Effects of nafamostat mesilate, a protease Inhibitor, on ischemia/reperfusion-induced kidney injury in mice

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SUMMARY

Ischemia is induced when blood flow to an organ is interrupted, and re-establishing blood flow is essential to prevent ongoing hypoxic injury, although it paradoxically imparts further injury. Nafamostat mesilate (NM), a synthetic serine protease inhibitor, has been used in patients undergoing hemodialysis who are at a high risk of bleeding. To determine the protective effect of NM on ischemia-reperfusion injury (IRI) in a mouse renal IRI model, NM was administered as a pre- and post-treatment or during ischemia reperfusion and compared to a control group. Mice were bilaterally nephrectomized and subjected to 40 min of renal pedicle occlusion followed by 24 h reperfusion. NM (240 μg/kg) significantly improved kidney function and lowered serum creatinine and blood urea nitrogen levels. Consistently, NM inhibited collagen formation in kidney tissues. NM treatment attenuated the effects of ischemia/reperfusion on kidney tissues and significantly inhibited activation of Toll-like receptor 4, nuclear factor kappa-light-chain-enhancer of activated B cells-phospho-65 (NF- kB-p65), phospho-inhibitor of NF-κβa, and inducible nitric oxide synthase (iNOS). NM treatment also decreased expression of Bcl-2, Caspase-3 and Bax in kidney tissues, which has been linked with induction of apoptosis in kidney tissues. Our studies suggest that NM may be a novel therapeutic agent to prevent and treat kidney IRI, in which iNOS and/or NF-κβ are upregulated. The exact regulatory mechanism and its functional significance require further elucidation.

Key words: Apoptosis – Gene expression – Ischemia-reperfusion injury – Nafamostat mesilate

INTRODUCTION

Ischemia-reperfusion injury (IRI) is an obligatory insult that occurs at the time of organ procurement and engraftment. Ischemia is induced when blood flow to an organ is interrupted (Roberts et al., 2014). Re-establishment of blood flow is essential in order to prevent hypoxic injury, but it paradoxically imparts further injury, called IRI. Warm ischemia time (WIT) is relatively short in brain-dead donors. However, it can be prolonged in donors following cardiac arrest (Roberts et al., 2013). Furthermore, low temperatures have a multitude of effects on mammalian cell biology and have required careful investigation to allow cold application during organ preservation. This has been the basis for the development of techniques currently used worldwide (Huang et al., 2009). The clinical ramifications of IRI include systemic inflammatory effects and organ dysfunction, increased graft immunogenicity, increased risk of delayed graft function, acute rejection, and chronic allograft dysfunc-

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tion (Roberts et al., 2013). Substantial evidence implicates purinergic signaling in the pathogenesis and endogenous response to IRI, so strategies targeting various aspects of the pathway could be of therapeutic potential. Nephron-sparing surgery helps maintain long-term renal function and prevents chronic kidney disease in patients with small renal masses (Janetschek et al., 2007). Extending WIT > 30 min during open or laparoscopic partial nephrectomy can lead to considerable renal injury and continues to be a limiting factor when performing larger, more complex partial nephrectomies. Renal damage is also proportional to increasing WIT due to a sudden lack of nutrients, severe hypoxia, and/or anoxia of the renal parenchyma (Gao et al., 2014; Versteilen, 2004). Thus, optimal postoperative function of the kidney is determined by the integrity of the renal parenchyma preserved and the extent and duration of ischemic injury (Guibert et al., 2011). Acute kidney injury (AKI) occurs in approximately 18% of hospitalized patients, and a higher incidence rate is reported in children (Goldstein, 2012; Singbartl et al., 2012). Additionally, AKI has a higher likelihood of developing into chronic kidney disease and end-stage renal disease (Chawla et al., 2012; Ishani et al., 2009). Renal IRI is a major cause of AKI (Kinsey et al., 2011; Mao et al., 2013). IRI primarily affects the kidney cortex, particularly the proximal tubular epithelial cells (PTCs). The pathology of IRI is complex, involving inflammation, hemodynamic alterations, injury of the PTCs, followed by a restorative process to recover epithelial differentiation and function (Sun et al., 2016).

Nafamostat mesilate (NM) is a synthetic serine protease inhibitor that has been used in patients undergoing hemodialysis who are at high risk of bleeding because of the short half-life of NM (Choi et al., 2015). Although a few studies have reported the effectiveness of NM as a substitute for heparin in patients with a high risk of bleeding, there is limited clinical experience with NM and few reports are available on its safety and efficacy (Baek et al., 2012). Moreover, limited clinical data have been generated from randomized trials on NM use in patients with a bleeding tendency. Therefore, we performed this randomized study to evaluate the efficacy and safety of NM in mice with IRI. We hypothesized that the inhibitory effects of NM on inducible nitric oxide synthase (iNOS) and/or nuclear factor kappa beta (NF-κβ) may protect against renal IRI by decreasing apoptosis protein levels and injury induced by IRI in a mice model of renal I/R.

MATERIALS AND METHODS

Animals and tissue preparation

Six-to-eight-week-old male C57BL/6 mice (weight, 20-25 g) were purchased from the Orient Co, Ltd (Charles River Korea, Seoul, Korea). The animals were bred under specific pathogen-free conditions and maintained under standard laboratory conditions with a 12-hour light/dark cycle and free access to food and water. All animal experiments were approved by the appropriate Institutional Review Boards of the Seoul National University Bundang Hospital (BA1607-205/044-01) and conducted in accordance with National Institutes of Health Guide for the Care Use of Laboratory Animals (NIH publication No. 86-23, revised in 1996).

Surgical procedure

The mice were anesthetized with 50-60 mg/kg of pentobarbital sodium by intraperitoneal injection. Pentobarbital solution was diluted with sterile saline to have a concentration of 5 mg/ml for injection. Shortly after pentobarbital injection, 50 μg/kg of buprenorphine was administered subcutaneously for relief from pain and distress. After pentobarbital and buprenorphine injections, the hair on both sides of the mouse was removed with the hair clipper. The skin in the surgical area was then wiped clean with 70% alcohol swab.

Surgery

Immediately after the skin preparation, the mouse is placed on the homeothermic blanket of a homeothermic monitor system and covered by sterile gauze. The body temperature is monitored through a rectal probe and controlled in the range of 36.5-37°C. Surgery will not be started until the body temperature is stabilized at the set-point, and the mouse is in deep anesthesia and thus does not respond to pain induced by toe pinch. The mouse is placed on the thermostatic station laying on the right side. The skin and muscle on the left flank side are cut open along the back to expose the left kidney. The incision is positioned at 1/3 of the body from the back of the mouse and the incision size is 1-1.5 cm along the back. The kidney is then pushed out from the cut with sterile cotton swabs to expose the renal pedicle. Dissection of the pedicle tissue is done with ultra-fine-point tweezers to remove the tissue around the renal pedicle to expose the blood vessels for renal pedicle clamping. After the preparation, the left kidney is returned to the abdomen cavity. The right renal pedicle is prepared by a similar surgical procedure, but the incision is closer to the rib due to the different position of the right kidney. After the pedicle preparation, both kidneys are returned back to their original positions in the abdomen cavity.

Mouse model of renal ischemia-reperfusion Injury

The mice were induced by clamping both renal pedicle clamping for 40 min, and sham operations were performed using a similar surgical procedure except for clamping of the renal pedicles. At 24 h after IRI, the animals were sacrificed, blood was collected by intracardiac puncture and both kidneys were perfused with PBS and processed for molecular and histological examinations. NM (240

µg/kg) or the same volume of vehicle was injected intraperitoneally three times at 48 and 24 h before ischemia, and 30 min after ischemia.

Renal function analysis

For assessing renal function, blood urea nitrogen (BUN) and serum creatinine (Scr) were measured with ELISA kits (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions.

Assessment of renal injury

Blood samples clotted overnight at 4°C before centrifugation at 3000 g for 10 min for collection of serum. Serum creatinine (Scr) was measured using the autoanalyzer (HITACHI , Tokyo, Japan).

Immunohistochemistry

Kidney tissues were incubated overnight in 10% buffered formalin. Four-micron sections were cut from paraffin embedded tissue blocks and deparaffinized in xylene and rehydrated in graded alcohols (100-70%). Heat antigen retrieval was achieved by boiling of tissue sections in antigen retrieval solution pH 6.0 or pH 9.0 (Dako, Carpinteria, CA, USA) for 10 min in microwave prior to incubation at 4°C overnight with primary antibodies against Bcl-2 (sc-526 [1:100], Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After incubation with the appropriate biotinylated secondary antibodies for 30 min at 4°C and subsequently with a streptavidin (Dako, Kyoto, Japan), color development was done using 3-amino-9-ethylcarbazole or DAB as a chromogen and counterstained with hematoxylin. The photographs were processed through an image analysis program (anaylySIS) and the degree of staining densities was measured by image J. In order to verify the differences within each group, we implemented Duncan's multiple range test.

Masson trichrome staining

Masson trichrome staining was carried out in accordance with well-characterized protocols. Briefly, kidney tissue sections were deparaffinized and hydrated in distilled water prior to a 1-hour treatment in Bouin's fixative (Richard-Allan Scientific; Catalog #NC9674780) at 56°C. Sections were washed in running distilled water until clear, and then stained in Weigert's iron hematoxylin (Richard-Allan Scientific; Catalog #NC9231529) for 10 min. Following a 10 min wash in running water, sections were stained in Biebrich scarlet-acid fuchsin (Richard-Allan Scientific; Catalog #NC9424144) for 2 min. Sections were rinsed in distilled water followed by a 10 min differentiation in phosphomolybdic-phosphotungstic acid (Richard-Allan Scientific; Catalog #NC9443038). Aniline blue (Richard-Allan Scientific; Catalog #NC9684104) was used as a counterstain for 10 min, and then sections were differentiated in 1% acetic acid for 3 min. Sections were dehydrated through a series of graded alcohols back to xy-

lene, and then coverslipped and sealed using Cytoseal XYL (Richard-Allan Scientific). It is used in order to observe the degree of collagen fiber penetrating using light microscopy. The photographs were processed through an image analysis program (anaylySIS) and the degree of collagen fiber was measured by image J. To verify the differences within each group, we implemented Duncan's multiple range test.

Western Blot Analysis

The tissue was homogenized in 10 mM Tris HCl buffer, pH 7.4 containing 0.5 mM EDTA, pH 8.0, 0.25 M sucrose, 1 mM PMSF, 1 mM Na4VO3 and a protease inhibitor cocktail (Roche-Boehringer-Mannheim, Mannheim, Germany). After centrifugation, the supernatant fluids were resolved using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked in TBS with 5% non-fat dry milk at room temperature for 1 h in 0.1% Tween 20 and incubated with the appropriated primary antibodies, including iNOS, p-IkBa and NF-kB-p65 (ab15323 [1:1000]; ab12135 [1:1000]; ab16502 [1:1000], Abcam, Cambridge, Massachusetts), TLR4, Bax, Bcl-2, Caspase-3 and Actin (sc-293072 [1:1000]; sc-526 [1:2000]; sc-492 [1:2000]; sc-65497 [1:2000]; sc-1616 [1:2000], Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), at 4°C overnight. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology Inc., Danvers, MA, USA) for 1 h at room temperature. After washing, the membrane was visualized by a chemiluminescent reaction using an ECL-detection kit system from GE Healthcare (formerly Amersham Bioscience, Piscataway, NJ, USA).

Statistical Analysis

Results were expressed as the mean ± standard deviation. Differences between all other parameters for the three groups were evaluated by ANOVA, followed by multiple-group comparisons. An unpaired two-tailed t-test and Mann-Whitney test were used, and a P value < 0.05 was considered statistically significant. SPSS 14.0 for windows (SPSS, Chicago IL, USA) was used for all statistical analyses.

RESULTS

Biochemical results. The serum biochemical analysis demonstrated a significant increase in serum blood urea nitrogen (BUN) and serum creatinine (Scr) in the IRI group compared with those in the control group at 1 day, 2 days, and 3 days (Fig. 1A-F). NM decreased serum of BUN and Scr levels in compared with those in the IRI group at 1-3 days (Fig. 1).
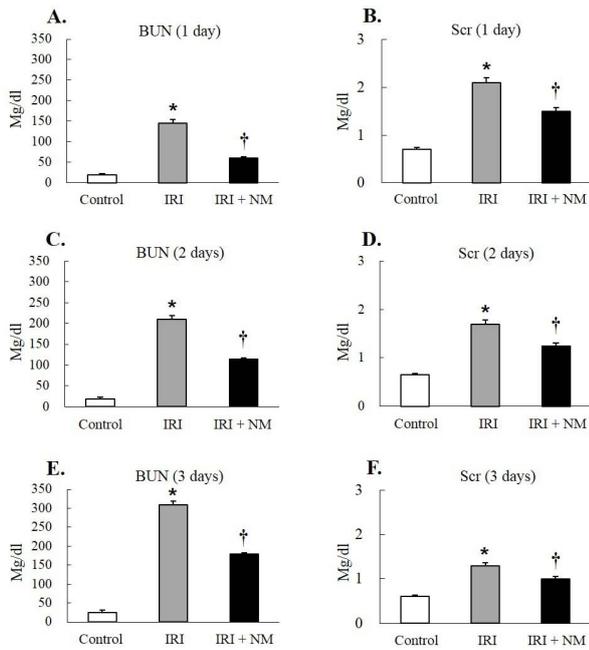
Fig 1. Effect of Nafamostat mesilate treatment on serum BUN and Scr levels in IRI mice at 1 day (A, B), 2 days (C, D), and 3 days (E, F). BUN and Scr were lower in the C group and the IRI + NM group compared to the IRI group. White bars = control group (n = 7); gray bars = IRI group (n = 7); black bars = IRI + NM group (n = 7). *P < 0.05 vs. C group, †P < 0.05 vs. IRI group. C, control group; IRI, Ischemia reperfusion injury group; IRI + NM, Ischemia reperfusion injury + Nafamostat mesilate group.

NM reduces kidney collagen deposition. To evaluate the effect of NM on the development of renal fibrosis and remodeling, kidney samples were collected 3 days after IRI to investigate kidney histology. Kidney cortex and medulla stained with Masson's Trichrome showed more pronounced collagen deposition in mice with IRI compared to that in control and animals treated with NM (Fig. 2).

Immunohistochemistry analysis. Ischemia/reperfusion-induced changes in Bcl-2 expression were analyzed in areas of the kidney where Bcl-2-positive cells were located. The overall Bcl-2-positive distribution pattern seemed to be preserved in renal IRI-induced mice compared with that of the control mice. The area of Bcl-2-positivity decreased significantly in the NM group compared with that in the IRI group (Fig. 3).

Western blotting for apoptosis signaling. This experiment was designed to investigate whether NM changed Bax, Caspase-3 and Bcl-2 protein expression levels in kidney tissues. Bax, Caspase-3 and Bcl-2 protein immunoreactivity decreased significantly in the NM group compared with that in the IRI group (Figs. 4A, 4B). The actin bands indicate protein loading in the same sample (Fig. 4).

Western blotting for toll-like receptor (TLR)-4-NF-kB signaling. This experiment was designed to investigate whether NM changed TLR-4-NF-kB signaling levels in kidney tissues. TLR-4, phospho-inhibitor of NF-κβa (p-IkBa), NF-kB-p65, and iNOS protein immunoreactivity decreased significantly in the NM group compared with that in the IRI group (Figs. 5A, 5B). The actin bands indicate protein loading in the same sample (Fig. 5).
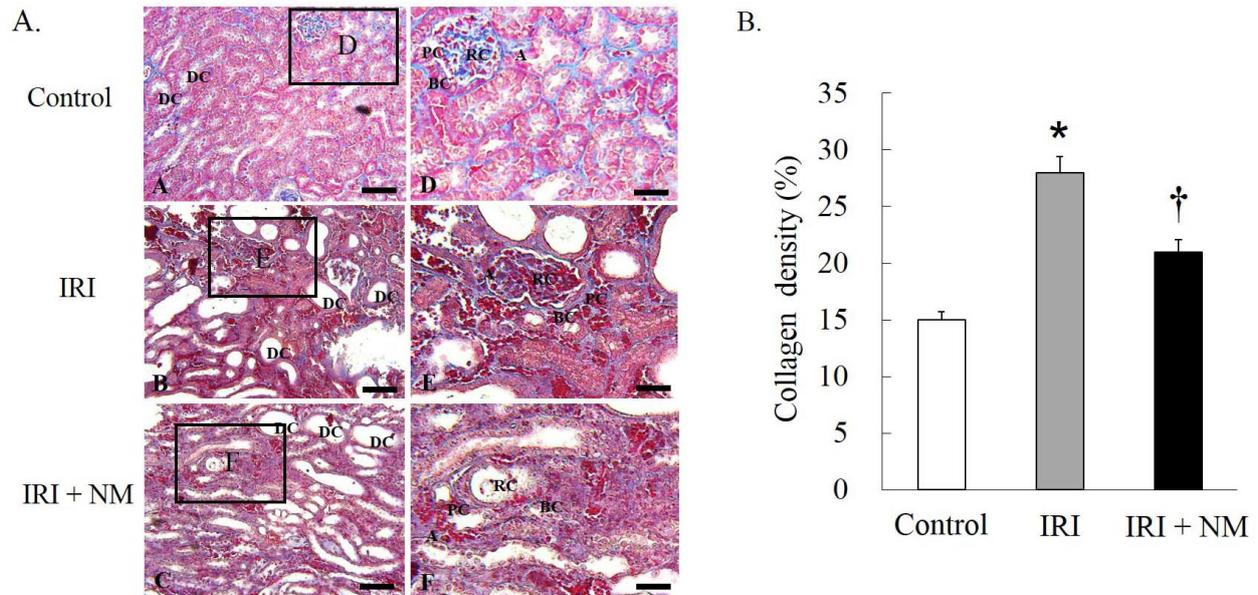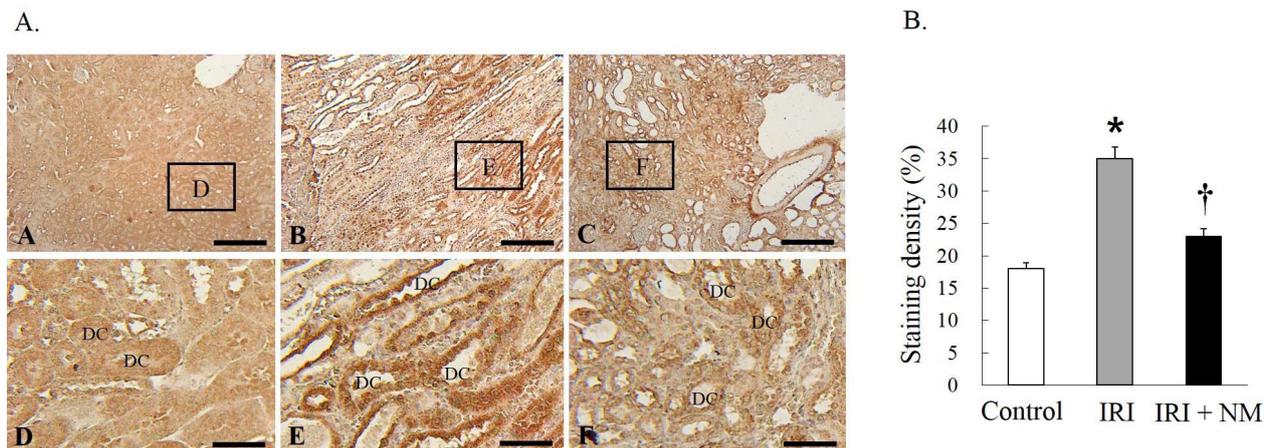

Fig. 2.- Representative images of kidney tissues stained with Masson's Trichrome from C group (A), IRI group (B), and IRI + NM group (C) at 3 days after Nafamostat mesilate treatment in IRI mice. Panels (D), (E) and (F) are high power views of panels (A), (B) and (C); fibrosis is colored blue. *P <0.05 vs. C group, †P < 0.05 vs. IRI group. C, control group; IRI, Ischemia reperfusion injury group; IRI+NM, Ischemia reperfusion injury + Nafamostat mesilate group. Scale bars= 50 μm (A, B, C), 20 μm (D, E, F).

A.

B.

Fig 3. Localization of Bcl-2-immunoreactive cells in the kidney tissues at 3 days. Immuno- histochemical expression reveals that the positive cells of Bcl-2 is significantly higher in the IRI group than that in the C groups. However, immunohistochemical expression reveals that the positive cells of Bcl-2 is significantly lower in the IRI + NM group than that in the IRI groups. DC; distal convoluted tubule. *$P < 0.05$ vs. C group, [†]$P < 0.05$ vs. IRI group. C, control group; NM, Nafamostat mesilate group; IRI, Ischemia reperfusion injury group; IRI + NM, Ischemia reperfusion injury + Nafamostat mesilate group. Scale bars = 50 μm (A, B, C, D).
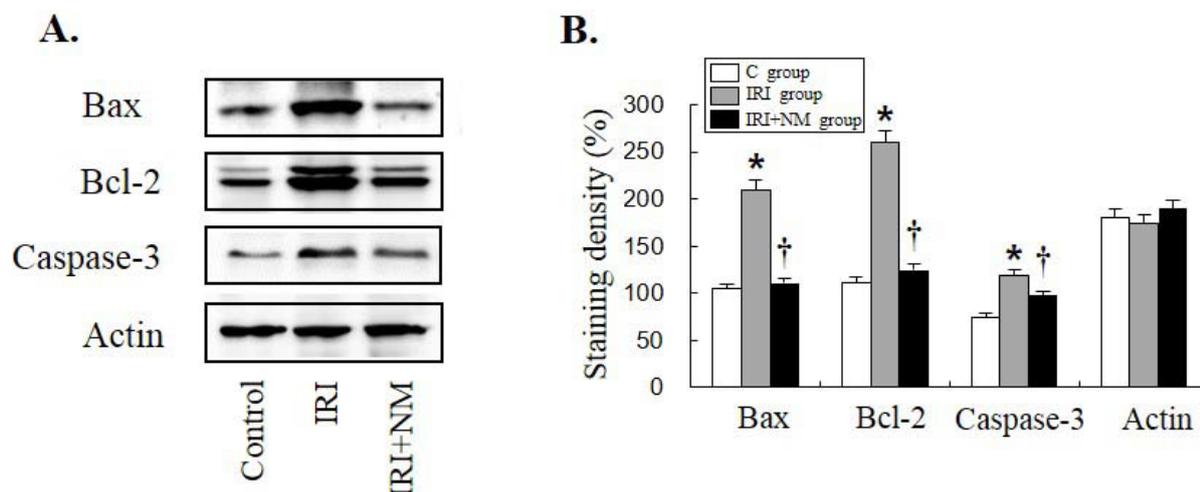
A.

B.

Fig 4. Changes of of Bax, Caspase-3 and Bcl-2 protein expression levels after Nafamostat mesilate treatment in IRI mice. The protein expressions of Bax, Caspase-3 and Bcl-2 at 3 days were significantly increased in the IRI group compared to the C groups. However, the protein expressions of Bax and Bcl-2 were decreased in the IRI + NM group compared to the IRI group. *$P < 0.05$ vs. C group, [†]$P < 0.05$ vs. IRI group. C, control group; IRI, Ischemia reperfusion injury group; IRI + NM, Ischemia reperfusion injury + Nafamostat mesilate group.

DISCUSSION

Our study examined the effects of NM on renal IRI using histopathology and biochemical and molecular indices. The protective effect of NM on hepatic IRI has been shown in previous studies (Horiuchi et al., 2001; Miyagi et al., 2004; Na et al., 2016). In addition, the protective effects of NM on neural damage and myocardial IRI have been reported (Chen et al., 2014; Kwon et al., 2015; Schwertz et al., 2008). This I/R study is clinically quite important, as the kidney is the first organ affected influenced during renal surgery or any stimulus that disrupts kidney hemodynamics. The results of our study shows that TLR-4, p-IkBa, and NF-κβ-p65 expression increased in response to I/R damage along with free oxygen radicals and iNOS in parallel. We verified our hypothesis that NM would benefited IRI. We supported the biochemical kidney function findings through a histopathological study, and showed that NM corrected parameters disrupted by IRI. Acute kidney damage is one of the most important causes of mortality in intensive care units, and the early diagnosis and treatment of acute kidney damage is important for correcting the progress of kidney disease (Schrier et
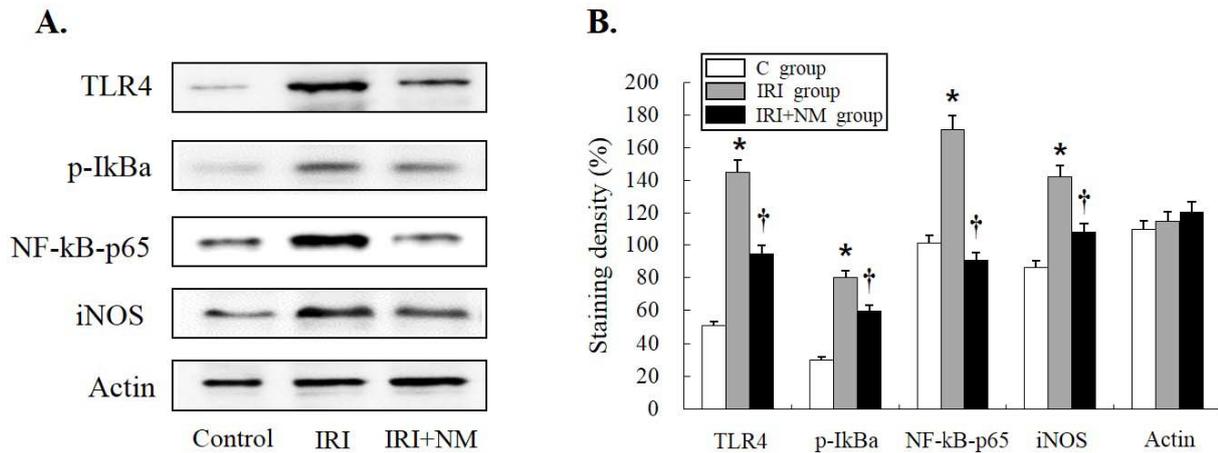
A.

B.

Fig 5. Western immunoblot analysis of the levels of expression of TLR4, p-IkBa, NF-kB-p65, iNOS and Actin immuno-reactivity after Nafamostat mesilate treatment in IRI mice. The semi-quantitative analysis confirms that IRI mice exhibited increased levels of immunoreactivity of TLR4, p-IkBa, NF-kB-p65 and iNOS in kidney tissues (n = 4-5 per group). However, the protein expressions of TLR4, p-IkBa, NF-kB-p65 and iNOS were decreased in the IRI + NM group compared to the IRI group. *P <0.05 vs. C group, †P < 0.05 vs. I/R group. C, control group; IRI, Ischemia reperfusion injury group; IRI + NM, Ischemia reperfusion injury + Nafamostat mesilate group.

al., 2004). Previously, it reported that 60 min kidney ischemia resulted in a higher number of kidney inflammatory gene changes at both 6 and 36 h compared with 30 min ischemia (Grigoryev et al., 2008). At 6 h, Significance Analysis of Microarrays identified 55 IRI-affected inflammatory genes after 60 min of ischemia compared with 31 genes after 30 min of ischemia, including 24 genes that were responsive to both mild and severe ischemia (Grigoryev et al., 2008). Similarly, at 36 h 54 genes were affected by 60 min of ischemia compared with 28 genes affected by 30 min of ischemia, including 20 genes that were sensitive to both mild and severe ischemia. 60 min ischemia induced sustained inflammation-associated expressional changes in 31 genes, of which 11 and 20 genes were previously identified as associated with kidney I/R and kidney inflammation, respectively (Grigoryev et al., 2008). The best-known diagnostic methods for detecting kidney damage are serum BUN and Scr levels (Waikar et al., 2007). We found that BUN and Scr levels increased during IRI. The global gene expression profiling of injured mouse kidney revealed significant severity- and time-dependent association of the inflammatory pathway with AKI. The injury severity-dependent changes in gene expression were correlated with the Scr and kidney morphology of AKI. Scr were significantly increased severe IRI (60 min ischemia) compared with sham, whereas 30 min ischemia did not lead to significant change in Scr (Grigoryev et al., 2008).

Severe tissue and organ damage occurs during I/R and restarting the blood supply (Grace, 1994). Ischemia stops the oxygen support necessary for tissues to survive and maintain their functions; thus, ischemia occurs in every tissue. However,

ischemia in the kidney is very important clinically. Many conditions, such as kidney transplantation, partial nephrectomy, and hydronephrosis, lead to kidney ischemia and result in renal damage (Anaya-Prado et al., 2002; Waikar et al., 2007). In addition, reperfusion damage occurs when reperfusion develops following ischemia. Re-oxygenation of oxygen-starved tissues generates oxidative stress (Masztalerz et al., 2006). Hydroxyl NO and superoxide promote oxidative stress and lead to cellular damage (Radi et al., 1991; Szabo et al., 1996). In our study, iNOS increased in the IRI model but decreased increased in the NM treated groups. NO creates a complex with angiotensin and reactive oxygen species, and these pathways are involved in the physiopathology of several diseases (Li et al., 2007). NO has important characteristics in renal circulation and urine formation, and one study showed that iNOS expression increases during renal IRI (Adachi et al., 1997; Kim et al., 2010). Several studies have shown that iNOS increases during IRI (Chatterjee et al., 2003; Mark et al., 2005). In addition, we found that iNOS expression decreased with NM treatment. IRI also increases the release of NF-κβ in kidney tissues (Camara- Lemarroy et al., 2009; Liu et al, 2010). One study showed that captopril inhibits the NF-κβ signaling pathway and decreases iNOS (He et al., 2007). These studies indicate that IRI increases NF-κβ and that NM reduce this increase in NF-κβ, which may decrease NO production and free oxygen radicals to inhibit NF-κβ expression. We showed that NM has important effects against IRI-induced damage. NM decreased NO and NF-κβ levels, and IRI-induced renal damage was inhibited at the biochemical, histopathological, and molecular levels. Activation

of the NF-κβ pathway is involved in the pathogenesis of chronic inflammatory diseases, and other inflammatory diseases. Altered regulation of NF-κβ may be involved in other diseases, in which the inflammatory response is at least partially involved. Finally, abnormalities in the NF-κβ pathway are also frequently seen in human cancers. Therefore, therapeutic strategies to prevent prolonged activation of the NF-κβ pathway have been discussed (Jorens et al., 1992), and NF-κβ inhibitors have great pharmaceutical potential in cancers, HIV-1 infection, and a wide variety of inflammatory diseases (Makarov, 2000; Schmitz et al., 2001). NM is effective and safe for patients with disseminated intravascular coagulation, which can be associated with septic-shock-associated acute kidney injury. NM improves coagulopathy and may also suppress overexpression of iNOS in patients with sepsis. Furthermore, NM suppresses other bioactive molecules, such as inflammatory cytokines and enzymes, such as matrix metalloproteinases (Nakatsuka et al., 2000), which are all associated with activating NF-κβ. Therefore, clinical application of NM should be investigated for various diseases such, as I/R, graft rejection, inflammatory diseases, autoimmune diseases, and infections, in which NF-κβ and/or iNOS are upregulated. However, more work to understand the function and role of NM in the NF-κβ-iNOS signaling pathway is required.

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