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Critical role of Kupffer cells in the management of diet-induced diabetes and obesity

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ABSTRACT

The aim of this study was to investigate the role of Kupffer cell in glucose metabolism and hepatic insulin sensitivity in mice. Both phagocytic activity and secretory capacity of Kupffer cells were blunted 24 h after $GdCl_3$ administration. Glucose tolerance – evaluated following an oral glucose tolerance test (OGTT) – was higher in $GdCl_3$-treated mice whereas fasting insulinemia and HOMA-IR index decreased. The improvement of glucose tolerance and hepatic insulin signalling pathway after inhibition of Kupffer cells was supported by a lower hepatic gluconeogenic enzyme expression and a higher phosphorylation of Akt upon insulin challenge. Moreover, fasting hyperglycemia, insulin resistance and impaired glucose tolerance – induced by high fat (HF) diet – were improved through chronic administration of $GdCl_3$. Interestingly, the inhibition of Kupffer cell exerted antiobesity effects in HF-fed mice, and lowered hepatic steatosis. Therefore, strategies targeting Kupffer cell functions could be a promising approach to counteract obesity and related metabolic disorders.

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Introduction

Obesity is strongly associated with the development of insulin resistance, type 2 diabetes and related alterations. It has been widely established that insulin resistance precedes the development of overt hyperglycemia [1]. Impaired peripheral insulin sensitivity decreases insulin-stimulated glucose uptake principally in the skeletal muscle and reduces insulin capacity to inhibit lipolysis in adipose tissue [1,2]. In the liver, insulin resistance is translated into impaired suppression of glucose production – which largely accounts for hyperglycemia and glucose intolerance – as well as a higher lipid synthesis and storage. These events link obesity and metabolic syndrome to non-alcoholic fatty-liver disease (NAFLD) [1–3]. Increased adiposity promotes macrophage infiltration and activation in adipose tissue, perpetuating local inflammation and causing insulin resistance [4–6]. Compared with the other organs, the liver has one of the largest resident populations of macrophages, i.e. Kupffer cells. Kupffer cells produce various inflammatory mediators including cytokines (TNF-α), prostaglandins and reactive oxygen species, namely through NADPH-oxidase or inducible NO-synthase (iNOS) activities [7,8]. It is known that Kupffer cell dysfunction contributes to the pathogenesis of NAFLD [9,10]. However, the role of Kupffer cells in the regulation of the liver metabolism and in the occurrence in metabolic disease remains enigmatic. To address this critical question, we propose a physiological approach to assess whether Kupffer cells are involved in glucose metabolism and insulin sensitivity in the liver. Gadolinium chloride ($GdCl_3$) is often used as tool for studying the role of Kupffer cells, since it significantly and drastically decreased fixed macrophage activity and number in the liver tissue [11–16]. We have studied the acute and chronic effect of specific inhibition of Kupffer cells by $GdCl_3$ on glucose homeostasis on the one hand, and on the development of diet-induced obesity (DIO), on the other hand.

Materials and methods

Effects of a single dose of $GdCl_3$ in mice fed a normal chow diet. C57bl6/J male mice (9-week-old, Charles River Laboratories, France) were housed in a controlled environment (12-h daylight cycle, light out at 6 pm) with free access to food (normal chow diet AO4) and water. After 1 week for acclimatisation, mice received an i.p. injection of either NaCl 0.9% (CT) or $GdCl_3$ (20 mg/kg). The assessment of phagocytosis and functionality of Kupffer cells, the oral glucose tolerance test (OGTT), the analysis of the expression of genes coding key enzymes and regulators of glucose metabolism and the determination of insulin-stimulated serine phosphorylation of Akt were performed 24 h after $GdCl_3$ or saline injection as described below.

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Effects of chronic administration of $GdCl_3$ in mice fed a high fat diet. C57bl6/J male mice (9-week-old, Charles River Laboratories, France) were housed in a controlled environment (12-h daylight cycle, light out at 6 pm) with free access to food (normal chow diet AO4) and water. After 1 week for acclimatisation, control mice (CT) were fed a standard diet (A04, UAR, Villemoisson-sur-Orge, France), whereas HF-treated mice received a diet containing 49.5% fat – corn oil and lard – (g/100 g of total dry diet), 37% protein, 10% cellulose, 3.5% mineral/vitamins mixture. The energy content of the HF diet was 72% of fat, 28% protein and <1% carbohydrates. During the dietary treatment, mice received also an i.p. injection of either NaCl 0.9% or $GdCl_3$ (20 mg/kg) twice a week. OGTT was performed on 6 h fasted-mice after 3 weeks of treatment. Mice were killed by cervical dislocation after 4 weeks of treatment and after a 5 h period of fasting; blood samples and tissues were harvested for further analysis.

Test for phagocytic function of Kupffer cells. Mice were anesthetised (ketamine/xylazine i.p., 100 and 10 mg/kg, respectively) 24 h after $GdCl_3$ or saline injection. Colloidal carbon challenge was performed through portal injection of 100 μl/10 g body weight Pelikan® No. 17 dissolve in saline 1:9. After 10 min, 20 μl of blood was obtained from vena cava and added into 2 ml of 0.1% Na_2CO_3 solution and absorbance (OD) was read at 600 nm. Pieces of liver, spleen, white adipose tissues (epididymal and visceral) were fixed in formalin for further histological studies.

Oral glucose tolerance test (OGTT). OGTT was performed on 6 h fasted-mice, 24 h after a single $GdCl_3$ or saline injection as well as after chronic $GdCl_3$ or saline administration. Glucose was administered orally (3 g/kg of glucose, 66% glucose solution) and blood glucose determined using a glucose metre (Roche Diagnostics) on 3.5 μl of blood collected from the tip of the tail vein both before (−30 and 0 min) and after glucose load (15, 30, 60, 90, 120 min). Twenty microlitres of blood was sampled 30 min before and 15 min after the glucose load to assess plasma insulin concentration. Insulinoresistance index (HOMA-IR) was calculated by multiplying fasting glycemia (mM) and fasting insulinemia (pM) divided by 22.5.

Real-time quantitative PCR. Mice were killed by cervical dislocation 24 h after a single $GdCl_3$ or saline injection as well as after chronic $GdCl_3$ or saline administration. Liver and subcutaneous adipose tissue were carefully dissected and immersed in liquid nitrogen and stored at −80 °C. Total RNA was isolated using Tri-Pure isolation reagent kit (Roche Diagnostics Belgium, Vilvoorde). cDNA was prepared by reverse transcription of 1 μg total RNA using the Kit Reverse transcription System (Promega, Leiden, The Netherlands). Real-time PCRs were performed with the StepOnePlus real-time PCR system and software (Applied Biosystems, Den Ijssel, The Netherlands) using SYBR-Green for detection. RPL19 RNA was chosen as housekeeping gene. Primer sequences for the targeted mouse genes are available on request (audrey.neyrinck@uclouvain.be). All samples were run in duplicate in a single 96-well reaction plate and data were analysed according to the 2-ΔCT method [6]. The identity and purity of the amplified product was checked through analysis of the melting curve carried out at the end of amplification.

Insulin signalling upon insulin challenge. Mice were anesthetised (ketamine/xylazine i.p., 100 and 10 mg/kg, respectively) 24 h after $GdCl_3$ or saline injection, and after a 12 h period of fasting. Insulin was injected in the portal vein (5 UI) and liver were dissected, quickly immerse in liquid nitrogen and stored at −80 °C. Liver phosphorylated proteins of Akt (S473) were measured with a multiplex kit (Bio-Rad, Nazareth, Belgium) using Luminex technology (Bio-Plex, Bio-Rad, Nazareth, Belgium). Total target assay for Akt was performed through the same technology according to the manufacturer's instructions. Lysates of liver tissue were prepared using the Bio-Plex lysis kit (Bio-Rad, Nazareth, Belgium) according to the manufacturer's instructions. Protein concentration of the lysates was adjusted to 200 μg/ml for the assay and was determined using the Bradford method.

Statistical analysis. Results are presented as means ± SEM. Statistical analysis was performed by ANOVA and Student *t* test (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered as statistically significant.

Animal experiments were approved by the local ethics committee and housing conditions were as specified by the Belgian Law of 14 November 1993 on the protection of laboratory animals (agreement No. LA 1230314).

Results

Kupffer cell functions after $GdCl_3$ injection

After a single i.p. injection of $GdCl_3$, phagocytic activity by hepatic resident macrophages is strongly reduced as shown by both a higher colloidal carbon accumulation in the blood (Fig. 1A) and histological analysis of the cellular colloidal carbon uptake in the liver (Fig. 1B). Of note, the colloidal carbon uptake is not modified by $GdCl_3$ treatment in spleen and is undetectable in white adipose tissues (data not shown). Liver expression of TNF-α, NADPH-oxidase and iNOS is lower after $GdCl_3$ administration than in control mice (Fig. 1C, D and E, respectively). We did not observe any change for TNF-α expression in white adipose tissue (data not shown).

Glucose tolerance and hepatic insulin sensitivity after inhibition of Kupffer cells

Fasting glycemia is similar in $GdCl_3$-treated and control mice (7.1 ± 0.4 and 7.4 ± 0.3 mM, respectively; Student *t* test: $p > 0.05$) whereas fasting insulinemia is lower in $GdCl_3$-treated mice as compared to control mice (110 ± 16 versus 180 ± 23 mM, respectively; Student *t* test: $^{*}p < 0.05$). $GdCl_3$-treated mice display a better glucose tolerance than saline-injected mice (Fig. 2A and B). Interestingly, insulinoresistance index, estimated through HOMA-IR calculation, decreases after $GdCl_3$ administration (Fig. 2C). Furthermore, insulin-stimulated serine phosphorylation of Akt is higher in the liver of mice treated with $GdCl_3$ as compared to control mice, whereas the phosphorylation state of the protein remains unchanged in the absence of insulin challenge (Fig. 2D). The expression of several genes in the liver that regulate glucose metabolism has been examined (Fig. 2E). $GdCl_3$-treatment does not significantly change the expression of (1) a glucose responsive transcription factor named ChREBP (carbohydrate responsive element-binding protein) playing a crucial role in regulating short-term carbohydrate metabolism and fat synthesis; (2) the sterol regulatory element-binding protein-1c (SREBP-1c) emerging as a major mediator of insulin action on glycolytic and lipogenic gene expression; (3) GLUT-2 as a marker for glucose uptake from the liver; (4) GK, an enzyme which catalyses the phosphorylation of glucose to glucose 6-phosphate, a key step of glycolysis, glycogen synthesis and pentose phosphate pathway; (5) PEPCK, a key enzyme involved in gluconeogenesis. In $GdCl_3$-treated mice, only G6Pase was significantly down-regulated, thus suggesting a targeted modulation of gluconeogenesis at the crucial last step (release of glucose from glucose-6-phosphate).

Effect of chronic Kupffer cell inhibition on HF diet-induced impaired glucose tolerance and insulinoresistance

HF diet induces diabetes after 3 weeks of dietary treatment, as shown by a higher fasting hyperglycemia (Fig. 3A), a higher fasting insulinemia (Fig. 3B) and a higher index of insulinoresistance (HOMA-IR, Fig. 3C) as compared to mice fed normal chow diet.

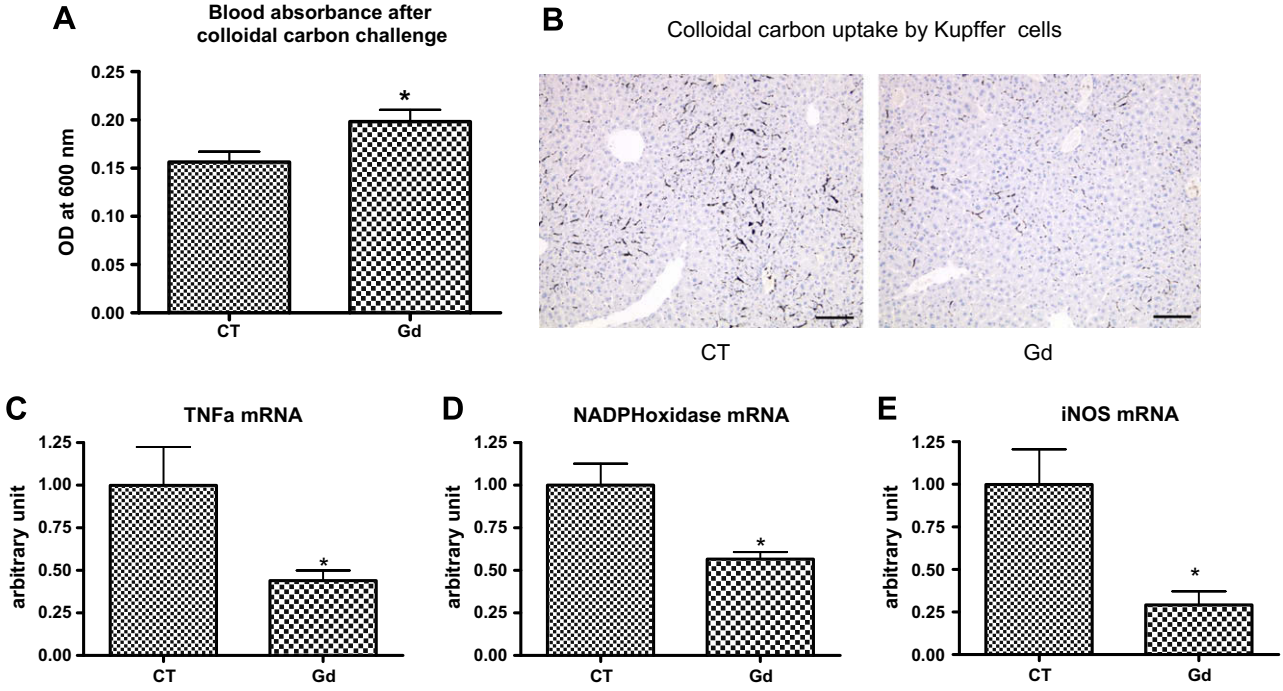

Fig. 1. Kupffer cell functions 24 h after $GdCl_3$ injection (Gd, 20 mg/kg) or saline injection (CT). (A) Blood absorbance at 600 nm, 10 min after colloidal carbon injection in the portal vein, $n = 4$. (B) Distribution of colloidal carbon in the liver 10 min after colloidal carbon injection in the portal vein, $n = 4$ (bar = 100 μm); the carbon is mainly taken up in Kupffer cells (dark spots). (C–E) Hepatic expression of plasma tumour necrosis factor-alpha (TNF-α), NADPH-oxidase and inducible NO-synthase (iNOS), $n \geqslant 7$. Data are means ± SEM. The Student t test was used for statistical analysis: $^{*}p < 0.05$.

Importantly, chronic administration of $GdCl_3$ decreases fasting glycemia and HOMA-IR index in mice fed a HF diet. In addition, the development of glucose intolerance observed upon HF diet, is completely abolished after Kupffer cell inhibition (Fig. 3D and E). Moreover, the better glucose tolerance observed following chronic $GdCl_3$ administration is also observed upon normal chow diet. We

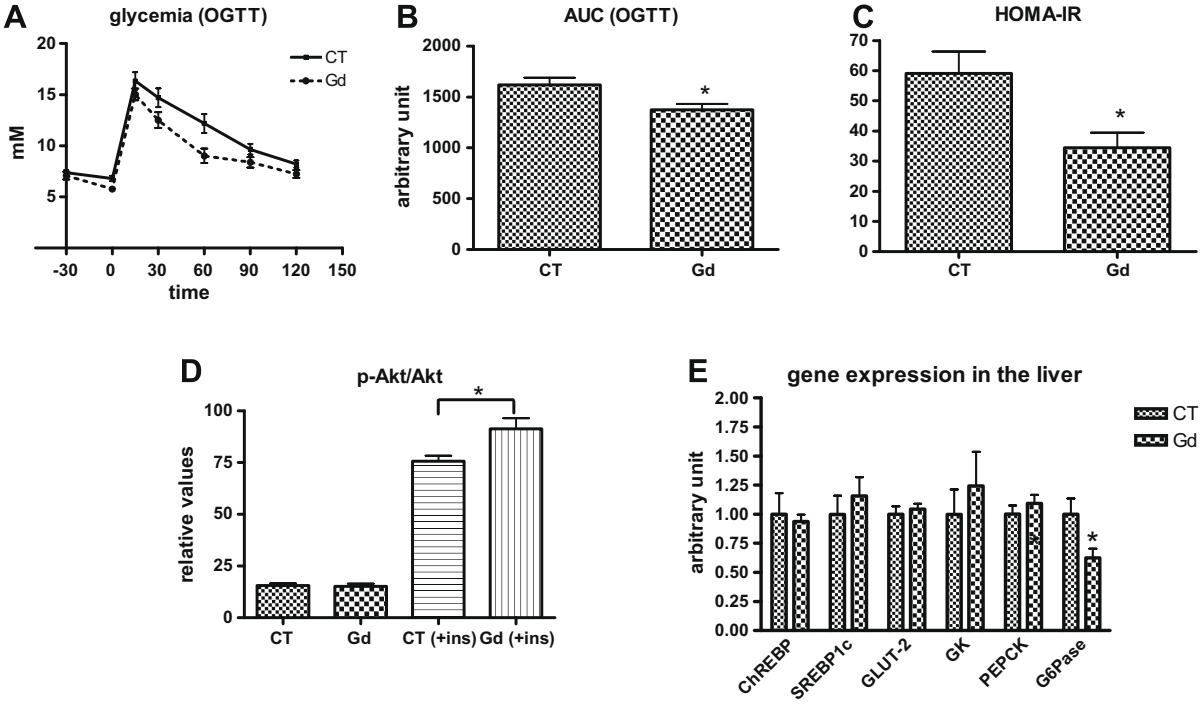

Fig. 2. Glucose tolerance, insulin resistance, hepatic insulin sensitivity and expression of genes that regulate glucose metabolism 24 h after $GdCl_3$ injection (Gd, 20 mg/kg) or saline injection (CT). (A) Plasma glucose following an oral glucose load (3 g/kg), $n \geqslant 7$. (B) Area under curve (AUC) of the glucose excursion after the oral glucose load (3 g/kg), $n \geqslant 7$. (C) Insulinoresistance index (HOMA-IR) calculated by multiplying fasting glycemia (mM) and fasting insulinemia (pM), divided by 22.5, $n \geqslant 7$. (D) Akt phosphorylation (Ser473) in the liver after insulin challenge (portal i.v. injection, 5 UI), using Luminex technology, $n \geqslant 7$. (E) Hepatic expression of key genes in the liver that regulate glucose metabolism, $n \geqslant 7$. Data are means ± SEM. The Student t test was used for statistical analysis: $^{*}p < 0.05$.

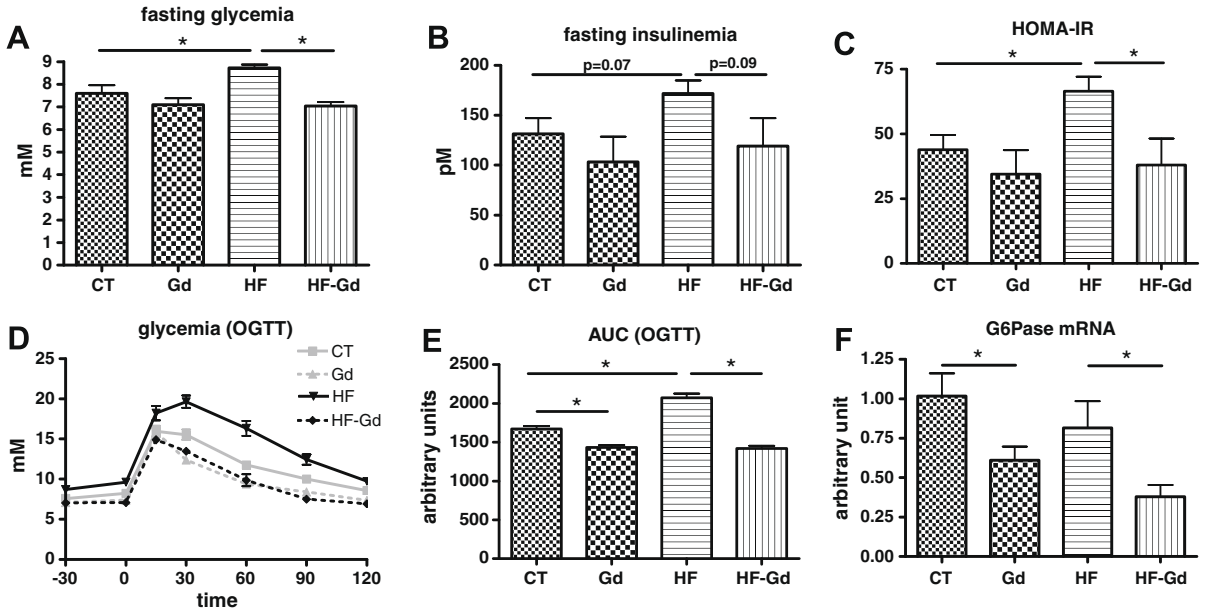

Fig. 3. Fasting glycemia and insulinemia, glucose tolerance, insulin resistance and hepatic gluconeogenic enzyme in mice fed a high fat diet (for HF and HF-Gd groups) or normal chow diet (for CT and Gd groups) for 3 weeks (A–E) or 4 weeks (F); $GdCl_3$ (20 mg/kg) was injected twice a week for Gd and HF-Gd groups whereas saline was injected twice a week for CT and HF groups. (A) Fasting glycemia, $n \geqslant 7$. (B) Fasting insulinemia, $n \geqslant 5$. (C) Insulinoresistance index (HOMA-IR), $n \geqslant 5$. (D) Plasma glucose following an oral glucose load (3 g/kg), $n \geqslant 7$. (E) Area under curve (AUC) of the glucose excursion after the oral glucose load (3 g/kg), $n \geqslant 7$. (F) Hepatic expression of glucose-6-phosphatase (G6Pase), a gluconeogenic enzyme, $n \geqslant 6$. Data are means ± SEM. In (D), ANOVA was used for statistical analysis: $^{*}p < 0.05$ for time, treatment and interaction. In (A–C), (E) and (F), the Student t test was used for statistical analysis: $^{*}p < 0.05$.

have observed that the expression of G6Pase mRNA is lower after Kupffer cell inhibition, whatever the dietary treatment, suggesting decreased hepatic capacity of glucose output (Fig. 3F).

Effect of chronic Kupffer cell inhibition on HF-induced body weight gain and metabolic alterations

The total caloric intake is higher in HF-treated group compared to chow diet (Fig. 4A). Mice fed with a chow diet, i.e. CT and Gd groups, gain similar amounts of weight during the experiment (Fig. 4B). Food intake remains unchanged after $GdCl_3$ treatment (Fig. 4A). High fat diet increases significantly body weight gain (0.98 ± 0.27 and 1.90 ± 0.25 g for CT and HF mice, respectively; Student t test: $^{*}p < 0.05$ and Fig. 4B). Furthermore, development of epididymal, visceral and subcutaneous fat mass is significantly higher in HF-group as compared to CT group (Fig. 4C, D and E). Interestingly, upon HF dietary treatment, chronic Kupffer cell inhibition decreased body weight gain by about 29% as compared to HF (Fig. 4B); this effect is accompanied by a lower fat mass development (Fig. 4C, D and E). HF diet feeding increases also hepatic triglycerides by about 24% as compared to CT group (Student t test: $p > 0.05$) (Fig. 4F). A significant increase of SREBP1c expression (Fig. 4G), but no modification of FAS mRNA (Fig. 4H) occurred in HF-fed mice versus controls. Importantly, the chronic inhibition of Kupffer cells abolished the triglyceride accumulation in the liver and decreased the expression of FAS and SREBP1c (the effect on SREBP1c being not significant) (Fig. 4F, G and H).

Discussion

Studies in obese and diabetic *fa/fa* rats or *ob/ob* mice provided the first clue that Kupffer cells dysfunction might be involved in the pathogenesis of NAFLD [9,10]. Furthermore, our previous studies suggest that Kupffer cells play a role in the physiological regulation of lipid metabolism of the adjacent hepatocytes [11,17]. More recently, several studies suggest that, similar to the paracrine role of macrophages in obese adipose tissue, the activation of pro-inflammatory pathways in Kupffer cells could be involved in the development of hepatic insulin resistance upon obesity [18–20]. What is their role and relevance in obesity-induced metabolic disease? To address this question, we determined whether the short- and long-term inhibition of Kupffer cells participate to the regulation of glucose tolerance and hepatic insulin sensitivity. Moreover, in the long-term study, we have also analysed the impact of Kupffer cell inhibition on glucose tolerance, adiposity and steatosis in DIO.

$GdCl_3$, a specific inhibitor of Kupffer cells, is often used as tool for studying the role of Kupffer cells [11–16]. Furthermore, we have previously demonstrated *in vitro* that inflammatory mediators released by the liver tissue (TNF-α, PGE_2 and nitrites) are decreased after $GdCl_3$-treatment, independently of the presence of inflammatory stimulus [8]. In the present study, we demonstrate that the phagocytic and functionality of Kupffer cells are inhibited after administration of $GdCl_3$ without acting on resident macrophages in other organs, such as the spleen or the white adipose tissue, as previously described [11,12,14]. We observed that mice display a better glucose tolerance when Kupffer cells were acutely or chronically inhibited. In the short-term study, we observed a higher insulin-dependent activation of Akt. The inhibition of Kupffer cell caused also a decrease in expression of hepatic G6Pase, the final "gate" for release of hepatic glucose through gluconeogenesis [21,22]. Altogether, these data suggest that Kupffer cell depletion may improve both glucose tolerance by decreasing the expression of the key gene regulating hepatic gluconeogenesis and by improving insulin signalling pathway in the liver tissue under physiological conditions. This effect is relevant in both short- and long-term inhibition of Kupffer cells. Are those effects relevant in pathological conditions such as development of metabolic disorders associated to obesity? In order to address this question, we induced obesity, impaired glucose tolerance, insulin resistance together with metabolic disorders (fat accumulation in adipose tissues and liver) in mice by feeding them a HF diet [6,23,24]. In order to evaluate

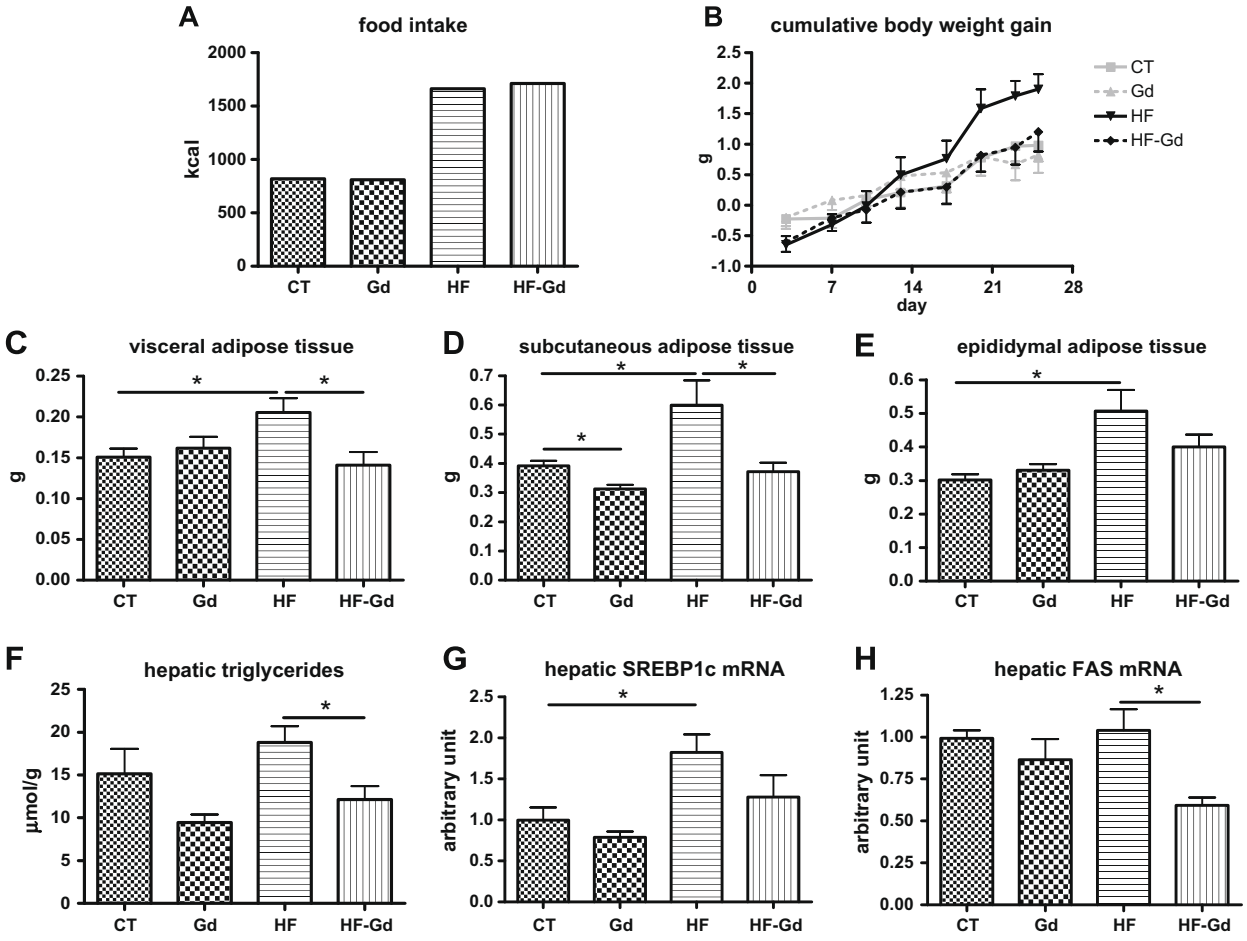

Fig. 4. Food intake, body weight gain, fat mass, hepatic triglycerides and lipogenic gene expression in the liver of mice fed a high fat diet (for HF and HF-Gd groups) or normal chow diet (for CT and Gd groups) for 4 weeks; $GdCl_3$ (20 mg/kg) was injected twice a week for Gd and HF-Gd groups whereas saline was injected twice a week for CT and HF groups. (A) Total food intake for four mice, $n = 2$. (B) Cumulative body weight gain, $n \geqslant 7$. (C) Visceral, (D) subcutaneous and (E) epididymal adipose tissue weight, $n \geqslant 6$. (F) Hepatic triglycerides, $n \geqslant 6$. (G, H) Hepatic expression of SREBP-1c and FAS, $n \geqslant 6$. Data are means ± SEM. In (B), ANOVA was used for statistical analysis: $^{*}p < 0.05$ for time and treatment; $p > 0.05$ for interaction. In (C–H), the Student t test was used for statistical analysis: $^{*}p < 0.05$.

the role played by Kupffer cells in the nutritional HF model for diabetes and obesity, we treated mice chronically by $GdCl_3$ [15,16]. With this approach, we demonstrated for the first time that Kupffer cell inhibition completely abolished HF-induced fasting hyperglycemia and glucose intolerance. Furthermore, HF-induced insulin resistance, indicated by the HOMA-IR index, was improved by inhibition of Kupffer cell functions. The hepatic glucose output coming from both gluconeogenic and glycogenolytic pathways seemed to be blunted by Kupffer cell depletion, as shown by the lower hepatic expression of G6Pase in $GdCl_3$-treated mice.

Although additional studies such as hyperinsulineamic–euglycemic clamp with glucose tracers will be required to assess the peripheral and hepatic insulin sensitivity as well as the hepatic glucose production, our findings define a crucial and direct role for the hepatic resident macrophages in the pathogenesis of diabetes, glucose intolerance and insulin resistance induced by HF diet.

It is well known that HF-diet induced obesity and increased adiposity but also causes ectopic fat deposition in a variety of tissues, which can result in insulin resistance in peripheral tissues such as liver [6,25]. Consistent with this notion, reductions in body weight and adiposity observed in Kupffer cell-depleted mice were accompanied by a decrease in the liver triglycerides. Both hyperinsulinemia and hyperglycemia, the most obvious clinical parameters modified in the insulin resistant state, *per se*, are factors that concur to stimulate *de novo* hepatic lipogenesis, namely by activation of the transcription factor SREBP-1c mediating most of insulin's effects on lipogenesis. In the present study, we demonstrated that the chronic depletion of Kupffer cell functions decreased FAS and SREBP-1c expression in the liver of mice fed a HF diet. Thus, Kupffer cells seem to play a role in the management of both molecular and functional events related to insulin action in liver.

We conclude that Kupffer cells play a critical role in the glucose metabolism since its depletion may improve hepatic insulin response and may lower gluconeogenesis by acting on G6Pase expression, with, as consequence, a better glucose tolerance. Furthermore, inhibition of Kupffer cell functions exerts antidiabetic and antiobesity effects in HF-fed mice that are related to decreased expression to lipogenic genes in liver together with a suppression of hepatic expression of a key gene of gluconeogenesis (G6Pase). Therefore, strategy aimed to deplete Kupffer cell functions could be promising therapeutic target for the treatment of obesity and/or diabetes and related metabolic disorders such as NAFLD.

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