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Chlorogenic acid alleviates autophagy and insulin resistance by suppressing JNK pathway in a rat model of nonalcoholic fatty liver disease

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Non-alcoholic fatty liver disease (NAFLD) is one of the leading causes of chronic liver diseases around the world and commonly associated with insulin resistance and hyperlipidemia. Chlorogenic acid (CG) was reported to have insulin-sensitizing activity and exert hypocholesterolemic and hypoglycemic effect. However, the involvement of CG in NAFLD remains far from being addressed. In this study, a high-fat diet-induced NAFLD rat model was used to investigate the biological roles and underlying mechanism of CG in NAFLD. The results showed that high-fat diet-fed rats exhibited an increase in body weight, glucose tolerance, liver injury, insulin resistance, as well as autophagy and C-Jun N-terminal kinase (JNK) pathway. Nevertheless, all these effects were alleviated by CG treatment. Moreover, angiotensin treatment in CG group activated the JNK pathway, and promoted autophagy, insulin resistance, and liver injury. In conclusion, our findings demonstrated that CG ameliorated liver injury and insulin resistance by suppressing autophagy via inactivation of JNK pathway in a rat model of NAFLD. Therefore, CG might be a potential application for the treatment of NAFLD.

Keywords. Autophagy; chlorogenic acid; insulin resistance; JNK; NAFLD

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is deemed as the most frequent chronic hepatic disorder in children and adolescent in developed countries, leading to a broad spectrum of severe pathologies, such as steatosis, nonalcoholic steatohepatitis (NASH), cirrhosis, and even hepatocellular carcinoma (Amato *et al.* 2017). NAFLD is considered as a hepatic manifestation of metabolic syndrome due to its association with hypertension, obesity, dyslipidemia and type 2 diabetes (Lim *et al.* 2015). A prevalent hypothesis for NAFLD development points out that insulin resistance, as the "first-hit" to the liver, elicits the onset of second hits, such as oxidative stress, inflammation, apoptosis, and autophagy (Polyzos *et al.* 2012). Indeed, insulin sensitizers have been demonstrated to be able to improve biochemical and histological features of NAFLD (Ozturk and Kadayifci 2014). In addition, autophagy is also reported to be linked to liver physiology and pathogenesis (Czaja 2016). In recent years, NAFLD has reached epidemic proportions with the rapid increase of morbidity (Cimini *et al.* 2017). However, there is no effective therapeutic approach for the treatment of NAFLD. Suitable animal models offer the possibility to investigate

the mechanisms involved in the complex pathophysiological processes and progression of NAFLD.

Natural polyphenols have been proposed to treating various metabolic disorders, including NAFLD (Salomone *et al.* 2016). Chlorogenic acid (CG), an ester of caffeic acid and quinic acid, is a phenolic metabolite extracted from plants, fruits, vegetables, and coffee drinks (Azuma *et al.* 2000). In addition to its strong antioxidative characteristic, CG was reported to have many other biological properties, such as antimicrobial (Zhu *et al.* 2004), anti-inflammatory (Jiang and Dusting 2003) and insulin-sensitizer activities (Ma *et al.* 2015). CG can improve lipid and glucose metabolism by facilitating triglyceride (TG) and free fatty acids (FFA) clearance and increasing insulin sensitivity (Li *et al.* 2009). However, the involvement of CG in NAFLD and its possible mechanism remain unknown.

C-Jun N-terminal kinases (JNKs; including JNK1, JNK2 and JNK3 isoforms), can be activated by inflammatory cytokines and FFA (Hirosumi *et al.* 2002; Sethi and Hotamisligil 1999). It has been documented that activation of JNK pathway resulted in lipid accumulation and liver damage (Schattenberg *et al.* 2006). The JNK signaling transduction pathway, as a link between inflammation and metabolic diseases, was also revealed to be implicated in the pathogenesis and development of NAFLD and obesity-driven insulin resistance (Liu and Rondinone 2005; Solinas and Becattini 2017). More importantly, a previous study reported that inactivation of JNK inhibited autophagy and alleviated insulin resistance in a NAFLD rat model (Yan *et al.* 2017). Therefore, this study attempted to discuss whether CG was able to attenuate insulin resistance and inhibit NAFLD development by regulating JNK signaling pathway.

2. Materials and methods

2.1 *Animals and treatments*

Healthy female Sprague–Dawley rats (age, 8–9 weeks; weight, 197 ± 4 g) were obtained from Beijing Huafukang Biological Science and Technology Stock Co. Ltd (Beijing, China). All rats were kept at a well controlled temperature (22 ± 2 °C) and humidity (50 ± 5%) in a pathogen-free facility with a 12 h light/dark cycle and standard diet. After one-week acclimation, the rats were randomly distributed into 3 different dietary groups (n = 6/group): normal diet group (NG), high-fat diet group (HG), and chlorogenic acid group (CG). The rates in NG group were fed with normal chow diet and administrated with normal saline by oral gavage three times a week from the fourth week. The HG rats were fed with high-fat diet to induce NAFLD and received equal amount of normal saline by oral gavage three times a week from the fourth week. In CG group, the rats fed a high-fat diet were treated with CG (50 mg/kg; Sigma, St Louis, MO, USA) in normal saline by oral gavage three times a week from the fourth week. After 20 weeks, rats were fasted overnight and then euthanized with a high dose of phenobarbitone via intraperitoneal injection. The blood, muscle, adipose and liver sample were collected and stored at −80 °C for subsequent analysis. In addition, the rats in CG group were further subcutaneously injected with anisomycin (150 mg/kg body weight) diluted in an equivalent volume of 0.9% saline. All animal experimental protocol was carried out with approval of Animal Care and Use Committee of Shaanxi Provincial People's Hospital.

2.2 *Biochemical analyses*

The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (T-CHO) and triglyceride (TG) were measured using commercially available kits (Accurex, Mumbai, India) and a multifunctional biochemistry analyzer (AU600; Olympus, Tokyo, Japan). The serum level of tumor necrosis factor (TNF)-α was detected by an enzyme-linked immunosorbent assay (ELISA)-based commercial kit (Assaypro, St. Charles, MO, USA). Serum level of FFA was assayed using a commercial kit (Bioassay System, Hayward, CA, USA). Insulin level was determined with a rat insulin radioimmunoassay kit (Linco Research Inc, St Charles, MO, USA).

2.3 *Glucose tolerance and insulin resistance tests*

Glucose tolerance tests were performed after the rats in different groups (NG, HG, CG) were treated for 20 weeks. The rats received an intraperitoneal administration of glucose (2.0 g/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) or insulin (1.5 IU/kg of body weight; R&D systems, Minneapolis, MN, USA) following 6 h fasting. At 15, 30, 60 and 120 min after glucose injection, blood samples were harvested from tail vein and subjected to a glucometer (Roche diagnostic GmbH, Germany) for glucose level measurement. Plasma insulin levels were measured using the commercial ELISA kits (Crystal Chem, Downers Grove, IL, USA). The The livers were excised for western blot analysis of phospholyrated insulin receptor (IR)β-subunit (IRβ) (p-IRβ), phospholyrated IR substrate-1 (IRS-1) (p-IRS-1) (Ser307) and phospholyrated protein kinase B (PKB/Akt) (p-Akt) after 30 min of injection.

2.4 *Western blot analysis*

Proteins were extracted from liver tissues using lysis buffer (Beyotime, Haimen, China) and quantified by a BCA Protein Assay Kit (Beyotime). A total of 20 μg protein sample was

separated by 8% SDS-PAGE and blotted onto PVDF membrane. After blocked with 5% nonfat powdered milk in TBST for 1 h, the membranes were then incubated with appropriate primary antibodies at 4 °C overnight, followed by probed with secondary antibody conjugated to horseradish peroxidase. The protein signals were visualized using an ECL detection system. The primary antibodies including Atg3, Atg5, Beclin-1, LC3-I, LC3-II, p-IRβ, IRβ, p-IRS-1 (Ser307), IRS-1, p-Akt, and Akt were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

2.5 *Statistical analysis*

All experimental results are displayed as mean ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test or one-way analysis of variance by SPSS statistical software (version 10.0; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was defined as statistically significant.

3. Results

3.1 *CG treatment alleviated liver injury and insulin resistance in a rat model of NAFLD*

First, the body weight of rats in different groups was measured once every 4 weeks for 20 weeks. As shown in figure 1A, the body weight of rats in HG group was significantly higher than that in NG group after 4 weeks of high-fat diet feeding, while the rat body weight in CG group was obviously decreased compared to HG group. To further explore the effect of CG on insulin resistance in NAFLD, the blood glucose and insulin levels in rats of NG, HG, and CG groups were determined at 0, 15, 30, 60, and 120 min after intraperitoneal injection with glucose or insulin. The results displayed that glucose and insulin levels in HG group were markedly higher at 15, 30, 60, and 120 min after injection of glucose than NG group, which was dramatically attenuated by CG administration (figure 1B), suggesting that CG treatment alleviated glucose tolerance in HG rats. Additionally, insulin tolerance test showed that CG treatment also ameliorated insulin resistance in HG rats (figure 1F). We further analyzed the effect of CG on liver function. The serum levels of liver function parameters including TG, T-CHO, ALT, and AST (figure 1C) were obviously increased in HG group compared with NG group, however, this effect was strikingly suppressed after CG treatment. Additionally, the serum levels of TNF-α (figure 1D) and FFA (figure 1E) were also remarkably elevated in HG group when compared to NG group, while CG administration relieved this influence. All these data demonstrated that CG treatment alleviated liver damage and insulin resistance in a high-fat diet-induced NAFLD rat model.

3.2 *CG treatment attenuated autophagy, insulin resistance, and suppressed JNK pathway in a rat model of NAFLD*

It has been reported that high-fat diet could activate the JNK pathway in NAFLD. To further explore the effect of CG on high-fat diet-induced JNK activation, the protein levels of p-JNK1 and JNK1 were determined in muscle, adipose and liver samples from rats of NG, HG and CG groups by western blot. As a result, the relative protein level of p-JNK1/JNK was significantly improved in the muscle, adipose and liver samples of rats in HG group, while CG treatment effectively suppressed high-fat diet-induced increase of p-JNK1/JNK1 level (figure 2A), suggesting that CG treatment suppressed activation of the JNK pathway. To evaluate the effect of CG on autophagy in the rat model of NAFLD, the protein levels of autophagy-related markers LC3-I, LC3-II, Beclin-1, Atg3, and Atg5 in the liver samples of rats were analyzed. As shown in figure 2B, the conversion from LC3-I to LC3-II, as well as the expressions of Beclin-1, Atg3, and Atg5 were all markedly improved in HG group, which were strikingly repressed after CG treatment, indicating that CG treatment hindered autophagy in NAFLD. The insulin resistance was assessed by detecting the expressions of p-IRβ, IRβ, p-IRS-1 (Ser307), IRS-1, p-Akt, and Akt after the rats of NG, HG and CG group were fasted overnight and intraperitoneally administered with 1.5 U/kg insulin. As presented in figure 2C, the expression levels of p-IRβ, p-IRS-1, and p-Akt in the liver samples were markedly increased in high-fat diet-fed rats of HG group, whereas this effect was mitigated by CG treatment (figure 2C). Taken together, these results revealed that CG treatment restrained high-fat diet-triggered autophagy, insulin resistance, and JNK pathway in a NAFLD rat model.

3.3 *CG suppressed autophagy and alleviated insulin resistance by inhibiting the JNK pathway in a rat model of NAFLD*

To confirm the biological role of CG in the pathological process of NAFLD was mediated by the JNK pathway, angiotensin was subcutaneously injected into rats in CG group to activate the JNK pathway. Western blot analysis showed that anisomycin administration significantly promoted the phosphorylation of JNK1 in rats of CG group (figure 3A). The effect of anisomycin on autophagy was examined by detecting the protein levels of LC3-I, LC3-II, Beclin-1, Atg3, and Atg5. Anisomycin treatment obviously increased the ratio of LC3-II/LC3-I, and enhanced the expression of Beclin-1, Atg3, and Atg5 in the liver samples of rats in GC group (figure 3B). Furthermore, anisomycin treatment also increased the phosphorylation of Akt, IRS-1 and IRβ in the liver samples of rats in GC group (figure 3C).

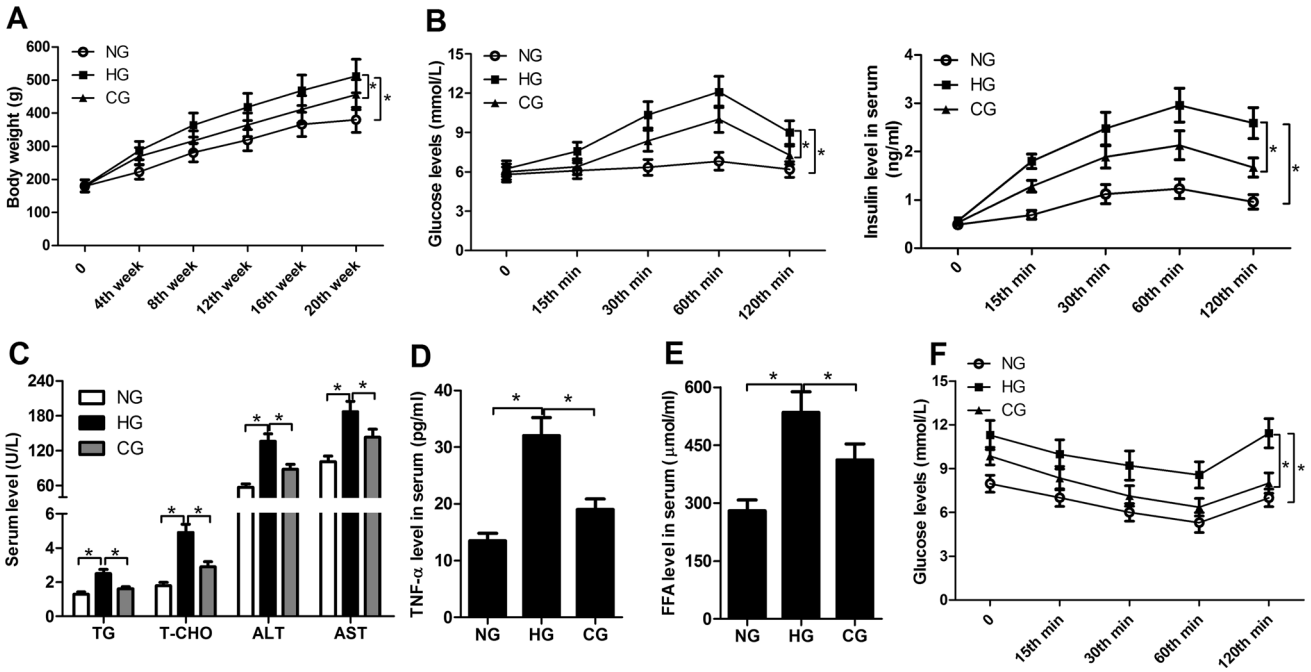

Figure 1. CG alleviated high-fat diet-induced liver injury and insulin resistance in NAFLD rat model. (**A**) Body weight of rats in NG, HG, and CG groups was measured every 4 weeks for 20 weeks. (**B**) Blood glucose and insulin levels of rats in NG, HG, and CG groups were determined at 0, 15, 30, 60, 120 min after intraperitoneal injection with glucose. The serum levels of TG, T-CHO, ALT, and AST (**C**); TNF-α (**D**), and FFA (**E**) in rats of NG, HG, and CG groups were measured. (**F**) Blood glucose levels of rats in NG, HG, and CG groups were determined at 0, 15, 30, 60, 120 min after intraperitoneal injection with insulin. *$P < 0.05$.

Together, we concluded that CG suppressed autophagy and alleviated insulin resistance by inhibiting JNK pathway in a NAFLD rat model.

3.4 *CG attenuated liver injury by suppressing JNK pathway in a rat model of NAFLD*

To confirm the effect of JNK activation on liver injury in NAFLD, the serum levels of TG, T-CHO, ALT, AST, TNF-α, FFA and insulin in the rats of CG group subcutaneously injected with anisomycin were also measured. As illustrated in figure 4A-4D, anisomycin treatment significantly improved the serum levels of TG, T-CHO, ALT, AST, TNF-α, FFA and insulin in the rats of CG group. Together, these results demonstrated that CG attenuated liver injury by retarding JNK pathway in a rat model of NAFLD.

4. Discussion

It is well known that NAFLD is the primary cause of progressive liver disease worldwide. Emerging evidence suggests that NAFLD onset is characterized by an abnormal fat accumulation and inflammation in hepatic cells (Xiang *et al.* 2016). Insulin resistance and autophagy are demonstrated to be closely involved in the pathological progression of NAFLD (Dongiovanni *et al.* 2017; Mao *et al.* 2016). Although great efforts have been made in the etiology of NAFLD, the detailed pathological mechanisms of NAFLD have yet to be identified. In the present study, it is demonstrated that CG treatment alleviated high-fat diet-induced liver injury, autophagy, and attenuated high-fat diet-induced insulin resistance in the rat model of NAFLD. Furthermore, the related beneficial effects of CG on NAFLD were mediated by the JNK pathway.

CG, the major component in coffee, exerts beneficial effects on glucose and lipid metabolism disorders as well as weight gain induced by high fat diet (McCarty 2005; Wan *et al.* 2013). Ma *et al.* reported that CG treatment repressed high-fat diet-induced hepatic steatosis and insulin resistance in mice (Ma *et al.* 2015). Ghadieh *et al.* demonstrated that oral administration of CG abated high-fat diet-induced body weight gain and insulin resistance in mice (Ghadieh *et al.* 2015). In accordance with these previous studies, our study suggested that CG treatment ameliorated high-fat diet-induced liver injury and insulin resistance in a rat model of NAFLD. Previous documents have clarified the dysregulated autophagy in the liver of NAFLD patients and NAFLD murine model (Li *et al.* 2014; Ma *et al.* 2013). Our study also found that CG treatment reduced autophagy-related gene expression in a rat model of NAFLD, suggesting that

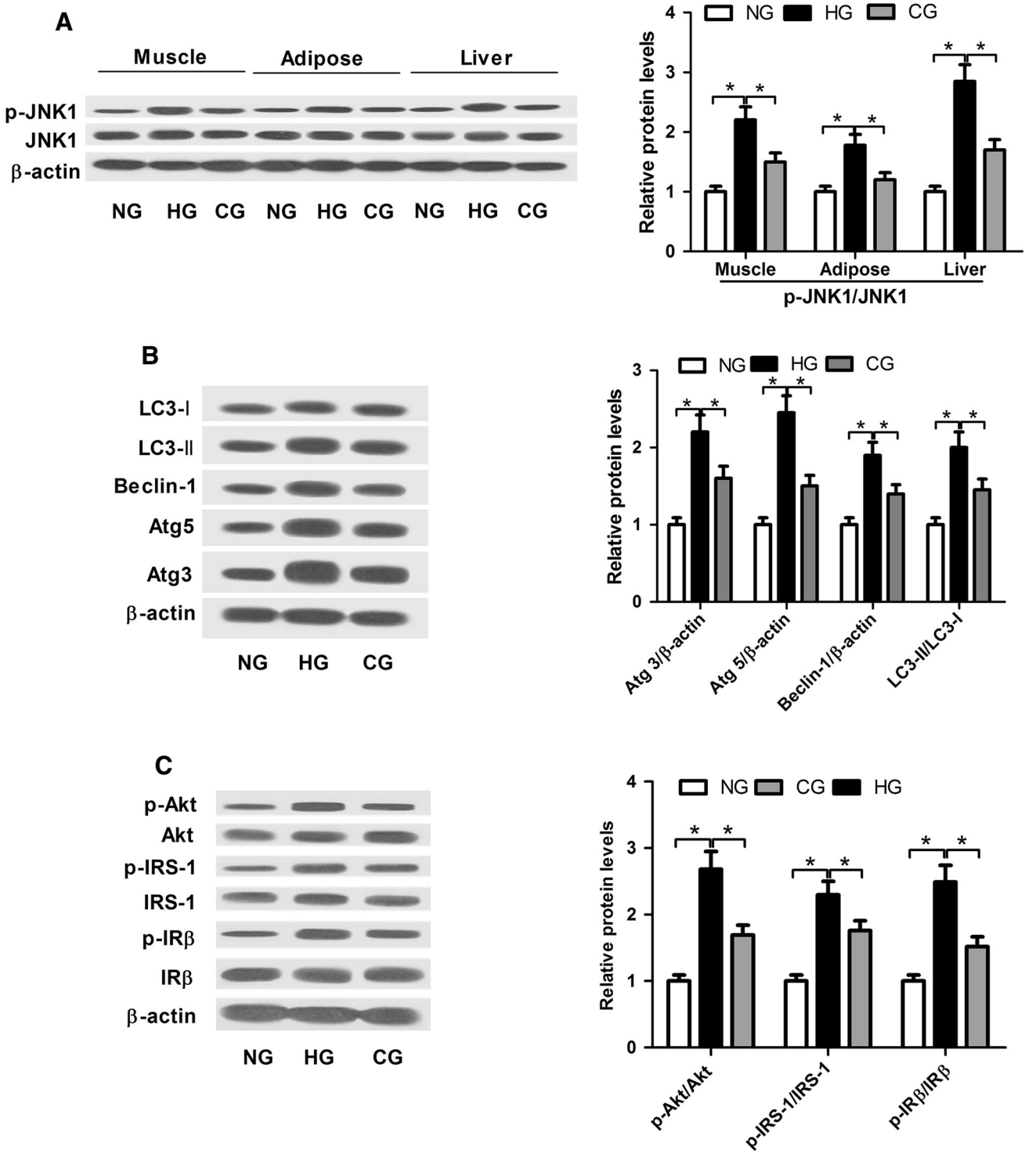

Figure 2. CG inhibited high-fat diet-induced autophagy, insulin resistance, and JNK pathway in NAFLD rat model. (**A**) Western blot analysis for p-JNK1 and JNK1 in the muscle, adipose and liver samples of rats in NG, HG, and CG groups. (**B**) Western blot analysis for LC3-I, LC3-II, Beclin-1, Atg3, and Atg5 in the liver samples of rats in NG, HG, and CG groups. (**C**) Western blot analysis for p-IRβ, IRβ, p-IRS-1 (Ser307), IRS-1, p-Akt, and Akt in the liver samples of rats in NG, HG, and CG groups. *$P < 0.05$.

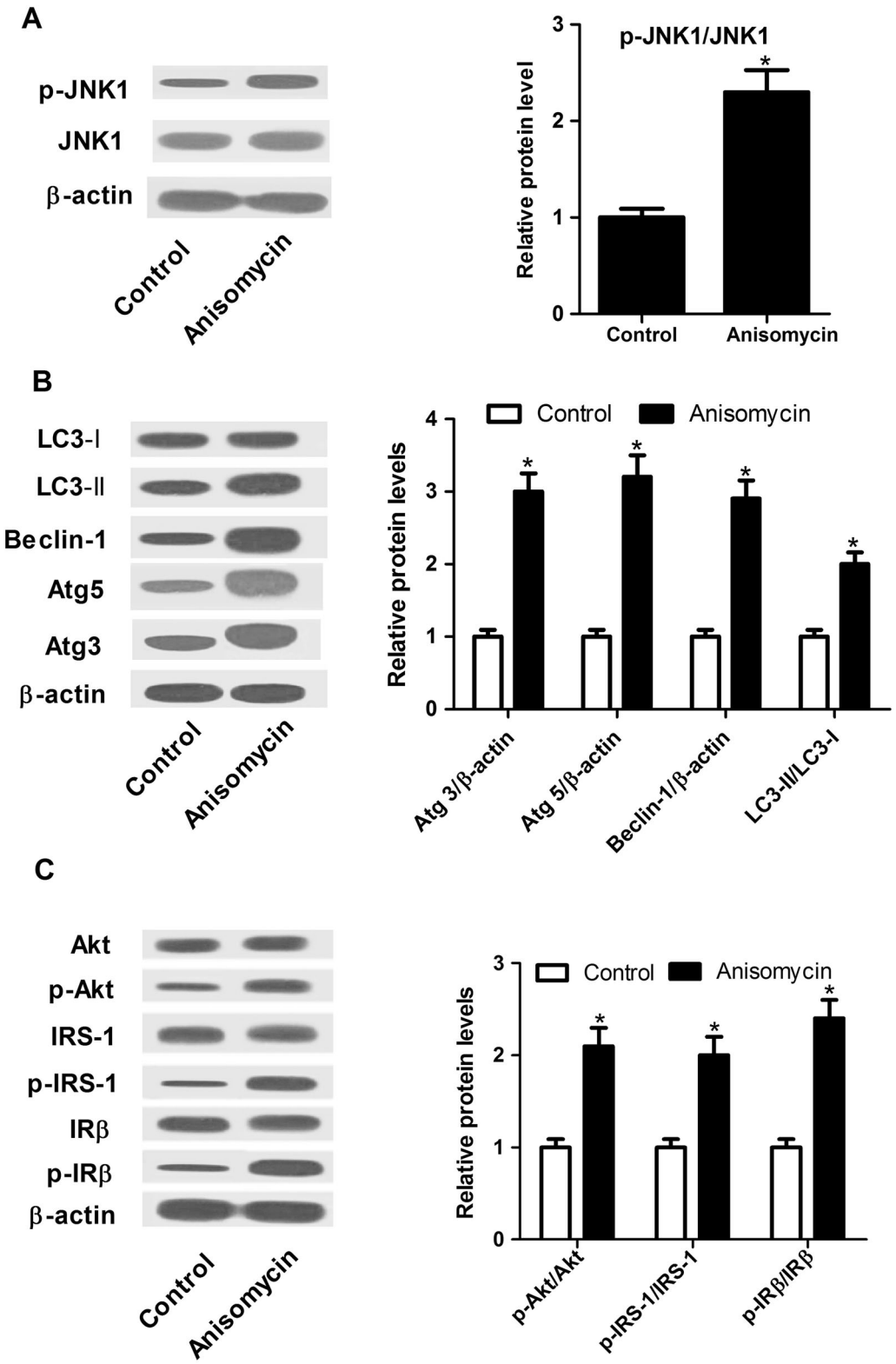

Figure 3. Angiotensin reversed CG-induced inhibition on autophagy and insulin resistance in NAFLD rat model. After the rats in CG group were subcutaneously injected with 150 mg/kg anisomycin, the protein levels of p-JNK, JNK (**A**), LC3-I, LC3-II, Beclin-1, Atg3, and Atg5 (**B**), p-IRβ, IRβ, p-IRS-1 (Ser307), IRS-1, p-Akt, and Akt (**C**) in the liver were estimated by Western blot. $^{*}P < 0.05$.

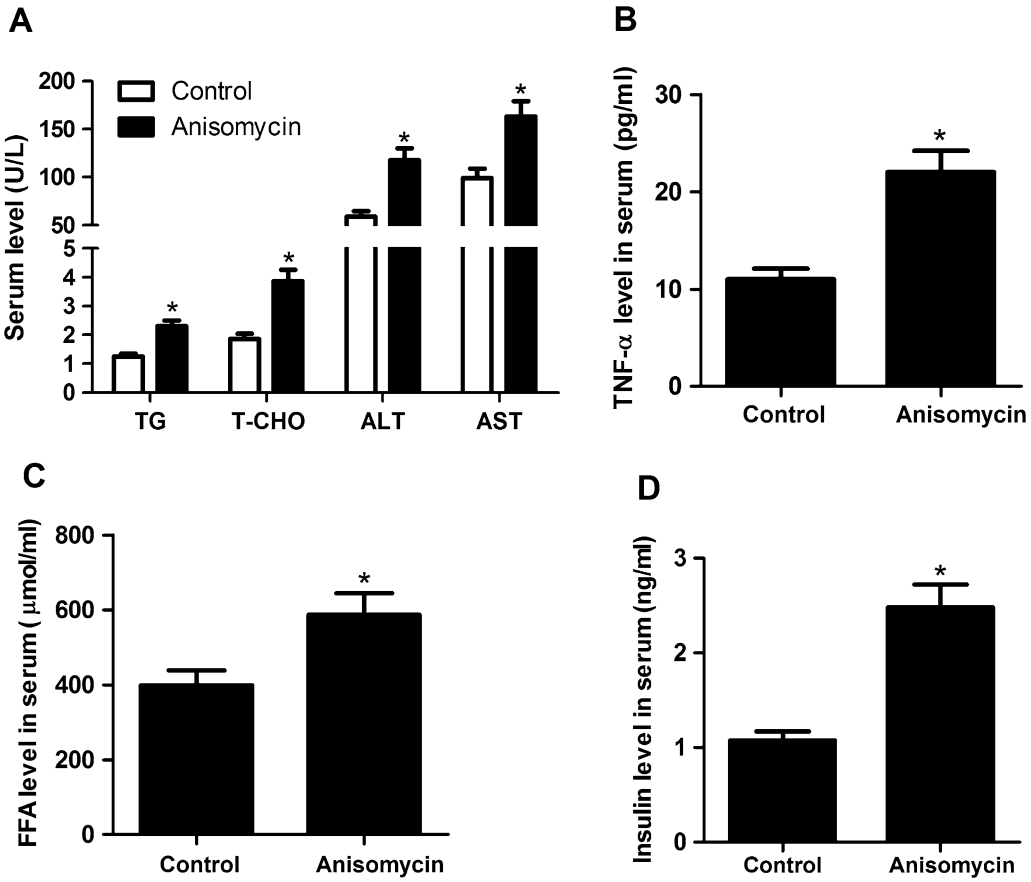

Figure 4. Angiotensin abated the protective effect of GC on liver injury in NAFLD rat model. After the rats in CG group were subcutaneously injected with 150 mg/kg anisomycin, the serum levels of TG, T-CHO, ALT, and AST (**A**); TNF-α (**B**), FFA (**C**) and insulin (**D**) were also measured. *$P < 0.05$.

CG blocked high-fat diet-induced autophagy. Taken together, these results indicated that CG treatment suppressed the progression of NAFLD by ameliorating high-fat diet-induced insulin resistance and autophagy. However, Mubarak *et al.* revealed that supplementation of a high-fat diet with CG resulted in increased insulin resistance and impaired fatty acid oxidation (Mubarak *et al.* 2013), discrepant with our findings. CG administration, dose, and mice strain may explain this variation.

JNK has been confirmed as a vital mediator for obesity and obesity-related disruption to metabolic homeostasis (Pal *et al.* 2016). JNK1 was identified as a crucial molecular link between obesity, inflammation, insulin resistance and disorders of glucose homeostasis in NAFLD (Kodama and Brenner 2009). It was previously reported that JNK1 and JNK2 both mediated insulin resistance in high-fat diet-fed mice, but the JNK isoforms had variant influence on steatohepatitis, with JNK1 aggravating steatosis and hepatitis and JNK2 repressing hepatocyte cell death by preventing the mitochondrial death pathway (Singh *et al.* 2009). Notably, JNK inhibition was demonstrated to suppress autophagy and attenuate insulin resistance in a rat model of NAFLD (Yan *et al.* 2017). In this study, it is found that CG treatment suppressed the phosphorylation of JNK1 in the muscle, adipose and liver samples of high-fat diet-fed rats, indicating that CG treatment restrained the JNK pathway. In addition, the study further clarified that activation of JNK signaling pathway by anisomycin increased autophagy and insulin resistance. Furthermore, JNK overturned the protective effect of CG on NAFLD.

In summary, it is demonstrated that CG treatment attenuated high-fat diet-induced liver injury, inhibited autophagy and ameliorated insulin resistance in a rat model of NAFLD, which was mediated by suppressing JNK pathway. Therefore, CG might be a good choice for the treatment of NAFLD. However, further clinical experiments are required to be conducted to confirm the biological roles of GC in NAFLD.

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