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ORIGINAL ARTICLE

Intermedin ameliorates IgA nephropathy by inhibition of oxidative stress and inflammation

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Abstract IgA nephropathy (IgAN) is the most frequent form of glomerulonephritis worldwide. The role of oxidative stress and inflammation in the pathogenesis of IgAN has been reported. Intermedin (IMD) is a newly discovered peptide that is closely related to adrenomedullin. We have recently reported that IMD can significantly reduce renal ischemia/reperfusion injury by diminishing oxidative stress and suppressing inflammation. The present study was designed to explore whether IMD ameliorates IgAN via oxidative stress- and inflammation-dependent mechanisms. Our results showed that IMD administration resulted in the prevention of albuminuria and ameliorated renal pathomorphological changes. These findings were associated with (1) decreased renal TGF-β1 and collagen IV expression, (2) an increased SOD level and reduced MDA level, (3) the inhibition of the renal activation of NF-κB p65 and (4) the downregulation of the expression of inflammatory factors (TNF-α, MCP-1 and MMP-9) in the kidney. These results indicate that IMD in the kidney protects against IgAN by reducing oxidative stress and suppressing inflammation.

Keywords Intermedin · IgA nephropathy · Oxidative stress · Inflammation

Introduction

Immunoglobulin A nephropathy (IgAN) is the most common form of glomerulonephritis worldwide and one of the main causes of end-stage renal disease (ESRD) [1, 2]. Recent studies have indicated that IgAN may cause slowly progressive chronic renal impairment, eventually leading to ESRD. Approximately 25–30 % of patients will require renal replacement therapy within 20–25 years [3, 4]. The costs of dialysis and kidney transplantation increase yearly and have become heavy burdens on patients and the society. Thus, it is of great importance to find a novel and efficient way to treat IgAN.

Previous reports have indicated that reactive oxygen species (ROS) and mononuclear leukocyte infiltration in the kidney play important roles in the pathophysiology of both IgAN patients [5–8] and animal models of this disease [9–11]. ROS can accelerate the activation of the transcription factor nuclear factor-kappa B (NF-κB) and stimulate the expression of cytokines, including tumor necrosis factor-alpha (TNF-α), matrix metalloproteinase-9 (MMP-9) and monocyte chemoattractant protein-1 (MCP-1), which has been shown to play a critical role in the recruitment of leukocytes to injury sites and their adherence to endothelium [12–15]. Furthermore, the infiltration of leukocytes can also enhance tissue damage via positive feedback by increasing the production of ROS. Thus, the inactivation of ROS might be an effective strategy to combat the progression of IgAN.

Intermedin (IMD), which is also called adrenomedullin-2 (ADM2), is a novel member of the calcitonin gene-

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related peptide (CGRP) superfamily [16, 17]. IMD is distributed in a wide variety of tissues, including the brain, heart, lung, gastrointestinal tract, pituitary and kidney [18, 19]. It has been noted for its protective roles in tissue injuries, such as injuries of the central nervous, pulmonary, cardiovascular and renal systems [20, 21]. A recent study by our group has demonstrated that IMD reverses renal ischemia/reperfusion (I/R)-induced oxidative stress and ROS release in vivo and in vitro [22]. In addition, in many animal disease models, this protein has been shown to have protective effects on some tissues and cells via the inhibition of oxidative stress [23–26]. Thus, the effects of the inhibition of oxidative stress by IMD on the progression of IgA nephropathy (IgAN) and the mechanisms underlying this process have not been widely investigated.

The aim of the present study was to test the hypothesis that IMD prevents the development of IgAN through the inhibition of oxidative stress and accompanying inflammatory processes, including the activation of NF-κB, TNF-α, MMP-9 and MCP-1 expression in an IgAN rat model.

Materials and methods

Animal model

Six-week-old male Sprague–Dawley rats (specific pathogen-free) weighing 150 g (±10) were obtained from the Experimental Animal Center of Shanxi Medical University and housed under stable environmental conditions (temperature of 22 ± 1 °C with a 12-h dark period). The rats were given free access to tap water and fed a standard laboratory diet. The protocol was approved by the Institutional Animal Care and Use Committee of Shanxi Medical University. All rats were active and had glossy hair before the start of the experiments.

The rats ($n = 40$) were divided randomly into the following groups: control with solvent treatment (control), control with IMD treatment (control + IMD), IgA nephropathy model with solvent treatment (IgAN) and IgA nephropathy model with IMD treatment (IgAN + IMD). The IgAN rat model was established in our previous study [27]. Briefly, rats were administered bovine gamma globulin (BGG) 0.1 % (Sigma, St Louis, MO, USA) with 6 mM HCl in tap water for 8 weeks, followed by tail intravenous injection of 1 mg BGG daily for 3 successive days. For the control group, the BGG was replaced with saline. The IgAN model was established at the end of 12 weeks. No other rat deaths occurred during the whole experimental process.

After the establishment of the IgA nephropathy model, the IMD-treated animals were subcutaneously administered IMD (100 ng/kg/h) in normal saline by a mini-osmotic pump (ALZET model 2004 osmotic pump; ALZA Corp., CA, USA) for 3 weeks.

Hepatic and renal function

Coomassie Brilliant Blue was utilized to measure 24-h urinary protein levels. Renal and hepatic functions were analyzed by measuring serum creatinine, urea nitrogen, total protein, albumin, alanine amino transferase and aspartate amino transferase levels with an autoanalyzer (Beckman Instruments, Palo Alto, CA).

Morphologic analysis by light microscopy

After fixation with 10 % paraformaldehyde, paraffin-embedded transverse kidney slices were sectioned at 3 μm and stained with hematoxylin and eosin (HE) and periodic acid–schiff (PAS).

Immunohistochemistry

Consecutive kidney sections (5 μm) were deparaffinized and rehydrated with a gradient of ethanol concentrations, and antigen retrieval was performed by high-pressure repair for 2 min. The sections were incubated in 3 % H_2O_2 for 20 min to block endogenous peroxidase and then in 1.5 % blocking serum for 30 min to block nonspecific staining. After antigen retrieval, the kidney sections were incubated with polyclonal rabbit antirat TGF-β, collagen IV, NF-κB, TNF-α, MMP-9 and MCP-1 antibodies (each at a 1:100 dilution, Santa Cruz Biotechnology, CA, USA), followed by incubation with biotinylated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and finally with avidin-conjugated horseradish peroxidase. An isotype-matched control antibody was used as a negative control.

All images were acquired using an Olympus BX51 clinical microscope and a DP70 digital camera and software (Olympus, Tokyo, Japan) as described previously [28]. Briefly, the image was analyzed using Image-Pro Plus software (Media Cybernetics). For each glomerulus, the sum integrated optical density (IOD SUM) of positive area (brown) was calculated automatically by the software and divided by the total positive area of the glomerulus. At least 10 random glomeruli from randomly chosen kidney sections for each rat and the average mean density (IOD SUM/area) were determined. The relative density was quantified as fold expression relative to control rats.

Reverse transcription polymerase chain reaction and real-time polymerase chain reaction

Total RNA was extracted from renal cortex tissue using Trizol (TAKARA, Dalian, China). Complementary DNA

(cDNA) was obtained from total RNA using an SYBR Green I assay and a Stratagene M3000 Sequence Detection System (Stratagene, USA). Gene sequences were identified in GenBank to design specific primers (Table 1). Amplifications were carried out using a 96-well plate in 20-μL reaction volumes under the following conditions: 95 °C for 5 min, followed by 95 °C for 5 s and 60 °C for 30 s for 40 cycles. For each assay, a standard curve was determined alongside the examined samples. Gene expression was quantified using a modification of the 2-ΔΔct method.

Western blot analysis

The cortical tissues of the rat kidneys were extracted in lysis buffer (KeyGEN Biotech, Nanjing, China). Equal amounts of protein (50 μg), as determined using a BCA assay kit (KeyGEN Biotech, Nanjing, China), were separated on 10 % SDS–polyacrylamide gels and were electrophoretically transferred to a nitrocellulose filter membrane (Millipore, Bedford, MA). After blocking, the membranes were incubated overnight at 4 °C with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 dilutions. After being washed three times, a fluorescein-linked secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:3000 dilution was added, and the membranes were incubated at room temperature for 2 h. The specific bands were visualized by fluorography using an enhanced chemiluminescence kit (Pierce, Rockford, USA). The relative density was quantified using a Quantity One analysis system (Bio-Rad, California, USA).

Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content

For the measurements of SOD activity and the products of lipid peroxidation (MDA), rat kidneys were homogenized in ice-cold 20 mM Tris–HCl buffer (pH 7.4). The SOD activity and MDA level were determined with commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, P.R. China).

Table 1 Primers for real-time PCR

Gene	Primer sequences (5′ to 3′)	
TGF-β1	Forward	CATTGCTGTCCCGTGCAGA
	Reverse	AGGTAACGCCAGGAATTGTTGCTA
Col-IV	Forward	GGGGTCGGGCTGGGAGTGAT
	Reverse	GCTGGCCGTCCATACCCGTG
MMP-9	Forward	AGTTTGGTGTCGCGGAGCAC
	Reverse	TACATGAGCGCTTCCGGCAC
TNF-α	Forward	AACTCCAGCCGG
	Reverse	GTTCAGCAGGCAGAAGAGGATT
MCP-1	Forward	CAGCCAGATGCAGTTAATGCC
	Reverse	AGCCGACTCATTGGGATCAT
β-actin	Forward	TGGCTCCTAGCACCATGAAG
	Reverse	GCTCAGTAACAGTCCGCCTAGA

Statistical analysis

The results are expressed as the mean ± SD. Data were analyzed by one-way ANOVA. A $P < 0.05$ was considered statistically significant.

Results

IMD attenuated proteinuria in IgA nephropathy rats

The rats with IgA nephropathy showed significant increases in urine protein levels beginning on week 3, and these levels continued to increase until the end of the study (29.11 ± 5.14 mg/24 h, 15 weeks) when the animals were killed (Fig. 1). The level of proteinuria did not increase after week 12 in the IgAN + IMD rats, and the final proteinuria level was deceased to 15.52 ± 2.76 mg/day, which was significantly lower than that in the untreated animals. The proteinuria level of the normal control group remained consistently low (week 15: 2.41 ± 0.34 mg/24 h).

Although the urine protein levels significantly increased in the IgAN rats, no evidence of renal or liver function damage was observed during the entire study period. Only creatinine slightly increased in the IgAN rats compared with the control rats on the 15th weekend (39.5 ± 2.22 vs.

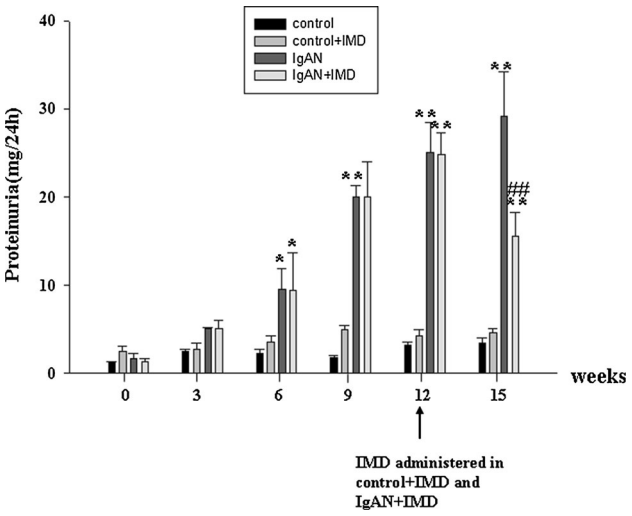

Fig. 1 IMD attenuated proteinuria in IgA nephropathy rats. The data are presented as the mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus IgAN

29.30 ± 4.61 μmol/L, $P < 0.05$). The body weight changes of the rats in each group did not significantly differ (data not shown). In addition, the conditions of the mice did not decline significantly during the experiment, and no significant hair loss or appetite change was observed.

Pathomorphological changes in renal tissue

Light microscopy, as shown in Fig. 2a, b, revealed marked glomerular proliferation of mainly mesangial and focal cells as well as interstitial (mainly periglomerular) mononuclear leukocyte infiltration in the kidneys of the IgAN rats compared with the normal control group. In contrast, all of these renal pathological abnormalities were markedly reduced in the IgAN + IMD group. In addition, there was no significant difference in the intensity of IgA deposition in the glomerulus between the IgAN and IgAN + IMD groups (data not shown), indicating that the IMD treatment had no effect on immune complex deposition.

IMD decreased the expression of TGF-β1 and the deposition of collagen IV in IgAN rats

As shown in Fig. 3a, b, compared with the normal control animals, the IgAN rats were characterized by a marked increase in TGF-β1 expression (IgAN vs. control, $P < 0.01$). These effects were substantially decreased in the IgAN + IMD rats (33.3 %, IgAN + IMD vs. IgAN, $P < 0.05$). The representative immunohistochemistry images are shown in Fig. 3c, d. Collagen IV deposition was 2.54-fold higher in the IgAN glomerular sections compared with the normal controls. IMD distinctly lowered glomerular collagen IV deposition by 58 % (IgAN + IMD vs. IgAN, $P < 0.01$).

To further confirm our findings, we measured the mRNA expression levels of TGF-β1 and collagen IV (Fig. 3e), which were consistent with the above results.

IMD attenuated oxidative stress-induced injury in IgAN rats

It has also been reported that renal damage in IgA nephropathy is associated with an increase in the expression of intrarenal ROS [29]. SOD inactivates ROS, thereby playing a protective role in IgAN [30]. To understand the possible mechanism involving IMD in IgA nephropathy rats, we measured the activity of SOD. As shown in Table 2, we found that SOD activity in the kidney was significantly decreased in the IgAN rats and that IMD treatment significantly increased SOD activity. Malondialdehyde (MDA) is a well-accepted marker of oxidative stress. As expected, the IgAN rats had significantly higher levels of MDA than the normal control rats, and this effect was significantly reduced in the IgAN + IMD rats (Table 2). These results suggest that IMD reduces oxidative stress in IgAN rats.

NF-κB activation associated with IgAN and IMD led to effective inhibition of its phosphorylation

Activation of the NF-κB pathway has been implicated in the acceleration and progression of IgAN [31, 32]. As shown in Fig. 4a, b, compared with the normal control group, the IgAN rats showed the significantly increased renal nuclear translocation of NF-kB p65 compared to the normal control rats ($P < 0.01$). IMD significantly suppressed the synthesis of NF-kB in the renal tissues of the IgAN rats. Moreover, the Western blotting results demonstrated that NF-kB p65 was greatly increased in the

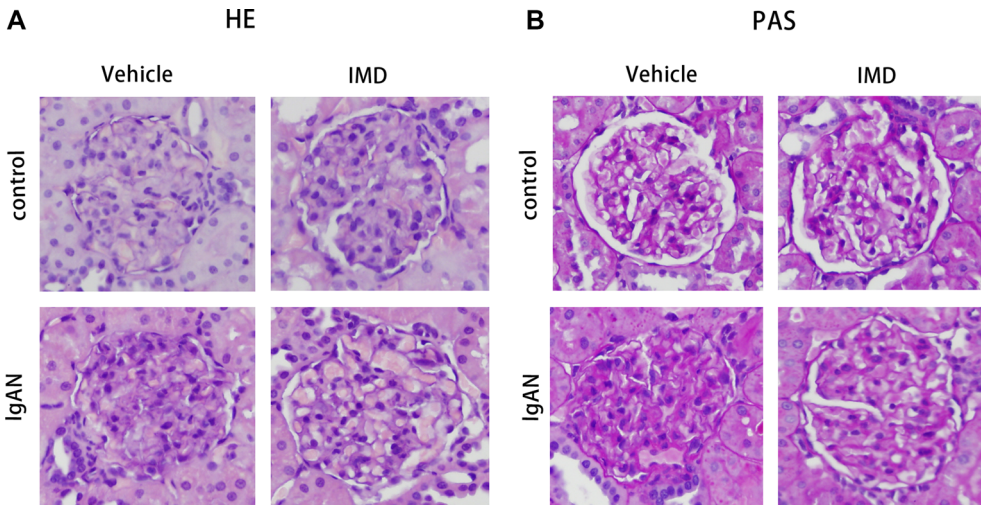

Fig. 2 Pathomorphological changes in renal tissue. **a** HE staining, **b** PAS staining. Original magnification, 400×

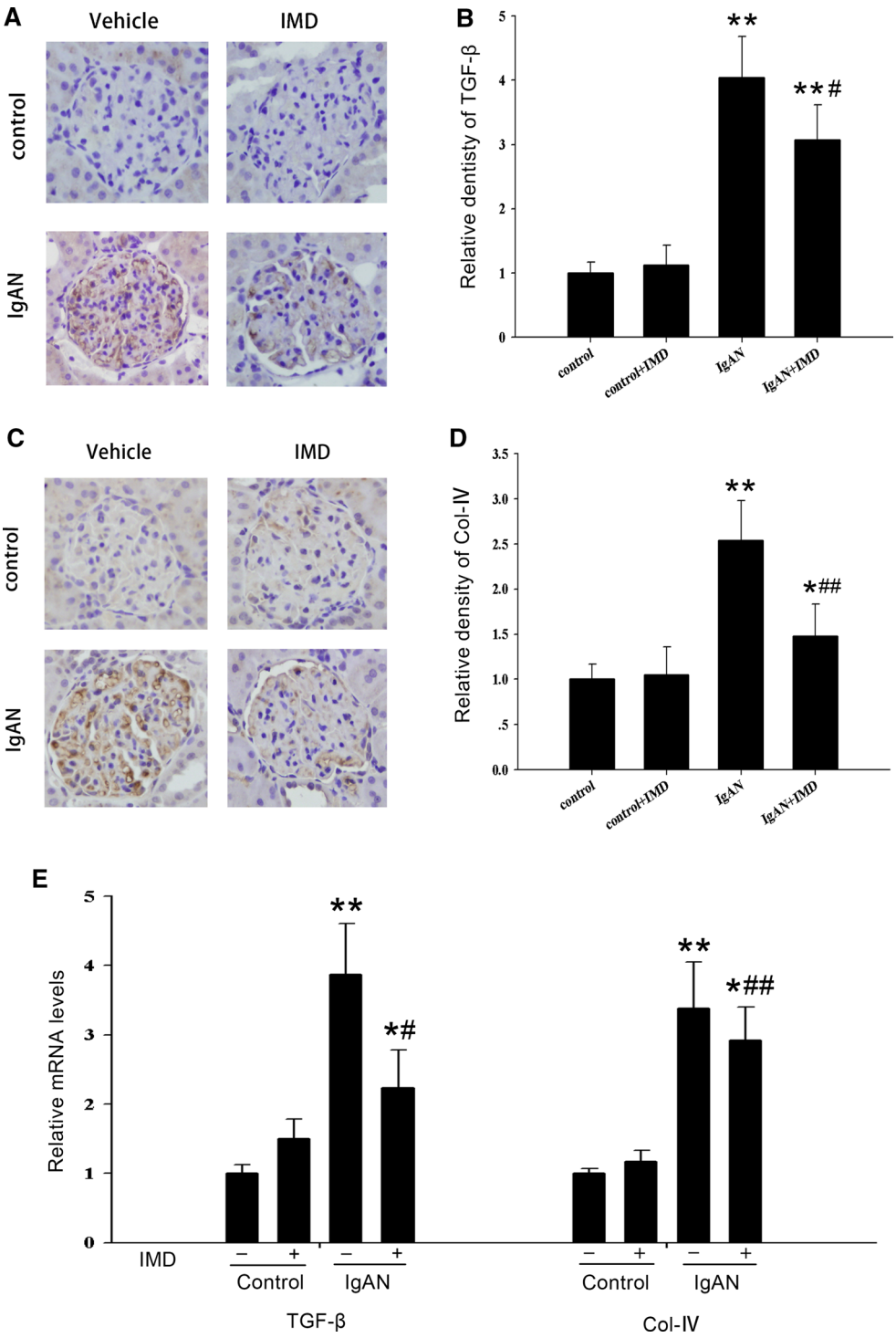

Fig. 3 Effects of IMD on markers of glomerular fibrosis in IgAN rats. Glomerular expression of TGF-β (**a**, **b**) and collagen IV (**c**, **d**) as shown by immunohistochemical staining. The *arrowheads* in the stained *panels* indicate positive staining. **e** The mRNA expression levels of TGF-β and collagen IV were determined by real-time PCR. The values represent the mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus IgAN. Original magnification, 400×

Table 2 Superoxide dismutase (SOD) activity and malondialdehyde (MDA) concentration in rats

	Control	Control + IMD	IgAN	IgAN + IMD
SOD activity (U mg^{-1})	77.15 ± 21.47	69.71 ± 16.20	57.14 ± 8.35**	64.29 ± 7.55*#
MDA content (nmol mg^{-1})	12.37 ± 4.92	13.23 ± 5.88	23.86 ± 5.49**	17.12 ± 5.12*#

The data are presented as the mean ± SD. * $P < 0.05$ and ** $P < 0.01$ versus control; # $P < 0.05$ versus IgAN

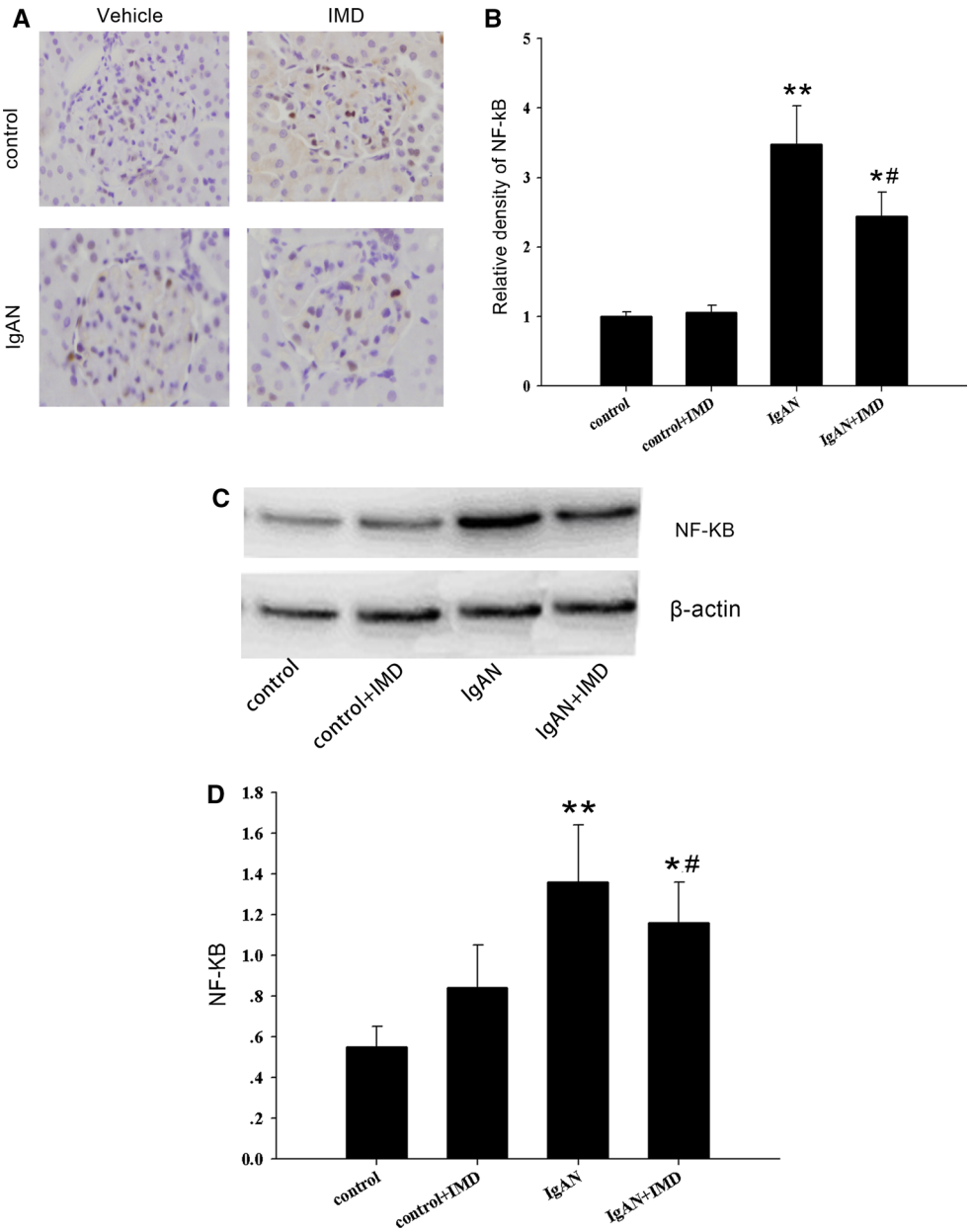

Fig. 4 Nuclear translocation of NF-kB p65 in IgAN rats. **a** Representative photographs of immunohistochemical staining sections show the nuclear translocation of NF-kB p65 (**a**, **b**). The *arrowheads* in the stained *panels* indicate positive staining. Western blot was used to analyze NF-kB p65 levels (**c**, **d**). The *bar graph* shows the mean ± SD from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus IgAN. Original magnification, 400×

IgAN rats compared to the normal control rats ($P < 0.01$) and that this effect was markedly inhibited in the IgAN + IMD rats ($P < 0.05$) (Fig. 5c), which indicated that IMD can regulate the NF-κB pathway activities.

Effects of IMD on TNF-α, MCP-1 and MMP-9 in IgAN rats

The immunohistochemistry images shown in Fig. 5a–f provide a characteristic overview of the impact of IMD on cytokine levels in IgAN rats. The expression levels of the cytokines TNF-α, MCP-1 and MMP-9 were almost negative in the renal tissues of the normal control group rats. However, the IgAN rats showed significantly increased levels of all three cytokines. In the IgAN + IMD rats, significant reductions were observed in the expression of TGF-β1 (56.4 %, IgAN + IMD vs. IgAN, $P < 0.01$), MCP-1 (46.5 %, IgAN + IMD vs. IgAN, $P < 0.05$) and MMP-9 (58.1 %, IgAN + IMD vs. IgAN, $P < 0.01$). The mRNA expression of TNF-α, MCP-1 and MMP-9 confirmed these results (Fig. 5g).

Discussion

In the present study, we showed that IMD/ADM2 can ameliorate renal pathomorphological changes and prevent the further increase in proteinuria in IgAN rats. Our data

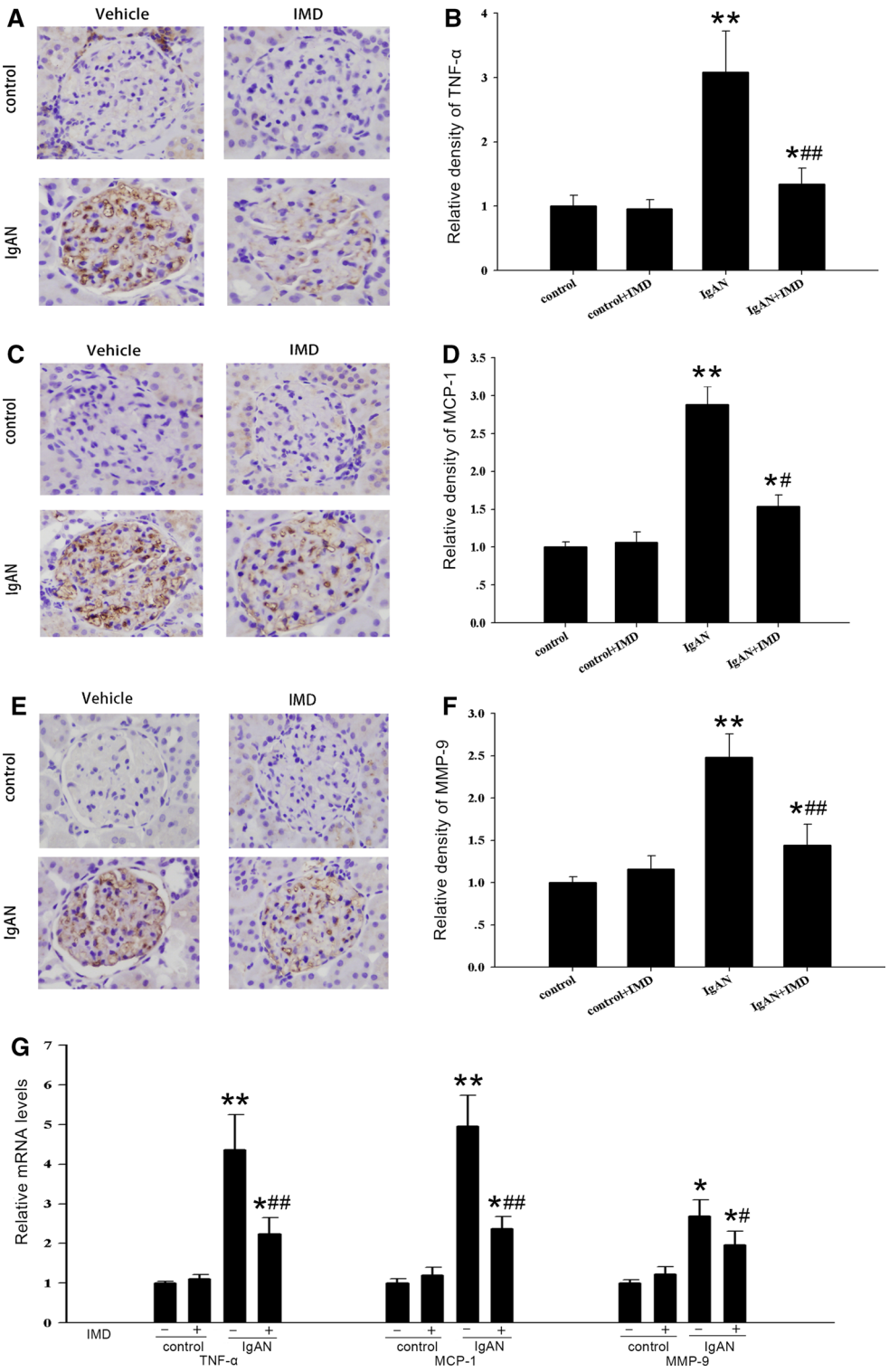

Fig. 5 Effects of IMD on cytokines in IgAN rats. Glomerular expression of TNF-α (**a**, **b**), MCP-1 (**c**, **d**) and MMP-9 (**e**, **f**) as shown by immunohistochemical staining. The arrowheads in the stained *panels* indicate positive staining. **g** The mRNA expression levels of TNF-α, MCP-1 and MMP-9 were determined by real-time PCR. The values represent the mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus IgAN. Original magnification, 400×

suggest that the beneficial effects of IMD on IgAN rats mainly occur through the inhibition of oxidative stress and NF-kB activation as well as the reduction in inflammatory cytokine expression in the kidney.

As a member of the calcitonin/CGRP family, IMD has been shown to have pathophysiological effect in multiple disease processes involving the circulatory and renal systems [25]. IMD augments cardiac contractility [33],

inhibits collagen synthesis, attenuates the proliferation of cardiac fibroblasts [34] and reverses renal ischemia/reperfusion [35].

IMD/ADM2 prevented an additional increase in proteinuria and protected kidney function in rats with IgAN. In our IgAN model, despite the presence of heavy proteinuria, the animals had normal renal function that remained stable for several months. These findings were consistent with clinical symptoms that have been reported for human IgAN [36]. The serum creatinine levels in the IgAN group were slightly higher than those in the control group only at week 15. These data proved that substantial proteinuria for a long period of time could affect kidney function. On the 15th weekend of this experiment, renal pathologies in the IgAN group included glomerular proliferation (mainly mesangial and focal cells) as well as increases in interstitial mononuclear leukocyte infiltration and proteinuria, and pathological lesions were markedly reduced in the IgAN + IMD group. Moreover, the levels of TGF-β and Col-IV were higher in the IgAN group than in the control group. Compared with the IgAN group, the level of proteinuria was reduced, renal pathological lesions were ameliorated, and the expression levels of TGF-β and Col-IV were decreased in the IgAN + IMD group. These results suggest that IMD/ADM2 protected the kidneys of the IgAN rats by improving their structures and functions. To determine the underlying protective mechanism, we explored the effects of IMD on the three main inflammatory mediators, TNF-α, MCP-1 and MMP-9, and on macrophage infiltration in the kidneys of IgAN rats.

IMD/ADM2 can help to prevent the development of renal lesions in IgAN rats in a progressive manner, which may occur due to the inhibition of oxidative stress. Oxidative stress caused by the increased production of ROS and compromised antioxidant activity are major pathogenic factors in the development [37–39] and progression [40, 41] of glomerular disorders, including IgAN [41]. Consistent with this concept, we found that SOD activity was significantly reduced, and the MDA concentration was increased in the IgAN group. This increased oxidative stress was associated with impaired renal function and histological changes [42], as evidenced by a marked increase in serum creatinine and characteristic renal morphological changes. However, IMD/ADM2 administration significantly inhibited the increase in MDA level in the IgAN rats, augmented SOD activity in the injured kidneys and inhibited lipid peroxidation. These data suggest that a decrease in oxidative stress caused by the activity of IMD/ADM2 may contribute to the attenuation of renal pathology in IgAN rats.

It is well recognized that inflammation is the manifestation of oxidative stress [43] and that the pathways that generate inflammatory factors, such as cytokines, are all induced by this type of stress [44]. ROS act as important second messengers and participate in numerous cellular functions through the regulation of redox-sensitive transcription factors, including NF-κB and AP-1 [45–47], orchestrating the expression of multiple inflammatory genes that have been recognized to be important in IgAN, such as TNF-α, MMP-9 and MCP-1. Inflammation itself results in oxidative stress in IgAN. MCP-1 is considered to play a major role in the progression of IgAN because it recruits mononuclear leukocytes to lesion sites [48, 49] and because the deletion of macrophages ameliorates severe renal inflammatory disorders [14]. Consistent with these findings, the upregulation of TNF-α, MCP-1 and MMP-9 following a marked increase in the severity of the histopathology of renal lesions and the characteristic infiltration of periglomerular macrophages was observed in the IgAN + IMD animals compared to the IgAN animals. These results indicate that IMD can reduce cytokine expression and renal lesions, probably due to its antioxidative properties, leading to a reduction in inflammation.

Collectively, our results demonstrate that IMD in the kidney confers protection against IgAN, apparently by reducing oxidative stress and suppressing inflammation. Further investigations of the interactions between oxidative stress and NF-kB and the precise mechanisms involved in the effects of IMD in the development of IgAN will aid in the assessment of this peptide as a potential candidate for maintaining remission in IgAN patients.

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Conflict of interest We have no conflicts of interest to declare.

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