Jixuecao (Herba Centellae Asiatica) alleviates mesangial cell proliferation in IgA nephropathy by inducing mitofusin 2 expression

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Abstract

OBJECTIVE: To investigate the effect of mitofusin 2 (Mfn2) and its downstream signaling pathway on glomerular mesangial cells (GMCs) proliferation in IgA nephropathy (IgAN), as well as the mechanism of action of Jixuecao (Herba Centellae Asiatica, HCA) in the treatment of IgAN.

METHODS: Adenovirus-mediated Mfn2 gene transfection and Mfn2 expression were analyzed by real-time polymerase chain reaction (PCR) and Western blotting. IgA1 induced the proliferation of GMCs, which were then treated with HCA. Cell proliferation was detected with cell counting kit-8 (CCK-8), and Mfn2 expression was analyzed by real-time PCR and western blotting. An IgAN animal model was also established and treated with HCA. GMCs proliferation was detected by hematoxylin-eosin staining, mitochondrial structure was analyzed by electron microscopy, mitochondrial function was determined by the Clark oxygen electrode method, and the expression of Mfn2, Phospho-extracellular regulated protein kinases1/2 (P-ERK1/2), Cyclin-dependent kinase 2 (CDK2), Phospho-p27 (p-p27), and cyclin A was analyzed by Western blotting.

RESULTS: In vitro, HCA inhibited GMCs in a concentration-dependent manner in association with the upregulation of Mfn2 expression. The overexpression of Mfn2 inhibited IgA1-induced GMCs proliferation and elevated the effect of HCA. In vivo, treatment with HCA could alleviate albuminuria and creatinine and GMCs proliferation. These effects were related to the upregulation of Mfn2, p-p27 and inhibition of p-ERK1/2, CDK2, and cyclin A. Mitochondrial swelling, vacuolar degeneration, and reduction of respiratory control rate were identified in IgAN, but HCA could improve these mitochondrial features.

CONCLUSION: HCA inhibited GMCs proliferation via the upregulation Mfn2 and the inhibition of Ras-Raf-ERK/MAPK. We revealed that changes of mitochondrial structure and function are associated with IgAN, but HCA can improve these mitochondrial features.

Keywords: Centella; Glomerulonephritis, IGA; Mesangial cells; Mitofusin 2

INTRODUCTION

IgA nephropathy (IgAN) is the most common form of
primary glomerulonephritis. Nearly 25%-30% of affected patients go on to develop end-stage renal disease. The disease is characterized by the mesangial deposition of polymeric IgA (plgA), followed by the proliferation of mesangial cells, increased synthesis of extracellular matrix, and infiltration of immune cells. Thus, the inhibition of mesangial cell proliferation plays an important role in the progression of this disease. Many medicines have been used to treat IgAN, such as glucocorticoids and cytotoxic drugs. However, these have numerous side effects, such as infection, liver function damage, leukocytopenia, and femoral head necrosis.

Jixuecao (Herba Centellae Asiaticae, HCA) is a perennial herbaceous creeping plant with kidney-shaped leaves, which is found in China and other Southeast Asian countries. As a common Traditional Chinese Medicine, the earliest record of HCA is in Shen Nong Materia Medica (A.D. 102-200 during the E. Han Dynasty), describing its use to treat evil wounds, carbuncles, and abscesses, among others. Clinical practitioners of Traditional Chinese Medicine have explored the use of HCA and revealed its efficacy in treating chronic glomerulonephritis. HCA compound is an agent that we developed and used in the treatment of chronic nephritis for many years. Previously, our small-sample clinical trials showed that HCA compound can decrease proteinuria and improve renal function in IgAN.

Our earlier work also showed that HCA compound can inhibit rat glomerular mesangial cells (GMCs) proliferation induced by IgA1 and reduce IL-6 and TGF-β1 mRNA/protein expression. Moreover, animal experiments revealed that it can reduce IgA immune complex deposition, and limit mesangial proliferation and podocyte fusion, thereby protecting against IgAN, and reducing albuminuria and hematuria. However, the mechanism and pathway through which HCA compound affects chronic glomerulonephritis are still unknown. HCA is the main drug in HCA compound, which is widely used to repair scars in a clinical context and as a kind of oxidant. It can reduce oxidative stress and ameliorate diabetes and nerve/myocardial injury, among others. However, less research has been performed on HCA’s effects on kidney disease. In the present study, we thus explore the precise role of HCA in the treatment of IgAN.

Mitofusin 2 (Mfn2) is a recently discovered multifunctional protein widely expressed in the heart, skeletal muscle, liver, brain, kidney, and other tissues and organs. In 2004, Chen et al. demonstrated that Mfn2 is a powerful regulator of cell proliferation in vivo and in vitro, and it is an endogenous Ras inhibitor. The overexpression of rat Mfn2 was shown to overtly suppress mitogenic stimulus-evoked vascular smooth muscle cell (VSMC) proliferation in culture and block balloon injury-induced restenosis in vivo via inhibition of the Ras-Raf-MEK-ERK/MAPK signaling pathway. In 2007, Chen et al. further demonstrated that Mfn2 displays a profound proapoptotic effect in VSMCs. The upregulation of Mfn2 triggers marked apoptosis in cultured VSMCs via inhibition of the Ras-Pi3K-Akt cell survival pathway and subsequent activation of the mitochondrial death pathway. Notably, the profound anti-proliferative and proapoptotic effects of Mfn2 are independent of its functional role in mitochondrial fusion. Bach et al. reported that the expression of Mfn2 was decreased in diabetic patients. Tang et al. demonstrated that Mfn2 expression was inhibited significantly in diabetic rats and further showed that Mfn2 overexpression was associated with decreased kidney volume (as indicated by KW/BW) and reduced proteinuria and ACR in diabetic rats, indicating that Mfn2 slowed the progression of diabetic nephropathy (DN) and suggesting its potential as a therapeutic target for the treatment of early-stage DN. It was also reported that abnormal expression of Mfn2 occurs in high-glucose-induced rat GMCs, while the overexpression of Mfn2 could alleviate GMCs proliferation and increase apoptosis. Moreover, it was shown that Mfn2 may exert its activities through the MAPK/ERK and PI3K/Akt signaling pathways in connection with proliferation and apoptosis.

Given this background, we conclude that Mfn2 is associated with the proliferation and apoptosis of GMCs in DN, although only limited studies have been performed on the relationship between Mfn2 and IgAN. Therefore, we sought to investigate the effects of Mfn2 on IgAN and to explore whether Mfn2 is a therapeutic target of HCA in the treatment of IgAN.

**METHODS**

**Cell culture**

The rat GMCs strain was provided by the China Center for Culture Collection (Peking Union Medical College, Beijing, China). The cells were cultured in minimal essential medium (MEM) containing Earle’s balanced salt solution (MEM/EBSS) supplemented with 10% fetal bovine serum (FBS: Invitrogen, Carlsbad, CA, USA), streptomycin (100 μg/mL), and penicillin (100 IU/mL) at 37 °C in 95% air/5% CO2. Rat GMCs used in the experiments were sampled from five to seven passages.

**Gene transfer**

An adenovirus encoding the complete Mfn2 open reading frame (AdMfn2) and a control adenovirus encoding the green fluorescent protein open reading frame (AdGFP) were constructed by Vector Gene Technology Ltd. (Beijing, China). A ViraBind Adenovirus Purification Kit (Cell Biolabs, SandraDiego, CA, USA) was used for multiplication and purification of the viruses. The cells were infected with the indicated adenovirus multiplicity of infection in MEM containing 0.5% FBS. After viral adsorption, the GMCs were incubated at 37 °C in 95% air/5% CO2.
IgAN animal model
Twenty-five healthy male Sprague-Dawley rats weighing 180-220 g were purchased from the Experimental Animal Center of Zhejiang Chinese Medical University. The rats were randomly divided into an IgAN group (n = 30) and a normal control group (CG) (n = 7). Rats in the IgAN group were administered 200 mg/kg bovine serum albumin by gavage every other day for 12 weeks. Additionally, 0.05 mg of lipopolysaccharide was injected into the caudal vein during the 6th and 8th weeks. Twice per week, 0.1 mL of carbon tetrachloride and 0.5 mL of castor oil were subcutaneously injected for a total of 12 weeks.15 Rats in the control group were administered sterile distilled water by gavage. Equal volumes of saline were injected into the caudal vein and subcutaneously. Starting from the 4th week, IgAN groups received the intragastric administration of HCA [high group (HG): 90 mg·kg⁻¹·d⁻¹ and low group (LG): 45 mg·kg⁻¹·d⁻¹] and sterile distilled water (2 mL/d). CG was administered with sterile distilled water at 2 mL/d until sacrifice. To measure 24-h urinary albumin excretion, albumin concentrations in the urine were determined using mouse albumin enzyme-linked immunosorbent assay kits, in accordance with the manufacturer’s instructions.

Renal pathology
Immunofluorescence and optical microscopy were used to examine renal tissue. Kidney slices were fixed in 10% formalin, embedded in paraffin wax, cut into 5-μm sections, and stained with hematoxylin and eosin. The tissues were evaluated by light microscopy. The rest of each fixed specimen was used for immunofluorescence detection of IgA, IgG, and C3.

Immunohistochemical staining
Immunohistochemical analyses were performed on paraffin-embedded renal sections (3 μm). The sections were incubated overnight at 4 °C with primary antibodies specific for Mfn2. After washing with PBS, the secondary antibody was applied. The signals were developed with DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA, USA). All immunohistochemical analyses were repeated at least three times, for which representative images are presented.

Real-time quantitative polymerase chain reaction (PCR)
Mfn2 RNAs were extracted from GMCs and renal tissue using Trizol reagent (Invitrogen), in accordance with the manufacturer’s instructions. After DNase treatment (Promega), RNA was reverse-transcribed to first-strand cDNA using a RevertAid First Strand cDNA Synthesis Kit (Fermentas MB, Vilnius, Lithuania). Cycling and real-time detection were performed with the ABI PRISM 7900 Sequence Detection System. Gene-specific primers (show in Table 1) were designed using Vector NTI (Invitrogen). The amplification was performed for 45 cycles at 94 °C for 1 min, 56 °C for 45 s, and 72 °C for 1 min, followed by a final extension for 10 min at 72 °C.

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<th>Gene</th>
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<tr>
<td>Mfn-2</td>
<td>F 5′-ATGATAGACGGCTTGAA-3’</td>
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<tr>
<td></td>
<td>R 5′-CGACTCCCTCTTTGTA-3’</td>
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<tr>
<td>β-actin</td>
<td>F 5′-CTTAGTTGCGTTACACCTTTC-3’</td>
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<td></td>
<td>R 5′-CACCTTACCGTTCCAGTTT-3’</td>
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Notes: transcriptional abundance is expressed as fold increase relative to a control value calculated using the 2⁻ΔΔCt method.

Western blot analysis
Antibodies against Mfn2 and β-tubulin were obtained from Sigma (St. Louis, MO, USA). Antibodies against P-ERK1/2, cyclin A, CDK2, p-p27,GADPH were obtained from Cell Signaling Technologies (Danvers, MA, USA). These proteins were examined by western blotting. Total protein was isolated from cells and tissues after 30 min on ice. After centrifugation (12 000 rpm, 15 min, 4 °C), the supernatant was collected and the protein concentration was measured using the bicinchoninic acid protein assay (Thermo Scientific, Waltham, MA, USA). Equal amounts of protein (50 μg) were separated by SDS-PAGE and transferred onto PVDF membranes. After blocking nonspecific binding sites for 3 h with 5% nonfat milk, the membranes were incubated overnight on ice with primary antibodies against Mfn2 (1: 500), P-ERK1/2 (1: 500), cyclin A (1: 400), CDK2 (1: 400), p-p27 (1: 400), β-tubulin (1: 250), GADPH (1: 250). After washing, the membranes were probed with appropriate secondary antibodies for 90 min on ice. Then, membrane enhanced chemiluminescence was conducted using a chemiluminescence detection kit.

Cell proliferation analyses
Cells were seeded in 96-well plates with a final total volume of 100 μL/well, and were treated with different interventions for 48 h. Then, 10 μL of Cell Counting Kit-8 (CCK-8) solution was added to each well. The plates were incubated at 37 °C for 2 h in the dark. The absorbance at 450 nm was measured using a microplate reader (Omega), and cell proliferation was calculated using the following equation: cell proliferation = (mean A₄₅₀ of interfered cells/mean A₄₅₀ of non-interfered cells) × 100%. To induce the proliferation of GMCs, cells were incubated with IgA1 as a test group. In accordance with the results of the CCK8 assay, we defined the optimal concentration and time of IgA1 for culture with GMCs to be 100 μL/mL for 24 h.

Mitochondrial separation and respiratory activity
The mitochondria were isolated by differential centrifugation, a mitochondrial suspension was made, the protein concentration was determined, and the mitochondrial activity was determined by the Clark oxygen elec.
trole. This involved calculating the RCR value, which is the ratio of S3 [the respiratory rate of mitochondria in the presence of substrate (succinic acid) and ADP] to S4 (the respiratory rate of the substrate when the ADP runs out). The RCR value can be used to evaluate the state of mitochondrial respiratory function, to evaluate the integrity of mitochondrial structure and function.

**Statistical analysis**

All values are expressed as mean ± standard error of mean. The results were statistically evaluated by one-way analysis of variance. The results in the same group were statistically evaluated by t-test, with \( P < 0.05 \) considered to represent statistical significance. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses.

**RESULTS**

**HCA prevented IgA1-induced GMCs proliferation by regulating Mfn2 expression**

To determine the effect of HCA on IgA1-induced GMCs proliferation, we used CCK8 to detect GMCs proliferation. As shown in Figure 1A, B in the test group (TG), IgA1 induced GMCs proliferation (1.17 ± 0.10) compared with that in the control group (CG) (1.02 ± 0.08, \( P < 0.05 \)). After we treated cells with HCA at different concentrations (HG: 0.2 mg/mL: LG: 0.1 mg/mL), GMCs proliferation was inhibited (LG: 1.05 ± 0.11: HG: 0.95 ± 0.10) compared with that in TG (\( P < 0.05 \)). In HG, the inhibitory effect was greater than that in the LG group (\( P < 0.05 \)), showing that the inhibitory effect of HCA on the proliferation of mesangial cells was concentration-dependent. We further detected the Mfn2 expression by real-time PCR. The results showed that, in TG, Mfn2 expression was downregulated compared with that in CG (\( P < 0.05 \)). After treatment with HCA, the expression increased in HG compared with that in TG (\( P < 0.05 \)), with a clear difference between the two groups. We also tested Mfn2 expression by western blotting and obtained the same results (Figure 1C-E).

**Overexpression of Mfn2 can inhibit IgA1-induced GMCs proliferation**

In our study, fluorescence microscopy was used to estimate the efficiency of exogenous gene transfection. The number of GFP-positive cells dramatically increased 12 h after transfection and peaked after 48 h, with 80% transfection efficiency. No statistically significant difference was observed between the AdGFP and AdMfn2 groups. To prove that the adenovirus-mediat-
Overexpression of Mfn2 can elevate HCA’s effect of inhibiting GMCs proliferation

Based on the above experiments, we further treated GMCs with HCA. We found that the proliferation of MGCs in the AdMfn2 + IgA1 + HCA group was inhibited more than that in the other groups (P < 0.05; Figure 2E). This means that the overexpression of Mfn2 can increase the effect of HCA on inhibiting GMCs proliferation.

HCA inhibited GMCs proliferation and ameliorated albuminuria in IgAN animal model

To determine the effect of HCA in an IgAN animal model, we established such a rat model for a tissue experiment. In this experiment, all of the rats in the IgAN group exhibited IgA, IgG, and C3 deposition in the mesangial region, as detected by immunofluorescence. As shown in Figure 3A, according to hematoxylin-eosin (HE) staining, there was clear GMCs proliferation in the IgAN group, compared with that in the control group (CG). In the medicine groups (HG and LG), treatment with HCA inhibited GMCs proliferation. There was no obvious difference in cell proliferation between HG and LG. Moreover, albuminuria and serum creatinine increased in the IgAN group compared with those in the CG. Meanwhile, after treatment with HCA, all of these variables decreased (P < 0.05; Figure 3B, C). These findings indicate that HCA can reduce mesangial cell proliferation, albuminuria, and serum creatinine in IgAN, although these effects were not concentration-dependent.
Through upregulating Mfn2 expression, HCA inhibited Ras-Raf-ERK/MAPK signaling pathway

To evaluate the underlying signal transduction mechanisms associated with proliferation and apoptosis in IgAN, we detected the Mfn2 expression and expression of related signaling proteins (P-ERK1/2, cyclin A, CDK2, and p-p27). We used real-time PCR and western blotting to detect the expression of Mfn2. Compared with that in CG, Mfn2 expression was decreased in the IgAN group \((P < 0.05)\). Meanwhile, treatment with HCA elevated Mfn2 expression, compared with that in the IgAN group \((P < 0.05)\). There was a clear difference in this variable between HG and LG (Figure 3D-F). We further detected Mfn2 expression by immunohistochemical staining, as shown in Figure 3F, which revealed that it was located in mesangial cells.

The Ras-Raf-ERK/MAPK signaling pathway is activated in response to a multitude of mitogenic stimuli and drives cell-cycle progression through the activation of cyclin-dependent kinases (CDKs). CDKs form holoenzymes with their regulatory subunits, the cyclins, resulting in phosphorylation of the tumor suppressor protein Rb and S-phase entry. ERK1/2 signaling is also necessary for the degradation or downregulation of CDK inhibitors, such as p21waf1 (p21) and p27kip1 (p27), particularly p27, thereby eliminating their growth-suppressive activities. Based on these findings, we detected P-ERK1/2, cyclin A, CDK2 and p-p27 expression, the results showed that, compared with that in CG, in the IgAN group, the expression of P-ERK1/2, cyclin A, and CDK2 was increased, while that of p-p27 was decreased \((P < 0.05)\). After treatment with

![Graphs and images showing experimental results.](image-url)

Figure 3 HCA inhibited GMCs proliferation and ameliorated albuminuria and serum creatinine in IgAN animal model

A: HE stain detection GMCs proliferation \((\times 400)\). A1: in control group; A2: in IgAN group; A3: in low group; A4: in high group. B, C: serum creatinine and 24 h albuminuria in different groups. D-E: detection Mfn2 expression by Western-blot. F: detection Mfn2 expression by real-time PCR. G: detection Mfn2 expression by Immunohistochemical stains \((\times 400)\). G1: in control group; G2: in IgAN group; G3: in low group; G4: in high group. CG: normal rats were treated only with distilled water (2 mL/d). IgAN group: IgAN animal models (treated with bovine serum albumin by gavage lipopolysaccharide and carbon tetrachloride injection) were treated with sterile distilled water (2 mL/d). HG: IgAN animal models were treated with HCA \((90 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1})\). LG: IgAN animal models were treated with HCA \((45 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1})\). Compared with CG, \(P < 0.05\); compared with IgAN, \(P < 0.05\); compared with HG, \(P < 0.05\). HCA: Jixuecao (Herba Centellae Asiaticae); GMCs: glomerular mesangial cells; HE: hematoxylin-eosin; real-time PCR: real-time polymerase chain reaction; IgAN: IgA nephropathy; HG: high group; LG: low group; CG: control group.
HCA, the expression of P-ERK1/2, cyclin A, and CDK2 was markedly reduced, while the expression of p-P27 was increased, compared with the levels in the IgAN group ($P < 0.05$). Between the groups administered HCA there was a significant difference in the expression of these proteins ($P < 0.05$). These results indicate that, by upregulating the expression of Mfn2, HCA can further inhibit the Ras-Raf-ERK/MAPK signaling pathway, thus having an effect of preventing GMCs proliferation in IgAN. This effect was shown to be concentration-dependent (Figure 4).

**DISCUSSION**

Mfn2 is embedded in the mitochondrial outer membrane and mediates mitochondrial fusion. Its deficiency or dysfunction is associated with many diseases, such as human neurodegenerative diseases, Charcot-Marie-Tooth type 2A, Parkinson’s disease, and Alzheimer’s disease. Mfn2 also plays an important role in regulating the proliferation and apoptosis of VSMCs through the MAPK and Ras-Pi3K-Akt pathways: notably, this is independent of its mitochondrial fusion function. Mounting evidence suggests that Mfn2 plays an important role in regulating the proliferation of a variety of cells. It has been widely published in cardiovascular proliferative diseases and cancers such as urinary bladder carcinoma, hepatocellular carcinoma, and breast carcinoma. Brook et al. studied the effects of Mfn2 in cisplatin-induced acute tubular necrosis and found that it has a protective influence on the
kidney by inhibiting excessive apoptosis of renal tubular epithelial cells. Moreover, Tang et al.\textsuperscript{23} found that the overexpression of Mfn2 could alleviate high-glucose-induced GMCs proliferation and increase apoptosis, which may contribute to reversing pathological changes in early diabetic nephropathy. However, few studies have focused on the connection between IgAN and Mfn2.

HCA has been widely used in the treatment of many diseases and other health conditions in a clinical context, such as scar healing, type 2 diabetes, and for cardioprotective purposes. Recent studies have revealed that HCA acts as an antioxidant, reduces the effects of oxidative stress, and modulates antioxidant and mitochondrial pathways to improve cognitive function in mice with Alzheimer’s disease. It may also affect mitochondrial biogenesis, which in conjunction with the activation of antioxidant response genes and normalizing calcium homeostasis probably contributes to cognitive and neuroprotective effects against amyloid-β toxicity.\textsuperscript{24,25} Maulidiani et al.\textsuperscript{26} showed that long-term treatment with HCA extract in an obese diabetic condition could reverse glucose, lipid, tricarboxylic acid cycle, and amino acid metabolic disorders back towards the normal state, and enhance the secretion of insulin by β cells. Si et al.\textsuperscript{27} also demonstrated that HCA attenuated fibrosis by blocking both transforming growth factor-β1/Smad and interleukin-6 signaling activation, to attenuate pathological cardiac structural remodeling and preserve cardiac function. Xiao et al.\textsuperscript{28} reported that HCA functioned as a Smad7 agonist and suppressed TGF-β/ Smad3-mediated renal fibrosis in a mouse model of obstructive nephropathy by inducing Smad7. HCA also has a key effect of anti-fibrotic activity. Another study also found that it can maintain mitochondrial morphology, playing an important role in inhibiting myocardial remodeling.\textsuperscript{27}

Increasing evidence indicates that the overexpression of Mfn2 suppresses cell proliferation and triggers apoptosis in cultured VSMCs by inhibiting the Ras-Raf-ERK/MAPK signaling pathway and the Ras-PI3K-Akt cell survival pathway.\textsuperscript{24,25} Based on the above studies, we further tested the proteinuria, renal pathology, Mfn2, and downstream-related protein expression in IgAN animal models. We found similar results: in the IgAN group, the proliferation of mesangial cells and the severity of proteinuria increased significantly, the expression of Mfn2 and downstream-related protein expression decreased significantly, and HCA treatment significantly reversed pathological changes in IgAN animal models. In addition, HCA treatment significantly improved mitochondrial structure and respiratory function in IgAN animal models.

Figure 5 HCA improved mitochondrial structure and respiratory function

A-C: clark oxygen electrode method was used to detected mitochondrial activity; D: detected mitochondrial structure by electron microscopy. D1: in control group; D2: in IgAN group; D3: in low group; D4: in high group. D1, D3, D4 magnification × 25000. In IgAN group Mitochondrial swell and the disappearance of mitochondrial ridge. The mitochondrial structure was normal in CG, HG and LG. CG: normal rats were treated only with distilled water (2 mL/d). IgAN group: IgAN animal models (treated with bovine serum albumin by gavage lipopolysaccharide and carbon tetrachloride injection) were treated with sterile distilled water (2 mL/d). HG: IgAN animal models were treated with HCA (90 mg·kg\textsuperscript{-1}·d\textsuperscript{-1}). LG: IgAN animal models were treated with HCA (45 mg·kg\textsuperscript{-1}·d\textsuperscript{-1}). Compared with CG, \(P<0.05\); compared with IgAN, \(P<0.05\). HCA : Jixuecao (Herba Centellae Asiaticae); RCR: respiratory control ratio; S3: State 3 respiration; S4: State 3 respiration; IgAN : IgA nephropathy; HG: high group; LG: low group; CG: control group.
proteinuria were significantly higher than in the control group and were negatively correlated with the expression of Mfn2. We further examined the downstream pathway proteins and discovered that the expression of Mfn2 in the IgAN group was significantly reduced, the expression of p-ERK1/2, cyclin A, and CDK2 was increased, and p-p27 expression was decreased. These results indicated that reduced expression of Mfn2 in IgAN may activate the Ras-Raf-ERK/MAPK signaling pathway, increase P-ERK1/2, and downregulate the cell cycle protein kinase inhibitor p-p27, prompting cyclin A expression and the rise of CDK2. This in turn prompts Rb phosphorylation, leading to the accumulation of Rb, which then promotes G1/S-phase transformation, cell cycle progression, and finally cell proliferation.

Against this background, we further treated rats with different concentrations of HCA and found that HCA could effectively inhibit the exacerbation of proteinuria and proliferation of mesangial cells in IgAN, and that the inhibitory effect occurred in a concentration-dependent manner.

With regard to the detection of Mfn2 and downstream proteins, we found that, after treatment with HCA, the expression of Mfn2 was significantly increased, p-ERK1/2, cyclin A, and CDK2 expression was downregulated, and p-p27 expression was increased. It is thus suggested that HCA can inhibit the Ras-Raf-ERK/MAPK signaling pathway by upregulating Mfn2 and play a role in the treatment of IgAN.

Mfn2 is an important protein for regulating mitochondrial structure. Previous studies revealed that the inhibitory effect of Mfn2 on cell proliferation is independent of the promotion of mitochondrial fusion. To determine whether the effect of promoting mitochondrial fusion of Mfn2 is also involved in the pathogenesis of IgAN, we performed electron microscopy analysis. This revealed, in the IgAN group, mitochondrial swelling, vacuolar degeneration, and disappearance of the mitochondrial ridge. We further characterized the mitochondrial respiratory function, which showed that the levels of S3, S4, and RCR in the IgAN group were significantly lower than those in the control group. Meanwhile, after treatment with HCA, we identified improvements in the changes of mitochondrial structure and function. The findings indicated that the downregulation of Mfn2 leads to abnormal mitochondrial structure and function, which is also involved in the occurrence and progression of IgAN. HCA thus plays an important role in improving mitochondrial structure and function.

In conclusion, our results demonstrate that HCA might inhibit mesangial cell proliferation in IgAN through regulating Mfn2 and the upstream signaling pathway Ras-Raf-ERK/MAPK. Our novel discoveries include that changes of mitochondrial structure and function are involved in the pathogenesis of IgAN and that HCA has an important role in improving these changes. These findings provide novel insight into the renoprotective effect of HCA as well as evidence supporting its use in a wide range of IgAN treatments.

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