

Original Article

Diosgenin glucoside protects against myocardial injury in diabetic mice by inhibiting RIP140 signaling

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Abstract: Myocardial injury is often observed during diabetes, but the nature physiological association is unclear. Here, we investigated the protective effective of diosgenin glucoside (DG), a pharmacologically active saponin extracted from *Tritulus terrestris* L., against myocardial injury in type 2 diabetic (db/db) mice and its molecular mechanism of action. Levels of serum and myocardial tissues, blood glucose and inflammatory cytokines, as well as cardiac function indicators, of db/db mice were measured, and DG's mechanism of action was evaluated by immunohistochemistry and Western blotting. We found that long-term DG treatment improved glucose tolerance and lipid profiles, reduced production of IL-1 β , IL-6, and TNF- α , and decreased serum levels of the cardiac injury indicators creatine kinase and lactate dehydrogenase. Interestingly, DG also inhibited RIP140 signaling, which normally regulates transcription of estrogen receptor genes and influences expression of proinflammatory cytokines. Our study revealed a novel mechanism of DG's anti-inflammatory effect against myocardial injury via RIP140 signaling modulation in diabetic mice.

Keywords: Diosgenin glucoside, myocardial injury, inflammation, RIP140, diabetes mellitus

Introduction

Diabetes mellitus (DM) is a metabolic disease with various causes and is characterized by chronic hyperglycemia associated with insulin secretion/action dysfunction [1]. The number of DM patients worldwide is expected to reach 592 million by 2035 [2]. Diabetes leads to abnormal metabolism of sugar, fat, and protein and subsequent serious complications, such as blindness, renal failure, liver injury, nerve injury, and atherosclerosis [3, 4]. Myocardial injury is a common co-morbidity of DM patients [5, 6] manifested by inflammation, hepatic fibrosis, and lipid accumulation [7]. Several studies have reported that anti-inflammatory agents exert protective effects against DM-associated myocardial injury [8], suggesting their utility in treating this condition.

Receptor-interacting protein 140 (RIP140) plays an important role in the mediating the inflammatory response of macrophages by pro-

moting expression of cytokines IL-6 and TNF- α via interaction with nuclear factor kappa B (NF- κ B) and cAMP response element binding protein (CREB) [9, 10]. Recently, RIP140 was implicated as a global signaling regulator in the diabetic environment and identified as a promising molecular target for treating DM [11]. In an experimental model of endotoxin tolerance, RIP140 specifically modulated production of NF- κ B-dependent proinflammatory cytokines [12].

Diosgenin glucoside (DG) is a saponin extracted from *Tritulus terrestris* L. with various pharmacological activities related to anti-inflammation and neuroprotection [13, 14]. However, to our knowledge, no study of DG's possible therapeutic effect against diabetic cardiomyopathy has been performed. Therefore, our research aims to evaluate the impact of DG on DM-associated myocardial injury and explore its molecular mechanism of action in a diabetic mouse model.

Materials and methods

Reagents and kits

Glucose and DG were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for detecting IL-1 β , IL-6, and TNF- α were purchased from Elabscience (Wuhan, China). Commercial kits for quantifying total triglycerides (TG), total cholesterol (TC), creatine kinase (CK), and lactate dehydrogenase (LDH), insulin were purchased from Jiancheng Bioengineering Institute (Nanjing, China), and primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Animals

Male C57BL/KsJ type 2 diabetic (db/db) mice and non-diabetic control (db/m) mice were purchased from the animal model research center of Nanjing University and placed under specific pathogen-free conditions. Mice were randomly divided into five groups (10 mice in each group): db/m group, db/db group, db/db + metformin (met, 400 mg/kg) group, db/db + DG (DG, 50 mg/kg) group, and db/db + DG (DG, 100 mg/kg) group. DG was administered orally. Treatments occurred daily for 12 weeks, during which animals had free access to food and water. Our animal welfare standards strictly followed the procedures for the care and use of experimental animals. All study procedures were reviewed and approved by Nanjing Medical University.

Oral glucose tolerance test (OGTT)

OGTT in all mice was conducted during the last week of the experiment. Briefly, mice fasted for 18 h and were then fed glucose (2 g/kg) orally. Blood samples were collected at 0, 30, 60, 90, and 120 min and centrifuged at 4°C (4000 \times g) for 10 min to obtain serum for glucose determination with an automatic analyzer.

Electrocardiogram (ECG) measurement

ECG was tested by BL-4200 biological function experimental system (Chengdu, China).

Histological analysis

Heart specimens were immediately excised, fixed in 4% paraformaldehyde (PFA), paraffin-

embedded, and cut into 5- μ m-thick sections. Embedded sections were de-paraffinized in xylene and absolute ethanol, stained with hematoxylin and eosin (H&E), and observed at 200 \times magnification with an optical microscope (Nikon, Tokyo, Japan).

Molecular assays of serum and cardiac tissue

Levels of serum glucose, TG, TC, CK, LDH, insulin were determined by commercial kits according to the manufacturer's instructions. Concentrations of IL-1 β , IL-6, and TNF- α in serum and cardiac tissue were determined via ELISA according to the manufacturer's protocol.

Quantitative RT-PCR

RNA was extracted using TRIzol reagent (Takara, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized via first-strand cDNA synthesis with a PrimeScript RT reagent Kit (Takara, Tokyo, Japan). RT-PCR was performed using the CFX 96 q-PCR system (Bio-Rad, California, USA). A SYBR Green RT-PCR Kit (Takara, Tokyo, Japan) was used for quantitative RT-PCR analyses. All reactions were performed in a 20 μ l reaction volume in triplicate. The relative expression levels of target genes were normalized against the level of GAPDH. The following primers were used for RT-PCR: RIP140 forward: 5'-TGTCTTAACCTA-CCTCGAAGGGT-3', reverse: 5'-CGTCTGGGTAC-TGACAGTGG-3'; NF- κ B forward: 5'-AGCGCG-GGGACTATGACTT-3', reverse: 5'-GCCCCGGTTA-TCAAAAATCGGAT-3'; I κ B α forward: 5'-TGAA-GGACGAGGAGTACGAGC-3', reverse: 5'-TGCA-GGAACGAGTCTCCGT-3'; GAPDH forward: 5'-TGGCCTTCCGTGTTCTTAC-3', reverse: 5'-GAG-TTGCTGTTGAAGTCGCA-3'.

Immunohistochemistry

Expression of RIP140 and p-NF- κ B in the heart of db/db mice was detected by immunohistochemical staining. Briefly, cardiac tissue sections were de-paraffinized as described above, microwaved in sodium citrate buffer, and washed with PBS. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide for 20 min, and sample were blocked with 5% goat serum for 20 min before incubation with primary antibodies against RIP140 (1:1,000) and p-NF- κ B (1:200) overnight at 4°C.

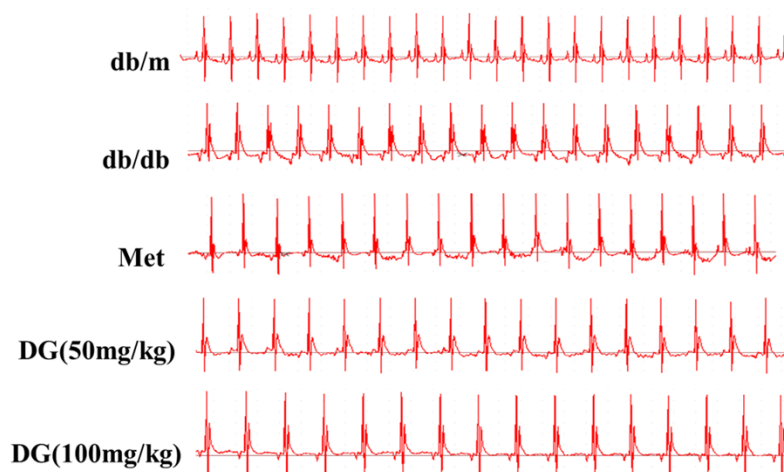


Figure 1. Effects of DG on ST segment elevation in control and diabetic mice. Electrocardiograms of indicated control and treatment groups are shown.

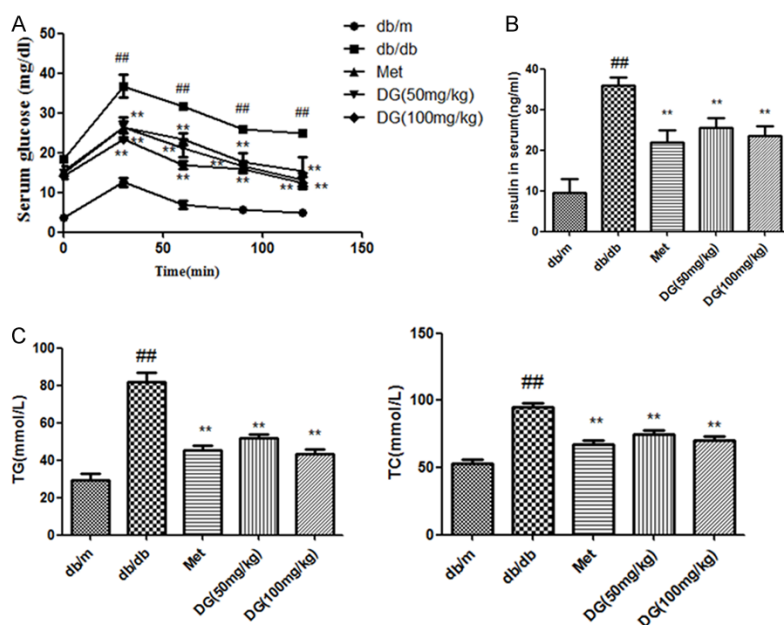


Figure 2. Effects of DG on diabetic biochemical parameters in control and diabetic mice. Measurements of serum glucose (A), insulin (B), and lipids (C) were obtained from diabetic mice and non-diabetic controls after 12 weeks of treatment with the indicated compound. All data are presented as means \pm SD. Compared with db/m mice: * $P < 0.05$, ** $P < 0.01$. Compared with db/db mice: * $P < 0.05$, ** $P < 0.01$. TG, triglycerides; TC, total cholesterol.

Next, sections were washed with PBS three times, treated with goat anti-rabbit IgG secondary antibody for 20 min, and incubated with horseradish peroxidase-labeled streptavidin working solution for 20 min. After three additional PBS washes, the samples were stained with 3-3' diaminobenzidine and hematoxylin. After dehydration, sections were fixed with neutral glue and observed microscopically.

Western blotting

Cardiac tissue was homogenized, washed with PBS, and dissolved in commercial RIPA buffer. After centrifugation at 12,000 rpm for 20 min, dissolved protein was obtained from the supernatant and quantified in a BCA protein assay (Beyonce, Nanjing, China). Equal amounts of sample protein were electrophoresed through a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a nitrocellulose membrane. After blocking, membranes were incubated overnight with primary antibodies at 4°C and incubated for 2 h at room temperature with a secondary antibody conjugated to horseradish peroxidase. Visualization of protein bands using enhanced chemiluminescence detection reagents and gel imaging system was then performed (Tanon Technology Co., Ltd., China). Protein expression levels were normalized to those of GAPDH as a control.

Statistical analysis

Data are expressed as means \pm standard deviations (SD) of at least three separate experiments. Statistical comparisons between experimental groups were performed by ANOVA with Tukey's multiple comparison test. A value of $P < 0.05$ indicated statistical significance.

Results

DG reverses ST segment elevation in db/db mice

ECG results for all treatment groups were shown in **Figure 1**. Compared with the control

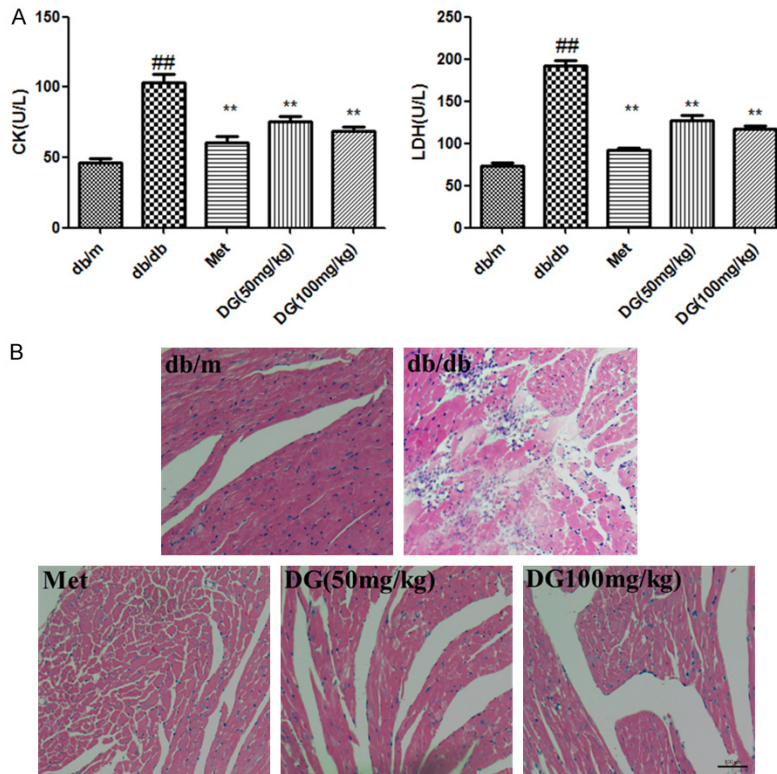


Figure 3. Indicators of physiological damage and changes in cardiac histopathology following DG treatment. A. Levels of creatine kinase (CK) and lactate dehydrogenase (LDH), which indicate cardiac muscle injury, were measured in the indicated treatment groups. All data are presented as means \pm SD. Compared with control mice: * $P < 0.05$, ** $P < 0.01$. Compared with db/db mice: * $P < 0.05$, ** $P < 0.01$. B. Sections of cardiac tissues from indicated mice were stained with hematoxylin and eosin and visualized at 200 \times magnification.

mg/kg DG exhibited improved levels (**Figure 3A**). Histopathological examination of H&E-stained tissues (**Figure 3B**) revealed more lipid accumulation and inflammation in db/db mice compared with non-diabetic controls, including infiltration of monocytes and neutrophils. However, animals treated with both doses of DG showed less inflammation, indicating that DG may improve DM-associated myocardial injury.

DG decreases levels of inflammatory cytokines in serum and cardiac tissue

As shown in **Figure 4A** and **4B**, levels of IL-1 β , IL-6 and TNF- α were markedly elevated in serum and cardiac tissues of db/db mice compared with db/m mice. Treatment with 50 or 100 mg/kg DG significantly decreased levels of these cytokines in both sample types.

group, the ST segment of db/db mice was increased. However, DG reversed this negative impact, indicating its protective effect under diabetic conditions.

DG improves levels of blood glucose, insulin, and lipids in db/db mice

As shown in **Figure 2A**, blood glucose levels of db/db mice were significantly higher than those of db/m mice, while both 50 and 100 mg/kg DG improved them. We also found that DG treatment restored serum insulin levels in db/db mice (**Figure 2B**). Moreover, 50 and 100 mg/kg DG significantly lowered DM-induced TC and TG elevation in db/db mice (**Figure 2C**).

DG reduces biochemical indicators of cardiac injury and direct pathological damage

Compared with db/m mice, CK and LDH levels in the db/db group were significantly higher, although db/db mice treated with 50 or 100

DG attenuates RIP140/NF- κ B signaling in db/db mice

The expression of RIP140/NF- κ B pathway-related mRNA and protein was measured to explore the underlying mechanism of inhibitory effect of DG on diabetes. The mRNA expression of RIP140, I κ B α , and NF- κ B was significantly increased in the db/db mice compared with the control group, as well as a markedly reduced in the DG treatment group (**Figure 5A**). Meanwhile, we observed increased expression of RIP140, p-NF- κ B, and p-I κ B α in cardiac tissue of db/db mice that was subsequently reduced after DG treatment (**Figure 5B**). Similarly, **Figure 5C** shows DG's significant attenuation of these signaling proteins in db/db mice as demonstrated by immunohistochemical staining.

Discussion

Although many interventions for cardiovascular diseases exist, such as thrombolytic therapy

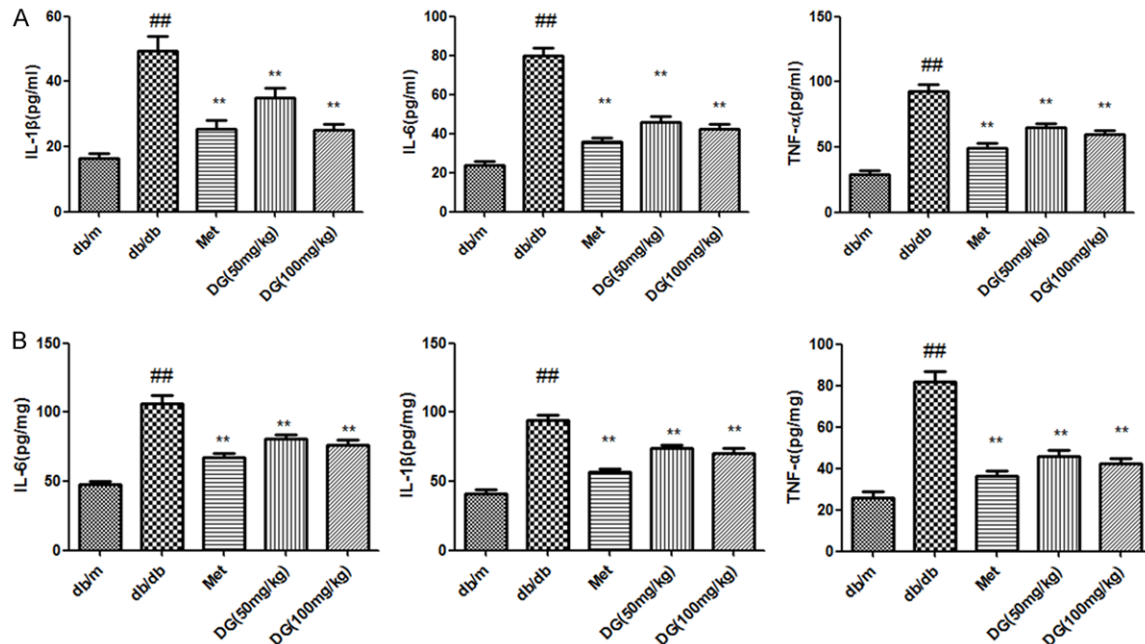


Figure 4. Levels of inflammatory cytokines in control and diabetic mice following DG treatment. Levels of serum (A) and cardiac tissue (B) inflammatory cytokines were measured after 12 weeks of the indicated treatment. All data are presented as means \pm SD. Compared with control mice: # P < 0.05, ## P < 0.01. Compared with db/db mice: * P < 0.05, ** P < 0.01.

and coronary artery bypass grafting, the curative effects of these procedures on diabetic cardiomyopathy are not ideal, necessitating further study of treatments for this condition [15, 16]. In this study, we confirmed that RIP140 activity plays an important role in diabetic cardiomyopathy, and DG exerts an anti-diabetic effect and improves cardiac inflammation by inhibiting RIP140/NF- κ B signaling pathways.

Dyslipidemia in DM is characterized by high levels of TG and LDL [17] and relates to the development of insulin resistance [18]. Thus, correcting dyslipidemia can reduce diabetes complications, including cardiac injury [19]. In our study, varying doses of DG significantly reduced serum TG and TC levels in diabetic mice, indicating that DG has a therapeutic effect on DM-induced lipid metabolism dysfunction. Similarly, chronic hyperglycemia is directly associated with multi-organ pathology, including cardiac damage, that can be detected by changes in certain plasma enzymes. We found that DG reduces both insulin resistance and hyperglycemia-induced cardiac injury in db/db mice as indicated by decreased levels of CK and LDH in these mice after DG treatment.

Cardiac inflammation involves the excessive production of cytokines, such as IL-1 β , IL-6, and TNF- α , which can lead to insulin resistance and indicate the severity of DM [20, 21]. In addition, increased serum IL-6 levels in various adipose tissues is a predictor of DM progression [22]. Increased expression of inflammatory cytokines and subsequent cardiac inflammation manifested as the upregulation of serum and cardiac tissue levels of IL-1 β , IL-6, and TNF- α in db/db mice, which was significantly reduced after DG treatment.

RIP140 plays an important role in promoting inflammatory cytokine production in macrophages and specifically interacts with NF- κ B and CREB to modulate expression of IL-6 and TNF- α [23, 24]. We found that DG improved lipid metabolism in db/db mice by inhibiting RIP140, whose upregulation correlates to diabetes complications in human patients and animal models. In addition, previous studies have shown that extracellular RIP140 binds cell surface receptors and promotes inflammation by inducing production of various inflammatory mediators, such as IL-1 β , IL-6, and TNF- α [25]. In our experiments, DG downregulated expression of these cytokines and RIP140, suggesting

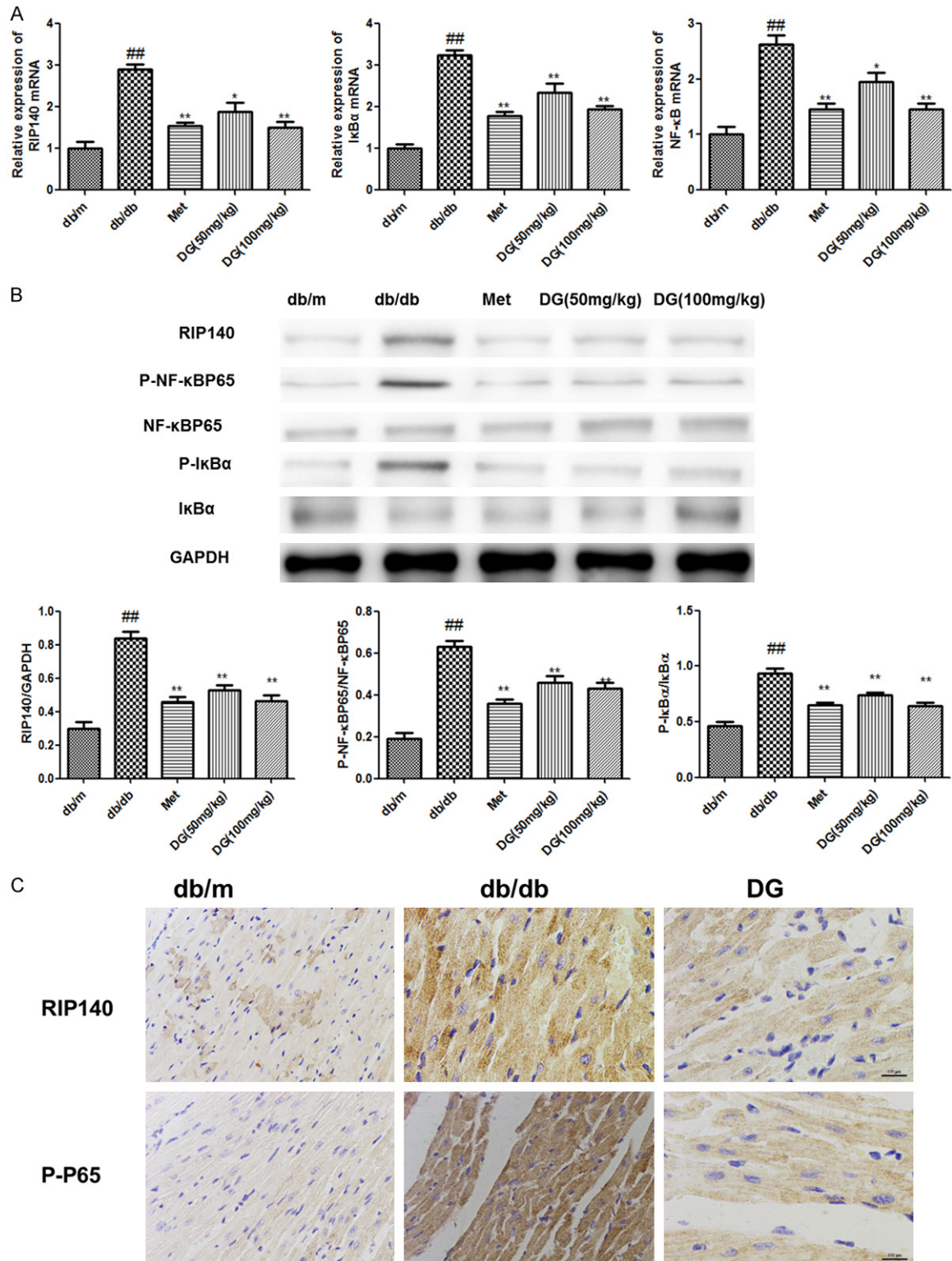


Figure 5. DG modulation of RIP140/NF-κB signaling in db/db mice. Expression of indicated mRNA (A) and proteins (B) involved in RIP140/NF-κB signaling pathways. GAPDH was used as an internal control. Comparisons of protein expression among indicated treatment groups of control and diabetic mice are shown. All data are presented as means \pm SD. Compared with control: $^*P < 0.05$, $^{##}P < 0.01$. Compared with model: $^*P < 0.05$, $^{**}P < 0.01$. (C) Immunohistochemical staining of cardiac tissues from indicated treatment groups illustrate presence localization of RIP140 and p-p65.

that RIP140 contributes to DM-associated inflammation.

Here, we studied the protective effects of DG on diabetic cardiomyopathy and found that the compound can significantly improve insulin resistance, hyperglycemia, and cardiac inflammation in db/db mice. Mechanistically, we demonstrated that DG exerts these effects by inhibiting RIP140/NF- κ B signaling. We conclude DG may be a novel therapy for reversing cardiac injury induced by DM.

Acknowledgements

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Disclosure of conflict of interest

None.

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