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Adipose tissue insulin resistance exacerbates liver inflammation and fibrosis in a diet-induced NASH model

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Abstract

Background: Insulin regulates various biological processes in adipocytes, and adipose tissue dysfunction due to insulin resistance in this tissue plays a central role in the development of metabolic diseases, including NAFLD and NASH. However, the combined impact of adipose tissue insulin resistance and dietary factors on the pathogenesis of NAFLD-NASH has remained unknown.

Methods and Results: 3′-phosphoinositide–dependent kinase 1 (PDK1) is a serine-threonine protein kinase that mediates the metabolic actions of insulin. We recently showed that adipocyte-specific PDK1 knockout (A-PDK1KO) mice maintained on normal chow exhibit metabolic disorders, including progressive liver disease leading to NASH, in addition to reduced adipose tissue mass. We here show that maintenance of A-PDK1KO mice on the Gubra amylin NASH (GAN) diet rich in saturated fat, cholesterol, and fructose exacerbates inflammation and fibrosis in the liver. Consistent with these histological findings, RNA-sequencing analysis of the liver showed that the expression of genes related to inflammation and fibrosis was additively upregulated by adipocyte-specific PDK1 ablation and the GAN diet. Of note, the reduced adipose tissue mass of A-PDK1KO mice was not affected by the GAN diet. Our results thus indicate that adipose tissue insulin resistance and the GAN diet additively promote inflammation and fibrosis in the liver of mice.

Conclusions: A-PDK1KO mice fed with the GAN diet, constitute a new mouse model for studies of the pathogenesis of NAFLD-NASH, especially that in lean individuals, as well as for the development of potential therapeutic strategies for this disease.

Abbreviations: ALT, alanine aminotransferase; A-PDK1KO, adipocyte-specific PDK1 knockout; AST, aspartate aminotransferase; Ctrl, control; ECM, extracellular matrix; eWAT, epididymal white adipose tissue; FoxO1, forkhead box protein O1; GAN, Gubra amylin NASH; H&E, hematoxylin-eosin; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, knockout; PDK1, 3′-phosphoinositide–dependent kinase 1; RT, reverse transcription; seq, sequencing.

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INTRODUCTION

NAFLD has become the most frequent cause of chronic liver disease.[1] NAFLD is actually a disease spectrum ranging from NAFL to NASH, which may progress to liver cirrhosis and HCC.[2] It is often associated with obesity and related comorbidities,[1–3] but it can also develop in individuals of normal or below normal body weight.[4–6] Although lean NAFLD has frequently been described in Asian populations, it is diagnosed in 10%–20% of nonobese individuals in the United States and other western countries.[4,7] Despite their apparent "healthier" phenotype, such lean individuals also manifest the spectrum of histopathologic features of NAFLD-NASH, including steatosis, lobular inflammation, hepatocyte ballooning, and fibrosis.[8]

Although the pathophysiology of NAFLD-NASH remains largely unknown, adipose tissue dysfunction is thought to play an important role in its development.[9] Various biological processes in adipocytes—including lipid handling, glucose uptake, and adipokine expression and secretion—are regulated by insulin,[10–12] and insulin resistance in adipose tissue has been associated with NAFLD-NASH and other metabolic disorders.[13,14] 3′-Phosphoinositide–dependent kinase 1 (PDK1) is a serine-threonine protein kinase that plays a central role in insulin action by phosphorylating and activating the downstream kinase Akt.[15] We have recently shown that adipocyte-specific PDK1 knockout (A-PDK1KO) mice have a reduced adipose tissue mass that results from dysfunction of this tissue and gives rise to systemic insulin resistance, an increased blood glucose level, and NAFLD-NASH during maintenance on normal chow,[16] a phenotype that resembles lean NASH in humans. We also found that adipocyte-specific ablation of forkhead box protein O1 (FoxO1) ameliorated NAFLD-NASH and other metabolic disorders in A-PDK1KO mice, suggesting that adipose tissue insulin resistance regulated by a PDK1-FoxO1 axis contributes to the development of NAFLD-NASH.[16]

To provide insight into the combined impact of dietary factors and adipose tissue insulin resistance on NAFLD-NASH progression, we have here examined the effect of the Gubra amylin NASH (GAN) diet rich in saturated fat, cholesterol, and fructose[17] on the development of NAFLD-NASH in A-PDK1KO mice. A-PDK1KO mice fed with the GAN diet for 16 weeks showed more pronounced inflammation and fibrosis in the liver compared with control mice fed with the GAN diet or with A-PDK1KO mice fed with normal chow. Of note, the reduced adipose tissue mass of A-PDK1KO mice was not affected by the GAN diet. A-PDK1KO mice fed with the GAN diet, therefore, provide a new mouse model of lean NASH. To identify potential molecular signatures of NASH progression, we performed RNA-sequencing (seq) analysis for the liver and evaluated the effects of adipocyte-specific PDK1 ablation and the GAN diet on hepatic gene expression.

METHODS

Animals

Pdk1-floxed mice[18] and *Adipoq-Cre* mice[19] were described previously and were bred to obtain A-PDK1KO mice. A-PDK1KO ($Pdk1^{F/F}$; *Adipoq-Cre*) and control ($Pdk1^{F/F}$) mice were housed at a temperature of 21 °C to 25 °C and on a 12-hour-light/12-hour-dark cycle in the animal facility at Kobe University Graduate School of Medicine. Male mice at 4 weeks of age were randomized to individual diet groups according to baseline body weight and were provided with free access to tap water and either normal chow (CE-2, CLEA Japan) or the GAN diet [40 kcal% fat (0% transunsaturated and 46% saturated fatty acids by weight), 22% fructose, 10% sucrose, and 2% cholesterol (D09100310, Research Diets)] for 16 weeks. All animal experiments were performed in accordance with the guidelines of the animal ethics committee of the Kobe University Graduate School of Medicine.

Analysis of metabolic parameters

Blood glucose concentration was measured with a Glutest kit (Sanwa Kagaku Kenkyusho). Plasma transaminase, triglyceride, nonesterified fatty acid, and cholesterol levels were determined with kits (Fujifilm Wako). Plasma insulin concentration was determined with an ELISA kit (Fujifilm Wako Shibayagi).

Assay of liver triglyceride content

Lipids were extracted from the liver as described,[20] and the concentration of triglyceride in the extract was determined with a kit (Fujifilm Wako).

Histology of adipose tissue

Tissue was fixed with 10% neutral buffered formalin and processed for staining of paraffin-embedded sections with hematoxylin-eosin (H&E) or F4/80 antibody (T-2028, BMA Biomedicals).

Liver histology

Serial sections of the liver were stained with H&E or Sirius red according to standard techniques. The steatosis (0–3), hepatocyte ballooning (0–2), and

inflammation (0–3) scores were determined from H&E-stained sections and were summed to give the total NAFLD activity score.[21] The extent of hepatic fibrosis was determined from Sirius red–stained sections and was scored as follows: stage 1, zone 3 perisinusoidal fibrosis; stage 2, zone 3 perisinusoidal fibrosis with portal fibrosis; stage 3, zone 3 perisinusoidal fibrosis and portal fibrosis with bridging fibrosis; and stage 4, cirrhosis.[22]

Quantification of liver collagen

The proportion of liver tissue stained with Sirius red was quantified by morphometric analysis with the use of ImageJ software in 5 randomly selected fields per section (×200 magnification), as described.[23] The hydroxyproline content in whole-liver specimens was quantified with a Hydroxyproline Assay Kit (STA-675, Cell Biolabs).

Reverse transcription and real-time PCR analysis

Total RNA was extracted from tissue with the use of an RNeasy Minikit (Qiagen) and was subjected to reverse transcription (RT) with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNA was subjected to real-time PCR analysis with SYBR Green PCR Master Mix in an ABI StepOne Plus real-time PCR system (Applied Biosystems). Relative mRNA abundance was determined by the standard curve method with normalization according to the amount of *Rplp0* mRNA. The sequences of the PCR primers are provided in Supplemental Table S1, http://links.lww.com/HC9/A289.

RNA-seq analysis

Purified RNA was subjected to mRNA amplification by 5′ template-switching PCR with the use of a Clontech SMART-Seq v4 Ultra Low Input RNA Kit (Clontech). Amplified cDNA was fragmented and ligated with dual-indexed barcodes with the use of a Nextera XT DNA Library Prep Kit (Illumina). Libraries were validated with an Agilent 4200 TapeStation, pooled, sequenced with the NovaSeq. 6000 system, and analyzed with DRAGEN Bio-IT Platform v3.6.3. Relative expression levels were evaluated on the basis of transcripts per million.

Pathway analysis

The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed with the use of DAVID (https://david.ncifcrf.gov).

Statistical analysis

Data are presented as means + SEM and were compared by 1-way ANOVA followed by Tukey test. A p-value of < 0.05 was considered statistically significant.

RESULTS

Effects of the GAN diet on glucose and lipid metabolism in A-PDK1KO mice

A-PDK1KO and control mice were fed either normal chow or the GAN diet for 16 weeks after weaning at 4 weeks of age (Figure 1A). Consistent with our previous findings,[16] A-PDK1KO mice showed increased blood glucose and plasma insulin concentrations compared with control mice during maintenance on normal chow (Figure 1B). While the GAN diet increased blood glucose and plasma insulin levels in control mice, it lowered them in A-PDK1KO mice (Figure 1B). The plasma concentrations of triglyceride and total cholesterol were increased in A-PDK1KO mice compared with control mice during feeding with normal chow (Figure 1C), again consistent with our previous results.[16] The GAN diet reduced the plasma triglyceride level in A-PDK1KO mice, whereas it had no effect on that of triglyceride in control mice (Figure 1C). The GAN diet increased the plasma total cholesterol level both in control mice and A-PDK1KO mice. The plasma concentration of free fatty acids did not differ between control and A-PDK1KO mice fed with normal chow and was not affected by the GAN diet (Figure 1C). These results suggested that the GAN diet has differential effects on glucose and lipid metabolism in A-PDK1KO mice and in control mice.

The GAN diet and adipocyte-specific PDK1 deficiency additively promote liver inflammation and fibrosis

We previously showed that A-PDK1KO mice fed with normal chow develop NASH with age.[16] We, therefore, next investigated the effect of the GAN diet on the development of liver disease in these mice. Maintenance of the GAN diet for 16 weeks increased liver weight in control mice and augmented the increase in liver weight apparent in A-PDK1KO mice fed with normal chow (Figure 2A, B). The plasma concentrations of aspartate aminotransferase and alanine aminotransferase were increased in control mice by the GAN diet and were further increased in A-PDK1KO mice by this diet (Figure 2C). Histological analysis revealed mild steatosis associated with ballooning degeneration of some hepatocytes in the liver of A-PDK1KO mice fed with normal chow (Figure 2D, E). Although the GAN diet exacerbated steatosis and hepatocyte ballooning in

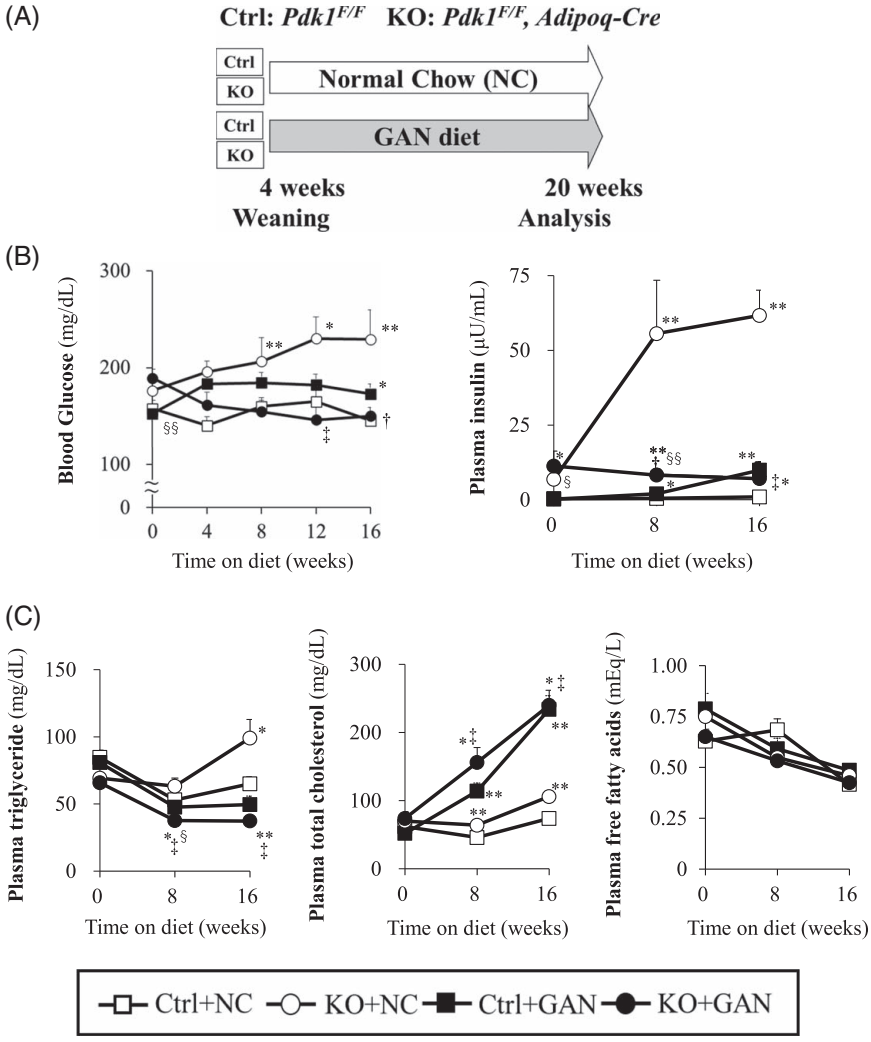

FIGURE 1 Effects of the GAN diet on glucose and lipid metabolism in A-PDK1KO mice. (A) Study protocol. Blood glucose and plasma insulin concentrations (B), and plasma triglyceride, total cholesterol, and free fatty acid concentrations (C) in the ad libitum fed state for male Ctrl and A-PDK1KO (KO) mice maintained on either NC or the GAN diet for 16 weeks (n = 10 mice for each group). All data are means + SEM. *$p < 0.05$, and **$p < 0.01$ versus control mice fed with NC; †$p < 0.05$ and ‡$p < 0.01$ versus A-PDK1KO mice fed with NC; §$p < 0.05$ and §§$p < 0.01$ versus control mice fed with the GAN diet (1-way ANOVA followed by Tukey test). Abbreviations: Ctrl, control; GAN, Gubra amylin NASH; KO, knockout.

A-PDK1KO mice, the extents of these conditions did not differ substantially between A-PDK1KO and control mice maintained on this diet (Figure 2D, E). The differences in steatosis score among the groups of mice were paralleled by those in liver triglyceride content with the exception that the GAN diet did not further increase triglyceride content for A-PDK1KO mice (Supplemental Figure S1A, http://links.lww.com/HC9/A290). Mild inflammation was apparent in about half of the A-PDK1KO mice fed with normal chow (Figure 2D, E), and stage 1 fibrosis, consisting of interstitial or periportal fibrosis, was evident in most of these mice (Figure 2F–H). While control mice fed with the GAN diet manifested hepatic inflammation and fibrosis, these conditions were more severe in A-PDK1KO mice fed with this diet (Figure 2D–H). Consistent with the fibrosis score, the hydroxyproline content of the liver was markedly increased in A-PDK1KO mice fed with the GAN diet compared with the other 3 groups (Figure 2I). Together, these results suggested that adipocyte-specific PDK1 ablation and the GAN diet additively promote inflammation and fibrosis in the liver.

The GAN diet and adipocyte-specific PDK1 deficiency additively upregulate hepatic gene expression related to inflammation and fibrosis

To understand how adipocyte-specific PDK1 deficiency and the GAN diet contribute to the development of

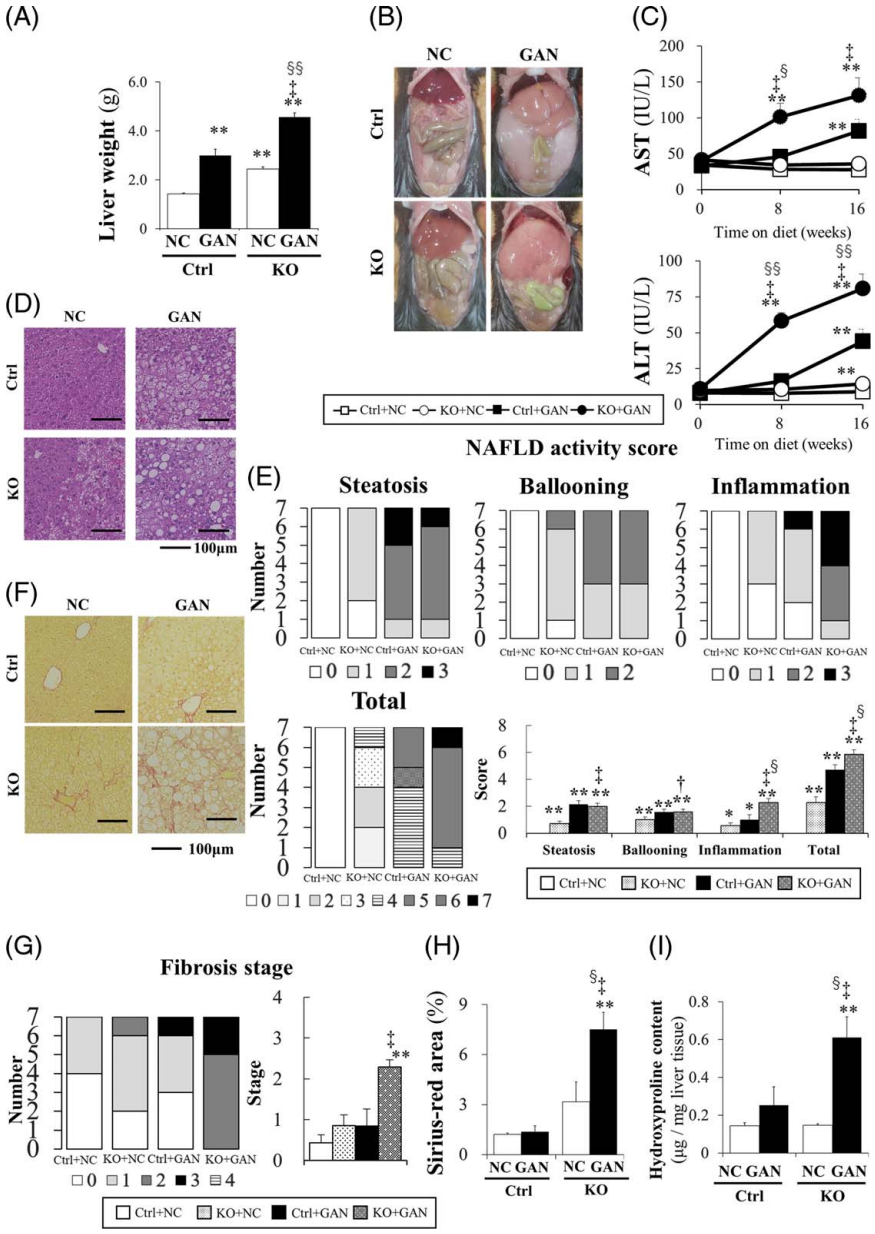

FIGURE 2 The GAN diet and adipocyte-specific PDK1 deficiency additively promote liver inflammation and fibrosis. (A–C) Liver weight (A), appearance of the liver (B), and plasma transaminase concentrations (C) for male control (Ctrl) and A-PDK1KO (KO) mice fed with either NC or the GAN diet for 16 weeks (n = 10 mice for each group). (D–H) Histopathologic assessment of liver sections from A-PDK1KO and control mice fed with NC or the GAN diet for 16 weeks (n = 7 mice per group). Representative H&E staining (D), the NAFLD activity score [steatosis score (0–3) ballooning score (0–2) inflammation score (0–3) and total score (0–8)] (E), representative Sirius red staining (F), fibrosis stage (0–4) (G), and percentage area positive for Sirius red staining (H) are shown. (I) Hydroxyproline content of the liver for A-PDK1KO and control mice after maintenance on NC or the GAN diet for 16 weeks (n = 10 mice per group). All quantitative data with the exception of mouse numbers are means + SEM. *$p < 0.05$ and **$p < 0.01$ versus control mice fed with NC; †$p < 0.05$ and ‡$p < 0.01$ versus A-PDK1KO mice fed with NC; §$p < 0.05$ and §§$p < 0.01$ versus control mice fed with the GAN diet (1-way ANOVA followed by Tukey test). Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; Ctrl, control; GAN, Gubra amylin NASH; KO, knockout; NC, normal chow.

NASH, we investigated gene expression profiles in the liver by RNA-seq analysis. We first analyzed changes in gene expression induced by the GAN diet in control and A-PDK1KO mice. The expression of 1619 genes was altered (> 2-fold or <0.5-fold change) by the GAN diet in both control and A-PDK1KO mice (Figure 3A). The KEGG pathway analysis revealed that these 1619 genes were enriched in various pathways, with the top 2 being cytokine-cytokine receptor interaction and chemokine signaling pathway (Figure 3A), both of which are implicated in the inflammation associated with NASH pathogenesis.[24,25] We next analyzed changes in gene expression induced by adipocyte-specific PDK1 deficiency in mice fed with the GAN diet or normal chow. The expression of 354 genes was altered (> 2-fold or <0.5-fold) by adipocyte-specific

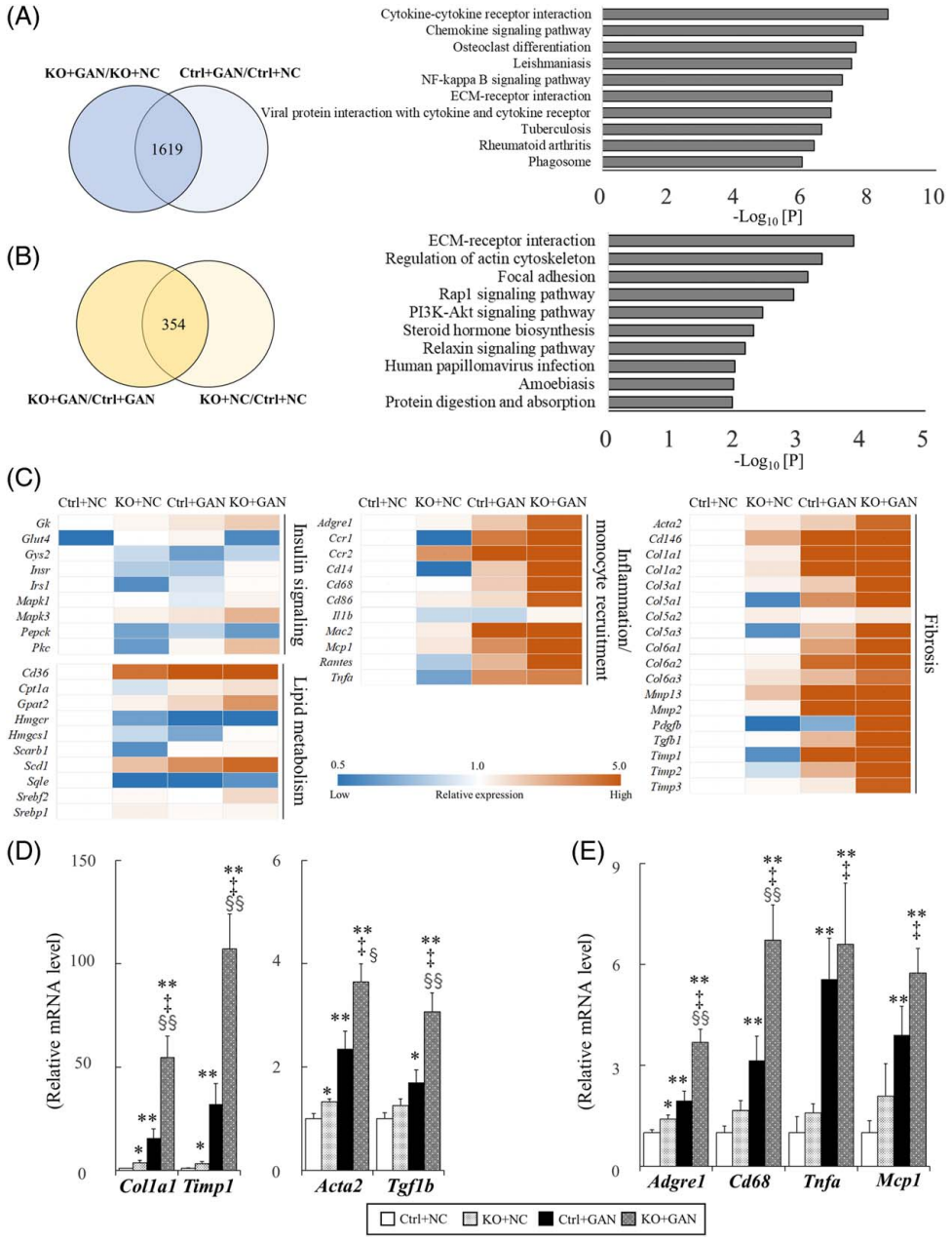

FIGURE 3 The GAN diet and adipocyte-specific PDK1 deficiency additively upregulate gene expression related to inflammation and fibrosis in the liver. (A, B) KEGG pathway analysis based on the results of RNA-seq analysis for the liver of male control (Ctrl) or A-PDK1KO (KO) mice fed with either normal chow (NC) or the GAN diet for 16 weeks (pooled sample from 10 mice for each group). The top 10 pathways enriched among genes whose expression was altered (>2-fold or <0.5-fold) by the GAN diet in control or A-PDK1KO mice (A) or by adipocyte-specific PDK1 deficiency in mice fed with normal chow or the GAN diet (B) are shown. (C) Heat maps for changes in the expression of NAFLD-NASH candidate genes as determined by the RNA-seq analysis. RT and real-time PCR analysis of the expression of genes related to fibrosis (D) or to inflammation (E) in the liver of A-PDK1KO or control mice after maintenance on normal chow or the GAN diet for 16 weeks (n = 10 mice for each group). Data are means + SEM. *$p < 0.05$ and **$p < 0.01$ versus control mice fed with normal chow; ‡$p < 0.01$ versus A-PDK1KO mice fed with normal chow; §$p < 0.05$ and §§$p < 0.01$ versus control mice fed with the GAN diet (1-way ANOVA followed by Tukey test). Abbreviations: Ctrl, control; ECM, extracellular matrix; GAN, Gubra amylin NASH; KO, knockout; NC, normal chow.

PDK1 deficiency in both the GAN diet– and normal chow–fed animals (Figure 3B). The KEGG pathway analysis showed that these 354 genes were enriched in pathways including extracellular matrix (ECM)–receptor interaction, regulation of actin cytoskeleton, and focal adhesion (Figure 3B), all of which are also implicated in NASH pathogenesis.[26] Collectively, these results, thus, suggested that various genes in the identified pathways may additively or independently contribute to NASH pathogenesis, including the fibrosis and inflammation induced by the GAN diet or adipocyte-specific PDK1 deficiency.

We next generated transcriptome signatures for various candidate genes implicated in NASH development

and progression with the results of our RNA-seq analysis.[27] Genes related to fibrosis or to inflammation or monocyte recruitment were upregulated by the GAN diet in control mice, and this effect was augmented by adipocyte-specific PDK1 deficiency (Figure 3C). We also confirmed the altered expression of the genes related to fibrosis—including *Col1a1*, *Timp1*, *Acta2*, and *Tgfb1*—or to inflammation, including *Adgre1*, *Cd68*, *Tnfa*, and *Mcp1*, by RT and real-time PCR analysis (Figure 3D, E). On the other hand, the expression of genes related to lipid metabolism did not show a consistent trend with regard to the effects of the GAN diet or adipocyte-specific PDK1 deficiency (Figure 3C). The expression of *Pparg*, *Srebp1*, *Fasn*, *Dgat2*, and *Gpat1* was significantly increased or tended to be increased by adipocyte-specific PDK1 deficiency, whereas the GAN diet downregulated the expression of these lipogenic genes in A-PDK1KO mice (Supplemental Figure S1B, C, http://links.lww.com/HC9/A290). These results suggested that the GAN diet and adipocyte-specific PDK1 deficiency additively promoted the hepatic expression of genes related to inflammation or fibrosis in association with the promotion of NASH pathogenesis, whereas they did not show a consistent effect on the expression of those related to steatosis.

The GAN diet does not affect adipose tissue inflammation or adipokine production in A-PDK1KO mice

Finally, we investigated whether the exacerbation by the GAN diet of NASH pathology due to adipocyte-specific PDK1 deficiency might be mediated by an effect on adipose tissue. As we showed in our previous study,[16] the weight of epididymal white adipose tissue (eWAT) was reduced in A-PDK1KO mice compared with control mice (Figure 4A). Although the GAN diet increased the weight of eWAT in control mice, it had no effect on that in A-PDK1KO mice (Figure 4A). Adipocyte size was also increased by the GAN diet in control mice but was unaffected in A-PDK1KO mice (Figure 4B). Chronic inflammation of adipose tissue plays an important role in the development of systemic insulin resistance and metabolic disease.[9] We, therefore, investigated whether the GAN diet might induce chronic inflammation in adipose tissue. Immunohistochemical staining of the macrophage marker F4/80 revealed no obvious macrophage infiltration in eWAT of A-PDK1KO mice fed with either normal chow or the GAN diet, whereas the GAN diet appeared to promote macrophage infiltration in adipose tissue of control mice (Figure 4C). Consistent with these immunohistochemical findings, the expression of proinflammatory cytokine and chemokine genes, such as *Tnfa*, *Il6*, and *Mcp1*, and that of the gene for *Adgre1* in eWAT were not affected by the GAN diet in A-PDK1KO mice (Figure 4D). Moreover, expression of the genes for the insulin-sensitizing adipokines adiponectin and leptin was not affected by the maintenance of A-PDK1KO mice on the GAN diet (Figure 4E). Collectively, these results suggested that the exacerbation of NASH pathology by the GAN diet in mice with adipocyte-specific PDK1 deficiency is independent of chronic inflammation or the expression of adiponectin or leptin genes in adipose tissue.

Adipocyte-specific PDK1 deficiency alters adipokine expression to exacerbate NASH pathology by the GAN diet

To reveal the mechanism of the additional effect of the GAN diet and adipocyte-specific PDK1 deficiency on NASH pathology, we investigated gene expression profiles in eWAT by RNA-seq analysis. Among >900 potential adipokines,[28] the expression of 50 genes was upregulated (≥ 1.4-fold change), and that of 47 genes was downregulated (≤ 0.7-fold change) by adipocyte-specific PDK1 deficiency in both GAN diet– and normal chow–fed animals (Figure 4F, Supplemental Table S2 and S3, http://links.lww.com/HC9/A291 S3, http://links.lww.com/HC9/A292). Thus, some of these adipokines, regulated by adipose tissue insulin signaling through the PDK1 pathway, might be involved in the pathology of NASH caused by adipocyte-specific PDK1 deficiency and the GAN diet.

DISCUSSION

We have previously shown that adipose tissue dysfunction in A-PDK1KO mice gives rise to the development of NAFLD-NASH during maintenance on normal chow.[16] The GAN diet, which is rich in saturated fat, cholesterol, and fructose, induces liver histopathologic changes that recapitulate the hallmarks of NASH.[17,29] We have here investigated the combined effects of adipose tissue insulin resistance and the GAN diet on NAFLD-NASH progression with the use of A-PDK1KO mice. We found that these mice fed with the GAN diet develop inflammation and fibrosis in the liver that is more severe and less variable in extent compared with A-PDK1KO mice fed with normal chow or with control mice fed with the GAN diet. Animals maintained on an experimental diet have previously been shown to develop NAFLD-NASH at different rates and of varying severity.[30] Furthermore, the time required for NASH development in animal models can be an obstacle to research. The GAN diet is most commonly used in diet-induced NASH models. While normal mice on the GAN diet for ~28 weeks exhibit NASH histology, including steatosis, inflammation, hepatocyte ballooning, and fibrosis,[17] A-PDK1KO mice fed with this diet for 16 weeks exhibit similar or more severe hepatic inflammation and fibrosis. A-PDK1KO mice fed with the GAN

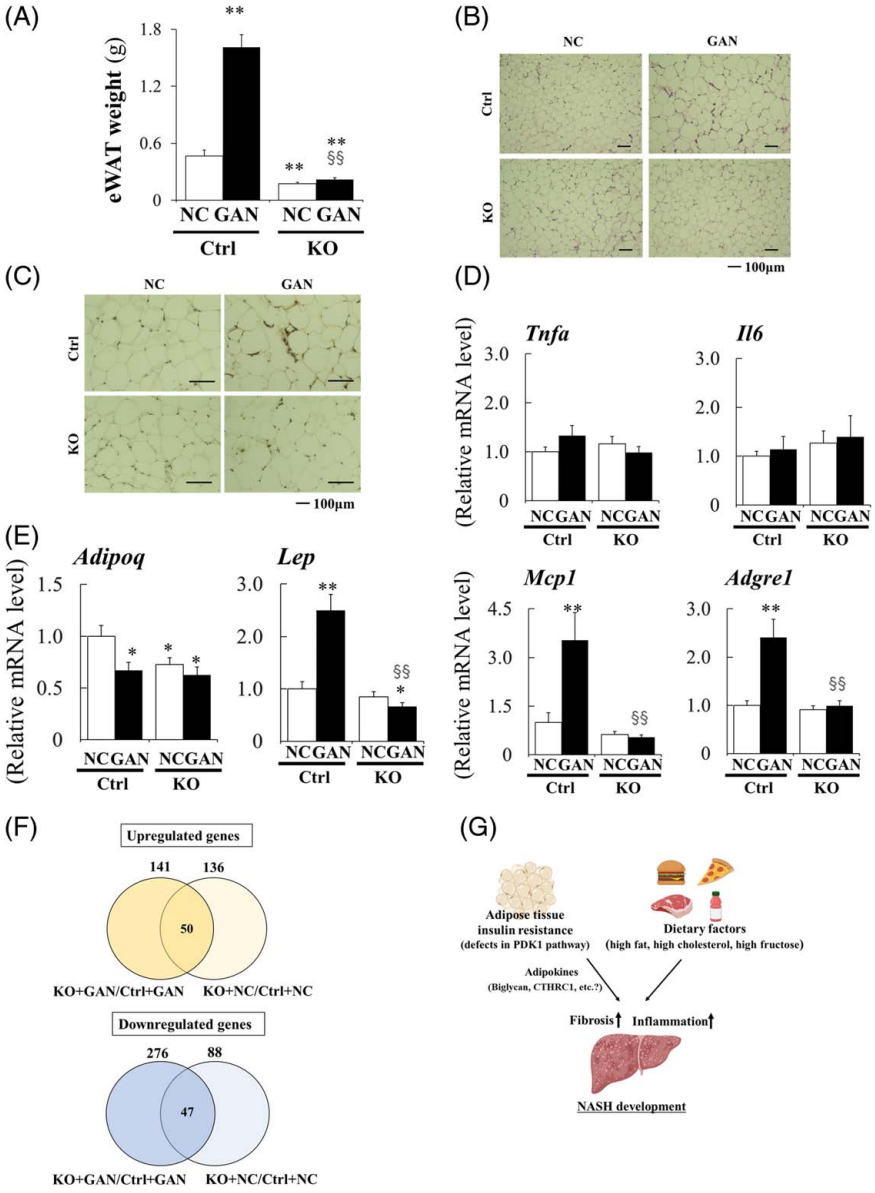

FIGURE 4 The evaluation of inflammation and adipokine expression in epididymal adipose tissue. (A) Weight of eWAT in male Ctrl and A-PDK1KO (KO) mice fed with either NC or the GAN diet for 16 weeks (n = 10 mice for each group). Representative hematoxylin-eosin staining (B) and F4/80 immunohistochemical staining (C) for eWAT of A-PDK1KO and control mice as in (A). (D) and (E) RT and real-time PCR analysis of the expression of genes related to inflammation or monocyte recruitment (D) or of adipokine genes (E) in eWAT of mice (n = 10 mice for each group) as in (A). (F) Venn diagram for the number of potential adipokine genes whose expression was increased (> 1.4-fold) or decreased (< 0.7-fold) by adipocyte-specific PDK1 deficiency in both GAN diet– and NC–fed mice in RNA-seq analysis of eWAT (pooled sample from 10 mice for each group). (G) Schematic summary showing that adipose tissue insulin resistance exacerbates liver inflammation and fibrosis in a diet-induced NASH model. All quantitative data are means + SEM. *$p < 0.05$ and **$p < 0.01$ versus control mice fed with NC; §§$p < 0.01$ versus control mice fed with the GAN diet (1-way ANOVA followed by Tukey test). Abbreviations: Ctrl, control; eWAT, epididymal white adipose tissue; GAN, Gubra amylin NASH; KO, knockout; NC, normal chow.

diet, thus, constitute a new mouse model that develops NAFLD-NASH with less histological variability and in a shorter period of time compared with existing models.

NAFLD is generally associated with obesity and related comorbidities, but it can also develop in the absence of obesity, in which case it is referred to as lean NAFLD. Although lean NAFLD has often been described for Asian populations, it has been diagnosed in 10%–20% of nonobese white individuals.[4] The pathological factors underlying the development of NAFLD in lean individuals remain unclear, but genetic factors, adipose tissue dysfunction, hormonal changes, reduced muscle mass, and gut dysbiosis have been implicated.[4–6,31] Indeed, adipose tissue insulin resistance that leads to adipose tissue dysfunction is thought to play an important role in the pathogenesis of lean NAFLD.[32] Studies of diabetic and weight-matched nondiabetic individuals and of nondiabetic men have

shown that adipose tissue insulin resistance correlates with hepatic fat accumulation, and this correlation is independent of body mass index and adipose tissue mass.[33,34] Insulin resistance in adipose tissue was also found to be associated with hepatic fat accumulation in addition to metabolic abnormalities, even in healthy nonobese Japanese men with a body mass index of <25 kg/m^2.[35] Adipose tissue insulin resistance is, thus, thought to contribute to the pathogenesis of hepatic fat accumulation independently of body weight and adipose tissue mass. The concept of metabolic dysfunction–associated fatty liver disease has recently been proposed for both obese and nonobese (normal weight or underweight) individuals,[36] with adipose tissue insulin resistance likely being an important contributor to the pathogenesis of this condition.

Functional enrichment analysis of genes whose expression in the liver was altered by the GAN diet revealed affected pathways to include those related to cytokine-cytokine receptor interaction and chemokine signaling pathway. Both of these pathways have been found to be upregulated in human NASH and are thought to play an important role in NASH pathophysiology, including inflammation and fibrosis.[24,25] On the other hand, the top pathway affected by adipocyte-specific PDK1 deficiency was ECM-receptor interaction. Excessive production of ECM gives rise to liver fibrosis. HSCs constitute the predominant ECM-producing fibroblasts in NAFLD-NASH models.[24] Adipose tissue insulin resistance due to PDK1 deficiency may, therefore, result in the production of some factor that links adipose tissue dysfunction to the activation of hepatic stellate cells. Pathways related to the regulation of actin cytoskeleton and to focal adhesion were also affected by adipocyte-specific PDK1 deficiency. Both of these pathways play important roles in intracellular processes in addition to defining the outward shape and appearance of a cell. Recent studies have also implicated changes in the cytoskeleton in the pathophysiology of NAFLD-NASH.[26] Cytoskeletal organization responds to changes in the extracellular environment, including those in the ECM and cytokines-chemokines.[37,38] Adipocyte-specific PDK1 deficiency and the GAN diet may, therefore, mutually promote inflammation and fibrosis in the liver through mechanisms related to interactions between the extracellular environment and the cytoskeleton in hepatocytes and nonparenchymal cells.

We investigated whether the exacerbation by the GAN diet of NAFLD-NASH pathology due to adipocyte-specific PDK1 deficiency is mediated through an effect on adipose tissue. However, the GAN diet did not affect adipose tissue mass or adipocyte size in A-PDK1KO mice. Adipokine imbalance is a key factor in NAFLD-NASH pathology.[39,40] However, although reduced blood levels of adiponectin have been associated with the pathogenesis of NAFLD-NASH,[41] we found that *Adipoq* expression in adipose tissue of A-PDK1KO mice was not affected by the GAN diet. Leptin also plays a role in the pathophysiology of NASH in addition to the regulation of energy metabolism,[42,43] but expression of the leptin gene in adipose tissue of A-PDK1KO mice was not altered by the GAN diet. Finally, the GAN diet had no effect on the expression of inflammatory cytokine and chemokine genes, including *Tnfa*, *Il6*, and *Mcp1*, or on that of *Adgre1* (examined as a marker of macrophage infiltration) in adipose tissue of A-PDK1KO mice even though chronic inflammation in adipose tissue plays an important role in the pathogenesis of metabolic disorders, including NAFLD-NASH.[9] Thus, well-known adipokines, including TNFα, IL6, MCP1, adiponectin, and leptin that may affect the development of NASH, are not likely to contribute to the additive effect of adipocyte-specific PDK1 deficiency on hepatic inflammation and fibrosis although some of these adipokines may be involved in the development of NASH by the GAN diet.

To elucidate the mechanism by which PDK1 deficiency in adipose tissue exacerbates the pathogenesis of NASH induced by a GAN diet, we examined alterations in the expression of potential adipokines in adipose tissue by comprehensive gene expression analysis using RNA-seq. We found a number of potential adipokines whose expression in this tissue was altered by PDK1 deficiency in both normal and GAN diets. Among these adipokines, factors such as C7, Elane, Krt18, Bgn, and Grem1, all of which were upregulated by adipocyte-specific PDK1 deficiency, have been reported to correlate their plasma levels or liver expression with the progression of NAFLD/NASH in humans.[44–48] In addition, it has been reported that genetic deletion of Bgn or Cthrc1, both of which were also upregulated by adipocyte-specific PDK1 deficiency, ameliorates drug-induced liver fibrosis in mice.[48,49] Both Biglycan, encoded by Bgn, and CTHRC1 have been shown to activate HSCs,[48,49] and activation of these cells is known to lead to increased ECM productions and reorganization of the actin cytoskeleton,[48,50] both of which are the top 2 pathways altered by adipocyte-specific PDK1 deficiency (Figure 3B). Therefore, these adipokines could link adipose tissue insulin resistance and histopathological alterations in the liver (Figure 4G). On the other hand, some of the 47 genes with reduced expression by adipocyte-specific PDK1 deficiency may act as adipokines that are protective against NASH progression. Although it is not known whether any of these factors are actually secreted from adipose tissue and delivered to the liver, it is possible that these factors may play important roles in the development of diet-induced NASH. The current study does not provide direct evidence to understand the mechanism of how adipocyte-specific PDK1 deficiency exacerbates the

pathogenesis of NASH, and further study with adipocyte-specific genetic deletion of these potential adipokines should shed light on the mechanism underlying NASH.

In summary, we have shown that adipose tissue insulin resistance and the GAN diet additively promote inflammation and fibrosis in the liver of mice. Given that both adipose tissue insulin resistance and dietary factors are thought to play important roles in the pathogenesis of human NAFLD-NASH, A-PDK1KO mice fed with the GAN diet constitute a new mouse model for studies of disease pathogenesis, especially for the genetically and environmentally susceptible population of lean individuals, as well as for the development of novel therapeutic strategies.

AUTHOR CONTRIBUTIONS

Yusei Hosokawa, Tetsuya Hosooka, and Wataru Ogawa designed experiments and analyzed the data. Yusei Hosokawa and Makoto Imamori performed experiments. Kanji Yamaguchi and Yoshito Itoh contributed to the histopathologic assessment of the liver. Yusei Hosokawa and Tetsuya Hosooka wrote the manuscript. Kanji Yamaguchi, Yoshito Itoh, and Wataru Ogawa contributed to the discussion.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

DATA AVAILABILITY

Original RNA-seq data have been deposited in the Gene Expression Omnibus database of NCBI (GSE 216128 and 224733).

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