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Reduced Adiponectin Signaling Due to Weight Gain Results in Nonalcoholic Steatohepatitis Through Impaired Mitochondrial Biogenesis

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Abstract

Obesity and adiponectin depletion have been associated with the occurrence of nonalcoholic fatty liver disease (NAFLD). The goal of this study was to identify the relationship between weight gain, adiponectin signaling, and development of nonalcoholic steatohepatitis (NASH) in an obese, diabetic mouse model. Leptin-receptor deficient ($Lepr^{db/db}$) and C57BL/6 mice were administered a diet high in unsaturated fat (HF) (61%) or normal chow for 5 or 10 weeks. Liver histology was evaluated using steatosis, inflammation, and ballooning scores. Serum, adipose tissue, and liver were analyzed for changes in metabolic parameters, messenger RNA (mRNA), and protein levels. $Lepr^{db/db}$ HF mice developed marked obesity, hepatic steatosis, and more than 50% progressed to NASH at each timepoint. Serum adiponectin level demonstrated a strong inverse relationship with body mass ($r = -0.82$; $P < 0.0001$) and adiponectin level was an independent predictor of NASH (13.6 μg/mL; $P < 0.05$; area under the receiver operating curve (AUROC) = 0.84). White adipose tissue of NASH mice was characterized by increased expression of genes linked to oxidative stress, macrophage infiltration, reduced adiponectin, and impaired lipid metabolism. HF $lepr^{db/db}$ NASH mice exhibited diminished hepatic adiponectin signaling evidenced by reduced levels of adiponectin receptor-2, inactivation of adenosine monophosphate activated protein kinase (AMPK), and decreased expression of genes involved in mitochondrial biogenesis and β-oxidation (*Cox4*, *Nrf1*, *Pgc1α*, *Pgc1β* and *Tfam*). In contrast, recombinant adiponectin administration up-

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regulated the expression of mitochondrial genes in AML-12 hepatocytes, with or without lipid-loading.

Conclusion—Lepr$^{db/db}$ mice fed a diet high in unsaturated fat develop weight gain and NASH through adiponectin depletion, which is associated with adipose tissue inflammation and hepatic mitochondrial dysfunction. We propose that this murine model of NASH may provide novel insights into the mechanism for development of human NASH.

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in the United States. It encompasses a wide spectrum of liver diseases ranging from benign steatosis to pathological nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis and liver cancer.[1] Although obesity and insulin resistance are major risk factors for NAFLD, the molecular mechanisms underlying the progression of this disease from simple steatosis to clinical NASH remain unclear.[2]

A commonly observed effect of increased weight gain and expansion of visceral fat is a decrease in the adipose tissue-specific hormone, adiponectin.[3,4] Depletion of this antiinflammatory and antidiabetic adipokine has been linked to the progression of NASH.[5] While many small-animal models of NASH have been created by high-caloric feeding of saturated[6–10] or unsaturated fats,[11–16] as well as through a combination of dietary and genetic factors,[7,19–21] the direct effects of weight gain on adiponectin depletion and NASH development have not been demonstrated in an animal model. Furthermore, *in vivo* and *in vitro* studies have demonstrated that saturated fats have other unique obesity-independent effects in the liver such as inflammation,[22] endoplasmic reticulum (ER) stress,[23] altered insulin signaling,[24] and increased apoptosis.[25] Thus, the effects of saturated fats and other common dietary components such as fructose[6,26] and cholesterol[6,27,28] which also have similar independent effects could confound an understanding of the relationship between weight gain, adiponetin signaling, and NASH. Comparatively, unsaturated fats are often demonstrated to have ameliorating effects in liver injury, especially monounsaturated fatty acids (MUFA) and omega (ω)-3 fatty acids.[29] In contrast, ω-6 fatty acids have been found to be detrimental or at least not beneficial,[29] and several studies suggest that a diet composed of a high ω-6 to ω-3[29,30] ratio may represent a frequently disregarded route to the development of NASH.

Other challenges in the development of small-animal models of NASH are consistency of feeding and the ability to reliably produce extensive weight gain in a suitable time period. Several models centered on high-fat diet consumption have failed to produce substantial weight gain[8,10,13,15–17] and exhibit infrequent NASH development,[8,16,17] while other models required over 20 weeks to develop the desired weight gain and histologic features.[6,9,19] A more elegant solution to consistent weight gain with minimal intervention is the use of hyperphagic mice, such as leptin- and leptin receptor-deficient mice (Lep$^{ob/ob}$ and Lepr$^{db/db}$, respectively) as increases in leptin signaling have previously been linked to adaptive feeding.[31]

The goal of the current study was to determine the role of adiponectin depletion in the development of NASH using a murine model which recapitulates the human NASH metabolic profile characterized by insulin resistance, diabetes, and obesity and the

histological features similar to human NASH such as inflammation, hepatocellular ballooning, and apoptosis by feeding a high-unsaturated fat diet to Lepr$^{db/db}$ mice and to corroborate the direct effects of adiponectin in AML-12 hepatocytes in the presence or absence of free-fatty acids.

Materials and Methods

Animals Studies

Five-week-old male C57BL/6J and B6.BKS(D)-*Lepr*^{db}/J diabetic mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were fed a normal chow diet (N) or a liquid high-fat diet (HF) (#712031, Lieber DeCarli Diet, Dyets, Bethlehem, PA; 71% energy from fat, 18% from protein, and 11% from carbohydrates; 1 Kcal/mL) *ad libitum* for either 5 or 10 weeks. Mice were weighed weekly and food consumption was monitored daily for mice on the HF diet. At the 5- and 10-week endpoints, fasting mice were sacrificed and serum and plasma samples were collected by cardiac puncture for assays of glucose, liver enzymes, and cytokines. All animal protocols were approved by the Benaroya Research Institute Animal Care and Use Committee.

Histological Analysis of Liver Tissue

Left and medial lobes of liver tissues were fixed in 10% formaldehyde saline solution and stained with hematoxylineosin and Masson's trichrome to assess fibrosis. Histological scoring was performed by a blinded hepatopathologist (M.M.Y.) with expertise in steatohepatitis by evaluating steatosis (0–3), inflammation (0–3), ballooning (0–2), and fibrosis (0–6) scores after the NASH Clinical Research Network (CRN) scoring system.[32]

Statistical Analysis

Statistical analysis for group comparisons was performed using Kruskal-Wallis, Mann Whitney, and Wilcoxon rank sum tests. Statistics were performed using the Pearson correlation test for continuous variables and the Spearman rank correlation for ordinal variables to determine associations. All analysis was performed using GraphPad Prism 5.0 (La Jolla, CA) and $P < 0.05$ was considered statistically significant.

See the Supporting Materials for a description of additional methods and results.

Results

Lepr$^{db/db}$ Mice Fed an Unsaturated Fat Diet Develop Marked Obesity and Hepatomegaly

Weight gain was assessed weekly in C57BL/6 and Lepr$^{db/db}$ mice. Mean HF diet consumption by Lepr$^{db/db}$ HF-fed mice was ~27 Kcal/day for the first 4 weeks, but ~23 Kcal/day for the final 6 weeks. This was significantly increased relative to C57BL/6 HF mice who consumed ~6.5 Kcal/day during most of the study period ($P < 0.001$, Fig. 1A).

Lepr$^{db/db}$ HF mice rapidly increased in mass at a rate of ~5.5 g/week for the first 5 weeks and ~1.5 g/week for the remainder of the study, with average mass ~62 g by week 8. Chow-fed Lepr$^{db/db}$ (N) mice increased in mass at ~1.5 g/week for the entire study and gained

~60% of their starting mass after 10 weeks (35–40 g final mass). C57BL/6 N and HF mice showed nearly identical weight gain at ~0.5 g/week throughout, with HF mice showing a slight increase only in the final week. Lepr$^{db/db}$ HF mice had significantly increased mass at every timepoint compared to Lepr$^{db/db}$ N mice while no differences were observed in body mass between C57BL/6 groups after 70 days (Fig. 1B). Liver mass was not significantly increased by HF feeding in C57BL/6 mice. Lepr$^{db/db}$ N mice had increased liver mass compared to C57BL/6 N mice at both timepoints, while Lepr$^{db/db}$ HF mice had hepatomegaly compared to genetic and dietary controls (Table 1).

HF-Fed Lepr$^{db/db}$ Mice Recapitulate the NASH Metabolic Phenotype

A panel of serum markers was examined to analyze liver injury, inflammation, and adipose tissue function (Table 1). Liver injury as determined by serum alanine transaminase (ALT) levels was not increased in C57BL/6 HF mice at either timepoint (Table 1). Lepr$^{db/db}$ HF mice showed greatly increased ALT levels at both timepoints compared to dietary and genetic controls. Glucose and insulin levels were substantially increased in all Lepr$^{db/db}$ mice compared to C57BL/6 controls, but showed no changes between chow and HF-fed animals within the C57BL/6 and Lepr$^{db/db}$ strains. Serum levels of the inflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin (IL)-6 were both increased in Lepr$^{db/db}$ N and HF mice by 10 weeks compared to their C57BL/6 dietary controls but not significantly elevated in Lepr$^{db/db}$ HF mice compared to Lepr$^{db/db}$ N. As expected, serum leptin was substantially elevated in all Lepr$^{db/db}$ mice compared to C57BL/6 controls. In both C57BL/6 and Lepr$^{db/db}$ animals, an HF diet sig nificantly increased leptin levels compared to chow-fed controls. Adiponectin levels were not significantly altered in C57BL/6 HF or Lepr$^{db/db}$ N mice compared to C57BL/6 N controls; however, Lepr$^{db/db}$ HF mice experienced ~25% and 33% reductions in adiponectin compared to Lepr$^{db/db}$ N mice at the 5 and 10 week timepoints, respectively.

Development of Obesity Results in NASH Histology and Increased Hepatic Apoptosis

Histological scoring for features of steatohepatitis were assessed as described in the Materials and Methods[32] and is shown in Table 2. C57BL/6 N and HF mice showed minimal histological activity with no steatosis at either timepoint. One Lepr$^{db/db}$ N mouse developed mild steatosis at 5 weeks, while two had mild steatosis by 10 weeks. In contrast, Lepr$^{db/db}$ HF mice developed moderate to severe steatosis with increased lobular inflammation and hepatocellular ballooning at both 5 and 10 weeks (50%, 60%, respectively, Fig. 1C; Supporting Fig. 1). Apoptotic nuclei were very infrequently observed in C57BL/6 N and HF mice with minor increases observed in Lepr$^{db/db}$ N mice. However, at both 5 and 10 weeks Lepr$^{db/db}$ HF mice exhibited increased apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining (Fig. 1D) compared to C57BL/6 HF and Lepr$^{db/db}$ N controls.

Reduced Serum Adiponectin Levels Are Associated With NASH Diagnosis

Mice with NAFL (n = 8) and NASH (n = 6) at 5 or 10 weeks were compared using Lepr$^{db/db}$ N mice without steatosis as controls as shown in Table 2 (n = 8; denoted as diabetes mellitus, DM). There were no significant differences between serum leptin levels among DM, NAFL, and NASH groups in the Lepr$^{db/db}$ mice. Remarkably, of all parameters tested,

only serum adiponectin differentiated the two hepatic phenotypes, NAFL and NASH. Adiponectin levels were significantly lower in NASH compared to NAFLD mice ($P = 0.046$, Fig. 2B) and correlated strongly to the proportional increase in body mass ($r = -0.82$, $P < 0.0001$, Fig. 2A). Furthermore, receiver-operator curve (ROC) analysis using adiponectin levels to predict NASH versus NAFL showed an area under the ROC (AUROC) (95% confidence interval [CI]) of 0.85 (0.63–1.0) ($P = 0.038$) with an 83.3% sensitivity and 71.4% specificity for adiponectin levels less than 13.29 μg/mL (Fig. 2C). Adiponectin levels were also useful for predicting NAFL versus DM with an AUROC (95% CI) of 0.88 (0.64–1.0) ($P < 0.02$, Fig. 2C) with a sensitivity of 85.7% and a specificity of 100% for adiponectin levels less than 17.19 μg/mL.

NASH Mice Exhibit Adipose Tissue Inflammation and Impaired Lipid Metabolism

Figure 3A shows relative mRNA levels of differentially expressed genes in the epididymal white adipose tissue (EWAT) of the Lepr$^{db/db}$ diagnosis groups. Adiponectin gene expression (*Adipoq*) and one of its transcription factors, CCAAT/enhancer-binding protein alpha (*C/ebpα*), were significantly reduced in NASH mice compared to DM mice, explaining the observed differences in serum levels. Triglyceride synthesis enzyme, diacylglycerol O-acyltransferase 2 (*Dgat2*) showed a reduction in NASH mice relative to the DM group. Furthermore, gene expression levels of stearoyl CoA desaturase (*Scd*1) and fatty acid synthase (*Fas*) were reduced in the NASH and NAFL groups relative to DM. Expression of hypoxia inducible factor 1 alpha (*Hif1α*) was increased in NAFLD and NASH mice. Erythropoeitin (*Epo*) gene expression was directly associated with serum adiponectin levels across all Lepr$^{db/db}$ mice ($r = 0.49$, $P < 0.001$; data not shown). The protein levels of Mac-2, an activated macrophage marker, was up-regulated in NASH mice (Fig. 3B) as was the expression level of macrophages markers, *CD68* and *F480/emr1*, and cytokines, *Ccl2* (also known as MCP-1) and *Tnfα* in the EWAT of NASH mice (Fig. 3C).

To further determine potential contributors to the distinct decrease in adiponectin levels observed in NASH mice, inter- (Fig. 2B) and intragroup (Supporting Table 2) regression analyses were performed between *Adipoq* mRNA and serum adiponectin levels against various genes in the adipose tissue. Interestingly, *Adipoq* expression showed a strong negative relationship with expression of inflammatory genes *Il6* ($r = -0.89$, $P = 0.02$) and *Crp* ($r = -0.92$, $P = 0.01$) within the NASH group alone and serum adiponectin levels were inversely correlated with expression of inflammatory genes *Nfkb1* ($r = -0.92$, $P = 0.01$), *Tnfα* ($r = -0.94$, $P = 0.005$) and directly associated with *C/ebpα* expression levels ($r = 0.95$, $P = 0.004$). There was also a positive association between expression of all these genes and final mass ($|r > 0.80|$, $P < 0.05$ for all) in NASH mice, but not within or across the other groups (data not shown).

Diminished Hepatic Adiponectin-adipoR2-AMPK Signaling in NASH Mice

To examine the downstream effects of hepatic adiponectin depletion, we evaluated protein and mRNA levels of the predominantly liver-specific adiponectin receptor-2 (AdipoR2)[33] and the activation status of its target effector, a serine threonine kinase-adenosine monophosphate activated protein kinase (AMPK) (Fig. 4A–C). The relative levels of AdipoR2 and phosphorylation status of AMPKα were reduced in the livers of NASH mice

relative to both DM and NAFL groups (Fig. 4A,B). Protein levels of liver kinase B1 (LKB1), one of the upstream protein kinases responsible for activating AMPK, were also decreased in NASH mice relative to NAFL and DM (Fig. 4B). Furthermore, the phosphorylation of Acetyl CoA carboxylase (ACCα), the downstream target of AMPK, was reduced in NASH mice relative to DM (Fig. 4B). Finally, protein and gene expression levels of sirtuin 1 (SIRT1), a regulator of hepatic energy metabolism, which is regulated by adiponectin and AMPK signaling,[34] were also significantly lower in the NASH mice relative DM (Fig. 4C).

Hepatic Mitochondrial Biogenesis Is Impaired in Livers of NASH Mice

We next examined the status of the mitochondrial biogenesis pathway, since the adiponectin-SIRT1-AMPK axis has been shown previously to influence mitochondrial signaling.[35] We found that gene expression levels of a class of important nutrient sensors and transcription factors, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1α*) and beta (*Pgc1β*),[36] and their downstream mitochondrial genes including Nuclear response factor (*Nrf1*), Transcription factor A, mitochondrial (*Tfam*), and cytochrome oxidase 4 (*Cox4*) were decreased in the NASH mice compared to DM and NAFL groups (Fig. 5A). Protein levels of PGC1α were also significantly reduced in the livers of NASH mice with respect to DM and NAFL mice (Fig. 5B).

Adiponectin Promotes Gene Expression Related to Adiponectin Signaling and Mitochondrial Gene Expression in Hepatocytes In Vitro

AML-12 cells were treated with recombinant full-length adiponectin (20 μg/mL) to examine the specific and individual effect of adiponectin. Adiponectin-treated hepatocytes showed an increase in expression levels of genes related to adiponectin signaling, such as *Adipor2*, *Ampkα*, and *Sirt1* (Fig. 6A). Furthermore, levels of mitochondrial biogenesis and β-oxidation related genes including *Cox4*, *Pgc1α*, and *Tfam* were also significantly elevated by adiponectin treatment relative to untreated controls (Fig. 6B). Adiponectin treatment also led to an increase in *Nrf1* expression levels, but the increase was not statistically significant ($P = 0.06$).

We also examined whether the effect of adiponectin on adiponectin signaling and mitochondrial gene expression was observed in the setting of free-fatty acid exposure in AML-12 hepatocytes. Cells were treated with 250 μM oleic acid (OA) for 24 hours with or without adiponectin in the presence of 1% fetal bovine serum (FBS) containing AML12 media. After 24 hours of treatment with OA, there was a significant reduction in the gene expression levels of *Adipor2*, *Ampkα*, *Pgc1α*, and *Tfam* relative to isopropanol-treated controls. Notably, adiponectin treatment was able to appreciably up-regulate the gene expression levels in the “steatotic” AML-12 cells also, partially mitigating the repression exerted by the free-fatty acid exposure (Fig. 6C).

We have also extended these studies to primary rat hepatocytes, and find that, as compared to OA-treated hepatocytes, those treated with recombinant adiponectin and OA showed significantly elevated levels of *AMPKα*, *SIRT1*, *PGC1α*, and *TFAM* (Supporting Fig. 2).

Discussion

We have shown that obese, diabetic mice fed a diet high in unsaturated fat are prone to rapid development of severe obesity, NAFLD and NASH. The single most important determinant of NASH development in this model appears to be adiponectin depletion, which develops after a critical level of weight gain. Significantly, the development of the NASH phenotype is rapid and was observed as early as 5 weeks, Lepr$^{db/db}$ HF mice developed steatohepatitis with inflammatory foci, consistent ballooning, increased apoptosis, and a metabolic phenotype resembling human NASH. No previous models to our knowledge have shown serum adiponectin as an independent predictor of NASH developing in concert with a threshold level of increase in body mass. Importantly, we also demonstrated that 10 weeks of *ad libitum* feeding of this diet to C57BL/6 mice does not lead to weight gain, liver damage, or artificially lowered adiponectin levels. However, one limitation of our experimental model is the severe leptin resistance present in this mouse model, which may limit the extrapolation of our findings to human NASH, wherein hepatic leptin resistance is also a feature, albeit to a lesser degree than this model.

Clinical observations have shown that obesity can lead to decreased serum adiponectin levels,[37] lower *Adipoq* expression in various fat depots,[38] and reduced hepatic levels of adiponectin receptor-2,[39] supporting our hypothesis for a central role for adiponectin depletion in the metabolic sequelae of obesity.

Previous studies have attempted to recapitulate human NASH using murine models of dietary and genetic models. However, several of these studies used diets containing saturated fats and additional factors (i.e., fructose or cholesterol), often over a period of 5–6 months.[6,9,19,28] While these complex models may emulate the common dietary combinations seen in a Western diet and reproduce the histologic features of NASH, they have limitations in interpreting their effect on NASH-induction pathways because their obesity-independent effects on inflammation, apoptosis, or ER stress may confound the influence of weight gain on the NASH phenotype.

A novel aspect of our model was the ability to produce NASH within 5 weeks using the same criteria as used for human studies.[32] Prior models using unsaturated fat feeding and genetically based hyperphagia were shown to develop hepatic fibrosis over a longer period of time,[12,20,21] suggesting that the model used in the current study may also recreate fibrosis associated with NASH over time.

In this study we demonstrated that the most important cause of NASH in obesity-related weight gain in a genetic model of obesity and diabetes is adiponectin depletion. While hypoadiponectinemia is an important feature of the metabolic syndrome, adiponectin depletion has not been reported in many earlier models of obesity and NAFLD/NASH.[8,10,11,13–16,18,20,21] Our model showed a significant decrease of 5–6 μg/mL in plasma adiponectin at both the 5- and 10-week time-points, which was clearly linked to advanced obesity. Not only was serum adiponectin distinctly lower in Lepr$^{db/db}$ HF mice at both timepoints, it was an independent predictor of both NAFL and NASH. Previous studies using adiponectin knockout mice, and the use of adiponectin supplementation-related studies

have provided useful lessons for the potential pathways of adiponectin action in protection against NASH progression. These mice are prone to development of hepatic steatosis, mitochondrial dysfunction, M1 macrophage activation, diminished M2 antiinflammatory markers, and steatohepatitis.[40–43] Mechanistic studies through knockout models and 3T3-L1 adipocytes revealed that the main therapeutic action of adiponectin is through the activation of the AMP-active protein kinase (AMPK) pathway.[33,44]

It should be pointed out that, while ω-3 fats have been associated with beneficial outcomes and ω-6 fats have been associated with worsening fatty liver disease in several animal studies,[29,30] NASH in human subjects has not been shown to develop from overfeeding polyunsaturated fatty acids (PUFAs), which is a caveat to the extrapolation of our findings to human NASH. Also, while it is known that there is a greater depletion of ω-3 fats compared to ω-6 fats in human NASH patients,[30,45] there is no clear evidence that consuming ω-6 PUFAs in amounts greater than ω-3 fats will lead to NASH in humans. Further, while some studies show that ω-3 PUFAs may improve hepatic steatosis in humans,[45] more detailed studies need to be done to determine the duration and dose to assess improvement of NASH.[45]

In our model, weight gain due to unsaturated HF-feeding of Lepr$^{db/db}$ mice led to visceral adipose tissue inflammation characterized by hypoxia, macrophage infiltration, and M1 activation, which could have contributed to the depletion of adiponectin.[46] Since adiponectin is known to exert multiple hepatoprotective effects such as regulating lipid metabolism, mitigating inflammation, and preventing cell death,[47] among others, its impaired production and signaling could have led to the major histological features of NASH observed in our mouse model: steatosis, inflammation, ballooning and apoptosis.

Adiponectin regulates hepatic energy homeostasis function through its receptors, adiponectin receptor-1(AdipoR1) and receptor-2 (AdipoR2), which signal through AMPK, to govern fatty acid oxidation, triglyceride synthesis, and mitochondrial biogenesis.[33,48] Mitochondrial function is further regulated by the transcription factors PGC1α, NRF1, and the mitochondrial transcription factor, TFAM. SIRT1, an NAD+ dependent deacytelase is also an important nutrient sensor and regulator of liver energy homeostasis and mitochondrial function, using PGC1α as one of its targets for deacetylation, thereby activating it.[34] In addition, AMPK regulates mitochondrial biogenesis not only through increased production, but also activation of PGC1α.[35]

In our NASH model, a critical threshold of weight gain led to hypoadiponectinemia and concomitant reduction of its liver-specific receptor, AdipoR2-exacerbating a feedforward “adiponectin resistance” in the liver leading to inactivation of the downstream effectors of its various target pathways: lipid metabolism and mitochondrial biogenesis. Strikingly, we show that protein levels of AdipoR2, LKB1, activated AMPKα, and mitochondrial regulators including PGC1α were reduced in the livers of NASH mice compared to NAFL mice, implying that adiponectin depletion-mediated impairment of mitochondrial biogenesis is a major factor in the transition from simple steatosis to NASH. Significantly, we also demonstrate that the direct addition of recombinant full-length adiponectin to the AML-12 hepatocytes *in vitro* stimulated genes associated with mitochondrial biogenesis. It is

noteworthy that we used a form of adiponectin, which has previously been demonstrated to form high molecular weight (HMW) oligomers. HMW oligomers of adiponectin has been shown to be the bioactive form of adiponectin capable of exerting its multiple beneficial effects on the liver.[47] Furthermore, we showed that while lipid-loading of AML-12 hepatocytes led to a decrease in the gene expression levels of *Ampkα*, *Adipor2*, *Pgc1α*, and *Tfam*, addition of adiponectin appreciably ameliorated the down-regulation exerted by lipid loading. Data from clinical studies also imply that mitochondrial dysfunction, indicated by mitochondrial structural defects, distinguishes NAFLD from NASH.[49] In support of our findings regarding the critical role of adiponectin-AMPK signaling in mitochondrial biogenesis, Galic et al.[50] recently showed hematopoietic AMPK deficiency causes reduced mitochondrial function and increased inflammation. Thus, our study links adiponectin depletion to hepatic mitochondrial dysfunction in an *in vivo* model.

These data provide support to the hypothesis that weight gain from an HF diet past a "tipping point" results in decreased adiponectin production from the adipose tissue, which then drives NASH by impaired mitochondrial β-oxidation of an increased fatty acid load in the setting of fatty liver.

In conclusion, we have successfully developed a small-animal model that accurately recapitulates the histological features of human NASH associated with obesity and diabetes. The rapid progression to NASH with consistent hepatocellular ballooning as early as 5 weeks makes our model attractive for interventional studies. Our model also provides insights into the development of NASH by obesity-induced adiponectin depletion and consequent dysregulation of hepatic lipid metabolism and mitochondrial biogenesis and highlights the direct effect of adiponectin in regulating hepatic mitochondrial biogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AdipoR2	Adiponectin receptor-2
AMPK	adenosine monophosphate activated protein kinase
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
PGC1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
SIRT1	sirtuin 1 (silent mating type information regulation 2 homolog 1)

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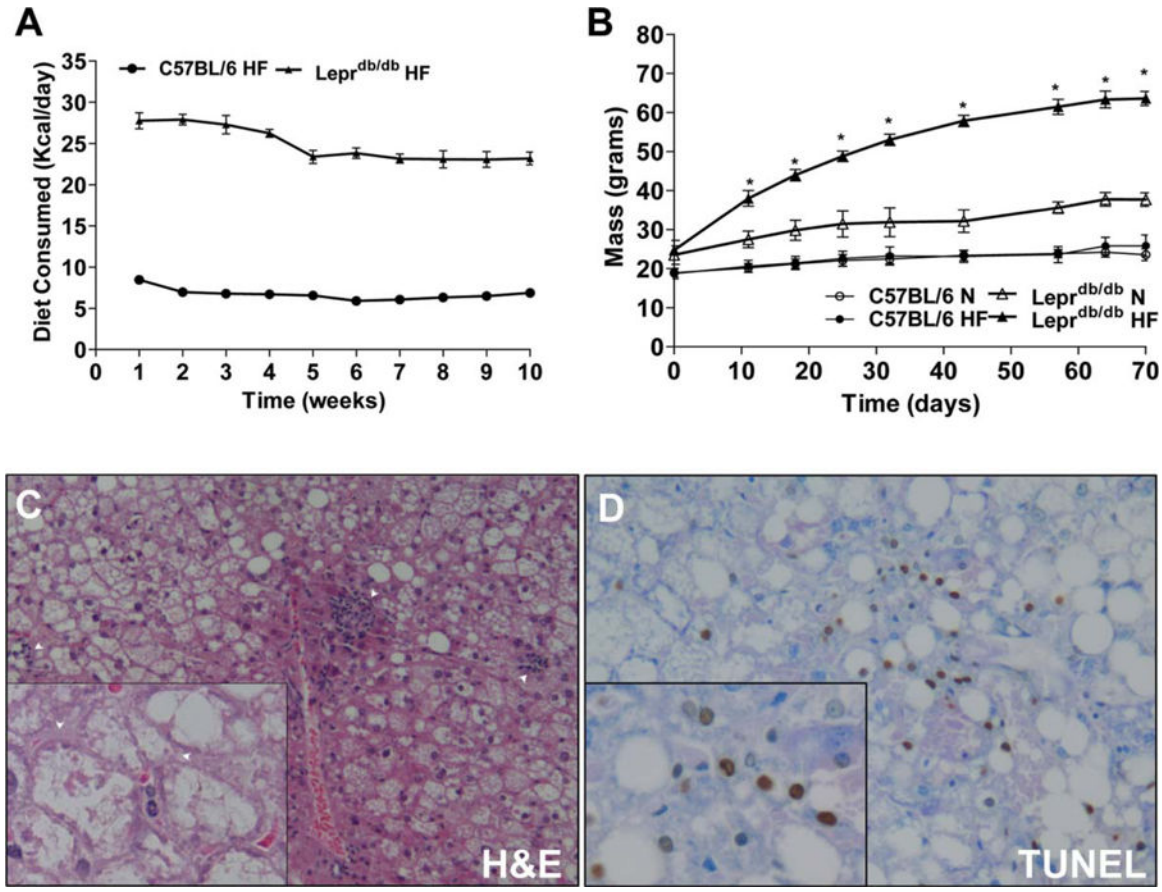

Fig. 1.

HF-fed Lepr$^{db/db}$ mice experience rapid weight gain, advanced NASH histology, and increased TUNEL staining. (A,B) Lepr$^{db/db}$ and C57BL/6 mice were put on a chow and HF diet and assessed weekly for food consumption and weight gain. (C) Representative micrograph of a Lepr$^{db/db}$ mouse on HF diet, showing hematoxylin and eosin (H&E) staining (C, 200× and 400×) and apoptosis by way of TUNEL staining (D, 200× and 400×). Arrows in (C) indicates lobular inflammation (200×), and ballooned hepatocytes (400×). *P < 0.05 versus Lepr$^{db/db}$ N Lepr$^{db/db}$, leptin-receptor deficient; N, normal chow diet; HF, high-fat diet; H&E, hematoxylineosin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

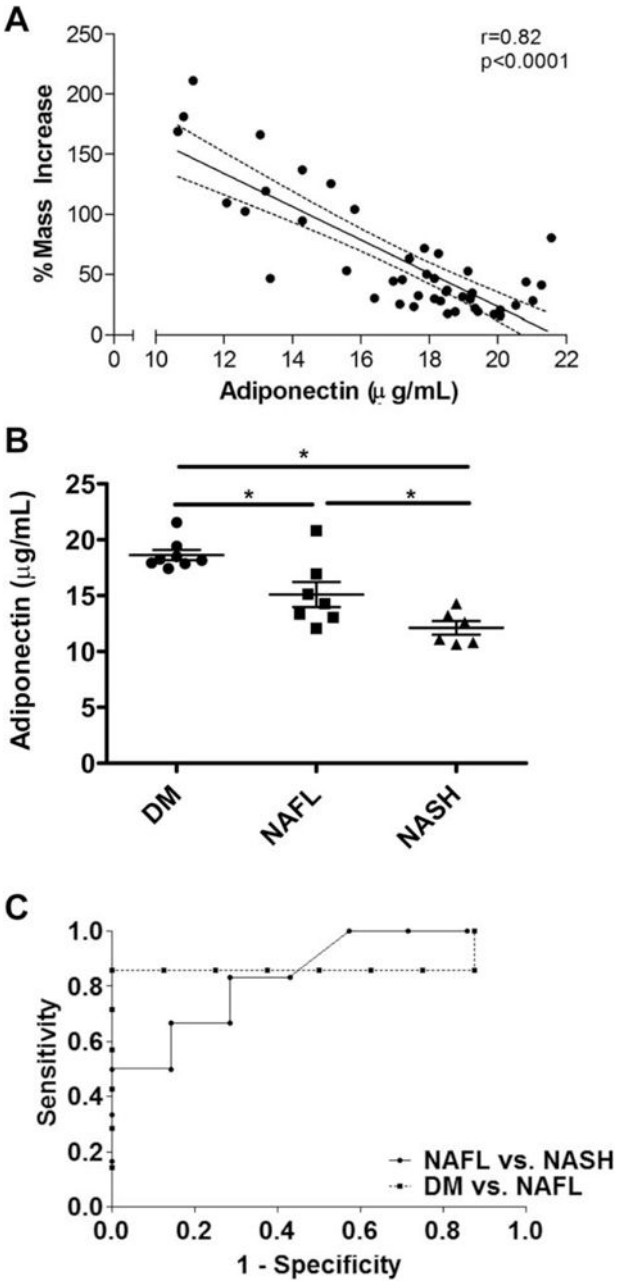

Fig. 2.

Mass increase results in adiponectin depletion and is an independent predictor of NASH. (A) Correlation between serum adiponectin and percent mass increase across all the mice in the study. (B) Serum adiponectin levels in DM, NAFL and NASH mice. **P* < 0.05, n = 6–8 each. (C) ROC analysis of serum adiponectin comparing DM, NAFL and NASH groups.

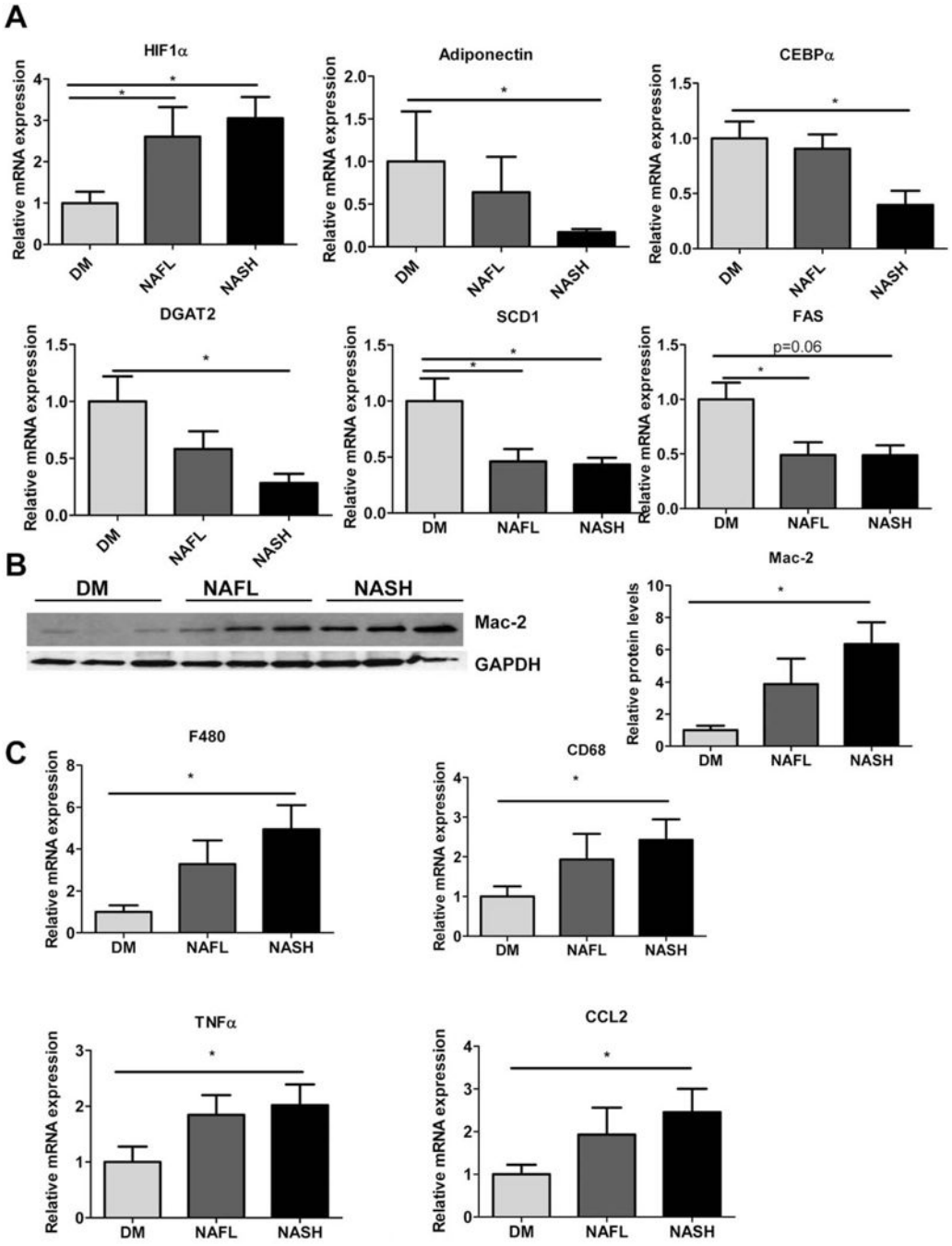

Fig. 3.

Adipose tissue dysfunction in NASH mice. (A) Relative mRNA levels of *Hif1a*, *Adipoq*, *C/ebpa*, *Dgat2*, *Scd1* and *Fas*. (B) Representative blot from adipose tissue lysates of Mac-2 (left) and densitometric quantitation (right) from DM, NAFL and NASH mice. (C) Relative mRNA levels of macrophage markers and inflammatory mediators: *F480*, *CD68*, *Tnfa* and *Ccl2* in the adipose tissue of DM, NAFL, and NASH mice. n = 6–8, $*P < 0.05$.

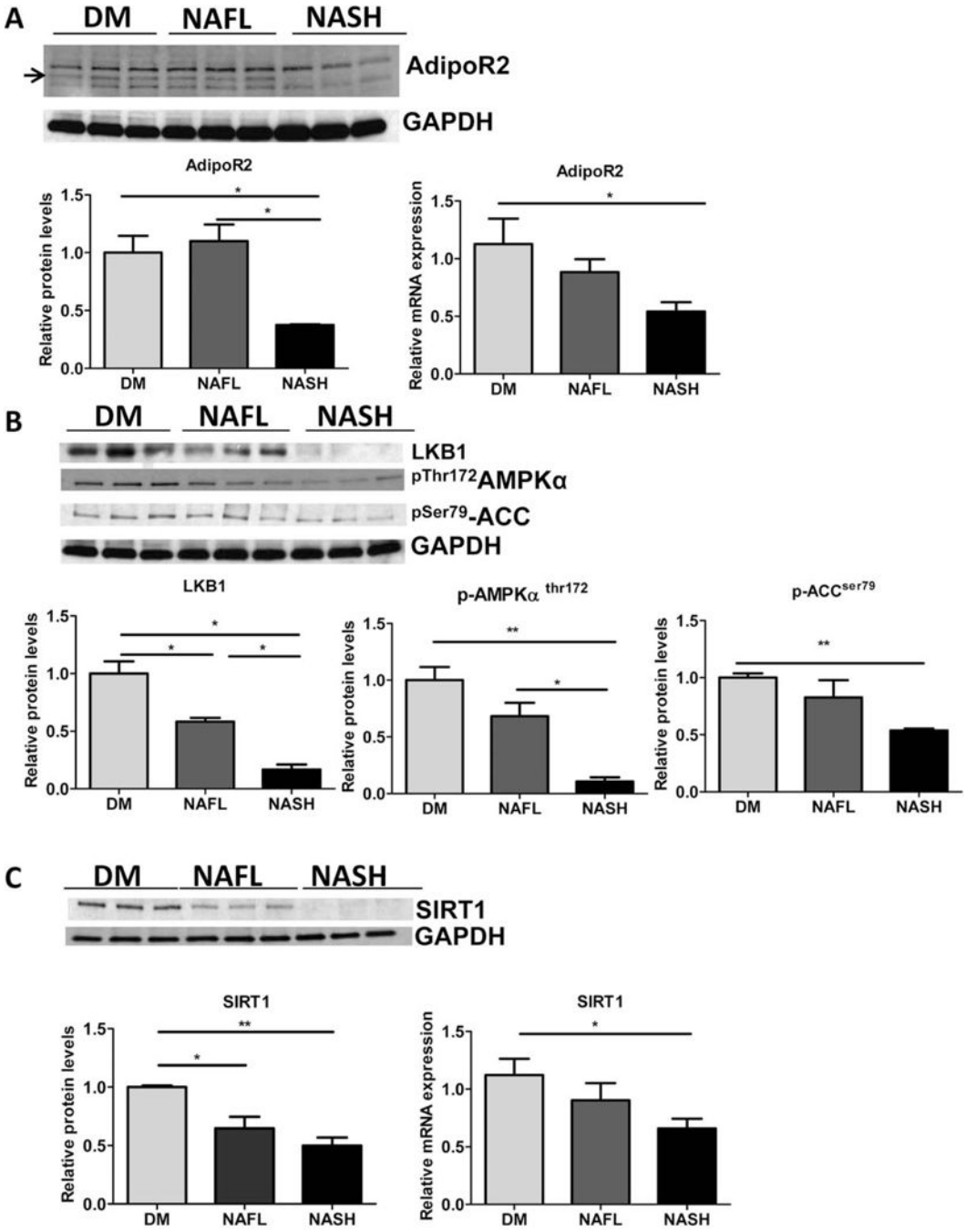

Fig. 4.

NASH mice experience decrease in hepatic adiponectin-AMPK signaling. Representative western blots (top) and densitometric quantitations of (A) adiponectin receptor-2 and GAPDH protein and relative mRNA expression analysis (below) (B). Representative western blots (top) and densitometric quantitations of LKB1, phosphorylated (Thr 172)-AMPKα protein levels, phosphorylated (Ser 79)-ACCα relative to GAPDH are shown (below). (C). Representative western blots (top), and densitometric quantitations of SIRT1 relative to GAPDH and relative gene expression in DM, NAFL and NASH groups of mice (below).

DM mice (n = 5), NAFL (n = 5), and NASH mice (n = 5), *$P < 0.05$, **$P < 0.01$ indicates statistical significance between the relevant groups shown.

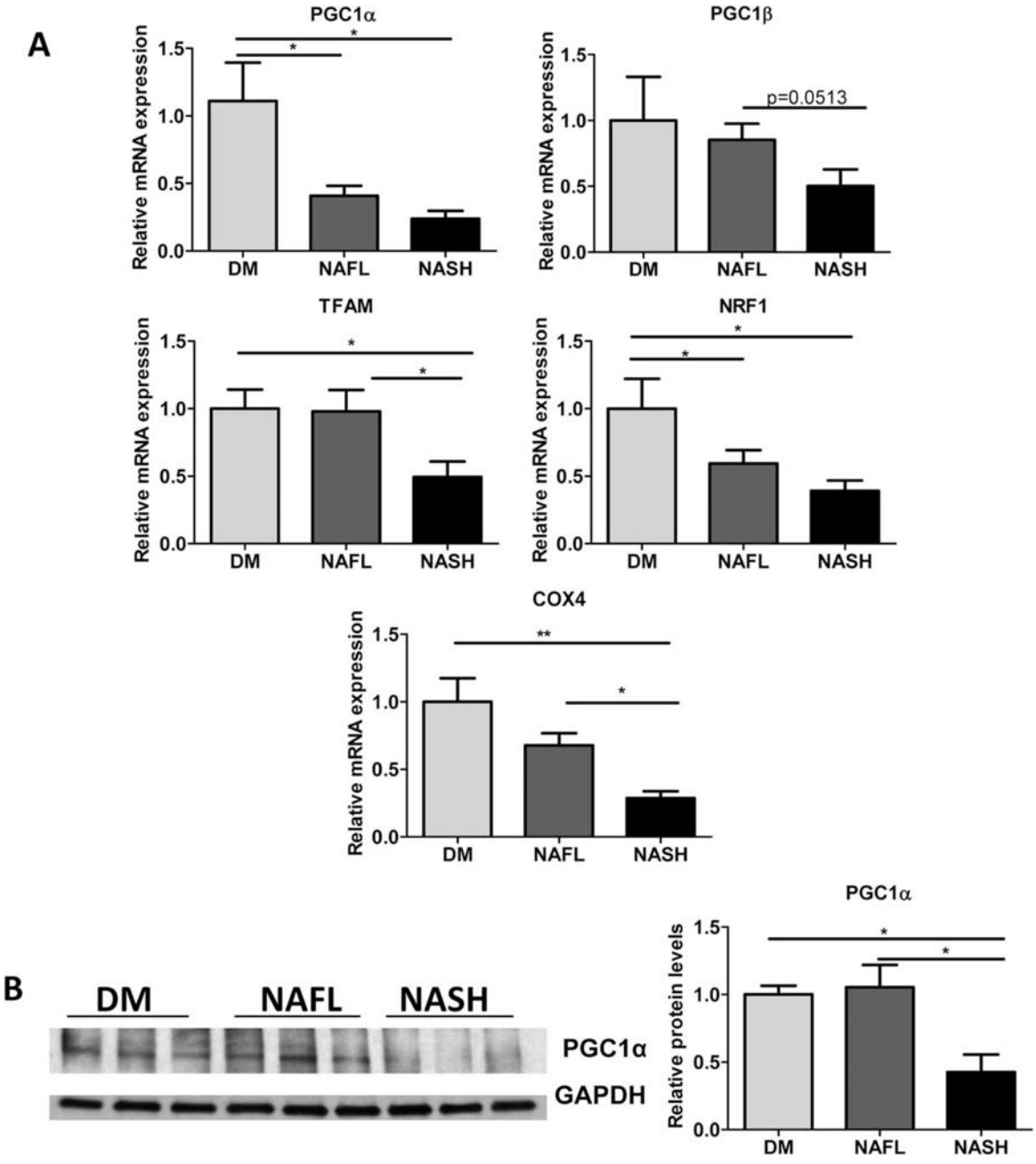

Fig. 5.

NASH mice experience hepatic mitochondrial dysfunction relative to DM and NAFL mice. (A) Relative mRNA expression levels of *Pgc1α*, *Pgc1β*, *Tfam*, *Nrf1* and *Cox4* were determined in DM, NAFL, and NASH mice. n = 6–8, $*P < 0.05$. (B) Protein levels of PGC1*α* relative to GAPDH protein (as loading control) were assessed in the livers of DM, NAFL, and NASH groups. n ≥ 5, $*P < 0.05$, $**P < 0.01$ indicates statistical significance between the relevant groups.

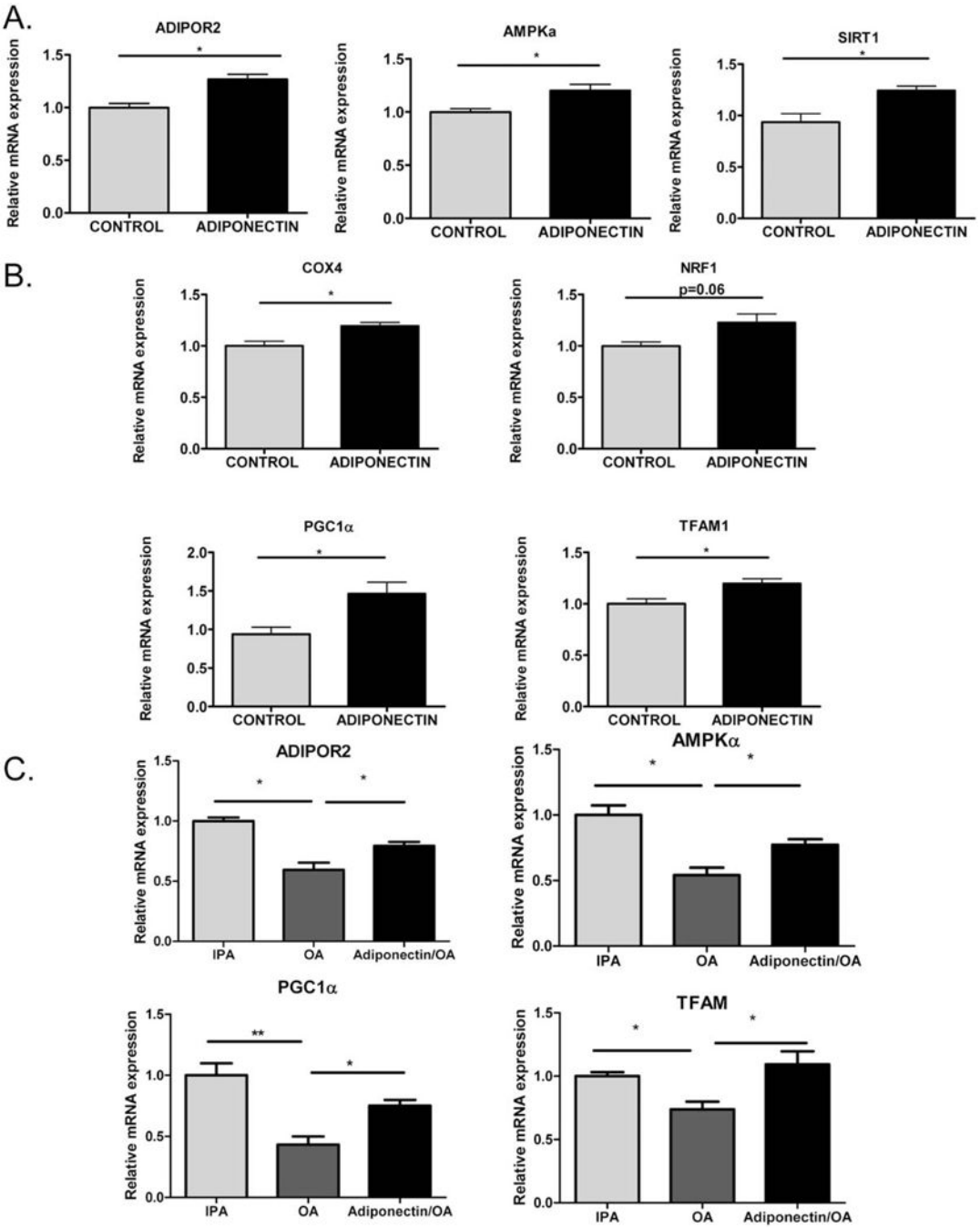

Fig. 6.

Adiponectin promotes gene expression of mitochondrial biogenesis pathway in hepatocytes. (A) Relative mRNA expression of *Adipor*2, *Ampkα*, and *Sirt*1 was determined from AML-12 hepatocytes treated with recombinant full-length adiponectin (20 μg/mL) for 24 hours in 1% FBS containing AML-12 media relative to untreated controls. n = 3. (B) Relative mRNA expression levels of *Cox4*, *Nrf1*, *Pgc1α*, and *Tfam* were determined from AML-12 hepatocytes treated with recombinant full-length adiponectin (20 μg/mL) for 24 hours in 1% FBS containing AML-12 media relative to untreated controls. n = 3. The

experiment was repeated three times and representative data are shown. (C) Adiponectin restores impaired adiponectin signaling and mitochondrial biogenesis-related gene expression levels in lipid-loaded AML-12 hepatocytes: AML-12 cells were treated with 250 μM OA for 24 hours with or without adiponectin (20 μg/mL) in 1% FBS containing AML-12 growth media. Control cells were treated with isopropanol (IPA, final concentration 0.5%). Relative mRNA expression levels of *Adipor2*, *Ampkα*, *Pgc1α*, and *Tfam* was determined. *$P < 0.05$ and **$P < 0.01$ indicates statistical significance.

Table 1

Effects of Diet and Genotype on Weight Gain and Metabolic Parameters After 5 and 10 Weeks of Dietary Administration

Time on Diet Genotype Diet	5 weeks				10 weeks			
	C57BL/6		Lepr$^{db/db}$		C57BL/6		Lepr$^{db/db}$	
	Chow (n=6)	High fat (n=6)	Chow (n=6)	High fat (n=5)	Chow (n=6)	High fat (n=6)	Chow (n=6)	High fat (n=6)
Weight gain								
Body mass, g	23.5 ± 0.2	24.8 ± 0.6	32.6 ± 1.8	54.4 ± 0.7*†	23.5 ± 0.6	25.9 ± 1.1	37.7 ± 0.7	63.6 ± 0.7*†
Liver mass, g	1.07 ± 0.03	1.06 ± 0.04	1.42 ± 0.14†	2.88 ± 0.07*†	1.07 ± 0.02	0.98 ± 0.04	1.99 ± 0.25†	2.92 ± 0.20*†
Metabolic parameters								
ALT, U/L	20.4 ± 1.9	38.8 ± 9.5	50.8 ± 4.5†	245.8 ± 1.6*†	48 ± 10.7	128.7 ± 58.3	96.8 ± 18.1	244.7 ± 29.9*†
Glucose, mg/dL	356 ± 19	464 ± 30	747 ± 45†	718 ± 51†	429 ± 53	483 ± 17	828 ± 52†	699 ± 75†
Insulin, pg/mL	415 ± 37	426 ± 70	2846 ± 631†	2333 ± 452†	200 ± 22	236 ± 67	3147 ± 910†	3189 ± 372†
TNFα, pg/mL	7.5 ± 0.4	8.6 ± 0.2	7.2 ± 1.0	10.5 ± 0.8	6.1 ± 0.4	7.9 ± 0.6	8.9 ± 0.8†	10.5 ± 0.6†
IL-6, pg/mL	5.4 ± 1.2	8.2 ± 1.5	11.9 ± 3.6	25.1 ± 7.0	4.8 ± 1.4	5.6 ± 0.8	12.3 ± 2.1†	14.1 ± 2.0†
Leptin, ng/mL	1.7 ± 0.2	7.7 ± 1.0*	50.3 ± 16.7†	319.2 ± 71.1*†	0.9 ± 0.1	8.2 ± 2.5*	83.4 ± 21.4†	274.1 ± 104.4*†
Adiponectin, μg/mL	19.4 ± 0.7	18.8 ± 0.3	18.2 ± 0.4	13.7 ± 0.6*†	18.4 ± 0.4	18.5 ± 0.9	18.2 ± 1.2	12.2 ± 0.8*†

Mean body mass, liver mass, alanine transaminase (ALT), glucose, insulin, tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), leptin and adiponectin. Values are expressed as mean ± SEM.

$^{*}P < 0.05$ vs. chow control;

$^{\dagger}P < 0.05$ vs. C57BL/6 control.

Table 2

Effects of Diet and Genotype on Liver Injury After 5 and 10 Weeks of Dietary Administration

Time on Diet Genotype Diet	5 weeks				10 weeks			
	C57BL/6		Lepr$^{db/db}$		C57BL/6		Lepr$^{db/db}$	
	Chow (n=6)	High fat (n=6)	Chow (n=6)	High fat (n=5)	Chow (n=6)	High fat (n=6)	Chow (n=6)	High fat (n=6)
Steatosis	0	0	0.2 ± 0.2	2.2 ± 0.3 *†	0	0	0.3 ± 0.2	2.8 ± 0.2 *†
Inflammation	0.2 ± 0.2	0	0	1 ± 0 *†	0	0.2 ± 0.2	0.3 ± 0.2	0.8 ± 0.3 *†
Ballooning	0	0	0	0.6 ± 0.2 *†	0	0	0	1.2 ± 0.3 *†
NAFLD Dx	0/6	0/6	1/6	2/5	0/6	0/6	2/6	3/6
NASH Dx	0/6	0/6	0/6	3/5	0/6	0/6	0/6	3/6
TUNEL, %	0.53 ± 0.05	0.50 ± 0.09	0.72 ± 0.23	2.32 ± 1.01 *†	0.29 ± 0.07	0.70 ± 0.24	1.30 ± 0.65	2.34 ± 0.54 *†

Means scores for steatosis, inflammation, ballooning, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) with the ratio of mice diagnosed with NAFLD and NASH. Values are expressed as mean ± SEM.

*$P < 0.05$ vs. dietary control;

†$P < 0.05$ vs. strain control.