

Adiponectin receptor agonist AdipoRon relieves endotoxin-induced acute hepatitis in mice

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Abstract

Background: Adiponectin is the most abundant adipokines that plays critical roles in the maintenance of energy homeostasis as well as inflammation regulation. The half-life of adiponectin is very short and the small-molecule adiponectin receptor agonist has been synthesized recently. In the present study, the potential roles of AdipoRon, an adiponectin receptor agonist, in a mouse model of lipopolysaccharide (LPS)/D-galactosamine (D-Gal)-induced acute hepatitis was explored.

Methods: BALB/c mice ($n = 144$, male) were divided into three sets. In set 1, 32 mice were randomized into four groups: the control group, the AdipoRon group, the LPS/D-Gal group, and the AdipoRon + LPS/D-Gal group. The mice in set 1 were sacrificed after LPS/D-Gal treatment, and the plasma samples were collected for detection of tumor necrosis factor- α (TNF- α). In set 2, the 32 mice were also divided into four groups similar to that of set 1. The mice were sacrificed 6 h after LPS/D-Gal injection and plasma samples and liver were collected. In set 3, 80 mice (divided into four groups, $n = 20$) were used for survival observation. The survival rate, plasma aminotransferases, histopathological damage were measured and compared between these four groups.

Results: AdipoRon suppressed the elevation of plasma aminotransferases (from 2106.3 ± 781.9 to 286.8 ± 133.1 U/L for alanine aminotransferase, $P < 0.01$; from 566.5 ± 243.4 to 180.1 ± 153.3 U/L for aspartate aminotransferase, $P < 0.01$), attenuated histopathological damage and improved the survival rate (from 10% to 60%) in mice with LPS/D-Gal-induced acute hepatitis. Additionally, AdipoRon down-regulated the production of TNF- α (from 328.6 ± 121.2 to 213.4 ± 52.2 pg/mL, $P < 0.01$), inhibited the activation of caspase-3 (from 2.04-fold to 1.34-fold of the control), caspase-8 (from 2.03-fold to 1.31-fold of the control), and caspase-9 (from 2.14-fold to 1.43-fold of the control), and decreased the level of cleaved caspase-3 (0.28-fold to that of the LPS/D-Gal group). The number of terminal deoxynucleotidyl transferase-mediated nucleotide nick-end labeling-positive apoptotic hepatocytes in LPS/D-Gal-exposed mice also reduced.

Conclusions: These data indicated that LPS/D-Gal-induced acute hepatitis was effectively attenuated by the adiponectin receptor agonist AdipoRon, implying that AdipoRon might become a new reagent for treatment of acute hepatitis.

Keywords: AdipoRon; Lipopolysaccharide; D-galactosamine; Acute hepatitis; Apoptosis

Introduction

Cumulative evidence suggests that adipose tissue not only plays a central role in the storage of energy, but also secretes a series of adipokines with extensive regulatory activities.^[1] Adiponectin is the most abundant adipokines that fulfills critical roles in the maintenance of energy homeostasis.^[2] Adiponectin strongly improves the sensitivity of insulin via suppressing gluconeogenesis; therefore, adiponectin has been regarded as a promising target for the treatment of diabetes.^[3] In addition, the beneficial metabolic action of adiponectin has attracted considerable attention in obesity, metabolic syndrome, non-alcoholic fatty liver disease (NAFLD), and atherosclerosis.^[4]

Besides its functions in metabolic regulation, adiponectin also plays an important role in the regulation of inflammation.^[5] In obese patients, the down-regulation of adiponectin has been found to be associated with the elevation of various inflammatory markers.^[6] Treatment with adiponectin also suppressed the production of pro-inflammatory cytokines and alleviated inflammatory injury in animals with sepsis-induced acute lung injury, ischemic acute kidney injury, and caerulein-induced acute pancreatitis.^[7-9] In addition, the anti-inflammatory activities of adiponectin have been observed in cultured macrophages and endothelial cells.^[10,11]

However, the short half-life of adiponectin limits its application.^[12] Therefore, AdipoRon, a small-molecule

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adiponectin receptor agonist with a long half-life, has been synthesized recently.^[12,13] Several studies have shown that AdipoRon mimics the beneficial effects of adiponectin in the intervention of diabetes and atherosclerosis.^[12,14] Treatment with AdipoRon also attenuated inflammatory injury in animal with spinal cord injury.^[15] Endotoxin, also known as lipopolysaccharide (LPS), is a representative stimulator of inflammation, which induces severe acute hepatitis in D-galactosamine (D-Gal)-sensitized mice.^[16] In this widely-used inflammatory model, the potential effects of AdipoRon on inflammatory response, hepatocyte apoptosis, liver lesions, and animal survival were investigated.

Materials and Methods

Materials

LPS, D-Gal, and AdipoRon were purchased from Sigma (St. Louis, MO, USA). The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) test kits were purchased from the Nanjing Institute of Bioengineering (Nanjing, China). An enzyme-linked immunosorbent assay (ELISA) kit was purchased from the Neo-Bioscience (Shenzhen, China). Cytoplasmic and nuclear protein extraction kits and caspase-3, caspase-8, and caspase-9 colorimetric assay kits were purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). An in situ detection kit for apoptosis was purchased from Roche (Indianapolis, IN, USA). Cleaved caspase-3 and β -actin antibodies were purchased from Abcam (Cambridge, MA, USA). A bicinchoninic acid (BCA) protein assay kit, horseradish peroxidase-conjugated goat anti-rabbit antibody and enhanced chemiluminescence (ECL) reagents were purchased from Pierce Biotechnology (Rockford, IL, USA).

Animals

Male BALB/c mice, approximately 6 to 8 weeks old, were used in this experiment, and all were obtained from the Animal Experimental Center of Chongqing Medical University. The mice weighed 20 to 25 g and were provided with sterile water and sterile food for 1 week in the specific pathogen-free animal room, with lights on for 12 h per day and controlled temperature of $24 \pm 2^\circ\text{C}$. After the mice adapted to the environment, the experiment was carried out. All animal experiments were approved by the Chongqing University Animal Ethics Committee.

LPS/D-Gal-induced liver injury and AdipoRon administration

LPS and D-Gal were both prepared in saline (0.9% NaCl) at concentration of 0.01 mg/kg for LPS and 700 mg/kg for D-Gal. All drugs were administered by intraperitoneal injection. AdipoRon was administered 30 min before modeling at a dose of 100 mg/kg dissolved in dimethylsulfoxide. The 144 mice were divided into three sets. In set 1, 32 mice were randomized into four groups ($n = 8$ for each group): (A) the control group: mice with intraperitoneal injection of the solvent; (B) the AdipoRon group: mice with intraperitoneal injection of AdipoRon; (C) the LPS/D-Gal group: mice with intraperitoneal injection of LPS/D-Gal; (D) the AdipoRon + LPS/D-Gal: LPS/D-Gal was injected

30 min after AdipoRon injection. The mice in set 1 were sacrificed by cervical dislocation 90 min after LPS/D-Gal treatment, and the plasma samples were collected for detection of tumor necrosis factor- α (TNF- α). In set 2, the 32 mice were also divided into four groups similar to that of set 1. The mice were sacrificed 6 h after LPS/D-Gal injection and plasma samples and liver were collected. In set 3, 80 mice (divided into four groups, $n = 20$) were used for survival observation. The mice were monitored for survival for at least 3 days every 12 hours.

Histopathological analysis

Liver tissue was fixed in 4% paraformaldehyde. Then, the sample was washed and placed in 70% alcohol. Next, samples were embedded in paraffin, stained with hematoxylin & eosin and sealed with resin. Histopathological analysis was carried out under a microscope. The degrees of the histological abnormalities were semi-quantitatively scored blindly according to the methods described in our previous publication.^[17] Briefly, the degree of liver injury was scored using a 0 (no lesion) to 3 (severe change) scoring method in 20 random fields at $400\times$ magnification per animal.

Measurement of liver enzymes

The treated mice were placed in an animal room with a constant temperature and humidity. A dose of 5 mL/kg of heparin was intraperitoneally injected 5.5 h after the model was established. Five hours and 50 min after the model was established, phenobarbital was used for abdominal anesthesia. Six hours after the model was established, blood samples were drawn from the retro-orbital vein, and each sample was approximately 0.5 to 1.5 mL. After centrifugation, the supernatant was removed and stored in a low-temperature freezer at -80°C . After collecting blood,

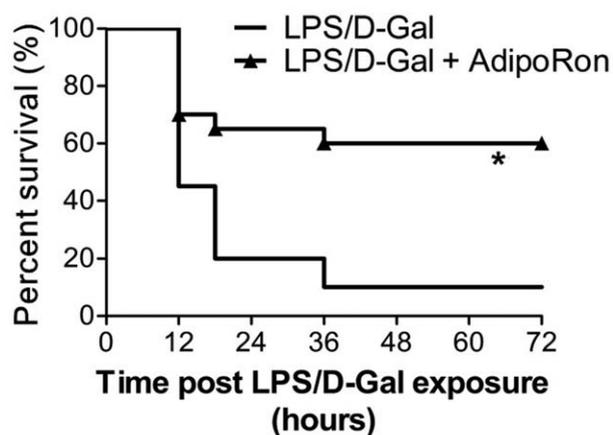


Figure 1: AdipoRon decreased mortality in LPS/D-Gal exposed mice. The 80 mice were divided into four groups ($n = 20$ per group). AdipoRon is injected 30 min before LPS/D-Gal exposure. The mortality rate of experimental animals is monitored every 12 h, the cumulative survival curve is expressed as a Kaplan-Meier survival curve. * $P < 0.01$ (the survival rate of the control group and the AdipoRon group were both 100%, and the data was not displayed). LPS/D-Gal: Lipopolysaccharide/D-galactosamine; TNF- α : Tumor necrosis factor- α .

the investigators followed the steps in the instructions of the test kits to measure AST and ALT levels.

Detection of TNF- α levels in plasma by ELISA

Animal models were established at 1.5 h. The modeling method was the same as that described above. Anesthesia was intraperitoneally injected 1 h and 20 min after modeling. Then, blood was collected 1.5 h after modeling. The remaining steps are the same as those in the 6-h model. Using an ELISA kit, a standard curve was drawn according to the instructions to calculate the concentration of TNF- α .

Detection of caspase-3, caspase-8, and caspase-9 activities in liver tissue using a caspase activity assay kit

The liver activity of caspase-3, -8, and -9 was determined and a colorimetric assay kit was used according to the

manufacturer's instructions. The brief procedure is as follows. First, the liver sample was placed in cell lysis buffer. The homogenate was centrifuged at $10,000 \times g$ for 1 min with the substrates for caspase-3, -8, and -9 for 90 min at 37°C . The activities of caspases were assessed according to the absorbance measured at 405 nm and normalized by the total protein concentration of the same sample.

Western blotting (WB) for detection of cleaved caspase-3 expression in liver tissue

Total protein was extracted from frozen liver samples according to the method described in the Protein Extract Kit. The protein concentration was determined using a bicinchoninic acid protein assay. The protein extract was separated on a 10% polyacrylamide sodium dodecyl sulfate gel and transferred to a nitrocellulose membrane.

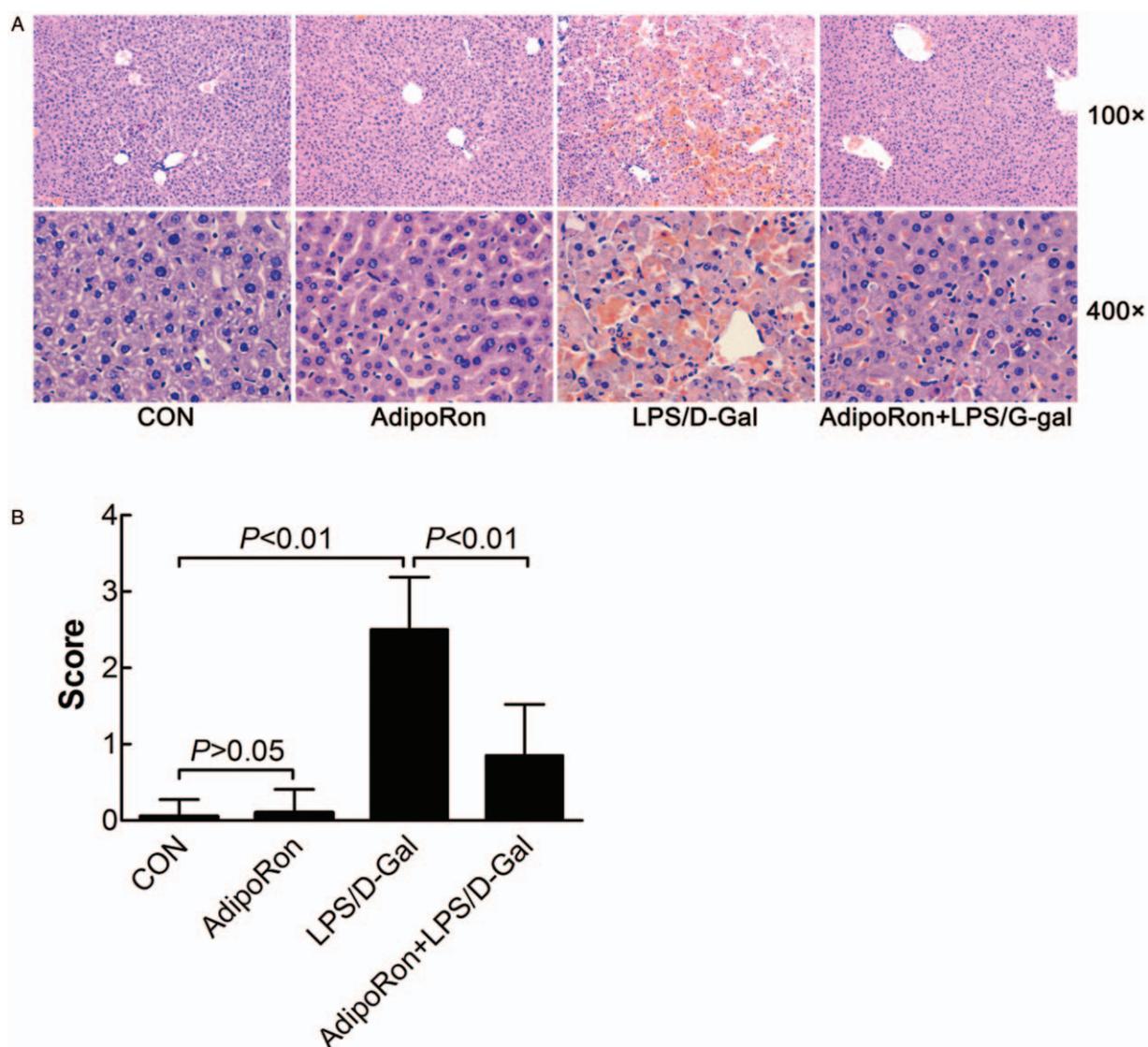


Figure 2: AdipoRon can alleviate LPS/D-Gal-induced liver histopathological damage. AdipoRon treatment alone has no significant effect on the normal tissue structure of liver tissue. LPS/D-Gal treatment can induce obvious congestion and severe tissue damage in liver tissue, while AdipoRon can significantly alleviate these pathological abnormal changes. Representative liver slices for each group were shown (A, original magnification, $\times 100$ and $\times 400$). The degree of liver damage was semi-quantitatively scored, 20 random fields per animal and eight animals per group (B). CON: control; LPS/D-Gal: Lipopolysaccharide/D-galactosamine.

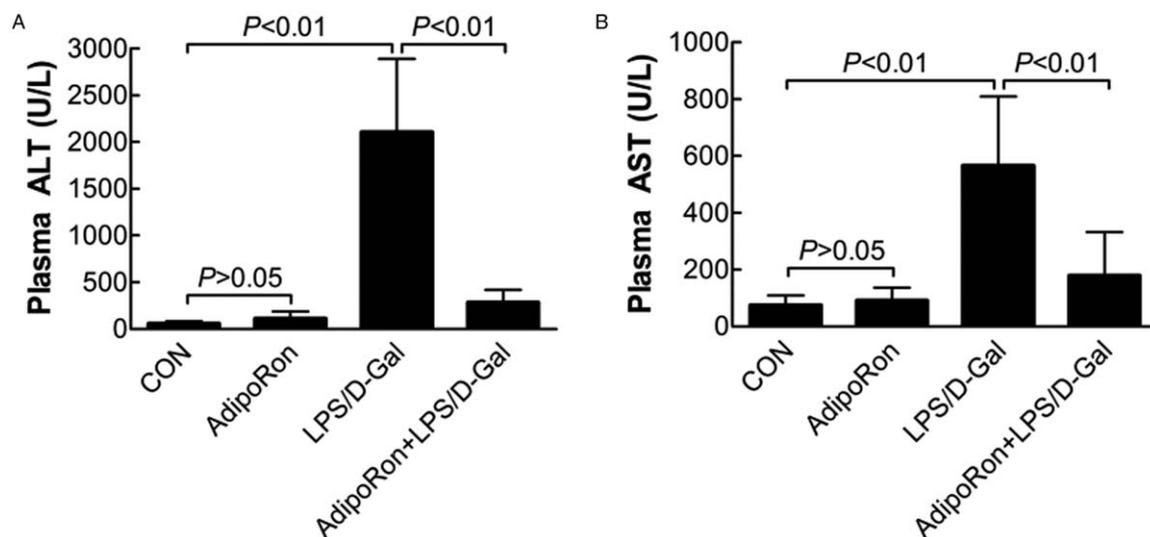


Figure 3: AdipoRon attenuates LPS/D-Gal-induced plasma ALT and AST elevation. There was no significant difference in ALT (A) and AST (B) levels between the control and AdipoRon treated mice. ALT (A) and AST (B) were significantly elevated in LPS/D-Gal-treated mice. AdipoRon intervention was effective in reducing plasma ALT (A) and AST (B) levels in hepatitis models ($n = 8$, $P < 0.01$). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LPS/D-Gal: Lipopolysaccharide/D-galactosamine.

The membrane was blocked with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.05% Tween-20, incubated with primary antibody overnight at 4°C and then incubated with the secondary antibody. Antibody binding was observed using an ECL system and a short X-ray exposure.

Terminal deoxynucleotidyl transferase-mediated nucleotide nick-end labeling (TUNEL) assay

Hepatocyte apoptosis was detected by an *in situ* cell death detection kit (Roche). Our procedure was carried out in accordance with the company's product specifications. The terminal transferase reaction ultimately produced a dark brown precipitate, and then, the sections were lightly counterstained with hematoxylin.

Survival analysis

The 80 BALB/c mice were randomly divided into four groups: the control group, the AdipoRon group, the LPS/D-Gal group, and the AdipoRon + LPS/D-Gal group. LPS, D-Gal, and AdipoRon were all prepared with a normal saline (0.9% NaCl) solution; the dose of LPS was 0.01 mg/kg, the dose of D-Gal was 700 mg/kg, and the dose of AdipoRon was 100 mg/kg. The drugs were administered via intraperitoneal injection. All of the mice were observed and recorded once every 12 h for 3 days.

Statistical analysis

All experimental data are expressed as mean \pm standard deviation. Differences between multiple groups were compared using one-way analysis of variance, and differences between groups were tested using the Tukey *post hoc* test. Survival rates were compared using the Kaplan-Meier curve for animal survival. Differences between the groups were considered statistically significant when $P < 0.05$.

Results

AdipoRon attenuated LPS/D-Gal-induced liver injury and mortality

In the survival curve analysis, the survival rate of hepatitis model mice was only 20% after 24 h, while the survival rate of the mice group after AdipoRon treatment was still as high as 60% at the same time and 72 h after modeling [Figure 1]. LPS/D-Gal caused severe histopathological changes, while AdipoRon significantly improved these histological changes [Figure 2]. Consistently, LPS/D-Gal induced an increase in ALT, AST (2106.3 ± 781.9 and 566.5 ± 243.4 U/L, respectively) in the liver injury model. With treatment of AdipoRon, the level of ALT decreased to 286.8 ± 133.1 U/L ($P < 0.01$), and the level of AST decreased to 180.1 ± 153.3 U/L ($P < 0.01$) [Figure 3A and 3B]. The above results indicated that AdipoRon can alleviate the degree of liver damage in the liver injury model and improve the survival rate of mice.

AdipoRon suppressed LPS/D-Gal-induced TNF- α

TNF- α is an important pro-apoptotic factor in LPS/D-Gal-induced acute hepatitis.^[18] The TNF- α level in plasma of each group was detected by ELISA and the results showed that the level of TNF- α in the AdipoRon treated group was not significantly different from that in the normal control group (91.4 ± 18.0 pg/mL *vs.* 90.8 ± 12.9 pg/mL). LPS/D-Gal induced a significant increase in TNF- α levels in plasma (328.6 ± 121.2 pg/mL, $P < 0.01$), whereas TNF- α levels were significantly reduced after AdipoRon treatment (213.4 ± 52.2 pg/mL, $P < 0.01$) [Figure 4].

AdipoRon inhibits LPS/D-Gal-induced hepatocyte apoptosis

In the LPS/D-Gal-induced liver injury model, a large number of TUNEL-positive apoptotic hepatocytes were observed in the liver tissue with the nuclei stained brown.

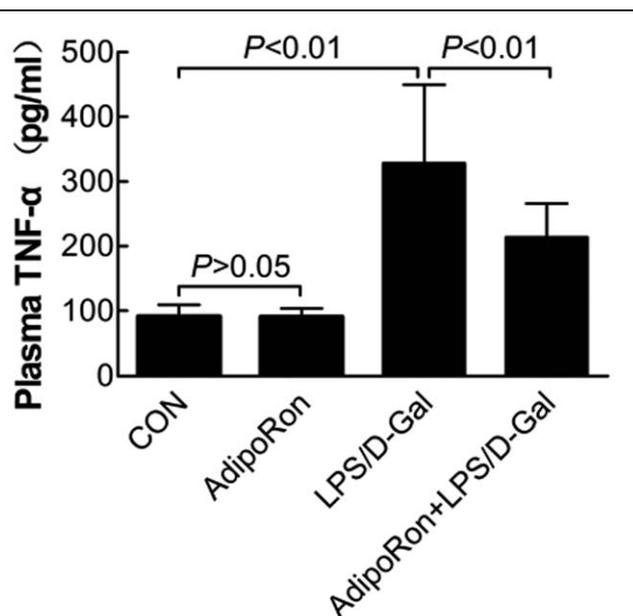


Figure 4: AdipoRon reduced the level of TNF- α in plasma of LPS/D-Gal exposed mice. This experiment used ELISA to detect the plasma in each group of mice. The level of TNF- α in the AdipoRon treated group was not significantly different from that in the normal control group ($n=8$, $P<0.05$). LPS/D-Gal induced a significant increase in TNF- α levels in plasma, whereas TNF- α was significantly lower after AdipoRon treatment ($n=8$, $P<0.01$). ELISA: Enzyme-linked immunosorbent assay; LPS/D-Gal: Lipopolysaccharide/D-galactosamine; TNF- α : Tumor necrosis factor-alpha.

After AdipoRon treatment, the number of TUNEL-positive cells in the nucleus was decreased, suggesting that AdipoRon inhibits LPS/D-Gal-induced hepatocyte apoptosis [Figure 5].

AdipoRon suppressed LPS/D-Gal-induced activation of caspases

Activation of caspase in apoptosis is a key molecular response.^[19] The activity of caspase-3, caspase-8, and caspase-9 in liver tissue was detected in this experiment. It was found that there was no significant difference in caspase activity between the normal control group and the AdipoRon treated group. In the liver tissue of the LPS/D-Gal model group, the activity of caspases was significantly higher than those of the control group (2.04-fold for caspase-3, 2.03-fold for caspase-8, and 2.14-fold for caspase-9), and AdipoRon treatment decreased the activity of caspase-3, caspase-8, and caspase-9 to 1.34-fold, 1.31-fold, and 1.43-fold of the control, respectively [Figure 6A–C]. This experiment also used WB to detect the protein level of cleaved caspase-3 in liver tissue. As shown in Figure 7, AdipoRon treatment can clearly down-regulate LPS/D-Gal-induced increase in the level of cleaved caspase protein (0.28-fold to that of the LPS/D-Gal group).

Discussion

Adiponectin is an important adipokine with a variety of regulatory activities in metabolism and inflammation.^[2,5] AdipoRon is a small-molecule adiponectin receptor

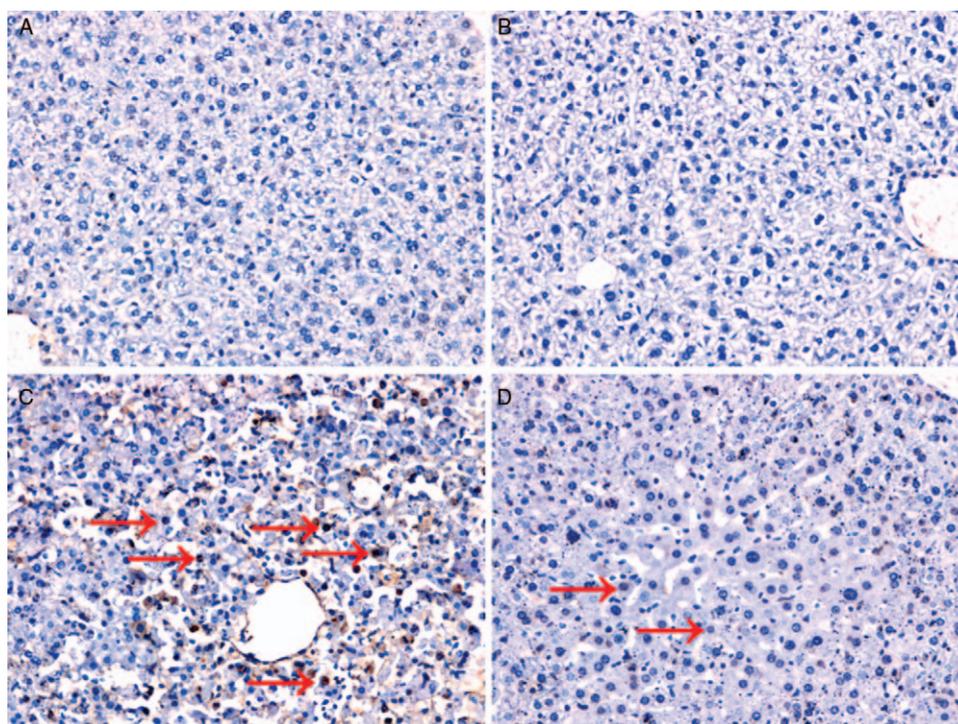


Figure 5: AdipoRon suppressed LPS/D-Gal-induced apoptosis of hepatocytes. Apoptotic cells were assayed by TUNEL assay; the positive cells showed dark brown nuclei and indicated by arrows. Representative liver slices for the control group (A), the AdipoRon group (B), the LPS/D-Gal group (C) and the AdipoRon + LPS/D-Gal group (D) were shown (original magnification, $\times 400$). LPS/D-Gal: Lipopolysaccharide/D-galactosamine; TUNEL: Terminal deoxynucleotidyl transferase-mediated nucleotide nick-end labeling.

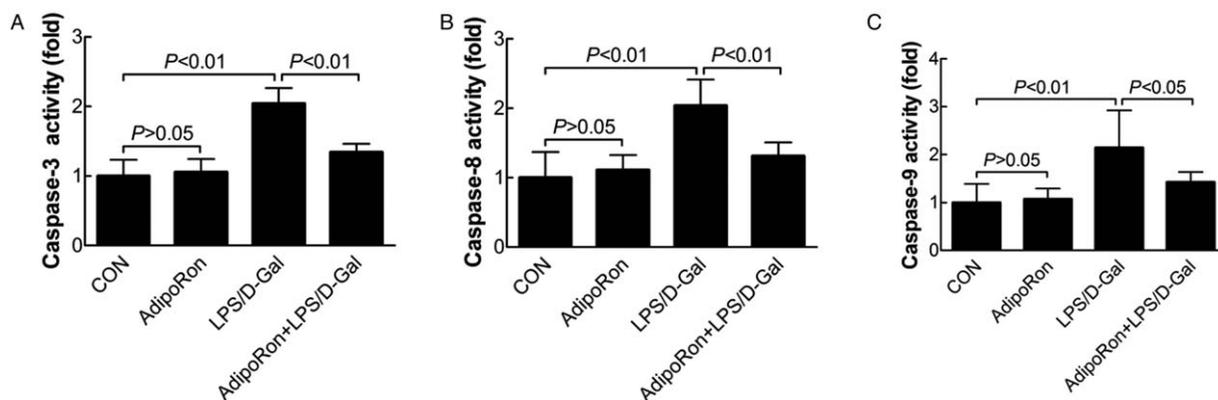


Figure 6: AdipoRon inhibits caspase activity in the LPS/D-Gal model. It was found that there was no significant difference in caspase activity between the normal control group and AdipoRon treated group. The activity of caspase in liver tissue of LPS/D-Gal model group was significantly enhanced ($n = 8$, $P < 0.01$), and AdipoRon treatment showed decreased caspase-3 (A), caspase-8 (B), and caspase-9 (C) activity ($n = 8$, $P < 0.01$). LPS/D-Gal: Lipopolysaccharide/D-galactosamine.

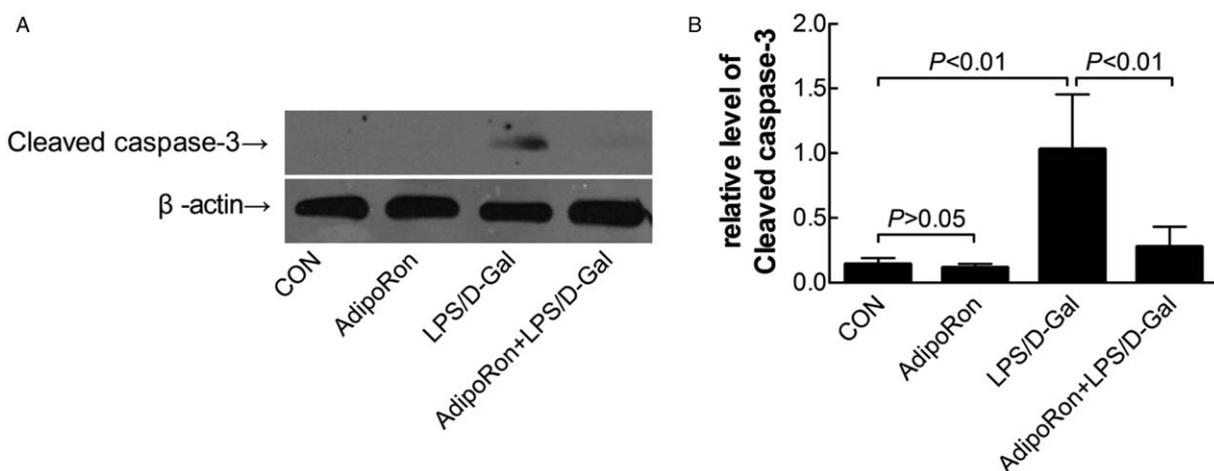


Figure 7: AdipoRon suppressed the levels of cleaved caspase-3 in LPS/D-Gal-induced mice. The levels of cleaved caspase-3 in the liver were determined by immunoblot analysis. AdipoRon treatment significantly down-regulated LPS/D-Gal-induced increase in cleaved caspase-3 protein levels. LPS/D-Gal: Lipopolysaccharide/D-galactosamine.

agonist with beneficial effects on diabetes and atherosclerosis.^[12,14] Previous study has reported that LPS/D-Gal induced more severe liver injury in adiponectin deficient mice.^[20] In the present study, we found that treatment with AdipoRon suppressed the production of TNF- α , attenuated hepatocyte apoptosis, alleviated liver injury, and improved the survival in LPS/D-Gal-exposed mice. Therefore, AdipoRon might have potential value for the pharmacological intervention of acute hepatitis.

In the LPS/D-Gal-induced hepatitis model, the induction of the pro-inflammatory cytokine TNF- α plays central roles in the development of liver injury.^[21] The anti-inflammatory properties of adiponectin have been well-documented in previous studies both *in vitro* and *in vivo*.^[7-11] In addition, the suppressive effects of adiponectin on TNF- α production have been confirmed in uric acid-insulted renal tubular epithelial cells, LPS-stimulated cardiomyocytes, and palmitic acid-exposed endothelial.^[11,22,23] In the present study, LPS/D-Gal-induced production of TNF- α

was suppressed by AdipoRon, which might contribute greatly to the beneficial outcomes in AdipoRon-treated animals.

Adiponectin not only has anti-inflammatory effects, but also has anti-apoptotic effects in various diseases. Studies have shown that adiponectin can attenuate vascular endothelial apoptosis and alleviate neuronal apoptosis.^[24-27] In line with the anti-apoptotic activities of adiponectin, AdipoRon suppressed post-ischemic myocardial apoptosis and diabetes-induced apoptosis in the kidney in experimental animal studies.^[28,29] In addition, treatment with AdipoRon also inhibited the apoptosis of glomerular endothelial cells induced by palmitate or high concentration of glucose.^[28,30] Consistently, treatment with AdipoRon inhibited the activation of hepatic caspases, suppressed the cleavage of caspase-3 and reduced the count of TUNEL-positive cells. Therefore, the protective benefits of AdipoRon in LPS/D-Gal-induced acute hepatitis might result from its anti-inflammatory and anti-apoptotic properties.

Interestingly, recent studies have revealed the correlation between adiponectin levels and hepatic disorders.^[31] And the decreased level of circulating adiponectin has been regarded as a critical risk factor for the progression of NAFLD and liver fibrosis.^[31] On the contrary, supplementary of recombinant adiponectin provided protective effects in mice with NAFLD and liver fibrosis.^[32,33] In addition, treatment with adiponectin also resulted in beneficial outcomes in experimental animals with liver ischemia-reperfusion injury or virus infection.^[34,35] The main limitation of the present study is whether the hepatoprotective effects of AdipoRon is exactly mediated by the adiponectin receptor is unclear. In addition, the downstreaming molecular mechanism underlying the hepatoprotective effects of AdipoRon/adiponectin remains to be further investigated.

Taken together, our study showed that treatment with AdipoRon reduced suppressed LPS/D-Gal-induced inflammatory response and hepatocyte apoptosis, resulting in alleviated liver injury and improved animal survival. Although the molecular mechanisms underlying the protective effects of AdipoRon in acute hepatitis remains to be investigated, our data suggest that AdipoRon might become a beneficial reagent for the treatment of acute hepatitis.

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Conflicts of interest

None.

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