Hepatocyte-specific, PPARγ-regulated mechanisms to promote steatosis in adult mice

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Abstract

Peroxisome proliferator-activated receptor γ (PPARγ) is the target for thiazolidinones (TZDs), drugs that improve insulin sensitivity and fatty liver in humans and rodent models, related to a reduction in hepatic de novo lipogenesis (DNL). The systemic effects of TZDs are in contrast to reports suggesting hepatocyte-specific activation of PPARγ promotes DNL, triacylglycerol (TAG) uptake and fatty acid (FA) esterification. As these hepatocyte-specific effects of PPARγ could counterbalance the positive therapeutic actions of systemic delivery of TZDs, the current study used a mouse model of adult-onset, liver (hepatocyte)-specific PPARγ knockdown (aLivPPARγkd). This model has advantages over existing congenital knockout models, by avoiding compensatory changes related to embryonic knockdown, thus better modeling the impact of altering PPARγ on adult physiology, where metabolic diseases most frequently develop. The impact of aLivPPARγkd on hepatic gene expression and endpoints in lipid metabolism was examined after 1 or 18 weeks (Chow-fed) or after 14 weeks of low- or high-fat (HF) diet. aLivPPARγkd reduced hepatic TAG content but did not impact endpoints in DNL or TAG uptake. However, aLivPPARγkd reduced the expression of the FA translocase (Cd36), in 18-week Chow- and HF-fed mice, associated with increased NEFA after HF feeding. Also, aLivPPARγkd dramatically reduced Mogat1 expression, that was reflected by an increase in hepatic monoacylglycerol (MAG) levels, indicative of reduced MOGAT activity. These results, coupled with previous reports, suggest that Cd36-mediated FA uptake and MAG pathway-mediated FA esterification are major targets of hepatocyte PPARγ, where loss of this control explains in part the protection against steatosis observed after aLivPPARγkd.

Key Words
- adult-onset hepatocyte-specific knockdown
- Cd36
- Mogat1
- LC/MS
- diet-induced steatosis

Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as excessive accumulation of fat (steatosis) within hepatocytes that is independent of alcohol intake. NAFLD increases the risk of diabetes, non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (Michelotti et al. 2013, Lade et al. 2014). Importantly, NAFLD is now
recognized as the leading cause of chronic liver disease in the US (Younossi et al. 2011) and the third most common reason for liver transplants (Zezos & Renner 2014). Given the negative association between NAFLD and human health, a concerted effort is being made to understand the cellular and molecular mechanisms that control hepatocyte triacylglycerol (TAG) content. It is clear that hepatocyte TAG content is dictated by the balance between fatty acid (FA) synthesis, uptake, esterification and oxidation, as well as TAG release via very-low-density lipoprotein (VLDL) (Browning & Horton 2004). A better understanding of the mechanisms controlling these processes could help to identify individuals with higher risk of NAFLD, as well as identify novel drug targets to design therapeutic strategies that prevent or reverse NAFLD progression.

Thiazolidinediones (TZDs) are synthetic agonists of the nuclear receptor, peroxisome proliferator-activated receptor γ (PPARγ) used to treat diabetes type 2 (Ahmadian et al. 2013). Treating patients with non-alcoholic steatohepatitis (an advance stage of NAFLD featured by elevated markers of liver injury and fibrosis) with TZDs reduces steatosis with variable effects on fibrosis (Belfort et al. 2006, Ratziu et al. 2008, Sanyal et al. 2010). The reduction in steatosis is associated with a reduction in hepatic de novo lipogenesis (DNL; Baysen et al. 2008). Similar to clinical studies, TZDs have also been shown to reduce hepatic fat content in rodent models (Nan et al. 2009, Gupta et al. 2010). However, in striking contrast to the global impact of TZDs: (1) hepatocyte-specific PPARγ expression is positively associated with fatty liver in humans (Pettinelli & Videla 2011) and mouse models (Rahimian et al. 2001, Gavrilova et al. 2003, Matsusue et al. 2003, Inoue et al. 2005); (2) adenoviral overexpression of a PPARγ transgene in the liver of high-fat (HF)-fed PPARα knockout or WT mice dramatically increases hepatic fat content (Yu et al. 2003, Bai et al. 2011); (3) congenital hepatocyte-specific knockout of PPARγ reduces hepatic fat content in mice fed a HF diet (Moran-Salvador et al. 2011), as well as in mice with fatty liver due to inactivating mutations in the leptin gene (ob/ob; Matsusue et al. 2003) or lipodystrophy induced by lack of adipocyte development (AZIP; Gavrilova et al. 2003). This disconnect between the impact of systemic TZD (PPARγ agonist) delivery compared to the impact of hepatic-specific alterations in PPARγ function can be attributed to the integrated effects of PPARγ on multiple target tissues. Specifically, TZDs increase systemic insulin sensitivity, which in turn reduces insulin demands. These changes are associated with an increase in adiponectin production by the adipocyte. Adiponectin in turn promotes hepatic fatty acid oxidation via phosphorylation of AMPK and ACC (Yamauchi et al. 2002, Xu et al. 2003), which suppresses DNL (Xu et al. 2003). Therefore, TZD’s effects on lowering steatosis are likely due to extra-hepatocyte mechanisms (Furnsinn & Waldhausl 2002).

Although the global therapeutic effects of TZDs have been largely positive, the direct actions of PPARγ on the hepatocyte could serve to counterbalance these effects. Therefore, to optimize the development and use of TZDs, it is important to understand the tissue (cell)-specific impact of PPARγ on lipid homeostasis. With respect to the hepatocyte, studies using hepatocyte-specific PPARγ-knockout models have led to the conclusion that PPARγ directly promotes hepatic fat accumulation by increasing lipid uptake, as well as promoting DNL (Gavrilova et al. 2003, Matsusue et al. 2003, 2014, Schadinger et al. 2005, Zhang et al. 2006). However, it remains unclear if the shifts in hepatic gene expression that support these conclusions are due directly to loss of hepatocyte PPARγ or to compensation by other hepatic genes during development and/or secondary to changes in the systemic metabolic milieu. Therefore, in the current study, we have employed a mouse model of adult-onset, hepatocyte-specific knockdown of PPARγ (aLivPPARγkd), that is generated by treating adult (10 weeks) PPARγfl/fl mice with an adenov-associated virus serotype 8 (AAV8) bearing a liver-specific thyroxine-binding globulin (TBG)-promoter driving a Cre recombinase transgene (AAV8-TBGp-Cre) vector. This model allows us to study the immediate impact of hepatocyte-specific loss of PPARγ in the adult liver and how this deficit influences liver function overtime under different dietary conditions. As indicated in this study and supported by accumulating evidence (Yang et al. 2014, Ashpole et al. 2016, Shin et al. 2016), injection of AAV8-TBGp-Cre is an efficient and reproducible method to knockdown a floxed allele only in hepatocytes, independent of age.

Analysis of changes in hepatic gene expression and circulating metabolites, combined with assessment of hepatic FA composition (gas chromatography/mass spectrometry (GC/MS)) and relative levels of TAG, diacylglycerols (DAG) and monoacylglycerols (MAG) (liquid chromatography/mass spectrometry (LC/MS)), indicate that adult-onset loss of hepatocyte PPARγ has little direct impact on DNL, FA oxidation, lipid uptake and TAG export. However, evidence indicates that hepatic PPARγ plays a primary role in regulating FA uptake, likely through regulating the expression of Cd36, and the MAG pathway, by regulating expression of Mogat1,
which esterifies FA to MAG to form DAG. Impairment of these pathways after the loss of hepatocyte PPARγ may explain, in part, the protection against age- and diet-induced steatosis.

Materials and methods

Animals

All mouse studies were approved by the IACUC of the Jesse Brown VA Medical Center and performed in accordance with the Guide for the Care and Use of Laboratory Animals. PPARγfl/fl (He et al. 2003) mice were purchased from Jackson Laboratories (Strain 004584, B3.129-Ppargtm2Rev/J, Bar Harbor, ME) and bred as homozygotes. Animals were housed in a temperature- (22–24°C) and humidity-controlled specific pathogen-free barrier facility with 12-h light/12-h darkness cycle (lights on at 06:00 h). Mice were fed a standard laboratory rodent chow diet (Formulab Diet 5008, Purina Mills, Richmond, IN, USA), unless otherwise indicated. Ten- to twelve-week-old male PPARγfl/fl littermate mice were randomized and injected in the lateral tail vein with 100 µL saline containing 1.5 × 10^{11} genome copies of an AAV8 bearing either a TBG-driven Cre recombinase (AAV8-TBGp-Cre, Penn Vector Core, University of Pennsylvania), which generates adult-onset hepatocyte (liver)-specific PPARγ-knockdown mice (aLivPPARγkd) or AAV8-TBGp-Null, which generates controls.

Chow-fed mice were killed in the post-absorptive state (4 h after food removal at 07:00 h), at 1 or 18 weeks after PPARγ knockdown. A separate group of PPARγfl/fl mice was fed a low-fat (LF) diet with 10% kcal fat (D12450B, Research Diets, Inc. New Brunswick, NJ, USA) from weaning onward. At 10–12 weeks of age, mice were injected with either AAV8-TBGp-Cre or AAV8-TBGp-Null, and half the mice in each group switched to a nutrient-matched 60% HF diet (D12450B, Research Diets, Inc.), whereas the remaining mice continued to receive a LF diet. Animals were maintained on their respective diets for 14 weeks and killed in the post-absorptive state.

Mice were killed by decapitation, and trunk blood was collected to determine blood glucose (Alphatrack2, Abbott), plasma insulin (Merckodia, Uppsala, Sweden), TAG, NEFA, cholesterol and 3β-hydroxybutyrate (Wako Diagnostics) levels following the manufacturer’s instructions. Liver and fat sub-depots were weighed. Livers were snap-frozen in liquid nitrogen and stored at −80°C. In a subset of mice killed 1 week after AAV8-TBGp-Null or AAV8-TBGp-Cre injection, multiple tissues were collected to assess AAV8-TBGp-driven Cre expression. A group of 10- to 12-week-old C57Bl6/J mice was injected with 1.5 × 10^{11} genome copies of an AAV8-TBGp-EGFP (Cat #AV-8-PV0146, Penn Vector Core, GFP as a reporter gene) and killed 1 week after to collect multiple tissues to assess GFP expression. Also, a piece of liver was fixed in 10% formalin to assess hepatocyte-specific expression of GFP.

Assessment of hepatic lipids

To assess the hepatic TAG content, neutral hepatic lipids were extracted in isopropanol and TAG measured as previously published (Cordoba-Chacon et al. 2014a).

To assess hepatic FA composition in mice fed LF and HF diets, total lipids were extracted using the Bligh and Dyer Method (Bligh & Dyer 1959). An aliquot of extracted lipids was transmethylated to quantify specific methyl esters of FA using GC/MS, as we previously reported (Kineman et al. 2016).

LC/MS was used to assess the relative content of hepatic TAG, DAG and MAG. Briefly, hepatic homogenates were spiked with standards (50 µg trinonadecadienoin glyceride (TAG-(19:2/19:2/19:2)), 50 µg dipentadecanoin glyceride (DAG-(15:0/15:0)) and 50 µg monononadecanoin glyceride (MAG-(19:0)); Nu-Chek, Waterville, MN, USA) and extracted using the Bligh and Dyer Method (Bligh & Dyer 1959). Aliquots were dissolved in 80:19.5:0.5 parts of methanol/chloroform/water to dilute the standards to a concentration of 0.25 µg/mL. Samples (10 µL) were injected using an Agilent 2600 UPLC into the AB Sciex 6500 Qtrap mass spectrometer (Agilent Technologies) without chromatography separation. The flow rate of mobile phase (methanol/chloroform/water 80:19.5:0.5 v/v) containing 0.1% of NH$_4$COOH was set to 200 µL/min. Electrospray ionization-mass spectrometry (ESI-MS) was performed in positive multiple reaction monitoring (MRM) mode for the quantitative and qualitative analysis. The spray voltage was 4.5 kV, the source temperature was set at 450°C. Mass spectra were acquired and recorded by Analyst software (AB Sciex, version 6.1). The major neutral lipids species known to be present in the liver tissues were analyzed in MRM mode, with the transition from its ammoniated ion (Q1) to the product ion derived from the loss of its ammoniated fatty acid (Q3) (Yang & Subbaiah 2015). Quantification of individual molecular species was performed from the relative intensities of the various species and the corresponding internal standards, respectively. Individual MRM of the internal standards was MAG-(19:0) Q1 390.4, Q3 75.1;
DAG-(15:0/15:0) Q1 558.5, Q3 299.3; TAG-(19:2/19:2/19:2) Q1 938.8, Q3 625.5.

Gene expression analysis

Hepatic and adipose tissue RNA was extracted using TRIzol Reagent (Life Technologies) and treated with RQ1 RNase-free DNase (Promega). DNA-free RNA was transcribed, and qPCR was performed as previously published (Cordoba-Chacon et al. 2014a, Kineman et al. 2016). qPCR primer sequences for PPARγ, carnitine palmitoyltransferase 1α (Cpt1α), adipose triglyceride lipase (Atgl), hormone-sensitive lipase (Hsl), sterol response element-binding protein 1c (Srebp1c), acetyl-CoA carboxylase 1 (Acc1), fatty acid synthase (Fasn), very-low-density lipoprotein receptor (Vldlr), lipoprotein-related protein 1 (Lrp1), monoacylglycerol acyltransferase 1 (Mogat1), Cre recombinase, GFP, cyclophilin A, β-actin and hypoxanthine-guanine phosphoribosyltransferase were previously published (Cordoba-Chacon et al. 2014a, 2015a, Kineman et al. 2016). Primer sequences for PPARα (NM_011144): Se: GGGAAGACCAGCAAACCC, As: GCAGTGGAGAACATGGGCT; acyl-CoA synthetase long-chain family member 1 (Acsl1, NM_007981): Se: AGTGGAAGAATCGGACCT; acyl-CoA synthetase acyltransferase 1 (Dgat1, NM_010046.2): Se: AGCTGTGGCCAGCATT; hepatic nuclear factor 4α (Hnf4α), Cre recombinase, GFP, cyclophilin A, β-actin and hypoxanthine-guanine phosphoribosyltransferase were previously published (Cordoba-Chacon et al. 2014a, 2015a, Kineman et al. 2016). Primer sequences for PPARγ (NM_010011.3): Se: CCACTGATTCTGTTGTGGAGC, As: GTCTCGAATTGCCTGAGTGG; monoglyceride acyltransferase 2 (Mogat2, NM_146035.2): Se: TGTGAAAACCTTGGAATATCGACA, As: CAGTCTCCAGCATGAAAATCC; diacylglycerol acyltransferase 1 (Dgat1, NM_0010046.2): Se: AGCTGTGGCC TTACTGGTTG, As: AGCAGCCCCACTGACCC.

Western blot

Livers were homogenized in extraction buffer pH 7.5, 50mM HEPES, 2mM EGTA, 2mM EDTA, 130mM NaCl, 10mM NaF, 20mM β-glycerophosphate, 2mM sodium pyrophosphate, 1mM sodium vanadate, 0.5mM PMSE, 0.1% nonidet P-40, 2mM benzamidine, 1mM TLCK, 10μg/mL leupeptin, 10% glycerol, with protease inhibitors (Complete, Roche), followed by sonication for 10s. Protein concentration was determined using Bradford reagent (Bio-Rad Laboratories). 100μg of denatured proteins were separated by SDS-PAGE (Mini-PROTEAN TGX Gels 10%, Bio-Rad Laboratories) and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat, dry milk in Tris-buffered saline with 0.05% Tween-20 for 1h at 25°C, and then incubated with primary antibodies overnight at 4°C (Rabbit anti-human PPARγ mAb (C26H12), 1/500; Rabbit anti-human Histone H3 mAb (D1H2), 1/1000 (Cell Signaling Technology)), washed and incubated with secondary antibodies for 2h at 25°C (Goat Anti-Rabbit IgG (H+L)-HRP Conjugate, 1/2000 (Bio-Rad Laboratories)). After washing, SuperSignal WestFemto Maximum Sensitivity Substrate (Thermo Scientific) was added and the light signal was detected and analyzed using a C-Digit Blot Scanner and Image Studio Lite Ver 3.1 (Li-Cor Biosciences, NE, USA).

Immunohistochemistry

Livers were fixed in formalin and paraffin embedded. 5μm sections were deparaffinized, hydrated in graded-ethanol/water solutions and then treated with 10mM citrate buffer at 125°C for 5’ (GFP, desmin and F4/80 staining) followed by 0.05% porcine trypsin in Tris-buffered solution (TBS) for 10min at 37°C (F4/80 staining only). Samples were blocked in TBS-containing 1.5% goat serum and 0.01% Tween-20 for 20’, and sections were incubated (4°C overnight in TBS 0.01% Tween-20) with mouse anti-GFP, 1:100 Cell Signaling #2955; rabbit anti-desmin, 1:50 Cell Signaling #5332; rat anti-F4/80 1:50 Biosciences (San Diego, CA, USA) #14-4801-82. Secondary antibodies (1:500 in blocking buffer): goat anti-rabbit IgG Alexa Fluor594 Cell Signaling #8889 (for desmin staining), goat anti-mouse IgG Alexa Fluor594 via free access.
Cell Signaling #8890 and goat anti-mouse IgG Fluor488 Cell Signaling #4408 (for GFP stainings), goat anti-rat IgG Alexa Fluor488 Cell Signaling #4416 (for F4/80 staining). Sections were mounted with Fluoroshield with DAPI (Sigma-Aldrich) and immunofluorescence was detected using a Olympus BX43 microscope (Olympus). Images were recorded using Olympus CellSens software (Olympus) and processed using ImageJ (NIH) and CellSens (Olympus).

Statistics

Student’s t-tests were performed to analyze the effect of AAV8-TBGp-Cre on PPARγ expression in liver, epididymal fat (eWAT) and inguinal fat (iWAT) (Fig. 1G). 2-Way ANOVA, followed by Bonferroni’s post hoc analysis, was used to determine the effect of aLivPPARγkd with age or the effect of aLivPPARγkd with diet. P values less than 0.05 were considered significant.

All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software).

Results

Validation of hepatocyte-specific expression of TBGp-driven transgene after AAV8 injection

Hepatocyte specificity expression of transgenes delivered by AAV8 and driven by TBG was confirmed using an AAV8-TGBp-EGFP reporter (Fig. 1A, B, C, D and E) that led to GFP-positive staining only in hepatocytes and not in other cell types, including vascular endothelial cells, connective tissue surrounding vessels, cholangiocytes of bile ducts (Fig. 1A and B), hepatic stellate cells (HSC; stained positive (red) for desmin (Fig. 1C)) and macrophages, (stained positive (red) F4/80 (Fig. 1D)). AAV8-TBgp-driven GFP and Cre expression was detected in hepatic but not in extrahepatic tissues.

![Figure 1](http://joe.endocrinology-journals.org)

**Figure 1**

Hepatocyte specificity expression of AAV8-TBgp-driven transgene. Hepatocyte-specific expression of GFP in AAV8-TBgp-EGFP-injected wild-type mice (green, A and B). GFP expression was absent in non-hepatocyte cells (yellow arrows) in sinusoids (Sn), central vein (CV), portal vein (PV), bile duct (BD) or artery (HA). TBGp-GFP was not expressed in hepatic stellate cells (HSC, desmin +, red, C) or macrophages (Mac, F4/80+, red, D). Sections were counterstained with DAPI (blue nuclei, A, B, C and D). Hepatic GFP (E) and Cre (F) expression was detected only in liver extracts of AAV8-TBgp-EGFP- and AAV8-TBgp-Cre-injected mice, respectively. To confirm the hepatocyte-specific activity of Cre recombinase, PPARγfl/fl mice were injected with AAV8-TBgp-Cre and expression of PPARγ was reduced in hepatic extracts but not adipose tissue (G). Ctx, cortex; eWAT, epididymal fat; Int, intestine; iWAT, inguinal fat. Asterisks indicate the difference between AAV8-TBgp-Cre-injected mice as compared to AAV8-TBgp-Null mice. *P<0.05; **P<0.0001. n=3–6 mice/group.
Hepatic PPARγ-mediated diet-induced steatosis

(Fig. 1E and F). Expression of PPARγ was reduced in liver but not in adipose tissue (Fig. 1G). Interestingly, a modest increase of PPARγ expression was detected in eWAT of aLivPPARγkd mice.

Effects of hepatocyte-specific PPARγ knockdown on metabolic endpoints in adult mice (aLivPPARγkd)

After 1 and 18 weeks of chow diet AAV8-TBGp-Cre injection resulted in a clear knockdown of hepatocyte PPARγ mRNA and protein at 1 week after injection that persisted after 18 weeks of AAV8 injection (Fig. 2A, B and C). Mouse PPARγ gene produces two isoforms PPARγ1 and PPARγ2 (NM_001127330.2 and NM_0111463). Three mRNA variants are described for PPARγ1 (variant 1: NM_001127330.2; variant 3: NM_001308352.1; variant 4: NM_001308354.1), but all encode for the same protein (isoform 1). In PPARγ1fl/fl mice two exons, previously described as exons 1 and 2 (He et al. 2003), are flanked byloxP sites. These exons span 227 and 169 bp respectively are located within the CDS of each variant and the qPCR primers employed in this study are placed in these two exons. Therefore, the qPCR result of PPARγ gene expression accounts for all variants and thereby the western blot results account for both isoforms. Of note, hepatic PPARγ mRNA levels modestly, but significantly, increased with age in control but not aLivPPARγkd mice (Fig. 2B and C).

To assess if hepatic lipid metabolism was altered in chow-fed aLivPPARγkd mice, we measured whole body, liver and adipose tissue (unilateral epididymal, inguinal and retroperitoneal fat depots) weights, hepatic TAG levels, as well as circulating blood glucose, plasma insulin, ketones, TAG, NEFA and cholesterol (Table 1). For the majority of endpoints examined, aLivPPARγkd had no effect in either age group. However, there was an overall inhibitory effect of aLivPPARγkd on hepatic TAG levels (P=0.0185), indicating hepatic PPARγ plays a role in hepatic lipid accumulation, even under standard feeding conditions.
Table 1 Impact of aLivPPARγkd on body, liver and fat depot (sum of the unilateral epididymal, inguinal and retroperitoneal fat depot) weight, hepatic TAG levels and circulating metabolic endpoints (blood glucose, plasma insulin, ketones, TAG, NEFA and NEFA), in chow-fed (top, 1 week and 18 weeks) and LF/HF-fed (bottom, 14 weeks) aLivPPARγkd mice and their littermate controls.

<table>
<thead>
<tr>
<th></th>
<th>1 week aLivPPARγkd – chow diet</th>
<th>18 weeks aLivPPARγkd – chow diet</th>
<th>Overall effect (P value) of aLivPPARγkd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>aLivPPARγkd</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>Liver (g/g BW)</td>
<td>25.36 ± 0.84</td>
<td>24.6 ± 0.64</td>
<td>31.81 ± 1.88</td>
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<td>Fat mass (g/g BW)</td>
<td>0.045 ± 0.0008</td>
<td>0.043 ± 0.0022</td>
<td>0.0308 ± 0.0011</td>
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<tr>
<td>Hepatic TAG (mg/g)</td>
<td>47.63 ± 6.987</td>
<td>38.63 ± 3.027</td>
<td>50.36 ± 4.611</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>187 ± 14.77</td>
<td>176.8 ± 18.87</td>
<td>190.4 ± 15.7</td>
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<tr>
<td>Plasma insulin (ng/µL)</td>
<td>1.59 ± 0.15</td>
<td>1.69 ± 0.12</td>
<td>1.88 ± 0.37</td>
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<td>Plasma ketones (µM)</td>
<td>372.1 ± 62.194</td>
<td>372.1 ± 57.92</td>
<td>153.9 ± 40.907</td>
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<tr>
<td>Plasma TAG (mg/dL)</td>
<td>91.26 ± 3.772</td>
<td>83.95 ± 5.728</td>
<td>61.35 ± 12.607</td>
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<td>Plasma NEFA (mEq/L)</td>
<td>1.05 ± 0.038</td>
<td>1.12 ± 0.063</td>
<td>1.06 ± 0.095</td>
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<td>Plasma cholesterol (mg/dL)</td>
<td>104.83 ± 5.568</td>
<td>107.83 ± 5.233</td>
<td>116.17 ± 8.003</td>
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</table>

14 weeks aLivPPARγkd – LF diet

<table>
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<tr>
<th></th>
<th>Control</th>
<th>LF diet</th>
<th>Overall effect (P value) of Diet</th>
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<tr>
<td>Body weight (g)</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>Liver (g/g BW)</td>
<td>30.88 ± 0.95</td>
<td>29.65 ± 0.6</td>
<td>49.52 ± 0.55</td>
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<td>Fat mass (g/g BW)</td>
<td>0.0376 ± 0.0007</td>
<td>0.0373 ± 0.0006</td>
<td>0.049 ± 0.001</td>
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<tr>
<td>Hepatic TAG (mg/g)</td>
<td>37.13 ± 9.114</td>
<td>41.27 ± 9.188</td>
<td>326.25 ± 16.65</td>
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<tr>
<td>Blood glucose (mg/dL)</td>
<td>182.6 ± 20.78</td>
<td>187.2 ± 9.14</td>
<td>218 ± 15.12</td>
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<tr>
<td>Plasma insulin (ng/µL)</td>
<td>1.39 ± 0.11</td>
<td>1.96 ± 0.3</td>
<td>11.27 ± 0.85</td>
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<td>Plasma ketones (µM)</td>
<td>232.3 ± 42.17</td>
<td>215.4 ± 30.85</td>
<td>348.71 ± 22.787</td>
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<tr>
<td>Plasma TAG (mg/dL)</td>
<td>52.78 ± 7.716</td>
<td>57.18 ± 7.479</td>
<td>35.8 ± 3.672</td>
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<tr>
<td>Plasma NEFA (mEq/L)</td>
<td>1.17 ± 0.05</td>
<td>1.1 ± 0.08</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td>Plasma cholesterol (mg/dL)</td>
<td>117.71 ± 12.99</td>
<td>104.57 ± 9.2</td>
<td>212.86 ± 37.85</td>
</tr>
</tbody>
</table>

After 14 weeks of LF or HF diet To further explore if hepatic lipid accumulation induced by excess dietary fat intake is altered by aLivPPARγkd, we fed a HF- or a nutrient-matched LF control diet to aLivPPARγkd mice and their littermate controls. After 14 weeks of either LF or HF feeding (Fig. 2D) hepatic PPARγ mRNA/protein expression remained suppressed in aLivPPARγkd mice, compared to controls (Fig. 2E and F).

Consistent with previous reports (Inoue et al. 2005, Moran-Salvador et al. 2011, Yamazaki et al. 2011), HF feeding in control mice increased hepatic PPARγ expression, compared to those fed a LF diet (Fig. 2E and F). Also, in control mice, HF feeding increased body, liver and fat mass weight and elevated hepatic TAG content and plasma glucose, insulin and cholesterol levels, but plasma TAG and NEFA were reduced (Guo et al. 2009, Obrowsky et al. 2013, Cordoba-Chacon et al. 2014b, 2015b, Horakova et al. 2016), likely due to elevated insulin levels under these conditions. aLivPPARγkd, in LF-fed mice, did not impact any of the metabolic endpoints examined (Table 1), including food intake (data not shown). However, HF-fed aLivPPARγkd mice showed reduced relative liver weight, and this was associated with a reduced hepatic TAG content (Table 1). Despite reduced hepatic TAG levels, there were no differences in post-absorptive blood glucose, plasma insulin and TAG levels in HF-fed aLivPPARγkd mice, compared to diet-matched controls (Table 1). However, normal glucose and insulin levels in the post-absorptive mouse may not be indicative of the whole-body glucose homeostasis, which requires dynamic evaluation by glucose and insulin tolerance tests. Of note, plasma ketones and cholesterol were reduced in HF-fed aLivPPARγkd mice, whereas plasma NEFAs were increased as compared to HF-fed control littermates (Table 1).
Association between aLivPPARγkd-mediated alterations in hepatic gene expression and metabolic endpoints

It has been previously reported that congenital liver-specific knockout of PPARγ alters the expression of a number of genes related to the regulation of hepatic TAG levels (Gavrilova et al. 2003, Matsusue et al. 2003, Moran-Salvador et al. 2011). However, from these studies, it is difficult to determine which changes may be due to the direct actions of hepatocyte PPARγ and which may be altered by secondary changes that occur overtime. Therefore, qPCR was used to screen changes in expression of key genes related to fatty acid oxidation, intrahepatic TAG hydrolysis, hepatic TAG export, DNL, hepatic lipid uptake and TAG synthesis, in liver samples from chow-fed (1 and 18 weeks after aLivPPARγkd) and HF-fed (14 weeks after aLivPPARγkd) mice. The data shown in Fig. 3 are expressed as fold-change in hepatic gene expression in aLivPPARγkd mice, compared to PPARγ-intact controls (set at 0), within age and diet, whereas Supplementary Table 1A and B (see section on supplementary data given at the end of this article) provides absolute values. There were small, but significant changes in the expression of a number of genes in aLivPPARγkd mice, compared to PPARγ-intact controls within specific groups. However, collective examination of this data set provides insight into which genes (pathways) are major targets of PPARγ, that could explain why aLivPPARγkd protects against hepatic fat accumulation.

De novo lipogenesis Although experimental evidence suggests that hepatocyte PPARγ promotes liver fat accumulation by regulating the expression of genes important for DNL (Matsusue et al. 2003, 2014), aLivPPARγkd did not significantly reduce the expression of DNL genes (Sreb1c, Acc1, Fasn, Elov6, Scd1) across age or diet. However, we could not exclude the fact aLivPPARγkd could indirectly mediate the activity of DNL enzymes, independent of changes in gene expression. Therefore, to estimate the impact of aLivPPARγkd on hepatic DNL, we used GC/MS to measure FA composition in livers of mice fed either a LF or HF diet. Supplementary Table 2 provides
data for all FA detected, whereas Fig. 4A shows absolute levels of palmitic acid (16:0), palmitoleic acid (16:1(n-7)) and linoleic acid (18:2(n-6)). The absolute levels of these FAs were significantly reduced in HF-fed aLivPPARγkd mice as compared to HF-fed controls, consistent with the reduction in hepatic TAG content. Specific ratios of these FA (Fig. 4B) have been shown to be indicative of DNL (SCD-1 index 16:1/16:0) and DNL index, 16:0/18:2 (Sevastianova et al. 2012, Silbernagel et al. 2012, Lee et al. 2015, Kineman et al. 2016). As previously reported, HF diet reduces the rate of DNL (Duarte et al. 2014) and expression of DNL proteins (Benard et al. 2016), as reflected by significant reduction in both the SCD-1 and DNL index, as well as the reduction in DNL gene expression (Acc1, Fasn, Elovl6 and Scd1; Supplementary Table 1B). Independent of diet, loss of hepatocyte PPARγ did not influence these indices (Fig. 4B). Taken together, these results suggest that hepatic PPARγ plays a minimal role in directly regulating hepatic DNL.

**Hepatic TAG export, FA oxidation and lipid uptake**  A reduction in hepatic fat content observed in aLivPPARγkd mice could be due to an increased rate of hepatic VLDL production or a decreased rate of TAG lipoprotein clearance. However, examination of genes critical for these processes were not consistently altered by aLivPPARγkd (Fig. 3) and circulating TAG in aLivPPARγkd did not differ from PPARγ-intact controls, within age and diet group (Table 1). It could also be possible that the reduced TAG content observed in livers of aLivPPARγkd mice is due to an increase in intrahepatic TAG hydrolysis and FA oxidation. However, the fact that the expressions of Atgl, Hsl, Mgl1, PPARα, Acs11, Cpt1α, Hnf4α, Fgc1α and Cyp4a10 were not increased across groups (Fig. 3), whereas HF-aLivPPARγkd mice exhibited a decrease in plasma ketones (Table 1), suggests that hepatic FA oxidation is actually reduced. It is possible that this reduction in FA oxidation/ketogenesis is secondary to a reduction in intrahepatic FA availability due to a reduction in FA uptake as aLivPPARγkd reduced the expression of hepatic Cd36 (FA translocase), where this was associated with an increase in circulating NEFA in HF-fed mice (Table 1). In fact, Cd36 has been previously shown to be a PPARγ target gene (Tontonoz et al. 1998). However, it should be noted that a significant reduction in Cd36 was not

![Figure 4](image_url)
observed in aLivPPARγkd after 1 week of knockdown, or in LF-fed mice, suggesting that under these conditions, the low level of PPARγ is not sufficient to maintain Cd36 expression or that the full expression requires additional factors that are activated with age or diet, such as LXR and PXR (Zhou et al. 2008).

**TAG synthesis**  TAG, DAG and MAG are generated by esterification of newly synthesized FA or extrahepatic FA into acylglycerol backbones, as illustrated in Fig. 5A. In the liver, it is thought that the primary source of TAG is via the glycerol-3-phosphate (G3P) pathway, that generates DAG (Coleman & Mashek 2011, Mashek 2013), which serves as a substrate of DAG transferases 1 and 2 (Dgat1/2). However, it is becoming evident that the MAG pathway (i.e. generation of DAG from MAG via MAG transferases 1 and 2 (Mogat1/2 in mice)), may be a relevant pathway (i.e. generation of DAG from MAG via MAG transferases 1 and 2 (Dgat1/2)).

Hepatic PPARγ-mediated diet-induced steatosis

**Discussion**

Hepatocyte PPARγ has been defined as a steatogenic factor (Rahimian et al. 2001, Gavrilova et al. 2003, Matsusue et al. 2003, Inoue et al. 2005, Moran-Salvador et al. 2011, Pettinelli & Videla 2011), whereas others suggest that its activation decreases hepatic steatosis (Belfort et al. 2006, Ratziu et al. 2008, Sanyal et al. 2010). The fact that PPARγ is the target of TZDs that are used in TAG synthesis

![Diagram](https://example.com/diagram.png)

**Figure 5** aLivPPARγkd reduces HF diet-induced hepatic TAG and DAG levels, whereas increasing MAG levels, independent of diet, indicative of impaired hepatic MAG pathway activity in aLivPPARγkd mice. (A) Schematic representation of acylglycerol synthesis by glycerol-3-phosphate (G3P) pathway that produces DAG by subsequent re-esterification of FA in G3P and lysophosphatidic acid (LPA) or by monoacylglycerol (MAG) pathway that produces DAG after re-esterification of FA in MAG. (B) Relative hepatic TAG, DAG and MAG levels assessed by liquid chromatography/mass spectrometry (LC/MS) in control (open columns) and aLivPPARγkd (close columns) mice. TAG, DAG and MAG are shown as relative values of LF-fed controls. Asterisks indicate differences between control and aLivPPARγkd. ***P<0.0001. Letters indicate differences between LF- and HF-fed mice within group. †P<0.01; ‡P<0.0001. n=5–6 mice/group.
the treatment of diabetes and NAFLD raises the question if the hepatic-specific agonism of PPARγ is well understood (Ahmadian et al. 2013). Therefore, we sought to determine which molecular processes are the primary targets for hepatocyte PPARγ that promotes TAG accumulation. We have taken advantage of the novel adult-onset, hepatocyte-specific PPARγ knockdown (aLivPPARγkd) model that allowed us to study the early events altered by aLivPPARγkd (1 week of knockdown), and how this deficit influences liver function overtime under different dietary conditions. Our approach has benefits over existing congenital knockout models because it avoids compensatory changes that could occur with embryonic knockout and therefore better models the consequence of manipulating PPARγ function in adults, where NAFLD/NASH typically develops. As discussed in detail below, our primary findings are that hepatic PPARγ has minimal direct effects on hepatic DNL, TAG uptake, TAG export or FA oxidation, but plays a major role in upregulating genes/pathways critical for hepatic FA uptake- and MAG pathway-mediated FA esterification.

Congenital hepatocyte-specific PPARγ knockout has been reported to be associated with a reduction of hepatic expression of genes critical for DNL (Gavrilova et al. 2003, Matsusue et al. 2003, Yu et al. 2003, Moran-Salvador et al. 2011). Also, use of antisense oligonucleotide strategy to suppress elevated hepatic PPARγ in apoB/BATtess mice, reduced hepatic TAG accumulation, as well as the expression of DNL genes and DNL rate as assessed by 3H2O incorporation into TAG-associated FA (Zhang et al. 2006). In addition, overexpression of PPARγ in a hepatic cell line (AML-12; Schadinger et al. 2005) was shown to increase SREBP1c and Fasn expression that was associated with an increase in 14C-acetate incorporation into TAG. Finally, overexpression of PPARγ in some (Yu et al. 2003), but not all (Bai et al. 2011) in vivo models, increased the expression of genes associated with DNL. In contrast to these reports, in the aLivPPARγkd model system, expression of DNL genes were not suppressed in any conditions tested, consistent with the lack of an effect on DNL indices (16:1/16:0 and 16:0/18:2). In fact, we have recently reported that although PPARγ expression was associated with TAG accumulation in a model of DNL-generated steatosis (Kineman et al. 2016), adult-onset hepatic PPARγ knockdown in this steatotic model did not reduce hepatic TAG content or FA indices of DNL (Cordoba-Chacon et al. 2015a). The question arises, why are our current results counter to that previously reported by others? We might speculate that any changes observed in the expression of DNL genes in congenital liver-specific PPARγ-knockout models could be secondary to changes that occur due to compensation during development or systemic metabolic changes that occur overtime. Also, the use of antisense oligonucleotides (ASO) to acutely suppress enhanced PPARγ expression in vivo (Zhang et al. 2006) is not a hepatocyte-specific approach, and therefore, a reduction in PPARγ in other cell types could contribute to the phenotype observed. Finally in vivo (Zhang et al. 2006) or in vitro (Schadinger et al. 2005) overexpression of PPARγ may have off-target effects. Therefore, we can conclude that in the context of adult metabolic function, loss of hepatic PPARγ does not directly control hepatic DNL.

Early work by Gavrilova and coworkers and Matsusue and coworkers who crossbred the congenital liver-specific PPARγ model with a lipodystrophic model (AZIP; Gavrilova et al. 2003), and ob/ob mice (Matsusue et al. 2003), respectively, observed a reduction in hepatic TAG content associated with elevated plasma TAG and thus concluded that hepatic PPARγ was critical to maintain hepatic TAG uptake. However, in chow-fed WT mice (Matsusue et al. 2003, Moran-Salvador et al. 2011) and HF-fed (Moran-Salvador et al. 2011) mice with congenital liver-specific PPARγ knockout, as well as in our HF-fed aLivPPARγkd model, circulating TAG did not differ from PPARγ-intact controls, despite the dramatic reduction in hepatic TAG content. Also, there were no decreases in the expression of genes known to be critical in hepatic TAG uptake in mouse livers, including low-density lipoprotein receptor (Ldlr; Ishibashi et al. 1994, Havel & Hamilton 2004) and hepatic lipase (HL; Havel & Hamilton 2004, Freeman et al. 2007). However, it should be noted that the expression of very low-density lipoprotein receptor (Vldr), a known PPARγ target in adipocytes (Tao et al. 2010), was significantly reduced in HF-fed aLivPPARγkd mice. The expression of hepatic Vldr is normally low, but is increased in mouse models of fatty liver (also observed with HF feeding in this study, see Supplementary Table 1), and whole-body knockout of Vldr reduces ER stress and HF diet-induced hepatic steatosis (Jo et al. 2013). However, a liver-specific role of Vldr in hepatic TAG uptake remains to be determined. Although the role of PPARγ in regulating hepatic TAG uptake remains to be further explored, our current results coupled with previous reports, do provide compelling evidence that hepatic PPARγ promotes hepatic FA uptake by regulating the expression of Cd36. Specifically, in both aged and HF-fed aLivPPARγkd mice, expression of Cd36 was reduced. Cd36 has been shown to be a direct target of PPARγ (Tontonoz et al. 1998) and its
expression is increased in steatotic livers. In the current study, a reduction in Cd36 levels in aLivPPARγkd mice was only observed with age or HF feeding, consistent with the fact that in addition to PPARγ, LXR and PXR are also required for the full expression of Cd36 (Zhou et al. 2008). Although global Cd36 knockout does not protect against high fructose-induced hepatic steatosis (Hajti et al. 2002) or prevent fatty liver in ob/ob mice (Nassir et al. 2013), a more recent report supports a liver-specific role of Cd36 in FA uptake. Specifically, congenital liver-specific Cd36 knockout reduced steatosis in liver-specific Jak2 knockout mice that led to an increase in plasma NEFA (Wilson et al. 2015). Also in that same study, liver-specific Cd36-knockout mice with intact hepatic Jak2, dramatically reduced HF diet-induced steatosis that was associated with a reduction in hepatic FA uptake as measured by hepatic accumulation of BODIPY-FA in vivo (Wilson et al. 2015). However, loss of hepatic Cd36 did not entirely prevent hepatic FA uptake, which could be mediated by other facilitated transport mechanisms or passive diffusion (Glatz et al. 2010), indicating the reduction in CD36 after aLivPPARγkd is only in part responsible for the reduction in hepatic TAG content.

Of the selected genes examined in this study, the expression of Mogat1 was the most sensitive to PPARγ loss. As previously reported, the expression of Mogat1 in the lean mouse liver is low, but increases in association with TAG accumulation, similar to that observed for PPARγ (Lee et al. 2012, Hall et al. 2014, Soufi et al. 2014, Yu et al. 2016), as we also observed in the current study. In addition, hepatic expression of Mogat1, as well as PPARγ, is elevated in humans with NAFLD (Hall et al. 2012, Yu et al. 2015). There is an ongoing debate regarding the physiologic role hepatic Mogat1 plays in TAG synthesis as it was originally thought that the glycerol-3-phosphate pathway, not the MAG pathway, is the dominant route to form TAG in the liver (Coleman & Mashek 2011, Mashek 2013). In fact, one laboratory could not detect hepatic MOGAT activity (Cortes et al. 2009) and recently reported that global Mogat1 knockout in the ob/ob or Agpat2KO mice did not impact steatosis (Agarwal et al. 2016). However, hepatic MOGAT activity has been detected by other laboratories (Yen et al. 2002, Hall et al. 2012). Importantly, the increase in Mogat1 expression observed in HF-fed and ob/ob mice is associated with an elevation of hepatic MOGAT activity, which is reduced by Mogat1 ASO ip treatment (Hall et al. 2014, Soufi et al. 2014). The Mogat1-ASO injections reduced hepatic TAG accumulation in one study (Soufi et al. 2014), but not the other (Hall et al. 2014). However, it was acknowledged that the knockdown of Mogat1 was not hepatocyte specific, and actually led to a reduction in Mogat1 expression in the adipose tissue that could offset any hepatocyte-specific effect (Soufi et al. 2014). In strong support of a physiologic role of hepatic Mogat1 in maintaining hepatic TAG levels, adenoviral shRNA-Mogat1 delivery (that is preferentially taken up by the liver) (Lee et al. 2012), and nonviral siRNA-Mogat1 delivery (Hayashi et al. 2014), reduced Mogat1 expression and hepatic TAG levels in HF-fed mice. The results of the present study indicate that PPARγ is necessary to maintain Mogat1 activity, as well as Mogat1 expression, based on the increase in hepatic MAG levels in aLivPPARγkd mice. Interestingly, it has been postulated that Mogat1 may prefer extrahepatic (dietary FA) over those produced by hepatic DNL, to synthesize DAG (Steneberg et al. 2015). This would be consistent with our observation that although MAG levels were increased in LF-fed aLivPPARγkd mice, DAG and TAG levels were normal, where in this context, hepatic DAG and TAG are mainly derived from DNL. However, in HF-fed aLivPPARγkd mice (where the bulk of FA is coming from the diet), a reduction in DAG and TAG levels was observed.

Taken together, results suggest that hepatocyte PPARγ expression in adult livers is not essential to maintain the expression of DNL genes, but it is essential to induce the expression of genes important in hepatic FA uptake (Cd36) and re-esterification (Mogat1). Certainly, other pathways may be mediated by hepatic PPARγ that were not revealed by our targeted approach. Nonetheless, our current data, coupled with previous reports suggest that impairment of the Cd36-mediated FA uptake and FA esterification by MAG pathway could explain in part the protection against steatosis observed in aLivPPARγkd mice.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-16-0447.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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