Insulin Suppresses Ubiquitination via the Deubiquitinating Enzyme USP14, Independent of Proteasome Activity in H4IIEC3 Hepatocytes.

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ABSTRACT
Ubiquitin-proteasome dysfunction contributes to obesity-related metabolic disorders such as diabetes and fatty liver disease. However, the regulation of ubiquitin-proteasome activity by insulin remains to be elucidated.

Here, we show that prolonged insulin stimulation activates proteasome function even though it reduces the ubiquitinated proteins in H4IIEC3 hepatocytes. Looking for a pathway by which insulin inhibits ubiquitination, we found that hepatic expression of ubiquitin-specific protease 14 (USP14) was upregulated in the liver of patients with insulin resistance. Indeed, the USP14-specific inhibitor IU1 canceled the insulin-mediated reduction of ubiquitinated proteins.

Furthermore, insulin induced endoplasmic reticulum (ER) stress, which was canceled by IU1, suggesting that USP14 activity is involved in insulin-induced ER stress. Co-stimulation with insulin and IU1 for 2 h upregulated the nuclear translocation of the lipogenic transcription factor, sterol regulatory element binding protein-1c (SREBP-1c), upregulated the expression of the lipogenic gene, fatty acid synthase (Fasn), and repressed the gluconeogenic genes.

In conclusion, insulin activates proteasome function even though it inhibits protein ubiquitination by activating USP14 in hepatocytes. USP14 activation by insulin inhibits mature SREBP-1c while upregulating ER stress and the expression of genes involved in gluconeogenesis. Further understanding mechanisms underlying the USP14 activation and its pleiotropic effects may lead to therapeutic development for obesity-associated metabolic disorders such as diabetes and fatty liver disease.

SIGNIFICANCE STATEMENT
This study shows that insulin stimulation inhibits ubiquitination by activating USP14, independent of its effect on proteasome activity in hepatocytes. USP14 also downregulates the nuclear translocation of the lipogenic transcription factor SREBP-1c and upregulates the expression of genes involved in gluconeogenesis. Since USP14 is upregulated in the liver of insulin-resistant patients, understanding mechanisms underlying the USP14 activation and its pleiotropic effects will help develop treatments for metabolic disorders such as diabetes and fatty liver.

Introduction
Obesity is closely associated with hepatic insulin resistance and fatty liver diseases, however, the causal relationship among these remains debated. One of the key pathways linking obesity to insulin resistance is proteasome dysfunction in the liver (Otoda et al. 2013a; Yalcin and Hotamisligil 2013). In eukaryotic cells, ubiquitin-proteasome and autophagy-lysosome systems are considered as two main intracellular protein degradation pathways. Proteasome selectively degrades substrates which bind
ubiquitin chains and contribute to cell homeostasis. We previously found that liver proteasome activity is reduced by approximately 30–40% in mouse models of obesity (db/db mice, ob/ob mice, and C57BL6 mice fed a high-fat diet), resulting in endoplasmic reticulum stress leading to insulin resistance, Forkhead box O (FOXO) activation leading to enhanced gluconeogenesis, and SREBP-1c activation leading to fatty liver (Otoda et al. 2013a). Therefore, proteasome activity may be a potential therapeutic target for obesity-associated metabolic disorders such as insulin resistance, diabetes, and fatty liver disease. However, molecular mechanisms regulating proteasome function have not yet been fully understood.

Recently, USP has been reported to be involved in glucose metabolism (Forand et al. 2016; Hashimoto et al. 2022), lipid metabolism (Ni et al. 2020), type 2 diabetes mellitus (Bai et al. 2021), and nonalcoholic fatty liver disease (An et al. 2017; Zhao et al. 2018). Proteasome transiently interacts with proteasome-interacting proteins (PIPs), which are not proteasome subunits but are involved in the function. USP14 is one of the PIPs and controls proteasome degradation negatively by the activity to trim ubiquitin chains on substrates. Lee et al. demonstrated that a small-molecule inhibitor of USP14, IU1, enhances proteasome activity (Lee et al., 2010). However, the role of this pathway in obesity and diabetes remains unclear.

Overnutrition promotes insulin secretion directly stimulated by nutrients and as compensation for insulin resistance associated with obesity and fatty liver. In the present study, we have investigated insulin-mediated regulation of ubiquitin-proteasome function, which may be critical for developing ubiquitin-targeting therapies against metabolic disorders.

**Materials and Methods**

**Chemicals and Reagents.** Antibodies against ubiquitin, phospho-Akt (Ser473), Akt, Bip, CHOP, β-actin, and Lamin A/C were purchased from Cell Signaling Technology (Beverly, MA). The antibody against p-IRE1α was from Novus Biologicals (Littleton, CO). Antibodies against SREBP-1 were purchased from BD Pharmingen. Antibody against ubiquitinated protein was purchased from Proteintech. Antibodies against GAPDH were purchased from Abcam (Cambridge, England). Human recombinant insulin was obtained from Sigma (Louis, MO). Bortezomib, a proteasome inhibitor, was purchased from Selleck Chemicals (Houston, TX). IU1, a small-molecule inhibitor of USP14, was generously provided by Daniel Finley (Harvard Medical School, Boston, MA) and purchased from Sigma (Louis, MO). T0901317, a liver X receptor (LXR) agonist, was purchased from Wako Pure Chemical Industries (Osaka, Japan). Proteasome 20S and Proteasome 26S human purified protein were purchased from ENZO life sciences (Farmingdale, NY).

**Cell Culture and Treatment.** The rat hepatocytes cell line H4IIEC3, human hepatocytes cell line HepG2, and mouse hepatocytes cell line Hepal-6 were cultured with 4.5g/L high glucose Dulbecco modified Eagle medium (DMEM) (Nacalai tesque, Inc.) supplemented with 10% fetal bovine serum (FBS) (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. All studies were performed using cells from 80-90% confluent cultures.

For experiments using only insulin, the hepatocytes were incubated with or without 100 nM insulin for 24 h in DMEM in the presence of 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. After treatment, the cells were washed with phosphate-buffered saline (PBS) once and were stimulated with 1ng/mL insulin for 15 min.

For experiments using IU1, USP14 specific inhibitor, during insulin treatment, the hepatocytes were pre-incubated with 75 µM IU1 or DMSO for 20 min in DMEM including 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. And then, after washed with PBS once, the cells were cultured with or without 100 nM insulin, 75 µM IU1, and 50 nM bortezomib for 2 h in DMEM in the presence of 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

Cell Harvest. At the indicated times, these cells were washed with PBS once, harvested in ice-cold PBS, and then collected by centrifugation at 4°C. The pellets of the cells were incubated for more than 1 h at -80°C, and the cells were lysed by being passed 4 times through a 26-gauge needle with the syringe in ice-cold lysis buffer containing 50 mM Tris-HCl (PH 7.5), 150 mM NaCl, 5 mM MgCl2, 10% glycerol (v/v), 1 mM DTT, 2 mM ATP, 1% complete protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. The lysates were centrifuged at 4°C to remove insoluble materials. Nuclear and cytoplasmic extracts were obtained using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit according to the manufacturer’s protocol (Thermo Fisher). Protein concentrations were determined using the Lowry Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Proteasome Activity Assay. For evaluation of proteasome activity by luminescence-based assay, the whole-cell lysates as described above and Proteasome-Glo 3-Substrate Cell-Based Assay System (Promega, Madison, WI) were used. The values of luminescence were corrected by protein concentration. For in-gel proteasome activity assay, the whole-cell lysates, as described above, were separated on NuPAGE 3-8% Tris-Acetate Gel (Thermo Fisher Scientific) at 4°C, using the 2x native loading buffer containing 250 mM Tris-HCl (PH 7.5), 10% (v/v) Glycerol, and 0.007% (w/v) Xylene cyanol. After electrophoresis, the gels were incubated in 25 mM Tris-HCl (PH 7.4), 10 mM MgCl2, 0.5 mM ATP, 0.5 mM DTT, and 50 µM Suc-LLVY-AMC (succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; Peptide Institute, Osaka, Japan) at 37°C for 60 min. The fluorescent signal was photographed using ChemiDoc Touch Imaging System (Bio-Rad).

Western Blot Analysis. 2x Sample Buffer Solution (2ME+) (WAKO) was added to the whole-cell lysates as described above to denature proteins, and each sample was boiled at 95°C for 5 min. The proteins were subjected to 5-20% SDS-PAGE and transferred to PVDF membranes using the iBlot® 2 Gel Transfer System (Thermo Fisher Scientific). The membranes were blocked in PVDF Blocking Reagent for Can Get Signal® (Toyobo, Osaka, Japan) for an hour at room temperature. After that, they
were incubated with specific primary antibodies overnight at 4°C, washed in a buffer containing 50 mM Tris, 150 mM NaCl, and 0.1% Tween 20, pH 7.5, and incubated with the secondary, HRP-labeled antibodies. Can Get Signal Immunoreaction Enhancer Solutions 1 and 2 (Toyobo) were used to dilute primary and secondary antibodies, respectively. All images were visualized with ChemiDoc Touch Imaging System. Densitometry analysis of blotted membranes was performed using Image Lab Software (Bio-Rad Laboratories).

**Quantitative RT-PCR.** Total RNA was extracted from the cultured hepatocytes using a High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). The reverse transcription of 100 ng of total RNA was performed using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Quantitative RT-PCR (qRT-PCR) was performed using TaqMan probes (Fasn: Rn01463550_m1 (rat), Mm00662319_m1 (mouse), FASN: Hs01005622_m1; Acaca: Rn00733747_m1 (rat), Mm01304258_m1 (mouse), ACACA: Hs01046047_m1 (human); Scd1: Rn00594894_g1 (rat), Mm00772290_m1 (mouse), SCD: Hs01682761_m1 (human); Pck1: Rn01529014_m1 (rat), PCK1: Hs00159918_m1 (human); G6pc: Rn00565347_m1 (rat), G6PC: Hs02802676_m1 (human); Usp14: Rn01236255_m1 (rat); Gapdh Control Reagents: 4352338E (rat), 4352932E (mouse), GAPDH Control Reagents: 402869 (human) and the StepOne Plus real-time PCR system (Life Technologies), as described previously (Nakamura et al. 2009).

**siRNA Transfection in H4IIEC3 Hepatocytes.** H4IIEC3 hepatocytes were grown transiently transfected with 10 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. Stealth siRNAs RSS307309, RSS307310, and RSS353978 (Thermo Fisher) were used for Usp14-specific siRNAs. Med GC12935300 (Thermo Scientific) was used for the negative control siRNA. Twenty-four hours after transfection, the cells were followed by total RNA extraction.

**Human Studies.** Liver samples for DNA chip analysis were obtained from 21 patients with type 2 diabetes and 11 subjects with normal glucose tolerance using ultrasonography-guided biopsy needles (Hirofumi Misu et al. 2010). Of these 12 data (7 patients with type 2 diabetes and 5 subjects with normal glucose tolerance) were used in this study. Insulin sensitivity was tested using the glucose clamp method (Defronzo, Tobin, and Andres 1979). Insulin sensitivity was measured in terms of the metabolic clearance rate of glucose (MCR of glucose) in mg/kg/min. Detailed methods and clinical information about these subjects are described previously (H. Misu et al. 2007; Hirofumi Misu et al. 2010; Takamura et al. 2008).

**Statistical Analysis.** All data were analyzed using the Japanese Windows Edition of Statistical Package for Social Science (SPSS) version 28 (IBM corporation, NY) or GraphPad Prism version 9 (San Diego, California). Data were expressed as mean ± standard error and analyzed using Student's t-test or one-way ANOVA with posthoc analysis by the Turkey method. Differences with a P value of less than 0.05 were considered statistically significant.
RESULTS

**Prolonged Stimulation with Insulin Activates Proteasome Activity.** It is reported that insulin inhibits proteasome activity (Brown and Goldstein 1997b; Foretz et al. 1999a). However, because long-term insulin exposure induces insulin resistance in the hepatocytes (Hirano et al. 2001a), we first addressed various insulin treatment conditions on proteasome activity in hepatocytes. H4IIEC3 hepatocytes were cultured with or without insulin for 24 h, and the cells were stimulated with insulin again for 15 min (Figure 1A). Akt phosphorylation at Ser 473 (p-Akt) in the cells by insulin stimulation was greater with long-term stimulation (Figure 1B, a vs. b) than with short-term stimulation (Figure 1B, a+c vs. a+d). Short-term stimulation after long-term stimulation with insulin did not enhance Akt phosphorylation (Figure 1B, b+e vs. b+f). On the other hand, chymotrypsin-like, trypsin-like, and caspase-like proteasome activities were enhanced by long-term insulin stimulation (Figure 1C, a vs. b) but not by short-term stimulation (Figure 1C, a+c vs. a+d). Long-term stimulation with insulin followed by short-term stimulation did not enhance proteasome activity, while it enhanced proteasome activity compared to short-term stimulation alone (Figure 1C, b+e vs. b+f). In addition, long-term insulin stimulation enhanced proteasome activity in a concentration-dependent manner (Figure 1D). These results indicate that long-term but not short-term insulin stimulation enhances proteasome activity and an insulin downstream Akt phosphorylation.

**Insulin signaling is involved in the expression of hepatic USP14 in humans.** Akt (Ser473) phosphorylates and activates USP14 and thereby inhibits the ubiquitin-proteasome proteolysis by trimming the ubiquitin chains on substrate proteins (Sundqvist et al. 2005a) (Figure 2A). USP14 also interacts with FASN and increases FASN stability (Liu et al. 2018). Consequently, overexpression of USP14 promotes hepatic triglyceride accumulation, while inhibition of USP14 ameliorates hepatic lipidosis, hyperglycemia, and insulin resistance (Botolin et al. 2006a). In search for the molecular targets responsible for long-term insulin treatment-mediated proteasome activation, we analyzed the relationship between clinical characteristics and hepatic expression of the *USP14* gene using the data obtained in ultrasonography-guided percutaneous needle liver biopsies from 21 people with type 2 diabetes and 11 non-diabetic subjects (Ruiz et al. 2014a). As shown in Table 1, hepatic *USP14* mRNA levels were negatively correlated with metabolic clearance rate (MCR) of glucose (Figure 2B) and positively correlated with acetyl-CoA carboxylase alpha (*ACACA*), stearoyl-CoA desaturase (*SCD*), and sterol regulatory element binding transcription factor 1 (*SREBF1*), which are expressed downstream of Akt phosphorylation (Figure 2C).

**Prolonged Insulin Stimulation Inhibits Ubiquitination via USP14.** We evaluated the accumulation of ubiquitinated proteins (Figure 3A and C-E) and proteasome 26S-specific activity by using the native PAGE and in-gel chymotrypsin activity to investigate the involvement of insulin and USP14 in the ubiquitin-proteasome system (Figure 4A-D). Insulin reduced the accumulation of ubiquitinated proteins in H4IIEC3 hepatocytes. The pharmacological effects of a selective small molecule inhibitor of USP14
(IU1) (Lee et al. 2010a) reversed the insulin-induced reduction in ubiquitinated proteins (Figure 3A). Furthermore, siRNA-mediated suppression of Usp14 (Figure 3B) canceled the insulin-induced reduction in ubiquitinated proteins, similar to the pharmacological effects of IU1 (Figure 3C). Similar findings were observed in human HepG2 cells but not in mouse Hepa1-6 cells (Figures 3D and E). Similarly, proteasome activity was examined by in-gel assay (Figures 4A and B). Insulin elevated 20S proteasome activity, and proteasome inhibitor bortezomib inhibited 26S proteasome activity. IU1 (Figures 4A) and Usp14 siRNA (Figure 4B) did not directly affect either of the 20S and 26S proteasome activities. Similarly, USP14 did not affect proteasome activity in HepG2 hepatocytes and Hepa1-6 hepatocytes (Figures 4C and D). These results indicate that insulin reduces ubiquitinated proteins by activating USP14. Insulin directly enhances proteasome activity, as shown in Fig. 1C, independently of USP14.

**USP14 activity is related to the ER stress induced by insulin.** We previously showed that obesity impairs proteasome function and thereby causes ER stress (Otoda et al. 2013b). Other groups reported that insulin induces ER stress in the liver (Xu, Shan, Lee, Zhu, Zhang, Sun, Liu, Shi, et al. 2015a). Based on these findings, we have addressed whether USP14-mediated regulation of ubiquitination is involved in insulin-mediated ER stress. Western blotting was conducted to estimate ER stress markers, including CHOP, phosphorylated IRE1α (p-IRE1α), and BiP/GRP78 in H4IIEC3 hepatocytes treated with insulin, IU1, Usp14 siRNA, and bortezomib for 2 h. Insulin elevated CHOP, which was canceled by IU1 (Figure. 5A) but not with Usp14 siRNA (Figure. 5B). On another front, in HepG2 hepatocytes and Hepa1-6 hepatocytes, CHOP protein was found to be further elevated by IU1 (Figure 5C and D).

**USP14 activity relieves the amount of mature nuclear SREBP-1c.** Insulin induces the expression of lipogenic genes in the liver via SREBP-1c (Foretz et al. 1999b). SREBP-1c, a member of the SREBP family of transcription factors, is well known to enhance the transcription of genes involved in de novo lipogenesis and fatty acid synthesis (Brown and Goldstein 1997a). SREBP-1c in the nucleus is degraded by the ubiquitin-proteasome system (Botolin et al. 2006b; Hirano et al. 2001b; Sundqvist et al. 2005b). We previously reported that mice with proteasome dysfunction show elevated levels of cleaved/mature SREBP-1c in the nucleus (Otoda et al. 2013a). To investigate whether inhibition of ubiquitination by insulin contributes to the accumulation of SREBP-1c in the nucleus, we assessed SREBP-1c protein levels in the nuclear fraction after treatment with insulin and IU1 for 2 h in H4IIEC3 hepatocytes. As shown in Figure 6A, IU1 treatment, together with insulin, promoted the nuclear translocation of SREBP-1. Usp14 siRNA did not promote the SREBP-1 nuclear translocation (Figure 6B).

We then examined whether insulin and Usp14 also enhance the expression of crucial lipogenic enzyme genes such as fatty acid synthase (FAS, encoded by Fasn in rats and mice, FASN in humans), acetyl-CoA carboxylase 1 (ACAC1, encoded by Acaca in rat and mouse, ACACA in humans), and stearoyl-CoA desaturase-1 (SCD-1, encoded by Scd1 in rat and mouse, SCD in humans) in hepatocytes. H4IIEC3 hepatocytes cells were treated with insulin and IU1 for 2 hours. Insulin upregulated genes involved in lipogenesis, which were not affected by IU1 (Figure 7A) or Usp14 siRNA treatment (Figure 7B). IU1
tended to upregulate the expression of lipogenic enzyme genes in HepG2 hepatocytes (Figure 7C), whereas IU1 downregulated lipogenic enzyme gene expression in Hepa1-6 hepatocytes (Figure 7D).

**USP14 activity is associated with the expression of gluconeogenic genes.** Next, we analyzed the expression of the key enzymes involved in gluconeogenesis, which are negatively regulated by SREBP-1c (Ruiz et al. 2014b; Yamamoto et al. 2004). Insulin remarkably downregulated mRNAs for phosphoenolpyruvate carboxykinase 1 (PKC-1/PEPCK, encoded by *Pck1* in rats) and glucose-6-phosphatase catalytic subunit (G6Pase encoded by *G6pc* in rat). IU1 treatment with insulin further suppressed the expression of gluconeogenic genes (Figure 8A). On the other hand, *Usp14* siRNA did not affect genes involved in gluconeogenesis (Figure 8B). Insulin also downregulated PCK-1/PEPCK (encoded by *PCK1* in humans) and G6Pase (encoded by *G6PC* in humans) in HepG2 cells, which were canceled by IU1 (Figure 8C).

**Discussion**

Several lines of evidence show that USP14 inhibits proteolysis of 26S proteasome via trimming ubiquitin chains (Lee et al. 2010b) and that USP14 is activated by p-Akt (Ser473)-mediated phosphorylation (Xu, Shan, Lee, Zhu, Zhang, Sun, Liu, Liang, et al. 2015). Insulin is well known to phosphorylate Akt at Ser473. In the present study, long-term insulin stimulation phosphorylated Akt more than short-term stimulation. Accordingly, long-term, but not short-term, insulin stimulation elevated proteasome activity. Long-term insulin stimulation enhanced proteasome activity concentration-dependently.

So far, the relationship between insulin signaling and the ubiquitin-proteasome system has remained insufficiently investigated. Bennett et al. previously reported that insulin reduces proteasome activity by dissociating an insulin-degrading enzyme from the proteasome (Bennett, Hamel, and Duckworth 2000). In this study, we discovered a novel pathway through which insulin inhibits ubiquitination independent of its effect on proteasome activity. In our comprehensive human hepatic gene expression database (Misu et al., 2007, 2010; Takamura et al., 2008) (Table 1), we found that insulin signaling is associated with hepatic expression of *USP14* (Figure 2). Indeed, long-term than short-term insulin stimulation reduces the accumulation of ubiquitinated proteins via activating USP14 in hepatocytes (Figure 3A-E). On the other hand, insulin-mediated proteasome activation was not mediated by USP14 activity (Figure 4A-D). To our knowledge, this is the first study to show that insulin signaling plays a critical role in inhibiting ubiquitination through the activation of USP14 in hepatocytes (Figure 9).

Next, we investigated metabolic outcomes affected by the insulin-mediated inhibition of ubiquitination. Insulin induces ER stress (Birkenfeld et al. 2011). USP14 inhibits ER-associated degradation and is involved in ER stress (Nagai et al. 2009). In addition, we previously reported that proteasome dysfunction results in ER stress in the liver (Otoda et al. 2013a). In the present study, insulin elevated ER stress in hepatocytes, which was suppressed by IU1, suggesting that USP14 activity is involved in insulin-induced
ER stress in hepatocytes (Figure 5A-D). Given that insulin has a protein anabolic action, it is reasonable that insulin suppresses ubiquitination via activating USP14 and independently activates proteasome function.

We previously observed the increased mature SREBP-1c protein in the nucleus in the liver of mice with proteasome dysfunction (Otoda et al. 2013a). SREBP-1c in the nucleus is degraded by the ubiquitin-proteasome system (Botolin et al. 2006b; Hirano et al. 2001b; Sundqvist et al. 2005b). In the absence of insulin, activated glycogen synthase kinase 3 phosphorylates SREBPs (Kim et al. 2004). The phosphorylation helps ubiquitin ligase SCF^{Fbw7} to bind SREBPs, which promotes ubiquitination and 26S proteasome-mediated degradation of SREBPs (Sundqvist et al. 2005b). In the present study, the USP14 inhibitor IU1 did not directly affect proteasome activity (Figure 4A). Nevertheless, IU1 elevated the insulin-induced accumulation of mature SREBP-1c in the nucleus (Figure 6A). This effect might be an off-target effect of IU1. Alternatively, it may be possible that insulin-activated USP14 inhibits SREBP-1 nuclear translocation, the underlying mechanisms of which should be investigated in the future.

Despite the increased SREBP-1c protein levels in the nuclear fraction, insulin treatment for 2 hours upregulated \textit{Fasn} expression but not other lipogenic gene expressions such as \textit{Acaca} and \textit{Scd1}. So far, it remains unclear how IU1 regulates lipogenic gene expression in H4IIEC3 hepatocytes (Figure 7A). In addition, SREBP-1c negatively regulates gluconeogenic genes through a cross-talk with HNF-4α interference with PGC-1 recruitment (Ruiz et al. 2014b; Yamamoto et al. 2004). In agreement with these findings, SREBP-1c may be involved in the insulin- and IU1-mediated downregulation of gluconeogenic \textit{PCK-1} and \textit{G6pc} genes (Figure 8A-C). USP14 activation by long-term insulin reduces mature SREBP-1c in the nucleus and elevates ER stress and gluconeogenic gene expression via deubiquitination (Figure 9). Xu et al. reported that USP14 interacts not only with proteasome but also with lower molecular weight proteins and that USP14 has some phosphorylation sites other than S432 mainly enhanced by phosphorylated Akt (Ser473) (Xu, Shan, Lee, Zhu, Zhang, Sun, Liu, Shi, et al. 2015b). In the present study, USP14 was upregulated in the liver of people with insulin resistance (Figure 2) and may directly enhance hepatic gluconeogenesis. IU1 strongly suppressed gluconeogenesis in cell experiments (Figure 8A). These results suggest the possibility that USP14 is directly involved in the upregulation of gene expression in liver glucose metabolism.

Finally, in the present study, chronic treatment with insulin elevated proteasome activity even though it reduced ubiquitinated proteins through USP14 activation in hepatocytes (Figure 1C-D, 3A-E, and 4A-E). Possibly, ubiquitination and proteasome activity may occur independently of each other (Orlowski & Wilk, 2003). In particular, proto-oncoproteins and oncosuppressive proteins are known to be ubiquitin-independent proteasome substrates (Hwang et al., 2011; Jariel-Encontre et al., 2008).

In summary (Figure 9), insulin activates proteasome function even though it inhibits protein ubiquitination by activating USP14 in hepatocytes. Furthermore, USP14 activation by insulin inhibits mature SREBP-1c while upregulating ER stress and the expression of genes involved in gluconeogenesis.
Further understanding mechanisms underlying the USP14 activation and its pleiotropic effects may lead to therapeutic development for obesity-associated metabolic disorders such as diabetes and fatty liver disease.

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Conflict of Interest
The authors declare that there are no conflicts of interest with the contents of this article.

Authorship Contributions:
Participated in research design: Kamoshita, Ishii, Tahira, Kikuchi, Matsumoto, Takamura.
Conducted experiments: Kamoshita, Ishii, Tahira, Kikuchi, Abuduwaili.
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Wrote or contributed to the writing of the manuscript: Kamoshita, Ishii, Tahira, Takamura.

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Foretz, Marc et al. 1999. “MOLECULAR AND CELLULAR BIOLOGY ADD1/SREBP-1c Is Required in the Activation of Hepatic Lipogenic Gene Expression by Glucose.”


**ABBREVIATIONS:** ubiquitin-specific protease 14 (USP14); ER, endoplasmic reticulum; SREBP-1c, sterol regulatory element binding protein-1c; FASN, fatty acid synthase; FOXO, forkhead box o; PIPs proteasome-interacting proteins; HOMA-IR, homeostatic model assessment-insulin resistance; HbA1c, hemoglobin A1c; FPG, fasting plasma glucose; MCR, metabolic clearance rate; ACACA, acetyl-CoA carboxylase alpha; SCD, stearoyl-CoA desaturase; SREBF1, sterol regulatory element binding transcription factor 1

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**Figure Legends**

**Fig. 1.** Long-term insulin stimulation elevates proteasome activity. (A) Schematic view of the representation of insulin treatment condition. H4IIEC3 hepatocytes were cultured with or without 100 nM insulin for 24 h (long-term exposure), and then they were stimulated by 1 ng/ml insulin again for 15 min (short-term stimulation). The groups of cells were named (a)-(f) according to the kind of insulin treatment. (B) Western blotting analysis of p-Akt levels. The values from densitometry of four independent experiments were normalized to the level of total Akt and expressed as the mean fold increase over
control as cells of the group (a) (n = 4). (C) Chymotrypsin-like, trypsin-like, and caspase-like proteasome activity assay (n = 4). (D) Concentration-dependent proteasome activity during 2-hour insulin treatment (n = 4). * p < 0.05, ** p < 0.01.

Fig. 2. (A) USP14 activation was responsible for the insulin-mediated inhibition of the ubiquitin-proteasome system. A: Explanatory diagram showing that USP14 regulates proteolysis of the ubiquitin-proteasome system. 26S proteasome is composed of PA700 and 20S proteasome. A ubiquitinated protein as a substrate first binds the PA700 due to its ubiquitin chains and then is translocated to the 20S proteasome, which has the chymotrypsin-like activity and degrades it. USP14 activity helps the substrate to escape from the 26S proteasome because of the deubiquitinating activity and has the proteolysis inhibited. (B) Hepatic USP14 mRNA levels had a negative correlation with insulin sensitivity in humans. Liver biopsy specimens were obtained from 12 human subjects (seven subjects with type 2 diabetes, ●; and five subjects without diabetes, ○), and we used DNA chip analysis and performed glucose clamp experiments on these subjects. Spearman's correlation coefficient (r) = -0.734; p = 0.007 (two-sided test). MCR: metabolic clearance rate of glucose. (C) USP14 expression vs. ACACA expression: Spearman's correlation coefficient (r) = 0.373; p = 0.036 (two-sided test). USP14 expression vs. SCD expression: Spearman's correlation coefficient (r) = 0.430; p = 0.014 (two-sided test). USP14 expression vs. SREBF1 expression: Spearman's correlation coefficient (r) = 0.441; p = 0.012 (two-sided test).

Fig. 3. Insulin suppresses ubiquitination via USP14. (A) The amount of ubiquitinated proteins accumulated in the H4IIEC3 hepatocytes treated for 2 h with or without 100 nM insulin, 75 μM IU1, and 50 nM bortezomib. After treatment, proteins were extracted and, western blotting was conducted (n = 4). (B) Knockdown of Usp14 using siRNA in H4IIEC3 hepatocytes (n = 4). (C) Quantification of ubiquitinated proteins in H4IIEC3 hepatocytes using Usp14 siRNA #3 (n = 4). (D) Quantification of ubiquitinated proteins in HepG2 hepatocytes treated for 2 h with or without 1 μM insulin, 75 μM IU1, and 50 nM bortezomib (n = 4). (E) Quantification of ubiquitinated proteins in Hepa1-6 hepatocytes treated for 2 h with or without 100 nM insulin, 75 μM IU1, and 50 nM bortezomib (n = 4).

Fig. 4. USP14 inhibitor IU1 did not affect proteasome activity by insulin. (A) The chymotrypsin-like activity of the 20S and 26S proteasome in the H4IIEC3 hepatocytes treated for 2 h with or without 100 nM insulin, 75 μM IU1, and, 50 nM bortezomib. After native-PAGE was conducted to separate 26S proteasome from 20S proteasome, Suc-LLVY-AMC hydrolysis by proteasome in the gel was used to visualize the activity. The values from densitometry of four independent experiments were expressed as the mean fold increase over as control proteasome 20S human purified protein 1 μg. (B) Quantification of proteasome activity in H4IIEC3 hepatocytes using Usp14 siRNA #3
(n = 4). (C) Quantification of proteasome activity in HepG2 hepatocytes treated for 2 h with or without 1 μM insulin, 75 μM IU1, and 50 nM bortezomib (n = 4). (D) Quantification of proteasome activity in Hepa1-6 hepatocytes treated for 2 h with or without 100 nM insulin, 75 μM IU1, and 50 nM bortezomib (n = 4). * p < 0.05, ** p < 0.01. IU1: a specific inhibitor of USP14's catalytic activity; bortezomib (Bor): a proteasome inhibitor.

Fig. 5. USP14 activity was related to ER stress. (A) ER stress markers including phosphorylated IRE1α (p-IRE1α), CHOP, and BiP in western blotting. H4IIEC3 hepatocytes were treated with 100 nM insulin, 75 μM IU1, and 50 nM bortezomib for 2 h. The extracts were subjected to SDS-PAGE and immunoblotting. (B) Quantification of ER stress markers in H4IIEC3 hepatocytes using Usp14 siRNA #3. (C) Quantification of ER stress markers in HepG2 hepatocytes treated for 2 h with or without 1 μM insulin, 75 μM IU1, and 50 nM bortezomib (n = 4). (D) Quantification of ER stress markers in Hepa1-6 hepatocytes treated for 2 h with or without 100 nM insulin, 75 μM IU1, and 50 nM bortezomib (n = 4).

Fig. 6. USP14 activity was related to the SREBP-1c nuclear translocation. (A) Intracellular localization of SREBP-1c in western blotting. H4IIEC3 hepatocytes were treated with 100 nM insulin and 75 μM IU1 for 2 h. The extracts were subjected to SDS-PAGE and immunoblotting. (B) Quantification of SREBP-1c intracellular localization in H4IIEC3 hepatocytes using Usp14 siRNA #3. The values from densitometry of four independent experiments were expressed as the mean fold increase over insulin-free cells as controls. * p < 0.05, ** p < 0.01. IU1, a specific inhibitor of USP14's catalytic activity; bortezomib (Bor), a proteasome inhibitor; T0901317, liver X receptor (LXR) agonist.

Fig. 7. Effect of USP14 on lipogenic gene expression. (A) H4IIEC3 hepatocytes were treated with 100 nM insulin and 75 μM IU1 for 2 h. Fasn, Acaca, and Scd1 gene expression in H4IIEC3 hepatocytes (n = 4). Acc1 is encoded by Acaca. Fas is encoded by Fasn. Scd1 is encoded by Scd1. Expression values were normalized to Gapdh mRNA expression. (B) Quantifying lipogenic enzyme gene expression in H4IIEC3 hepatocytes using Usp14 siRNA #3. (C) Quantifying lipogenic enzyme gene expression in HepG2 hepatocytes treated for 2 h with or without 1 μM insulin, 75 μM IU1, and 50 nM bortezomib (n = 4). ACC1 is encoded by ACACA. FAS is encoded by FASN. SCD is encoded by SCD. Expression values were normalized to GAPDH mRNA expression. (D) Quantifying lipogenic enzyme gene expression in Hepa1-6 hepatocytes treated for 2 h with or without 100 nM insulin, 75 μM IU1, and 50 nM bortezomib (n = 4).

Fig. 8. USP14 activity contributed to the activation of gluconeogenic gene expression. (A) Pck1 and G6pc gene expression in H4IIEC3 hepatocytes (n = 4). PCK-1/PEPCK is encoded by Pck1. G6Pase is encoded by G6pc. Expression values were normalized to Gapdh mRNA expression. (B) Quantifying
gluconeogenic gene expression in H4IIEC3 hepatocytes using Usp14 siRNA #3. (C) Quantifying gluconeogenic gene expression in HepG2 hepatocytes treated for 2 h with or without 1 μM insulin, 75 μM IU1, and 50 nM bortezomib (n = 4). PCK-1/PEPCK is encoded by PCK1. G6Pase is encoded by G6PC. Expression values were normalized to GAPDH mRNA expression. * p < 0.05, ** p < 0.01. IU1, a specific inhibitor of USP14's catalytic activity.

Fig. 9. Scheme of regulatory mechanism for ubiquitination, ER stress, SREBP-1c nuclear translocation, and expression of gluconeogenic gene expression through USP14 activity by insulin in H4IIEC hepatocytes.
Table 1 Clinical characteristics of the study subjects.

<table>
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<tr>
<th></th>
<th>Non-diabetes</th>
<th>Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>21</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>6/5</td>
<td>15/6</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.9 ± 12.7</td>
<td>53.0 ± 9.7*</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.5 ± 4.6</td>
<td>24.4 ± 4.1</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.2 ± 1.1</td>
<td>3.1 ± 2.6</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.1 ± 0.5</td>
<td>7.4 ± 1.6**</td>
</tr>
<tr>
<td>Fasting plasma glucose (mg/dl)</td>
<td>90.1 ± 8.3</td>
<td>143.4 ± 50.0**</td>
</tr>
<tr>
<td>Metabolic clearance rate (mg/kg/min)</td>
<td>7.7 ± 2.2</td>
<td>5.5 ± 2.2</td>
</tr>
</tbody>
</table>

Data are mean ± SD, * p < 0.05 vs. non-diabetes, ** p < 0.01 vs. non-diabetes
Figure 1

A

long-term exposure  
(24 hours)  
short-term stimulation  
(15 minutes)

(a) vehicle  
(b) insulin  
(c) vehicle  
(d) insulin  
(e) vehicle  
(f) insulin

B

p-Akt/t-Akt

**  

C

Chymotrypsin-like activity

**  

Trypsin-like activity

Caspase-like activity

**  

D

Chymotrypsine-like activity

**  

Trypsine-like activity

Caspase-like activity

**  

The figure illustrates the effects of long-term exposure and short-term stimulation on various activities. The results show significant differences in p-Akt/t-Akt, Chymotrypsin-like activity, Trypsin-like activity, and Caspase-like activity across different groups.

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Figure 2

A. Diagram showing the positive and negative regulation of ubiquitinated protein degradation.

B. Scatter plot showing the correlation between USP14 expression and MCR.

C. Scatter plots showing the correlation between USP14 expression and ACACA, SCD, and SREBF1 expression.

- ACACA expression: $r = 0.373$ ($p = 0.036$)
- SCD expression: $r = 0.430$ ($p = 0.014$)
- SREBF1 expression: $r = 0.441$ ($p = 0.012$)
Figure 5

A

CHOP  
p-IRE1α  
Bip  
β-actin

insulin  - + + + +  
IU1  - + + + +  
Bor  - - - - +

B

CHOP  
p-IRE1α  
Bip  
β-actin

insulin  - + + + +  
siRNA  - + + + +  
Bor  - - - - +

C

HepG2

CHOP  
p-IRE1α  
Bip  
β-actin

insulin  - + + + +  
IU1  - + + + +  
Bor  - - - - +

D

Hepa 1-6

CHOP  
p-IRE1α  
Bip  
β-actin

insulin  - + + + +  
IU1  - + + + +  
Bor  - - - - +
Figure 7

(A) *Fasn*, *Acaca*, and *Scd1* mRNA expression in response to insulin and IU1 treatments in HepG2 cells.

(B) *Fasn*, *Acaca*, and *Scd1* mRNA expression in response to insulin and siRNA treatments in HepG2 cells.

(C) mRNA expression of *FASN*, *ACACA*, and *SCD* in HepG2 and Hepa1-6 cells under insulin and IU1 treatments.

(D) *Fasn*, *Acaca*, and *Scd1* mRNA expression in response to insulin and IU1 treatments in Hepa1-6 cells.

Legend:
- **: p = 0.097
- *: p = 0.062
- **: p = 0.091

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Figure 8

A

Pck1

G6pc

mRNA expression

mRNA expression

insulin - - + +
IU1 - + - +

B

Pck1

G6pc

mRNA expression

mRNA expression

insulin - - + +
siRNA - + - +

C

HepG2

PCK1

G6PC

mRNA expression

mRNA expression

insulin - - + +
IU1 - + - +

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Figure 9

USP14

Insulin → Proteasome

pUSP14

IU1

Protein deubiquitination

ER stress   SREBP-1c   Gluconeogenesis