Original Article
4-Hydroxyisoleucine improves hepatic insulin resistance by restoring glycogen synthesis *in vitro*

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Abstract: Introduction/Aims: Hydroxyisoleucine (4-HIL), derived from fenugreek seeds, improves insulin resistance in 3T3-L1 adipocytes and L6 myotubes. However, the effects of 4-HIL on liver glycogen synthesis and hepatic insulin resistance have not been described. The aim of this study was to investigate the effects of 4-HIL on glycogen synthesis in a tumor necrosis factor-α (TNF-α)-induced insulin resistance model using HepG2 cells. Materials and methods: HepG2 cells were divided into eight groups: control; TNF-α; and 5, 10, or 20 μM 4-HIL without or with TNF-α. Glycogen and protein expression were evaluated using a glycogen assay kit and western blotting, respectively. Results: Glycogen levels did not differ between the 4-HIL groups and control ($P>0.05$), but were decreased significantly in the TNF-α group ($P<0.05$), indicating the establishment of insulin-resistant HepG2 cells. Adding 20 μM 4-HIL to TNF-α-treated cells increased glycogen levels ($P<0.05$). Relative to the control group, the P-IRS-1/IRS-1 and P-JNK/JNK ratios were increased ($P<0.001$) in the TNF-α group, whereas the P-AKT/AKT and P-GSK/GSK ratios were decreased ($P<0.001$). When 20 μM 4-HIL was added to TNF-α-treated cells, the P-IRS-1/IRS-1 and P-JNK/JNK ratios decreased ($P<0.001$ and $P<0.05$, respectively), whereas the P-AKT/AKT and P-GSK/GSK ratios increased ($P<0.05$ and $P<0.001$). Conclusions: 4-HIL directly or indirectly reversed TNF-α reduced glycogen levels by inhibiting JNK and IRS-1 (Ser^{307}) phosphorylation and increasing AKT (Ser^{473}) and GSK-3 phosphorylation. These findings demonstrate that 4-HIL modulates hepatic insulin resistance at the molecular level, and suggest that it is a novel potential therapeutic agent for the treatment of insulin resistance in patients with diabetes.

Keywords: Fenugreek, JNK signaling pathway, insulin resistance, 3T3-L1 adipocytes, L6 myotubes

Introduction

Insulin resistance (IR) is the main pathophysiological characteristic of type 2 diabetes. IR can develop in hepatocytes, muscle cells, kidney cells, adipocytes, endothelial cells, and pancreatic β-cells [1]. Because the liver is a site of insulin action, hepatic IR can have significant effects on the body [2]. Increased hepatic glucose production and decreased glycogen synthesis, which are manifestations of hepatic IR, lead to increased blood glucose levels [3]. Many factors contribute to the pathogenesis of IR, including IRS family members [4-6], glucose transporter protein (GLUT) [7, 8], the phosphorylation of intracellular glucose, oxidative stress [9], free fatty acids (FFAs) [10-12], and adipokines [13, 14].

Studies have indicated that cytokines can also induce IR. Tumor necrosis factor-α (TNF-α) was the first cytokine implicated in the induction of IR [15]. TNF-α inhibits glycogen synthase in the IR state via the JNK signaling pathway: phosphorylation of JNK (P-JNK) and IRS-1 (P-IRS-1) increases, whereas the phosphorylation of AKT (P-AKT) and GSK (P-GSK) decreases, thus reducing glycogen synthesis [16].

Fenugreek (*Trigonellafoenum-graecum*) is an annual herb that grows primarily in the Mediterranean and countries such as Ukraine, India, and China. Owing to its immunomodulatory and anti-oxidizing effects, it is used as a dietary supplement to treat wounds and several conditions including diabetes, high cholesterol levels, inflammation, and gastrointestinal com-
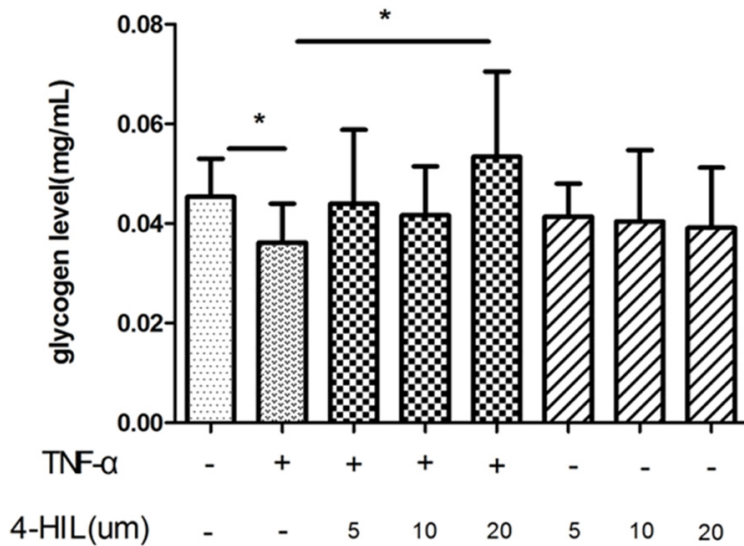
Figure 1. 4-HIL increases the TNF-α-induced reduction in glycogen levels. 4-HIL promoted glycogen synthesis in HepG2 cells. HepG2 cells (5×10^{6}) were treated with TNF-α or 4-HIL at the indicated concentrations for 24 h. Data are presented as means ± SDs; *$P<0.05$ vs. TNF-α group.

plaints [17]. 4-Hydroxyisoleucine (4-HIL) is derived from fenugreek seeds, which are composed of 80% free amino acids. 4-HIL is comprised of (2S, 3R, 4S) isomers (~90%) and (2R, 3R, 4S) isomers [18]. Some studies have indicated that (2S, 3R, 4S)-4-HIL enhances islet β-cell insulin secretion and improves IR, although the mechanism behind these effects is unclear [19-23]. Recently, it was reported that 4-HIL ameliorates FFA-induced IR in skeletal muscle cells by inhibiting the production of palmitate-induced reactive oxygen species (ROS) and ROS-associated inflammation [24]. We demonstrated previously that 4-HIL decreases soluble TNF-α expression and IRS-1 (Ser[307]) phosphorylation and increases GLUT4 expression, leading to enhanced insulin sensitivity in 3T3-L1 adipocytes [25]. However, the effects of 4-HIL on liver glycogen synthesis, its ability to modulate TNF-α-induced hepatic IR, and the underlying mechanisms behind these effects have not been described.

In this study, an insulin-resistant cell model was established by treating HepG2 cells with TNF-α. The cells were then exposed to different concentrations of 4-HIL to determine the effects of 4-HIL on hepatic glycogen synthesis and IR, and investigate the molecular mechanisms underlying these effects.

Materials and methods

Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), a high-glucose medium purchased from HyClone (Beijing, China), was stored at 4°C. Fetal bovine serum (FBS) was obtained from Gibco (Beijing, China) and stored at -20°C. TNF-α (human) was purchased from ProSpec-Tany (East Brunswick, NJ, USA). Antibiotics (penicillin and streptomycin solution) were purchased from HyClone. 4-HIL was purchased from Sigma (St. Louis, MO, USA). Novolin R was purchased from Novo Nordisk (Bagsvaerd, Denmark). JNK, IRS-1, Akt, GSK, P-JNK, P-Akt (Ser[473]), P-IRS-1 (Ser[307]), and P-GSK rabbit anti-mouse antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). β-actin antibody was purchased from Wuhan Antgene Biotechnology Co., Ltd. (China). Goat anti-rabbit IgG (H + L) were purchased from Beijing 4A Biotech Co., Ltd. (China). All antibodies were used at the dilutions recommended by the manufacturer.

Cell culture

HepG2 cells are used commonly as a hepatocyte model for studies of insulin resistance [16, 26, 27]. Importantly, this cell line was characterized in detail previously, and the authors concluded that HepG2 cells exhibited most of the cellular features of primary hepatocytes, and that they retained the ability to synthesize all cellular proteins, except for C-reactive peptide [28]. HepG2 cells were grown at 37°C with 5% CO_2 in DMEM containing 10% FBS, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin.

Induction of IR and analysis of glycogen content

HepG2 cells were divided into eight groups: control, 5 μM 4-HIL, 10 μM 4-HIL, 20 μM 4-HIL, TNF-α, TNF-α + 5 μM 4-HIL, TNF-α + 10 μM 4-HIL, and TNF-α + 20 μM 4-HIL. HepG2 cells were exposed to 10 ng/mL TNF-α without or with different concentrations of 4-HIL for 21 h.
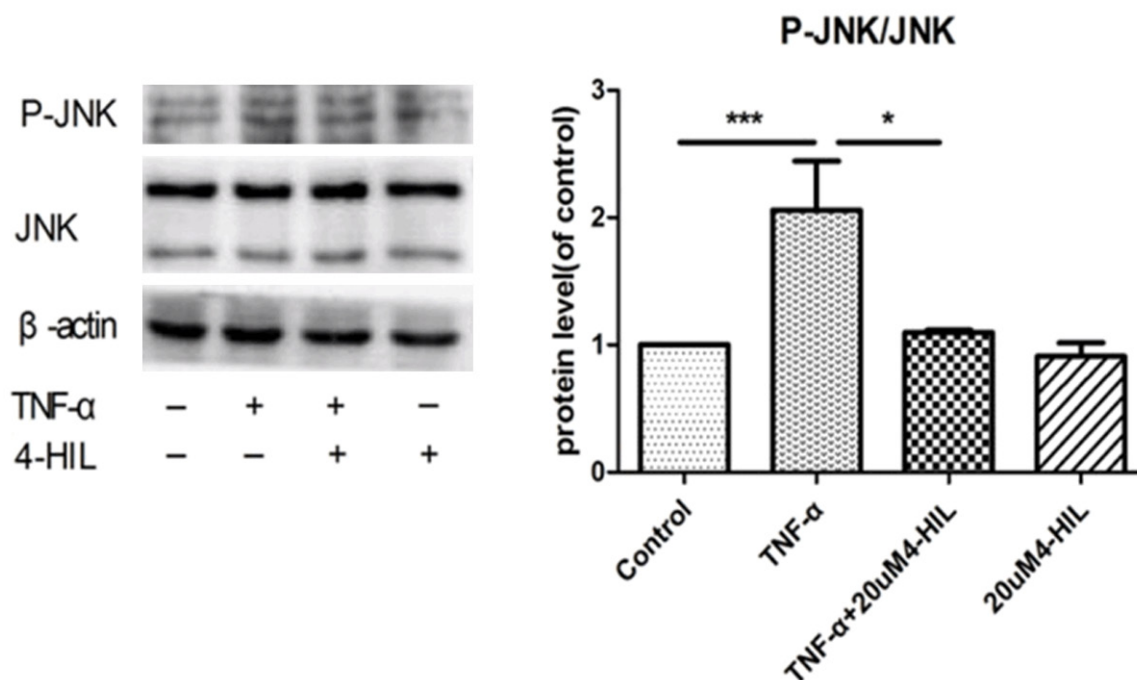
Figure 2. 4-HIL reduced phospho-JNK levels. Treatment with 4-HIL led to the reduced expression of phospho-JNK. HepG2 cells (2×10^6) were incubated with TNF-α alone or TNF-α plus 20 μM 4-HIL for 24 h. Whole-cell extracts were prepared to assess the phosphorylation of JNK. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading (***$P<0.001$ and *$P<0.05$ vs. TNF-α group).

Insulin (10 nM; Novo Nordisk) was added thereafter and incubated for another 3 h. The glycogen level in each group was assessed using a glycogen assay kit (Nanjing Jiancheng Bioengineering Institute, China), and the results were normalized to a standard curve and expressed as milligrams of glycogen per milliliter of protein, as per the manufacturer's instructions.

Western blotting

Cell lysates (20-30 μg of protein) were separated using 8% SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA), which were blocked using either 5% nonfat dried milk (for total proteins) or BSA (for phospho-specific antibodies), and incubated overnight at 4°C in the presence of specific antibodies. The blots were incubated with the antibodies described in *Reagents and antibodies* above at the dilutions recommended by the manufacturers, and signals were detected using ECL (Beyotime, Shanghai, China). To assess insulin signaling proteins, cells were treated with 10 nM insulin (Novo Nordisk) for 10 min before proteins were collected. Western

blots were quantified using ImageJ software (NIH, MD, USA).

Statistical analysis

All values were calculated as the means ± SEMs unless otherwise indicated. Student's *t*-test and one-way ANOVA test were used to determine significance. A *p*-value of <0.05 was considered significant.

Results

4-HIL increases the TNF-α-induced reduction in glycogen levels

Figure 1 shows the glycogen level in the following eight groups of HepG2 cells: control, 5 μM 4-HIL, 10 μM 4-HIL, 20 μM 4-HIL, TNF-α, TNF-α + 5 μM 4-HIL, TNF-α + 10 μM 4-HIL, and TNF-α + 20 μM 4-HIL. The glycogen level was significantly lower ($P<0.05$) in the TNF-α group compared with the control group. The addition of 4-HIL to TNF-α-treated cells increased the glycogen level ($P<0.05$, TNF-α + 20 μM 4-HIL group vs. TNF-α group). However, in the absence of TNF-α 4-HIL itself had no effect on the glycogen level compared with the control group.
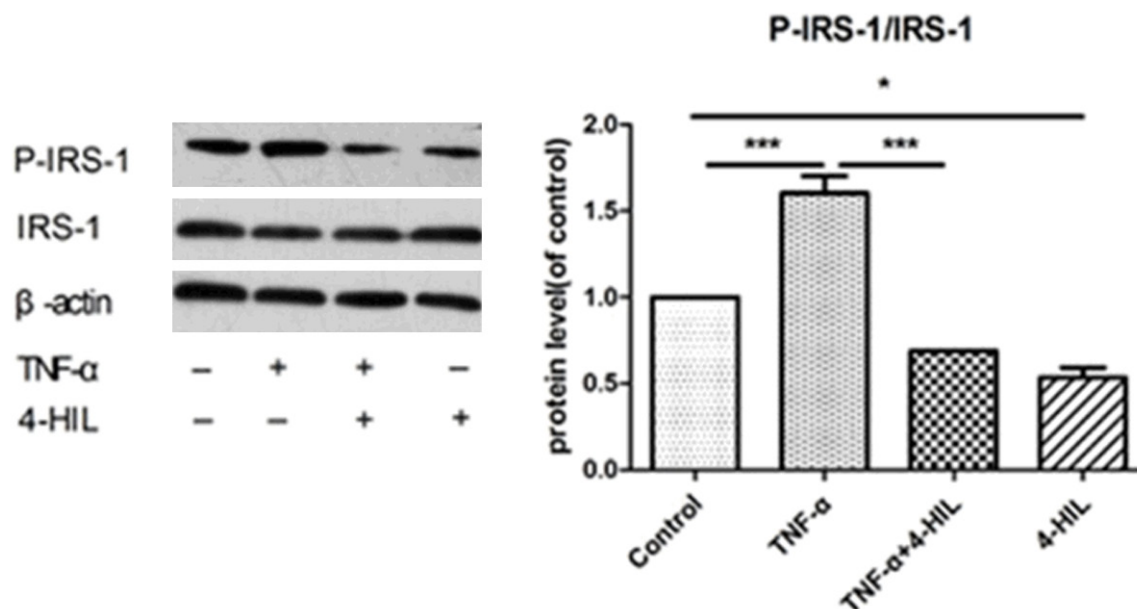
Figure 3. 4-HIL reduced phospho-IRS-1 levels. Treatment with 4-HIL led to the reduced expression of phospho-IRS-1 (Ser[307]). HepG2 cells (2×10^6) were treated with TNF-α alone or TNF-α plus 20 µM4-HIL for 24 h. Whole cell extracts were prepared to assess the expression of phospho-IRS-1. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading (***$P<0.001$ and *$P<0.05$ vs. TNF-α group).

4-HIL modulates the TNF-α-induced changes in insulin signaling

To assess the expression of insulin signaling proteins using western blotting, HepG2 cells were divided into four groups (control, 4-HIL, TNF-α, and TNF-α + 4-HIL). 4-HIL was used at a concentration of 20 µM because this had the maximal effect on glycogen levels (**Figure 1**). **Figure 2** shows the expression of P-JNK and JNK proteins. As expected, the levels of total JNK were unchanged by treatment with TNF-α and/or 4-HIL. The P-JNK/JNK ratios were similar in the control and 20 µM 4-HIL groups, but the ratio was markedly higher in the TNF-α group ($P<0.001$). However, the P-JNK/JNK ratio was significantly lower in the TNF-α + 4-HIL group than in the TNF-α group ($P<0.05$).

Figure 3 shows the expression of P-IRS-1 and IRS-1 proteins. The P-IRS-1 (Ser[307])/IRS-1 ratio was higher in the TNF-α group compared with the control group ($P<0.001$). The addition of 20 µM 4-HIL to TNF-α-treated cells significantly reduced the P-IRS-1 (Ser[307])/IRS-1 ratio ($P<0.001$ vs. the TNF-α group). Moreover, the P-IRS-1 (Ser[307])/IRS-1 ratio was significantly lower ($P<0.05$) in the 20 µM 4-HIL group than in the control group.

The expression of phospho-AKT (Ser[473]) and AKT proteins is shown in **Figure 4**. TNF-α treatment markedly decreased the P-AKT (Ser[473])/AKT ratio ($P<0.001$ vs. the control group). The ratio was significantly higher in the TNF-α + 4-HIL group than in the TNF-α group ($P<0.05$). In addition, 4-HIL alone slightly increased the P-AKT (Ser[473])/AKT ratio, although the difference between the 4-HIL group and the control group was not statistically significant.

The expression of phospho-GSK-3α/β (Ser[21/9]) and GSK proteins was also evaluated (**Figure 5**). TNF-α treatment decreased the P-GSK-3α/β (Ser[21/9])/GSK ratio ($P<0.001$ vs. the control group). The addition of 20 µM 4-HIL to TNF-α-treated cells restored the ratio to control levels ($P<0.001$ vs. the TNF-α group). In addition, 4-HIL alone slightly increased the P-GSK-3α/β (Ser[21/9])/GSK ratio, although the difference between the 4-HIL group and the control group was not statistically significant.

Discussion

TNF-α, a major inflammatory cytokine secreted by macrophages, is involved in cell proliferation, differentiation, survival, and apoptosis [29, 30]. Abnormal increases in TNF-α stimu-
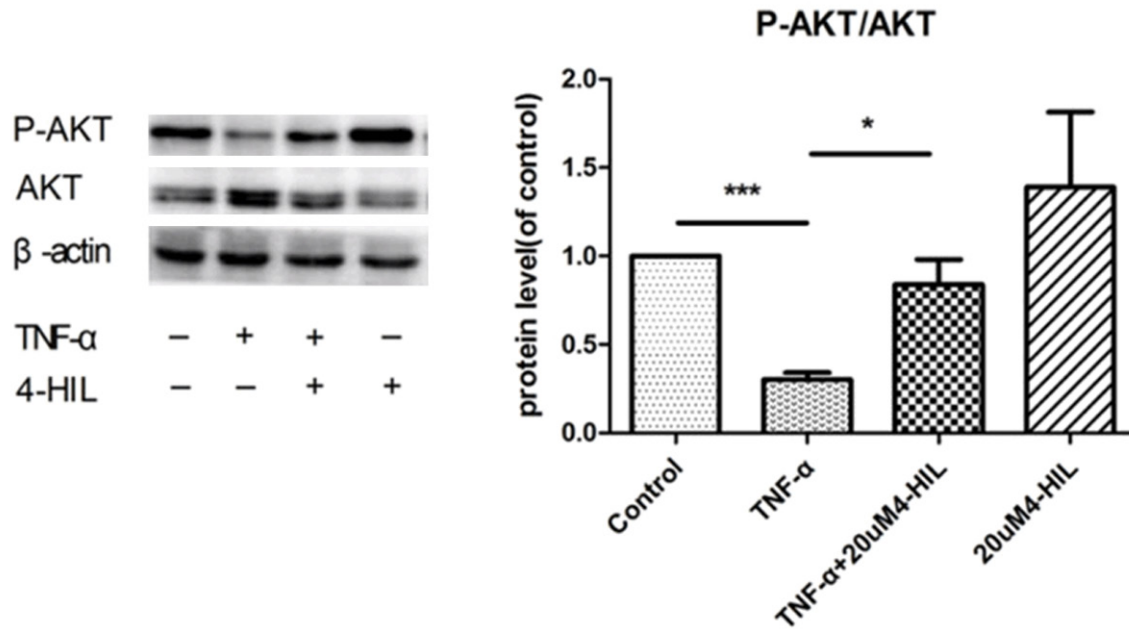
Figure 4. 4-HIL reduced phospho-AKT levels. Treatment with 4-HIL led to the reduced expression of phospho-AKT (Ser[473]). HepG2 cells (2×10^6) were treated with TNF-α alone or TNF-α plus 20 μM4-HIL for 24 h. Whole cell extracts were prepared to assess the expression of phospho-AKT. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading (***$P<0.001$ and *$P<0.05$ vs. TNF-α group).
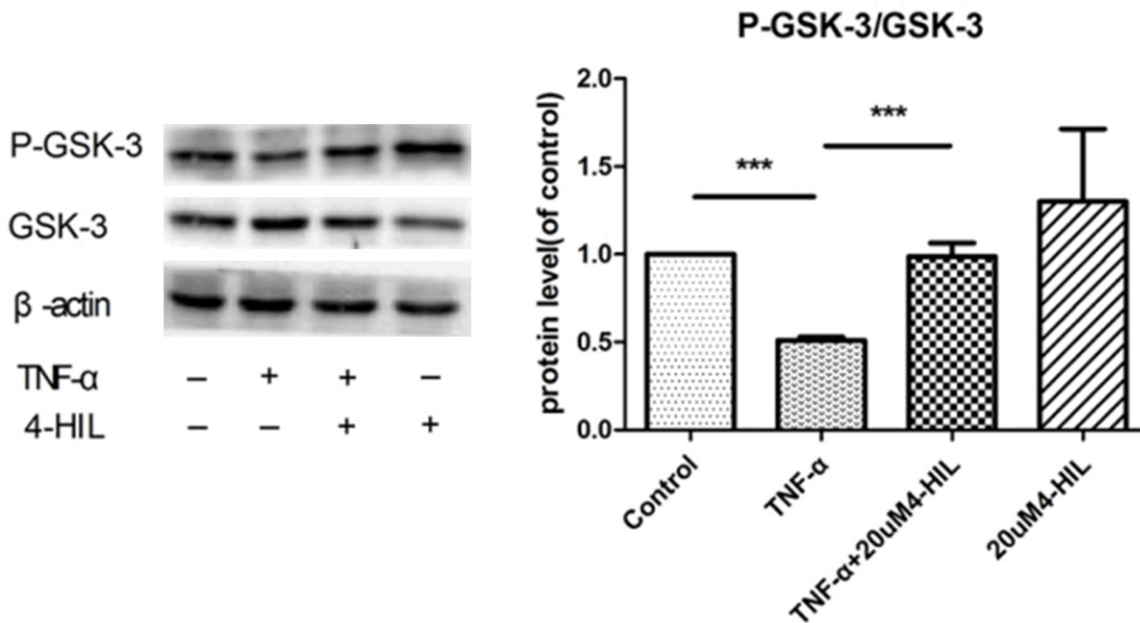

Figure 5. 4-HIL reduced phospho-GSK-3 levels. Treatment with 4-HIL led to the reduced expression of phospho-GSK-3α/β (Ser[21/9]). HepG2 cells (2×10^6) were treated with TNF-α alone or TNF-α plus 20 μM 4-HIL for 24 h. Whole-cell extracts were prepared to assess the expression of phospho-GSK-3α/β. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading (***$P<0.001$ vs. TNF-α group).

late the production of cytokines, which promote the activation of various inflammatory signaling pathways [31]. TNF-α is involved in the develop-

ment of obesity-related insulin resistance, and TNF-α levels are closely related to the severity of IR [32, 33]. Studies have demonstrated that
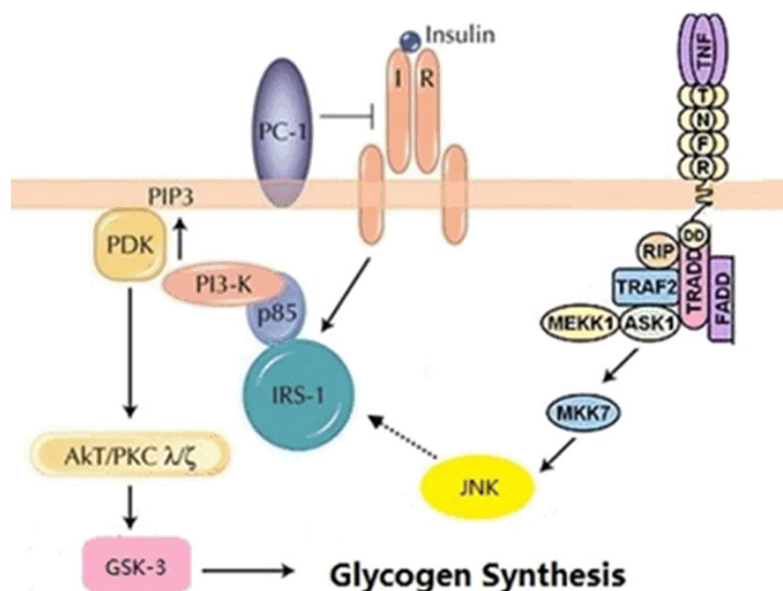
Figure 6. The mechanism by which TNF-α inhibits glycogen synthesis.

[24]. In an additional study, Rawat et al demonstrated that 4-HIL ameliorated IR in streptozotocin-treated rats and L6 myotubes via an AMPK- and AKT-dependent pathway in the liver [35]. Therefore, 4-HIL elicits multiple effects on insulin metabolism in a variety of tissues. The current study expands on these previous observations by highlighting at least some of the mechanisms of action of 4-HIL in hepatocytes. Further studies will expand on these observations and assess the ability of 4-HIL to reduce or prevent TNF-α-induced IR in vivo.

TNF-α increases the expression of phosphorylated JNK in 3T3-L1 adipocytes and worsens FFA-induced IR. However, inhibiting JNK phosphorylation might improve IR [34]. A reduction in hepatic glycogen synthesis is an important manifestation of IR. Li et al reported that TNF-α acts on JNK, which further affects the expression of IRS-1, AKT, and GSK, which subsequently reduces hepatic glycogen synthesis [16]. The mechanism by which TNF-α decreases hepatic glycogen synthesis is shown in **Figure 6**. The current study demonstrated that 4-HIL successfully inhibited the effects of TNF-α on inhibiting glycogen synthesis by preventing JNK and IRS-1 phosphorylation and increasing Akt and GSK-3 phosphorylation, and thereby improving hepatic IR. Therefore, these results suggest that 4-HIL might protect against inflammation-induced IR.

4-HIL promotes insulin secretion in a glucose-dependent manner in vitro. Furthermore, 4-HIL might improve IR within the liver, skeletal muscle, and adipose tissue [19-23]. Recently, Maurya et al reported that 4-HIL ameliorates FFA-induced IR in L6 myotubes [24]. In their study, 4-HIL enhanced insulin sensitivity by inhibiting the ability of palmitate to decrease the insulin-induced activation of the insulin receptor pathway. In addition, 4-HIL suppressed the production of ROS and ROS-associated inflammation induced by palmitate

We demonstrated previously that 4-HIL downregulates TNF-α expression by inhibiting TNF-α-converting enzyme and promoting tissue inhibitor of metalloproteinase, thus suppressing IRS-1 (Ser^{307}) phosphorylation and increasing GLUT4 expression [25]. These findings confirmed that 4-HIL could improve insulin resistance, but it remained unclear whether 4-HIL affected hepatic glycogen synthesis. Glycogen synthesis in the liver is highly complex, and involves multiple biological pathways and processes. In this study, we found that 4-HIL reversed TNF-α-induced reduction of hepatic glycogen levels, thus improving hepatic IR. These findings expand our knowledge of the effects of 4-HIL on hepatic IR, and also reveal at least some of the underlying mechanisms behind the pharmacological effects of 4-HIL in improving hepatic IR. As mentioned above, 4-HIL also modulates IR in other tissues such as adipose tissue and skeletal muscle. Therefore, future studies will focus on these tissues to increase our understanding of the mechanism of action of 4-HIL in other settings that mimic the in vivo environment, as well as on the hepatic IR induced by other factors including oxidative stress, free fatty acids, and other cytokines.

Conclusions

This study assessed the effects of 4-HIL on hepatic glycogen levels and IR, and also investi-

gated the molecular mechanisms underlying these effects. We found that 4-HIL reversed the TNF-α-induced reduction in glycogen levels in HepG2 cells via the JNK signaling pathway. These results suggest a novel role for 4-HIL in alleviating insulin resistance at the molecular level, and suggest that 4-HIL warrants attention in future studies in vivo as a potential anti-diabetogenic agent.

Acknowledgements

Disclosure of conflict of interest

None.

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