Increased liver AGEs induce hepatic injury mediated through an OST48 pathway

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The protein oligosaccharyltransferase-48 (OST48) is integral to protein N-glycosylation in the endoplasmic reticulum (ER) but is also postulated to act as a membrane localised clearance receptor for advanced glycation end-products (AGE). Hepatic ER stress and AGE accumulation are each implicated in liver injury. Hence the objective of this study was to increase the expression of OST48 and examine the effects on hepatic function and structure. Groups of 8 week old male mice (n = 10–12/group) over-expressing the gene for OST48, dolichyl-diphosphooligosaccharide-protein glycosyltransferase (DDOST+/-), were followed for 24 weeks, while randomised to diets either low or high in AGE content. By week 24 of the study, either increasing OST48 expression or consumption of high AGE diet impaired liver function and modestly increased hepatic fibrosis, but their combination significantly exacerbated liver injury in the absence of steatosis. DDOST+/- mice had increased both portal delivery and accumulation of hepatic AGEs leading to central adiposity, insulin secretory defects, shifted fuel usage to fatty and ketoacids, as well as hepatic glycogen accumulation causing hepatomegaly along with hepatic ER and oxidative stress. This study revealed a novel role of the OST48 and AGE axis in hepatic injury through ER stress, changes in fuel utilisation and glucose intolerance.

Chronic liver disease is increasing globally as a result of obesity and diabetes, with non-alcoholic fatty liver disease (NAFLD), the most common liver disorder1. However, NAFLD is asymptomatic and most individuals do not progress to non-alcoholic steatohepatitis (NASH). The development of NASH is postulated to be the result of a ‘second hit’ environmental factor2.

Glucose intolerance and dyslipidaemia which are often characteristic of NAFLD3, facilitate the non-enzymatic post-translational modification of proteins, forming advanced glycation end products (AGEs)4, which accumulate in tissues and contribute to liver fibrogenesis5. Excessive exposure to exogenous sources of AGEs, such as from food, can also exacerbate liver injury in the absence of glucose intolerance6. This is likely via simple diffusion through the epithelium if cleaved6, although AGE specific receptors may facilitate receptor-mediated gastro-intestinal trafficking. Once absorbed, AGEs are directed to the liver through the portal vein and excreted via the gall bladder by hepatic sinusoidal Kupffer and endothelial cells7, and by renal clearance8.

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Results

Generation of a ubiquitous OST48 knock-in mouse model. OST48 knock-in mice (ROSA26tm1(DDOST)) hereon termed as DDOST+/−) were heterozygous in their expression of human DDOST at the ROSA26 locus under the control of the ubiquitin promoter (Fig. S1A). There was a significant increase in hepatic OST48 gene (DDOST) expression (Fig. S1B), whilst endogenous gene expression (Ddost) was unaffected (Fig. S1C). Targeted proteomics identified that 32 week old DDOST+/− mice did not show a significant change in total OST48 protein in the gut using the major peptides detected (Fig. S1D). However, there is a substantial increase in OST48 protein in each major section of the gut than compared to an average of 5 differently tissue locations (Fig. S1D). Moreover, in fractionated liver tissue taken from DDOST+/− mice at the same time point, there was a significant increase in plasma membrane localisation of DDOST+/− fed on a high AGE diet (Fig. S1E). This increase in plasma membrane OST48 protein localisation and content in liver taken from DDOST+/− high AGE fed mice was not seen in the cytosol (data not shown). The small intestine (duodenum, jejunum and ileum) had a high relative abundance of OST48 protein when compared with other tissues (Fig. S1D).

In the absence of steatosis, hepatic injury was evident in DDOST+/− mice and this was exacerbated by a high AGE diet. Hepatomegaly was evident in DDOST+/− mice irrespective of the diet (Fig. 1A). H&E staining indicated that wild-type mice on a high AGE content diet had some hepatocellular ballooning, with modest rarefaction of the cytoplasm (Fig. 1B). All DDOST+/− mice exhibited hepatocellular enlargement and ballooning, clusters of lobular plasma cells, increased inflammatory infiltration and an abundance of rarefied cytoplasm in hepatocytes (Fig. 1B), as well as increases in hepatic fibrosis exhibited by increased Sirius Red staining and positive α-SMA staining, respectively (Fig. 1C and D). Although there was no substantial differences in Sirius Red staining between high AGE fed WT mice and DDOST+/− mice (Fig. 1C). We could however observe a gradual exacerbation of α-SMA staining in high AGE fed DDOST+/− mice (Fig. 1D). Plasma concentrations of the liver enzymes ALT and AST were also increased in DDOST+/− mice (Fig. 1E), and plasma ALP concentrations were the highest in DDOST+/− mice fed a high AGE diet, suggesting the presence of hepatocellular damage.

Hepatocellular ballooning is often indicative of lipid droplet accumulation in the liver. Despite the presence of vascular fibrosis, there were no differences among groups in hepatic Oil Red O staining for lipid droplets (Fig. 1F; bottom; $P = 0.0246$) associated with high AGE dietary intake. There was also a modest decrease (−30.9% low AGE diet and −15.9% high AGE diet, $P = 0.0516$, 2-way ANOVA) in hepatic ceramide content when comparing the DDOST+/− genotype with the WT mice however, this did not reach statistical significance.

DDOST+/− mice exhibited increased absorption of AGEs in the liver. The two-hit model of a high AGE diet and DDOST+/− mice exhibited increased deposition of both CML and OST48 in hepatic tissue sections (Fig. 2A). Oral administration of near-infrared labelled AGEs using IVIS/MR imaging, suggested prolonged exposure of AGEs in the liver of DDOST+/− mice (Fig. 2B). We further identified that hepatic AGE deposition was greater in mice fed a high AGE diet and in DDOST+/− mice irrespective of dietary alterations (Fig. 2C). Although WT mice fed a high AGE diet had similar deposition of AGEs in the liver, it was identified that circulating AGE concentrations were reduced in all DDOST+/− mice as compared with high AGE fed littermate WT mice (Fig. 2D) indicating an increase in trafficking and exposure of AGEs from the circulation in the liver.
Despite the absence of steatosis $DDOST^{+/-}$ mice had increased adiposity in addition to increased physical activity. There were no differences in body weight or lean body mass at either the beginning (Fig. S2A and B) or the end of the study (Fig. S2D and E) among mouse groups. Body fat mass was increased in $DDOST^{+/-}$ mice as compared to their respective WT group (Fig. 3A; left), and this was more pronounced with high AGE feeding at the study end. Indeed, adiposity, measured as the intra-abdominal fat pads (mesenteric and omental fat pads), was increased in $DDOST^{+/-}$ mice as compared to WT littermates (Fig. 3A; right). At the beginning of the study, there were no significant differences in adiposity between WT and $DDOST^{+/-}$ mice (Fig. S2C). Increased adiposity in $DDOST^{+/-}$ mice was not a consequence of reduced physical activity (Fig. 3B; left), nor of increased food intake (Fig. S2F). In fact, $DDOST^{+/-}$ mice exhibited increased locomotor activity during both dark/awake and light/sleep phases (Fig. 3C; right), irrespective of diet.
Hepatic injury associates with ER stress, oxidative stress and activated inflammasome. Confocal microscopy, showed co-localisation of GRP-78 with OST48 (Fig. 4A). Unbiased proteomic profiling of hepatic tissue using SWATH-MS, identified increases in key proteins involved in ER stress, specifically GRP78 (Fig. 4B; top) and EIF3A (Fig. 4B; bottom) in our two-hit model of OST48 over-expression and high AGE feeding. The anti-oxidant enzymes SOD1/SODC (Fig. 4C; top) and SOD2/SODM (Fig. 4C; bottom) were increased in DDOST+/− mice irrespective of the diet consumed. DDOST+/− mice fed a low AGE diet also had increases in the redox responsive GPX1 (Fig. S3A; left) and aconitase/ACON (Fig. S3A; right) when compared to low AGE fed WT mice. Furthermore, we also observed a significant ($P=0.034$) effect of a high AGE diet on increased 8-isoprostane levels in the urine (Fig. 4D). DDOST+/− mice fed a high AGE diet also had significant increases in the inflammatory proteins CD47 (Fig. S3B; left) and HA1D (Fig. S3B; right).

Defects in N-glycosylation do not explain the hepatic injury seen in DDOST+/− mice fed on a high AGE diet. Prolonged ER stress culminating in liver injury is often associated with impaired ER function leading to changes in glycosylation occupancy on proteins. We therefore investigated whether DDOST+/− mice altered hepatic protein synthesis N-glycosylation. Fifty-six glycosylation sites were identified on mouse plasma proteins (Fig. S4A) with six indicating partial modification by N-glycosylation (Fig. S4B), but there were no differences among groups in glycosylation occupancy on plasma proteins (Fig. S4A), nor in plasma proteins specifically glycosylated and secreted by the liver (Fig. S4C).

Hepatic injury was associated with changes in fatty acid metabolism. Using continuous indirect calorimetry, we determined that DDOST+/− mice had decreased respiratory exchange ratios during both the sleep/light (Fig. 5A; left) and active/dark (Fig. 5A; right) phases. This downward shift in RER suggested a
above. 2-way ANOVA and multiple comparison of genotype, diet and interaction by Bonferroni’s post hoc test. Compared with WT littermates. There were no differences in fed blood glucose concentrations (Table 1), or in insulin measured 24 hour physical activity and caloric intake at 24 weeks post-dietary intervention in WT (+) and DDOST+/- mice fed on either a high AGE diet (closed circles) or a low AGE diet (open circles). (B) Total locomotor activity in the horizontal plane measured by infrared beam breaks. (C) The line graphs show average hourly heat generation over the 24-hour period. The bar graphs on the right show the average physical activity over the entire 12-hour light/sleep (left) and dark/active (right) period. Data represented as means ± SD (n = 4–9/group). *P < 0.05, student’s t-test, α (genotype effect) P < 0.05, β (diet effect) P < 0.05, as per figure above. 2-way ANOVA and multiple comparison of genotype, diet and interaction by Bonferroni’s post hoc test.

proportional shift towards the use of free fatty acids and/or ketones for ATP generation as compared to wild-type littermates consuming either a low/high AGE diet. In support of this, an RER of 0.9, seen in DDOST+/- mice on a high AGE diet during their active cycle, demonstrates a ~65:35 of carbohydrate:fat/ketone oxidation as compared with WT mice fed a low AGE diet, (RER of 0.98; ~93:7 carbohydrate:fat/ketone oxidation)22. During the light cycle, DDOST+/- mice fed a high AGE diet had an RER of 0.88, demonstrating a large shift towards fatty acid and ketone oxidation (~59:41 carbohydrate:fat/ketone oxidation) as compared with low AGE fed WT mice (RER of 0.95; ~83:17 carbohydrate:fat/ketone oxidation). Moreover, we observed significant decreases in heat generation during both the sleep/light (Fig. 5B; left) and active/dark phases (Fig. 5B; right) specifically associated with a high AGE diet (P < 0.0001) in both WT and DDOST+/- mice.

The gene expression of key enzymes involved in fatty acid utilisation by the liver were altered in DDOST+/- mice fed a high AGE diet (Fig. 5C). Specifically, there was a significant increase in Pparα gene expression in DDOST+/- mice fed a high AGE diet (Fig. 5C; top) as compared with other groups. The gene expression of Lepr (Fig. 5C; bottom) was decreased by both the high AGE diet and in all DDOST+/- mice. DDOST+/- high AGE fed mice, also showed a decrease in the gene expression of both Acadvl (Fig. S5; left) and Acadm (Fig. S5; right), encoding enzymes involved in the oxidation of medium and long chain fatty acids.

Using SWATH-MS proteomics, significant increases in proteins associated with fatty acid oxidation, peroxisome proliferator-activated receptor (PPAR) protein signaling and fatty acid transport/export were demonstrated in DDOST+/- mice fed a high AGE diet when compared with WT low AGE fed mice (Fig. 5D). Specifically, the fatty acid oxidation enzymes ACOX1 and ACBP were increased, as well as the transport proteins FABP5 and FABPL, and the HDL core protein APOA1. The other proteins associated with lipid metabolism examined were not significantly altered.

**DDOST+/- mice exhibit impaired glucose tolerance at a young age.** Young (8 week old) DDOST+/− mice had increases in fasted blood glucose concentrations (Table 1 and Fig. S6A), lower fasting insulin concentrations and decreases in first phase insulin secretion (Table 1 and Fig. S6B) during ipGTT as compared with WT littermates. There were no differences in fed blood glucose concentrations (Table 1), or in insulin tolerance tests (Fig. S6C) among young mice.

**Adult DDOST+/− mice exhibited glucose intolerance which is augmented by high AGE dietary intake.** By the end of the study, all DDOST+/− mice had elevated glycated haemoglobin (Fig. 6A) and lower fasted plasma insulin concentrations (Fig. 6B), without differences in fasted or fed plasma glucose concentrations as compared with WT mice (Table 1). However, high AGE fed DDOST+/− mice also had increased 2 hour plasma glucose concentrations following a 2 g/kg glucose bolus (ipGTT), as compared with other groups (Fig. S7A and B). While on a high AGE diet, WT mice were less glucose tolerant as compared with low AGE fed mice (Fig. S7C).

In response to an insulin bolus (ipITT), DDOST+/− mice fed a high AGE diet had higher plasma glucose concentrations than other groups (Fig. 6C). Fasting plasma glucagon concentrations were not increased (Table 1), but hepatic glycogen storage was increased by 30–40% in DDOST+/− mice (Fig. 6D). When compared with
the WT low AGE fed group, all mice also had lower protein intensities of 1,4-alpha-glucan-branching enzyme (GLGB), a facilitator of glycogenolysis (Fig. 6E), consistent with greater levels of liver glycogen described above.

The gene expression of the gluconeogenic enzyme, pyruvate carboxylase kinase (Pck2; Fig. 6F) was increased by high AGE dietary feeding. Pathway analysis of proteins involved in GNG showed that hepatic proteins involved in oxidative phosphorylation were elevated in all mouse groups when compared with WT low AGE fed mice (Fig. 6G).

**DDOST+/-** mice on a high AGE diet exhibit increased amino acid metabolism. Increased gluconeogenesis and hepatic glucose release to potentially sustain an associated increased physical activity can be further substantiated by the evident increased circulating concentrations of the amino acids alanine (Fig. 7A) and serine (Fig. 7B) and the loss of glucogenic glycine in **DDOST+/-** mice (Fig. 7A). Plasma concentrations of the essential amino acids lysine and histidine (Fig. 7C) were also increased in **DDOST+/-** mice. Plasma concentrations of both tyrosine and tryptophan were also increased in the high AGE fed **DDOST+/-** mice when compared with other mouse groups (Fig. 7D). Pathway analyses showed an increase in protein associated with amino acid metabolism in high AGE fed **DDOST+/-** mice (Fig. 7E). There were no other changes observed in circulating concentration of other amino acids (Fig. S8).

**Discussion**

Our discovery of the accumulation of AGEs in the liver through an OST48 mediated pathway of uptake and clearance is novel. Despite the unconventional pathway of liver injury (normally the presence of steatosis leading to the onset of fibrosis) in our **DDOST+/-** mice, this allowed us to identify other metabolic factors that may contribute...
an important role in the development of liver injury\textsuperscript{23}. A recent study in children with NAFLD demonstrated that the presence of early portal inflammation on biopsy was independently associated with severity of liver fibrosis and metabolic risk factors for type 2 diabetes, in particular waist circumference\textsuperscript{24}. Indeed in that study, central adiposity was the only non-invasive variable associated with biopsy proven portal inflammation. These findings are consistent with the present study, where central adiposity was seen in concert with early liver injury and portal fibrosis in \textit{DDOST}+/− mice fed a high AGE diet.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{Post-diet modification} & \textbf{WT} & \textbf{DDOST}+/− & \textbf{Two-Way ANOVA} \\
\textbf{AGE\textsuperscript{low}} & \textbf{AGE\textsuperscript{high}} & \textbf{AGE\textsuperscript{low}} & \textbf{AGE\textsuperscript{high}} & \textbf{G} & \textbf{D} & \textbf{G-D} \\
\hline
Fed Glucose (mmol/L) & 15.70 ± 1.80 & 13.77 ± 1.53 & 15.66 ± 1.38 & 13.95 ± 1.18 & 0.96 & 0.23 & 0.94 \\
Fasting Glucose (mmol/L) & 10.65 ± 0.46 & 9.417 ± 1.13 & 9.913 ± 0.76 & 10.03 ± 0.47 & 0.94 & 0.49 & 0.96 \\
Fasting Insulin (ng/ml) & 1.255 ± 0.41 & 1.837 ± 0.53 & 0.9438 ± 0.27 & 0.7999 ± 0.14 & 0.05 & 0.51 & 0.28 \\
Fasting Glucagon (pg/ml) & 1366 ± 187.8 & 2087 ± 287.6 & 1552 ± 80.13 & 1677 ± 279.8 & 0.61 & 0.09 & 0.93 \\
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\end{tabular}
\caption{Biochemical measurements in mice post-diet modifications (32 weeks of age). Mean and standard deviation for biochemical measurements of each genotype during pre- and post-diet modification. Post-diet modification significance levels were determined by two-way ANOVA, testing the effect of genotype and diet. Differences between variables identified by Bonferroni’s post hoc test. Bold \textit{P} values indicates significant effect of at least < 0.05. G: Genotype; D: Diet; G-D: Interaction.}
\end{table}

\textbf{Figure 5}. \textit{DDOST}+/− mice have a shift towards increased fatty acid utilization. (A) CLAMS apparatus measured respiratory exchange rate at 24 weeks post-diet modification. Average difference in respiratory quotient corrected to lean body mass measured by indirect calorimetry during the entire 12-hour light/sleep (left) and dark/active (right) period. (B) Average difference in heat production measured by indirect calorimetry during the entire 12-hour light/sleep (left) and dark/active (right) period. (C) Real-time PCR of liver tissue targeting genes of interest \textit{Pparα} (top) and \textit{Lepr} (bottom) (\(n = 4–8\) per group in triplicate). (D) Heat map representation of SWATH-MS proteomics data for enzymatic pathways involved in fatty acid oxidation. Significant proteins are represented as the Log2 fold change where red indicates a decreased and blue indicates an increase in protein concentrations. Data represented as means ± SD (\(n = 4–9\)/group). For proteomics, MSstatsV3.5.1 determined significant (\(P < 0.05\)) log fold changes in the protein intensities between the selected experimental group and the WT low AGE diet group. *\(P < 0.05\), student’s t-test. Genotype effect \(P < 0.05\), (diet effect) \(P < 0.05\), 2-way ANOVA and multiple comparison of genotype, diet and interaction by Bonferroni’s post hoc test.
Figure 6. Hepatic fibrosis associated with glucose intolerance, increased glucose synthesis and storage in high AGE fed DDOST+/− mice. (A) Glycated haemoglobin. (B) Fasting plasma insulin concentrations. (C) Plasma glucose curve over 120 mins following a 1IU/kg insulin bolus (ipITT) and area-under-the-curve (AUC) analysis. (D) Hepatic glycogen measured in liver tissue. (E) SWATH-MS protein intensities for the glycogen storage pathway protein (GLGB). (F) qPCR of Pck2, the enzyme involved in gluconeogenesis. (G) Heat map representation of SWATH-MS proteomics data for enzymatic pathways involved in GNG. Significant proteins are represented as the Log2 fold change where red indicates a decreased and blue indicates an increase in protein concentrations. Data represented as means ± SD (n = 4–9/group). For proteomics, MSstatsV3.5.1 determined significant (P < 0.05) log fold changes in the protein intensities between the selected experimental group the WT low AGE diet group. *P < 0.05, student’s t-test. Genotype effect P < 0.05, (diet effect) P < 0.05, 2-way ANOVA and multiple comparison of genotype, diet and interaction by Bonferroni’s post hoc test.

Figure 7. DDOST+/− mice exhibit increased ketogenesis. (A) Concentrations of serum amino acids. (B) Heat map representation of SWATH-MS proteomics data for enzymatic pathways involved in ketogenesis. Significant proteins are represented as the Log2 fold change where red indicates a decreased and blue indicates an increase in protein concentrations. Data represented as means ± SD (n = 4–9/group). For proteomics, MSstatsV3.5.1 determined significant (P < 0.05) log fold changes in the protein intensities between the selected experimental group the WT low AGE diet group.
steatosis and proceed directly into fibrogenic pathways would require further investigation into a high fat diet model. Despite this caveat what was particularly interesting was that central adiposity and liver injury persisted in DDOST+/− mice fed a high AGE diet, despite them partaking in greater levels of physical activity. This interesting finding reiterates the complexity of the relationship between metabolic factors and liver injury, given that exercise is a feasible intervention to improve liver fibrosis in patients with NAFLD35 and relates to adiposity in NAFLD36.

The liver is a node which controls systemic glucose and lipid fluxes in the body, balancing these against its own energy requirements22. These processes are influenced heavily by pancreatic islet function via the endocrine hormones insulin and glucagon26 as well as other hormones. In the present study, abnormalities in insulin secretion and elevations in glycated haemoglobin were seen in the context of hepatic injury and increased fuel oxidation, particularly of fatty acids in high AGE fed DDOST+/− mice. This is in agreement with previous evidence that a shift in hepatic fuel utilisation towards fatty acids results in liver damage25. The increased utilisation of fatty acids for β-oxidation, and likely increased fatty acid export/transport proteins, may also explain the lack of hepatic fat accumulation seen in DDOST+/− mice fed a high AGE diet. Surprisingly, this led to an increase in central adiposity, despite increased physical activity and the absence of changes in caloric intake and lean body mass. There also appeared to be an excess supply of circulating glucose generated from hepatic gluconeogenesis which may be required by the skeletal muscle to facilitate the increases in physical activity seen in DDOST+/− mice, rather than for thermogenesis. This is also supported by the decreases in heat generation seen in AGED−R1 mice fed a high AGE diet. A futile cycle of energy generation in adipocytes has been linked to reduced body fat storage36, however it has not been previously linked to the development of liver fibrosis.

In summary, this group of studies revealed a previously unappreciated role of the OST48-AGE axis within the body. Indeed, the data suggest that the “trafficking role” of OST48 in normal hepatic physiology is not solely involved in protein N-glycosylation or elimination (detoxification/clearance) of AGEs but could also alter gastrointestinal uptake through the small intestine of AGEs leading to changes in glucose homeostasis, hepatic function and fuel utilisation. The physiological significance of this should form the basis of future studies. In the context of a high AGE diet, increases in OST48 expression exacerbated liver injury leading to fibrosis, likely via known pathways of injury including oxidative and ER stress. Further studies into the role of hepatic and gastrointestinal OST48 mediated AGE uptake could lead to a novel approach to minimise liver injury.

Materials and Methods

Animal Model. C57BL/6 mice (The Jackson Laboratory, United States) were genetically modified via the Cre-loxP recombination system (Ozgene, WA, Australia) and ubiquitous genetic knock-in of the human gene encoding OST48 (DDOST) at the ROSA26 locus with removal of the delivery neomycin cassette, as described in Supporting Experimental Procedure 1 and Supporting Fig. 1. DDOSTfloX mice are referred to as the wildtype (WT) background and DDOSTheterozygousCreOff mice are labelled as the genetic OST48 heterozygous knock-in mouse model (DDOST+/−). Eight week old male DDOST+/− and littermate controls (WT) were randomised to be fed either AIN-93G (low AGE diet; Specialty Feeds, Perth, Australia)37 or baked AIN-93G (1 hour at 100°C; high AGE diet), which contained a 5-fold higher content of the AGEs, N(ε-carboxymethyl)lysine (CML), N(ε)-(carboxyethyl)lysine and methylglyoxal32 for 24 weeks. Heat labile vitamin contents (vitamin A and thiamine) were not decreased by the heating protocol32. Mice were allowed access to food and water ad libitum and were maintained on a 12 hour light-dark cycle at 22°C. All mouse experiments were performed following approval from the AMREP Animal Ethics Committee and as per guidelines from the National Health and Medical Research Council of Australia.

Plasma AGE measurement. CML concentrations in plasma were measured by an in-house indirect ELISA as previously described35.

Human and mouse OST48 ELISAs. Mouse and human OST48 concentrations were determined in hepatic cortical membrane and cytosolic fractions by commercial ELISAs (Cloud-Clone Corp., Houston, United States), according to the manufacturer’s specifications.

Liquid Chromatography-Mass Spectrometry (LC-MS/MS). As previously described34, proteins were extracted from whole liver tissue samples using guanidine denaturing buffer (6 M guanidinium, 10 mM DTT and 50 mM Tris-HCl). Reduced cysteines were alkylated with acrylamide, and quenched with excess DTT. Proteins were precipitated in 4 volumes of 1:1 methanol:aceton and digested with trypsin. Peptides were desalted and analysed by Information Dependent Acquisition LC-MS/MS as described45 using a Prominence nanoLC system (Shimadzu, NSW, Australia) and Triple TOF 5600 mass spectrometer with a Nanospray III interface (SCIEX). SWATH-MS analysis was performed46 and analysed with MStats as previously described. Differentially abundant proteins were analysed using DAVID37.

Histology and Immunofluorescence. Haematoxylin and Eosin, Masson’s Trichrome and Sirius Red (Sigma-Aldrich, United States) stains were completed on 10% Buffered formalin fixed paraffin sections. Collagen content of the liver was quantified histologically using computerised quantification of picrosirius red staining, as described previously35. Oil-Red-O (Sigma-Aldrich, USA) staining was performed on frozen OCT embedded cryosections as previously described35. All sections were visualized on a slide scanner (Virtual Slide System VS120, Olympus, Tokyo, Japan) and viewed in the supplied program (OlyVIA Build 10555, Olympus, Tokyo, Japan). Briefly, for immunofluorescence staining, 4% PFA fixed frozen liver sections were dual stained with both anti-CML (1:125 dilution; ab30917; Abcam, United Kingdom) and OST48 (1:100 dilution; sc25558; Santa Cruz biotechnologies, United States). Dual staining on paraffin buffered formalin fixed sections was with GRP78 (1:50...
Serum and urine biochemistry and analysis of hepatic lipids. The plasma concentrations of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were measured by an autoanalyzer (Beckman Instruments, USA). Hepatic diacylglycerols (DAGs) and ceramides were extracted and quantified by thin layer chromatography and a liquid scintillation analyzer (LS6500; Beckmann Coulter Inc., CA, USA) as previously described. Lipid peroxidation was examined by urinary 8-isoprostane in a competitive ELISA (Oxford Biomedical Research, United States), according to the manufacturer’s specifications.

Glucose and insulin tolerance tests. Intraperitoneal glucose tolerance tests (ipGTT) were performed following a 2 g/kg D-glucose bolus as previously described. Insulin was determined by rat/mouse insulin ELISA (RnD systems, MN, United States). Intraperitoneal insulin tolerance test (ipITT), to determine glucose output was performed using a 1 IU/kg bolus of Humolog fast-acting insulin (Eli Lilly, IN, United States). Area under the curve was calculated using the trapezoidal rule (GraphPad Software, CA, United States).

Indirect calorimetry and assessment of body composition. Whole body composition was measured at 32-weeks of age in conscious, but physically restrained mice using an EchoMRI™ 3-in-1 body composition analyzer (EchoMRI, TX, USA). A cohort of mice were allocated for repeated measures of energy expenditure by indirect calorimetry (VO₂, VCO₂) normalised to lean body mass, locomotor/physical activity and rate of energy expenditure (heat generation) using a Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, OH, United States). Data was collected over a 24-hour cycle period, post-acclimatisation to the cages for a 12-hour period.

Liver glycogen content. As previously described, total liver glycogen content was determined using a glucose oxidase/peroxidase assay procedure and absorbance was analysed on a UV-1700 PharmaSpec UV-vis spectrophotometer (Shimadzu, NSW, Australia).

Quantitative real-time PCR. Messenger RNA was purified and was used (1 µg) to synthesize cDNA using SuperScript first-strand synthesis system (Invitrogen). Quantitative real-time PCR was performed using pre-designed TaqMan Gene Expression Assays® for Acadm, Acadvl, Acox1, ADRB2, C2l2, Colla1, Col3a1, Ddost, DDOST, G6pc, Gegr, Got1, Gyk, Kcnma1, Lepr, Pck1, Pck2, Ppara, Slc27a4 and Slc37a4 (Supporting Experimental Procedure 2; Life Technologies, Mulgrave, VIC, Australia) in ViiA™ 7 real-time PCR system (Applied Biosystem, Darmstadt, Germany). Gene expression levels were calculated after normalisation to an endogenous multiplexed control (18S) using the ΔΔCT method as previously described and expressed as relative fold change compared to the wild-type mice fed a high AGE content diet. The results are represented as mean ± CV.

Amino acid measurements. Approximately 40 µl of serum was mixed with 160 µl of an extraction solution (3:1 ratio of analytical grade methanol to ddH₂O), centrifuged at 16,000 × g for 10 minutes and the supernatant removed for processing. Amino acids were measured by HPLC as described in Chacko et al.

Statistical analysis. Results are expressed as mean ± SD (standard deviation), and analyzed by 2-way ANOVA followed by post hoc testing for multiple comparisons using the Bonferroni method unless otherwise specified. α (genotype effect) P < 0.05; β (diet effect) P < 0.05, β (interaction effect) P < 0.05 were reported. For comparison between groups as required, a two-tailed unpaired Student’s t-test was used. For SWATH-MS, MStatSv3.5.1 was used to detect differentially abundant proteins estimating the log-fold changes between compared conditions of the chosen experimental group and with the WT mice fed a low AGE diet group. For all calculations a P < 0.05 was considered as statistically significant.

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Author Contributions
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M.R.P., M.A.S., C.H., D.M., K.C.S., P.K., M.P.H., M.J.W., B.L.S. assisted with the experiments conducted. F.Y.T., K.C.S., M.T.C., M.A.F., J.M.F. contributed to the design of the mouse model. P.A., B.L.S. and J.M.F. contributed to the design and direction of the study. All authors reviewed the manuscript.

**Additional Information**

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