Chapter 2

Hepatic inflammation does not underlie the predisposition to insulin resistance in dyslipidemic LDL receptor knockout mice

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Abstract

Chronic inflammation is considered a causal risk factor predisposing to insulin resistance. However, evidence is accumulating that inflammation confined to the liver may not be causal to metabolic dysfunction. To investigate this, we assessed if hepatic inflammation explains the predisposition towards insulin resistance in low-density lipoprotein receptor knock-out (Ldlr⁻/⁻) mice. For this, wild type (WT) and Ldlr⁻/⁻ mice were fed a chow diet, a high fat (HF) diet or a high fat, high cholesterol (HFC) diet for 2 weeks. Plasma lipid levels were elevated in chow-fed Ldlr⁺/⁺ mice compared to WT mice. Although short-term HF or HFC feeding did not result in body weight gain and adipose tissue inflammation, dyslipidemia was worsened in Ldlr⁺/⁺ mice compared to WT mice. In addition, dyslipidemic HF-fed Ldlr⁻/⁻ mice had a higher hepatic glucose production rate than HF-fed WT mice, while peripheral insulin resistance was unaffected. This suggests that HF-fed Ldlr⁻/⁻ mice suffered from hepatic insulin resistance. While HFC-fed Ldlr⁻/⁻ mice displayed the anticipated increased hepatic inflammation, this did neither exacerbate systemic nor hepatic insulin resistance. Therefore, our results show that hepatic insulin resistance is unrelated to hepatic inflammation, indicating that hepatic inflammation does not play a causal role in triggering hepatic insulin resistance.
Introduction

Obesity is linked to many deleterious health consequences, including insulin resistance, type 2 diabetes (T2D) and the metabolic syndrome, a group of metabolic risk factors predisposing to T2D and cardiovascular disease. Low-grade, chronic inflammation is considered as one of the most important mechanisms explaining the etiology of insulin resistance, T2D, and the metabolic syndrome [1]. However, evidence is accumulating that inflammation when confined to the liver may not be causal to metabolic dysfunction in obesity (for review see [2]). For instance, we recently demonstrated that hepatic inflammation does not contribute to insulin resistance in TNFR1-non-sheddible mice expressing a mutated TNFR1 ectodomain incapable of shedding and dampening the hepatic inflammatory response [3]. Furthermore, we showed that cholesterol-induced hepatic inflammation does not advance the development of systemic insulin resistance in male Ldlr^-^ mice [4]. Consistent with the outcome of these gain-of-function studies, others have shown that reduced hepatic inflammation not necessarily corresponds to enhanced insulin sensitivity in mice [5,6], further indicating that factors other than hepatic inflammation may be causal in triggering insulin resistance.

Dyslipidemia, provoked by elevated plasma low-density lipoprotein (LDL) cholesterol and/or very low-density lipoprotein (VLDL) triglycerides levels and decreased high-density lipoprotein (HDL) cholesterol levels, may be such a causal factor in the development of insulin resistance [7]. Indeed, several studies have shown that dyslipidemia is an independent predictor of insulin resistance and T2D [8,9]. Furthermore, lipid-lowering drugs have been shown to exhibit a positive effect on insulin sensitivity [10]. Nevertheless, dyslipidemia may also occur as a result of insulin resistance since hepatic lipogenesis, in contrast to gluconeogenesis, remains sensitive to insulin [11]. This leads to an increased production of plasma lipids due to overstimulation of insulin receptor pathways caused by hyperinsulinemia [11]. Hampered by the co-existing nature of dyslipidemia and obesity its exact role in the etiology of insulin resistance therefore remains ill defined.

To further elaborate on these studies, we assessed the extent to which hepatic inflammation may explain the reported predisposition towards insulin resistance in dyslipidemic Ldlr^-^ mice [12]. Furthermore, the rapid development of dyslipidemia [13,14] and hepatic inflammation [14,15] in these mice, allows us to investigate their effect on insulin resistance before alterations in body weight occur. We opted to use female mice only as they confer a natural resistance against diet-induced obesity. This is of particular importance as adiposity drives the metabolic phenotype in most studies.
and differences in insulin resistance have been shown to disappear after matching the mice for body weight [16]. Our data show that hepatic inflammation is not a causal factor in the development of hepatic insulin resistance in Ldlr−/− mice. Thus, in line with the studies mentioned above, but contrasting with the current dogma, our data do not support a role for hepatic inflammation in triggering insulin resistance.

**Research Design and Methods**

**Animals and diets**
Age-matched (12-16 weeks) female Ldlr−/− mice on a C57BL/6J background and wild type (WT) C57BL/6J mice were used for all experiments. Ldlr−/− mice were bred inhouse and WT mice were purchased from Charles River (France). Mice were placed on a standard rodent chow diet, a high fat (HF) diet (containing 21% fat from milk butter and 0.02% cholesterol; Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France) or a high fat, high cholesterol (HFC) diet (containing 21% fat from milk butter and 0.2% cholesterol; Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France) for a period of 2 weeks with ad libitum access to food and water. Mice were housed individually and kept on a 12-hour light/12-hour dark cycle. Animals were anesthetized by isoflurane during all surgical operations and discomfort was minimized as much as possible. All animal experiments were approved by the ethics committee of the University of Groningen, which adheres to the principles and guidelines established by the European Convention for the Protection of Laboratory Animals.

**Oral glucose tolerance test and intraperitoneal insulin tolerance test**
Mice were fasted for 6 hours before performing an oral glucose tolerance test (OGTT) or an insulin tolerance test (ITT). For the OGTT, a glucose bolus of 2 g/kg body weight of 20% glucose solution was given by gavage. For the ITT, an insulin dose of 0.3 U/kg body weight was injected intraperitoneally. Glucose levels were measured with a One Touch Ultra glucose meter before the test and at 15, 30, 60, 90 and 120 minutes after gavage or injection. In addition, fasted insulin levels were measured with an ultrasensitive insulin ELISA kit (Alpco Diagnostics, Salem, NH). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasted insulin and glucose levels (fasted insulin (μU/ml) x fasted glucose (mmol/liter) /22.5).
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**Hyperinsulinemic-euglycemic clamp**

A hyperinsulinemic-euglycemic clamp (HIEC) was performed in conscious mice as described previously [17], with a modified protocol. In brief, mice were canulated in the right \textit{vena jugularis} to allow infusion of fluids for a HIEC. They were allowed to recover for 5-7 days before the HIEC was started. Before the HIEC, mice were fasted overnight for 9 hours and placed in experimental cages. Mice were infused at a rate of 0.10 ml/h for 4 hours with a solution containing 1\% bovine serum albumin (Sigma Aldrich, Zwijndrecht, the Netherlands), 30\% glucose (3\% [U-13C] glucose; 27\% glucose), 110 mU/ml insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark), and 40 μg/ml somatostatin (Eumedica NV, Brussels, Belgium). To maintain euglycemia, a 30\% glucose solution was infused (3\% [U-13C] glucose; 27\% glucose) via a second line and pump speeds were adjusted to the needs of the animal. Every 15 min, a blood sample was taken from the tail vein to determine plasma glucose levels, and every 30 minutes, a blood spot was collected on filter paper for gas chromatography-mass spectrometry (GC-MS) analysis.

**Gas chromatography-mass spectrometry analysis and calculations**

Extraction of glucose from blood spots and GC-MS analysis of extracted glucose were performed according to Van Dijk \textit{et al.} [18]. Hepatic glucose production and metabolic clearance rate were calculated from GC-MS results using mass isotopomer distribution analysis as previously described [18].

**Blood and tissue collection**

The mice were fasted for 6 hours before being sacrificed. Tissues were rapidly removed, snap frozen in liquid nitrogen, and stored at -80\(^\circ\)C until further analysis. For histology, tissues were frozen or fixed in paraformaldehyde and embedded in paraffin. Blood was collected by a heart puncture and separated by centrifugation (3000 g, 10 min, 4\(^\circ\)C). Plasma was decanted and frozen at -20\(^\circ\)C.

**Lipid analysis**

For hepatic triglyceride and cholesterol measurements, lipids were extracted from frozen livers according to the method of Bligh and Dyer [19]. Hepatic and plasma triglyceride and cholesterol levels were measured using commercially available kits from Roche (Mannheim, Germany). Hepatic free cholesterol levels were determined using a commercially available kit from DiaSys (Holzheim, Germany). For diacylglycerol (DAG)
determination, lipids were extracted from frozen-crushed livers with MeOH:CHCl₃ (1:2) and separated by thin-layer chromatography. Lipids were visualized with CuSO₄ and quantified by comparing the density to a standard amount of DAG.

**Immunoblot analysis**

Frozen tissues were homogenized for Western Blot analysis. Protein concentration was equalized and proteins were separated with SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE Healthcare Life Sciences, Diegem, Belgium). Membranes were incubated overnight at 4°C with an antibody against pAKT (Ser473, Cell Signaling Technology, Leiden, the Netherlands) or AKT (Cell Signaling Technology) in 5% bovine serum albumin. The following day, membranes were incubated with a secondary antibody containing horse-radish peroxidase (Goat-anti-rabbit: Bio-Rad, Veenendaal, the Netherlands). To visualize the immune complex, membranes were treated with enhanced chemiluminescence reaction reagent and a picture was taken using Gel Doc XR+ Imaging system (Bio-Rad). Protein bands were analyzed using Image Lab 3.0.1 (Bio-Rad).

**Gene expression**

To isolate RNA, liver biopsies were homogenized in Qiazol reagent and RNA was isolated according to the manufacturer's procedure (Qiagen, Venlo, the Netherlands). Adipose tissue RNA was isolated using a commercially available kit (Qiagen). From liver and adipose tissue RNA, cDNA was synthesized for RT-PCR using a commercially available kit (Bio-Rad). RT-PCR was performed using Sybr Green Supermix (Bio-Rad). The following primer sequences were used for RT-PCR: Tnфа, forward CATCTTCTCAAATTTCAGTGACA, reverse TGGGAGTAGACAAGGTACAAC; Mcp₁, forward GCTGAGGAGCTACAAGAGGATCA, reverse ACAGACCTCTCTCTTGAGCTTGG; Cd68, forward TGACCTGCTCTCTCTAGGCCTACA, reverse TCACGATTGCAAGAGAAATGG; Cd11b, forward TCAGAGAATGTCCTCAGCAG, reverse TGAGACAACCTCTCTCATCTC; Ppia forward TTCCTCCTTTCCAGAAAATTCCA, reverse CCGCCAGTGCCATTATGG.

**Histological analysis**

Paraffin-embedded adipose tissue biopsies were sectioned at 4 μm and stained with hematoxylin-eosin. Frozen liver sections of 5 μm were used to stain for the macrophage marker CD68 (FA11, Abcam, Cambridge, UK).
Statistical analysis
Mann-Whitney tests or ANOVAs were performed using Graph-Pad Prism 5.0 (San Diego, USA) to determine the differences between groups. To ensure that the assumption of homogeneity of variances was met, this was tested before performing an ANOVA. P-values < 0.05 were considered significant. Values are expressed as mean ± SEM and group sizes are indicated in the figure legends.

Results

Elevated plasma levels of cholesterol and triglycerides in Ldlr<sup>-/-</sup> mice fed a HF- and HFC-diet
Since dyslipidemia is an independent predictor of insulin resistance, we first assessed plasma lipid levels of WT and Ldlr<sup>-/-</sup> mice fed a chow, HF or HFC diet for 2 weeks. While body weight did not differ between both genotypes (Fig. 1A), plasma triglyceride (Fig. 1B) and cholesterol levels (Fig. 1C) were significantly elevated in Ldlr<sup>-/-</sup> mice regardless of the diet. However, their levels were most pronounced in Ldlr<sup>-/-</sup> mice fed a HF- and HFC-diet, as reflected by a close to 20-fold increase in plasma triglyceride levels (Fig. 1B) and a bigger than 10-14 fold increase in plasma cholesterol levels (Fig. 1C) compared to WT mice fed a similar diet. No differences were observed in plasma FFA levels (data not shown). In addition, glucose (Fig. 1D) and insulin levels (Fig. 1E) were significantly elevated in Ldlr<sup>-/-</sup> mice fed a HF diet compared to WT mice on the same diet. In Ldlr<sup>-/-</sup> mice fed a HFC diet only a trend towards an increase was observed. Likewise, the HOMA-IR was significantly elevated in Ldlr<sup>-/-</sup> mice fed a HF diet, whereas a trend towards an increase was observed in Ldlr<sup>-/-</sup> mice fed a HFC diet (Fig. 1F), confirming the reported predisposition towards insulin resistance in Ldlr<sup>-/-</sup> mice [12].

Increased hepatic inflammation in Ldlr<sup>-/-</sup> mice fed a HFC diet
To validate the degree of hepatic inflammation in WT and Ldlr<sup>-/-</sup> mice fed a chow, HF and HFC diet, we performed CD68 immunostaining and measured the expression of the pro-inflammatory genes Cd68, Cd11b, Tnfa and Mcp1 in livers of WT and Ldlr<sup>-/-</sup> mice fed a chow, HF and HFC diet. As expected, only Ldlr<sup>-/-</sup> mice fed a HFC diet showed an increased staining of CD68 in the liver (Fig. 2A), indicating an increased number of macrophages in their livers. Consistent with the histological analysis of the liver, Ldlr<sup>-/-</sup>
mice on a HFC-diet showed marked levels of hepatic inflammation (Figs. 2B-E). Following 2 weeks of HFC feeding, these mice showed a 5-fold and a 2-fold increase in the mRNA levels of the macrophage markers Cd68 and Cd11b, respectively (Figs. 2B-C) compared to WT mice. In addition, a 5-fold increase in the expression of the cytokine Tnfa (Fig. 2D) and a 10-fold increase in the expression of the chemokine Mcp1 (Fig. 2E) were observed compared to HFC-fed WT mice. We did not observe any differences in hepatic inflammatory gene expression between the genotypes in chow-fed mice. Apart from a 2-fold increase in Cd68 expression in Ldlr−/− mice fed a HF-diet compared to WT mice (Fig. 2B), no significant difference was observed in hepatic Cd11b, Tnfa or Mcp1 expression between these mice (Figs. 2C-E).

**Figure 1.** Circulating levels of lipids, glucose and insulin in Ldlr−/− mice fed a chow, HF, or HFC diet for 2 weeks. Body weight of WT and Ldlr−/− mice fed a chow, high-fat (HF) or high-fat cholesterol (HFC) diet was determined at time of sacrifice (A) (n = 12). Plasma triglyceride (B), cholesterol (C), glucose (D) and insulin (E) levels were measured in blood obtained following a 6-hour fast (n = 5-6). (F) The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasted insulin and glucose levels (n = 6). Data are expressed as means ± SEM for WT mice (white bars) and Ldlr−/− mice (black bars). * p < 0.05 vs WT.
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Absence of adipose tissue inflammation in Ldlr⁻/⁻ mice fed a HFC diet

Despite marked inflammation in the livers of Ldlr⁻/⁻ mice fed a HFC-diet, hematoxylin and eosin staining of white adipose tissue sections did not show signs of inflammation (Fig. 3A). In addition, no significant changes in the gene expression of the inflammatory markers Cd68, Cd11b, Tnfa and Mcp1 were found in white adipose tissue (Figs. 3B-E), confirming the absence of adipose tissue inflammation in Ldlr⁻/⁻ mice fed any of the given diets.

FIGURE 2. Hepatic inflammation in Ldlr⁻/⁻ mice fed a HFC diet. (A) Representative pictures of frozen liver sections stained with CD68 were taken from WT and Ldlr⁻/⁻ mice fed a chow, high-fat (HF) or high-fat cholesterol (HFC) diet (n = 5-6). RNA was isolated from liver tissue and the expression of the pro-inflammatory genes Cd68 (B), Cd11b (C), Tnfa (D) and Mcp1 (E) was determined by real-time PCR and expressed as fold induction (n = 5). Data are expressed as means ± SEM for WT mice (white bars) and Ldlr⁻/⁻ mice (black bars). * p < 0.05 vs WT.
FIGURE 3. Absence of adipose tissue inflammation in Ldlr⁻/⁻ mice fed a chow, HF, or HFC diet for 2 weeks. Representative pictures of paraffin-embedded white adipose tissue sections stained with hematoxillin-eosin (A) were taken from WT and Ldlr⁻/⁻ mice fed a chow, HF or HFC diet (n = 5-6). RNA was isolated from white adipose tissue and the expression of the pro-inflammatory genes Cd68 (B), Cd11b (C), Tnfα (D) and Mcp1 (E) was determined by real-time PCR and expressed as fold induction (n = 11-12). Data are expressed as means ± SEM for WT mice (white bars) and Ldlr⁻/⁻ mice (black bars).

Hepatic insulin resistance in dyslipidemic Ldlr⁻/⁻ mice is unrelated to hepatic inflammation

To investigate the degree of systemic insulin resistance in dyslipidemic Ldlr⁻/⁻ mice with or without hepatic inflammation, we performed an OGTT and an ITT in Ldlr⁻/⁻ mice fed a chow, HF- and HFC-diet. The OGTT (Fig. 4A) and ITT (Fig. 4B) did not detect differences
between the groups, suggesting that 2 weeks of HF and HFC feeding did not induce notable changes in glucose and insulin tolerance between the mice. We next performed a hyperinsulinemic-euglycemic clamp (HIEC) to distinguish between hepatic and peripheral insulin resistance. The glucose infusion rate (GIR; Fig. 4C) and the metabolic clearance rate of glucose (MCR; Fig. 4D) did not differ between the mice fed a chow, HF- or HFC-diet, confirming the similar glucose curves observed during the OGTT and the ITT. However, we observed a significant increase in hepatic glucose production in Ldlr\(^{-/-}\) mice fed a HF diet compared to HF-fed WT mice (Fig. 4E). These findings indicate that Ldlr\(^{-/-}\) mice fed a HF-diet suffered from hepatic insulin resistance, while peripheral insulin resistance remained unaffected. Hepatic insulin resistance was not observed in HFC-fed Ldlr\(^{-/-}\) mice, even though they had high levels of hepatic inflammation and a similar degree of dyslipidemia in comparison to Ldlr\(^{-/-}\) fed a HF-diet. Although clear hepatic insulin resistance was observed in Ldlr\(^{-/-}\) mice fed a HF-diet, the Ser473 phosphorylation of AKT (Fig. 4F) in the liver was not affected.

**Differences in hepatic lipid accumulation cannot explain hepatic insulin resistance**

Since the accumulation of lipid species in the liver has been associated with the development of hepatic insulin resistance [20-22], we measured hepatic lipid accumulation in WT and Ldlr\(^{-/-}\) mice fed a chow, HF- or HFC-diet for 2 weeks. HF and HFC feeding increased hepatic triglyceride accumulation (Fig. 5A) to a similar extent in both genotypes. As expected, HFC-feeding increased total and free cholesterol accumulation (Figs. 5B-C). When comparing genotypes, only Ldlr\(^{-/-}\) mice on a chow diet showed a significant increase in total cholesterol (Fig. 5B). No significant differences were found in free cholesterol levels (Fig. 5C). In particular, DAGs have been associated with the development of hepatic insulin resistance [21]. However, DAG levels were similar in both genotypes and on all diets (Fig. 5D). In summary, these results suggest that differences in hepatic lipid accumulation cannot account for the hepatic insulin resistance observed in Ldlr\(^{-/-}\) mice.
Hepatic inflammation does not induce hepatic insulin resistance in lean Ldlr⁻⁻ mice. To assess systemic insulin resistance, we performed an oral glucose tolerance test (A) and an insulin tolerance test (B) in WT and Ldlr⁻⁻ mice fed a chow, high-fat (HF) or high-fat cholesterol (HFC) diet (n = 5-6). To distinguish between hepatic and peripheral insulin resistance, a hyperinsulinemic-euglycemic clamp was performed during which glucose infusion rate (GIR) (C), metabolic clearance rate (MCR) (D), and hepatic glucose production (HGP) (E) were determined (n = 5-7). (F) Phosphorylation status of AKT in liver tissues obtained from WT and Ldlr⁻⁻ mice fed a chow, HF or HFC diet sacrificed 15 min after saline (n = 5) or insulin injection (n = 7) and determined by Western Blot analysis. Data are expressed as means ± SEM for WT mice (white bars) and Ldlr⁻⁻ mice (black bars). * p < 0.05 vs WT.
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**Discussion**

This study was designed to determine the effect of hepatic inflammation on the development of insulin resistance in Ldlr−/− mice, while excluding body weight gain as a confounding factor. Our results show that Ldlr−/− mice develop hepatic insulin resistance within 2 weeks of HF-feeding, while peripheral insulin resistance remained unaffected. Our data also show that both systemic and hepatic insulin resistance is not more advanced in Ldlr−/− mice fed a HFC diet, even though these mice had increased levels of hepatic inflammation compared to both chow and HF-fed Ldlr−/− mice. These results illustrate that hepatic insulin resistance can develop prior to alterations in body weight gain. Moreover, our findings suggest that hepatic inflammation is not associated with the onset of hepatic insulin resistance during this time frame and indicate that hepatic inflammation cannot explain the predisposition towards insulin resistance in these Ldlr−/− mice.

Our results also suggest that dyslipidemia is not causal to the development of hepatic insulin resistance as the degree of dyslipidemia was identical amongst the HF- and HFC-fed Ldlr−/− mice (Figs. 1B-C) whereas hepatic insulin resistance was only observed...
in the HF-fed \textit{Ldlr}^{-/-} mice (Fig. 4E). This argues against a causal relationship in the well-established metabolic link between hyperglycemia and dyslipidemia. Indeed, dyslipidemia, at the clinical level, is associated with elevated plasma glucose levels and insulin resistance. Furthermore, patients diagnosed with familial combined hyperlipidemia have an increased incidence of insulin resistance and T2D [23-27]. Moreover, dyslipidemia is an independent predictor for the development of insulin resistance and T2D later in life. Nevertheless, there is a complex genetic regulation and metabolic interplay between lipid and glucose metabolism, as we have recently observed that the genetic predisposition to dyslipidemia is related to lower levels of fasting plasma glucose, HbA1c, and HOMA-IR [28]. Out of the 15 loci that are associated with both lipids and glucose-related traits independently, 8 (CETP, MLXIPL, PLTP, GCKR, APOB, APOE-C1-C2, CYP7A1, and TIMD4) did exert an opposite allelic effect on dyslipidemia and glucose traits [28].

In contrast to several publications that indicate that hepatic inflammation can cause insulin resistance [29,30], we found that hepatic inflammation did not advance the development of hepatic or peripheral insulin resistance in female \textit{Ldlr}^{-/-} mice. These results extend our previous findings in male \textit{Ldlr}^{-/-} mice [4] as we now also demonstrate that hepatic insulin resistance is unrelated to hepatic inflammation. An explanation for the lack of an effect of hepatic inflammation on insulin resistance may be found in the cell type driving inflammation. Cai et al. described how inflammation was induced by hepatocyte activation of IKK, and this resulted in hepatic and systemic insulin resistance [30]. Although not assessed in this paper, previous studies have shown that Kupffer cells become foamy in \textit{Ldlr}^{-/-} mice within 7 days of HFC feeding and may be responsible for the initiation of hepatic inflammation in this model [14,15]. Kupffer cells are thought to contribute to insulin resistance by the production of pro-inflammatory cytokines that inhibit insulin signaling in hepatocytes [31]. Nevertheless, there is conflicting evidence for the role of Kupffer cells in hepatic insulin resistance. Some papers report an amelioration of insulin resistance with a depletion of Kupffer cells [32,33], whereas others show deterioration in insulin resistance [34]. Moreover, depleting Kupffer cells after the induction of insulin resistance has no therapeutic effect on metabolic changes [5]. Therefore, the cell type driving the hepatic inflammation may be important in determining the effect on insulin resistance. Hepatocyte-derived inflammation may be more important than Kupffer cell activation in the development of insulin resistance, highlighting the need for more studies focusing on cell type-specific induction of inflammation.
The lack of an effect of hepatic inflammation on insulin resistance may also reflect a time-dependent effect. A recent paper reported that inflammation was only involved in diet-induced insulin resistance once obesity had been established and not during the onset of obesity [35]. When obesity is established, a crosstalk between adipose tissue and liver may start to play a role. Hence, a HFC diet induces insulin resistance in Ldlr-/- mice only after 24 weeks of HFC feeding, which is presumably caused by adipose tissue inflammation [36]. In another study, the pro-inflammatory cytokines secreted from adipose tissue were shown to be able to induce insulin resistance in hepatocytes [37]. In our animal model, hepatic inflammation was present for a period of 2 weeks (Fig. 2A), without increased body weight gain or adipose tissue inflammation (Figs. 2B and 3C) being present. This may not be long enough for hepatic inflammation to inhibit insulin signaling in the liver. Our data indicate that cholesterol-induced hepatic inflammation, in the absence of adipose tissue inflammation, is not enough to induce insulin resistance in hepatocytes. Within this 2-week time frame hepatic insulin resistance may be primarily caused by factors other than hepatic inflammation and dyslipidemia.

A few limitations of our study must be taken into account. While there is no doubt about the hepatic insulin resistance observed in Ldlr-/- fed a HF diet during the HIEC, we were not able to confirm these results by measuring phosphorylation of AKT in the livers of insulin injected mice. This may be explained by the many pathways and molecules that are involved in insulin signaling [38]. Interference with insulin signaling may take place at a different part of the insulin signaling cascade than at the level of AKT. In addition, while we excluded differences in hepatic lipid content that may affect hepatic insulin resistance, we cannot rule out that other changes that could occur in Ldlr-/- mice might contribute to their hepatic insulin resistance. Lack of the Ldlr may lead to differences in intracellular signaling cascades that could affect insulin signaling. However, in chow-fed Ldlr-/- mice, we observed no changes in either hepatic or peripheral insulin resistance, indicating that the effects on hepatic insulin resistance are not intrinsic to the Ldlr deficiency, but are related to the HF diet intervention in these mice. In this regard, we cannot explain why cholesterol addition to the HF diet confers protection against the development of hepatic insulin resistance in Ldlr-/- mice. This may be related to the growing evidence that suggest that statins are associated with an increased incidence of new-onset T2D [39]. As mechanisms explaining the potentially higher incidence of new onset T2D with statin therapy have not been defined, further studies are required to understand this effect.
Conclusions
In conclusion, our data show that neither hepatic inflammation nor dyslipidemia is causally related to the development of hepatic insulin resistance in Ldlr^{-} mice and suggest that both factors may occur as a consequence of metabolic dysfunction in obesity.

Declaration of interest
The authors declare that there is no conflict of interests regarding the publication of this paper.

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