Research Article

Oleanolic Acid Attenuates Insulin Resistance via NF-κB to Regulate the IRS1-GLUT4 Pathway in HepG2 Cells

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The aim of our study is to elucidate the mechanisms of oleanolic acid (OA) on insulin resistance (IR) in HepG2 cells. HepG2 cells were induced with FFA as the insulin resistance model and were treated with OA. Then the glucose content and the levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were analyzed. Moreover, protein expression of nuclear factor kappa B (NF-κB), insulin receptor substrate 1 (IRS1), and glucose transporter 4 (GLUT4) in cells treated with OA were measured by Western blot analysis. Additionally, IRS1 protein expression exposed to OA was detected after using pyrrolidine dithiocarbamate (PDTC). Our results revealed that OA decreased the glucose content in HepG2 cells in vitro. Moreover, OA reduced the levels of TNF-α and IL-6 and upregulated IRS1 and GLUT4 protein expression. Furthermore, OA also reduced NF-κB protein expression in insulin-resistant HepG2 cells. After blocking NF-κB, the expression of IRS1 protein had no obvious changes when treated with OA. OA attenuated insulin resistance and decreased the levels of TNF-α and IL-6. Meanwhile, OA decreased NF-κB protein expression and upregulated IRS1 and GLUT4 protein expression. Therefore, regulating the IRS1-GLUT4 pathway via NF-κB was the underlying mechanism of OA on insulin resistance.

1. Introduction

Most pathology ultimately arises from obesity's characteristic milieu of chronic low-grade inflammation and insulin resistance [1]. The inability of insulin to perform normal biological functions in vivo is called insulin resistance. In general, insulin resistance occurs when a certain concentration of insulin cannot effectively stimulate glucose uptake and utilization in the peripheral target organs. Thus the organism suffers from impaired glucose tolerance that ultimately leads to diabetes or other diseases [2–4]. Many studies have demonstrated that patients with insulin resistance (IR) have displayed an increased risk of developing diabetes, cardiovascular disease, and other diseases [5, 6].

Recent studies have found that metabolic diseases including obesity are a common cause of insulin resistance. Meanwhile, increases in FFA (sodium oleate, Figure 1) levels have been shown to occur in metabolic diseases [7–9]. Higher FFA levels could induce the body to secrete inflammatory cytokines, such as TNF-α or IL-6, which would be a low-grade inflammatory state. Inflammation induced by FFA plays a key role in insulin resistance [10, 11]. As the important target of the inflammatory pathways, NF-κB could disrupt IRS1 and downregulate the expression of IRS1 under conditions of insulin resistance [12, 13].

Oleanolic acid (OA), a naturally occurring triterpenoid (Figure 2), is the main effective active ingredient in many herbs such as glossy privet fruit and exists largely in food products (vegetable oils) [14].

Preliminary study found that OA had blood glucose-reducing effect and inhibited insulin resistance in diabetic rats [15]. OA decreased blood glucose, improved insulin resistance, and enhanced insulin signaling by inhibition of ROS and anti-inflammatory effect in diabetic mice [16]. OA also regulated the NF-κB signaling which exhibits high anti-inflammatory activity and was regarded as a potential NF-κB inhibitor [17]. Recently a study conducted by Li et al. demonstrated that OA ameliorated insulin resistance via...
2.1. Cell Culture. The human hepatocellular carcinoma cell line HepG2 was purchased from Land Unicomed. Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS at 37°C in a 5% CO₂ atmosphere. In all experiments, the cells were grown to 80–90% confluence.

2.2. Cell Viability Assay. The cytotoxicity of OA against the HepG2 cells was assessed by the MTT assay. The MTT viability assay was described previously [19]. Briefly, HepG2 cells were plated in 96-well plates at 1 × 10⁴ cells per well. After 24 h, HepG2 cells were treated with 10% heat-inactivated FBS (20 μL; 5 mg/mL in PBS) was added to each well to achieve a total reaction volume of 220 μL. After 4 h of incubation at 37°C and 5% CO₂, the media were then removed and 150 μL dimethyl sulfoxide (DMSO) was added to every well. After shaking for 10 min, the amount of purple formazan was measured by absorbance at 490 nm.

2.3. Induction of Insulin Resistance in HepG2 Cells and Glucose Utilization Experiments. The HepG2 cells were cultured and determined glucose utilization was performed as previously described [20]. Briefly, HepG2 cells were seeded on 24-well plates at 1 × 10⁵ cells/well and incubated for 24 h to reach maximal confluence. The cells were then cultured for 24 h in serum-free DMEM, 0.2% BSA, and 200 μmol/L sodium oleate in the absence or presence of OA (OA was dissolved in DMSO) or RSG. Next, cells were washed twice with PBS and incubated for 3 h in serum-free DMEM containing 25 mmol/L glucose and 1 × 10⁻⁹ mol/L insulin. The culture medium was collected. The content of glucose was quantified using a GOD-POD kit.

2.4. Enzyme-Linked Immunosorbent Assay of TNF-α and IL-6 Levels. Insulin resistance was induced in HepG2 cells as previously described. The culture medium was centrifuged at 14000 × g for 10 minutes at 4°C. The supernatant was then collected and stored at −80°C until analysis. The levels of TNF-α and IL-6 in the supernatant were measured using ELISA kits according to the manufacturer’s instructions.

2.5. Western Blot Analysis. Insulin resistance was induced in HepG2 cells as previously described. Cells were washed with ice-cold PBS and lysed with a RIPA lysis buffer. For Western blotting, protein samples (20 μg) of sodium oleate induced insulin-resistant HepG2 cells were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a PVDF membrane and incubated with primary antibody (anti-NF-κB, anti-IRS1, anti-GLUT4, or anti-GAPDH), followed by a secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG). The intensity of the immunoblot signal was assayed using Western Bright ECL spray and analyzed quantitatively using GeneTools software from Syngene (Syngene, Cambridge, UK).

To choose the most effective concentration of PDTC, the cells were incubated for 24 h in serum-free DMEM at the different dosages of PDTC or 0.2% BSA and 200 μmol/L sodium.
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The influence of OA on the cell viability in HepG2 cells was examined using the MTT reduction assay. The MTT assay results demonstrated no significant difference in cell viability of cells treated with OA at the concentrations of lower than 50 μmol/L. As the concentration of OA was 75 μmol/L, the cell viability was reduced by approximately 40% when compared to the control treatment (Figure 3).

3.1. Influence of OA on the Cell Viability in HepG2 Cells. The influence of OA on the cell viability in HepG2 cells was examined using the MTT reduction assay. The MTT assay results demonstrated no significant difference in cell viability of cells treated with OA at the concentrations of lower than 50 μmol/L. As the concentration of OA was 75 μmol/L, the cell viability was reduced by approximately 40% when compared to the control treatment (Figure 3).

3.2. Effect of OA on the Glucose Content of the Culture Media in Insulin-Resistant HepG2 Cells. The HepG2 cells were incubated for 24 h in serum-free DMEM containing 200 μmol/L sodium oleate, in either 0.5, 1, 5, 10, and 25 μmol/L OA or RSG (10 μmol/L). Next, the cells were incubated for 2 h in serum-free DMEM containing 200 μmol/L sodium oleate, and three dosages of OA were added. Next, the cells were incubated for an additional 24 h after preparing the protein samples in Western blot experiments and incubated with anti-NF-κB.

3.3. Effect of OA on the Levels of TNF-α and IL-6 in Insulin-Resistant HepG2 Cells. To examine the effect of OA on inflammatory cytokines, the levels of TNF-α and IL-6 in the culture media of insulin-resistance HepG2 cells were measured by ELISA. The HepG2 cells were incubated for 24 h in serum-free DMEM containing 200 μmol/L sodium oleate, in the 5, 10, and 25 μmol/L OA, or in RSG (10 μmol/L). The levels of TNF-α and IL-6 in insulin-resistant HepG2 cells were significantly higher compared with the control cells (P < 0.01). After treatment with OA (5, 10, and 25 μmol/L), the glucose content in the culture medium significantly reduced compared with the IR cells (P < 0.05). Rosiglitazone (RSG), which is the insulin sensitizer as a positive control drug, decreased the glucose content in the culture medium compared with the IR cells (P < 0.01). These results showed that OA attenuated insulin resistance in a dose-dependent manner (Figure 4).

3.4. Effect of OA on the Protein Expression of NF-κB, IRS1, and GLUT4 in Insulin-Resistant HepG2 Cells. To elucidate the mechanism of OA on insulin resistance in HepG2 cell, a...
Western blot analysis was used to measure the protein expression of NF-κB, IRS1, and GLUT4 in HepG2 cells. The cells were divided into six groups, including a Control group, IR group (200 μmol/L sodium oleate), RSG group (200 μmol/L sodium oleate and RSG 10 μmol/L), OA-25 μmol/L group (200 μmol/L sodium oleate, OA 25 μmol/L), OA-10 μmol/L group (200 μmol/L sodium oleate, OA 10 μmol/L), and OA-5 μmol/L group (200 μmol/L sodium oleate, OA 5 μmol/L). As shown in Figure 4, the expression of NF-κB protein in the IR group was significantly greater than the Control group (P < 0.01). IRS1 and GLUT4 protein expression in the IR group was significantly reduced compared with the Control group (P < 0.01). After treatment with OA at three dosages, the protein expression of NF-κB was significantly reduced compared with the IR group (P < 0.01). In addition, the expression of IRS1 and GLUT4 protein with OA at three dosages was significantly higher compared with the IR group (P < 0.01). RSG was also able to increase the expression of IRS1 and GLUT4 protein and reduce the expression of NF-κB protein compared with the IR group (P < 0.01) (Figure 6).

3.5. Effect of PDTC on NF-κB Protein Expression in HepG2 Cells. To select the most effective concentration of PDTC that blocked NF-κB expression using Western blot analysis, HepG2 cells were divided into six groups, including a Control group, 100 μmol/L PDTC group, 300 μmol/L PDTC group, 500 μmol/L PDTC group, 1000 μmol/L PDTC group, and IR group (200 μmol/L sodium oleate). The levels of NF-κB expression in all groups that were treated with PDTC were significantly lower than the Control group (P < 0.01). The expression of NF-κB in the IR group was significantly greater than the Control group (P < 0.01). As shown in Figure 5, the most effective concentration of PDTC that blocked NF-κB expression was 300 μmol/L (Figure 7).

3.6. Effect of OA on the Expression of IRS1 Protein in Insulin-Resistant HepG2 Cells with Blocking of the Expression of NF-κB

Figure 8(a). To study the IRS1 protein expression when PDTC exists or not, HepG2 protein samples were divided into four groups as follows: Control group, IR group (200 μmol/L sodium oleate), P + Control group (300 μmol/L PDTC), and P + IR group (300 μmol/L PDTC and 200 μmol/L sodium oleate). As shown in Figure 6(a), the expression of IRS1 protein in the P + Control group was significantly greater than the Control group (P < 0.01). In addition, the expression of IRS1 protein in P + IR group was significantly increased compared with the IR group (P < 0.01) and significantly reduced compared with the P + Control group (P < 0.05).

Figure 8(b). To evaluate the effect of OA on the IRS1 protein expression when PDTC exists or not, HepG2 protein samples were divided into nine groups as follows: Control group, IR group (200 μmol/L sodium oleate), OA-25 μmol/L group (200 μmol/L sodium oleate, 25 μmol/L OA), OA-10 μmol/L group (200 μmol/L sodium oleate, 10 μmol/L OA), OA-5 μmol/L group (200 μmol/L sodium oleate, 5 μmol/L OA), P + IR group (300 μmol/L PDTC, 200 μmol/L sodium oleate), P + OA-25 μmol/L group (300 μmol/L PDTC, 200 μmol/L sodium oleate and 25 μmol/L OA), P + OA-10 μmol/L group (300 μmol/L PDTC, 200 μmol/L sodium oleate and 10 μmol/L OA), and P + OA-5 μmol/L group (300 μmol/L PDTC, 200 μmol/L sodium oleate and 5 μmol/L OA). As shown in Figure 6(b), the expression of IRS1 protein in the IR group was significantly lower than the Control group (P < 0.01); the expression of IRS1 protein in the OA-25 μmol/L group, OA-10 μmol/L group, OA-5 μmol/L group, P + IR group, P + OA-25 μmol/L group, P + OA-10 μmol/L group, and P +
OA-5 μmol/L group was significantly greater than the IR group ($P < 0.01$). When compared to the three dosages of OA without PDTC, the expression of IRS1 protein in three dosages of OA with PDTC was reduced ($P < 0.05$) (Figure 8).

4. Discussion
This study demonstrated that OA attenuated insulin resistance in HepG2 cells, whose effect is possibly mediated through decreasing the levels of TNF-$\alpha$ and IL-6 and regulating the expression of IRS1 and GLUT4 protein via the NF-$\kappa$B protein.

Elevated levels of free fatty acids are thought to be the pathogenic factors causing metabolic disorders such as obesity and diabetes [21]. Oleate, an unsaturated fatty acid, can induce insulin resistance in HepG2 cells [22]. HepG2 cells were wildly used for studying insulin resistance [23]. When HepG2 cells were induced with insulin resistance, their responses to glucose were affected. The glucose content in insulin-resistant cells in culture medium increased compared with healthy cells [24]. In the present study, we used 200 μmol/L sodium oleate to induce insulin resistance in HepG2 cells. The glucose content in the IR group in culture medium was significantly greater than the Control groups. It showed that it is a suitable model of insulin resistance.

Oleanolic acid (OA) exerts multiple pharmacological actions including glycoregulatory, hepatoprotective, anti-inflammatory, and antioxidant effects and is used to treat chronic diseases, such as diabetes, liver injury, and hepatitis [25]. A study conducted by Wang et al. found that OA decreased the glucose content in the culture medium,
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Improved insulin resistance, protected beta-cell, and inhibited the mitochondrial apoptosis in beta-TG3 cells [26]. de Melo et al.'s study [27] showed that OA decreased blood glucose and improved glucose tolerance in mice. Our study results showed that OA decreased the glucose content in the culture medium at variable dosage in HepG2 cells. It demonstrated that OA affected the glucose utilization and attenuated insulin resistance in dose-dependent manner (Figure 2). Consistency with our study, Teodoro et al. [28] found that OA enhanced insulin secretion and increased the glucose utilization in pancreatic beta-cells in vitro.

High levels of FFA could induce insulin resistance as well as inflammation [29]. Much research has shown that OA had an anti-inflammatory effect and reduced the content of inflammatory cytokines, such as TNF-α and IL-6. Chai et al. found that OA decreased the level of IL-6 and TNF-α in the serum and liver of db/db mice [30]. Liu et al. showed that OA could inhibit the generation of the inflammatory factors TNF-α and IL-6 [31]. Nkeh-Chungag et al. found that OA exerted potent anti-inflammatory effects by inhibiting NO and PGE2 in RAW 264.7 cells [32]. Our results showed that OA decreased the levels of IL-6 and TNF-α in insulin-resistant HepG2 cells (Figure 3).

Mounting evidence has suggested that inflammatory processes were related to the pathogenesis of insulin resistance [33]. The NF-κB is a pivotal molecular mediator of insulin resistance [34]. Various inflammatory cytokines, including IL-6 and TNF-α, have been shown to activate NF-κB to cause insulin resistance [35]. Insulin resistance causes the impairment of IRS1 and GLUT4, which lead to obstacle of glucose utilization [36]. As mentioned above, OA has anti-inflammatory effects. It not only reduced the content of inflammatory cytokines but also reduced the expression of NF-κB and upregulated IRS1 protein expression. Kim et al. found that OA could disturb NF-κB activation in 3T3-L1 adipocytes by inhibiting inflammatory responses during adipocyte differentiation through blocking IL-6-TRAF6-NF-κB signaling [37]. Li et al.'s results showed that OA could upregulate IRS1 protein expression in adipose tissue in insulin-resistant rats. Our studies showed that insulin resistance induced by sodium oleate could lead to overexpression of NF-κB protein and that IRS1 and GLUT4 proteins were impaired. After treatment with OA, the expression of NF-κB protein was significantly reduced and the expression of IRS1 and GLUT4 protein was partially restored.

Pyrrolidine dithiocarbamate (PDTC) is a specific NF-κB inhibitor that becomes widely used [38]. In Zheng et al.'s study, PDTC was used to block NF-κB to explain the protective effects of chronic resveratrol treatment on vascular inflammatory injury in streptozotocin-induced type 2 diabetic rats [39]. To illustrate the point that OA could relieve the expression of the IRS1 and GLUT4 by blocking the expression of NF-κB, PDTC was used to block NF-κB in our study. We found that the expression of IRS1 protein in the cells of the IR group that were previously blocked by PDTC was significantly elevated compared with IR group cells without PDTC. After the addition of OA at three dosages, the expression of IRS1 protein in the OA groups was significantly higher than those in the IR group. The expression of IRS1 protein in the OA groups with PDTC was not increased as obviously as the OA groups that were not exposed to PDTC. Therefore, we thought that NF-κB was the potential key target of OA that relieved insulin resistance. According to the results of Figure 4, OA could reduce the expression of NF-κB protein and directly increase the expression of IRS1. As mentioned above, PDTC is a specific NF-κB inhibitor. Meanwhile, the expression of IRS1 would increase when NF-κB was blocked. Based on the above knowledge, we speculated that OA can relieve insulin resistance by directly affecting the expression of IRS1 protein. OA also affected the expression of IRS1 protein indirectly by regulating NF-κB. When NF-κB was blocked by PDTC, OA could not affect IRS1 by NF-κB. Thus, the effect of OA on the expression of IRS1 protein in cells was attenuated compared to those without PDTC.

5. Conclusions

In conclusion, our study indicated that OA could decrease insulin resistance by reducing the content of inflammatory cytokines in culture medium. Regulating the IRS1/GLUT4 pathway via NF-κB was the underlying mechanism of the effects of OA on insulin resistance. Our findings may provide new insights into the mechanisms underlying the effect of oleanolic acid in insulin-resistant cells.

Abbreviations

IR: Insulin resistance
OA: Oleanolic acid
Figure 8: Effect of OA on IRS1 protein expression with blocking of the expression of NF-κB. (a) (1) Control; (2) IR; (3) P + Control; (4) P + IR. (b) (1) Control; (2) IR; (3) OA-25 μmol/L; (4) OA-10 μmol/L; (5) OA-5 μmol/L; (6) P + IR; (7) P + OA-25 μmol/L; (8) P + OA-10 μmol/L; (9) P + OA-5 μmol/L. The protein expression of IRS1 was measured via Western blotting as described in the text. The figures represent one of three experiments with similar results. △P < 0.01 compared with the Control group; *P < 0.01 compared with the IR group; #P < 0.05 compared with the P + Control group; ♦P < 0.05 compared with the OA-25 μmol/L group; ♣P < 0.05 compared with the OA-10 μmol/L group; ♠P < 0.05 compared with the OA-5 μmol/L group.

Disclosure

This paper does not contain any studies on human or animal subjects performed by any of the authors.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors’ Contribution

Dr. Jiao Guo and Xuguang Hu designed the study. Ming Li and Zongyu Han carried out experiments. Weijian Bei and Xianglu Rong participated in the design of study. Dr. B. Han, MC Fang, L. Wan, and M. Wang have provided a lot of support and help. All authors have read and approved the final paper.
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