Forkhead box protein O1 (FoxO1) knockdown accelerates the eicosapentaenoic acid (EPA)-mediated Selenop downregulation independently of sterol regulatory element-binding protein-1c (SREBP-1c) in H4IIIEC3 hepatocytes

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Abstract. Selenoprotein P is upregulated in type 2 diabetes, causing insulin and exercise resistance. We have previously reported that eicosapentaenoic acid (EPA) negatively regulates Selenop expression by suppressing Srebf1 in H4IIIEC3 hepatocytes. However, EPA downregulated Srebf1 long before downregulating Selenop. Here, we report additional novel mechanisms for the Selenop gene regulation by EPA. EPA upregulated Foxo1 mRNA expression, which was canceled with the ERK1/2 inhibitor, but not with the PKA inhibitor. Foxo1 knockdown by siRNA initiated early suppression of Selenop, but not Srebf1, by EPA. However, EPA did not affect the nuclear translocation of the FoxO1 protein. Neither ERK1/2 nor PKA inhibitor affected FoxO1 nuclear translocation. In summary, FoxO1 knockdown accelerates the EPA-mediated Selenop downregulation independent of SREBP-1c in hepatocytes. EPA upregulates Foxo1 mRNA via the ERK1/2 pathway without altering its protein and nuclear translocation. These findings suggest redundant and conflicting transcriptional networks in the lipid-induced redox regulation.

Key words: Hepatocyte, Insulin resistance, Polyunsaturated fatty acid (PUFA), Selenoprotein P, Eicosapentaenoic acid (EPA)

SELENOPROTEIN P (SeP; ENCODED BY SELENOP IN HUMANS, Selenop IN RODENTS) is a secretory protein almost completely derived from the liver, termed hepatokine [1, 2]. SeP has an antioxidant effect through its intrinsic thioredoxin domain and by distributing selenium to intracellular glutathione peroxidases, glutathione peroxidase (Gpx) 1 and 4 [3, 4]. We rediscovered SeP as a hepatokine that is overproduced in type 2 diabetes, causing insulin [5] and exercise resistance [6]. Further, SeP contributes to impaired angiogenesis [7], hypoadiponectinemia [8], insulin secretory failure [9], and exacerbation of myocardial infarction [10]; all complications of diabetes and aging-related diseases. Therefore, SeP may be a potential therapeutic target against diabetes and aging.

EPA is widely used in clinical settings as a therapeutic drug for hypertriglyceridemia. In clinical trials, EPA
suppresses the onset of cardiovascular events in Japanese hypercholesterolemic patients [11, 12]. EPA is expected to be used clinically as a therapeutic agent for diabetes due to its anti-inflammatory effect. However, the effect of EPA on insulin resistance remains controversial [13]. Previously, we reported that EPA suppresses the cleavage, nuclear translocation, and promoter DNA binding of the sterol regulatory element-binding protein-1c (SREBP-1c), a master transcription factor regulating fatty acid synthesis, and thereby downregulating Selenop gene expression [14]. However, in that study, EPA downregulated Srebf1 long before downregulating Selenop, i.e., Selenop was suppressed from 12 hours after EPA treatment, whereas its transcription factor Srebf1 was suppressed from 3 hours after EPA treatment. Therefore, the start time of the downregulation of these two mRNAs is very different. We hypothesized that an unknown Selenop regulatory mechanism by EPA antagonizes SREBP-1c-mediated Selenop downregulation.

In addition to the SREBP-1c, the SELENOP promoter contains various transcription factor binding sites such as forkhead box proteins O1 (FoxO1), and FoxO3a [14-17]. FoxO is the transcription factor belonging to the forkhead box protein class O of the forkhead families. FoxO1 regulates systemic glucose metabolism by regulating gluconeogenic genes such as phosphoenolpyruvate carboxykinase (PEPCK) and the glucose 6 phosphatase (G6Pase) [18]. The transcriptional activity of FoxO1 is regulated by various post-translational modifications such as phosphorylation [19], acetylation [20, 21], ubiquitination [22], and methylation [23], affecting its intracellular localization and DNA binding affinity. Insulin phosphorylates and inactivates FoxO1 by exporting it from the nucleus to the cytoplasm [24]. Previously, we reported that the first-line anti-diabetic agent metformin downregulates Selenop expression through FoxO3a inactivation in H4IIEC3 hepatocytes [16]. Furthermore, Selenop gene expression is negatively regulated by insulin via inactivating FoxO1 [17]. Therefore, we speculated that FoxO might antagonize the suppression of SREBP-1c by EPA. We investigate in the present study the mechanisms underlying the regulation of Selenop gene expression by EPA in hepatocytes to prove this hypothesis. We demonstrate that EPA oppositely regulates Selenop expression via Foxo1 mRNA upregulation and SREBP-1c inhibition.

Results

Polyunsaturated fatty acid suppresses Selenop gene expression

First, we examined the effect of polyunsaturated fatty acids (PUFAs) on Selenop gene expression in rat hepatoma cell line H4IIEC3 cells. Consistent with our previous report [14], EPA (C20:5 n-3), docosahexaenoic acid (DHA, C22:6 n-3), and arachidonic acid (AA, C20:4 n-6) suppressed the Selenop mRNA expression at a concentration of 0.25 mM. Of note, only EPA suppressed Selenop gene expression at a concentration as low as 0.1 mM (Fig. 1A). Similarly, EPA, DHA, and AA suppressed Srebf1 and its target Fasn encoding fatty acid synthase (Fig. 1B and C). Since Selenop is upregulated by FoxO1 [15] and FoxO3a [16], we examined the Foxo1 and Foxo3a gene expression. Contrary to Srebf1 and Fasn, EPA, DHA, and AA upregulated Foxo1, but not Foxo3a (Fig. 1D and E). DHA and AA tended to upregulate Foxo1 expression more than EPA (Fig. 1D). EPA downregulated Selenop expression most potently compared to DHA and AA (Fig. 1A). Therefore, we focus on the modulation of Selenop by EPA in the following study. These results suggest that EPA negatively regulates Srebfp1 while it positively regulates Foxo1; as such, it exerts opposite action on Selenop expression in hepatocytes.

Foxo1 upregulates Selenop gene expression

We performed a knockdown experiment using siRNA to investigate whether Foxo1 antagonizes the EPA-induced Selenop suppression. Foxo1 siRNAs #1 and #2 efficiently downregulated both Foxo1 protein and Foxo1 mRNA in hepatocytes (Fig. 2A and B). EPA downregulated Selenop expression by 21% of the vehicle after 24 hours (Fig. 2C left). Foxo1 knockdown enhanced the EPA-mediated downregulation of Selenop up to 73–77% of the vehicle (Fig. 2C middle and right). Foxo1 knockdown did not affect Srebf1 expression (Fig. 2D). These results indicate that Foxo1 upregulates Selenop expression during EPA treatment.

EPA-induced Foxo1 upregulation is canceled with the ERK1/2 inhibitor

We examined the effects of the signal inhibitors on EPA-induced gene expression of Foxo1, Srebfp1, and Selenop to determine the upstream signaling of EPA-induced Foxo1 upregulation. EPA regulates glycolipid metabolism via a G protein-coupled receptor 120 (GPR120) [25, 26]. Therefore, we examined the interaction of GPR120-mediated signaling. However, there was no mRNA expression of GPR120, 119, or 30, which are EPA receptors, in H4IIEC3 hepatocytes (data not shown). Despite the absence of GPR120 expression, the EPA-induced upregulation of Foxo1 was further enhanced with the GPR120 inhibitor AH77614 without affecting Selenop and Srebfp1 expression (Fig. 3A). Extracellular signal-regulated kinase (ERK1/2) and protein kinase A (PKA) are the downstream effector kinases
Fig. 1  EPA, DHA, and AA suppressed Selenop gene expression in H4IIEC3 hepatocytes

A: Selenop, B: Srebf1, and C: Fasn gene expression was downregulated, and D: Foxo1 gene expression, but not E: Foxo3a was upregulated at 0.1 mM concentration EPA-treated for 24 hours in H4IIEC3 hepatocytes. Each gene expression was assessed by real-time PCR. Expression values were normalized to 18S rRNA. Data represent mean ± S.D. (error bars) (n = 3). Extreme outliers were excluded. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus vehicle-treated cells.
Fig. 2  EPA upregulated Selenop gene expression via FoxO1

H4IIEC3 hepatocytes were transfected with Foxo1 siRNAs or negative control (N.C.) siRNA for 48 hours. A: Intracellular localization of FoxO1 was assessed by Western blotting. Expression of mRNAs (B, Foxo1; C, Selenop; D, Srebf1) were assessed by a real-time PCR. Cells were treated with the 0.25 mM EPA for the indicated times. Expression values were normalized to Actb mRNA or 18S rRNA. Data represent mean ± S.D. (error bars) (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus vehicle-treated cells.
of the EPA receptor GPR120 [25, 27]. ERK1/2 and PKAc regulate FoxO1 positively and negatively through regulating its activity and mRNA expression [21, 28-32]. Therefore, we next examined whether ERK1/2 and PKAc signaling mediate the EPA-induced Foxo1 upregulation. The EPA-induced upregulation of Foxo1 was canceled with the ERK1/2 inhibitor FR180204 (Fig. 3B right) but not with the PKA inhibitor H-89 (Fig. 3C right), suggesting that EPA upregulates Foxo1 mRNA via the ERK1/2 pathway. Consistent with Foxo1, EPA-induced upregulation of Selenop was canceled with the ERK1/2 inhibitor FR180204 (Fig. 3B left), but not with...
the PKA inhibitor H-89 (Fig. 3C left).

**EPA does not activate FoxO1 protein**

We examined the time-dependent effect of EPA on FoxO1 protein nuclear translocation to determine whether EPA activates FoxO1 protein. The results were not what we expected. Unlike the mRNA upregulation, EPA did not elevate the nuclear translocation of FoxO1 (Fig. 4A). Consistent with this, treatment with an inhibitor of GPR120 (AH7614) did not alter the AMP-activated protein kinase (AMPK), ERK1/2, PKAc, and cAMP response element binding protein (CREB) phosphorylation of downstream signals of GPR120, respectively (Fig. 4B–E).

**ERK1/2 inhibitor does not affect EPA-induced FoxO1 protein activation**

EPA upregulates Selenop via ERK1/2-Foxo1 mRNA pathway. We next examined whether ERK1/2 signaling mediates EPA-induced FoxO1 protein activation. An ERK1/2 inhibitor FR180204 sufficiently inhibited phosphorylation of ERK1/2 only with 0 hour of EPA.
PKA inhibitor does not affect EPA-induced FoxO1 protein activation

Next, we examined whether PKAc signaling mediates EPA-induced FoxO1 activation. A PKA inhibitor, H-89, slightly inhibited phosphorylation of PKAc (Fig. 6A), but not its downstream target CREB (Fig. 6B). H-89 slightly activated ERK1/2 after 24 hours of EPA treatment (Fig. 6C). However, H-89 did not affect EPA-induced FoxO1 nuclear translocation (Fig. 6D).

Discussion

The present study demonstrates that EPA regulates Selenop gene expression via FoxO1 mRNA upregulation. Unexpectedly, EPA does not elevate protein levels and nuclear translocation of FoxO1. However, FoxO1 knock-down significantly promotes EPA-induced Selenop
suppression, suggesting Foxo1 involvement in Selenop upregulation. Based on these findings, we conclude that EPA-induced Foxo1 mRNA upregulation is indirectly involved in Selenop expression. We speculate that EPA may downregulate FoxO1 protein, which was antagonized by upregulating Foxo1 mRNA. Therefore, suppressing the EPA-induced Foxo1 mRNA upregulation might be a more potent way to downregulate Selenop expression. Based on the previous [14] and present study, we consider that the balance between SREBP-1c suppression and Foxo1 upregulation may determine fatty acid-mediated regulation of Selenop expression. EPA upregulates Selenop by upregulating Foxo1 mRNA and downregulates Selenop by inactivating SREBP-1c.

Among the PUFAs, EPA downregulates Selenop expression more than DHA and AA (Fig. 1A). Interestingly, DHA and AA tended to upregulate Foxo1 expression more (Fig. 1D) and downregulate Srebf1 less (Fig. 1B) than EPA, which may be why EPA most effectively suppresses Selenop. The underlying mechanism of how DHA and AA regulate Selenop expression is unknown. This finding provides new insights that the regulation of

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**Fig. 6** PKAc did not involve in EPA-mediated FoxO1 activation

A–C: H4IEEC3 hepatocytes were pre-treated with 10 μM H-89 for 20 minutes before treatment with 0.25 mM EPA. Proteins were extracted after indicated times of EPA treatment. Each protein level was then assessed by Western blotting. D: Intracellular localization of FoxO1 in H4IEEC3 hepatocytes treated with 10 μM H-89 for 20 minutes before treatment with 0.25 mM EPA. Proteins were extracted after indicated times of EPA treatment. Data represent mean ± S.E. (error bars) (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus vehicle-treated cells.
Selenop by DHA and AA, as in the EPA, may be determined by the balance between suppression of Srebf1 and upregulation of Foxo1.

ERK regulates FoxO1 activity both positively and negatively. ERK signaling inactivates FoxO1 via acetylation and phosphorylation [21, 28, 29]. Conversely, EPA upregulates Foxo1 through ERK phosphorylation and subsequent AMPK phosphorylation in the human endothelial stromal cells [30]. In the present study, the ERK1/2 inhibitor FR180204 canceled the EPA-induced Foxo1 mRNA without affecting protein levels and nuclear translocation of Foxo1 (Fig. 5D). These findings suggest that ERK1/2 is partly involved in the EPA-mediated Foxo1 regulation.

PKA also regulates Foxo1 both positively and negatively. PKA activates CREB and thereby upregulates Foxo1 in hepatocytes [31, 32]. In contrast, in vascular endothelial cells, PKA directly phosphorylates and inactivates Foxo1 [33]. Phosphorylated Foxo1 is excluded from the nucleus, ubiquitinated, and degraded by the proteasome [22]. PKA activates the proteasome by phosphorylating Rpt6, the ATPase in the 19Scap [34, 35]. Thus, PKA may inactivate Foxo1 via proteasome activation. The PKA inhibitor H-89 did not affect the EPA-induced Foxo1 protein activation in the present study (Fig. 6D). These findings suggest that PKAc is not involved in the EPA action on Selenop expression.

To date, whether ω-3 fatty acids activate or inactivate Foxo1 remains controversial. Ingestion of ω-3 fatty acids increases FOXO1 gene expression in peripheral blood mononuclear cells of patients with coronary artery disease [36], whereas EPA reduces the hepatic Foxo1 level in fa/fa Zucker rats [37]. Since various post-translational modifications regulate Foxo1 activity, the phosphatase PPA2 dephosphorylates Foxo1 and induces its nuclear translocation [38]. Another ω-3 PUFA, DHA activates PPA2 via GPR120 [39]. EPA and DHA upregulate a histone deacetylase SIRT1 gene expression and increase its activity [40]. SIRT1 deacetylates Foxo1 and thereby activates the transcriptional activity of Foxo1 [41]. In the present study, EPA upregulates Foxo1 mRNA (Fig. 1D); however, unexpectedly, it did not affect the protein level and nuclear translocation of Foxo1 (Fig. 4A). We interpret these findings that EPA may downregulate Foxo1 protein but keep its protein levels and nuclear translocation by upregulating Foxo1 mRNA. This might be the novel action of EPA on Foxo1 regulation.

In summary, Foxo1 knockdown accelerates the EPA-mediated Selenop downregulation independently of SREBP-1c in hepatocytes. EPA upregulates Foxo1 mRNA via the ERK1/2 pathway without altering its protein and nuclear translocation. The present study also suggests the yet unknown EPA signaling pathways independent of GPR120, ERK1/2, and PKAc leading to Foxo1 expression. These findings deepen our understanding of previously unrecognized redundant and conflicting transcriptional networks in lipid-induced redox regulation. By understanding the complex signaling, we can efficiently downregulate the hepatic expression of Selenop by suppressing Foxo1 expression to overcome insulin and exercise resistance.

**Experimental Procedures**

**Materials**

The antibodies against Foxo1 (C29H4), phospho-p44/42 MAPK (Erk1/2), p44/42 MAPK (Erk1/2), phospho-PKA C (Thr197), PKA C, phospho-AMPKα (Thr172), AMPKα, phospho-CREB (Ser133), CREB, HDAC2, and β-Actin were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against GAPDH was purchased from Abcam. Cis-5,8,11,14,17-EPA sodium salt and albumin from bovine serum lyophilized powder (essentially fatty acid-free; FFA-free BSA) were purchased from Sigma-Aldrich. Methyl-N-9H-xanthen-9-yl-benzenesulfonamide (AH7614) and 5-(2-Phenyl-pyrazolo[1,5-a]pyridine-3-yl)-1H-pyrazolo (3,4-c)pyridazin-3-ylamine (FR180204) were purchased from TOCRIS bioscience (Bristol, UK). N-[2-[[3-(4-bromophenyl)-2-propen-1-yl]amino]ethyl]5-isoquinolinesulfonamide, dihydrochloride (H-89) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

**Cell culture**

Rat hepatoma cell line H4IIEC3 was obtained from American Type Culture Collection (Manassas, VA). Cells were cultured with high glucose Dulbecco modified Eagle medium (DMEM; Invitrogen) containing 10% fetal bovine serum (Invitrogen), 2 mmol/litter of L-glutamine (Wako Pure Chemical Industries, Ltd.), 100 units/mL of penicillin, and 0.1 g/mL streptomycin (Wako Pure Chemical Industries, Ltd.). The cells were maintained at 37°C in a humidified incubator with 5% CO2. Cells were treated with the indicated concentrations of EPA, DHA, AA, and arachidic acid in the presence of 2% FFA-free BSA (Sigma). EPA, DHA, and AA were dissolved in 99.5% ethanol (final ethanol concentration was 0.25%). Arachidic acid was dissolved in 99.0% chloroform (final chloroform concentration was 0.25%). Each inhibitor was dissolved in dimethyl sulfoxide (DMSO) (final DMSO concentration was 0.01%). The medium containing each fatty acid was incubated at 45°C for 5 minutes.
Western blot
Treated cells were collected and lysed, as described previously [42]. Cell lysis buffer contains RIPA Lysis Buffer (MILLIPORE), complete Mini EDTA-free (Roche), and PhosSTOP (Roche) cocktail was added to H4IIEC3 hepatocytes, and the cells were collected. The cells were pulverized with ultrasonic waves, centrifuged at 14,500 rpm at 4°C for 30 minutes, and the supernatant was collected. Nuclear extraction was performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (ThermoFisher) according to the manufacturer’s protocol. Protein samples were applied to SDS-PAGE and transferred onto PVDF membranes using the iBlot Gel Transfer system (Invitrogen). The membranes were blocked using 5% skim milk or PVDF Blocking Reagent for Can Get Signal (Toyobo Co., Ltd., Osaka, Japan) for 1 hour, immunoblotted with primary antibody overnight, washed three times, and incubated with the secondary antibody labeled by HRP for 1 hour. Bands were visualized with the ECL Prime Western blotting Detection System (G.E. Healthcare) and ChemiDoc Touch Imaging System (Bio-Rad). Quantification by densitometric analysis of blotted membranes was performed using Image Lab software version 5.2.

Quantitative real-time PCR
Total RNA was extracted from cultured H4IIEC3 hepatocytes using High Pure RNA Isolation Kit (Roche Diagnostics) or ISOSPIN Cell & Tissue RNA (NIPPON GENE) according to each manufacturer’s protocol. The reverse transcription of 100 ng of total RNA was carried out using a high-capacity cDNA reverse transcription kit (Invitrogen), according to the manufacturer’s protocol. Quantitative real-time PCR was performed using TaqMan probes (Actb, 4352340E; 18S rRNA, 4318839; Foxo1, Rn01494868_m1; Selenop, Rn00569905_m1; Srebf1, Rn01495769_m1; Fasn, Rn01463550_m1) and the 7900HT Fast Real-Time PCR System (Life Technologies). The PCR conditions were set for one cycle at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute.

siRNA Transfection in H4IIEC3 Hepatocytes
H4IIEC3 hepatocytes were grown transiently transfected with 50 nM small interfering RNA (siRNA) duplex oligonucleotides using 1 μL of LipofectamineTM RNAiMAX (Life Technologies) by the reverse-transfection method according to the manufacturer’s instructions. siGENOME siRNA Reagents D-088495-01 and D-088495-03 were used for FoxO1-specific siRNAs. Med GC12935300 (Thermo Scientific) was used for the negative control siRNA. Twenty-four hours after transfection, the cells were followed by the extraction of total RNA.

Statistical analysis
All data were analyzed using the Japanese Windows Edition of Statistical Package for Social Science (SPSS) or GraphPad Prism version 8. One-way ANOVA tested significance with post-hoc analysis by the Bonferroni method. Differences with a p value of less than 0.05 were considered statistically significant.

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Conflict of Interest
There are no conflicts of interest to declare.

Author Contributions
K.K., K-A.I., and T.T. designed the research, analyzed the data, and wrote the manuscript. K.K., N.T-S., T.S., H.A., T.A., H.K.O., X.Y., Q.L., and C.M.G-M. performed the experiments. K.K., K-A.I., H.T., S.K., and T.T. interpreted the results of the experiments.

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