Original Article

Ethanol enhances selenoprotein P expression via ERK-FoxO3a axis in HepG2 cells

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Drinking alcohol is considered one of the risk factors for development of diabetes mellitus. Recently, it was reported that selenoprotein P levels in blood are increased by ethanol intake. However, the mechanism by which ethanol increases selenoprotein P has not been elucidated. The expression of selenoprotein P protein and its mRNA were increased in a concentration- and time-dependent manner when human liver-derived HepG2 cells were treated with ethanol. Levels of AMPK and JNK proteins, which have been known to regulate selenoprotein P transcription, were unchanged by ethanol treatment. However, the amount of nuclear FoxO3a, a transcription factor of SeP, was increased. This was associated with dephosphorylation of ERK1 but not ERK2. It was found that ERK1 was dephosphorylated by activation of dual-specific phosphatase 5 and dual-specific phosphatase 6. However, the phosphorylation of MEK by ERK phosphokinase was not affected by ethanol treatment. These results suggest that the ethanol-induced increase in SeP levels occurs by enhanced transcription of SeP mRNA via the DUSP5/6–ERK1–FoxO3a pathway.

***Key Words*: selenoprotein P, ethanol, ERK1, DUSP6, FoxO3a**

Drinking habits are related to cultural and religious backgrounds.[1] A low level of alcohol (EtOH) consumption has minor positive effects on health by reducing the relative risk of intracerebral hemorrhage and acute myocardial infarction compared with the risk from abstinence.[2] In contrast, excessive drinking can trigger alcoholism and heavy drinking for years can cause liver dysfunction, esophageal cancer, and other physical and mental damage.[3–5] For example, it was reported that excessive alcohol consumption led to 3 million premature deaths worldwide in 2016, accounting for 5.3% of all deaths.[6]

Ingested EtOH is absorbed from the stomach (20%) and small intestine (80%), and most of it is ultimately metabolized in the liver.[7] EtOH is initially metabolized by alcohol dehydrogenase 1B (ADH1B) and cytochrome P450 (CYP) 2E1 to produce acetaldehyde as an oxidation product. This toxic compound causes oxidative stress, mitochondrial damage, and acceleration of cytokine synthesis.[7] Acetaldehyde is later metabolized to acetic acid by aldehyde dehydrogenase 2 (ALDH2).[7] Detoxification of EtOH and acetaldehyde in the liver is important because they induce an increase of reactive oxygen species (ROS) in various tissues.[8] For example, ROS in microglial cells increase NADPH oxidase (NOX) and 20s proteasome activities, resulting in cell death.[9] EtOH uptake by hepatocytes promotes ROS generation and triglyceride synthesis by activating SREBP-1c and inhibiting β-oxidation via PPAR-γ suppression, thereby causing the accumulation of triglycerides.[10] Excessive EtOH consumption causes liver dysfunction through ROS production and triglyceride accumulation.[11,12]

Selenoprotein P (SeP) (encoded at *SELENOP* gene) is a major Se-containing protein which is mainly synthesized in the liver and secreted into the plasma.[13] (SeP contains 10 selenocysteine residues; selenocysteine is an analog of cysteine where sulfur has been replaced by selenium.) SeP has multiple functions in the body.[13] Studies have shown that the SeP plasma concentration in patients with diabetes is double that of healthy controls.[14] The excess SeP in plasma induces phenotypes of type 2 diabetes, such as insulin resistance and impaired insulin secretion from pancreatic beta cells.[14,15] It was recently reported that 30 g of EtOH consumption per day increases blood SeP levels without affecting liver function.[16]

SeP levels are regulated in various ways at both steps of transcription, and translation. Metformin, which is a one of therapeutic agents for diabetes, activate AMP-activated protein kinase (AMPK) by phosphorylation. Activated AMPK in turn phosphorylates FoxO3a, which is an SeP transcription factor, and translocates it to the extranuclear region, thereby repressing SeP transcription.[14,17] High glucose levels decrease SeP protein levels by inhibiting AMPK activity.[14] Eicosapentaenoic acid inhibits SeP transcription by inactivating SREBP-1 by an AMPK-independent pathway.[18] Carrageenan, a common food additive used to improve texture, phosphorylates JNK by induction of endoplasmic reticulum (ER) stress, and phosphorylated JNK enhances SeP transcription.[18] Noncoding RNA has been reported as a suppressor of the translation step. A long noncoding RNA-inhibitor of SeP translation (L-IST), which has a complementary sequence to the 3'UTR of SeP mRNA, inhibits SeP mRNA translation by inhibiting the binding of SeP mRNA to the ribosome.[19]

In this study, we investigated the mechanism by which EtOH increases SeP synthesis in hepatocytes. We found that EtOH increases SeP expression by enhancing the nuclear translocation of FoxO3a, through a novel regulatory mechanism of SeP expression via the ERK–FoxO3a pathway.

Materials and Methods

Chemicals. EtOH and other chemicals were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan) or Sigma-Aldrich (St. Louis, MO). All chemicals used in this work were of the highest commercial quality.

Cell culture. Human hepatoma HepG2 cells and mouse hepatoma Hepa1-6 were purchased from the American Type Culture Collection (Manassas, VA). Human hepatoma HC-4 Cells and human hepatoma LI-7 cells were purchased from the Cell Resource Center for Biomedical Research, Tohoku University. HepG2 cells and Hepa1-6 cells were cultured in Dulbecco's modified Eagle's medium (Fujifilm Wako Pure Chemical), while HC-4 cells and Li-7 cells were cultured in Eagle's minimal essential medium (Fujifilm Wako Pure Chemical) containing

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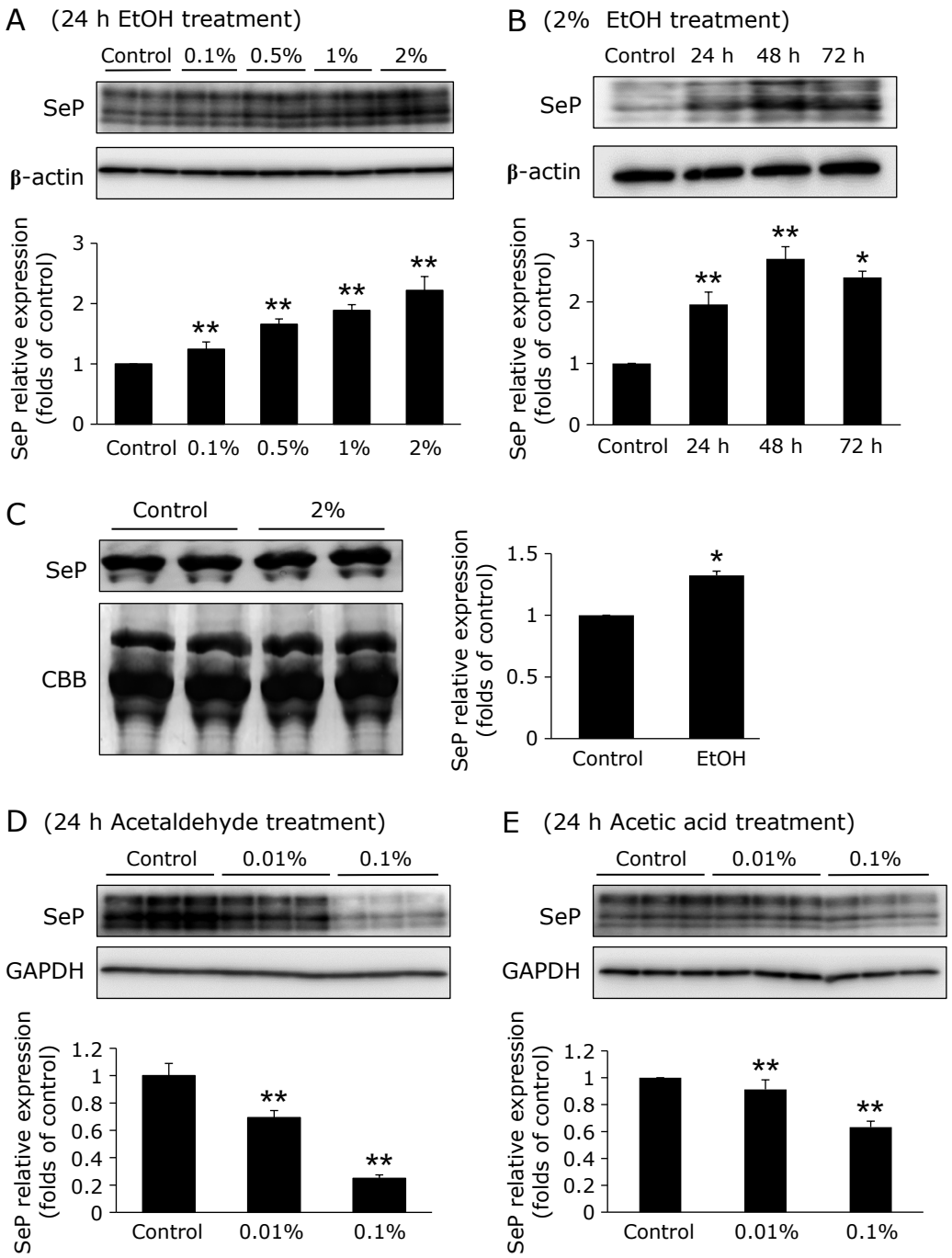

Fig. 1. EtOH increases SeP expression in HepG2 cells. HepG2 cells were treated with various concentrations of EtOH for various times (A and B). EtOH increased intracellular SeP protein levels in a concentration-dependent manner with the highest level under 2% EtOH (A). EtOH increased intracellular SeP protein in a time-dependent manner with the highest level at 48 h after EtOH treatment (B). EtOH at 2% increased secretion of SeP at 24 h treatment (C). HepG2 cells were treated with various concentrations of acetaldehyde and acetic acid (D and E). Data are means ± SD, $n = 3$. NS, not significant; *$p<0.05$; **$p<0.01$ by Tukey–Kramer test, ANOVA or Student's t test.

10% heat-inactivated fetal bovine serum (Nichilei, Tokyo, Japan) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA). All cells were cultured at 37°C under an atmosphere of 95% air and 5% CO_2.

Western blot analysis. A mixture of RIPA buffer, a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), and a phosphatase inhibitor (PhosSTOP; Roche, Basel, Switzerland) was used to extract proteins from the HepG2 cells by suspension at 4°C for 30 min. Undissolved cell residues were removed by centrifugation at 15,000 × g for 5 min. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as the reference standard. Proteins in each sample were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and identified by specific antibodies. These antibodies included anti-SeP,[20] anti-β-actin (1:10,000, A5441; Sigma-Aldrich), anti-GAPDH (1:40,000, AB8245; Abcam, Cambridge, UK), anti-pAMPK [1:1.000, #2535; Cell Signaling Technology (CST), Danvers, MA], anti-AMPK (1:1,000, #2532; CST), anti-pJNK (1:1,000, #9251; BD Biosciences, Tokyo, Japan), anti-JNK

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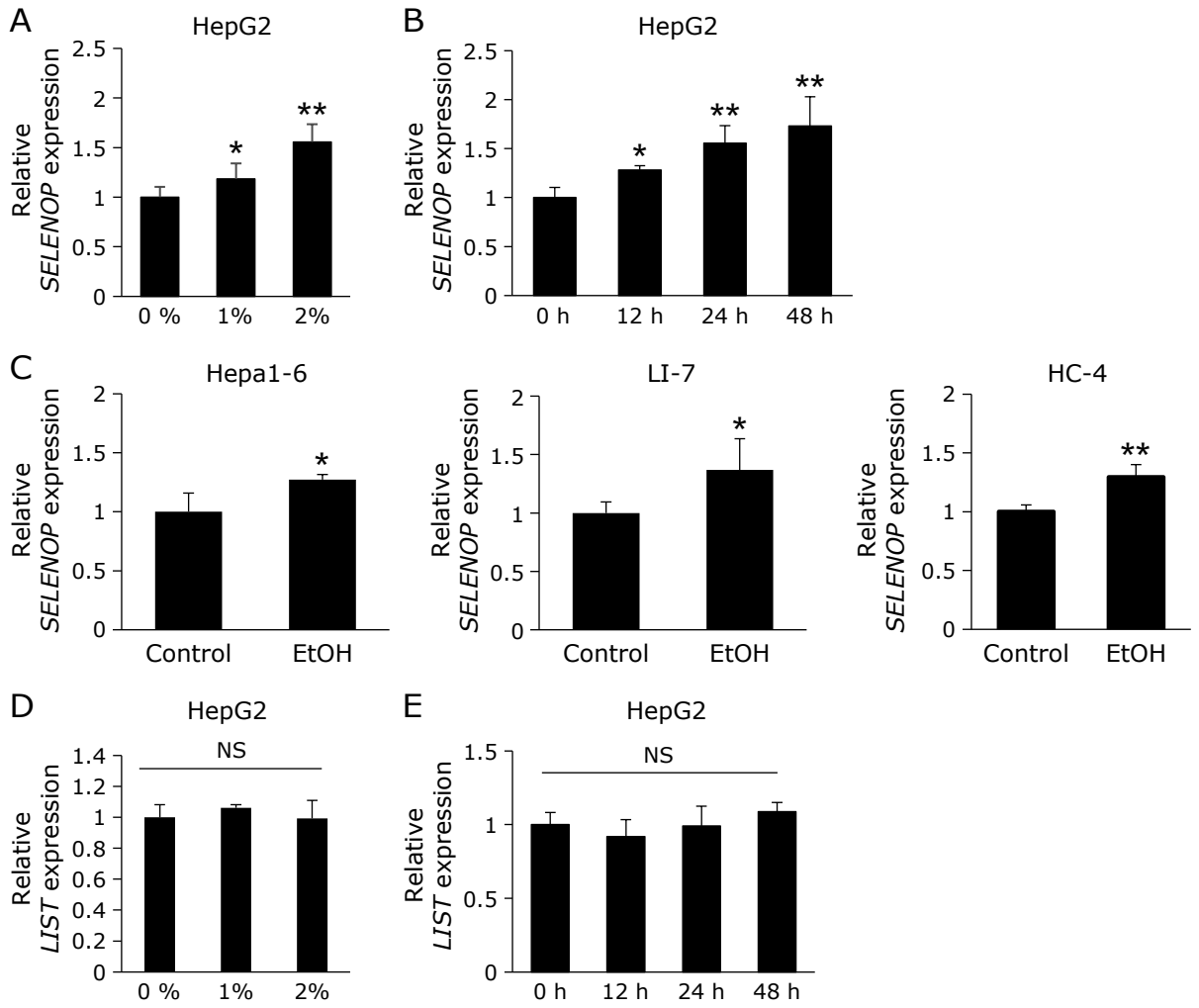

Fig. 2. EtOH increases SeP mRNA expression without changing L-IST in HepG2 cells. HepG2 cells were treated with various concentrations of EtOH for various times (A and B). EtOH increased SeP mRNA expression in a concentration-manner 24 h after treatment (A). EtOH at 2% increased SeP mRNA expression in a time-dependent manner (B). EtOH at 2% increased SeP mRNA in HC-4 cells, Hepa1-6 cells, and LI-7 cells (C). EtOH did not change L-IST expression in HepG2 cells. (D and E). Data are means ± SD, $n = 3$. NS, not significant; *$p<0.05$; **$p<0.01$ vs 0% or 0 h by Tukey–Kramer test, ANOVA or Student's t test.

(1:1,000, #9251; BD Biosciences), anti-pERK (1:1,000, #9102; CST), anti-ERK (1:1,000, #9102; CST), anti-SREBP-1 (1:1,000, sc-8984; Santa Cruz Biotechnology, Dallas, TX), anti-CHOP (1:1,000, #2895; CST), anti-FoxO3a (1:1,000, #2497; CST), anti-DUSP5 (1:1,000, ab200708; Abcam), lamin A/C (1:1,000, #2032; CST), and anti-DUSP6 (1:1,000, #50945; CST). Horseradish peroxide-conjugated secondary antibodies were obtained from Jackson Immuno Research (West Grove, PA). Coomassie brilliant blue staining was used as a control for protein loading in the quantification of secreted SeP. Bands were visualized with the ECL Prime Western Blotting Detection System (GE Healthcare, Chicago, IL) and LAS-3000 (Fujifilm, Tokyo, Japan).

Real-time PCR. Total RNA was isolated from cells using TRI Regent (Molecular Research Center, Cincinnati, OH). Isolated RNA was subject to reverse transcription using the PrimeScript RT Master Mix (Takara, Shiga, Japan). Real-time reverse transcription PCR was performed using the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) and a StepOneplus Real-Time PCR System (Thermo Fisher Scientific). The expression levels of target genes were normalized to the expression levels of RLP32. The sequences of the primers used were: human RPL32-F, CCCCTTGTGAAGCCCAAGA; human RPL32-R, TGACTGGTGCCGGATGAAC; human SeP-F, TGTG GAGCTGCCAGAGTAAAG; human SeP-R, CCACATTGCTG GGGTTGTCCTAT; human L-IST-F, GGGGAACTAGGAGCA ACAGC; human L-IST-R, AGACCTCCTTTGCTTGCATT, mouse RPL32-F, GAAACTGGCGGAAACCCA, mouse RPL32-R, GGATCTGGCCCTTGAACCTTC, mouse SeP-F, ACTCGTCA AAAGTCGTCCGT, mouse SeP-R, ACCACTGTCACTTTGCC CTC. RPL32 was used as the internal standard.

Luciferase assay. Human SeP promoter plasmids with the firefly luciferase gene were a gift from Dr. Takamura (Kanazawa University). Firefly luciferase and *Renilla* luciferase control plasmid (pRL-TK; Promega, Madison, WI) were cotransfected with FuGENE6 (Promega) according to the manufacturer's protocol. Stimulation with 2% EtOH was performed at 24 h post-transfection. At 24 h after EtOH stimulation, luciferase activities were measured using the Dual-Gro Luciferase Assay system (Promega) with a BioTek Synergy HTX microplate reader (Agilent, Santa Clara, CA).[17]

Nuclear fractionation. Cells were suspended in buffer [50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$] and subjected to repeated freeze–thaw cycles in liquid nitrogen and a water bath at 37°C. The cytosolic fractions were obtained from the supernatant after centrifugation at 1,000 × g for 10 min at

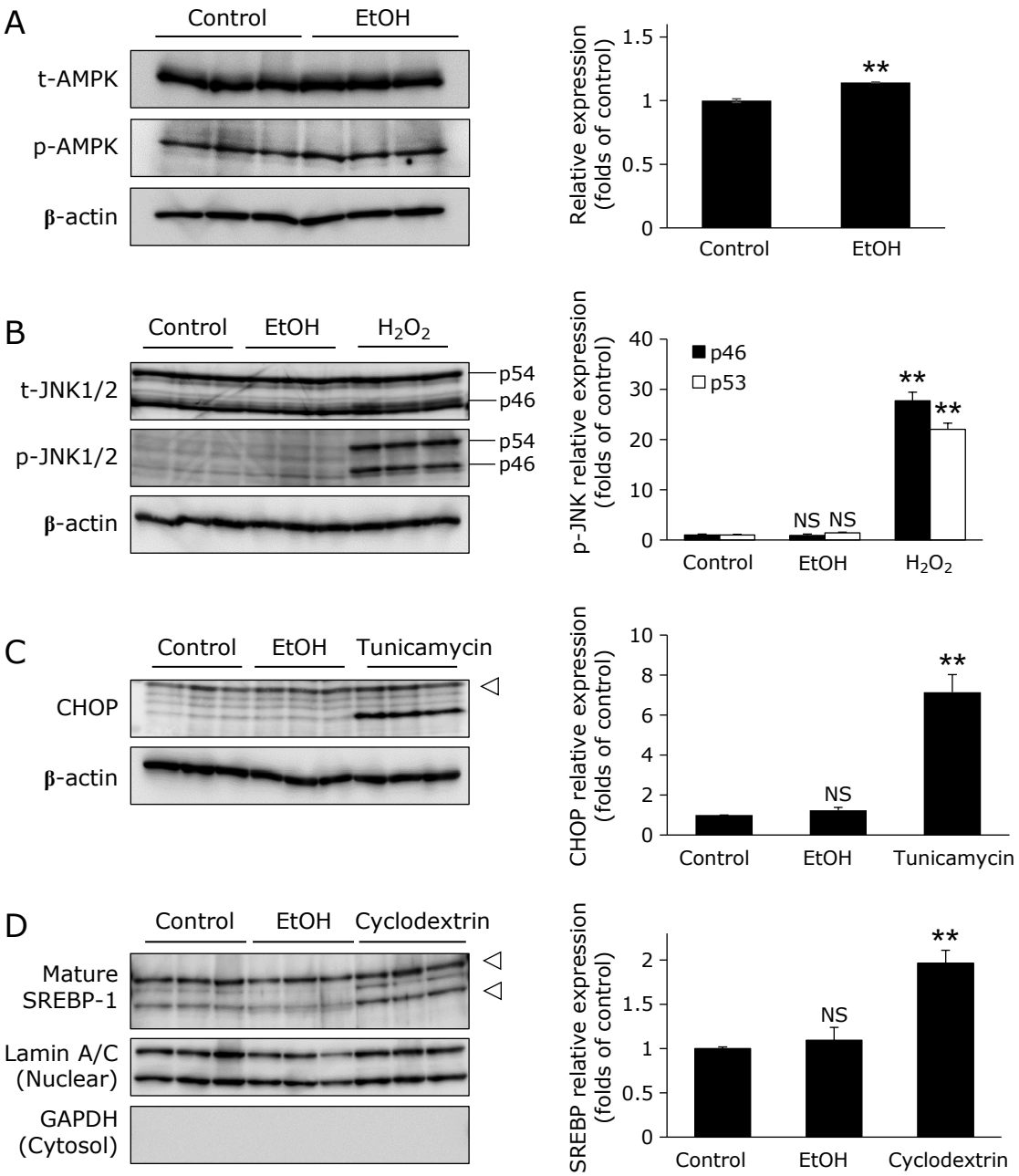

Fig. 3. AMPK, JNK, SREBP-1, and CHOP induction are not involved in the EtOH-induced increase in SeP expression. HepG2 cells were stimulated with 2% EtOH for 24 h. Phosphorylation of AMPK was increased with EtOH treatment (A). Phosphorylation of JNK and expression of CHOP showed no change (B and C). Nuclear mature SREBP-1 was not altered by EtOH treatment (D). Treatment with 100 mM H_2O_2 for 1 h (B), 2 mg/ml tunicamycin for 6 h (C), and 2 mM cyclodextrin for 24 h (D) were used as positive controls. White triangles represent nonspecific bands. NS, not significant. Data are means ± SD, $n = 3$. *p<0.05; **p<0.01 vs control by Tukey–Kramer test, ANOVA or Student's t test.

4°C. The pellet was suspended with suspension buffer containing 0.5% NP-40 and after centrifugation at 6,800 × g for 10 min at 4°C, the pellet was resuspended with RIPA buffer and used as the nuclear fraction. Protease inhibitors were added to each fraction. The distribution of each subcellular fraction was judged by protein standards such as lamin A/C (nuclear marker) and GAPDH (cytosolic marker).

Statistical analysis. Data are shown as the mean ± SD. Statistical comparisons were performed by the two-tailed unpaired Student's t test for data involving two groups or by analysis of variance for more than two groups with Tukey–Kramer multiple range comparisons. A probability value (p value) <0.05 was considered statistically significant. Statistical analyses were performed using Excel (Microsoft, Redmond, WA) and SPSS (IBM SPSS, Armonk, NY) software.

Results

EtOH increased SeP protein expression in a time- and concentration-dependent manner. The expression of SeP in HepG2 cells by exposure to EtOH was increased by treatment with between 0.1% and 2% EtOH for 24 h (Fig. 1A). SeP protein expression induced by 2% EtOH increased in a time-dependent manner to 48 h (Fig. 1B). Secretion of SeP from EtOH-stimulated

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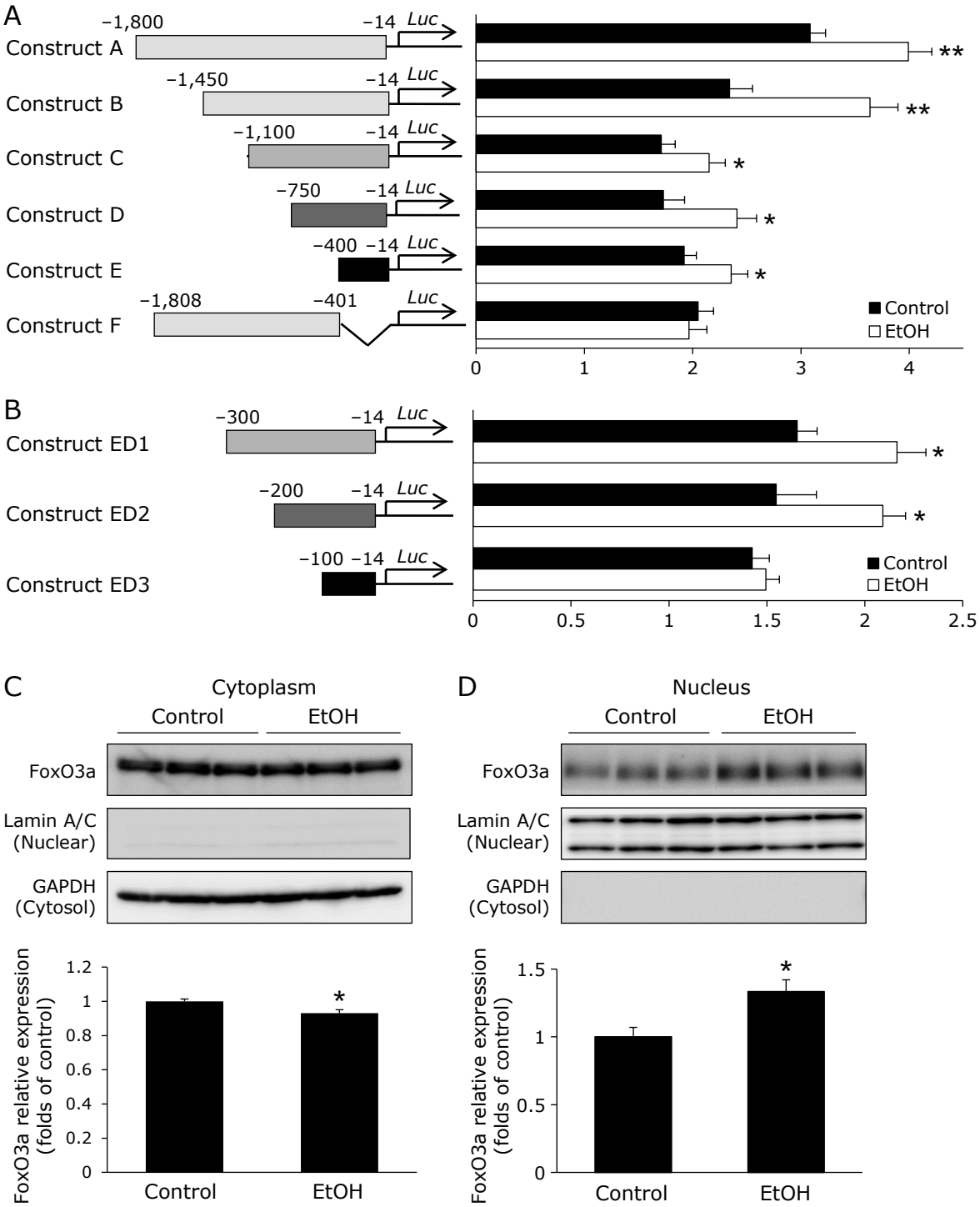

Fig. 4. Promoter activity of SeP is activated by EtOH. Nine deletion mutants of the SeP promoter were constructed to determine the nature of the EtOH-response region in the SeP promoter sequence. HepG2 cells were cotransfected with each reporter plasmid and control reporter plasmid for 24 h and then treated with 2% EtOH for 24 h. Signals were normalized by the control reporter plasmid. Promoter activity of Constructs A to E and Construct EΔ1 and EΔ2 were increased by EtOH treatment. In contrast, Constructs F and EΔ3 were unaffected (A and B). FoxO3a in both nucleus and cytosol was evaluated with Western blotting. Nuclear FoxO3a was increased and cytosolic FoxO3a was decreased in EtOH-treated HepG2 cells (C and E). Data are means ± SD, $n = 3$. *p<0.05; **p<0.01 vs control by Student's t test.

HepG2 cells increased with increasing intracellular proteins levels (Fig. 1C). Additionally, we studied the effects of metabolites of EtOH, acetaldehyde and acetic acid on SeP expression and found that these metabolites caused decrease in SeP levels after 24 h treatment of HepG2 cells (Fig. 1D and E). These results indicated that SeP expression in HepG2 cells was induced by EtOH but not by acetaldehyde or acetic acid.

EtOH increased SeP mRNA transcription. Expression levels of SeP mRNA and of L-IST (noncoding RNA that specifically inhibits SeP mRNA translation)[20] were analyzed in HepG2

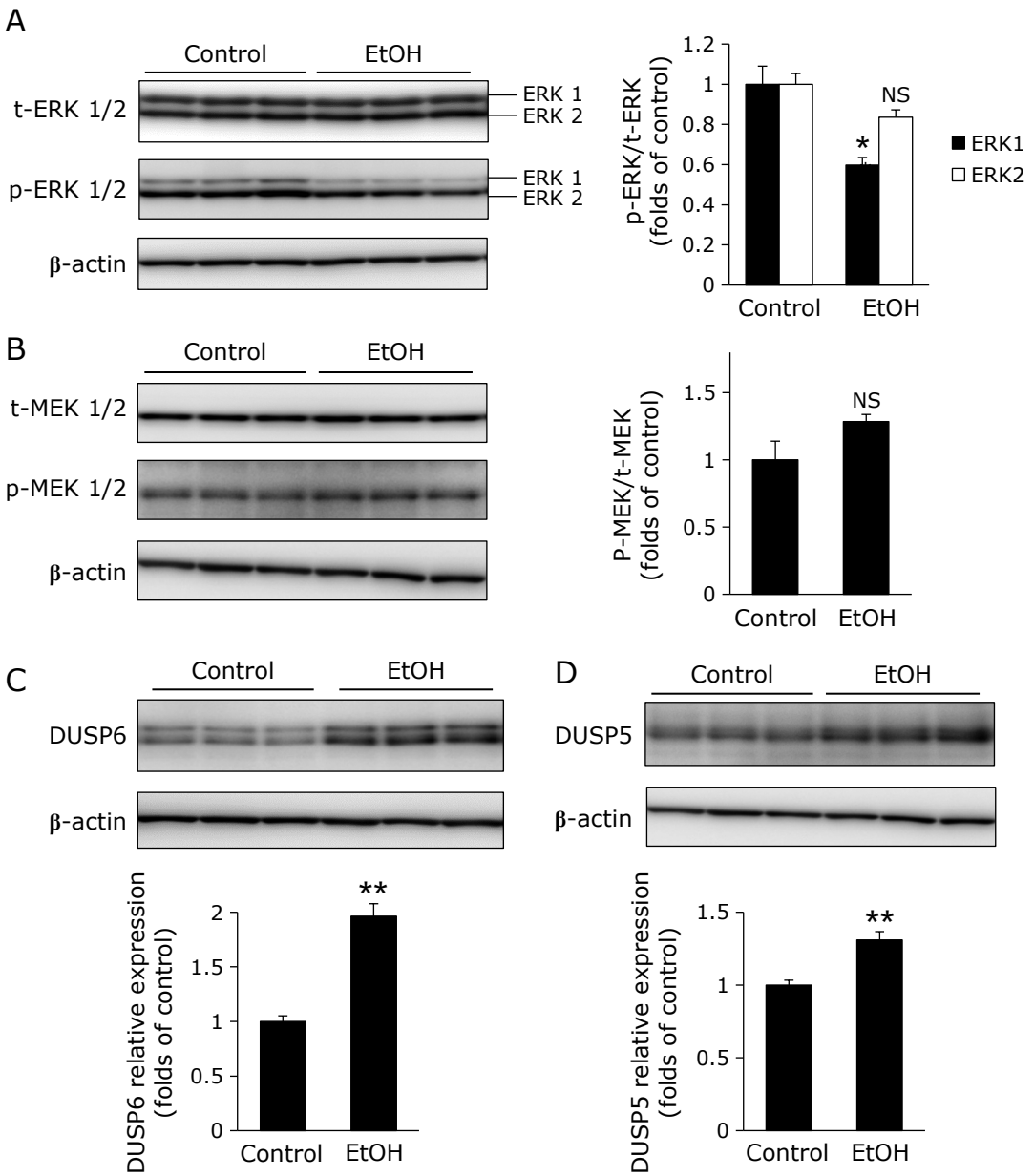

Fig. 5. EtOH activates FoxO3a by inhibiting ERK phosphorylation. HepG2 cells were treated with 2% EtOH for 24 h. (A) Phosphorylation of ERK1 was increased but not phosphorylation of ERK2 (A). Phosphorylation of MEK was unchanged (B). DUSP5 and DUSP6 were increased by EtOH treatment (C and D). Data are means ± SD, $n = 3$. NS, not significant; *$p<0.05$; **$p<0.01$ vs control by Student's t test.

cells. SeP mRNA levels were increased by EtOH stimulation in a time- and concentration- dependent manner (Fig. 2A and B). In addition, EtOH also increased SeP mRNA expression in other liver-derived cell lines such as HC-4 cells, LI-7 cells, and Hepa1-6 cells (Fig. 2C). However, the expression levels of L-IST were not changed by time or EtOH concentration (Fig. 2D and E).

EtOH-induced increase in SeP translation was not associated with of AMPK, JNK and CHOP, or SREBP-1. AMPK, JNK, SREBP-1, and CHOP regulate SeP mRNA transcription.[13] We investigated the association between these factors and the expression of SeP mRNA by EtOH stimulation. Phosphorylation of AMPK was increased in HepG2 cells stimulated with 2% EtOH for 24 h (Fig. 3A). However, activation of AMPK by metformin suppressed SeP transcription in H4IIEC3 rat hepatocytes and HepG2 cells. Therefore, the phosphorylated AMPK increased by EtOH is not likely to be related to expression of SeP by EtOH. Phosphorylation of JNK was increased by H_2O_2 treatment but not by EtOH stimulation (Fig. 3B). Similarly, the expression of CHOP was increased by treatment with tunicamycin, a CHOP inducer, but not by EtOH treatment (Fig. 3C). The mature form of SREBP-1 translocates from the cytoplasm to the nucleus and activates the transcription of fatty acid metabolism genes. The activated form of SREBP-1 in the nucleus was increased by treatment with 2 mM cyclodextrin but not by EtOH treatment (Fig. 3D).

FoxO3a was associated with EtOH-induced SeP expression. A luciferase assay was performed to identify the EtOH-response sequence regulating EtOH-induced expression of SeP. The enhancement of promoter activity of construct A through to construct E but not construct F was observed after EtOH treatment (Fig. 4A), indicating that the EtOH-response sequence of the SeP promoter lies in construct E. Next, we constructed

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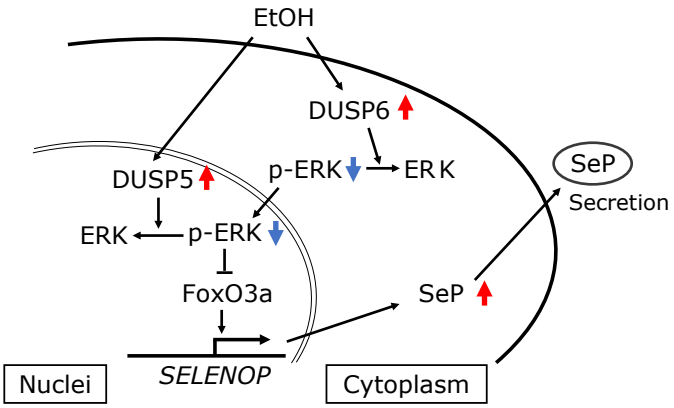

Fig. 6. Scheme of SeP expression by EtOH in the HepG2 cells. ERK induce FoxO3a phosphorylation and translocation from the nucleus to the cytoplasm. EtOH reduces ERK phosphorylation by increasing DUSP5/6, thereby suppressing the phosphorylation activity of ERK to FoxO3a and thus enhancing SeP expression.

additional deletion mutants of construct E, named constructs EΔ1 to EΔ3. Constructs EΔ1 and EΔ2 were increased by EtOH treatment (Fig. 4B). In contrast, construct EΔ3 was not increased by EtOH (Fig. 4B), indicating that the EtOH-response sequence is localized in the 100 bp to 200 bp region in the SeP promoter. Takayama *et al.*[17] reported that the 100 bp to 200 bp region has a FoxO3a binding sequence. Therefore, we investigated the involvement of FoxO3a in EtOH-induced SeP expression. Because an increase in nuclear FoxO3a levels enhances transcription of SeP mRNA,[17] we examined the protein levels of FoxO3a in the cytosolic and nuclear fractions. Cytosolic FoxO3a decreased with EtOH stimulation (Fig. 4C) whereas nuclear FoxO3a increased (Fig. 4D).

FoxO3a activity was increased by DSUP5 and DUSP6. FoxO3a is translocated from the nucleus to the cytoplasm when it is phosphorylated by phosphorylated ERK.[21] Therefore, we investigated phosphorylation of ERK1 and ERK2. In EtOH-stimulated HepG2 cells, phosphorylation of ERK1 decreased, but phosphorylation of ERK2 was unchanged (Fig. 5A). Next, we determined the activity of MEK, which phosphorylates ERK1. In HepG2 cells treated with 2% EtOH, phosphorylation was not increased (Fig. 5B). Finally, we analyzed dual-specificity phosphatase 5 (DUSP5) and DUSP6, which are phosphatases of ERK1. Expression of both phosphatases was increased in HepG2 cells treated with 2% EtOH (Fig. 5C and E).

Discussion

We have shown that EtOH increases the expression of SeP in HepG2 cells derived from liver. The increase in SeP levels is associated with the DUSP5/6–ERK–FoxO3a signal rather than the previously reported AMPK–FoxO3a pathway (Fig. 6).

FoxO3a has been shown to be a major transcription factor for SeP expression. Takayama *et al.*[17] reported that metformin inhibits SeP mRNA transcription by phosphorylation of FoxO3a, and transfers to the cytosol via activation of AMPK. In the present study, it was shown that EtOH stimulation increases the amount of nuclear FoxO3a, resulting in activation of the transcriptional SeP promoter (Fig. 4A). Previous studies have reported that FoxO3a is phosphorylated by upstream signals such as phosphorylated JNK and ERK as well as phosphorylated AMPK.[22] In this study, EtOH induced a significant increase in pAMPK (Fig. 3A). Increased phosphorylation of AMPK suppresses the expression of SeP; therefore, increase of pAMPK is not associated with the increase in SeP by EtOH treatment.[17] We also found that EtOH treatment decreased ERK1 phosphorylation but not ERK2 phosphorylation (Fig. 5A). EtOH reduces phosphorylation of ERK1 in the brain of EtOH-treated rats[23] and inhibits differentiation of human embryonic stem cells to hepatocytes by suppressing ERK activity.[24] These findings and our results suggest that EtOH treatment inhibits ERK1 activity in a variety of cells.

The activity of ERK is enhanced by phosphorylation by MEK and is inhibited by dephosphorylation by two phosphatases, DUSP6 in the cytosol and DUSP5 in the nucleus.[25–27] Phosphorylated ERK translocates into the nucleus and promotes the transcription of target genes.[28] Nuclear ERK acts as not only a direct regulator of transcription by forming complexes with ELK1 and PHF8,[29] but also as a signaling factor to phosphorylate FoxO3a.[21] We observed increased expression of DUSP5 and DUSP6 in HepG2 cells stimulated with EtOH (Fig. 5C and D). In contrast, EtOH treatment did not decrease the level of phosphorylated MEK (Fig. 5B). Thus, the DUSP5/6–ERK1–FoxO3a pathway, but not the MEK–ERK–FoxO3a pathway, may be involved in the EtOH-induced transcriptional enhancement of SeP mRNA. Previous reports have shown that EtOH causes DNA damage and increases p53.[30] Since p53 is known to enhance transcription of DUSP6,[31] the p53-DUSP6 pathway may be involved in EtOH induced DUSP6 expression. However, DUSP6 expression is reduced in the cerebral cortex of rats exposed to EtOH vapor for 7 weeks.[32] Therefore, the effect of long-term EtOH consumption on activation of DUSP6–ERK in the liver must be examined in detail.

Alcohol intake is one of the major risk factors for the development of diabetes mellitus. People with high activity of ALDH2, which metabolizes acetaldehyde to acetic acid, experience less discomfort after drinking than those with low ALDH2 activity.[33] Therefore, people with high ALDH2 activity tend to drink more and are at a higher risk of developing diabetes mellitus.[34] In addition, the risk of developing diabetes increases in relation to the amount of alcohol consumption for lean people, such as Japanese adults with a body mass index (BMI) <22.[35] Interestingly, the relationship between the increased risk of diabetes development and alcohol consumption is not observed for more obese people with a BMI >25.[35] Thus, the influence of risk factors for diabetes mellitus such as obesity may be greater than EtOH-induced SeP expression at high BMI. Thus, an SeP increase induced by EtOH consumption may be involved in the increased risk of developing diabetes in leaner populations.

We have demonstrated that EtOH treatment increases SeP expression in HepG2 cells. EtOH treatment activates FoxO3a and promotes SeP transcription by suppressing ERK1. Suppression of ERK activity is associated with an increase in DUSP5/6 protein levels, not increased MEK activity (Fig. 6). These results suggest that the DUSP5/6–ERK–FoxO3a pathway may be involved in the mechanism of diabetes development induced by alcohol consumption. DUSP5/6–ERK–FoxO3a signaling may be a new therapeutic target for SeP-related diseases, such as diabetes induced by high alcohol intake.

Author Contributions

JC and YM designed the study. JC performed the experiments, and JC and NN analyzed the data. YM wrote the initial draft of the manuscript. JC and NN critically reviewed the manuscript. All authors read and approved the final version and agreed to submit it to Journal of Clinical Biochemistry and Nutrition.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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