Proteasome Dysfunction Mediates Obesity-Induced Endoplasmic Reticulum Stress and Insulin Resistance in the Liver

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Chronic endoplasmic reticulum (ER) stress is a major contributor to obesity-induced insulin resistance in the liver. However, the molecular link between obesity and ER stress remains to be identified. Proteasomes are important multicatalytic enzyme complexes that degrade misfolded and oxidized proteins. Here, we report that both mouse models of obesity and diabetes and proteasome activator (PA)28-null mice showed 30–40% reduction in proteasomal activity and accumulation of polyubiquitinated proteins in the liver. PA28-null mice also showed hepatic steatosis, decreased hepatic insulin signaling, and increased hepatic glucose production. The link between proteasome dysfunction and hepatic insulin resistance involves ER stress leading to hyperactivation of c-Jun NH2-terminal kinase in the liver. Administration of a chemical chaperone, phenylbutyric acid (PBA), partially rescued the phenotypes of PA28-null mice. To confirm part of the results obtained from in vivo experiments, we pretreated rat hepatoma-derived H4IEC3 cells with bortezomib, a selective inhibitor of the 26S proteasome. Bortezomib causes ER stress and insulin resistance in vitro—responses that are partly blocked by PBA. Taken together, our data suggest that proteasome dysfunction mediates obesity-induced ER stress, leading to insulin resistance in the liver.

Obesity is a major cause of insulin resistance and contributes to the development of type 2 diabetes (1). Growing evidence suggests that chronic endoplasmic reticulum (ER) stress in the liver is a major contributor to obesity-induced insulin resistance (2–4). However, the molecular mechanisms linking obesity and ER stress are not fully understood.

We previously identified metabolic pathways that are significantly altered by obesity in the livers of people with type 2 diabetes by analyzing comprehensive gene expression profiles using DNA chips (5). We found that genes involved in ubiquitin-proteasome pathways were coordinately upregulated in obese individuals. Proteasomes play fundamental roles in processes that are essential for cell viability (6).

Eukaryotic cells contain several types of proteasomes. Core 20S proteasomes (20S) have binding sites for the regulatory particles proteasome activator (PA)700 and PA28 (7). PA28 is found in both previously described PA28–20S–PA28 complexes and PA700–20S–PA28 complexes, which also contain PA700 (8). The PA28 family comprises three members: α, β, and γ. PA28α encoded by the PSME1 gene and PA28β encoded by the PSME2 gene form a heteropolymer, which is mainly located in the cytoplasm, whereas PA28γ encoded by the PSME3 gene forms a homopolymer that predominantly occurs in the nucleus (9). The association of the PA28 with the 20S may play a role in antigen processing by modulating peptide cleavage in the 20S (10,11), but it appears that the PA28 may play a greater role in intracellular protein degradation than in antigen processing (12). Recently, it was reported that PA28α overexpression enhances ubiquitin-proteasome system–mediated degradation of abnormal proteins (13).

It has been reported that fatty acids, insulin (14), and oxidative stress (15) inhibit proteasome activity in cultured hepatocellular carcinoma (Hep)G2 cells. However, it remains to be determined whether liver proteasome function is dysregulated in obesity and type 2 diabetes.

Based on these findings, we hypothesized that proteasome dysregulation in the liver is involved in the development of hepatic insulin resistance in obesity and type 2 diabetes. To test this hypothesis, we generated PA28α,-PA28β,PA28γ triple-knockout (PA28 KO) mice as a model of impaired proteasome function and investigated their metabolic phenotypes.

RESEARCH DESIGN AND METHODS

Human studies. This study was approved by the ethics committee of Kanazawa University. Liver biopsy specimens were obtained from 21 patients with type 2 diabetes (15 men and 6 women; mean age 53.0 ± 2.1 years, BMI 24.4 ± 0.9 kg/m2, fasting plasma glucose 7.94 ± 0.59 mmol/L, HbA1c 7.3 ± 0.3%, and alanine aminotransferase 34.4 ± 5.5 IU/L) admitted to Kanazawa University Hospital between 2000 and 2003 as previously described (5,16). Statistical analyses of DNA chip gene expression data were performed as previously described.
FIG. 1. Proteasome dysfunction in mouse models of obesity. A: Gene expression and proteasome activity in the livers of patients with type 2 diabetes and obesity. Coordinate upregulation of genes involved in proteasome degradation pathways in the livers of type 2 diabetic patients with obesity compared with those without obesity. GenMAPP (Gene MicroArray Pathway Profiler [http://www.genmapp.org]) was used to annotate the pathway with expression ratios for the genes involved. The fold changes presented beside the gene names are for obese versus nonobese patients. Genes significantly upregulated in obesity ($P < 0.05$) are shown in red; genes analyzed whose expression was not significantly altered in obesity are shown in gray.

B: Graphs showing associations between homeostasis model assessment of insulin resistance (HOMA-IR) and the mRNA expression of subunits of PA28: PA28$^a$ (PSME1), PA28$^b$ (PSME2), and PA28$^g$ (PSME3). Data were normalized according to 18S mRNA level.

C: Comparison of liver proteasome activity in 32-week-old C57BL6 mice fed STD and HFD for 28 weeks (from 4 to 32 weeks of age), in WT and $db/db$ mice (20 weeks old), and in WT mice and $ob/ob$ mice (20 weeks old). Proteasome activity was estimated by measuring chymotrypsin-like activity.

D: Immunoblotting for ubiquitinated proteins.
Animal experiments. PA28 KO mice were generated by cross-breeding PA28α−/− PA28β−/− double-KO mice (18) and PA28y KO mice (19). Genetic background of both lines of KO mice is almost completely homologous to that of C57BL/6J mice because they were backcrossed to C57BL/6J mice for at least eight generations. C57BL/6J mice and db/db mice were obtained from Sankyo Laboratory Services (Tokyo, Japan), and genetically diabetic male db/db mice were obtained from Charles River (Tokyo, Japan). The study protocol was reviewed and approved by the animal care and use committee of Kanazawa University. All mice were housed in specific pathogen-free facilities, maintained under a 12-h light/dark cycle, fed a standard rodent food diet (Oriental Yeast) (STD) or rodent food containing 60% fat (Research Diet) (HFD) for up to 28 weeks, and provided with water ad libitum. Sodium 4-phenylbutyrate (PBA, Enzo Life Sciences, Farmingdale, NY) was mixed in drinking water at a concentration of 4 mg/mL and administered ad libitum for 3 weeks as previously described (20).

Gene expression analyses in mice. We used Genopat DNA chips (21) (Mitubishi Rayon, Tokyo, Japan [http://www.mrr.co.jp-genome/e/index.html]) and RT-PCR to identify significant changes in hepatic gene transcription. Total RNA was isolated from C57BL/6J Mini Kit (Tokyo, Japan) and converted into cDNA using a cDNA synthesis kit (Applied Biosystems, Tokyo, Japan) as previously described (22). Quantitative real-time PCR analysis was performed on an ABI Prism 7000 Sequence Detection System (Invitrogen) using TaqMan gene expression assays (Applied Biosystems) or the SYBR Green Master Mix (Takara Bio, Otsu, Japan) as described previously (23). The primers for mouse α1-acid glycoprotein (Acg1) were as follows: forward, 5′-TGGAGGAGCCCAACACACA-3′; reverse, 5′-TGGACTGACGTGGCAGAAAG-3′. The primers for mouse X-box binding protein 1 (Xbp1) were as follows: forward, 5′-AACAGAAGTGACCGCAGACAGTCG-3′; reverse, 5′-GAGGTCTTAACTAGGGCTTGTG-3′. The primers for mouse C/EBP homologous protein (Chop) were as follows: forward, 5′-TATCTAACCCCAGGAAACG-3′; reverse, 5′-GGGACCTGACACTGTGTTT-3′. TaqMan gene expression assays (Applied Biosystems) were used for Psmes1 (Hs00396820_m1), Psmes2 (Hs01501608_g1), Psmes3 (Hs01501267_m1), Psmes4 (Hs00990651_m1), Psmes5 (Mm00966949_m1), Psmes6 (Mm00975196_m1), Psmes7 (Mm01247058_m1), and glucose-6-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology) was used as a control for protein loading.

Electron microscopy. Liver samples were fixed in 4% buffered formalin and embedded in Tissue-Tek OCT compound (Sakura Finetek USA) and paraffin for histological analysis. The formalin-fixed and paraffin-embedded section (5 μm) was processed routinely for hematoxylin-eosin staining. Ultrastructural evaluation using electron microscopy (JEOL, Tokyo, Japan) was used to further confirm the morphological features of the liver samples. Liver sections of control mice were processed as described above and served as a control. Liver sections were examined using a JEM-1200 electron microscope (JEOL, Tokyo, Japan).

RESULTS

Expression of genes involved in proteasome pathways are altered in the livers of patients with type 2 diabetes and obesity. We previously identified metabolic pathways that are altered in association with obesity in the livers of
FIG. 2. PA28 KO mice show impaired proteasome function and accumulation of polyubiquitinated proteins in the liver. A: Mice were genotyped by PCR analysis as previously described (18,19). Genomic DNA extracted from mouse tails was analyzed by PCR. Primers used for genotyping were 5′-TTTCCTGTACGTGACTTCCATCCTGTTG-3′ (primer 1), 5′-GGTCCACATACAATAAAGACATGGGCTG-3′ (primer 2), and 5′-GATTGTGGTCCTCCTGCAACGCCTAAA-3′ (primer 3). Primers 1 and 3 amplified 1.2-kb fragments from the wild-type PA28a allele. Primers 1 and 2 amplified 2.2-kb fragments from the mutant allele. Primers used for genotyping were 5′-CCGGGACAATAAGACACATCACTC-3′ (primer 1), 5′-TTGTCCCTCCCTCCAGTCTAA-3′ (primer 2), and 5′-GATCCCCTCAGAAGAACTCGTCAA-3′ (primer 3). Primers 2 and 3 amplified 0.9-kb fragments from the wild-type PA28g allele. Primers 1 and 3 amplified 1.9-kb fragments from the mutant allele. B: RT-PCR analysis of total RNA prepared from liver of PA28 KO mice (n = 7). Transcripts of mouse PA28a, -b, and -g were examined by quantitative RT-PCR. Data were normalized according to GAPDH mRNA level and presented as a value relative to that for WT mice. C: Western blot analysis of extracts prepared from the liver of PA28 KO mice. The blot was probed with the anti-PA28a, anti-PA28b, and anti-PA28γ antibodies. D: Body weights of WT and PA28 KO mice fed an STD or the HFD. ○, WT mice fed the STD (n = 5); ■, WT mice fed the HFD (n = 4); □, PA28 KO mice fed the STD (n = 4); ▲, PA28 KO mice fed the HFD (n = 4). Data represent means ± SE. E: Comparison of liver proteasome activity in WT (n = 13, STD; n = 4, HFD) and PA28 KO (n = 11, STD; n = 4, HFD) mice. Data represent means ± SE. *P < 0.05. F: Proteasome activity was evaluated in the muscles isolated from 32-week-old WT mice (n = 5) and PA28 KO mice (n = 4). The activity was normalized to the total protein content. Data represent means ± SE. *P < 0.05. G: Western blot analyses of total ubiquitinated proteins in the livers of WT and KO mice. Livers were isolated from 32-week-old WT and PA28 KO mice fed the STD or the HFD for 28 weeks (from 4 to 32 weeks of age). Quantitation of ubiquitinated proteins levels is normalized to GAPDH and is represented as means ± SE. *P < 0.05.
FIG. 3. PA28 KO mice show proteasome dysfunction, glucose intolerance, and attenuated insulin signaling in the liver. A: Blood glucose concentrations and serum insulin levels measured in an overnight fasting state at 20 weeks of age. WT (n = 9, STD; n = 5, HFD) and PA28 KO (n = 6, STD; n = 4, HFD) mice. Data represent means ± SE. *P < 0.05. B: Intraperitoneal glucose tolerance tests (IPGTT). C: Intraperitoneal insulin tolerance tests (IPITT). Blood glucose level measured during the intraperitoneal glucose and insulin tolerance tests at 20 weeks of age. WT mice fed the STD, n = 5; WT mice fed the HFD, n = 4; PA28 KO mice fed the STD, n = 4; PA28 KO mice fed the HFD, n = 4. Data represent means ± SE. *P < 0.05. **P < 0.01. D: Insulin sensitivity was assayed by using a hyperinsulinemic-euglycemic clamp study in WT (n = 8) and PA28 KO (n = 10) mice. Left panel: Glucose infusion ratio. Middle panel: HGP before and after insulin clamp. Right panel: Glucose disposal rate. Data represent means ± SE. *P < 0.05. E: Equal amounts of protein in total lysates of liver and muscle were immunoblotted (IB) with anti-p-Akt (Ser473) and anti-Akt antibodies. p-Akt values of insulin-injected fasted mice were displayed relative to those of saline-injected mice. L-liver; M—muscle.
patients with type 2 diabetes (5,16). Clinical characteristics of study obese and nonobese subjects are shown in Supplementary Table 1. The hepatic expression of genes involved in a proteasome pathway, the gene encoding PA28α (P < 0.05), was coordinately upregulated in diabetic patients who were obese compared with those who were not obese (Fig. 1A). To further strengthen the significance of the hepatic expression of the genes for PA28s, we analyzed an independent new sample set of the human liver using RT-PCR. Clinical information of the subjects is described in Supplementary Table 2. As shown in Fig. 1B, mRNA levels of genes encoding PA28α, -β, and -γ in the human liver correlated positively with an insulin resistance index (homeostasis model assessment of insulin resistance). Therefore, hepatic expression of PA28s was associated with insulin resistance in humans. We next analyzed hepatic gene expression in a mouse model of diet-induced obesity (HFD mice) using a custom-made array, Metabolic Chip (21). Consistent with human data, the hepatic expression of genes involved in the proteasome pathway was coordinately upregulated in C57BL/6 mice fed the HFD compared with those fed the STD. Of these, the gene encoding PA28β (Psm2) was significantly upregulated in mice fed the HFD compared with those fed the STD (Supplementary Table 3). These findings prompted us to test the hypothesis that proteasome degradation pathways are involved in the development of obesity-induced insulin resistance in the liver.

**Liver proteasome activity is reduced in animal models of type 2 diabetes and obesity.** Next, we examined liver proteasome activity in animal models of type 2 diabetes and obesity. Proteasome activity was measured using a substrate for chymotrypsin-like activity. Unexpectedly, liver proteasome activity was reduced by ~30–40% in genetically obese ob/ob mice, diabetic db/db mice, and C57BL/6 mice fed the HFD (Fig. 1C). As a consequence, accumulation of ubiquitinated proteins was increased in the liver of C57BL/6 mice fed the HFD, db/db mice, and ob/ob mice (Fig. 1D). These results suggest that liver proteasome activity is reduced in animal models of type 2 diabetes and obesity. Coordinate upregulation of genes involved in the ubiquitin-proteasome pathway in obese patients and mouse models of type 2 diabetes and obesity may compensate for impaired proteasome function.

**PA28 KO mice show impaired proteasome function and accumulation of polyubiquitinated proteins in the liver.** To test the hypothesis that proteasome dysregulation in the liver is involved in the development of hepatic insulin resistance in obesity and type 2 diabetes, we generated a mouse model of impaired proteasome function: PA28 KO mice. We first performed a genomic DNA PCR analysis to confirm that the mutant mice expressed the messages of neither the PA28α nor the PA28γ gene (Fig. 2A). Knockout of the Psmel, Psm2, and Psm3 in the liver of PA28 KO mice was confirmed both by RT-PCR and by Western blotting analyses (Fig. 2B and C). Up to 30 weeks after birth, PA28 KO mice were indistinguishable in appearance and growth from age-matched wild-type (WT) mice (Fig. 2D).

When fed the STD, PA28 KO mice had 35% reduced hepatic proteasome activity compared with WT mice (Fig. 2E). Proteasome activity was also reduced by ~50% in the skeletal muscle of PA28 KO mice compared with that of WT mice (Fig. 2F). These results are consistent with previous observations in mouse embryonic fibroblasts from PA28α−/−/PA28β−/− mice (18). The degree of proteasome inactivation in the livers of PA28 KO mice corresponded with that in the livers of db/db mice and C57BL mice fed the HFD (Fig. 1C and Fig. 2E). Thus, the PA28 KO mouse appears to be an appropriate animal model that mimics the proteasome dysfunction observed in obesity and type 2 diabetes. When fed the HFD, PA28 KO mice tended to have further reduced hepatic proteasome activity compared with WT mice (P = 0.086) (Fig. 2E).

For assessment of the impact of proteasome dysfunction in the liver, polyubiquitinated proteins were detected by Western blotting (Fig. 2G). The HFD increased the accumulation of ubiquitinated proteins in the liver in both WT and PA28 KO mice. As expected, more polyubiquitinated proteins accumulated in the livers of PA28 KO mice compared with those of WT mice for both STD-fed and HFD-fed mice. Accumulation of the ubiquitinated proteins in the liver of PA28 KO mice fed HFD was significantly increased compared with those fed STD, suggesting that HFD further impairs proteasome function in PA28 KO mice.

**PA28 KO mice show glucose intolerance and attenuated insulin signaling in the liver.** To examine the metabolic effects of proteasome dysfunction in vivo, we performed glucose and insulin loading tests. Fasting glucose levels were similar in PA28 KO mice and WT mice (Fig. 3A). However, PA28 KO mice showed glucose intolerance after glucose loading (Fig. 3B), which was further exacerbated by the HFD (Fig. 3B). While serum insulin levels were significantly higher in PA28 KO mice compared with WT mice (Fig. 3A), there was no significant difference in insulin tolerance test results (Fig. 3C), suggesting the existence of hepatic insulin resistance in the PA28 KO mice.

Because the PA28 KO mouse appears to be a model that mimics the proteasome dysfunction observed in HFD-fed mice, subsequent experiments were performed only in mice fed the STD. To identify the responsible organ that contributes to insulin resistance in PA28 KO mice, we next performed hyperinsulinemic-euglycemic clamp experiments and Western blot analysis of the insulin-signaling pathway. As shown in Fig. 3D, endogenous glucose production was significantly increased in PA28 KO mice, whereas peripheral glucose disposal tended to be increased. In the liver of PA28 KO mice, insulin-induced phosphorylation of Akt at Ser473 was impaired (Fig. 3E) in association with marked reduction in IRS-2 protein levels (Fig. 3F) and enhanced serine phosphorylation of IRS-1 compared with WT mice (Fig. 3G). Insulin-induced increment in IRS-1–associated p85 subunit of PI3K was slightly impaired in the liver of PA28 KO mice compared with that of WT mice (Fig. 3G). On the other hand, in the skeletal muscle of the PA28 KO mice, insulin-induced phosphorylation of Akt at Ser473 was rather enhanced.

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**mice (WT mice fed the STD, n = 7; PA28 KO mice fed the STD, n = 7). Data represent means ± SE. *P < 0.05. F: Expression of mRNAs encoding Irs2 in the livers of 12-week-old WT and PA28 KO mice analyzed by quantitative real-time RT-PCR. Expression values were normalized to 18S mRNA. Data represent means ± SE (n = 7 per group). *P < 0.05. Liver lysates from mice were immunoblotted with anti–IRS-2 antibody. G: Liver lysates from mice were immunoprecipitated (IP) using anti–IRS-1 antibody bound to protein A agarose. The immunoprecipitates were immunoblotted with anti–p-IRS-1 (Ser307) and p85. Representative results from five mice of each genotype are shown. Right panel: densitometry quantitation of IRS-1 to p85 signal ratio is shown. Data represent means ± SE. *P < 0.05.**
compared with that of WT mice (Fig. 3E). These findings indicate that PA28 deficiency impairs insulin signaling mainly in the liver and thereby induces systemic glucose intolerance in vivo.

**PA28 KO mice show hepatic steatosis.** In insulin-resistant states, the hyperinsulinemia drives hepatic lipogenesis via a SREBP-1c pathway (26). Oil-red O staining of liver tissue sections revealed a slight hepatic steatosis in
the PA28 KO mice fed STD (Fig. 4A). Triglyceride content was significantly increased in the liver of PA28 KO mice (Fig. 4B). To understand the molecular basis underlying the enhanced hepatic steatosis in PA28-null livers, we analyzed gene expression of SREBP-1c that regulates lipid biosynthesis. As shown in Fig. 4C, expression of Srebf1 was modestly increased in the PA28-deleted livers compared with control livers. Furthermore, the levels of cleaved/active SREBP1c were much more increased in the liver of the PA28 KO mice compared with that of WT mice (Fig. 4D). Indeed, mRNA expression of Acc1, a target of SREBP-1c, was upregulated in the liver of the PA28 KO mice compared with that of WT mice (Fig. 4C). Nuclear SREBP-1c, but not membrane-bound precursor of SREBP-1c, was significantly increased in the livers of PA28 KO mice compared with those of WT mice (Fig. 4D).

**PA28 deficiency–induced proteasome dysfunction activates an unfolded protein response and increases ER stress in the liver.** To investigate the mechanisms underlying proteasome dysfunction–induced insulin resistance in the liver, we evaluated morphological changes in the liver through histological examination. Hematoxylin-eosin staining of liver tissues showed that hepatocytes were indistinguishable in the two groups (Fig. 5A). Next, we examined morphological changes in organelles within hepatocytes by electron microscopy. Electron micrographs revealed massive expansion of the ER in the livers of PA28 KO mice (Fig. 5B), suggestive of an unfolded protein response (UPR) (27). ER stress is caused by the accumulation of unfolded and misfolded proteins in the ER lumen and is associated with several human diseases (28,29). In addition, proteasome inhibitors have been reported to induce ER stress in cultured primary rat hepatocytes (30). Therefore, to examine whether PA28 deficiency–induced proteasome dysfunction causes ER stress in the liver, we analyzed the expression patterns of several molecular indicators of ER stress in liver extracts from 12-week-old WT and PA28 KO mice fed the STD (Fig. 5C and D). Proteasome dysfunction induced by PA28 gene deletion resulted in ER stress in the liver tissues of lean mice, as evidenced by increased levels of Grp78, CHOP, p-PERK, p-eIF2α, and p-IRE1α, as well as ER stress–inducible mRNAs encoding CHOP and the spliced form of XBP-1 (XBP-1s), compared with WT mice (Fig. 5C and D). XBP-1s protein amounts in nuclear fractions increased significantly in the liver tissue of PA28 KO mice compared with that of WT mice (Fig. 5C). Hyperactivation of JNK through phosphorylation is another marker of ER stress (31) and plays a role in linking ER stress and insulin resistance (32). Phosphorylation of JNK and its downstream target c-Jun was significantly increased in the livers of PA28 KO mice compared with those of WT mice (Fig. 5B). Based on these findings, it might be possible that general accumulation of polyubiquitinated proteins or accumulation of some specific substrate for PA28s promotes a UPR and ER stress in the livers of PA28 KO mice.

**Proteasome dysfunction–induced ER stress and insulin resistance are partly blocked by a chemical chaperone.** To confirm that proteasome dysfunction causes ER stress and insulin resistance at the cellular level, we pretreated rat hepatoma-derived H4IIEC3 cells with bortezomib, a selective inhibitor of the 26S proteasome. Bortezomib concentration-dependently increased levels of UPR molecular markers, such as BiP, CHOP, and phosphorylated forms of IRE1α and JNK (Fig. 5A). Gluconeogenic genes, such as Pck1 and Igfbp1, which are targets of FoxO1 were upregulated in the livers of PA28 KO mice compared with those of WT mice (Fig. 5C). These findings suggest that impaired degradation of FoxO1 is one of the candidate pathways leading to impaired suppression of gluconeogenic genes and increased hepatic glucose production in PA28 KO mice.

**Proteasome inhibition causes ER stress and insulin resistance in vitro—responses that are partly blocked by a chemical chaperone.** To test the effect of the chemical chaperone PBA on H4IIEC3 cells treated with bortezomib, a selective inhibitor of the 26S proteasome. Bortezomib concentration-dependently increased levels of UPR molecular markers, such as BiP and CHOP, and phosphorylated forms of IRE1α and JNK (Fig. 5A). Bortezomib also reduced insulin-stimulated serine phosphorylation of Akt at Ser473 in H4IIEC3 hepatocytes in a concentration-dependent manner (Fig. 5B). To address whether ER stress is responsible for proteasome dysfunction–induced insulin resistance, we tested the effect of the chemical chaperone PBA on H4IIEC3 cells treated with bortezomib. PBA partly prevented the accumulation of BiP and CHOP, the phosphorylation of JNK and the impaired phosphorylation of Akt at Ser473 (Fig. 5D) induced by bortezomib. These findings indicate that proteasome dysfunction at least partly accelerates insulin resistance via ER stress in hepatocytes.

**DISCUSSION**

Our study shows that liver proteasome activity is reduced by ~30–40% in genetic and dietary models of obesity and diabetes with coordinate upregulation of genes involved in the ubiquitin-proteasome pathway. In concert with our findings, recent systematic analyses of liver tissue in obese mice also revealed increased proteasome components (36,37), which may compensate for impaired proteasome function. To test a hypothesis that proteasome dysfunction may be a primary event that links obesity and insulin resistance in the liver, we established a mouse model of impaired proteasome function by deleting PA28 genes. The livers of PA28 KO mice showed impaired proteasome function similar to that observed in mouse models of...
FIG. 5. Deletion of PA28 genes causes ER stress in the liver. A: Hematoxylin-eosin–stained liver sections from PA28 KO and WT mice. Scale bar, 200 μm. B: Electron microscopic analyses of the ER in livers from WT and PA28 KO mice. The highlighted region of the upper panel is shown at a higher magnification in the lower panel. Scale bars: 10 μm (upper panel) and 1 μm (lower panel). C: Western blotting for the ER stress–associated markers GRP78, CHOP, p-PERK, p-eIF2α, and p-IRE1α in the livers of 12-week-old male WT and PA28 KO mice. Upper right panel: Each expression level was quantified (n = 3 per group). Data represent means ± SE. *P < 0.05. Lower right panel: Western blotting for nuclear spliced form of XBP-1s protein amounts in the livers of 12-week-old male WT (n = 5) and PA28 KO (n = 5) mice. Lamin A/C served as internal control. Data represent means ± SE. *P < 0.05. D: Expression of mRNAs encoding CHOP and XBP-1s in the livers of WT (□) and PA28 KO (■) mice. Expression values were normalized to Actb mRNA. Data represent means ± SE (n = 4–7 per group). *P < 0.05. E: Western blotting for p-JNK, p-c-Jun, and total JNK in the livers of WT and PA28 KO mice.
obesity and type 2 diabetes, accumulation of ubiquitinated proteins and damaged organelles, ER stress, JNK activation, and insulin resistance (Supplementary Fig. 2). A chemical chaperone PBA administration almost completely alleviated proteasome dysfunction–mediated insulin resistance, confirming the critical role of ER stress in the development of insulin resistance under proteasome dysfunction.

Accumulating evidence suggests that obesity promotes ER stress, which is detected as enhanced UPR signaling, that activates JNK and impairs insulin signaling at the level of IRSs in adipose tissue and the liver (2,4). However, the link between obesity and ER stress has remained unclear. We propose that obesity-associated proteasome dysfunction induces ER stress in the liver, as PA28 KO mice showed accumulation of polyubiquitinated proteins and massive expansion of the ER in the liver, probably due to a reduced capacity for proteasome-mediated degradation of ubiquitinated proteins. This is the first in vivo evidence of proteasome dysfunction–induced insulin resistance mediated by ER stress in the liver. Furthermore, we showed that the selective proteasome inhibitor bortezomib increases ER stress and thereby activates JNK in a cultured hepatocyte cell line.

Yang et al. (38) recently reported that hepatic autophagy is downregulated in the livers of ob/ob mice and that defective autophagy in Atg7 KO mice causes ER stress and hepatic insulin resistance. Therefore, it is possible that both proteasome-mediated protein degradation and autophagy-mediated protein degradation are impaired in the livers of obese individuals, further exacerbating ER stress.

Proteasome function seems to be altered differently in different tissues. Streptozotocin-induced hyperglycemia impairs proteasome activity in the liver and kidney (39,40), whereas proteasome activity is enhanced in the wasted muscle of obese diabetic db/db mice (41). Taken together, these results indicate that obesity predominantly induces proteasome dysfunction in the liver. This clarifies the previous finding that ER stress causes insulin resistance in the liver together with the adipose tissue (2) and brain (42,43). Mechanisms underlying enhanced insulin sensitivity in the skeletal muscle of PA28 KO mice should be investigated in the future.

Why obesity impairs proteasome function in the liver remains unclear. One possible link may be overnutrition-induced oxidative stress. Visceral adiposity in obesity causes excessive flux of free fatty acids into the liver via

FIG. 6. Effects of a chemical chaperone, PBA, administration on proteasome dysfunction–induced ER stress and insulin resistance in PA28 KO mice. A–C: WT and PA28 KO mice were administered mixed PBA (4 mg/mL) through drinking water for 3 weeks. A: Electron microscopic analyses of the ER in livers from WT and PA28 KO mice administered orally with or without PBA. The highlighted region of the left panel (magnification ×5,000) is shown at a higher magnification in the right panel (magnification ×20,000). Scale bars: 10 μm (left panel) and 1 μm (right panel). B: IRE1α phosphorylation and CHOP levels in the livers of WT and PA28 KO mice. C: WT and PA28 KO mice fed STD were starved overnight and injected with insulin (10 IU/kg i.p.). Equal amounts of protein in total lysates of liver and muscle were immunoblotted with anti-p-Akt (Ser473) and anti-Akt antibodies. p-Akt values of insulin-injected fasted mice values were displayed relative to those of saline-injected mice. Data represent means ± SE (n = 3 per group). *P < 0.05. t-, total.
the portal vein, resulting in oxidative stress in the liver (44). The saturated fatty acid palmitate induces excessive production of reactive oxygen species in mitochondria, activates JNK, and causes insulin resistance at the level of IRSs in hepatocytes (44,45). In addition, genes involved in oxidative phosphorylation are upregulated in parallel with insulin resistance in patients with type 2 diabetes who are obese compared with those who are not obese (5,16). Severe oxidative stress causes the covalent modification of 20S proteasome subunits, thereby reducing proteasome activity in the liver and in cultured hepatocytes (15). On the other hand, PA28α overexpression protects against oxidative stress in cultured cardiomyocytes, likely through enhancing the removal of oxidized proteins (46). PA28α and PA28β proteins interact with each other. The degradation rate of PA28β was also significantly decreased by PA28α overexpression in cultured cardiomyocytes (46). These findings suggest that obesity-associated mitochondrial reactive oxygen species and oxidative stress may impair proteasome function. Interestingly, expression of genes involved in the proteasome pathway, including PA28 genes, is increased in the liver tissues of obese mice compared with controls. A recent report compellingly demonstrated that upregulation of the immunoproteasome by interferons not only plays a previously recognized role in helping antigen presentation but also facilitates the removal of damaged proteins generated by interferon-induced oxidative stress (47). We speculate that these are compensatory mechanisms because the hybrid proteasome is better equipped to degrade misfolded proteins than is the conventional 20S proteasome. It was previously shown that the association of the 11S, also known as PA28 or REG, increases the peptidase activities of the 20S (48). PA28 KO mice may therefore be a suitable model for the development of therapies for proteasome dysfunction–associated diseases and metabolic abnormalities.

In the current study, PA28 KO mice showed hepatic steatosis associated with upregulated Srebf1 and Acc1 and increased cleaved/active SREBP-1c (Supplementary Fig. 2). There may be a cross-talk between ER stress pathways and hepatic lipogenesis. Lee et al. (49) reported that theIRE1/XBP1 pathway induces expression of critical genes
FIG. 8. The proteasome inhibitor bortezomib (BZ) induces ER stress and insulin resistance in H4IIEC3 cells. A and B: H4IIEC3 cells were treated with the indicated concentrations of bortezomib (in DMEM supplemented with 10% FBS) for 24 h. After washing, cells were serum starved for 16 h and then treated with insulin (1 nmol/L) or phosphate-buffered saline for 15 min. Cells were solubilized, and equal amounts of proteins were analyzed by Western blotting using BiP-, p-IRE1α–, CHOP-, p-JNK–, total JNK (t-JNK), p-Akt–, and total Akt (t-Akt)-specific antibodies. C and D: H4IIEC3 cells were pretreated or not for 24 h with 2 mmol/L PBA and then treated with the indicated concentrations of bortezomib (in DMEM supplemented with 10% FBS) for 24 h. Cells were washed, serum starved for 16 h, and treated with insulin (1 nmol/L) or phosphate-buffered saline for 15 min. Cells were solubilized, and equal amounts of proteins were analyzed by Western blotting using BiP–, CHOP–, p-JNK–, total JNK–, p-Akt–, and total Akt–specific antibodies. Blots of p-Akt were quantitated densitometrically and expressed as ratios to total Akt (n = 4 for each condition). Relative density is mean ± SE fold increase over control. *P = 0.05 vs. treatment with insulin alone.
involved in fatty acid synthesis, such as *Acc1*. In addition, the PERK/eIF2α pathway decreases Insig1 protein translation, which increases cleaved/active SREBP-1c (50). In concert with these reports, we observed enhanced phosphorylation of IRE1α, PERK, and eIF2α and increased protein level of XBP-1s in the liver of PA28 KO mice. Indeed, our results are compatible with a model in which the ubiquitin-proteasome system degrades the amount of the endogenous nuclear SREBP's but not the precursors (51).

XBP-1s directly binds FoxO1 and promotes its protein degradation via the proteasome (52). In the current study, XBP-1s protein was increased in the liver of PA28 KO mice, probably owing to increased phosphorylation of IRE1α, an endonuclease for *XBP1* gene. Even in such condition, FoxO1 protein amounts dramatically increased in total cell lysates as well as in cytoplasmic and nuclear fractions, probably owing to proteasome dysfunction in the liver of PA28 KO mice. In addition, hepatic insulin resistance caused by ER stress/JNK pathway and increased SREBP-1c that downregulates IRS-2 further accumulates FoxO1 in the nucleus, leading to induction of genes involved in gluconeogenesis such as *Pepck1* (Supplementary Fig. 2).

A limitation of the current study is the fact that we cannot rule out the possibility of altered insulin secretion in the PA28 KO mice. In vitro studies suggest that the ubiquitin-proteasome system plays a role in insulin secretion by maintaining the normal function of voltage-dependent calcium channels (53). Recently, the ER-associated degradation (ERAD)/ubiquitin/proteasome system was reported to be compromised in β cells of obese patients with type 2 diabetes (54), which is compatible with our observation in the liver. Therefore, it might be possible that PBA ameliorated proteasome dysfunction–induced inhibition of glucose-stimulated insulin secretion in the current study (Supplementary Fig. 1). Indeed, PBA was reported to ameliorate β-cell dysfunction in nondiabetic obese humans infused with fatty acids (55). On the other hand, proteasome activity is rather enhanced in the skeletal muscle of obese diabetic db/db mice (41). Therefore, future research should involve the tissue-specific regulation of proteasome function in obesity and diabetes.

In conclusion, proteasome function is impaired in obesity, which contributes to the development of hepatic insulin resistance and steatosis via activating JNK and SREBP-1c by ER stress. Proteasome dysfunction also increases total and nuclear FoxO1 that enhances hepatic gluconeogenesis (Supplementary Fig. 2). Therefore, proteasome dysfunction may be a primary event linking obesity and ER stress–induced insulin resistance in the liver.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan (to T.T. and S.K.). S.K. has received research grants from Takeda Pharmaceutical Company. T.T. and S.K. have a collaborative association with Mitsubishi Rayon developing Genopal DNA chips. No other potential conflicts of interest relevant to this article were reported.

T.Oto, researched the data, contributed to discussion, and wrote the manuscript. T.Taka, conceived and designed the experiments, researched the data, contributed to discussion, wrote the manuscript, and reviewed and edited the manuscript. H.M. and T. Ota contributed to discussion.

S.M. contributed to discussion and reviewed and edited manuscript. H.H., H.T., A.K., T.K., K.R.S., F.L., T.Taka., S.K., Kls., Y.K., K.Iw., K-i.K., M.U., Y.T., M.Y., and K.To. researched data. S.I. contributed to discussion. K.Ta. and S.K. contributed to discussion and reviewed and edited manuscript. T.Taka. is the guarantor of this work, had full access to all the data in the study, and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank Maki Wakabayashi, Yoko Hashimoto, and Shima Kitakata of the Department of Disease Control and Homeostasis, Kanazawa University Graduate School of Medical Science, for technical assistance.

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