Dysfunctional Glycogen Storage in a Mouse Model of α_1 -Antitrypsin Deficiency

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Autophagy is an intracellular pathway that contributes to the degradation and recycling of unfolded proteins. Based on the knowledge that autophagy affects glycogen metabolism and that α_1 -antitrypsin (AAT) deficiency is associated with an autophagic response in the liver, we hypothesized that the conformational abnormalities of the Z-AAT protein interfere with hepatocyte glycogen storage and/or metabolism. Compared with wild-type mice (WT), the Z-AAT mice had lower liver glycogen stores ($P < 0.001$) and abnormal activities of glycogen-related enzymes, including acid α -glucosidase ($P < 0.05$) and the total glycogen synthase ($P < 0.05$). As metabolic consequences, PiZ mice demonstrated lower blood glucose levels ($P < 0.05$), lower body weights ($P < 0.001$), and lower fat pad weights ($P < 0.001$) compared with WT. After the stress of fasting or partial hepatectomy, PiZ mice had further reduced liver glycogen and lower blood glucose levels (both $P < 0.05$ compared WT). Finally, PiZ mice exhibited decreased survival after partial hepatectomy ($P < 0.01$ compared with WT), but this was normalized with postoperative dextrose supplementation. In conclusion, these observations are consistent with the general concept that abnormal protein conformation and degradation affects other cellular functions, suggesting that diseases in the liver might benefit from metabolic compensation if glycogen metabolism is affected.

Keywords: autophagy; partial hepatectomy; fasting; glycogen degradation

α_1 -Antitrypsin (AAT), a 52-kD, 394-amino acid single-chain glycoprotein produced primarily by liver hepatocytes, functions as the major circulating inhibitor of neutrophil elastase (1, 2). The common Z-AAT mutation (glu342lys, referred to as "PiZ", for "protease inhibitor Z") results in a systemic deficiency of AAT (1, 2). The consequences of this single amino acid substitution are profound, including emphysema secondary to deficiency of lung antielastase protection and cirrhosis resulting from the toxic effects of the accumulation of the abnormal Z-AAT molecules in the liver (1–5).

The liver disease associated with AAT deficiency is a prototype of conformational diseases, disorders due to misfolding of proteins, with consequent aberrant intermolecular protein aggregation (2–7). In contrast to the normal M-type AAT, which rapidly transits through the endoplasmic reticulum (ER) and is efficiently secreted by hepatocytes, the Z-AAT form of AAT accumulates in the hepatocyte ER secondary to abnormal folding of the Z molecule, resulting in interaction between the reactive center loop of one Z-AAT molecule with the β -pleated

CLINICAL RELEVANCE

These data signal that dysregulation of energy metabolism in patients with α_1 -antitrypsin deficiency may result in a subtle shift in the baseline metabolic state with potential relevance to chronic and critical care of this patient cohort.

sheet of a second, causing loop-sheet polymerization and consequent accumulation of Z-AAT in the ER (3, 4, 8–10).

The understanding of the mechanism of the liver disease associated with AAT deficiency has been significantly advanced by studies of the "PiZ" mouse, a transgenic murine model of the Z form of AAT by Woo and colleagues (11, 12) and Perlmutter and colleagues (13–19). Recent studies demonstrated that Z-AAT accumulation in the ER exhibits a specific cellular response, including initiation of the ER stress response, activation of proapoptotic caspases, and autophagy as one of the major features (5, 16, 18–21). Autophagy is thought to be a specific cellular response activated by ER accumulation of Z-AAT, whereby cytosol and intracellular organelles are first sequestered from the cytoplasm into autophagosomes, allowing them to be degraded subsequently within lysosomes (22, 23). These observations support the general hypothesis that accumulation of misfolded proteins is associated with dysfunction of diverse cellular pathways (3, 4, 24, 25). Based on these concepts and the knowledge that among other changes, autophagy interferes with glycogen storage and/or metabolism (26–29), we hypothesized that the conformational abnormalities resulting in Z-AAT accumulation and consequent cellular response interfere with hepatocyte glycogen storage and/or metabolism, similar to other models of autophagy (26–29). To assess this hypothesis, we evaluated a variety of glycogen-related metabolic parameters in PiZ transgenic mice. The data reveal that PiZ mice have lower liver glycogen stores, lower blood glucose levels, lower body weights, and lower fat pad weights than wild-type mice and abnormal activities of glycogen-related enzymes, including acid α -glucosidase and glycogen synthase. Strikingly, the PiZ mice have reduced survival after partial hepatectomy, but show survival comparable to that of wild-type mice with dextrose supplementation after surgery. Together, these data add to the general concept of cellular metabolic dysfunction resulting from the conformational abnormalities of proteins, and raise the interesting possibility that patients with AAT deficiency might benefit from metabolic compensation of the dysfunction in glycogen metabolism in the liver.

MATERIALS AND METHODS

Experimental Animals

Transgenic PiZ mice with the human Z-AAT gene on a C57Bl/6 background (kindly provided by D. Perlmutter, University of Pittsburgh School of Medicine, Pittsburgh, PA), were maintained on 12-hour dark/light cycles and provided with water and standard rodent diet *ad libitum*. Littermates were separated by sex at the age of 4 weeks. Sex-matched wild-type C57Bl/6 mice (Jackson Laboratories, Bar Harbor,

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ME or Taconic, Inc., Germantown, NY) were used as controls. To confirm expression of human AAT in PiZ mice, blood was collected by tail vein puncture and allowed to clot for 1 hour at 23°C, followed by centrifugation at $10,000 \times g$ for 5 minutes to collect serum. Quantitative assessment of human Z-AAT in mouse serum was performed using an AAT ELISA kit (ALPCO Diagnostic, Windham, NH) with a standard curve generated using a purified human AAT standard (courtesy of M. Brantly, University of Florida, Gainesville, FL) (30).

The high-expressing PiZ mice were identified by high Z-AAT level in the serum ($2.3 \pm 0.5 \mu\text{M}$), compared with low-expressing mice that had an average Z-AAT level of $5.2 \pm 4.9 \text{ nM}$. Z-AAT was not detected in wild-type mice. High Z-AAT-expressing mice were selected for extensive characterization and comparison to wild-type mice as described below. Low Z-AAT-expressing mice received a selective characterization as detailed in the online supplement. Briefly, histologic analysis showed that low Z-AAT-expressing mice had few, if any, periodic acid-Schiff (PAS)-positive globules in the liver; both histologic and biochemical data demonstrated a reduced level of hepatocyte glycogen; and low Z-AAT-expressing mice exhibited good viability after partial hepatectomy (see Table E1 and Figure E1 in the online supplement). In summary, low Z-AAT-expressing mice presented an intermediate phenotype, and therefore high Z-AAT-expressing mice were selected for further analysis in this study.

Tissue Glycogen Stores

To evaluate glycogen stores, liver was harvested from randomly fed wild-type ($n = 8$) and PiZ mice ($n = 8$) under 2% isoflurane anesthesia (Baxter, Deerfield, IL). Tissue was fixed overnight at 4°C in PBS with 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA), and then transferred to 80% ethanol followed by processing through a graded series of alcohols and xylene before paraffin embedding. Sections (5 μm) were deparaffinized and stained with hematoxylin and eosin (H&E) to show gross morphology of the tissue. To evaluate the glycogen content of the tissue, the method of Lillie and Greco was used (31). Glycogen content was indicated by the comparative PAS staining with or without prior amylase treatment (HistoServ, Germantown, MD).

To evaluate quantitative glycogen levels in liver tissue and left gastrocnemius muscle, tissues were harvested from randomly fed wild type ($n = 6$) and PiZ mice ($n = 6$) as described above, frozen quickly in liquid nitrogen and stored at -80°C until further assessment. The methods of Roehrig and Allred (32) were used which described a quantitative evaluation of glycogen levels in muscle and liver tissue using a direct enzymatic procedure. The test is based on incubation of liver homogenate with amyloglucosidase, which degrades glycogen to glucose, and the glucose was then determined enzymatically by the use of glucose oxidase and peroxidase (33). Frozen tissue samples were cut in small pieces with a razor blade and weighed. Samples were homogenized (Tissue-Tearor; Biospec Products, Bartlesville, OK) in 100 mM sodium acetate, pH 5.0, for 15 seconds, transferred to a boiling water bath for 5 minutes, and homogenized again for 15 seconds as reported (31). The samples were then incubated overnight at 22°C on a shaker in 100 μl of H_2O (background) or H_2O with amyloglucosidase (50 units/ml, amyloglucosidase from *Aspergillus niger*; Sigma, St. Louis, MO). After a brief centrifugation ($12,000 \times g$, 5 min, 4°C) to remove nuclei and unbroken tissue, the concentration of glucose liberated by the amyloglucosidase was determined using a commercial glucose oxidase kit (Sigma), whereby glucose is oxidized to gluconic acid and hydrogen peroxide with glucose oxidase. Tissue glycogen levels were calculated by determining the difference between the glucose level with and without amyloglucosidase incubation and normalized to wet tissue weight. Purified glycogen (type IX from bovine liver; Sigma) was used to generate a standard curve (0.063–1.0 mg) to ensure that measured glucose levels were within the linear range of detection of the assay.

Glycogen Metabolism

Key enzymes involved in glycogen metabolism were evaluated in liver tissue of randomly fed mice. After harvesting liver tissue as described above, tissue was frozen rapidly in liquid nitrogen and stored at -80°C until further processing.

Glycogen synthase activity ($n = 3$ each group) was analyzed using a radioactive method initially described by Thomas and colleagues

(34) with minor modification as reported by DePaoli-Roach and colleagues (35). The method quantifies the incorporation of [^{14}C] glucose from UDP[^{14}C]glucose into glycogen (35). The glycogen synthase exists in two enzymatically interconvertible forms: the activity of active glycogen synthase is measured in the absence of glucose-6-phosphate and the activity of total glycogen synthase is quantified in the presence of glucose-6-phosphate. Liver tissue (50–100 mg) was homogenized in 500 μl homogenizing buffer (50 mM Tris-HCl [pH 7.8], 300 mM ethylenediamine tetraacetic acid [EDTA], 12 mM ethylene glycol tetraacetic acid [EGTA], 100 mM potassium fluoride, and 50 mM 2-mercaptoethanol). The homogenate was centrifuged once ($8,000 \times g$, 2°C, 15 min), and the supernatant was collected and spun again ($8,000 \times g$, 2°C, 15 min). The supernatant from the second spin was collected, and the protein concentration using a bicinchoninic acid (BCA) protein concentration kit (Pierce, Rockford, IL) and adjusted to equal protein levels (4 mg protein/ml). The reaction was started after mixing 90 μl liver homogenate with reaction mixture (50 mM Tris-HCl [pH 7.8], 15 mM EDTA, 0.6 mM EGTA, 50 mM potassium fluoride, 7 mg/ml bovine liver glycogen, 4.4 mM UDP[^{14}C] glucose, specific radioactivity 200 cpm/nmol) in the absence or presence of 7.2 mM glucose-6-phosphate. The reaction was performed at 23°C for exactly 10 minutes and terminated by pipetting 50 μl of the reaction mixture on a $2 \times 2 \text{ cm}$ 31 ET filter paper (Whatman, Florham Park, NJ), which was immediately submerged in 66% ethanol prechilled to -20°C to precipitate the glycogen. The residual glucose was removed by washing three times in cold 66% ethanol with slow stirring for 10 minutes followed by 60-minute and 30-minute washings at 23°C. The filter paper was then rinsed in acetone for 10 minutes, dried, and counted in a liquid scintillation counter using glass scintillation vials containing 5 ml biodegradable counting cocktail (Research Product International, Mount Prospect, IL) and counted with a multi-purpose scintillation counter (Beckman Instruments, Fullerton, CA). Blank filters were run in parallel during the experiment to establish a background level of counts. Heat-denatured samples (5 min, 99°C) were used as negative controls (not shown).

Glycogen phosphorylase activity ($n = 4$ each group) was assayed in the direction of glycogen breakdown using spectrophotometric determination of the rate of NADPH formation in crude liver extracts using a method coupled to phosphoglucomutase and glucose-6-phosphate dehydrogenase as described by Maddaiah and coworkers (36). Liver tissue (50–100 mg) was placed into 500 μl of cold sample buffer (4°C, 100 mM glycylglycine, 100 mM sodium fluoride, 1 mM EDTA, pH 6.8) and homogenized. The homogenate was cleared with two centrifugations at $8,000 \times g$, 2°C, 15 minutes as described above. The protein concentration of the final supernatant was adjusted to 2 mg/ml. For the activity assay, 250 μl phosphate assay buffer (51 mM potassium phosphate, 0.2% glycogen type III from rabbit liver, 1.4 mM MgCl_2 , 0.1 mM EDTA, 0.44 mM nicotinamide adenine dinucleotide phosphate [NADP^+], 0.0003% α -D-glucose 1,6-diphosphate, 1 U glucose-6-phosphate dehydrogenase, and 1 U phosphoglucomutase, pH 6.8) was added to wells of a Costar 96-well UV plate (Corning Technology, Corning, NY) in the presence or absence of 1.6 mM adenosine 5'-monophosphate and the reaction was initiated by adding 10 μl of sample. Glycogen phosphorylase activity was determined spectrophotometrically at 340 nm at intervals of 2 minutes for a period of 60 minutes at 37°C. A purified phosphorylase A from rabbit muscle (Sigma) was used for each experiment to generate a standard curve (0.03–2.0 U/ml). The activity of glycogen phosphorylase A was plotted as the average rate of reaction and was linear under these conditions throughout the 20-minute reaction. Heat-denatured samples (5 min, 99°C) were used as negative controls (data not shown).

Acid α -glucosidase activity ($n = 5$ each group) was measured fluorometrically with the artificial substrate 4-methylumbelliferyl- α -D-glucopyranoside (4MUG; Sigma), which was initially established by Salafsky and Nadler (37) to quantify the activity of acid α -glucosidase in liver tissue and fibroblasts for the diagnosis of Pompe's disease. We followed the modified protocol of Bijvoet and colleagues (38), who measured the activity of acid α -glucosidase at a suboptimal pH of 3.6 to reduce the interference of neutral glucosidase isoenzymes. Liver tissue (50–100 mg) was placed into 250 μl 4°C ice cold phosphate-buffered saline pH 7.4, homogenized, and centrifuged twice ($8,000 \times g$, 2°C, 15 min). Supernatant protein concentration was adjusted to 1 mg protein/ml. Tissue homogenates (30 μl) were added to a 30- μl substrate

solution containing 5 mM 4MUG in 0.2 M sodium citrate buffer, pH 3.6 at 37°C. The incubation was stopped after precisely 1 hour by addition of 600 μ l of 0.2 M glycine buffer, pH 10.8. Liberated 4-methylumbelliferone was measured (excitation 365 nm, emission 445 nm) with a Spectramax M5 UV meter (Molecular Devices, Sunnyvale, CA) in black-wall 96-well UV plates (Apogent, Hudson, NH). Purified α -glucosidase from rice (dilution between 0.78 U/ml and 50 U/ml; Sigma) was used as a standard for each experiment. Heat-denatured samples (5 min, 99°C) were used as negative controls (not shown).

Blood Glucose Levels

Blood glucose levels of randomly fed wild-type and PiZ mice were measured at a consistent time of day (4:00–5:00 P.M.) with a glucometer (Precision Xtra, Medisense; Abbott Laboratories, Abbott Park, IL). Blood glucose levels were evaluated in an age-matched group ($n = 10$ wild type, $n = 14$ PiZ; evaluation of all mice aged between 15 and 18 wk at one single time point) and in a body weight-matched group ($n = 6$ wild type, $n = 11$ PiZ; evaluation of all mice with a body weight between 22.0 and 24.9 mg). Values for single animals at single time points are reported as the mean of three independent determinations.

Body Weight and Fat Pads

Body weights of wild-type and PiZ mice were measured with a closed scale (Ohaus, Pine Brook, NJ). Mice fed *ad libitum* were weighed in three age groups: 10 to 11 weeks ($n = 10$ wild type, $n = 5$ PiZ), 19 to 20 weeks ($n = 10$ wild type, $n = 9$ PiZ), and 24 to 25 weeks ($n = 10$ wild type, $n = 11$ PiZ). Epigonadal fat pads were removed *en bloc* after careful removal of bladder and testis under 2% isoflurane anesthesia in 29- to 30-week-old randomly fed mice ($n = 5$ both groups) as described by Plum and coworkers (39).

Stress by Partial Hepatectomy and Fasting

Two strategies were used to stress the liver of the PiZ mice: partial hepatectomy and fasting as initially reported by the group of Perlmutter (16, 18). Partial hepatectomies were performed between 4:00 and 7:00 P.M. using randomly fed wild-type and PiZ mice in the age between 16 and 21 weeks under 2% isoflurane anesthesia. To avoid a bias during surgery, mice of different groups underwent hepatectomies in random order. The liver was mobilized through a median laparotomy, and the anterior right and left lobes together with the posterior left lobe (in total corresponding to a two-thirds partial hepatectomy) were resected *en bloc* after tightening the ligature over the vascular hilia at hepatic base. The liver lobes were immediately frozen in liquid nitrogen after removal and stored at -80°C . After checking the abdominal cavity for residual bleeding, the abdomen was closed in two layers with 3.0 silk ties. The animals were maintained in a heated room until recovery from anesthesia and were supplied with water and standard diet *ad libitum*. Resected liver lobes were used for morphologic and biochemical evaluation of tissue glycogen as described above. Twenty to twenty-four hours after partial hepatectomy, remaining liver lobes (right middle, right posterior) were resected for morphologic and biochemical evaluation of tissue glycogen ($n = 9$ wild type, $n = 5$ PiZ) under isoflurane anesthesia. One cohort of PiZ mice were treated as described above, but in addition mice received a single intraperitoneal administration of 500 μ l saline ($n = 8$) or 10% dextrose ($n = 8$) immediately after surgery. Survival rates were followed over 7 days in comparison to wild-type mice treated with saline ($n = 8$).

To stress mice by fasting, chow was withheld from age-matched wild-type and PiZ mice for 18 hours (fasts initiated between 7:00 and 9:00 P.M.). On the next day, the blood glucose levels were measured ($n = 15$ wild type, $n = 9$ PiZ) with a glucometer as described above. To evaluate liver glycogen level, liver tissue ($n = 10$ wild type, $n = 6$ PiZ) was harvested and tissue glycogen was quantified as described above.

Statistics

All data are reported as mean $\pm$ SE. A two-tailed Student's *t* test with unequal variance was used to compare conditions with *P* values less than 0.05 (indicative of a significant difference). Survival was assessed using Kaplan-Meier analysis.

RESULTS

Glycogen Stores in Liver and Muscle

The gross morphology of the livers of the PiZ mice appeared similar to that of wild-type mice. Histologic examination revealed an intact hepatic architecture in wild-type and PiZ mice (Figures 1A and 1B), except spherical cytoplasmic inclusions, also referred to as “globules,” within the hepatocytes of the PiZ mice (Figures 1D and 1F), similar to other reports (18). The globules were positive (bright magenta) when stained with the PAS stain (Figure 1D), and this was resistant to amylase treatment (Figure 1F). In contrast, the livers of wild-type mice did not contain PAS-positive inclusions (Figures 1C and 1E). The liver parenchyma in wild-type mice changed from PAS-positive (Figure 1C) to PAS-negative with amylase treatment (Figure 1E), but the liver parenchyma in PiZ mice were PAS-negative before amylase treatment, suggesting an absence of glycogen in the cytoplasm of hepatocytes (compare Figures 1C and 1D with Figures 1E and 1F). As previously described (12, 18), there were also lymphocytic infiltrations in the livers of the PiZ mice (not shown). Increased PAS-positive globules and decreased glycogen in PiZ mouse livers appeared to correlate with the level of Z-AAT expression in each PiZ mouse.

Consistent with these morphologic observations, liver tissue from PiZ mice demonstrated a significantly lower level of glycogen than liver tissue from wild-type mice (82% decrease from the mean level in wild-type controls, $P < 0.001$; Figure 1G). To evaluate the tissue specificity of this observation, glycogen levels were quantified in muscle tissue of randomly fed mice as a negative control. Wild-type and PiZ mice contained comparable levels of glycogen ($P > 0.9$; Figure 1H).

Glycogen Metabolism

Glycogen metabolism is influenced by the supply of glucose 1-phosphate, activation of glycogen synthase and the rates of glycogen catabolism by glycogen phosphorylase and acid α -glucosidase (Figure 2A) (40). To evaluate the hypothesis that glycogen metabolism was disturbed in PiZ mice, each of these enzymes was assessed in randomly fed mice. The glycogen synthase activity measured in the absence of glucose-6-phosphate reflects the activity of the active glycogen synthase, which was unchanged in wild-type and PiZ mice ($P > 0.4$; Figure 2B). In the presence of glucose-6-phosphate, inactive glycogen synthase is converted to active glycogen synthase, making this condition an indicator of total synthase protein. PiZ mice exhibited a 3-fold reduction in total glycogen synthase activity compared with wild-type mice (77% decrease compared with the mean level in wild-type controls, $P < 0.01$; Figure 2B), suggesting a limited capacity of glycogen synthase activity in total in PiZ mice. Under normal physiologic conditions, glycogen phosphorylase is the major catabolic enzyme in glycogen (40). Neither active glycogen phosphorylase levels (measured in the absence of supplemental AMP) nor total glycogen phosphorylase levels (measured in the presence of supplemental AMP) were changed in PiZ mice relative to wild-type mice ($P > 0.5$; Figure 2C). It is known that activity of acid α -glucosidase in lysosomes of hepatocytes contributes to the glycogen degradation in the liver (26–29). The liver of PiZ mice exhibited a mild but significant elevation in enzyme activity relative to wild-type mice (46% increase compared with mean wild-type level, $P < 0.05$; Figure 1D).

Blood Glucose Levels

Based on the knowledge that hepatic glycogen stores are important to maintain plasma glucose levels (40), we evaluated blood glucose levels. In age-matched 14- to 18-week-old randomly fed

Figure 1. Comparison of liver glycogen and muscle glycogen of randomly fed wild-type and PiZ mice. Liver and gastrocnemius muscle were harvested from 18- to 21-week-old wild-type or PiZ mice. (A) Wild-type liver, hematoxylin and eosin (H&E). (B) PiZ liver, H&E. (C) Wild-type liver, periodic acid Schiff (PAS). (D) PiZ liver, PAS. (E) Wild-type liver, PAS with amylase treatment. (F) PiZ liver PAS with amylase treatment. (G) Glycogen levels in liver of wild-type ($n = 8$) and PiZ mice ($n = 8$). (H) Glycogen levels in muscle of wild-type ($n = 6$) and PiZ mice ($n = 6$). For A–F, bar = 200 μm . For G and H, data shown are mean $\pm$ SE.

mice, PiZ mice revealed a mild but significant decrease in blood glucose levels compared with wild-type mice (13% decrease from mean wild-type level, $P < 0.05$; Figure 3A). However, PiZ mice were significantly lighter than wild-type mice at this time point (24% decrease from mean wild-type weight, $P < 0.01$; data not shown). To exclude the possibility that blood glucose levels varied as a function of weight, blood glucose levels were determined in body weight-matched mice. When matched by weight, the PiZ mice still exhibited lower blood glucose levels compared with wild-type mice (17% decrease from mean wild-type level, $P < 0.01$; Figure 3B). To verify lower body weight of PiZ mice, we evaluated body weights in different age groups (Table 1). The weights of wild-type and PiZ mice were indistinguishable in 10- to 11-week-old mice ($P > 0.8$). In 19- to 20-week-old mice, PiZ mice were significantly lighter than wild-type mice (10% decrease from mean wild-type weight, $P < 0.001$). The difference was greater in the oldest group of 24- to 25-week-old mice (16% decrease from mean wild-type weight, $P < 0.0001$). Epigonadal fat pad weight, an indicator of total body fat in mice (39), was reduced in the PiZ mice, with 29- to 30-week-old PiZ mice having almost half the amount of fat pads standardized to body weight compared with wild-type mice (48% decrease from mean wild-type weight, $P < 0.001$; Table 1).

Glycogen Stores in Liver and Blood Glucose Levels after Fasting

Liver glycogen plays an important role in blood glucose homeostasis, especially during periods of stress (40). Given the decreased ability of PiZ mice to store glycogen, we hypothesized that the response of PiZ mice to physiologic stress would be impaired. As one model of stress requiring use of liver glycogen, wild-type and PiZ mice were fasted for 18 hours, and liver tissue was collected for analysis.

H&E staining of fasted wild-type and PiZ mice showed normal hepatic architecture without signs of hepatocyte injury (Figures 4A and 4B). PAS staining showed a significant depletion of glycogen in wild-type mice reminiscent of the pattern observed in post-hepatectomy livers, with scattered cells retaining PAS-positive cytoplasm (Figure 4C, arrows). After fasting, PiZ mice livers maintained brightly stained, PAS-positive inclusions (Figure 4D). Quantitative analysis of glycogen levels in liver showed that both fasted wild-type (76% decrease from the mean observed in randomly fed mice) and PiZ mice (89% decrease from the mean observed in randomly fed mice) had decreased glycogen levels compared with randomly fed mice ($P < 0.05$, both comparisons, data not shown). Amylase treatment did not make a significant change in the overall appearance of the PAS stain reflecting the depletion of glycogen in the liver tissues (Figures 4E and 4F). Comparison of fasting wild-type to PiZ mice demonstrated that the wild-type mice still had higher amounts of glycogen stored in the liver (92% decrease from the mean wild-type value, $P < 0.001$; Figure 4G). To assess glucose homeostasis in wild-type and PiZ mice after fasting, blood glucose levels were measured. Randomly fed PiZ mice had lower blood glucose levels compared with wild-type mice after 18 hours of fasting (27% decrease from the mean wild-type value, $P < 0.01$; Figure 4H).

Glycogen Stores in Liver and Blood Glucose Levels after Partial Hepatectomy

In a second stress model, wild-type and PiZ mice underwent a two-thirds partial hepatectomy, and after 24 hours livers were assessed morphologically and biochemically for glycogen content.

A general morphologic assessment 24 hours after partial hepatectomy showed the presence of vacuoles in hepatocytes of both wild-type and PiZ mice, suggesting liver injury (Figures 5A

Figure 2. Glycogen metabolism in wild-type and PiZ mice. Livers from randomly fed mice were homogenized, normalized to same protein concentration, and assessed as described in MATERIALS AND METHODS. (A) Schematic of glycogen synthesis and degradation pathways. (B) Glycogen synthase activity was quantified in the livers of wild-type ($n = 3$) and PiZ mice ($n = 3$) in the absence and presence of glucose-6-phosphate. (C) Glycogen phosphorylase activity was evaluated in the livers of wild-type ($n = 4$) and PiZ mice ($n = 4$) in the absence and presence of AMP. (D) Glycogen acid α -glucosidase activity was assayed in the livers of wild-type ($n = 5$) and PiZ mice ($n = 5$).

and 5B). PAS staining suggested a significant depletion of glycogen in wild-type mice, with scattered cells retaining PAS-positive cytoplasm (Figure 5C, *arrows*). The livers of the PiZ mice after partial hepatectomy continued to harbor the brightly stained, PAS-positive inclusions, likely reflecting the aggregated Z-AAT (Figure 5D). The relative depletion of glycogen was reflected in the failure of amylase treatment to change the PAS staining pattern (Figures 5E and 5F). Quantification of glycogen levels in both wild-type and PiZ mice verified lower glycogen stores in PiZ mice after partial hepatectomy (95% decrease from wild-type level, $P < 0.001$; Figure 5G). Blood glucose

levels were assessed in wild-type and PiZ mice after partial hepatectomy. Randomly fed PiZ mice had lower blood glucose levels compared with wild-type mice after partial hepatectomy (95% decrease from wild-type level, $P < 0.05$; Figure 5H).

Survival after Partial Hepatectomy

After partial hepatectomy, PiZ mice were observed to be lethargic and prone to tremors, consistent with the concept that the PiZ mice may exhibit severe physiologic consequences of stress resulting from low glycogen and low blood glucose levels before surgery. To test this hypothesis, the survival of mice after partial hepatectomy was observed. Three groups of mice were studied: (I) wild-type mice receiving saline immediately after

Figure 3. Blood glucose levels of randomly fed wild-type and PiZ mice. Blood glucose levels were measured at the same time of day (4:00–5:00 P.M.). Data are presented for groups of mice based on comparable age or body weight. (A) Blood glucose levels in age-matched (14- to 18-wk-old) wild-type ($n = 10$) and PiZ mice ($n = 14$). (B) Blood glucose level in body weight-matched (22–25 g) wild-type ($n = 6$) and PiZ mice ($n = 11$). Data shown are mean $\pm$ SE.

TABLE 1. BODY WEIGHT AND EPIGONADAL FAT PAD WEIGHT IN WILD-TYPE AND PIZ MICE

Parameter	Age	Randomly Fed		
		Wild Type	PiZ	P Value
Body weight (g)	10–11 wk*	24.7 $\pm$ 0.7	24.5 $\pm$ 0.1	> 0.8
Body weight (g)	19–20 wk†	30.5 $\pm$ 0.6	27.3 $\pm$ 0.1	< 0.001
Body weight (g)	24–25 wk‡	34.5 $\pm$ 0.9	28.9 $\pm$ 0.6	< 0.0001
Epigonadal fat pads (g)	29–30 wk§	2.1 $\pm$ 0.2	0.9 $\pm$ 0.1	< 0.01
Epigonadal fat pads (% of body weight)	29–30 wk§	6.2 $\pm$ 0.3	3.2 $\pm$ 0.2	< 0.001

* Wild type $n = 10$, PiZ $n = 5$.

† Wild type $n = 10$, PiZ $n = 9$.

‡ Wild type $n = 10$, PiZ $n = 11$.

§ Wild type $n = 5$, PiZ $n = 5$.

Figure 4. Comparison of glycogen stores and blood glucose levels in wild-type and PiZ mice after fasting. Livers and blood samples were harvested from mice 18 hours after fasting. (A) Wild-type liver, H&E. (B) PiZ liver, H&E. (C) Wild-type liver, PAS. Arrows indicate traces of remaining glycogen after stress. (D) PiZ liver, PAS. (E) Wild-type liver, PAS with amylase treatment. (F) PiZ liver, PAS with amylase treatment. (G) Glycogen levels in liver in wild-type ($n = 10$) and PiZ mice ($n = 6$). (H) Blood glucose levels in age-matched wild-type ($n = 15$) and PiZ mice ($n = 9$) after fasting. For G and H, data shown are mean $\pm$ SE (bar = 200 μ m).

Figure 5. Comparison of glycogen stores and blood glucose levels in wild-type and PiZ mice after partial hepatectomy. Livers and blood samples were harvested from mice 24 hours after a two-thirds partial hepatectomy. (A) Wild-type liver, H&E. (B) PiZ liver, H&E. (C) Wild-type liver, PAS. Arrows indicate traces of remaining glycogen after stress. (D) PiZ liver, PAS. (E) Wild-type liver, PAS with amylase treatment. (F) PiZ liver, PAS with amylase treatment. (G) Glycogen levels in liver of wild-type ($n = 9$) and PiZ mice ($n = 5$). (H) Blood glucose levels in age-matched wild-type ($n = 4$) and PiZ mice ($n = 3$) after partial hepatectomy. For G and H, data shown are mean $\pm$ SE (bar = 200 μ m).

surgery, (2) PiZ mice receiving saline immediately after surgery, and (3) PiZ mice receiving saline supplemented with 10% dextrose immediately after surgery. Survival in wild-type mice was high (88%), with the only death in this group occurring less than 24 hours after surgery, likely secondary to trauma rather than metabolism (Figure 6). Consistent with the observed dysfunction in glycogen stores and metabolism in the PiZ mice, the PiZ mice had a markedly decreased survival after partial hepatectomy, with deaths occurring less than 48 hours after surgery ($P < 0.01$ compared with wild-type mice receiving saline). Of interest, for PiZ mice with low blood serum Z-AAT levels, survival after partial hepatectomy was not significantly different from wild type mice, an observation consistent with the mild morphologic phenotype in the livers of this group (see Figure E2). In contrast, when the PiZ mice received dextrose supplementation, they had a striking improvement in survival (63%, $P < 0.01$ compared with PiZ mice receiving saline), supporting the conclusion that deaths among PiZ mice that received saline were linked to inability to maintain blood glucose levels.

DISCUSSION

The presence of unfolded proteins in cells can induce autophagy, an intracellular pathway that can apparently contribute to the degradation and recycling of unfolded proteins. Based on the knowledge that the PiZ murine model of AAT deficiency mimics the conformational abnormalities of the Z protein in the hepatocyte and evokes autophagy (17, 41, 42), and that autophagy interferes with the glycogen metabolism (26–29), we hypothesized that the conformational abnormalities of the Z-AAT protein in the PiZ mouse induce dysfunction in hepatocyte metabolic pathways involving glycogen. The data in the current study are consistent with this hypothesis. The liver glycogen levels are significantly lower in the PiZ mice, and PiZ mice demonstrated abnormal activities of glycogen-related enzymes, including glycogen synthase and acid α -glucosidase. As metabolic consequences, the PiZ mice have lower blood glucose levels and body weights than wild-type controls, as well as a disproportionate loss of body fat. When the PiZ mice are stressed with partial hepatectomy or fasting, their liver glycogen and blood glucose levels are lower than those of similarly stressed wild-type mice. Strikingly, the PiZ mice have a higher mortality after partial hepatectomy, but consistent with the hypothesis of liver glycogen metabolism dysfunction, the PiZ mice have normal survival if they are supplemented with parenteral dextrose after surgery. Together, these observations are consistent with the concept that the conformational abnormalities of the Z protein evoked subtle changes in cellular metabolism that had systemic implications, suggesting that diseases might benefit from metabolic compensation of the dysfunction in glycogen metabolism in the liver.

The data in this report point to several possible changes in glycogen metabolism that might have contributed to glycogen degradation. The observed loss of glycogen in PiZ mice might have been caused by decreased glycogen synthesis, since PiZ mice exhibited a 3-fold reduction in glycogen synthase (as measured by total tissue enzyme activity) compared with wild-type mice, possibly resulting in a limited capacity for glycogen synthesis in PiZ mice. However, when assaying only the active pool of glycogen synthase as opposed to the total pool of the enzyme, no difference between PiZ mice and wild-type mice was observed. The decreased glycogen content could also have resulted from an increase of acid- α glucosidase activity; however, the magnitude of that increase (< 2 -fold) was modest. Interestingly, there are reports in the literature suggesting that autophagy might be connected to an induction of acid- α

Figure 6. Survival of wild-type and PiZ mice after partial hepatectomy. Mice underwent a two-thirds partial hepatectomy and were treated immediately after surgery with an intraperitoneal administration of saline or 10% dextrose. The Kaplan-Meier plot shows survival for wild-type mice after saline injection ($n = 8$), PiZ mice after saline administration ($n = 8$), and PiZ mice after dextrose injection ($n = 8$).

glucosidase activity leading to a glycogen hydrolyzation in autophagosome (26, 28, 29, 43, 44). However, further experiments with other models of autophagy are mandatory, since the role of acid- α glucosidase in autophagy is controversial and not fully understood. Nevertheless, other potential mechanism beside the autophagy may also explain the results. High expression of AAT might cause changes in liver expression independent of the misfolding of Z-AAT. To test this hypothesis, the liver glycogen level of transgenic mice that overexpress M-AAT (PiM mice) should be assessed. Similarly, overexpression of any misfolded protein (or more precisely, any misfolded protein in the secretory pathway) might disrupt liver metabolism. However, transgenic models are widely used for the study of mutant proteins, and the PAS-positive liver globules are not a typical pathology. After another line of reasoning, Perlmutter and colleagues have provided evidence for mitochondrial degradation in cells expressing Z-AAT, raising the possibility that more complex regulatory feedback mechanisms may account for decreased intracellular glycogen in PiZ mice (4, 19, 37). Recently, it has been demonstrated that glucose administration improved survival of caveolin-1-knockout mice after partial hepatectomy (45). The caveolin-1-knockout mice have defects in lipid homeostasis and cell proliferation after partial hepatectomy (45). In addition to the observation here that accumulation of body fat in PiZ mice was reduced, prior reports have noted differences between fat accumulation in the livers of PiZ mice and wild-type mice after physiologic stress (18). Therefore, potential links between lipid metabolism and glycogen regulation in PiZ mice may be warranted.

As part of the present study, the PiZ mice were stressed to determine if the deficit in glycogen storage might have significant consequences for the mice. Two stress models were employed, both of which have previously been applied to PiZ mice with reports of outcomes that differ from those of wild-type mice. Teckman and coworkers (18) showed that PiZ mice had a significantly decreased tolerance for prolonged fasting compared

with wild-type mice, and Rudnick and colleagues (16) reported an increased mortality after partial hepatectomy compared with wild-type mice. Although the prior study demonstrated a slightly higher survival after partial hepatectomy in PiZ mice compared with the present study, the two studies agree that PiZ mice are at greater risk of mortality compared with wild-type mice after partial hepatectomy. In both studies, PiZ mice after surgery became lethargic, had tremors, and died within 48 hours. Both fasting and partial hepatectomy resulted in decreased liver glycogen and decreased blood glucose levels in both wild-type and PiZ mice. Reasoning that the severe consequences resulted from a suboptimal glycogen supply, blood sugar was artificially raised by dextrose supplementation after surgery and the PiZ mice had a markedly improved post-surgical clinical course after partial hepatectomy, thus confirming the fact that PiZ mice have a significant alteration of their carbohydrate metabolism.

The hepatotoxicity associated with AAT deficiency is thought to result from a gain-of-toxic-function mechanism (41). In humans, variable expressivity of hepatotoxicity is observed (6, 7), leading to the suggestion that genetic modifiers and/or environmental variables may play a significant role in the development of liver disease (41). Based on the data presented here, an appreciation of the hepatocyte metabolic changes induced by Z-AAT might be one factor in understanding how a subset of AAT-deficient patients could be predisposed to liver disease. At this point, AAT deficiency is not connected to any glycogen disorder. However, the classic phenotype of a patient with AAT deficiency, which includes a thin, relatively underweight individual, has always been attributed to the extra exertion during breathing for these individuals. This study raises the interesting possibility that a metabolic shift in the liver may contribute to the phenotype. If this were to be true, the changes that one might expect to observe in humans would be far less dramatic than the PiZ mouse phenotype reported here, since PiZ mice express multiple copies of the mutant Z-AAT, whereas human patients with AAT deficiency carry only two copies of mutant AAT.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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