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Original research article

The mechanism of sirt1 reducing albumin induced renal podocyte injury in mice

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Abstract

Background and Purpose: *This study investigates the protective effects of sirt1 on albumin-induced podocyte damage, focusing on the regulatory role of HIF-1α. Podocyte injury contributes significantly to proteinuria, a marker of chronic kidney disease (CKD) progression. Understanding the sirt1/HIF-1α pathway may provide insights into novel therapeutic strategies for CKD.*

Materials and Methods: *Cultured mouse podocytes were divided into control, albumin-induced, sirt1 agonist, and sirt1 inhibitor groups. RT-qPCR was used to evaluate the expression of sirt1 and HIF-1α mRNA. Protein levels of Nephrin, podocalyxin, and TRPC6 were assessed through Western blot and immunofluorescence techniques to determine podocyte damage.*

Results: *The albumin-induced group showed decreased Sirt1 and increased HIF-1α levels, while sirt1 agonists reversed these changes, indicating that the kidney protective effect. In contrast, sirt1 inhibitors worsened podocyte injury. Western blot and immunofluorescence confirmed a significant reduction in Nephrin and podocalyxin and an increase in TRPC6 protein expression in albumin-treated groups, which was mitigated by sirt1 activation.*

Conclusions: *Sirt1 protects against albumin-induced podocyte damage by modulating HIF-1α, suggesting its potential as a therapeutic target for proteinuria and CKD management. This study provides a foundational understanding of sirt1's role in podocyte protection and highlights the need for further research to explore its clinical applications in preventing CKD progression.*

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INTRODUCTION

Proteinuria serves as both a marker of kidney damage and an independent risk factor for the progression of glomerular diseases. Dysfunction in the mechanical or charge function of the glomerular filtration barrier results in the development of proteinuria. Podocytes, situated on the outer side of the glomerular basement membrane, are highly specialized terminal cells (*1*). These podocytes form interdigitating foot processes that create crucial slit diaphragms, essential components of the glomerular filtration barrier. Among the extensively studied podocyte slit diaphragm protein complexes are Nephrin and podocin. Additionally, other podocyte structures and phenotypic proteins also contribute significantly to the occurrence and progression of proteinuria. Podocalyxin, a sialomucin with a substantial negative charge, is exclusively expressed on the surface of podocytes and vascular endothelial

cells in the kidney. It plays a role in regulating podocyte structure and maintaining slit diaphragm integrity, with changes in podocalyxin closely linked to proteinuria *(2)*. Transient Channel Receptor Potential Cation 6 (TRPC6), another vital component of the podocyte slit diaphragm, interacts with other proteins to uphold normal podocyte function *(3)*. Silent Information Regulator 1 (sirt1), a silent information regulator, is known to play a role in various physiological and pathological processes including cellular metabolism, aging, and apoptosis *(4)*. It has been shown to inhibit the epithelial-mesenchymal transition of renal tubular epithelial cells, providing protection against diabetic nephropathy-induced renal fibrosis *(5)*. While sirt1 is considered a promising target for preventing and treating kidney injury, the specific molecular mechanisms involved are still not fully understood. Hypoxia Inducible Factor-1α(HIF-1α) is closely linked to chronic kidney disease (CKD) progression through chronic hypoxia (*6*). Several studies have highlighted the relationship between renal hypoxia and CKD progression, with HIF-1α being a key focus in kidney disease research. Some research has indicated that HIF-1α is a downstream target gene of sirt1, and that sirt1 can inhibit the activation of HIF-1α *(7)*. YAN Guohua *et al. (8)* proposed that the sirt1/HIF-1α pathway may regulate inflammation and fibrosis in glomerular mesangial cells. Despite the use of glucocorticoids, immunosuppressants, and biological agents to manage proteinuria in CKD patients, treatment outcomes remain suboptimal. Challenges such as incomplete remission of proteinuria, recurrence, and progression to end-stage renal disease persist. Therefore, there is an urgent need to identify new upstream signaling pathways to address these issues at their core. There is currently a lack of research on how sirt1 regulates HIF-1α in proteinuria-induced podocyte injury. This study aims to explore the protective role of sirt1 in proteinuria-induced podocyte injury through the regulation of HIF-1α. The study will contribute to the field of a new theoretical foundation for treating proteinuria in patients with chronic kidney disease, with the goal of early intervention to prevent the progression of CKD.

MATERIALS AND METHODS

Materials

Cells

Murine podocyte cell line: purchased from Guangzhou Bohui Biotechnology Co., Ltd.

Reagents (kits)

10% fetal bovine serum, RPMI 1640 medium (Gibco, USA); Trizol, SYBR Green Master Mix (Invitrogen, USA); cDNA synthesis kit (ThermoFisher, USA); mRNA primers (RiboBio, Guangzhou, China); lysis buffer, anti-nephrin antibody (Abcam, UK, Cat No:ab72908); PVDF membrane (Bio-Rad, USA), anti-Podocalyxin antibody(TRC,Canada,Cat No:AN06597), anti-TRPC6 antibody(Jingkang Bioengineering Co., Ltd, shanghai,China,Cat No: JK261186).

Methods

Cell Grouping

Murine podocyte cell lines were cultured in RPMI 1640 medium with 10% fetal bovine serum at 37°C. The differentiated podocytes were then divided into several groups: control group; albumin group (5 g/L final concentration, albumin dissolved in deionized water); albumin + sirt1 agonist group (1 μmol/L final concentration of sirt1 agonist SRT1720, dissolved in DMSO); sirt1 agonist group; albumin + sirt1 inhibitor group (1 μmol/L final concentration of sirt1 inhibitor EX527, dissolved in DMSO); sirt1 inhibitor group. Each group underwent a 72-hour stimulation period with six replicates per group.

RT-PCR analysis

Trizol buffer containing DNase I was utilized to extract total RNA from cultured cells. Following assessment of RNA integrity, RNA concentration was determined using a nucleic acid microprotein detector. Subsequently, cDNA was synthesized using a cDNA synthesis kit, and target genes were amplified with SYBR Green Master Mix, utilizing human GAPDH as an internal control. The PCR products were then analyzed through 1.2% agarose gel electrophoresis at 76V for 50 minutes. Semi-quantitative analysis of sirt1 and HIF-1α mRNA was conducted using a gel imaging system.

sirt1 forward primer (F):
5'-TGACCTCCTCATTGTTATTGGG-3'

sirt11 reverse primer (R):
5'-GGCATATCTCGCCACCTAACCT-3'

HIF-1α F: 5'-AAGTCTAGGGATGCAGCACG-3'

HIF-1α R: 5'-AGATGGGAGCTCACGTTGTG-3'

GAPDH F: 5'-CCAGGTGGTCTCCTCTGA-3'

GAPDH R: 5'-GCTGTAGCCAAATCGTTGT-3'

Protein expression analysis

Lysis buffer containing PMSF was added to cultured cells for total protein extraction, followed by measurement of protein concentration. Subsequently, proteins were separated using SDS-PAGE and transferred to PVDF membranes. The membranes underwent blocking with a blocking solution for 1 hour, followed by overnight incubation with primary antibodies at 4°C. Post incubation, the membranes were washed with TBST on a shaker at room temperature and then exposed to specific secondary antibodies for 1-2 hours. Gel Pro analyzer 4 software was used for the analysis of protein imprinting bands. The antibodies utilized in this study were anti-

nephrin, anti-Podocalyxin, and anti-TRPC6, goat anti rabbit secondary antibody labeled with horseradish peroxidase.

Immunofluorescence

Cells in culture dishes were fixed with 4% paraformaldehyde at room temperature for 20 minutes, treated with 0.5% TritonX-100 for 20 minutes, and blocked with 5% BSA at room temperature for 20 minutes. Subsequently, mouse anti-human monoclonal Nephrin antibody (1:100) or mouse anti-human monoclonal Podocalyxin antibody (1:100), along with anti-TRPC6 antibody, were added, and the cells were incubated overnight at 4°C. Following this, secondary antibody incubation was carried out at 37°C for 2 hours, followed by four washes with PBS. DAPI was utilized for nuclear staining for 30 seconds, followed by washing with tap water, and mounting with a suitable medium for microscopy. The images were captured using a confocal microscope (Beijing Ruike Zhongyi Technology Co., Ltd). In each group, 10 random fields of view were selected, and the images were analyzed using Image-Pro Plus 6.0 software. The average fluorescence intensity was calculated as the ratio of the cumulative optical density value to the measured area of each field of view.

Statistical Analysis

Data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were presented as mean ± standard deviation. Group comparisons were conducted using one-way ANOVA, with pairwise comparisons analyzed using the least significant difference (LSD) t-test. Statistical significance was defined as a P value < 0.05.

RESULTS

Effects of albumin, sirt1 agonist, and sirt1 inhibitor on sirt1 and HIF-1α mRNA

The RT-PCR results indicated that the sirt1 mRNA levels were significantly lower in the albumin-induced group, albumin + sirt1 agonist group, albumin + sirt1 inhibitor group, and sirt1 inhibitor group compared to the blank control group, while the HIF-1α mRNA levels were significantly higher (P<0.05). No statistically significant difference in sirt1 and HIF-1α mRNA levels was observed in the sirt1 agonist group compared to the blank control group. When compared to the albumin-induced group, the sirt1 mRNA levels increased significantly in the albumin + sirt1 agonist group and sirt1 agonist group, while HIF-1α mRNA levels decreased significantly (P<0.05). Conversely, the sirt1 mRNA levels decreased significantly in the albumin + sirt1 inhibitor group, with a significant increase in HIF-1α mRNA levels (P<0.05). No statistically significant difference was found in sirt1 and HIF-1α mRNA levels in the Sirt1 inhibitor group compared to the albumin-induced group. Furthermore, the sirt1 mRNA levels were significantly lower and HIF-1α mRNA levels were significantly higher in the albumin + sirt1 inhibitor group compared to the albumin + sirt1 agonist group. Please refer to Figures 1 and 2 for more details.

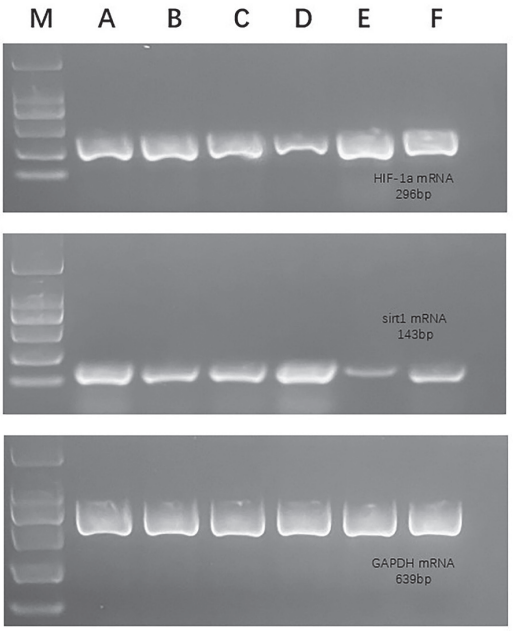

Figure 1. *Expression of sirt1 and HIF-1α mRNA in each group of cells. (M) molecular weight marker; (A) blank control group; (B) albumin-induced group; (C) albumin + sirt1 agonist group; (D) sirt1 agonist group; (E) albumin + sirt1 inhibitor group; (F) sirt1 inhibitor group (subsequent groupings are represented by these numbers).*

Effects of albumin, sirt1 agonist, and sirt1 inhibitor on podocyte-related proteins

Western blot analysis revealed significant differences in the protein levels of Nephrin, podocalyxin, and TRPC6 among different treatment groups. Specifically, Nephrin and podocalyxin levels were significantly lower in the albumin-induced group, albumin + sirt1 agonist group, albumin + sirt1 inhibitor group, and sirt1 inhibitor group compared to the blank control group (P<0.05), while TRPC6 levels were significantly higher. No statistically significant differences were observed in the protein levels of Nephrin, podocalyxin, and TRPC6 in the sirt1 agonist group compared to the blank control group. Furthermore, compared to the albumin-induced group, the albumin + sirt1 agonist group and sirt1 agonist group showed significantly increased levels of Nephrin and podocalyxin, with a decrease in TRPC6 levels (P<0.05). Conversely, the albumin + sirt1 inhibitor group exhibited decreased levels of Nephrin and podocalyxin, along with an increase in TRPC6 levels (P<0.05). No significant dif-

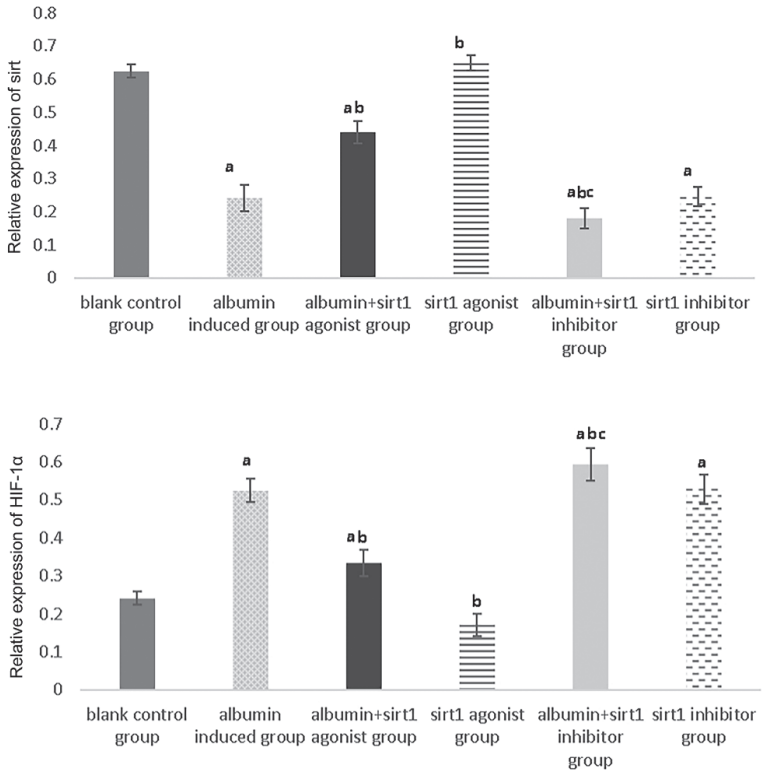

Figure 2. *Relative expression of sirt1 and HIF-1α mRNA in each group of cells. Note: Compared with the blank control group, a: P<0.05; compared with the albumin-induced group, b: P<0.05; compared with the albumin + sirt1 agonist group, c: P<0.05.*

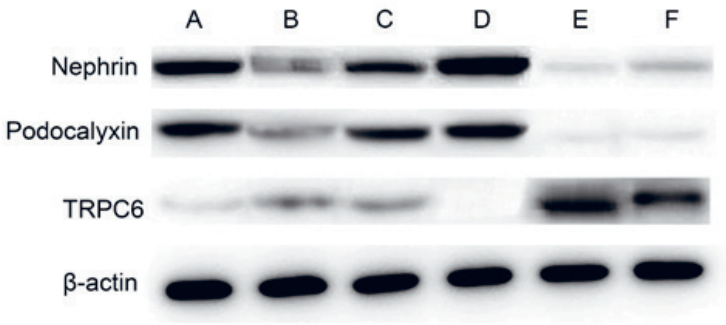

Figure 3. *Expression of podocyte-related proteins in each group of cells.*

ferences were found in the protein levels of Nephrin, podocalyxin, and TRPC6 in the sirt1 inhibitor group compared to the albumin-induced group. Specifically, Nephrin and podocalyxin levels were significantly lower, while TRPC6 levels were significantly higher in the albumin + sirt1 inhibitor group compared to the albumin + sirt1 agonist group. Please refer to Figures 3 and 4 for more details.

Immunofluorescence results revealed that the protein levels of Nephrin and podocalyxin were significantly lower in the albumin-induced group, albumin + sirt1 agonist group, albumin + sirt1 inhibitor group, and sirt1 inhibitor group, while the protein level of TRPC6 was significantly higher compared to the blank control group (P<0.05). There was no statistically significant difference in the protein levels of Nephrin, podocalyxin, and TRPC6 in the sirt1 agonist group when compared to the blank control group (P≥0.05). Furthermore, when compared to the albumin-induced group, the albumin + sirt1 agonist group and sirt1 agonist group showed significant increases in the protein levels of Nephrin and podocalyxin, with a significant decrease in TRPC6 levels (P<0.05). Conversely, the albumin + sirt1 inhibitor group exhibited significantly decreased levels of Nephrin and podocalyxin,

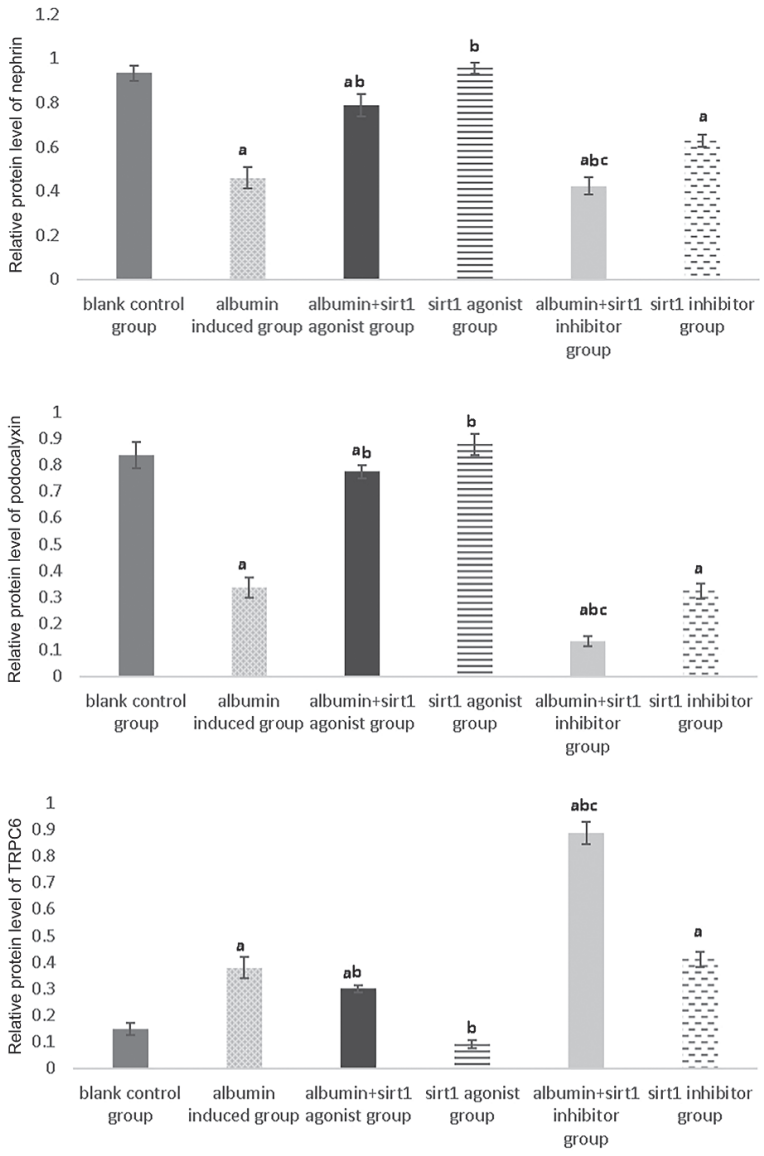

Figure 4. *Relative expression of podocyte-related proteins in each group of cells detected by western blot. Note: Compared with the blank control group, a: P<0.05; compared with the albumin-induced group, b: P<0.05; compared with the albumin + Sirt1 agonist group, c: P<0.05.*

along with increased levels of TRPC6 (P<0.05) when compared to the albumin-induced group. No significant differences were observed in the protein levels of Nephrin, podocalyxin, and TRPC6 in the sirt1 inhibitor group compared to the albumin-induced group (P≥0.05). Notably, the protein levels of Nephrin and podocalyxin were significantly lower in the albumin + sirt1 inhibitor group compared to the albumin + sirt1 agonist group. Please refer to Figures 5 and 6 for visual representation.

DISCUSSION

Podocyte injury is a key factor in the development of proteinuria and can indicate the progression of the condition *(9)*. The number and integrity of podocytes are crucial in maintaining normal glomerular filtration. Studies have shown that reduced expression of podocyte proteins like nephrin and podocin can lead to proteinuria in various kidney disease models *(10)*. *In vitro* studies have demonstrated that high glucose can induce podocyte migration, increase albumin permeability, upregulate MCP-1, and decrease nephrin expression *(11)*. Podocalyxin plays a role in the morphogenesis and differentiation of neonatal podocytes by affecting the formation of microvilli, foot processes, and slit septa, which are essential for glomerular filtration *(12)*. Mice lacking podocalyxin show impaired foot processes and clefts, leading to anuria and death shortly after birth *(13)*. TRPC6, a non-selective cation channel expressed in the kidney, can contribute to podocyte pathology through mechanisms like calcium

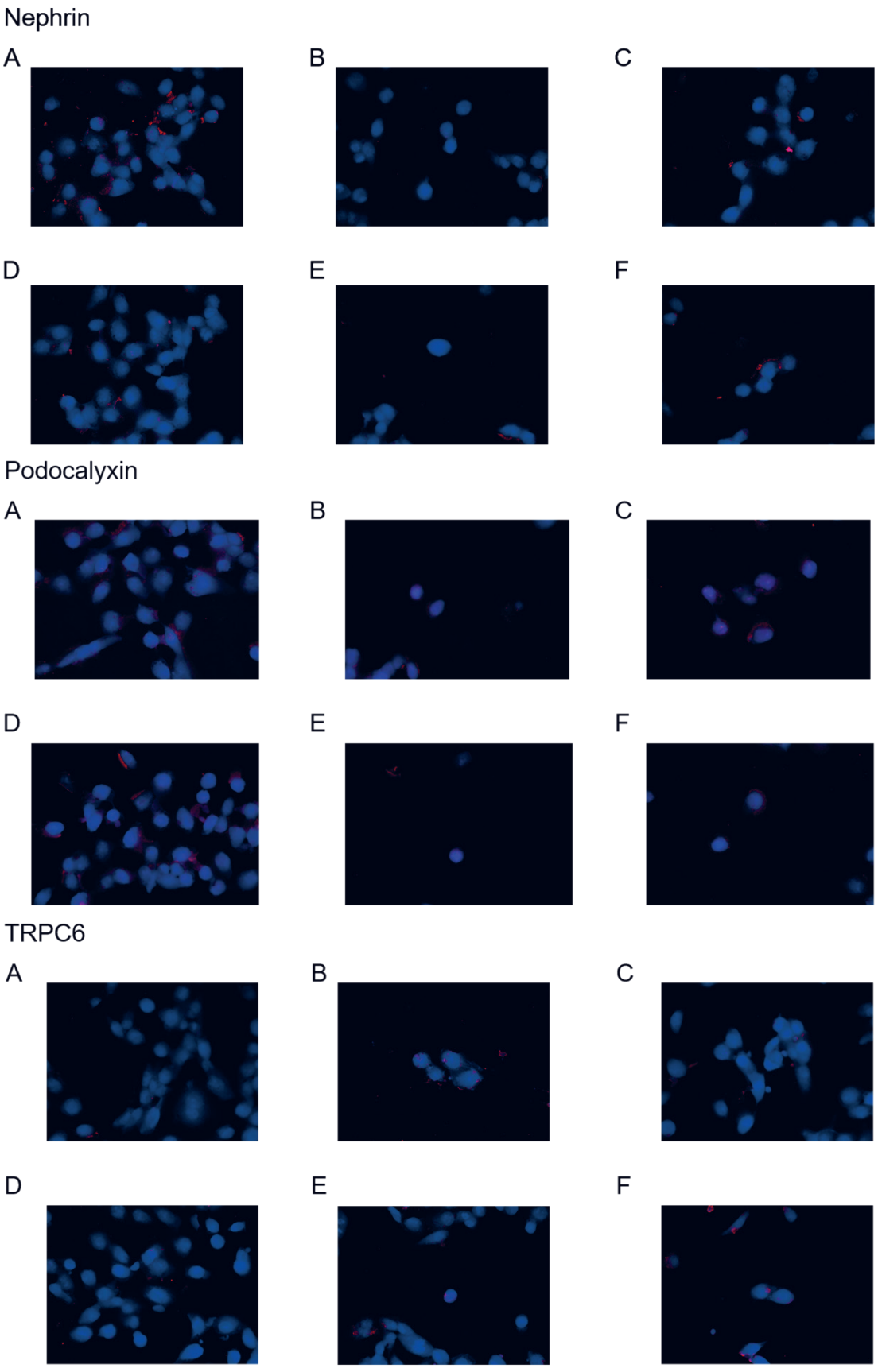

Figure 5. *Fluorescence expression of podocyte-related proteins in each group of cells (fluorescent microscope *100).*

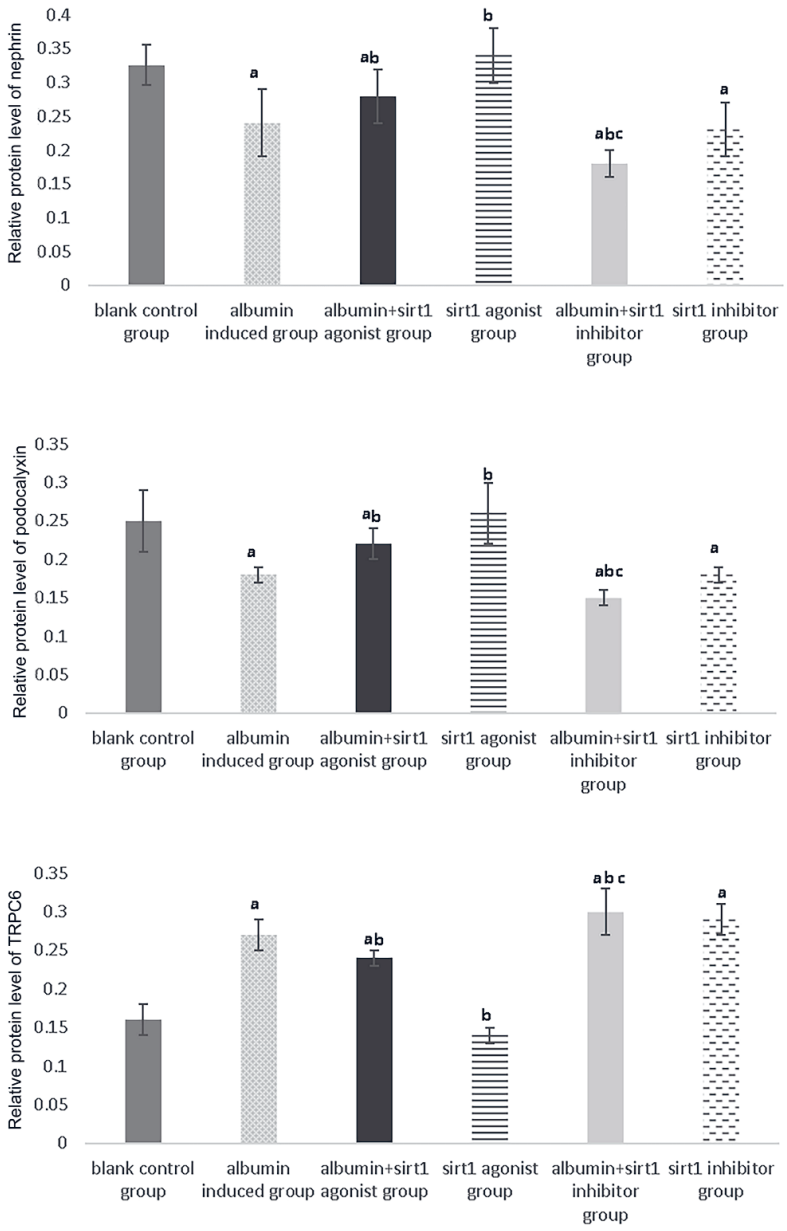

Figure 6. *Relative expression of podocyte-related proteins in each group of cells detected by immunofluorescence. Note: Compared with the blank control group, a: P<0.05; compared with the albumin-induced group, b: P<0.05; compared with the albumin + Sirt1 agonist group, c: P<0.05.*

homeostasis disruption, damage to slit membrane structure and cytoskeleton, and induction of mitochondrial dysfunction *(14)*. The author's research shows that stimulation of *in vitro* podocytes with albumin results in down-regulation of Nephrin and podocalyxin protein expression, while TRPC6 expression is upregulated.

Sirt1, a nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase belonging to class III histone deacetylase, has been shown in recent years to play a role in reducing oxidative stress, inflammation, fibrosis, and apoptosis to prevent kidney damage *(14, 15)*. Managing proteinuria and slowing the progression of kidney disease has long been a challenge for clinicians, prompting nephrologists to explore alternative therapeutic strategies such as targeting sirt1. Alagal *et al.* demonstrated that kaempferol alleviated DOX-induced nephropathy by up-regulating renal sirt1 mRNA and protein expression *(16)*, while reducing levels of renal tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6), as well as nuclear factor κB (NF-κB). The study also showed that the sirt1-specific inhibitor EX-527 reversed the effects of kaempferol. Additionally, the protective effect of sirt1 on podocytes has been investigated by Liu *et al. (17)*, with findings indicating that sirt1 down-regulation in podocytes treated with high glucose can be mitigated by MiR-

138 binding to sirt1 and reducing podocyte-specific damage markers like Desmin. Furthermore, grape seed proanthocyanidin B2 has been shown to inhibit high glucose-induced podocyte apoptosis and increase the expression of nephrin and podocalyxin proteins by activating AMPK and increasing sirt1 protein levels *(18)*. In this research, the author discovered that in an *in vitro* model of albumin-induced podocytes, the use of sirt1 inhibitors led to a significant down-regulation of podocyte-related proteins Nephrin and podocalyxin, while the protein expression of TRPC6 was notably up-regulated. Conversely, the administration of a sirt1 agonist resulted in the opposite effect. However, the specific mechanism through which sirt1 contributes to podocyte protection and the reduction of proteinuria remains unknown.

In the pathophysiological process of kidney disease, chronic hypoxia is involved in the entire process of kidney disease progression. HIF is a major transcription factor that adapts to multiple hypoxic responses. HIF-1α and HIF-2α are the two main subtypes of HIF with different expression sites. HIF-1α is expressed in tubular epithelial cells and promotes cellular adaptability in response to hypoxia. In a podocyte ablation model, HIF-1α has been shown to induce glomerulosclerosis through interaction with Smad3 *(19)*. Research reports also show that blocking HIF-1α can prevent the development of kidney disease in type 1 diabetic mice *(20)*. Previous studies reported that sirt1 mediates a wide range of cellular responses through its deacetylation activity to target numerous transcription factors, such as p53, forkhead box O3 (Foxo3), NF-κB, HIF-1α, etc. *(21)*. Ryu *et al. (22)* observed 5-week-old (young) and 24-month-old (old) C57Bl/6J mice and found that the expression of sirt1 in the kidneys of aging mice decreased, HIF-1α activity increased, and extracellular matrix (ECM) protein increase and cell apoptosis; *in vitro* experiments also found that in HK-2 cells, the sirt1 inhibitor sirtinol and siRNA-mediated knockdown of sirt1 enhanced HIF-1α activity, leading to cell apoptosis and ECM accumulation. During the period of hypoxia, the cells were given sirt1 agonist found that sirt1 directly interacts with HIF-1α, leading to deacetylation of HIF-1α and inhibiting HIF-1α activity. The author's research shows that the expression of HIF-1α mRNA and protein is significantly up-regulated in podocytes cultured with albumin induced by sirt1 inhibitor, while the expression of HIF-1α mRNA and protein is significantly down-regulated by administration of sirt1 agonist.

In conclusion, this study highlights the role of albumin in inducing podocyte injury, with sirt1 showing potential to reduce renal injury by upregulating Nephrin and Podocalyxin while downregulating TRPC6 expression in podocytes. Through the use of sirt1-specific agonists and inhibitors, it was observed that sirt1's renal protective effect may involve the reduction of HIF-1α. However, further investigations using bioinformatics tools is necessary to identify the binding site between sirt1 and HIF-1α. The study focused on *in vitro* molecular research, necessitating future in-depth exploration through animal experiments to verify sirt1's potential in reducing proteinuria for clinical applications.

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