CsA downregulates Selenop via a STAT3-FoxO1 axis

Cyclosporine A downregulates selenoprotein P expression via a STAT3-FoxO1 pathway in hepatocytes in vitro

Xingyu Yao1*, Hiroaki Takayama1,2*, Kyoko Kamoshita1, Hein Ko Oo1, Ryota Tanda1, Kaisei Kato1, Kiyo-Aki Ishii3, and Toshinari Takamura1

1 Department of Endocrinology and Metabolism, Kanazawa University Graduate School of Medical Sciences
2 Life Sciences Division, Engineering and Technology Department, Kanazawa University
3 Department of Integrative Medicine for Longevity, Graduate School of Medical Sciences, Kanazawa University

* These authors contributed equally to this work
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Running title: CsA downregulates Selenop expression via a STAT3-FoxO1 axis

Corresponding author: Toshinari Takamura, Department of Endocrinology and Metabolism, Kanazawa University Graduate School of Medical Sciences, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan. Tel.: 81-76-265-2711; Fax: 81-76-234-4214; E-mail: ttakamura@med.kanazawa-u.ac.jp

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Abbreviation: forkhead box protein O1 (FoxO1); signal transducer and activator of transcription 3 (STAT3); reactive oxygen species (ROS); sterol regulatory element-binding transcription factor 1c (SREBP-1c); hepatocyte nuclear factor 4 alpha (HNF4A); retinoic-acid-inducible gene I (RIG-I)
Cyclosporine A (CsA) is a worldwide applied immunosuppressant for preventing graft rejection and autoimmune diseases. However, CsA elevates oxidative stress leading to liver injuries. The present study aimed to clarify the mechanisms underlying the CsA-mediated oxidative stress. Among the redox proteins, CsA concentration-dependently downregulated *Selenop* encoding selenoprotein P (SeP), a major circulating antioxidant protein reducing reactive oxygen species (ROS), in hepatocytes cell lines and primary hepatocytes. The luciferase assay identified the CsA-responsive element in the *SELENOP* promoter containing a putative binding site for FoxO1. The CsA-mediated suppression on the *SELENOP* promoter was independent of NFAT, a classic target repressed by CsA. A ChIP assay showed that CsA suppressed the FoxO1 binding to the *SELENOP* promoter. *Foxo1* knockdown significantly downregulated *Selenop* expression in H4IIEC3 cells. Furthermore, CsA downregulated FoxO1 by inactivating its upstream signal transducer and activator of transcription 3 (STAT3). Knockdown of *Stat3* downregulated *Foxo1* and *Selenop* expression in hepatocytes. These findings revealed a novel mechanism underlying CsA-induced oxidative stress via downregulating the STAT3-FoxO1-*Selenop* pathway in hepatocytes.
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SIGNIFICANCE STATEMENT

Our study shows that CsA downregulates Selenop, an antioxidant protein, via suppressing the STAT3-FoxO1 pathway in hepatocytes, possibly one of the causations of CsA-induced oxidative stress in hepatocytes. The present study sheds light on the previously unrecognized CsA-redox axis.
INTRODUCTION

Cyclosporine A (CsA) is well known as a potent immunosuppressive agent that inhibits calcineurin activity. Therefore, CsA has been clinically applied in various organ transplant patients to prevent graft rejection (Kahan, 1992; Borel et al., 1996). Moreover, CsA is used for treating autoimmune diseases such as systemic lupus erythematosus, myasthenia gravis, psoriasis, and atopic dermatitis. However, CsA induces several side effects, including nephrotoxicity (Tirkey et al., 2005; Lai et al., 2017), cardiotoxicity (Miller, 2002), and hepatotoxicity (Wolf et al., 1997; Rezzani et al., 2005). Korolczuk et al. reported that the CsA-treated rat liver shows increased oxidative stress, necrosis of hepatocytes with mononuclear cell infiltration, and marked mitochondrial damage, suggesting that CsA-induced oxidative stress may play a crucial role during the CsA hepatotoxicity (Korolczuk et al., 2016). Durak et al. reported that CsA reduces the net hepatic antioxidant capacity in rabbits (Durak et al., 2004). Nevertheless, another report indicated that the high dose of CsA treatment enhances the activity of Cu/ZnSOD and MnSOD in rat hepatocytes (Andrés and Cascales, 2002). These inconsistent alterations in antioxidant proteins might involve both direct action of CsA and response to the CsA-mediated oxidative stress, the molecular mechanisms underlying which remain largely unknown.

Selenium, an essential micronutrient, plays pivotal roles in many biological systems, including the central nervous system, male reproductive function, muscle function, thyroid function, and immune function (Brown and Arthur, 2001; Roman et al., 2014). Moreover, selenium-containing proteins, collectively called selenoproteins, have been demonstrated to...
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mediate T cell proliferation and differentiation through an antioxidant mechanism (Shrimali et al., 2008). Selenoprotein P (SeP, encoded by SELENOP in humans) is the most abundant circulating selenoprotein mainly secreted from the liver. SeP functions as an antioxidant protein directly through its intrinsic thioredoxin moiety and indirectly by supplying selenium inside the cells for producing antioxidant glutathione peroxidases (Takamura, 2020).

*SELENOP* promoter activity involves transcription factors forkhead box-containing protein O (FoxO), sterol regulatory element-binding transcription factor 1c (SREBP-1c), and hepatocyte nuclear factor 4 alpha (HNF4A) (Takayama et al., 2014; Tajima-Shirasaki et al., 2017, Murai et al., 2019; Takamura, 2020). In addition, *SELENOP* mRNA is upregulated during hepatitis C virus infection via CCAAT-enhancer-binding protein (C/EBP)-a, and directly interacts with the virus sensor retinoic-acid-inducible gene I (RIG-I) protein, and impairs RIG-I-mediated type I interferon antiviral immunity (Murai et al., 2019). These findings suggest the involvement of SeP in immunity and redox.

Signal transducer and activator of transcription 3 (STAT3) is a transcriptional factor that plays a key molecular function in the differentiation of T helper 17 T cells and plays a role in various autoimmune diseases (Yang et al., 2007). STAT3 promotes liver cancer development by inducing inflammation (Yu et al., 2009). Consistently, STAT3 is highly phosphorylated in most of the hepatocellular carcinoma but not in the normal hepatocytes (Calvisi et al., 2006; Al Zaid Siddiquee and Turkson, 2008, He et al., 2010). Furthermore, STAT3 possesses anti-apoptotic properties via redox-dependent and independent pathways. STAT3 upregulates redox-associated protein Ref-1, reduces ROS, and thereby inactivates
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caspase-9, an apoptosis-related gene. Besides, STAT3 directly upregulates anti-apoptotic proteins, B cell lymphoma-extra large (Bcl-XL) and B cell lymphoma 2 (Bcl-2), via a redox-independent mechanism (Haga et al., 2003). Consequently, inflammation, oxidative stress, and apoptosis may interact and exacerbate liver injury. Although a previous report indicates that CsA inhibits STAT3 activity in the lung cancer cells (Shou et al., 2016), the effect of CsA on STAT3 in the hepatocytes remains unclear.

In the present study, we investigated the effects of CsA on the expression of the genes involved in redox. We found that CsA downregulates SELENOP expression and investigated the molecular mechanisms in hepatocytes.
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Materials and Methods

*Materials* - Antibodies against FoxO1 (#2880), β-Actin (#4967), FoxO3a (#12829), STAT3 (#12640), pSTAT3 (#9145) and Lamin A/C (#2032) were purchased from Cell Signaling Technology (Beverly, MA). The antibody against GAPDH (8245) was purchased from Abcam. The antibody against human SeP (BD1) was kindly provided by Prof. Saito (Tohoku Univ.) (Saito et al., 2001)

*Cell Culture* - The rat hepatoma cell line H4IIEC3 and the human hepatoma cell line HepG2 were purchased from American Type Culture Collection (Manassas, VA). As described previously, primary mouse hepatocytes were obtained (Okada et al., 2017).

Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies Corporation, Carlsbad, CA), containing 10% fetal bovine serum (SIGMA-Aldrich, St. Louis, MO), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (FUJIFILM Wako), 2 mmol/l L-Glutamine (FUJIFILM Wako Pure Chemical Corporation, Ltd.; Osaka, Japan), was used for cell culture. Cells were incubated at 37°C with 5% CO₂ in a humidified atmosphere for proliferation.

*Quantitative RT-PCR* - The ISOSPIN Cell & Tissue RNA (FUJIFILM Wako) kit was used to extract total RNA from cultured hepatocytes. Reverse transcription was performed using a high-capacity cDNA reverse transcription kit (ThermoFisher Scientific, Waltham, USA). Quantitative reverse transcription (RT)-PCR was performed using TaqMan probes (*ACTB*: Rn00666383_m1, *18s rRNA*: Rn01446790_g1, *Foxo1*: Rn01494868_m1, Mm00490676_m1, *Selenop*: Rn00569951_m1, Mm00486048_m1, *Stat3*: Rn00690715_m1, Mm01219775_m1, *Gpx1*: Rn00577994_g1, Mm00656767_g1, *Sod1*: Rn00566938_m1, Mm01344233_g1, *Sod2*: Rn00583832_g1, Mm01224678_g1).
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Rn00566942_g1, Mm00449726_m1, *Cat*. Rn00560930_m1, *Pck1*: Rn01529014_m1) and the 7900HT fast real-time PCR system (ThermoFisher Scientific). All protocols were based on the manufacturer’s instructions.

*Generation of Plasmid Constructs*- The reporter vectors used for the *SELENOP* promoter region were constructed previously (Takayama et al., 2014; Tajima-Shirasaki et al., 2017). Many lengths of *SELENOP* promoter fragments were amplified from normal human genomic DNA and subcloned into the pGL3-basic vector (Promega, Madison, WI). QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used to generate the FoxO1 and NFAT -binding-site-deficient vectors. The DNA sequences were confirmed before the experiments were performed. Putative transcriptional binding sites in the sequence of CsA-responsive elements were searched by using the JASPAR database (https://jaspar.genereg.net/).

*Transfection and Luciferase Reporter Gene Assay*- Firefly luciferase promoter construct DNA (0.4 µg/well) and control renilla luciferase plasmid pGL 4.74 (0.01 µg/well) were transfected into H4IIEC3 cells in a 24-well plate using FuGENE6 (1.2 µl/well; Promega). Luciferase activity was measured by the Dual-Luciferase assay system (Promega)(Honda et al., 2011).

*siRNA transfection*- Stealth RNAi™ Small interfering RNA (siRNA) targeting rat Stat3 (Cat no. 1330001) and Negative Control, Med GC (Cat no. 12935300) was purchased from ThermoFisher Scientific. According to the manufacturer’s instructions, 10 nM of
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siRNAs were transfected into H4IIEC3 cells cultured in a 24-well plate using 1 µL of Lipofectamine™ RNAiMAX (ThermoFisher Scientific) via the reverse transfection method.

**Western blotting**- Whole-cell lysates were prepared, as described previously (Takayama et al., 2014; Tajima-Shirasaki et al., 2017). Nuclear/cytoplasmic extractions were obtained using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (ThermoFisher Scientific). Polyvinylidene difluoride membranes (PVDF) were blocked in Can Get Signal blocking agent (TOYOBO Co., Ltd., Osaka, Japan). The membranes were then incubated with specific primary antibodies followed by washing and incubation with appropriate secondary horseradish peroxidase (HRP)-labeled antibodies. Bands were visualized with the Clarity™ Western ECL Substrate (Bio-Rad). Densitometric analysis of blotted membranes was performed using Image Lab (Bio-Rad) software.

**Chromatin immunoprecipitation assay**- The ChIP-IT Express Enzymatic Kit (Active Motif, Carlsbad, CA) was used to perform the chromatin immunoprecipitation (ChIP) assay according to the manufacturer’s instructions and the previous report (Takayama et al., 2014). The following PCR primers were designed to amplify the -644 bp to -493 bp sequence of the SELENOP promoter, including the putative FoxO binding site: Forward 5’-GCAGTGGTCCTGTGAAATCC -3’ and Reverse 5’-AGGTGCTAGCTTAGCCCACA -3’.

**Animals**- 7-week-old C57BL/6J male mice were purchased from NINOX Labo Supply, Inc. (Ishikawa, Japan). All animals were housed in a 12-hour light/dark cycle and were fed with normal chow. CsA was dissolved in 10% DMSO and 90% corn oil mixture and
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administered both intraperitoneally and orally to mice. Although CsA is available for oral administration in clinical treatment, a review concludes that intraperitoneal injection is a sufficient route to examine the effect of a compound in the pharmacological and proof-of-concept animal studies because it maintains the therapeutic bioavailability whereas the oral route has the potential possibility of degradation or modification of compound conformation (Al Shoyaib et al., 2020). Indeed, CsA is often intraperitoneally administrated in animal studies in previous reports (Provencher and Gascon-Barré, 2002; Ciechomska et al., 2005; Colgan et al., 2005). Vehicle or 50 mg/kg/day of CsA was administrated intraperitoneally for 3 days. After 12 hours of fasting, the mice were sacrificed to obtain the liver sample.

We also tested the effect of a single oral boot of 10 or 50 mg/kg of CsA in mice after 12 hours of fasting (Uchida et al., 2018). The mice were sacrificed to obtain the liver sample after 8 hours of treatment.

Stattic, a STAT3-specific inhibitor, was dissolved in a 10% DMSO and 90% corn oil mixture. Vehicle or 10 mg/kg of stattic was administrated intraperitoneally following overnight fasting. Six hours after injection, mice were sacrificed to obtain the liver sample.

This study was performed in accordance with the Guidelines on the Care and Use of Laboratory Animals, issued by Kanazawa University. The protocol was approved by the ethical committee of Kanazawa University (approval no. 194112).

Statistical Analyses- Significance was tested using a one-way analysis of variance (ANOVA) with the Bonferroni method. Statistically significant differences at a P-value of
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less than 0.05. For the box-and-whisker plot graphs, the medians were indicated by the center lines; the 25th and 75th percentiles were shown as the box limit; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, and outliers are represented by dots.

RESULTS

Cyclosporin A suppresses Selenop gene expression - To explore how CsA contributes to the induction of oxidative stress in the liver, we examined the effect of CsA on the expression of antioxidant proteins genes such as glutathione peroxidase (Gpx1), catalase (Cat), Cu/Zn-superoxide dismutase (Sod1), Mn-superoxide dismutase (Sod2), and Selenop in H4IIEC3 rat hepatoma cells. The results indicated that CsA decreased Selenop gene expression in a concentration-dependent manner, while there were no marked changes in other antioxidant enzymes (Fig. 1A). CsA downregulated Selenop gene expression in mouse primary hepatocytes (Fig. 1B) and human HepG2 cells (Fig. 1C). Consistent with these findings, CsA repressed SeP protein levels in the cultured media of HepG2 cells in a concentration-dependent manner either 24- or 48-hours treatment (Fig. 1D and Suppl. Fig. 1). CsA also suppressed time-dependent accumulation of SeP in the culture media for 24 hours. (Fig. 1E and Suppl. Fig. 2). These data indicate that CsA suppresses Selenop expression specifically among various antioxidant proteins in the hepatocytes.

NFAT is not involved in the CsA-mediated downregulation of SELENOP promoter activity – To investigate the molecular mechanism underlying the CsA-mediated downregulation of Selenop mRNA expression, we assessed the effect of CsA on SELENOP promoter activity by using reporter vectors constructed previously (Takayama et al., 2014).
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As shown in Fig. 2A, CsA at 4 μM suppressed the luciferase activity of mutant (Mut)-A, Mut-B, Mut-C, and full-length SELENOP promotor, but not that of the Mut-D (Fig. 2A). These results indicate that Mut-C contains the CsA-responsive element of the SELENOP promoter. The nuclear factor of activated T-cell (NFAT) is the transcriptional factor known as the target of calcineurin. CsA inhibits calcineurin and thereby inactivates NFAT activity in T cells (Flanagan et al., 1991). As previously reported (Al-Taie et al., 2002), we found a putative NFAT binding site in the Mut-C (Fig. 2B, Bold). Therefore, we tested whether the NFAT binding site mediates the action of CsA on the SELENOP promoter activity. However, neither deletion nor mutation of this site abolished the CsA-induced suppression of the promoter activity (Fig. 2C). These results indicate that the NFAT binding site is not involved in the CsA-induced repression of SELENOP promoter activity.

Cyclosporine A downregulates SELENOP promoter activity via downregulating Foxo1 expression – To determine a putative CsA-responsive element in a narrow range, we constructed additional deletion mutants based on Mut-C. As the result, both Mut-CΔ2 and Mut-CΔ3 were lost the response to CsA (Fig. 3A). This result indicated that the CsA-responsive element exists in the lacked sequence that overlapped between Mut-CΔ2 and Mut-CΔ3 (-570 to -561 in SELENOP promoter, Fig. 3B, underlined). In this CsA-responsive region, a putative FoxO1-binding site was identified according to the transcription factor database (Fig 3B, bold). To determine whether the putative FoxO1 site acts as a functional responsive element, we constructed another two additional mutant luciferase vectors with point mutation or deletion at the site (Fig. 3B). The luciferase activity assay revealed that this
putative FoxO1 binding site is essential for CsA response in Mut-C (Fig. 3C). Next, we conducted a ChIP assay to examine the direct association between FoxO1 and a specific region of the SELENOP promoter in HepG2 cells. Treatment with CsA almost completely suppressed the FoxO1 binding to the SELENOP promoter (Fig. 3D and Suppl. Fig. 3). We have previously identified another FoxO binding site in the Mut-D (Takayama et al., 2014). To confirm whether CsA affects FoxO interaction to this site, we performed ChIP PCR using the primers that detect FoxO binding site on Mut-D. Considering the reduction of gross FoxO1 protein levels by CsA treatment in the cultured hepatocyte, CsA decreases the binding of FoxO1 to the Mut-D site even though the repression is relatively weaker than that observed on the Mut-C site (Suppl. Fig. 4). Since CsA did not alter Mut-D activity, this reduction did not relate to CsA action. Moreover, knockdown of Foxo1 significantly downregulated Selenop mRNA levels in the H4IIEC3 cells (Suppl. Fig. 5). Also, Foxo1 knockdown canceled the CsA-mediated downregulation of the Selenop gene in H4IIEC3 cells (Fig. 3E). These results indicate that CsA downregulates SELENOP promoter activity through downregulating Foxo1 expression.

CsA represses Foxo1 by inactivating STAT3 - Following experiments indicated that CsA treatment reduced the protein amount of Foxo1 (Fig. 4A and Suppl. Fig. 6) in both cytoplasm and nucleus (Fig. 4B and Suppl. Fig. 7). Suppression of Foxo1 expression by CsA was observed at mRNA levels as well (Fig. 4C). It is reported that STAT3 promotes FoxO expression in T cells (Oh et al., 2011) and CsA inhibits STAT3 phosphorylation in cancer cells (Bauer et al., 2009, Shou et al., 2016). We hypothesized that CsA downregulates Foxo1 via a STAT3 - FoxO1 axis.
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expression via STAT3 inactivation based on the previous knowledge. CsA treatment reduced
STAT3 phosphorylation levels and nuclear translocation in H4IIEC3 cells (Fig. 4A and B)
without affecting Stat3 mRNA expression (Fig. 4D). Knockdown of Stat3 downregulated
Foxo1 and Selenop (Fig. 4E), whereas colivelin, a widely used specific STAT3 activator,
upregulated Foxo1 and Selenop genes (Fig. 4F). These results indicate that CsA inactivates
STAT3 and thereby downregulates Foxo1 and Selenop.

Administration of CsA or Stat3 inhibitor to mice- To assess the CsA action on
hepatic Selenop expression in vivo, we administrated 50 mg/kg/day of CsA intraperitoneally to
7-week-old male C57BL/6J mice for three days. The mice administered CsA showed
decreased bodyweight, fasting plasma glucose levels, and food intake (Suppl. Fig. 8A-C). The
starvation response genes, such as Selenop, Foxo1, and gluconeogenic Pck1 were upregulated
(Suppl. Fig. 8D and E). Intraperitoneal administration of CsA tended to elevate FoxO1 and
p-STAT3 levels in vivo (Suppl. Fig. 8F and G). Oral administration of CsA at 10 or 50 mg/kg
did not affect fasting glucose level and Selenop expression in the liver (Suppl. Fig. 9).

Then we tested a STAT3-specific inhibitor, stattic, administration to mice and
assessed Selenop mRNA expression in the liver. The results indicated that stattic
administration significantly suppressed liver Foxo1 and Selenop mRNA expression even
without alteration in plasma glucose levels (Fig. 4G and H).
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DISCUSSION

The present *in vitro* study revealed CsA-dependent Selenop downregulation in the hepatocytes and its underlying molecular mechanism. We identified a CsA-responsive element in the *SELENOP* promoter that contains the FoxO1 binding site. CsA downregulates Foxo1 expression as a consequence of STAT3 inactivation. Therefore, considering SeP as a vital antioxidant protein, CsA might induce oxidative stress by downregulating Selenop expression in hepatocytes.

The finding that CsA downregulates Selenop expression evokes novel machinery of immunosuppression by CsA. Selenium plays a pivotal role in maintaining optimal immune function (Brown and Arthur, 2001; Ibs *et al*., 2003). During innate immune responses, selenium levels and selenoproteins positively regulate macrophages' migration and the phagocytic functions (Safir *et al*., 2003; Carlson *et al*., 2010). During adaptive immune responses, the antioxidant function of selenoproteins is related to T cell immunity (Shrimali *et al*., 2008). Mice with T cells lacking selenoproteins exhibit reduced pools of mature T cells and a defect in T cell-dependent antibody responses. On the other hand, we reported that *SELENOP* mRNA is upregulated in hepatitis C infection and directly inhibits RIG-I-mediated type I interferon responses against RNA virus infections (Murai *et al*., 2019). Therefore, reduced Selenop expression may partially contribute to the CsA-mediated immune dysfunction, which requires further investigation.

Although previous research reveals a separate FoxO binding site on the *SELENOP* promoter (Speckmann *et al*., 2008, Takayama *et al*., 2014), the current study identified a
novel FoxO1 binding site critical for the CsA-mediated downregulation of the *SELENOP* promoter activity. The distinct regulatory mechanism appears to be associated with each FoxO site because CsA treatment did not alter Mut-D promoter activity, containing the previously identified FoxO1 site (Speckmann *et al.*, 2008; Takayama *et al.*, 2014). Interactions with unique co-factors and/or differences in the cis-element compatibility with other FoxO family members, such as FoxO3a, may determine the specificity of each site. The primary target of CsA is calcineurin, which is a calcium- and calmodulin-dependent serine/threonine protein phosphatase. Calcineurin dephosphorylates the transcription factor NFAT in T cells (Flanagan *et al.*, 1991). Since the *SELENOP* promoter involves the NFAT binding site (Al-Tae *et al.*, 2003), we examined the effect of CsA on this element. However, neither deletion nor mutation of this site abolished the CsA-mediated downregulation of the *SELENOP* promoter activity. Therefore, we consider that NFAT is not involved in the CsA-mediated downregulation of SELENOP promoter activity.

Unfortunately, we could not confirm the *in vivo* effect of CsA on the *Selenop* expression in the liver due to the biases possibly derived from the CsA-mediated weight reduction and starvation-like responses in mice. The current dose of CsA was used for mice in the previous report (Yuan *et al.*, 2015), which is not so different from clinically used initial doses of CsA (10-15 mg/kg) for humans (McMillan, 1989). Possibly due to the weight reduction and starvation-mediated activation of FoxO1 and STAT3, CsA rather upregulated *Selenop* expression in the liver. Because CsA is clinically administrated orally, we also tested the effect of oral CsA administration in mice (Suppl. Fig. 9). Oral administration of CsA did
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not affect hepatic Selenop expression in mice. Based on these findings, it is difficult to confirm CsA's effects on Selenop expression in the liver in vivo in the current study because CsA acts directly and indirectly on the liver. Therefore, we used a STAT3-specific inhibitor, stattic, in mice instead of CsA. Stattic administration significantly suppressed Foxo1 and Selenop mRNA expression in the liver even without alteration of plasma glucose levels, supporting our conclusion that CsA downregulates Foxo1 via inactivating STAT3.

CsA upregulated Gpx1, Sod1, Sod2, together with Selenop, in the mice liver (Suppl. Fig. 8H). The previous study also reported that high doses of CsA treatment increased Cu/ZnSOD and MnSOD gene expression in primary rat hepatocytes (Andrés and Cascales, 2002). However, our in vivo finding seems exceptional and may function as compensation against oxidative stress because CsA treatment did not affect the expression of these genes, except for Selenop in the cultured hepatocytes (Fig. 1A). Therefore, we conclude that the effect of CsA on Selenop is cell-autonomous and specific among the redox-related genes.

In the current study, loss-of-function experiments, such as knockdown of Foxo1 or Stat3, support our conclusion that CsA downregulates Selenop via a STAT3-FoxO1 pathway in hepatocytes in vitro. However, due to the low transfection efficiency of the constitutive-active STAT3 vector and the unchanged net FOXO1 amount owing to the downregulation of endogenous rat Foxo1 gene expression in human FOXO1 overexpressed H4IIEC3 cells (data not shown), we were unable to confirm the machinery by the gain-of-function experiments in the current study. Collectively, our data support a correlative relationship between CsA action and the STAT3-FoxO1 axis, although a causative role needs
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to be further investigated. In addition, CsA-mediated systemic effects on the liver may affect the Selenop expression. The time course of experiments with mice or human studies may deepen our understanding in the future.

In summary, the present study sheds light on the previously unrecognized CsA-redox axis. CsA downregulates Selenop via suppressing the STAT3-FoxO1 pathway in hepatocytes, which may lead to the CsA-induced oxidative stress in the hepatocytes.
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Authorship contributions:

Participated in research design: Takayama, Ishii, Takamura

Conducted experiments: Yao, Takayama, Kamoshita, Hein Ko Oo, Tanida

Contributed new reagents or analytic tools: Takayama, Kato

Performed data analysis: Yao, Takayama

Wrote or contributed to the writing of the manuscript: Yao, Takayama, Ishii, Takamura
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Footnotes:

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• Toshinari Takamura, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan E-mail: ttakamura@med.kanazawa-u.ac.jp

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**FIGURE LEGENDS**

**FIGURE 1.** CsA regulated *Selenop* expression in the hepatocytes

* A: CsA downregulated *Selenop* mRNA expression but not *Gpx1*, *Cat*, *Sod1*, and *Sod2* in H4IIEC3 cells. Hepatocytes were treated with the indicated concentrations of CsA for 24 hours. Data were represented by scatter (n = 4). *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus vehicle-treated cells. 

* B and C: CsA downregulated *Selenop* mRNA expression in primary and HepG2 cells. 

* D and E: CsA repressed SeP expression levels in the culture medium. HepG2 cells were treated with the 8 or 16 µM CsA for 24 or 48 hours (D) or 8 µM CsA for 1, 3, 6, 12, and 24 hours (E). Culture medium and whole-cell lysates were collected. Repeated data were obtained from 3 independent experiments. The black arrow indicates the predicted positions of human SeP.

**FIGURE 2.** NFAT binding site is not involved in the CsA-mediated suppression of *SELENOP* promoter activity

* A: Structures and luciferase activities of promoter-deletion mutants. The thin lines indicate the deleted sequences within the constructs. Each normal- or mutant- *SELENOP* reporter vector and the renilla luciferase control vector were co-transfected to H4IIEC3 cells for 24 hours and then the cells were treated with 4 µM CsA for 48 hours. Signals were normalized to those for the control reporter vector (n = 6). 

* B: Structures of NFAT-deletion or mutant vectors. Dashed lines or bold indicate the sequences deleted or mutated within the constructs.

* C: H4IIEC3 cells were co-transfected with each Mut-C mNFAT or ΔNFAT and the renilla luciferase control vector for 24 hours and then followed by 48 hours of 4 µM CsA treatment. n = 8; *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus vehicle-treated cells.

**FIGURE 3.** Identification of the FoxO1 binding site on the *SELENOP* promoter

* A: Structures and luciferase activities of promoter-deletion mutants. Each normal- or mutant- *SELENOP* reporter vector and the renilla luciferase control vector were co-transfected to H4IIEC3 cells for 24 hours and then the cells were treated with 4 µM CsA for 48 hours. (n = 5) 

* B: Structures of FoxO1-deletion or mutant vectors. Dashed lines or bold indicate the sequences deleted or mutated within the constructs.

* C: Deletion or mutation of the putative FoxO1 binding site inhibited the CsA-mediated repression of *SELENOP* promoter activity. Cells were co-transfected with each Mut-C mFoxO1 or ΔFoxO1 and the renilla luciferase control vector for 24 hours and then followed by 48 hours of 4 µM CsA treatment.

* D: Chromatin immunoprecipitation assay of HepG2 cells treated with CsA. After 6 hours of starvation, cells were treated with 8 µM CsA for 6 hours. Anti-FoxO1 or normal IgG antibodies were used to precipitate chromatin samples. Repeated data were obtained from 3
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independent experiments. E: H4IIEC3 cells were transfected with Foxo1 siRNA or negative control siRNA (NC) for 48 hours, followed by 8 μM CsA treatment for 24 hours. Expression values were normalized to 18s rRNA mRNA levels. n = 3; *, P < 0.05, **, P < 0.01; versus control group.

FIGURE 4. CsA downregulated FoxO1 expression by downregulating Stat3

A: CsA reduced FoxO1 and p-STAT3 protein levels. H4IIEC3 cells were treated with the low to high concentrations of CsA for 24 hours. B: FoxO1 and STAT3 protein levels in cytoplasm and nucleus. Cytoplasmic and nuclear fractions were isolated immediately after 24 hours of 4 μM CsA treatment. Repeated data were obtained from 3 independent experiments. C and D: Foxo1 and Stat3 gene expression under CsA treatment. H4IIEC3 cells were treated with the indicated concentration of CsA for 24 hours. Data were represented by scatter (n = 4). E: siRNA-induced knockdown of Stat3. H4IIEC3 cells were transfected with Stat3 siRNA or NC siRNA for 48 hours. n = 3; *, P < 0.05, versus control group. F: Colivelin, a STAT3 activator, contributed to the upregulation of Selenop and Foxo1 gene expression. H4IIEC3 cells were treated with 0.01μM Colivelin for 24 hours. n = 4; *, P < 0.05, versus control group. G: No alteration of fasting plasma glucose (FPG) between vehicle and 10 mg/kg stattic-treated mice after administration for 6 hours. H: Suppression of Selenop and Foxo1 mRNA expression in the liver of stattic-treated mice. n = 6,7; *, P < 0.05, versus vehicle group.
Figure 1

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Figure 2

A

SELENOP Promoter Luc+

Normal

Mut-A

Mut-B

Mut-C

Mut-D

SELENOP promoter activity (Relative change)

Vehicle
CsA 4μM

B

Mut-C

Mut-C mNFAT

Mut-C ΔNFAT

NFAT binding site

C

SELENOP promoter activity (Relative change)

Vehicle
CsA 4μM
Figure 3
Figure 4

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Cyclosporine A downregulates selenoprotein P expression via a STAT3-FoxO1 pathway in hepatocytes in vitro

Xingyu Yao, Hiroaki Takayama, Kyoko Kamoshita, Hein Ko Oo, Ryota Tanida, Kaisei Kato, Kiyo-Aki Ishii, and Toshinari Takamura

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Supplementary figure 1. Full western blot images for Fig. 1D
The red rectangle means the representative area shown in the figure.

Supplementary figure 2. Full western blot images for Fig. 1E
The red rectangle means the representative area shown in the figure.
Supplementary figure 3. The results from other two independent samples for Fig. 3D.

Supplementary Figure 4. The effect of CsA on FoxO element in Mut-D of human SELENOP promoter
Chromatin immunoprecipitation assay of HepG2 cells treated with CsA. After 6H starvation, HepG2 cells were treated with CsA for 6 hours. Chromatin samples were precipitated with anti-FoxO1 or normal IgG and amplified using primers for the Mut-D FoxO region of the human SELENOP promoter. (PCR primers were forward, 5’-GCACCTGCTACT- TTCTTTAAGTTG-3’; reverse, 5’-CACACGAGCAGC-ACTCTGATATTTAT-3’)

Supplementary figure 5. Knockdown efficiency of Foxo1 siRNA in H4IIEC cells.
H4IIEC3 cells were transfected with Foxo1 siRNA or negative control (NC) siRNA for 48 hours. Expression values were normalized to 18s rRNA mRNA levels. n = 6; **, P < 0.01, ***, P < 0.001.
Supplementary figure 6. Full western blot images for Fig. 4A
The red rectangle means the representative area shown in the figure.

Supplementary figure 7. Full western blot images for Fig. 4B
The red rectangle means the representative area shown in the figure.
Supplementary Figure 8. CsA administration to mice

A-C: Reduction of body weight, fasting plasma glucose (FPG), and food intake in the CsA-treated mice. D: Induction of Selenop, Foxo1, and Stat3 mRNA expression in the liver of CsA-treated mice. E: Gene expression of Pck1. F and G: Protein expression of FoxO1, pSTAT3, and total STAT3 in the mice liver. Western blotting of the liver samples from individual mice (F) and densitometry analysis (G). H: Gpx1, Sod1, and Sod2 in the mice liver. Seven-week-old male C57BL/6J mice were intraperitoneally administered 50 mg/kg CsA (dissolved in 10% DMSO and 90% corn oil) or vehicle (10% DMSO and 90% corn oil mixture) for 3 days. Followed by overnight fasting, the mice were sacrificed, and liver samples were collected. Liver RNA and proteins were analyzed by real-time PCR and western blot. n = 10,5; *, P < 0.05, **P<0.01 and ***P<0.001 versus corn oil-injected mice.
Supplementary Figure 9. Oral administration of CsA to mice
Fasting plasma glucose (A) and gene expression of Selenop in the mice liver (B) after oral CsA administration to mice. Seven-week-old male C57BL/6J mice were orally administered 10 or 50 mg/kg CsA (dissolved in 10% DMSO and 90% corn oil) or vehicle (10% DMSO and 90% corn oil mixture) followed by 12 hours fasting. The mice were sacrificed after 8 hours of treatment, and liver samples were collected.