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GW9662, a peroxisome proliferator-activated receptor gamma antagonist, attenuates the development of non-alcoholic fatty liver disease

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ABSTRACT

Background and aims: Insulin resistance is among the key risk factors for the development of non-alcoholic fatty liver disease (NAFLD). Recently, it has been reported that GW9662, shown to be a potent peroxisome proliferator-activated receptor gamma (PPARγ) antagonist, may improve insulin sensitivity in settings of type 2 diabetes. Here, we determined the effects of GW9662 on the development of NAFLD and molecular mechanisms involved.

Methods: Female C57BL/6J mice were pair-fed either a liquid control diet (C) or a fat-, fructose- and cholesterol-rich diet (FFC) for 8 weeks while either being treated with GW9662 (1 mg/kg body weight; C+GW9662 and FFC+GW9662) or vehicle (C and FFC) i.p. three times weekly. Indices of liver damage and inflammation, parameters of glucose metabolism and portal endotoxin levels were determined. Lipopolysaccharide (LPS)-challenged J774A.1 cells were treated with 10 μM GW9662.

Results: Despite similar caloric intake the development of NAFLD and insulin resistance were significantly attenuated in FFC+GW9662-treated mice when compared to FFC-fed animals. Bacterial endotoxin levels in portal plasma were almost similarly increased in both FFC-fed groups while expressions of toll-like receptor 4 (*Tlr4*), myeloid differentiation primary response 88 (*Myd88*) and interleukin 1 beta (*Il1b*) as well as nitrite (NO_2^-) concentration in liver were significantly higher in FFC-fed mice than in FFC+GW9662-treated animals. In J774A.1 cells, treatment with GW9662 significantly attenuated LPS-induced expression of *Il1b*, interleukin 6 (*Il6*) and inducible nitric oxide synthase (*iNos*) as well as NO_2^- formation.

Conclusion: In summary, our data suggest that the PPARγ antagonist GW9662 attenuates the development of a diet-induced NAFLD and that this is associated with a protection against the activation of the TLR4 signaling cascade.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) often referred to as the hepatic manifestation of the metabolic syndrome [1,2], covers a wide spectrum of liver conditions ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis and even hepatocellular carcinoma [3]. Recent estimates suggest that by now ~1 billion individuals are affected by the disease worldwide [4,5]. Although NAFLD has been shown to be strongly associated with obesity and insulin resistance, other factors including genetic predisposition, dietary

Abbreviations: Adipoq, adiponectin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; C, control diet; DE, diet effect; DExGWE, interaction between diet effect and GW9662 effect; Fas, fatty acid synthase; FFC, fat-, fructose- and cholesterol-rich diet; G6pc, catalytic unit of glucose-6-phosphatase; GTT, glucose tolerance test; GW9662, PPARγ antagonist; Il, interleukin; GWE, GW9662 effect; Icam, intercellular adhesion molecule; iNOS, inducible nitric oxide synthase; Myd88, myeloid differentiation primary response 88; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NFκB, nuclear factor kappa B; NO, nitric oxide; NO_2^-, nitrite; NOS, nitric oxide synthase; Pepck1, phosphoenolpyruvate carboxykinase 1; PPARγ, peroxisome proliferator-activated receptor gamma; SEM, standard error of the means; Srebp1, sterol response element-binding protein 1; Tlr4, toll-like receptor 4; TNFα, tumor necrosis factor alpha.

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pattern and dyslipidemia are also thought to be critical in the development of the disease [6–9]. Indeed, even in non-obese NAFLD, dyslipidemia and insulin resistance have been shown to be among the key factors associated with the development of NAFLD (for overview see [10]). Also, results of several human and animal-based studies suggest that targeting dyslipidemia and insulin resistance, be it through lifestyle interventions or pharmaceuticals, may improve not only glucose tolerance but also the progression of NAFLD [11,12]. However, despite intense research efforts and recent advances, universally accepted treatment strategies besides lifestyle interventions, which mainly focusing on body weight reduction and increased physical activity, are still limited.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors encompassing a subfamily of the nuclear receptor family. Three isoforms of PPARs have been described: PPAR alpha (PPARα), PPAR beta/delta (PPARβ/δ) and PPAR gamma (PPARγ). These isoforms are all differentially expressed in several tissues including skeletal tissue, adipose tissue, liver, kidney, vascular endothelial cells and intestinal epithelial cells (for overview see [13]) and are involved in the regulation of a wide spectrum of metabolic pathways (for overview see [14]). Furthermore, it has been shown, that they are tightly regulated through endogenous ligands, e.g., dietary lipids, phosphatidylcholines and fatty acids from lipolysis [15]. PPARγ is known to play a pivotal role in the regulation of the expression of genes mainly involved in lipid and glucose metabolism but has also been described to modulate inflammation [16]. Indeed, randomized controlled clinical trials suggest that targeting PPARγ activity with specific agonists, e.g., rosiglitazone and pioglitazone may be beneficial and may improve NAFLD-related hepatic steatosis (for overview see [17,18] and [19,20]). Conversely, several studies in animal models of obesity and diabetes reported an increased level of hepatic PPARγ and that an activation of PPARγ may even exacerbate hepatic lipid accumulation [21,22]. For instance, it has been shown that PPARγ2 is upregulated in livers of mice with a high fat diet-induced NAFLD and that a treatment with rosiglitazone in obese mice with high hepatic PPARγ expression may even exacerbate liver steatosis [23]. In line with these findings, others reported that PPARγ antagonists may dampen hyperglycemia and hyperinsulinemia as well as tumor necrosis factor alpha (TNFα) plasma levels in ob/ob and PPAR $\gamma^{+/-}$ mice [24] and may suppress adipocyte differentiation [25]. Furthermore, the PPARγ antagonist GW9662 has been shown to attenuate the development of NAFLD in ob/ob mice [26] and to induce differentiation of macrophages to M2c-like cells [27].

Based on this background, the aim of the present study was to analyse the effect of GW9662 on the development of a diet-induced NAFLD and to determine molecular mechanisms involved.

2. Material and methods

2.1. Animals and treatment

Female eight weeks old C57BL/6J mice (Janvier SAS, Le-Genest-Saint-Isle, France) were housed in a specific-pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Female mice have been shown to be more susceptible to the development of fructose-induced steatosis [28]. Also the beginning of early signs of early stages of NASH was shown to be similar pronounced as in male mice [29]. Mice had free access to tap water at all times. All procedures were approved and registered by the local Institution for Animal Care and Use Committee (Federal Ministry Republic of Austria Education, Science and Research, Vienna, Austria). Mice were pair-fed a liquid control diet (C; 15.7 MJ/kg diet: 69 E% from carbohydrates, 12 E% from fat and 19 E% from protein; Ssniff, Soest, Germany) or a liquid fat-, fructose- and cholesterol-rich diet (FFC; 17.8 MJ/kg diet: 60 E% from carbohydrates, 25 E% from fat and 15 E% from protein with 50% wt/wt fructose and 0.16% wt/wt cholesterol; Ssniff, Soest, Germany) as detailed previously [30]. In addition, some of the mice were treated i.p. with the PPARγ antagonist GW9662 (1 mg/kg body weight) or vehicle three times weekly for 8 weeks. The route of administration and concentration of GW9662 was chosen based on previous studies of others [31,32]. Whereas GW9662 was given daily for a total of 21 days [31] or once 1 h before the end of the experiment [32], in the present study, GW9662 was administered three times weekly to lower the stress of the animals. While GW9662 can be solved in DMSO, and therefore, could be added to the liquid diet used in the present study, we chose to administer the antagonist by i.p. injection to ensure adequate dosing per mouse. Indeed, pharmacokinetic studies suggested that while GW9662 was not detectable in plasma, mainly amine metabolites of GW9662 were quantified in the plasma following oral dosing. Albeit GW9662 was rapidly cleared and distributed in tissue after i.v. injection, GW9662 was found in plasma at low concentration [33]. Moreover, GW9662 is a selective covalent and irreversible antagonist of full-length PPARγ with IC50 value of 3.3 nM [34]. And while results of in vitro binding studies suggest that GW9662 can also bind irreversibly to the ligand-binding domains of the other PPAR isoforms, the binding to PPARα and PPARδ is thought to be approximately 10- and 600-fold less potent, respectively, than to PPARγ (IC50 value of 32 nM for PPARα or 2 μM for PPARδ) [34]. The sample size was calculated based on previous findings [35], suggesting that group sizes of $n = 7–8$ would be sufficient (for C: $n = 8$, for C+GW9662: $n = 8$, for FFC: $n = 7$, for FFC+GW9662: $n = 8$). To ensure equal caloric intake, the liquid diet intake of mice in each group was assessed daily and the mean caloric intake per group per day was calculated. The amounts of diet and calories in the different groups were then adjusted to the group with the lowest daily caloric intake, and the group with the lowest caloric intake was fed ad libitum. In week 7, a glucose tolerance test (GTT) was performed. In brief, mice were fasted for 6 h. A glucose solution (2 mg/kg body weight) was injected i.p and the GTT was performed as detailed previously [35]. At the end of the experiment, animals were anesthetized with 100 mg ketamine and 16 mg xylazine/kg body weight. Blood was collected from the portal vein just prior to cervical dislocation. Liver was fixed in neutral-buffered formalin or snap-frozen for further analyses. The experimental set-up is summarized in Supplemental Fig. S1. All measurements were carried out in a randomized order.

2.2. Cell culture experiment

J774A.1 cells (DMSZ, Braunschweig, Germany) were cultured in Dulbeccos's Modified Eagle Medium (Pan Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (PAN Biotech, Aidenbach, Germany) and 1% penicillin and streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. At 80% confluency, cells were challenged with 50 ng/ml lipopolysaccharide (LPS, serotype O55:B5; Sigma-Aldrich GmbH, Steinheim, Germany) +/− 10 μM GW9662 or vehicle for 18 h. Cell culture supernatant was harvested and cells were lysed in PeqGOLD Trifast (VWR International GmbH, Vienna, Austria) for subsequent RNA isolation or lysed in a nitric oxide synthase (NOS) buffer supplemented with protease inhibitors for the measurement of NOS activity.

2.3. Evaluation of liver histology and blood parameter of liver damage

Liver sections (4 μm) were stained with hematoxylin & eosin (Sigma-Aldrich GmbH, Steinheim, Germany) and liver histology was assessed using NAFLD activity score (NAS) as detailed by Kleiner et al. [36]. Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma were measured in a routine laboratory (Veterinary Medical University of Vienna, Vienna, Austria).

2.4. Endotoxin assay and measurement of toll-like receptor 4 (TLR4) ligands

Portal endotoxin levels were measured with a commercially

available limulus amebocyte lysate assay (Charles River, Ecully, France) as previously described [37]. In addition, total TLR4 ligands in portal plasma were determined photometrically at 655 nm using a commercially available SEAP HEK Blue TLR4 cells assay (Invivogen, Toulouse, France) as described in detail before [38].

2.5. Griess assay and NOS activity

Nitrite levels (NO_2^-) in cell culture supernatant were determined using Griess assay (Promega, Madison, WI, USA). To determine NOS activity in cell lysates, a fluorometric NOS activity assay kit (abcam, Cambridge, UK) was used.

2.6. RNA isolation, cDNA synthesis and real-time PCR

RNA from liver and adipose tissue as well as cells was isolated with PeqGOLD Trifast as previously described [37] and reverse transcribed with a cDNA synthesis kit (Reverse Transcription System; Promega, Madison, WI, USA). Real-time PCR was performed using iTaq™ Universal SYBR® Supermix (Bio-Rad Ges.m.b.H, Vienna, Austria) to determine mRNA expression of the respective genes. The number of targets was determined with the comparative cycle threshold (CT) method. Primer sequences are shown in Supplemental Table S1.

2.7. Western blot analysis

Portal plasma protein (20 μg) was separated in a gel electrophoresis and transferred onto a PVDF membrane. Membranes were incubated with anti-adiponectin (Cell Signaling Technology) at 4 °C overnight, and subsequently incubated with a secondary antibody (anti-rabbit, Cell Signaling Technology). Band intensity was analysed with Super Signal West Dura Kit (Thermo Fisher Scientific) and analysed using ChemiDoc XRS system.

2.8. Statistics

Data are shown as means ± standard error of the means (SEM). Outliers were identified using Grubb's test. Statistical analyses were performed with GraphPad Prism 7.0 software (GraphPad Prism Software). Data were log-transformed when they were not normal distributed or in case of inhomogeneity of variances. Unpaired Students *t*-test was applied to analyse differences between two groups. Two-way ANOVA was performed to determine differences between different treatment groups followed by Tukey's post hoc test. A p value ≤ 0.05 was defined to be statistical different.

3. Results

3.1. Effect of the PPARγ antagonist GW9662 on liver damage and inflammation as well as on markers of glucose metabolism

While absolute body weight, body weight gain and caloric intake were similar between FFC-fed mice and FFC-fed mice concomitantly treated with GW9662, in the latter, the development of steatosis and inflammation was markedly attenuated with NAS being significantly lower than in FFC-fed mice (Table 1, Fig. 1A and B). However, NAS was still significantly higher than in both control groups. Also, percentage of hepatocytes showing fat accumulation was similar between the two FFC groups. In FFC-fed mice treated with vehicle, macrovesicular fat accumulation was highly prevalent, while no macrovesicular fat accumulation was found in FFC-fed mice treated with GW9662 (Fig. 1A). Also, inflammation was significantly more pronounced in livers of FFC-fed mice than in those concomitantly treated with GW9662 (Table 1). Number of neutrophils in liver tissue and ALT activity in plasma of FFC-fed mice treated with GW9662 were almost at the level of controls, being significantly lower than in FFC-fed mice. In contrast, AST activity was significantly higher in both FFC-fed groups when compared to their respective controls (Fig. 1C–E). Furthermore, mRNA expression of *F4/80* was significantly higher in FFC-fed animals compared to C+GW9962- and FFC+GW9662-fed animals while intercellular adhesion molecule (*Icam*) mRNA expression in livers of FFC-fed mice was only significantly higher than in livers of C- and C+GW9662-fed mice and by trend in livers of FFC+GW9662-fed mice ($p = 0.07$; Table 1). In line with these findings, mRNA expression of interleukin 1 beta (*Il1b*) in liver tissue was also significantly higher in livers of FFC-fed animals than in those of FFC+GW9662 and C+GW9662-fed mice (Fig. 1F). Interleukin 6 (*Il6*)

Table 1
Effect of the PPARγ antagonist GW9662 on caloric intake, body and liver weight as well as liver damage and inflammation in FFC-fed C57BL/6J mice.

	Diet and treatment groups				*p* (two-way ANOVA)		
	C	FFC	C+GW9662	FFC+GW9662	DEx GWE	GWE	DE
Caloric intake (kcal/mouse/d)	8.5 ± 0.1	8.9 ± 0.1	8.9 ± 0.1	9.0 ± 0.1	0.1270	0.0405	0.1342
Absolute body weight (g)	22.4 ± 0.6	22.3 ± 0.3	21.1 ± 0.2	22.1 ± 0.2	0.1499	0.0464	0.1736
Body weight gain (g)	2.9 ± 0.3	2.7 ± 0.5	1.9 ± 0.4	2.3 ± 0.4	0.4146	0.0871	0.7188
Liver weight (g)	1.0 ± 0.1	1.6 ± 0.0[a,c]	1.0 ± 0.1	1.5 ± 0.0[a,c]	0.15	0.88	<0.0001
Liver to body weight ratio (%)	4.4 ± 0.2	7.0 ± 0.1[a,c]	4.7 ± 0.1	6.5 ± 0.1[a,c]	0.0021	0.4676	<0.0001
Steatosis (NAS)	0.9 ± 0.1	2.4 ± 0.2[a,c]	0.9 ± 0.1	2.1 ± 0.2[a,c]	0.2466	0.3525	<0.0001
Inflammation (NAS)	0.2 ± 0.1	1.0 ± 0.0[a,c,d]	0.3 ± 0.1	0.5 ± 0.1	0.0531	0.0413	0.0020
Fasting blood glucose (mg/dl)	122.3 ± 2.7	127.9 ± 2.8	119.9 ± 3.1	127.1 ± 4.5	0.8105	0.6524	0.0717
Icam mRNA expression[#] (% of control)	100.0 ± 10.0	139.6 ± 18.4[a,c]	99.0 ± 9.8	105.7 ± 10.4	0.0796	0.0672	0.0241
F4/80 mRNA expression[#] (% of control)	100.0 ± 10	142.4 ± 17.3[c,d]	79.0 ± 9.1	88.9 ± 9.9	0.1795	0.0039	0.0348
Srebp1 mRNA expression[#] (% of control)	100.0 ± 22.2	161.1 ± 45.5[c]	53.4 ± 10.4	153.2 ± 33.0[c]	0.2812	0.1943	0.0061
Fas mRNA expression[#] (% of control)	100.0 ± 17.5	212.7 ± 33.0[a,c]	95.0 ± 22.7	150.5 ± 21.7	0.2411	0.1699	0.0015
Adiponectin[$] (pixel intensity × 10^6)	2.81 ± 0.5	1.22 ± 0.2[a]	2.08 ± 0.5	1.07 ± 0.1[a]	0.4468	0.2918	0.0004
Adipoq mRNA expression* (% of control)	100.0 ± 6.7	95.4 ± 6.5	97.3 ± 17.7	106.9 ± 16.6	0.6264	0.7943	0.8945

Data are shown as means ± SEM, n = 7–8, [a]$p \leq 0.05$ compared with mice fed a C diet, [c]$p \leq 0.05$ compared with mice fed a C diet and treated with GW9662, [d]$p \leq 0.05$ compared with mice fed a FFC diet and treated with GW9662; # measured in liver, $ measured in plasma, * measured in adipose tissue. *Adipoq*, adiponectin; C, control diet; DE, diet effect; DExGWE, interaction of diet effect and GW9662 effect; Fas, fatty acid synthase; FFC, fat-, fructose- and cholesterol-rich diet; GWE, GW9662 effect; Icam, intercellular adhesion molecule; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; PPARγ, peroxisome proliferator-activated receptor gamma; Srebp1, sterol response element-binding protein 1.

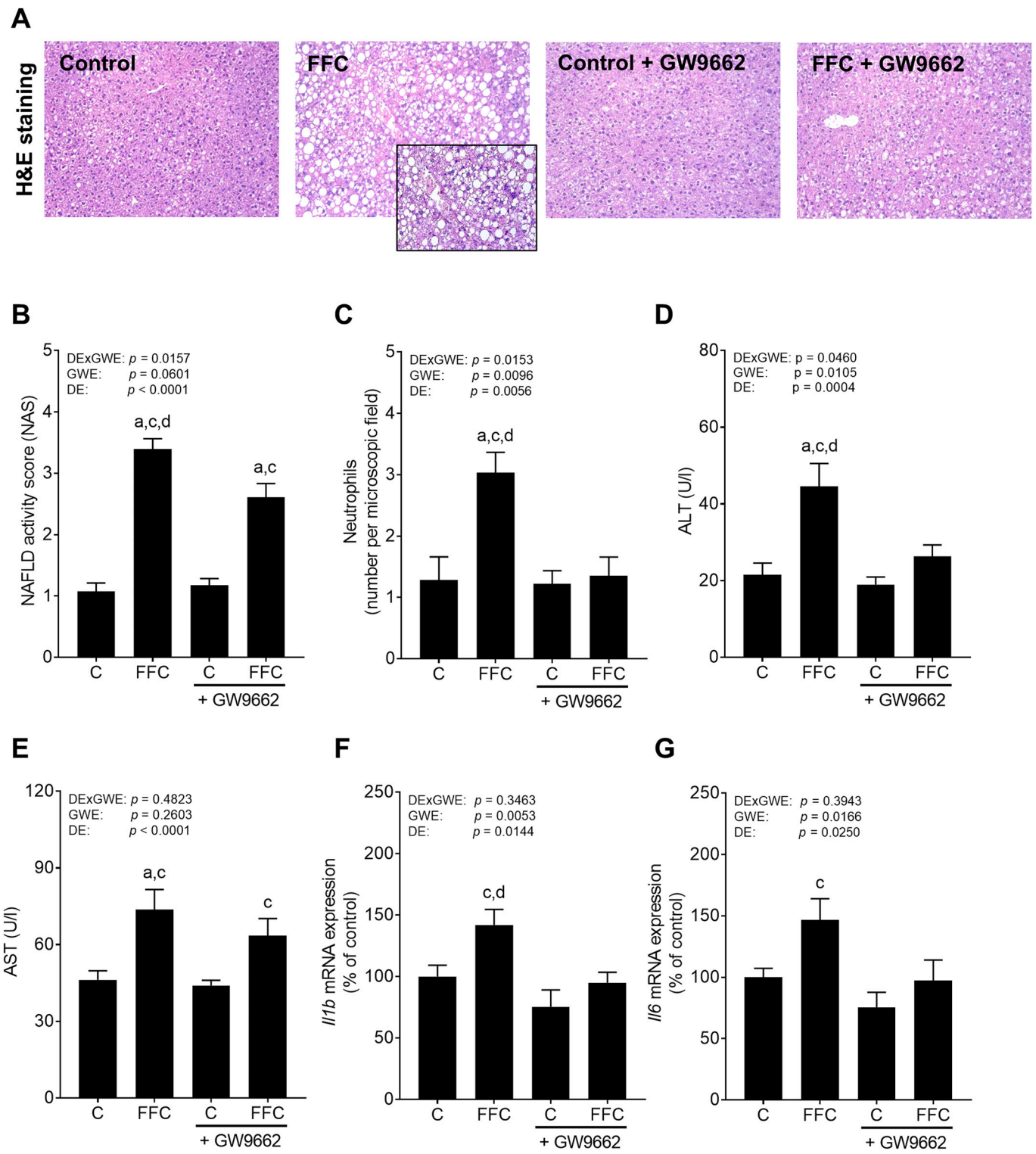

Fig. 1. Effect of the PPARγ antagonist GW9662 on indices of liver damage and inflammation in FFC-fed C57BL/6J mice. (**A**) Representative pictures of hematoxylin & eosin (H&E) staining in liver tissue (magnification 200× and 400×), (**B**) NAFLD activity score (NAS), (**C**) number of neutrophils, activities of (**D**) ALT and (**E**) AST in plasma. Hepatic mRNA expression of (**F**) *Il1b* and (**G**) *Il6*. Data are presented as means ± SEM, $n = 7–8$, except for transaminase activities and mRNA expression: $n = 6–8$. $^{a}p \leq 0.05$ compared with mice fed a C diet, $^{c}p \leq 0.05$ compared with mice fed a C diet and treated with GW9662, $^{d}p \leq 0.05$ compared with mice fed a FFC diet and treated with GW9662. ALT, alanine aminotransferase; AST, aspartate aminotransferase; C, control diet; DE, diet effect; DExGWE, interaction of diet effect and GW9662 effect; FFC, fat-, fructose- and cholesterol-rich diet; Il, interleukin; GWE, GW9662 effect; NAFLD, non-alcoholic fatty liver disease; PPARγ, peroxisome proliferator-activated receptor gamma.

mRNA expression was significantly higher in livers of FFC-fed mice than in C+GW9662-treated animals (Fig. 1G). Furthermore, the expression of sterol response element-binding protein 1 (*Srebp1*) in liver tissue was higher in both FFC-fed groups compared to C+GW9962-fed animals. In addition, mRNA expression of fatty acid synthase (*Fas*) was significantly higher in livers of FFC-fed mice compared to C- and C+GW9662-treated mice (Table 1). In contrast, *Fas* mRNA expression in liver of FFC-fed mice treated with GW9662 did not differ from that of control groups. Adiponectin levels were significantly lower in plasma of FFC- and FFC+GW9662-fed mice compared to C-fed animals whereas mRNA expression of adiponectin (*Adipoq*) in adipose tissue did not differ between groups as expression varied considerably within groups (Table 1).

Fasting glucose levels were similar between groups while area under the curve of GTT was significantly higher in FFC-fed mice when compared to all other groups. Similar differences were not found between FFC-fed mice treated with the PPARγ antagonist and controls (Table 1, Fig. 2A and B). Furthermore, the mRNA expression of the catalytic unit of glucose-6-phosphatase (*G6pc*) was significantly lower in livers of both FFC-fed groups and in those of C+GW9662-fed mice compared to C-fed mice (Fig. 2C). The mRNA expression of phosphoenolpyruvate carboxykinase 1 (*Pepck1*) was lower in FFC-fed and FFC+GW9962-fed mice when compared to C-fed animals (Fig. 2D).

3.2. *Effect of the PPARγ antagonist GW9662 on Pparg mRNA expression in liver and adipose tissue*

The mRNA expression of *Pparg1* in liver did not differ between groups (Fig. 3A). In FFC-fed mice, the development of macrovesicular liver steatosis with beginning inflammation was associated with a significant higher *Pparg2* mRNA expression in liver tissue than in controls (Fig. 3B). In mice concomitantly treated with the PPARγ antagonist GW9662, this induction of *Pparg2* mRNA expression was almost completely abolished with expression levels of the nuclear receptor being almost at the level of their respective control (Fig. 3B). In contrast, in adipose tissue neither mRNA expression of *Pparg1* nor *Pparg2* differed between groups regardless additional treatments (Fig. 3C and D).

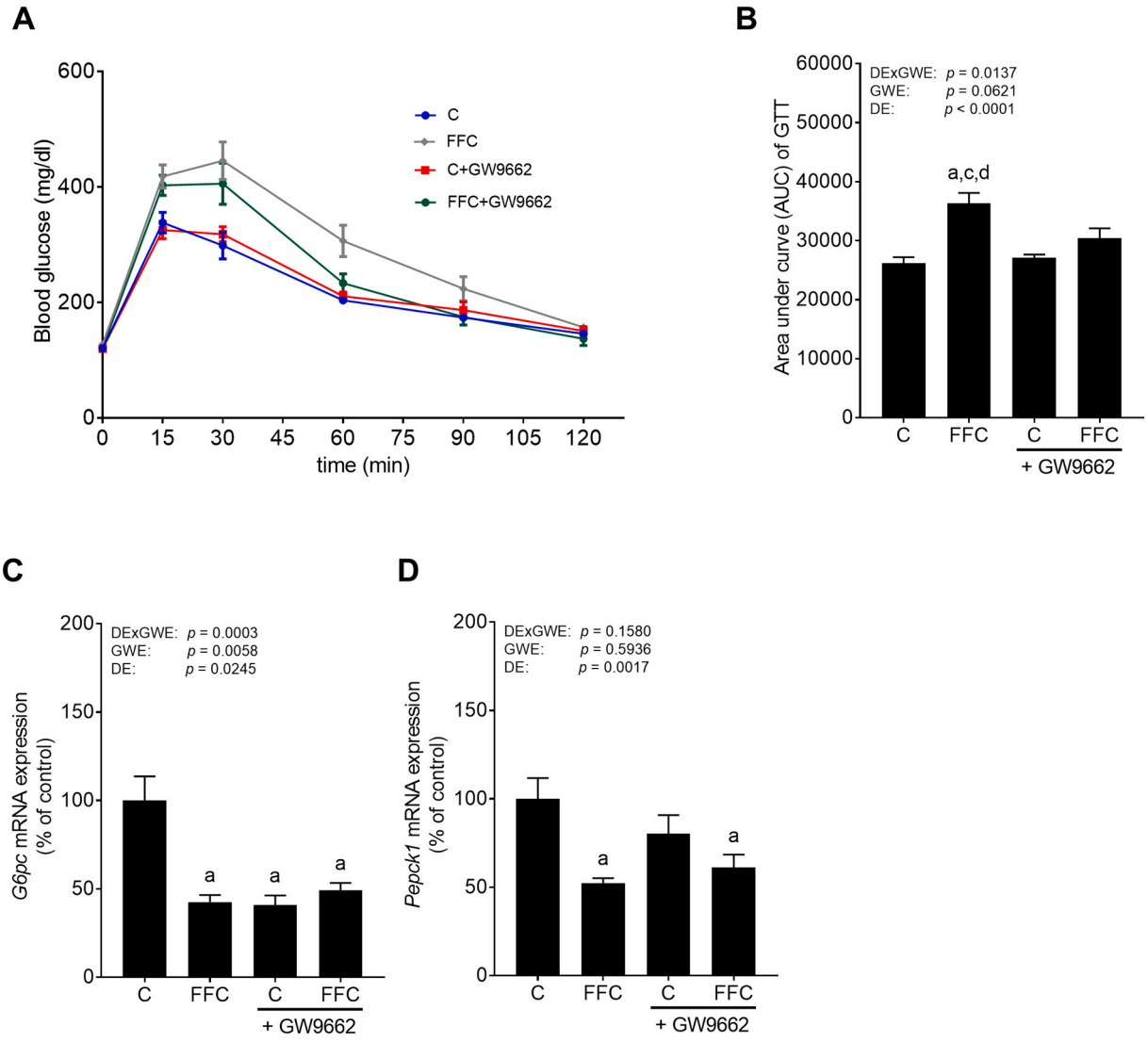

Fig. 2. Effect of the PPARγ antagonist GW9662 on glucose tolerance in FFC-fed C57BL/6J mice. (**A**) Blood glucose concentrations as well as (**B**) area under the curve (AUC) of blood glucose concentrations during glucose tolerance test (GTT), hepatic mRNA expression of (**C**) *G6pc* and (**D**) *Pepck1*. Data are presented as means ± SEM, $n = 7–8$, except for blood glucose: $n = 6–7$. $^{a}p \leq 0.05$ compared with mice fed a C diet, $^{c}p \leq 0.05$ compared with mice fed a C diet and treated with GW9662, $^{d}p \leq 0.05$ compared with mice fed a FFC diet and treated with GW9662. C, control diet; DE, diet effect; DExGWE, interaction of diet effect and GW9662 effect; FFC, fat-, fructose- and cholesterol-rich diet; G6pc, catalytic unit of glucose-6-phosphatase; GWE, GW9662 effect; Pepck1, phosphoenolpyruvate carboxykinase 1; PPARγ, peroxisome proliferator-activated receptor gamma.

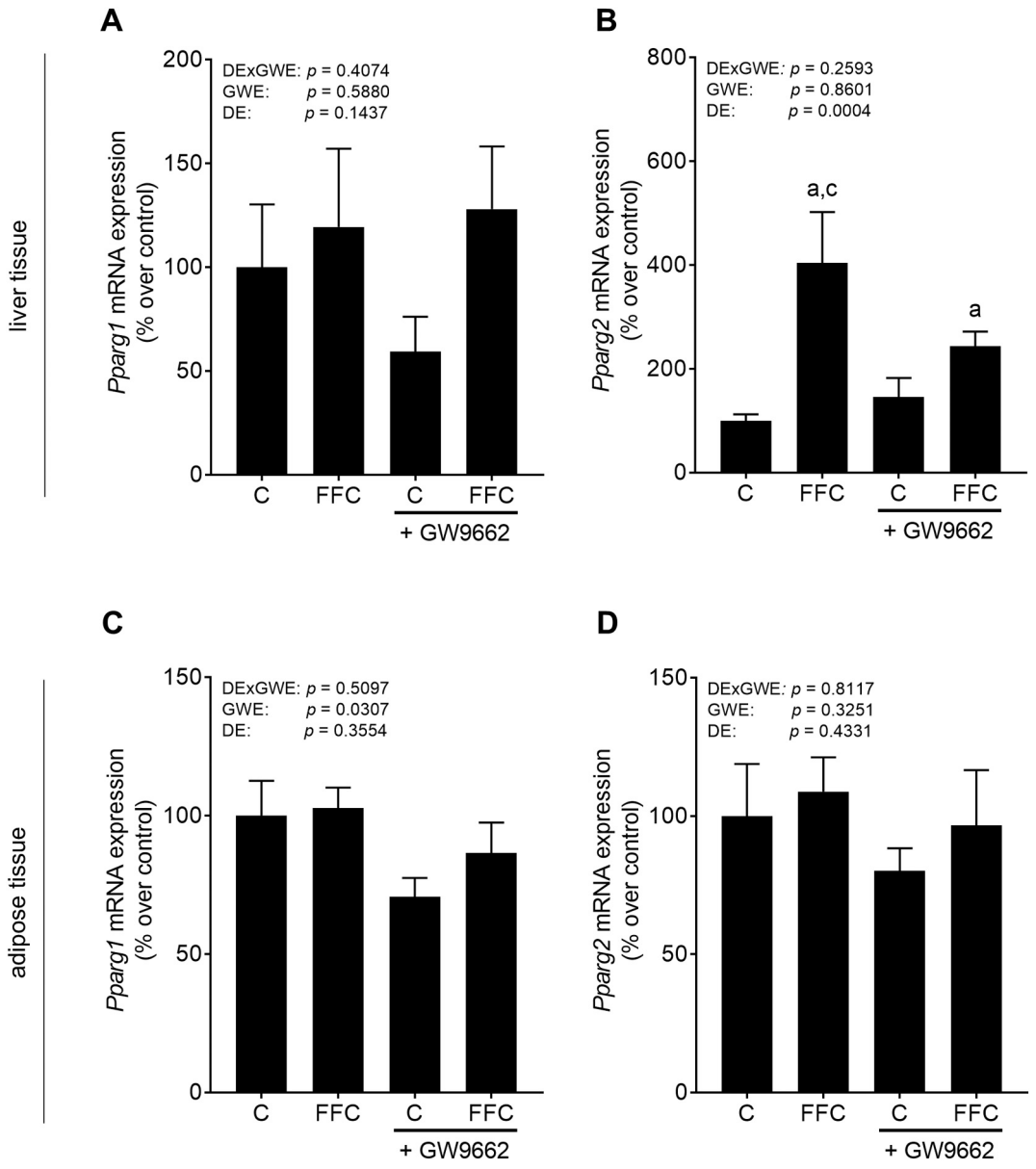

Fig. 3. Effect of the PPARγ antagonist GW9662 on *Pparg* mRNA expression in liver and adipose tissue in FFC-fed C57BL/6J mice. Expression of *Pparg1* and *Pparg2* mRNA in (**A**, **B**) liver tissue and (**C**, **D**) adipose tissue. Data are presented as means ± SEM, $n = 7–8$ for liver tissue, $n = 6–8$ for adipose tissue, $^{a}p \leq 0.05$ compared with mice fed a C diet, $^{c}p \leq 0.05$ compared with mice fed a C diet and treated with GW9662. C, control diet; DE, diet effect; DExGWE, interaction of diet effect and GW9662 effect; FFC, fat-, fructose-, and cholesterol-rich diet; GWE, GW9662 effect; Pparγ, peroxisome proliferator-activated receptor gamma.

3.3. Effect of the PPARγ antagonist GW9662 on markers of intestinal barrier function and TLR4 signaling

To determine if the protective effects of the PPARγ antagonist were associated with an effect on intestinal barrier function and the translocation of bacterial endotoxin shown to be critical in the development of NAFLD, and herein, especially inflammatory alterations in the liver [39], we determined bacterial endotoxin and total TLR4 ligand levels in portal plasma. Bacterial endotoxin levels and TLR4 ligand levels were significant higher in both FFC-fed groups than in controls (Fig. 4A and B). Interestingly, while hepatic *Tlr4* and myeloid differentiation primary response 88 (*Myd88*) mRNA expression were significantly higher in livers of FFC-fed animals when compared with all other groups, *Tlr4* and *Myd88* mRNA expression were both almost at the level of controls in FFC-fed mice concomitantly treated with GW9662 (Fig. 4C and D). In line with these findings, levels of NO_2^- in liver tissue were also significantly higher in FFC-fed mice than in all other groups. However, while being significantly lower than in FFC-fed mice, NO_2^- levels in liver tissue of FFC+GW9662-treated mice were still significantly higher than in both control groups (Fig. 4E).

3.4. Effect of the PPARγ antagonist GW9662 on inflammatory markers and nitric oxide (NO) synthesis in J774A.1 cells

To further determine if the PPARγ antagonist affects LPS-dependent activation of immune cells e.g., Kupffer cells and macrophages in the liver, J774A.1 cells were employed as a model of Kupffer cells and challenged with LPS in the presence or absence of GW9662. No differences were found between naïve cells and cells treated with GW9662 or vehicle. As expected, LPS treatment resulted in an induction of *Il1b*, *Il6* and inducible nitric oxide synthase (*iNos*) mRNA in cells. This induction was significantly attenuated in cells treated with GW9662 (Fig. 5A–C). In line with these findings, NO_2^- levels in medium and activity of NOS in cell lysates were both also significantly lower in LPS-challenged cells

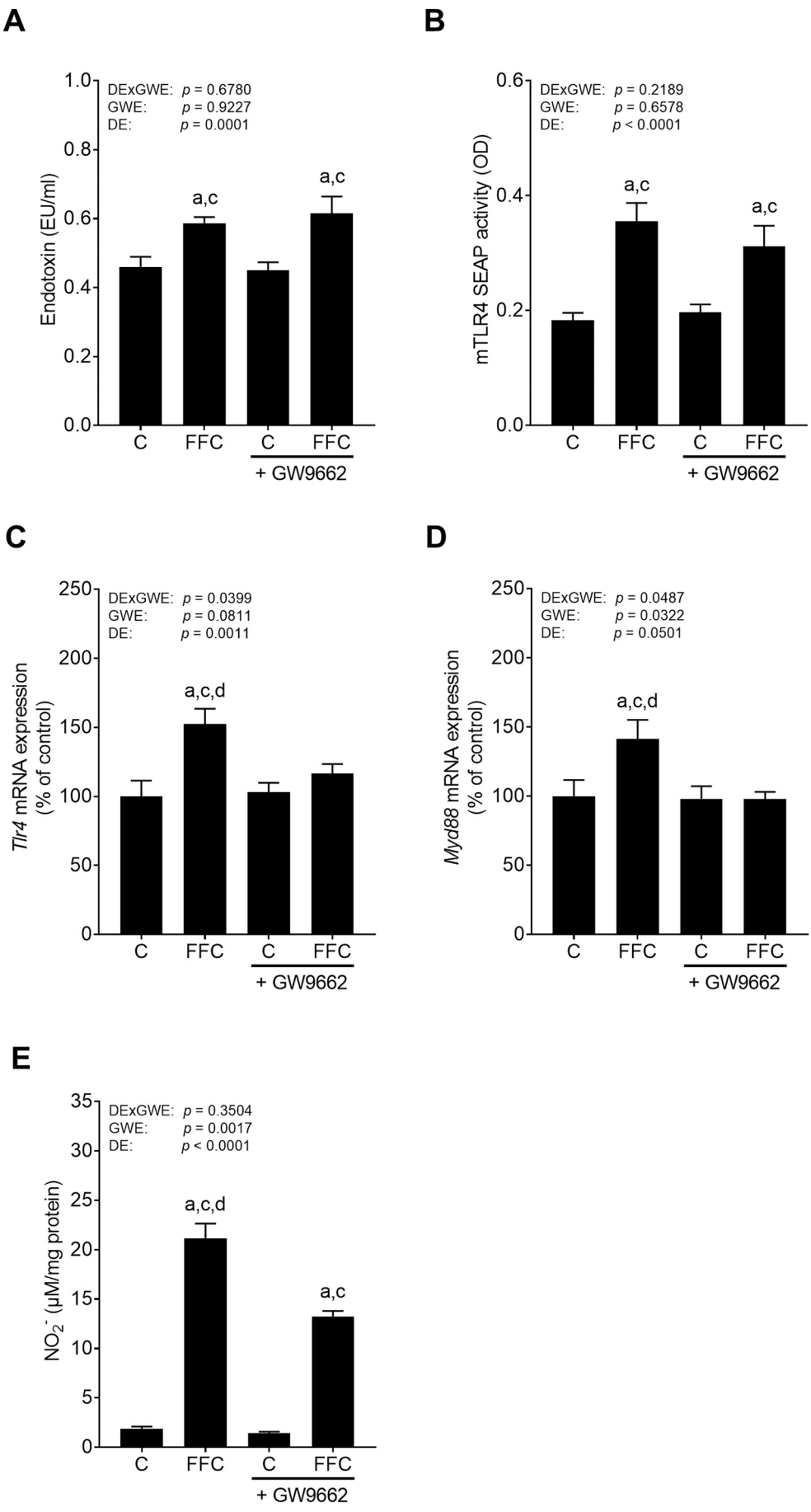

Fig. 4. Effect of the PPARγ antagonist GW9662 on markers of intestinal barrier function and parameters of the TLR4 signaling cascade and NO metabolism in livers of FFC-fed C57BL/6J mice. (**A**) Portal plasma endotoxin levels, (**B**) mTLR4 SEAP reporter activity, hepatic mRNA expression of (**C**) *Tlr4* and (**D**) *Myd88* as well as (**E**) NO_2^- concentration in liver tissue. Data are presented as means ± SEM, n = 7–8, except for mRNA expression: n = 6–8, for NO_2^-: n = 5–7 as some values were below the detection level. $^{a}p \leq 0.05$ compared with mice fed a C diet, $^{c}p \leq 0.05$ compared with mice fed a C diet and treated with GW9662, $^{d}p \leq 0.05$ compared with mice fed a FFC diet and treated with GW9662. C, control diet; DE, diet effect; DExGWE, interaction of diet effect and GW9662 effect; FFC, fat-, fructose- and cholesterol-rich diet; GWE, GW9662 effect; Myd88, myeloid differentiation primary response 88; NO, nitric oxide; NO_2^-, nitrite; PPARγ, peroxisome proliferator-activated receptor gamma; SEAP, secreted embryonic alkaline phosphatase; Tlr4, toll-like receptor 4.

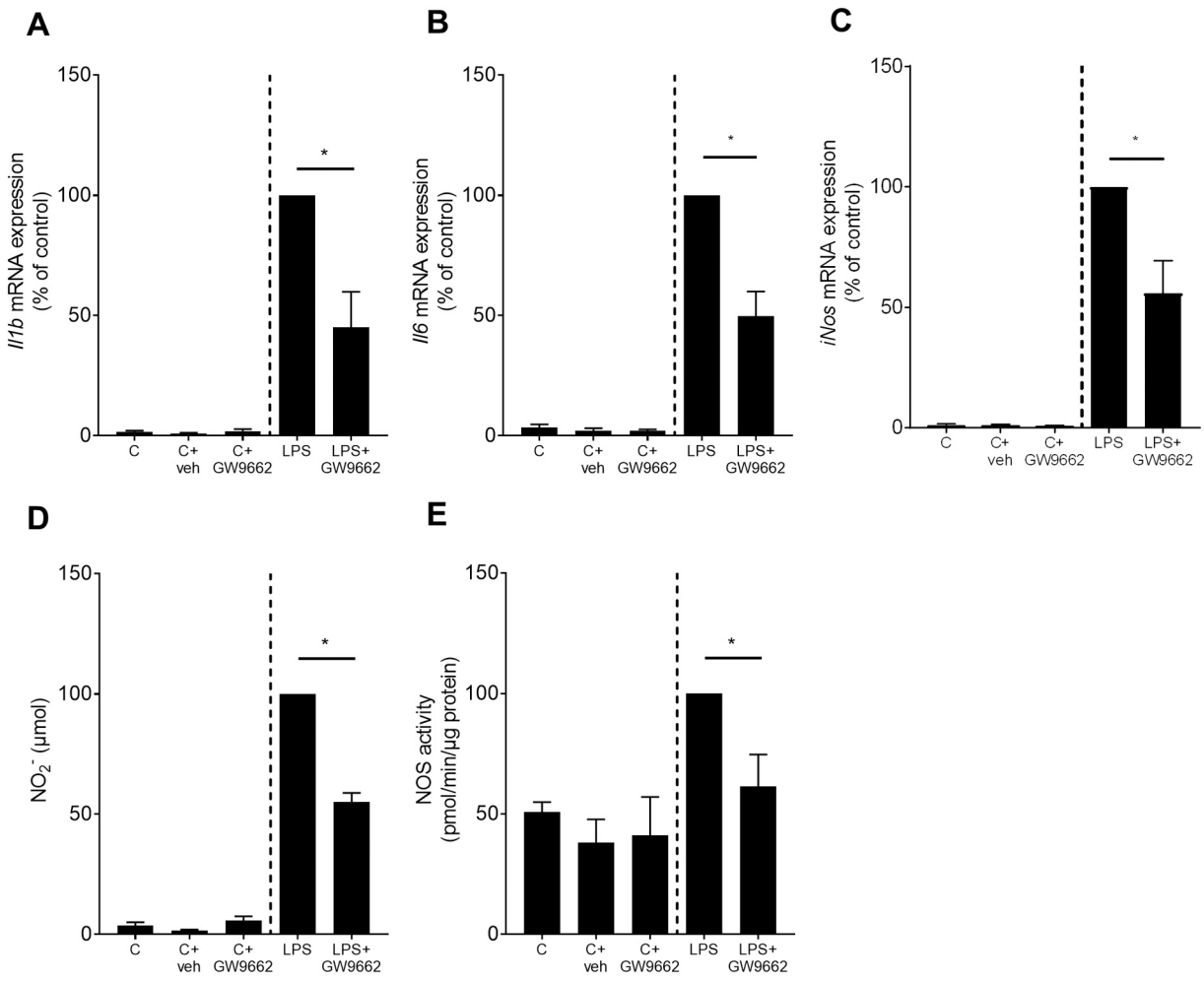

Fig. 5. Effect of the PPARγ antagonist GW9662 on proinflammatory markers and parameters of TLR4 signaling in LPS-challenged J774A.1 cells. mRNA expression of (**A**) *Il1b*, (**B**) *Il6* and (**C**) *iNos*, (**D**) NO_2^- levels in cell culture supernatant and (**E**) NOS activity in cell lysates in J774A.1 cells. Data are presented as means ± SEM, for NOS activity: $n = 3$, for mRNA expression: $n = 6$–7, for NO_2^-: $n = 6$. * $p \leq 0.05$. C, unstimulated cells; Il1b, interleukin 1 beta; Il6, interleukin 6; iNos, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO_2^-, nitrite; NOS, nitric oxide synthase; PPARγ, peroxisome proliferator-activated receptor gamma; veh, vehicle.

treated with the antagonist (Fig. 5D and E).

4. Discussion

Despite intense research efforts in the last decade, molecular mechanisms underlying the development of NAFLD are not fully understood and universally accepted therapies other than life-style interventions often afflicted with limited adherence and high relapse rates are not available. The role of PPARs, and herein, especially PPARγ and its isoform PPARγ2 in the development of NAFLD is still a matter of debate. In the present study, employing a pair-feeding model of diet-induced non-obese NAFLD, we assessed the effects of the PPARγ antagonist GW9662 on the development of NAFLD and insulin resistance. We previously showed that even in the absence of developing overweight or obesity, mice developed steatosis with early signs of inflammation within 8 weeks, and NASH and insulin resistance within 13 weeks of feeding a diet rich in fat, fructose and cholesterol [35,40]. In the present study, the development of steatosis with early signs of inflammation found in FFC-fed mice was associated with a significant increase in hepatic *Pparg2* mRNA expression while alterations alike were not found in adipose tissue. These findings are in line with those of others feeding mice a high fat diet [41]. Furthermore, the development of both NAFLD and insulin resistance was significantly attenuated in FFC-fed mice concomitantly treated with the PPARγ antagonist GW9662 when compared to FFC-fed mice. Indeed, the protective effects against the development of NAFLD were associated with lower numbers of neutrophils and *F4/80* mRNA expression being indicative of macrophages [42] as well as lower expression of pro-inflammatory cytokines e.g., *Il1b* while percentage of hepatocytes affected by fat accumulation was almost similar between groups. Our results are somewhat contrasting the findings of others reporting that PPARγ agonists like pioglitazone attenuated the development of NAFLD but also other liver diseases (for overview see [18,43]) and that a treatment with GW9662 abolished these protective effects (for overview [43]). As also recently reviewed by Gastaldelli et al., PPARγ agonists like pioglitazone may improve hepatic steatosis and inflammation, and herein, especially in NASH patients with type 2 diabetes. Furthermore, pioglitazone is also the only drug recommended for the treatment of diabetic NAFLD [18] and in line with the present study, the PPARγ antagonist GW9662 inhibited high-fat diet-induced obesity in rodent studies suggesting that this PPARγ antagonist may also possess therapeutic potential for metabolic diseases [44]. Furthermore, Zhang et al. reported that the oral treatment with GW9662 was associated with higher concentrations of triglycerides and free fatty acids in plasma and liver tissue compared to control groups and groups treated with agonists of PPARγ. In the same study, the treatment with an agonist of PPARγ resulted in an improvement of hepatic steatosis and inflammatory cell infiltration [45]. Differences between the results of the present study and those of Zhang et al. may have been related to

differences in models used (rats fed high-fat diet and high-sucrose drinking solution vs. liquid FFC diet pair-feeding of mice in the present study). In another study, the treatment with 4 mg GW9662/kg body weight daily for 8 weeks after feeding a high-fat diet for 16 weeks abolished the beneficial effects of the PPARγ agent ginsenoside on hepatic cell apoptosis [46]. Again, differences might have resulted from the dietary model employed as well as the duration of the feeding trials. Further studies are warrant to determine in which settings PPARγ agonist and PPARγ antagonist are beneficial or harmful. It has been discussed that the protective effects of PPARγ ligands might stem from an enhanced adiponectin synthesis in adipose tissue and that an activation of PPARγ with its specific ligands may reduce inflammatory responses by negatively interfering with nuclear factor kappa B (NFκB)-dependent transcription, thereby, also suppressing the production of TNFα and IL1β in monocytes and macrophages (for overview see [43] and [47,48]). In the present study, adiponectin levels in plasma were lower in FFC- and FFC+GW9662-fed animals compared to C-fed animals whereas the *Adipoq* expression in adipose tissue did not differ due to considerable variability in expression within groups. These data suggest that the protective effects found in the present study were not resulting from changes of adiponectin expression further suggesting that GW9662 might only have limited direct effects on adipose tissue. Contrasting these reports but being in line with the findings of the present study, Gao et al. also reported an overexpression of PPARγs, and herein, especially PPARγ2 in livers of mice fed a high fat diet [23]. This overexpression of the nuclear receptor went along with an increased fat accumulation in liver tissue and was even exacerbated when high fat diet-fed mice were treated with the PPARγ agonist rosiglitazone. PPARγ – when being ectopically overexpressed in hepatocytes – has also been shown to promote steatosis (for overview see [43]). Furthermore, adenovirus-mediated overexpression of PPARγ2 in hepatocytes has been shown to increase hepatosteatosis while a disruption of PPARγ signaling has been shown to decrease liver steatosis in ob/ob mice [49,50]. In the present study, we found no marked effects on the percentage of hepatocytes affected by fat accumulation in FFC-fed mice treated with GW9662; however, macrovesicular fat accumulation was almost not present in livers of FFC-fed mice concomitantly treated with the antagonist while being highly prevalent in FFC-fed mice treated with vehicle suggesting that fat synthesis might have been lower in livers of FFC-fed mice treated with GW9662 than in those treated with the vehicle. Supporting these findings, mRNA expression of *Srepb1* and *Fas* was higher in FFC-fed groups. Also, it might be that in our study, NAFLD was more progressed, as we used a diet combining fat, fructose and cholesterol while others only used a high fat diet or fed ob/ob mice. Still, together with the data of others, our data suggest that *Pparg2* is overexpressed in livers of mice with NAFLD and that the concomitant treatment with GW9662 protects mice from the development of NAFLD being associated with a suppression of the induction of *Pparg2*. These data by no means preclude that a treatment with PPARγ agonist might also prevent NAFLD as shown in several studies [45,46] but rather suggest that in settings of lean NAFLD, an intrinsic PPARγ overexpression in liver might have contrasting effects from those of a pharmacological activation of the receptor in the treatment of NAFLD. Interestingly, no changes in *Pparg1* and *2* mRNA expression were found in adipose tissue suggesting that the effect found in the present study might primerly related to alterations of *Pparg2* mRNA in liver tissue. Further studies are needed to delineate these differences but also to determine long-term effects of an oral treatment with GW9662 on the progression of NAFLD.

In the study of Nakano et al. GW9662 was shown to attenuate the development of high fat diet induced obesity while not affecting glucose tolerance but lowering fasting glucose levels [44]. Interestingly, in that study weight loss of mice was not associated with a lower food intake. These data are somewhat contrasting the findings of the present study, where we found no effects on body weight gain but a marked protection against the impaired glucose tolerance found in FFC-fed mice treated with vehicle. Differences between our study and that of Nakano et al. might be related to the differences in the feeding models used e.g., high fat diet ad libitum vs. pair-feeding a liquid diet rich in fat, fructose and cholesterol and doses of GW9662 used in the studies (0.1% (w/w) per 100 g diet as admixture vs. in the present study: 1 mg/kg body weight i.p.). Still, together with the data of Nakano et al., our study suggests that GW9662 may not only attenuate the development of NAFLD but also may improve glucose metabolism.

The development of NAFLD repeatedly has been shown to be associated with alterations of intestinal barrier function and increased bacterial endotoxin levels resulting in an activation of TLR4-dependent signaling cascade in the liver (for overview see [51,52]). In the present study, while finding a significant protection against inflammatory alterations that have been shown to be also associated with TLR4 signaling [53], no protection against the alterations of markers of intestinal barrier function were found between the two FFC groups. Also, concentrations of TLR4 ligands and bacterial endotoxin were similar between FFC groups irrespective of additional treatments while *Tlr4* and *Myd88* mRNA expression and NO_2^- concentrations in liver tissue, shown by us and others to be correspondingly increased in the presence of elevated bacterial endotoxin levels [54], were only found to be higher in livers of FFC-fed mice treated with vehicle. Both *Tlr4* and *Myd88* mRNA expressions have been shown to depend upon the activation of NFκB [55]. And while it has been suggested by the results of others that PPARγ agonists, also exhibited anti-inflammatory properties (for overview see [56]), attenuated the production of pro-inflammatory cytokines in human monocytes [57], recently, it has been shown that the PPARγ antagonists T0070907 can promote a shift in polarization from M1 to M2 in LPS-stimulated immunocells including an increase in M2 markers and a reduced expression of M1 markers such as IL1β, IL6, TNFα and iNOS [58]. These findings are in line with our findings employing LPS-stimulated J774A.1 cells concomitantly treated with GW9662. Indeed, in cells treated with the antagonist and challenged with LPS, expressions of *Il1b*, *Il6* and *iNos* mRNA as well as NO_2^- production were significantly lower than in cells only treated with LPS. Taken together, our data suggest that GW9662 may attenuate the development of NAFLD through mechanism involving a suppression of the activation of endotoxin-dependent TLR4/NFκB-dependent signaling. However, further studies are needed to determine the exact molecular mechanism underlying the suppression.

Our study is not without limitations that have to be considered when interpreting the data. Specifically, as already detailed above, GW9662 can also bind to the ligand-binding domains of PAPRα and PPARδ. However, binding is markedly less potent compared to binding of PPARγ [34]. The effect of GW9662 on NAFLD when administered through different routes might be different (i.p. in the present study vs. oral or i.v. dosing). Moreover, it has to be kept in mind that an ipGTT was performed to evaluate glucose tolerance while an oral GTT can be considered as the `physiological´ route of glucose. In the present study, an ipGTT was used as this kind of GTT is afflicted with less adverse effects than an oral administration of glucose by gavage [59]. Furthermore, in the present study, a model of early stage 'lean' NAFLD was employed. It remains to be determined if in settings of `obese´NAFLD or at late stages, effects alike are found.

5. Conclusion

Taken together, results of the present study suggest that *Pparg2* is overexpressed in livers of mice with diet-induced NAFLD and that the PPARγ antagonist GW9662 can protect mice from both, the development of fatty liver with early inflammatory alterations and insulin resistance. Our data further suggest that the protective effects of the antagonist are associated with a protection against the activation of the endotoxin-dependent activation of the TLR4 signaling cascade. Further studies are needed to determine if GW9662 also affects the development of later stages of the disease and if mechanisms alike are also found in humans.

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CRediT authorship contribution statement

Conceptualization, IB; data curation or formal analysis, ABa, KB, ABr, RS, FJ, DR, MJLP, IB; funding acquisition, IB; investigation, ABa, IB; supervision, IB; visualization, ABa, IB; writing original draft preparation, ABa and IB; writing – review and editing, ABa and IB. All authors have read and agreed to the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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