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Research Article

Ginsenoside Rb3 ameliorates podocyte injury under hyperlipidemic conditions *via* PPARδ- or SIRT6-mediated suppression of inflammation and oxidative stress

Heeseung Oh [a], Wonjun Cho [a], Seung Yeon Park [a, b], A.M. Abd El-Aty [c, d], Ji Hoon Jeong [a, b], Tae Woo Jung [a, *]

[a] Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul, Republic of Korea
[b] Department of Global Innovative Drugs, Graduate School of Chung-Ang University, Seoul, Republic of Korea
[c] Department of Pharmacology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
[d] Department of Medical Pharmacology, Medical Faculty, Ataturk University, Erzurum, Turkey

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ABSTRACT

Background: Rb3 is a ginsenoside with anti-inflammatory properties in many cell types and has been reported to attenuate inflammation-related metabolic diseases such as insulin resistance, nonalcoholic fatty liver disease, and cardiovascular disease. However, the effect of Rb3 on podocyte apoptosis under hyperlipidemic conditions, which contributes to the development of obesity-mediated renal disease, remains unclear. In the current study, we aimed to investigate the effect of Rb3 on podocyte apoptosis in the presence of palmitate and explore its underlying molecular mechanisms.
Methods: Human podocytes (CIHP-1 cells) were exposed to Rb3 in the presence of palmitate as a model of hyperlipidemia. Cell viability was assessed by MTT assay. The effects of Rb3 on the expression of various proteins were analyzed by Western blotting. Apoptosis levels were determined by MTT assay, caspase 3 activity assay, and cleaved caspase 3 expression.
Results: We found that Rb3 treatment alleviated the impairment of cell viability and increased caspase 3 activity as well as inflammatory markers in palmitate-treated podocytes. Treatment with Rb3 dose-dependently increased PPARδ and SIRT6 expression. Knockdown of PPARδ or SIRT6 reduced the effects of Rb3 on apoptosis as well as inflammation and oxidative stress in cultured podocytes.
Conclusions: The current results suggest that Rb3 alleviates inflammation and oxidative stress *via* PPARδ- or SIRT6-mediated signaling, thereby attenuating apoptosis in podocytes in the presence of palmitate. The present study provides Rb3 as an effective strategy for treating obesity-mediated renal injury.

1. Introduction

In modern society, the number of obese patients is rapidly increasing due to a lack of physical activity, poor eating habits, and overeating. Accordingly, the prevalence of various obesity-metabolic disorders, such as type 2 diabetes, cardiovascular disease, nonalcoholic fatty liver disease, and chronic kidney disease (CKD), is also increasing. In the same context, several studies also suggest that obesity plays a causative role in the development of metabolic syndrome [1]. Therefore, although improving obesity is a fundamental method of treating metabolic diseases, there are still no exact therapeutic methods.

CKD and chronic kidney failure reveal a gradual loss of renal function due to various risk factors, including oxidative stress and chronic low-grade inflammation caused by obesity. Chronic hyperlipidemia and insulin resistance result in the development of CKD in obesity [2]. Because glomerular filtration ability in the kidney mainly depends on podocyte conditions to maintain healthy renal function, several studies have focused on protecting podocytes against various cellular stresses, such as oxidative stress and inflammation [3]. Recently, Zhu et al reported that apoptosis in podocytes caused by mitochondrial oxidative stress plays a

* Corresponding author. Department of Pharmacology, College of Medicine, Chung-Ang University, 221, Heuksuk-dong, Dongjak-gu, Seoul, 06974, Republic of Korea.
E-mail addresses: abdelaty44@hotmail.com (A.M. Abd El-Aty), twjung@cau.ac.kr (T.W. Jung).

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causative role in the pathogenesis of kidney diseases, including podocytopathies [4]. Furthermore, fucoidan attenuates renal fibrosis by suppressing NLRP3 inflammasome-mediated apoptosis in podocytes [5]. Thus, finding substances that alleviate podocyte apoptosis under hyperlipidemic conditions by modulating inflammation and oxidative stress may be a potential therapeutic strategy for treating CKD.

Ginsenoside Rb3 is a pharmacologically active metabolite of saponins in ginseng [6]. It has demonstrated anti-inflammatory properties in several cell types. Xu et al showed that Rb3 prevents inflammation in macrophages treated with LPS by inhibiting the TLR4-dependent pathway [7]. Rb3 improves hepatic injury *via* PPARγ-mediated suppression of inflammation [8] and attenuates TNFα-induced inflammation in chondrocytes [9]. Rb3 also demonstrates antioxidative effects in various cell types. Wang et al have shown that Rb3 preserves endothelial function in the renal artery *via* suppression of oxidative stress [10]. Rb3 protects keratinocytes against UV-induced oxidative stress [11]. Fan et al reported that ginsenosides, including Rb3, can be therapeutic agents for treating metabolic syndrome and cardiovascular disease, which have inflammation as a primary risk factor [6]. However, the effects of Rb3 on inflammation, oxidative stress and apoptosis in podocytes under hyperlipidemic conditions have not been studied.

In the current study, we investigated the effects of Rb3 on inflammation, oxidative stress and apoptosis in cultured podocytes under hyperlipidemic conditions and explored related molecular signaling.

2. Materials and methods

2.1. Podocyte culture and treatment

CIHP-1 cells, the human podocyte cell line, were cultured in RPMI-1640 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1% antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin) (HyClone) at 37 °C in a humidified atmosphere containing 5% CO_2. Podocytes at passages 6 or 7 were used and confirmed to be free of contamination with mycoplasma using PCR detection. Sodium palmitate (Sigma, St. Louis, MO, USA) was conjugated with 5% bovine serum albumin (fatty acid-free grade; Sigma). Rb3 (Sigma) was dissolved in dimethyl sulfoxide (DMSO). CIHP-1 cells were treated with BSA-conjugated palmitate (400 or 200 μM) and Rb3 (0-10 μM) for 24 h. BSA was used as a vehicle control.

2.2. Protein expression analysis

Harvested experimental cells were suspended with PRO-PREP (iNtRON Biotechnology, Republic of Korea) and incubated at 4 °C for 1 h. The cell suspension was centrifuged at 13,000 rpm for 30 min at 4 °C to obtain supernatants as protein extracts. Protein samples were separated using 7 or 12% SDS–PAGE. Separated proteins trapped in SDS-gel were transferred to a nitrocellulose membrane. The protein sample-transferred membrane was blocked with 5% skim milk solution, probed with a primary antibody and then reacted with a matched secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The target protein signal was detected using enhanced chemiluminescence (ECL) buffers (Bio-Rad, Hercules, CA, USA). The first antibodies used in this study were as follows: anti-phospho-NFκB (1: 1,000), anti-NFκB (1: 3,000), anti-phospho-IκB (1: 1,000), anti-PPARδ (1: 2,000), anti-SIRT6 (1: 2,000), anti-SOD1 (1: 2,000), anti-nephrin (1: 2,000), anti-caspase 3 (1: 1,000), and anti-β-actin (1: 4,000), which were purchased from Santa Cruz Biotechnology. Anti-cleaved caspase 3 (1:2,000) was purchased from Cell Signaling (Danvers, MA, USA).

2.3. Suppression of gene expression

Small interfering (si) RNA transfection using Lipofectamine® 2000 (Invitrogen) was conducted to knock down gene expression following the manufacturer's directions. PPARα and SIRT6 siRNAs (Santa Cruz Biotechnology) were used to suppress the expression of each matched gene.

2.4. Cell viability determination

An MTT assay was applied to determine podocyte viability. Experimental podocytes were incubated with commercial MTT working solution (Sigma) for 4 h at 37°C [12]. The crystallized red formazan in cells was dissolved in DMSO after washing with PBS. The optical density was measured at 570 nm and presented as the cell viability level.

2.5. Hydrogen peroxide, mitochondrial complex I activity, and catalase activity assay

Hydrogen peroxide production assays were conducted using a hydrogen peroxide assay kit (Abcam) following the kit manual. Mitochondrial complex I activity was measured using a Complex I enzyme activity microplate assay kit (Abcam) following the manufacturer's directions. According to the manufacturer's directions, catalase activity levels were determined using a commercial colorimetric catalase assay kit (Abcam).

2.6. Cell fluorescence staining

Cellular ROS levels were determined using a DCFDA staining kit (Abcam). Selective mitochondrial staining was performed using Cytopainter (red) (Abcam). Staining experiments were conducted following the manufacturer's directions.

2.7. Secreted cytokine concentration determination

The release of TNFα and MCP-1 from cultured podocytes was measured using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's directions.

2.8. Caspase 3 activity assay

According to the manufacturer's directions, caspase 3 activity levels were determined using a commercial colorimetric caspase 3 assay kit (Abcam).

2.9. Statistical analyses

The levels in the graphs are expressed as the fold change of the highest value (means ± standard deviations). The experiments were carried out independently three or five times. Tukey tests were utilized for post hoc multiple comparisons (one-way ANOVA). All analyses were performed using Prism statistical program (version 8 for Windows; GraphPad, La Jolla, CA, USA).

3. Results

3.1. Rb3 prevents apoptosis in podocytes under hyperlipidemic conditions

Ginsenoside Rb3 is the main active panaxadiol of *Panax ginseng* [6] (Fig. 1A). Cell viability or detachment of podocytes pivotally determines glomerular filtration ability in the kidney [3]. As a preliminary experiment for optimizing cell treatment conditions,

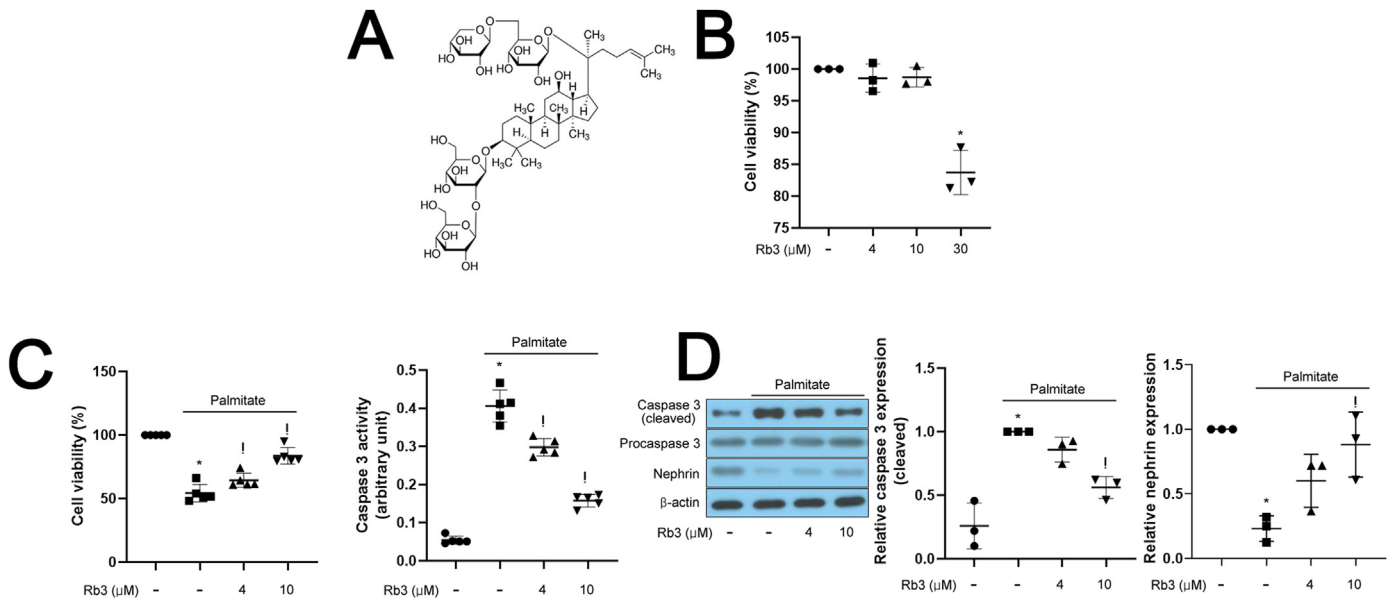

Fig. 1. Rb3 prevents apoptosis in cultured podocytes treated with palmitate. (A) Chemical structure of ginsenoside Rb3. (B) Cell viability assay in CIHP-1 cells treated with Rb3 (0-30 μM) for 24 h. (C) Cell viability and caspase 3 activity assay in palmitate (400 μM)- and/or Rb3 (0-10 μM)-treated CIHP-1 cells for 24 h. (D) Western blotting of cleaved and full-length caspase 3, and nephrin in palmitate (400 μM)- and/or Rb3 (0-10 μM)-treated CIHP-1 cells for 24 h. Means ± SDs were calculated from five or three independent experiments. Significance ($P < 0.05$) *: *vs* control, ! *vs* palmitate.

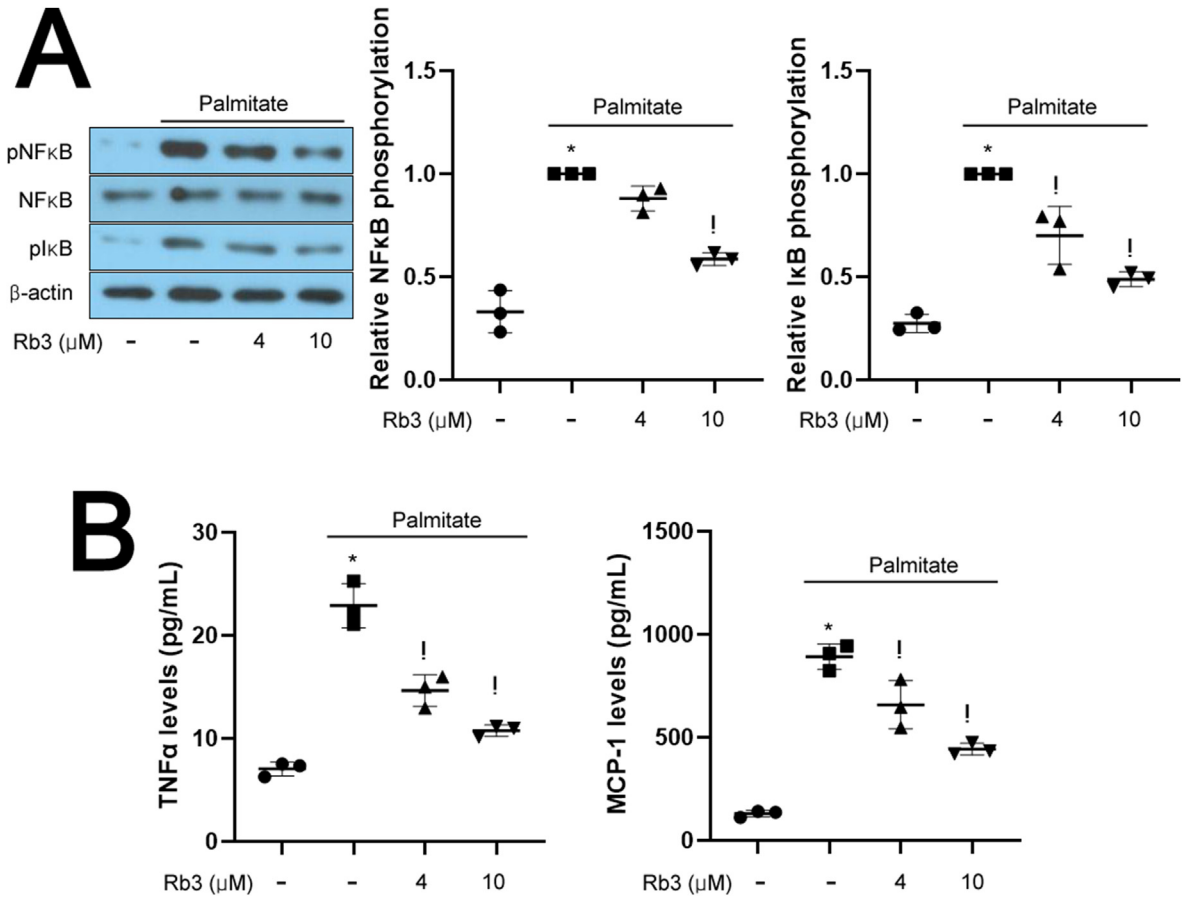

Fig. 2. Rb3 ameliorates inflammatory responses in palmitate-treated podocytes. (A) Western blotting of phosphorylated NFκB and IκB in CIHP-1 cells treated with palmitate (200 μM) and/or Rb3 (0-10 μM) for 24 h. (B) ELISA of TNFα and MCP-1 in the culture supernatant of CIHP-1 cells treated with palmitate (200 μM) and/or Rb3 (0-10 μM) for 24 h. Means ± SDs were calculated from three independent experiments. Significance ($P < 0.05$) *: *vs* control, ! *vs* palmitate. NFκB phosphorylation levels were normalized to total NFκB expression. IκB phosphorylation levels were normalized to β-actin expression.

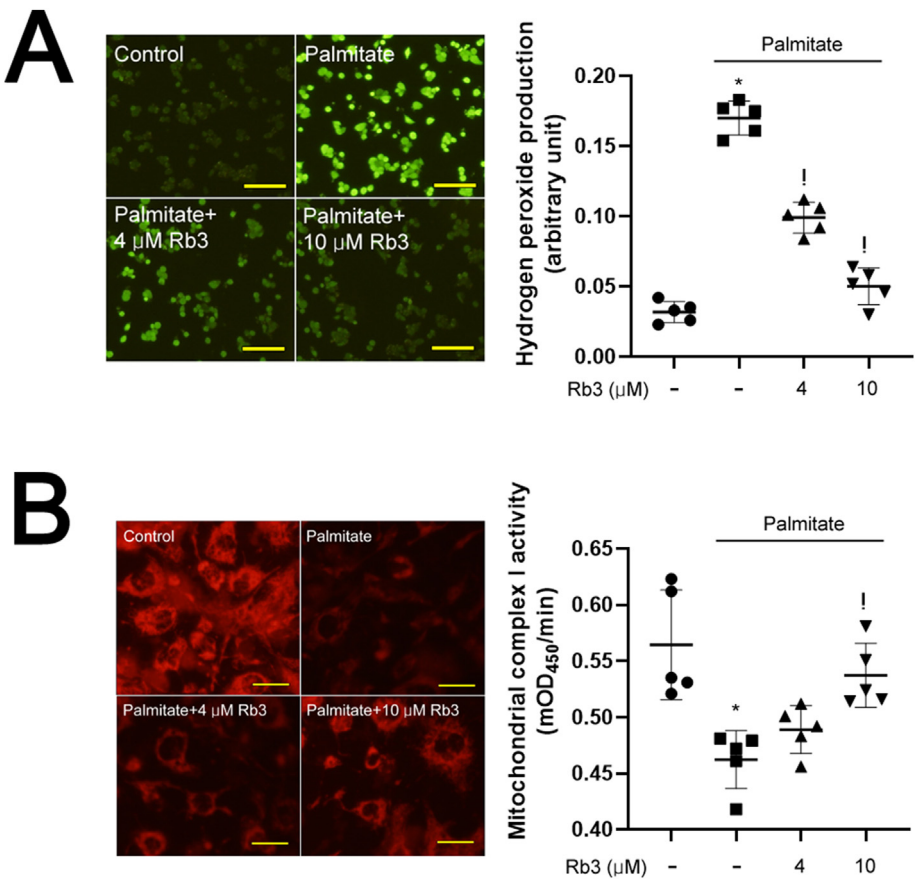

Fig. 3. Rb3 suppresses ROS production and restores mitochondrial function in podocytes treated with palmitate. DCFDA staining (Scale 125 μm) and hydrogen peroxide production assay (A), and mitochondria staining (Scale 50 μm) and complex I activity assay (B) in CIHP-1 cells treated with palmitate (200 μM) and/or Rb3 (0-10 μM) for 24 h. Means ± SDs were calculated from five independent experiments. Significance ($P < 0.05$) *: *vs* control, ! *vs* palmitate.

the toxicity of Rb3 in podocytes was investigated. Treatment with 30 μM Rb3 for 24 h significantly ($P < 0.05$) impaired cell viability in CIHP-1 cells (Fig. 1B). Palmitate was used to construct *in vitro* hyperlipidemic conditions [13]. Palmitate treatment (400 μM) reduced the cell viability and nephrin expression linked to podocyte injury [13] and increased caspase 3 activity and cleaved caspase 3 expression in CIHP-1 cells. However, Rb3 reversed these changes in a dose-dependent fashion (Fig. 1C and D).

3.2. Rb3 suppresses lipid-mediated inflammation in podocytes

Renal inflammation plays a vital role in developing obesity-related kidney dysfunction [14]. Elevated expression of inflammatory markers, such as phosphorylated NFκB and IκB (Fig. 2A), as well as TNFα and MCP-1 release (Fig. 2B), were detected in CIHP-1 cells treated with palmitate. However, additional treatment with Rb3 suppressed palmitate-induced inflammation in a dose-dependent fashion (Fig. 2A and B).

3.3. Rb3 ameliorates oxidative stress in palmitate-treated podocytes

Oxidative stress plays a pivotal role in developing renal injury in obesity [15]. Treatment with Rb3 alleviated cellular ROS levels and hydrogen peroxide production in palmitate-treated CIHP-1 cells (Fig. 3A). Palmitate treatment impaired mitochondrial accumulation and complex I activity, an indicator of mitochondrial respiration, whereas Rb3 ameliorated these reductions in CIHP-1 cells (Fig. 3B).

3.4. PPARδ or SIRT6 contributes to the effects of Rb3 on inflammation, oxidative stress and apoptosis in podocytes

PPARδ [16,17] and SIRT6 [18] have been documented to attenuate cell apoptosis and inflammation under hyperlipidemic conditions. Treatment with Rb3 dose-dependently increased PPARδ and SIRT6 expression as well as SOD1 expression and catalase activity in cultured CIHP-1 cells (Fig. 4A). Suppression of PPARδ or SIRT6 expression by each siRNA mitigated the effects of Rb3 on inflammation (Fig. 4B and C), oxidative stress (Fig. 4D and E) and apoptosis (Fig. 4F) in palmitate-treated CIHP-1 cells.

3.5. Rb3 independently regulates PPARδ and SIRT6 to regulate antioxidants

PPARδ or SIRT6 siRNA abrogated the effects of Rb3 on SOD1 expression and catalase activity in cultured CIHP-1 cells (Fig. 5A). However, PPARδ siRNA did not affect SIRT6 expression (Fig. 5B). Furthermore, SIRT6 siRNA did not influence SIRT6 expression in Rb3-treated CIHP-1 cells (Fig. 5C).

4. Discussion

The incidence of CKD related to obesity has been continuously increasing. Various studies have suggested solutions for obesity-

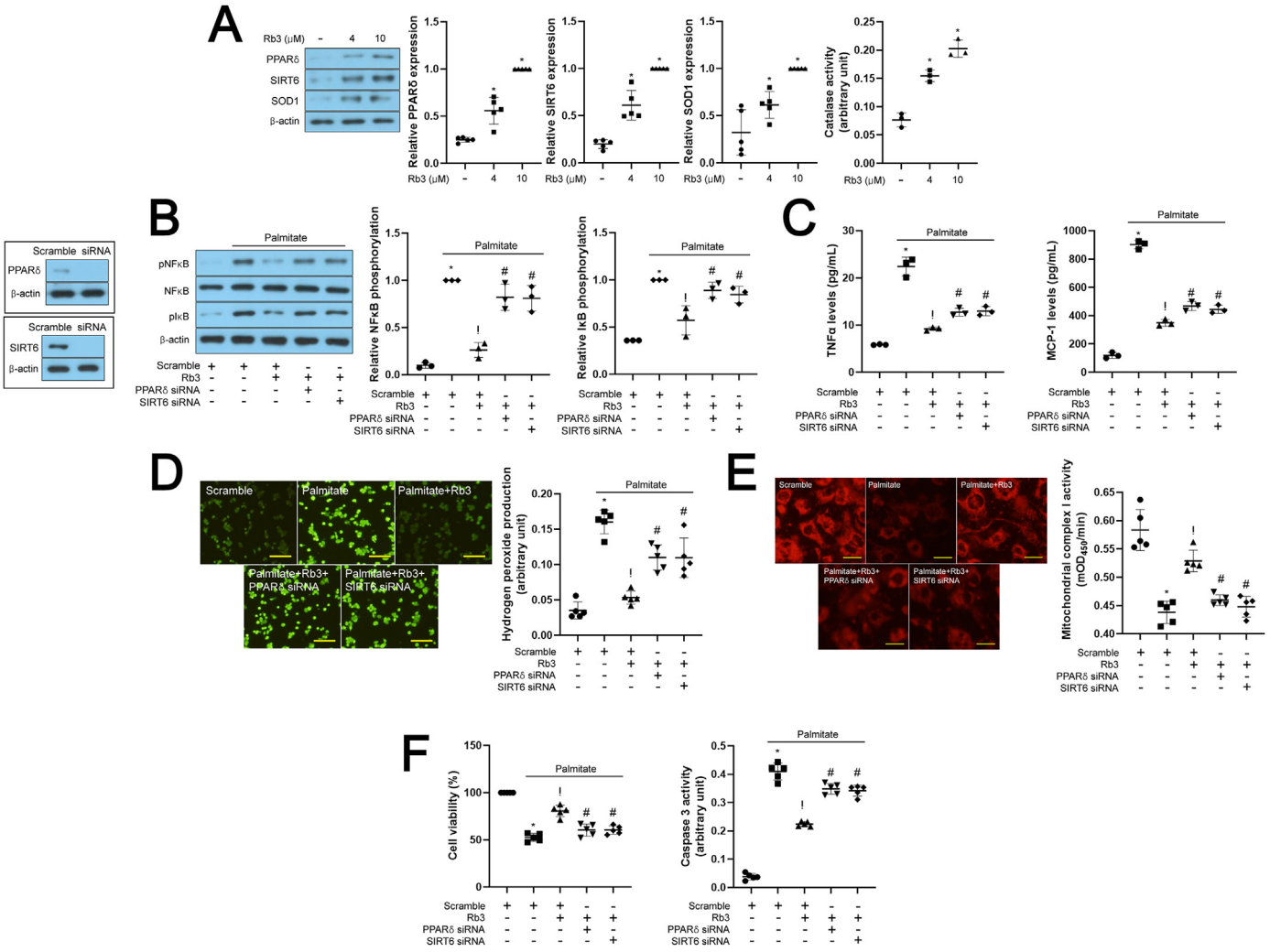

Fig. 4. PPARδ or SIRT6 contributes to the effects of Rb3 on inflammation, oxidative stress, and apoptosis in palmitate-treated podocytes. (A) Western blotting of PPARδ, SIRT6 and SOD1 and a catalase activity assay in CIHP-1 cells treated with Rb3 (0-30 μM) for 24 h. (B) Western blotting of phosphorylated NFκB and IκB in PPARδ- or SIRT6 siRNA-transfected CIHP-1 cells treated with palmitate (200 μM) and/or Rb3 (10 μM) for 24 h. (C) ELISA of TNFα and MCP-1 in the culture supernatant of PPARδ- or SIRT6 siRNA-transfected CIHP-1 cells treated with palmitate (200 μM) and/or Rb3 (10 μM) for 24 h. DCFDA staining (Scale 125 μm) and hydrogen peroxide production assay (D) and mitochondria staining (Scale 50 μm) and complex I activity assay (E) in PPARδ- or SIRT6 siRNA-transfected CIHP-1 cells treated with palmitate (200 μM) and/or Rb3 (10 μM) for 24 h. (F) Cell viability and caspase 3 activity assay in PPARδ- or SIRT6 siRNA-transfected CIHP-1 cells treated with palmitate (400 μM) and/or Rb3 (10 μM) for 24 h. Means ± SDs were calculated from three or five independent experiments. Significance ($P < 0.05$) *: *vs* control, ! *vs* palmitate, #: *vs* palmitate plus Rb3. NFκB phosphorylation levels were normalized to total NFκB expression. IκB phosphorylation levels were normalized to β-actin expression.

related CKD, but no definitive treatment has been developed. In this study, we propose the possibility of ginsenoside Rb3 as a therapeutic agent for obesity-related kidney disease by confirming the effect of Rb3 on podocytes based on an *in vitro* obesity model. Our three novel findings in the current study are as follows: 1) Rb3 prevented apoptosis in CIHP-1 cells treated with palmitate, 2) Rb3 ameliorated palmitate-induced inflammatory responses and oxidative stress, and 3) PPARδ- or SIRT6-mediated signaling plays a pivotal role in the effects of Rb3 on inflammation, oxidative stress, and apoptosis in palmitate-treated cultured podocytes.

Podocytes have a unique cytoskeletal structure associated with the fundamental ability of kidney filtration, which shows that the cell viability of podocytes dominates renal function [19]. Podocyte apoptosis enhances the loss of renal endothelial function, which is a hallmark of CKD. Furthermore, obesity increases the prevalence of CKD *via* obesity-mediated diabetes and hypertension as risk factors for CKD. Elevated serum free fatty acids in obesity, called hyperlipidemia, also cause renal damage *via* lipotoxicity to podocyte-mediated signaling [20,21]. Free fatty acid palmitate provokes inflammation and oxidative stress in podocytes [20]. In the same context, palmitate causes podocyte injury resulting in glomerular damage, thereby causing proteinuria [22]. The present study used palmitate to construct an *in vitro* hyperlipidemia model. We found that Rb3 treatment prevented inflammation, oxidative stress, and apoptosis in cultured podocytes under hyperlipidemic conditions. These results suggest the potential of Rb3 as a therapeutic agent for hyperlipidemia-mediated CKD in obesity.

PPARδ is a nuclear receptor that serves as a transcription factor regulating the expression of various genes associated with several cellular physiological events, such as inflammation, oxidative stress, lipid and glucose metabolism, and insulin signaling [23,24]. In a clinical study, reduced PPARδ expression levels in leukocytes of

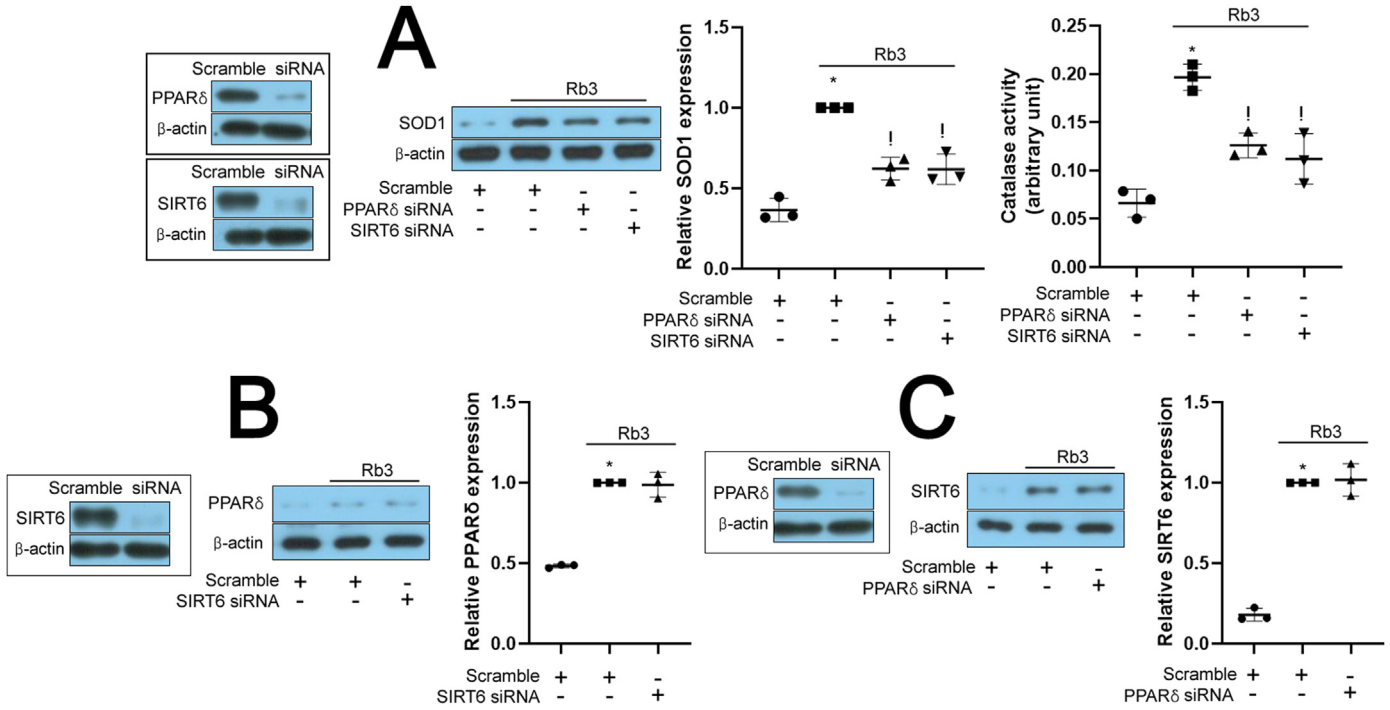

Fig. 5. Rb3 induces antioxidants *via* PPARδ- or SIRT6-mediated pathways. (A) Western blotting of SOD1 and catalase activity assay in PPARδ- or SIRT6 siRNA-transfected CIHP-1 cells treated with Rb3 (10 μM) for 24 h. (B) Western blotting of PPARδ in SIRT6 siRNA-transfected CIHP-1 cells treated with Rb3 (10 μM) for 24 h. (C) Western blotting of SIRT6 in PPARδ siRNA-transfected CIHP-1 cells treated with Rb3 (10 μM) for 24 h. Means ± SDs were calculated from three independent experiments. Significance ($P < 0.05$) *: *vs* control, ! *vs* Rb3.

obese individuals were observed [25], implying the possibility of impairment of immune function by obesity due to a lack of PPARδ activity. Several studies have demonstrated the suppressive effects of PPARδ on inflammation in several cell types. Jung et al reported that the myokine METRNL activates PPARδ signaling, thereby attenuating lipid-induced inflammation in the skeletal muscle of mice [16]. Conversely, deletion of PPARδ in Kupffer cells demonstrates reduced sensitivity to IL-4 and thus impaired inflammatory responses [26]. In clinical trials, PPARδ negatively correlates with proinflammatory cytokines in obese patients [25]. PPARδ is also involved in the regulation of oxidative stress and cell survival. PPARδ agonists attenuate apoptosis in hydrogen peroxide-treated cardiomyoblasts through suppression of oxidative stress [27]. Clinical studies have demonstrated that PPARδ activation alleviates oxidative stress in the skeletal muscle of overweight subjects [28]. Based on these reports, we hypothesized that PPARδ might contribute to the modulation of inflammation, oxidative stress, and apoptosis by Rb3. We found that Rb3 treatment dose-dependently augmented PPARδ expression in cultured podocytes in the current study. Moreover, PPARδ-specific siRNA reduced the effects of Rb3 on inflammation, oxidative stress, and apoptosis. These results suggest that PPARδ contributes to the beneficial effects of Rb3 in podocytes under hyperlipidemic conditions.

SIRT6, an NAD^+-dependent class III histone deacetylase, maintains chromatin and genome stability [29]. However, previous studies have demonstrated that SIRT6 also modulates cellular stresses, including inflammation and oxidative stress. He et al reported that overexpression of SIRT6 prevents inflammation through NRF2-mediated signaling in endothelial cells, whereas knockdown of SIRT6 enhances TNFα-mediated inflammatory responses [30]. Jung et al have shown that kynurenic acid improves inflammation in palmitate-treated cultured skeletal muscle cells and adipocytes *via* a SIRT6-dependent pathway [31]. SIRT6 contributes to the protective effect of melatonin on myocardial infarction injury in rats by suppressing oxidative stress [32]. Greiten et al reported that SIRT6 suppresses oxidative stress, thereby attenuating vascular dysfunction in animal models [33]. Therefore, we expected that SIRT6 might contribute to the anti-apoptotic effect of Rb3 in cultured podocytes treated with palmitate by simultaneously modulating inflammation and oxidative stress. This study found that Rb3 dose-dependently increased SIRT6 expression in CIHP-1 cells. siRNA-mediated knockdown of SIRT6 reduced the effects of Rb3 on inflammation, oxidative stress, and apoptosis in the presence of palmitate. These results reveal that Rb3 suppresses inflammation and oxidative stress, leading to the attenuation of podocyte apoptosis through SIRT6 signaling.

We next investigated the relationship between PPARδ and SIRT6 in the Rb3-mediated pathway. PPARδ siRNA did not affect Rb3-induced SIRT6 expression. Moreover, siRNA for SIRT6 also did not show any changes in PPARδ expression in CIHP-1 cells treated with Rb3. However, both PPARδ and SIRT6 siRNAs abolished the effects of Rb3 on antioxidants. These results suggest that Rb3 simultaneously but independently regulates PPARδ and SIRT6, resulting in the promotion of antioxidants.

5. Conclusion

In the present study, we demonstrate that Rb3 suppresses inflammation and oxidative stress, thereby improving podocyte apoptosis under hyperlipidemic conditions through independent dual PPARδ- and SIRT6-dependent pathways (Fig. 6). This study provides the basis of a therapeutic strategy for treating obesity-mediated CKD.

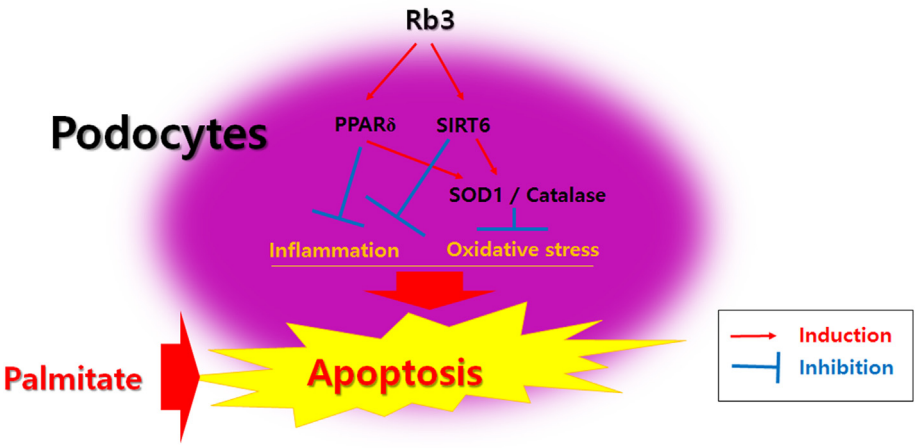

Fig. 6. Schematic diagram of the effects of Rb3 in podocytes under hyperlipidemic conditions.

Contributors' statement

Wonjun Cho: conceptualization; investigation; methodology. Seung Yeon Park: conceptualization, investigation, methodology. Heeseung Oh: conceptualization; investigation; methodology. Ji Hoon Jeong: conceptualization; roles/writing - original draft. Tae Woo Jung: conceptualization; data curation; formal analysis; funding acquisition; investigation; roles/writing - original draft. A. M. Abd El-Aty: roles/validation; writing - original draft; writing - review & editing. All authors approved the final version of the manuscript. All authors are responsible for the overall integrity of the work.

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Declaration of competing interest

The authors declared that they had no conflicts of interests.

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