7A1* gene in the liver [7]. However, treatment with FGF-15 did not result in increased SHP mRNA levels in the liver, suggesting that the SHP-mediated mechanism of FGF-15 in suppressing transcription of the *CYP7A1* gene may involve other than transcriptional regulation of the SHP gene [12]. Recent studies demonstrated that hepatic bile acids stabilize SHP protein through post-translational modifications to suppress bile acid biosynthesis in the liver [12]. Furthermore, it was demonstrated that bile acid-induced intestinal FGF-19 also increases stability of the exogenously expressed ad-flag-SHP protein without inducing transcription of the SHP gene in both mouse liver and human hepatoma cell line, HepG2 [12]. Thus, these studies suggested that increased stability of SHP protein may play an important role in increased SHP action

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to regulate bile acid homeostasis in the liver in response to elevated bile acids, in addition to the increased transcription of the SHP gene [12]. However, both the expression level and the degradation rate of the endogenous SHP protein in response to cholic acid and FGF-19 have not been reported either in mice or in cultured hepatoma cell line. This study examined the effects of cholic acid treatment on the endogenous SHP protein levels in mice and the effects of FGF-19 on the degradation rate of the endogenous SHP protein in HepG2 cell line.

Materials and Methods

Animal treatment

For cholic acid experiment, BALB/c mice (Harlan Sprague Dawley, 3.5-month old) were fed normal chow or chow supplemented with 0.5% cholic acid (TD05271, Harlan Teklad Co., Madison, WI, USA) for 6~24 hr. Four groups of mice (2 animals/group) were divided into control (normal chow), 6 hr, 12 hr, and 24 hr cholic acid treatment period. All mice were maintained under a conventional photoperiod regimen with 12 hr light (7 AM~7 PM) and 12 hr dark cycle. Animals were fasted for 6 hr before beginning the experimental diet in order to induce the sufficient amount of the experimental food consumption and mice from the same group were housed in the same transparent polycarbonate cage and allowed free access to water and either normal chow or chow supplemented with 0.5% cholic acid for the time period specified for each group. Body weight of each mouse was measured before and after the diet treatment and the average consumption of the cholic acid-supplemented chow was measured for each group.

Cell culture and reagents

Human hepatoma HepG2 (ATCC HB8065; American Type Culture Collection, Rockville, MD, USA) cells were grown in Dulbecco's modified Eagle's medium-F12 (1:1) phenol red-free medium. The medium was supplemented with 100 unit of penicillin and 100 ug of streptomycin sulfate/ml and 10% heat-inactivated charcoal-treated fetal bovine serum. The HepG2 cells were treated with either 50 μM chenodeoxycholic acid (CDCA; Sigma, St. Louis, MO, USA) or 50 ng/ml of FGF-19 for 2 hr and 5 hr respectively in serum-free medium and then treated with 10 ug/ml of cyclohexamide for 0~2 hr. The vehicles for CDCA and FGF-19 were ethyl alcohol and phosphate buffered saline (PBS), respectively.

Nuclear extract preparation

Liver tissue was collected from each mouse in cold PBS, minced with razer blade, and washed with ice-cold 1x SSC buffer (150 mM NaCl, 15 mM sodium citrate, pH 7.5). Minced tissue pieces were centrifuged at 3,000× *g* and 4°C for 2 min and the supernatant was removed. The pellet was added hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2% NP-40, 1 mM EDTA, 5% sucrose, 1 mM DTT, protease inhibitors, pH 7.9) and dounce homogenized. The released nuclei were quickly checked under the microscope to ensure for the proper nuclei preparation. The liver homogenate was layered on cushion buffer (10 mM Tris-HCl, 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 10% sucrose, 1 mM DTT, protease inhibitors, pH 7.5) and centrifuged at 3,000× *g* and 4°C for 5 min. After removing the supernatant, the nuclei pellet was resuspended in TEG buffer (50 mM Tris-HCl, 1 mM EDTA, 15% glycerol, 500 mM NaCl, 1 mM DTT, protease inhibitors, pH 8.0) and the nuclear extract was prepared by sonication on ice for 5 sec at 50% setting and incubating the sonicated solution on ice for 30 min. The nuclear debris were removed by ultracentrifugation at 200,000× *g* (Ti-70.1, Beckman) and 4°C for 30 min and the supernatant was then collected into new eppendorf tubes and stored in liquid nitrogen until western immunoblot analysis.

The nuclear extracts from cultured HepG2 cells were prepared as following. At the end of each time point of cyclohexamide treatment, the culture media were removed and the cells were washed, scraped with 10 ml ice-cold PBS and transferred into 15 ml conical tubes on ice. The cells were collected by centrifugation at 1,000× *g* and 4°C for 3 min, resuspended in a hypotonic buffer II (10 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM DTT, protease inhibitors, pH 8.0), and were allowed to swell by incubating on ice for 10 min. The swollen cells were then added with 10% NP-40 up to a final concentration of 0.25%, mixed and sheared by passing through the 22G needle in 1 ml syringe 10 times on ice. The released nuclei were collected by centrifugation at 960× *g* and 4°C for 5 min, resuspended in the nuclei extraction buffer (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, protease inhibitors, pH 8.0), and incubated on ice by rocking for 15 min. The nuclear extracts were collected by centrifugation at 20,000× *g* and 4°C for 10 min and transferring the supernatant into new eppendorf tubes. The extract samples were then added to equal volume of 2x sample buffer

(0.5 M Tris-HCl, 4% SDS, 20% glycerol, 0.2 M DTT, 0.001% bromophenol blue, pH 6.8), boiled for 5 min, and stored at -20°C until electrophoresis.

Western immunoblot analysis

Samples of nuclear extracts from mice and cultured HepG2 cells were separated by 10% SDS-PAGE and transferred to PVDF membrane (Amersham, USA). The membrane was first incubated with blocking buffer (100 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 5% skim milk, pH 7.2) at RT for 1 hr to prevent non-specific binding of antibodies. The blocked membrane was then incubated with rabbit anti-human SHP IgG primary antibody (SC-H160, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) 4°C overnight followed by donkey anti-rabbit IgG secondary antibody conjugated with horse radish peroxidase (Santa Cruz Biotechnologies) at RT for 50 min. The membrane was washed three times for 10 min each and peroxidase activity was detected following incubation with 4-chloro-1-naphtol, diaminobenzidine and hydrogen peroxide as described by the manufacturer's protocol (ECL Chemiluminescence, PIERCE, Rockford, IL, USA). Antibody for actin was purchased from Santa Cruz Biotechnologies.

Results

Consumption of cholic acid supplemented chow

The average body weights of mice in each group were between 24 and 26 g. The average amount of chow consumption containing cholic acid were different between different treatment groups of mice and there were no positive correlations between the amount of cholic acid consumption and the treatment time period, that is, the amount of cholic acid consumption was not higher in the group of 12-hr feeding time than the group of 6-hr feeding time (Table 1).

Table 1. The average body weight and the average amount in gram of cholic acid-supplemented chow consumption per mouse in each group. Control mice were fed normal chow and the body weights shown are from the measurement at the beginning of the experiment. (n=2 mice/group)

	Cholic acid-diet group			
	C	6 hr	12 hr	24 hr
Avg. BW (gram)	24.2	25.3	24.7	25.8
Avg. CA-diet consumption (gram) /mouse	0	2.15	1.76	3.37

Expression of the endogenous SHP protein in mouse liver by cholic acid diet

As reported in the previous studies [12], livers from mice fed normal chow expressed the endogenous SHP protein (Fig. 1). Although expression levels of the endogenous SHP protein did not change or decreased in the livers of mice fed cholic acid-supplemented chow for 6 hr from those fed normal chow, expression levels of the endogenous SHP protein increased in livers from mice fed cholic acid-supplemented chow for both 12 hr and 24 hr (Fig. 1A). Densitometry analysis of the band intensity indicates that the levels of the endogenous SHP expression were between 1.4 and 1.8 fold in livers collected from mice fed cholic acid-supplemented chow for both 12 hr and 24 hr except one mouse in 24 hr feeding group as compared to those from mice fed normal control chow (Fig. 1B). Based on the data from the amount of cholic acid consumption among different groups of feeding times (Table 1), it may be possible that different amount of consumption of the cholic acid

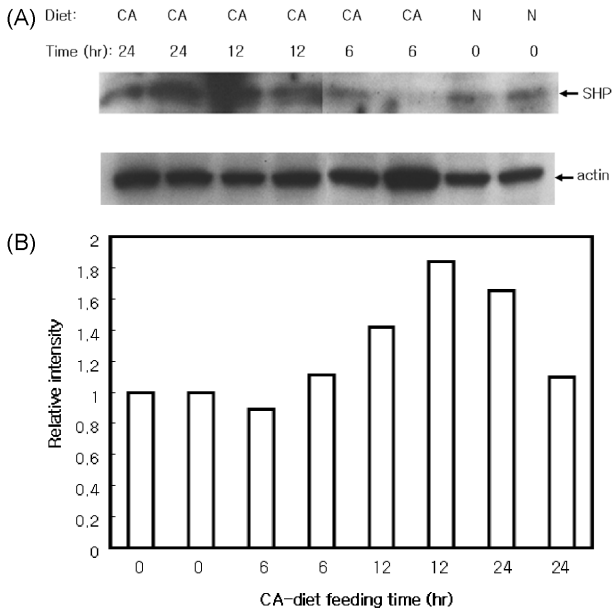

Fig. 1. Expression of the endogenous SHP protein in mouse liver by cholic acid diet. (A) Livers from mice fed a normal chow or mice fed a cholic acid-supplemented chow for 6~24 hr were collected. Endogenous levels of SHP was detected by Western analysis with polyclonal SHP antibody. Results from two sets of mice are shown for each treatment group. As a control, actin levels were also detected. (B) Band intensities were determined using ImageJ densitometry (BioRad, USA) and the average value for control samples was set to 1. The plotted graph shows band intensities relative to the control group (0 hr). CA: cholic acid-supplemented chow, N: normal chow.

may account for the variations in the level of the endogenous SHP protein detected in livers from different mice within a same treatment group (Fig. 1). In addition, another possibly more important factor that can contribute to the increased levels of the SHP protein in the liver in response to cholic acid may be the duration of time passed after exposure of the cholic acid rather than the amount of the cholic acid exposed to liver. For example, the fact that although the average amount of cholic acid consumption was even higher in the 6 hr feeding group than in the 12 hr treatment group, the levels of the endogenous SHP protein detected in the 6 hr feeding group was lower than in the 12 hr-fed mice may explain this possibility. These results suggest that cholic acid increases the levels of the endogenous SHP protein expressed *in vivo* of mice.

Degradation rate of the endogenous SHP protein in HepG2 cells by CDCA

Consistent with the previous report [12], the endogenous SHP protein was expressed in HepG2 cells (Fig. 2). However, treatment with CDCA did not increase levels of the endogenous SHP protein (Fig. 2A, B). This result is not in agreement with the previous report in which treatment with CDCA in HepG2 cells for 1.5 hr increased the level of the endogenous SHP protein. To examine the effect of CDCA treatment on the rate of degradation of the endogenous SHP, the endogenous SHP protein levels were examined after cyclohexamide treatment. Treatment with CDCA did not influence significantly the rate of degradation of the endogenous SHP protein in HepG2 cells. The half-life of the endogenous SHP protein was about 60 min in cells not treated with CDCA and was slightly extended more than 60 min in cells treated with CDCA (Fig. 2B). These results are also different from the previous studies in which CDCA reduced the exogenous flag-SHP protein degradation rate about four times as compared to that of vehicle treatment. Levels of actin as a loading control were not affected by cyclohexamide treatment (data not shown).

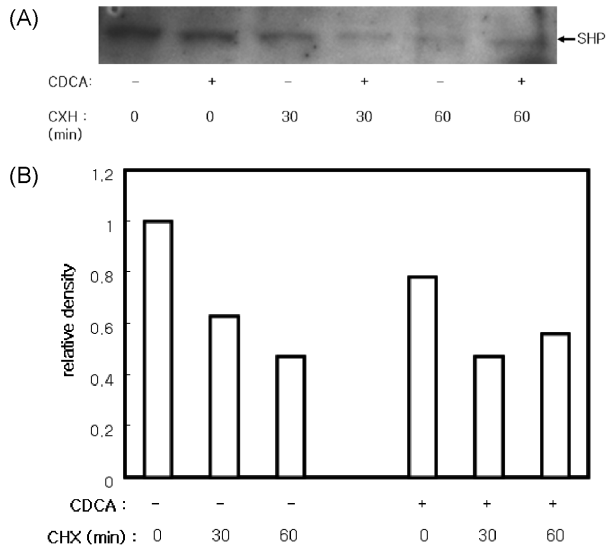

Fig. 2. Degradation rate of the endogenous SHP protein in HepG2 cells by CDCA and vehicle control. (A) HepG2 cells were treated with either vehicle (ethanol, −) or CDCA (50 uM, +) for 4 hr. and incubated with cyclohexamide (CHX) (10 ug/ml) for the indicated times. Endogenous levels of SHP protein were detected by Western analysis with anti-human SHP antibody. (B) Band intensities were measured by densitometry and the intensities relative to the 0-min CHX time point of vehicle control were plotted.

Degradation rate of the endogenous SHP protein in HepG2 cells by FGF-19

Treatment of FGF-19 decreased the rate of degradation of the endogenous SHP protein in human hepatoma cell line, HepG2 (Fig. 3A, B). When HepG2 cells were treated with

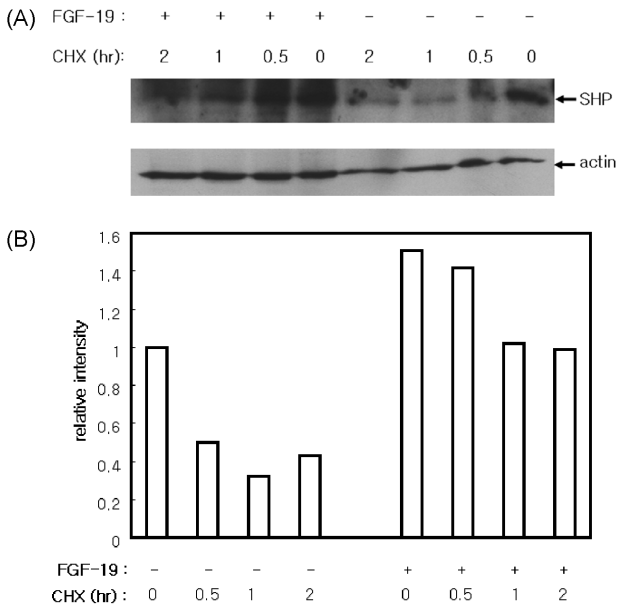

Fig. 3. FGF-19 decreases the degradation rate of the endogenous SHP protein in HepG2 cells. (A) HepG2 cells were treated with either PBS (-) or 50 ng/ml of FGF-19 in PBS (+) for 5 hr, followed by cyclohexamide treatment for the indicated times. Endogenous levels of SHP protein were detected by Western analysis with anti-human SHP antibody. As a control, actin levels were also detected. (B) Band intensities relative to the 0-min CHX time point of PBS control were plotted.

FGF-19 followed by cyclohexamide treatment, the half-life of the endogenous SHP protein was more than 2 hr whereas cells treated with PBS vehicle had significantly shorter half-life of about less than 30 min (Fig. 3B). These results suggest that FGF-19 decreases degradation rate of the endogenous SHP protein in HepG2 cells.

Discussion

Recent studies demonstrated that post-translational modifications play an important role in the regulation of SHP stability and function that mediates the negative feedback regulation of bile acid biosynthesis in order to maintain cholesterol and bile acid homeostasis in the liver. Hepatic bile acids dramatically stabilized the exogenously introduced flag-SHP protein in addition to increasing transcription of the gene via FXR in order to suppress the expression of the bile acid biosynthesis enzyme CYP7A1. Bile acid-induced intestinal FGF-19 also acts at the liver cells to inhibit hepatic bile acid biosynthesis by increasing protein stability of the exogenously introduced flag-SHP without increasing transcription of the gene.

This study further reports that cholic acid treatment increases levels of the endogenous SHP protein in mouse liver and that the intestinal FGF-19 decreases degradation rate of the endogenous SHP protein in HepG2 cells.

The increase in the levels of the endogenous SHP protein in mouse liver after feeding cholic acid-supplemented chow for 12~24 hr is in agreement with the previous report that when mice injected with ad-flag-SHP was treated with cholic acid-supplemented chow for 14 hr, levels of the exogenously expressed flag-SHP protein significantly increased. These results demonstrate that cholic acid increases the levels of SHP protein either exogenously or endogenously expressed *in vivo* of mice.

The discrepancy between the results of this study and those of the previous studies in the effects of CDCA on the endogenous SHP protein levels in HepG2 cells is not clear but may involve different factors. First, it may in part come from the different analysis methods. In the previous studies [12], the endogenous SHP levels were detected by immunoprecipitation, whereas this study detected SHP protein levels by immunoblotting directly on the samples of liver nuclear extract run on SDS-PAGE. Second, varying conditions of the hepatoma cell line and thus the different responsiveness to CDCA may be a contributing factor to the protein levels in response to CDCA treatment. And third, in the previous studies on the SHP stability [12], HepG2 cells were first infected exogenously with adenovirus-flag-SHP before treatment with cyclohexamide and the SHP protein levels were detected with M2 antibody instead of SHP antibody. Thus, these results may suggest that levels of the expression and/or degradation rate of the endogenous SHP protein in response to CDCA may be different from those of the exogenously infected flag-SHP in HepG2 cells.

The decreasing effect of FGF-19 on the degradation rate of the endogenous SHP protein in HepG2 cells are in consistent with the previous report on the exogenously expressed ad-flag-SHP protein.

In summary, these results suggest that cholic acids and FGF-19 increase the endogenous SHP protein levels in mouse liver and HepG2 cells, reapectively. In addition, this study further reports FGF-19 decreases degradation of the endogenous SHP protein in HepG2 cells.

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초록 : 생쥐의 간과 HepG2 세포에 있어서 내인성 small heterodimer partner (SHP)의 단백질 수준에 미치는 cholic acid/CDCA 및 FGF-19의 효과

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최근의 연구에서 생쥐에 장기간 서구식 사료를 급여했을 때 내인성 SHP 단백질의 수준이 증가함을 보고하였다. 또한 HepG2 세포배양을 통한 실험에서, CDCA 처리가 내인성 SHP 단백질의 수준을 증가시킬 뿐만 아니라 외인성으로 발현된 flag-SHP의 분해율을 감소시켰다. 그리고 HepG2 세포를 ad-flag-SHP로 유전자 형질전환 시켰을 때, 담즙산에 의해 유도되어진 소장 FGF-19이 외인성으로 발현된 flag-SHP 단백질의 반감기를 증가시켰다. 그러나 cholic acid와 FGF-19에 의한 내인성 SHP 단백질의 발현수준과 분해율은 생쥐 또는 배양된 간암세포주에서 아직 명확히 이해되고 있지 않다. 이 연구는 cholic acid의 처리가 생쥐에서 내인성 SHP 단백질의 수준에 미치는 영향과, FGF-19이 HepG2 세포주에서 내인성 SHP 단백질의 분해율에 미치는 영향을 조사하였다. 정상적인 사료를 급여한 대조군 생쥐에서의 내인성 SHP 단백질 수준과 비교하여, 0.5%의 cholic acid를 첨가한 사료를 급여한 생쥐에서는 12시간과 24시간의 처리기간 동안에 내인성 SHP 단백질의 수준이 증가하였다. 배양된 인간 간암세포주인 HepG2에서 CDCA의 처리는 CDCA를 처리하지 않은 대조군 세포주와 비교하여 내인성 SHP 단백질의 분해율을 유의성 있게 변화시키지 않았다. 한편 외인성 ad-flag-SHP 단백질에 대한 이전의 연구와 일치하게, HepG2 세포에 cyclohexamide를 처리하였을 때 FGF-19는 내인성 SHP 단백질의 분해율을 현저히 감소시켰다. 이러한 결과는 담즙산과 FGF-19 모두 생쥐의 간과 HepG2 세포주에서 내인성 SHP 단백질의 수준을 증가시킴을 제시한다.