Soluble receptor for advanced glycation end-products prevents unilateral ureteral obstruction-induced renal fibrosis

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Soluble receptor for advanced glycation end-products prevents unilateral ureteral obstruction-induced renal fibrosis

Directed by Professor Shin-Wook Kang

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I'd like to dedicate this paper to all of you with all of my heart.

Thanks everyone.

Chan Ho Kim

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ABSTRACT

Soluble receptor for advanced glycation end-products prevents unilateral ureteral obstruction-induced renal fibrosis

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Background: Accumulating evidence suggests that advanced glycation end-products (AGEs) and its receptor (RAGE) play an important role in extracellular matrix (ECM) accumulation, which is consistently observed in various renal diseases associated with progressive glomerulosclerosis and tubulointerstitial fibrosis. A recent study also demonstrated that high mobility group protein box1 (HMGB1), one of several ligands of RAGE, directly induced ECM accumulation in cultured renal tubular epithelial cells. Meanwhile, soluble RAGE (sRAGE) acts as a

decoy. Therefore, blocking RAGE signaling using sRAGE is considered a potential therapeutic candidate for renal fibrosis, but it has never been explored. The aim of this study was to investigate the therapeutic effects of sRAGE on HMGB1-induced tubular epithelial cell injury *in vitro* and unilateral ureter obstruction (UUO)-induced renal tubulointerstitial fibrosis *in vivo*.

Methods: *In vitro*, NRK-52E cells were cultured in DMEM media with or without HMGB1 (10 μg/ml). To examine the effect of sRAGE on HMGB1-induced tubular cell injury, the cells were also incubated with sRAGE (1 μg/ml). *In vivo*, twenty-four Sprague-Dawley rats were divided into 4 groups, each of which consisted of 6 rats: sham operation with diluent (Control); sham operation with sRAGE (4 μg/kg); UUO operation with diluent; and UUO operation with sRAGE. The animals were treated intraperitoneally with either diluent or sRAGE at 1 hr before and every 48 hr after sham or UUO surgery. Rats were sacrificed at 10 days after operation, and the left kidneys were removed. The expression of fibrosis-related molecules and RAGE-dependent signaling cascades, including the mitogen-activated protein (MAP) kinases and NF-κB, were evaluated by Western blot analysis in cultured NRK-52E cells and the kidneys. Masson's trichrome and immunohistochemical staining (IHC) for ED-1, fibronectin, and type I collagen were also performed with the kidneys.

Results: The protein expression of fibronectin, type I collagen, α-smooth muscle actin (α-SMA), and connective tissue growth factor were significantly increased in HMBG1-stimulated NRK-52E cells, and these increases were attenuated by sRAGE. The MAP kinase pathway and NF-κB were also activated in NRK-52E cells exposed to HMBG1, and sRAGE treatment significantly abrogated the activation of these pathways. Compared to sham-operated rats, RAGE and HMGB1 protein expression were significantly increased in the kidney of UUO rats. Renal expression of fibrosis-related molecules such as fibronectin, type I collagen, and α-SMA were also significantly increased in UUO rat compared to the control group. All these

changes were significantly ameliorated by the administration of sRAGE. IHC and Masson's trichrome staining also confirmed the anti-fibrotic effect of sRAGE in a UUO rat model. In addition, the activation of the MAP kinase pathway and NF-κB along with an increased number of infiltrated macrophages within the tubulointerstitium in the kidney of UUO rats was significantly attenuated by sRAGE treatment.

Conclusion: These findings suggest that RAGE plays a pivotal role in the pathogenesis of renal fibrosis and its inhibition by sRAGE may be a potential therapeutic approach to various kidney diseases associated with renal fibrosis.

Key words: receptor for advanced glycation end-products (RAGE), soluble RAGE, unilateral ureteral obstruction, renal fibrosis

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I. INTRODUCTION

Tubulointerstitial fibrosis, which is characterized by the accumulation of fibroblasts and extracellular matrix (ECM) within the interstitium of the kidney along with loss of functioning nephrons, is a major pathological feature of progressive chronic kidney disease (CKD).[1-3] Furthermore, interstitial inflammation and fibrosis within the kidney are significant risk factors for CKD progression towards end-stage renal disease.[4] Despite a number of therapeutic interventions have been tried, an effective therapy to mitigate renal fibrosis is still not established.

Accumulating evidence suggests that advanced glycation end-products (AGEs) play an important role in ECM accumulation, which is consistently observed in diabetic and non-diabetic renal diseases associated with progressive glomerulosclerosis and tubulointerstitial fibrosis.[5,6] Receptor for AGEs (RAGE), which belongs to the immunoglobulin superfamily,[7] is a multi-ligand transmembrane receptor with a capability to interact with several types of proteins, including AGEs, S100 proteins or calgranulins, the high-mobility group B1 (HMGB1, also known as 'amphoterin'), and the proteins with a β fibrillary structure.[6,8,9] Binding of RAGE with these molecules activates a series of signal transduction pathways and transcription factors, including the mitogen-activated protein (MAP) kinases and NF-κB, which leads to the production of reactive oxygen species and pro-inflammatory cytokines, and consecutively induces inflammation.[6,9] In addition, infiltrated inflammatory cells release profibrotic cytokines such as transforming growth factor (TGF)-β1, platelet-derived growth factor, and fibroblast growth factor, which then modify the biology of resident cells to elicit fibrogenesis.[2] Therefore, it has been suggested that RAGE has a central role to the pathogenesis of inflammatory and/or fibrotic diseases in non-diabetic as well as diabetic conditions.[10] Moreover, recent studies demonstrated that RAGE-HMGB1 interaction was a potential signaling event in the development of renal tubulointerstitial fibrosis.[11-13]

RAGE consists of an intracellular domain, a short transmembrane domain, and an extracellular domain known as soluble RAGE (sRAGE).[7] Since sRAGE has the same ligand-binding specificity as RAGE, it may act as a decoy by competitively binding with proinflammatory ligands and preventing them from activating membrane-associated RAGE signaling.[6,7,9] Recently, blocking RAGE signaling using sRAGE has been revealed to be a potential therapeutic candidate for various diseases associated with inflammation such as left ventricular hypertrophy, atherosclerosis, Alzheimer's disease, and arthritis.[14-18] Furthermore, inflammatory process was known to play an important role in the pathogenesis and progression of

tubulointerstitial nephritis.[2,3,19] HMGB1, one of several ligands of RAGE, was also found to directly induce ECM accumulation in cultured renal tubular epithelial cells.[11,13] Based on these findings, it is surmised that inhibition of RAGE signaling can provide a new renoprotective perspective on the progression of renal fibrosis. However, the role of RAGE and the consequences of its inhibition by sRAGE are not fully understood in terms of renal tubulointerstitial fibrosis.

Unilateral ureteral obstruction (UUO) is a well-established experimental tool to induce tubulointerstitial fibrosis in the obstructed kidney.[20-22] Previous studies have shown that the inflammatory cells infiltration and lymphocyte dysfunction are two major pathogenic mechanisms contributing to UUO-induced renal tubulointerstitial fibrosis.[23,24] Intracellular adhesion molecule-1 (ICAM-1), which mediates the recruitment and infiltration of macrophages, have also been demonstrated to be involved in this UUO-induced renal fibrosis.[22] In the present study, I aimed to investigate the therapeutic effects of sRAGE on HMGB1-induced renal tubular cell injury *in vitro* and UUO-induced renal tubulointerstitial fibrosis *in vivo*.

II. MATERIALS AND METHODS

1. Cell culture

Renal proximal tubular epithelial cells (NRK-52E) were used for cell culture experiments. NRK-52E cells were cultured in Dulbeco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 26 mM $NaHCO_3$ at 37°C in humidified 5% CO_2 in air. Subconfluent NRK-52E cells were serum restricted for 24 hr, after which the media were replaced by serum-free medium containing HMGB1 (10 μg/ml) (A&RT, Daejeon, Korea), sRAGE (1 μg/ml) (A&RT), or HMGB1 with sRAGE. At 24 hr after the media change, the cells were harvested and conditioned culture media were collected. All experimental groups were cultured in triplicate.

2. Animal studies

All animal studies were conducted using a protocol approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University College of Medicine, Seoul, Korea. Twenty-four Sprague-Dawley (SD) rats were divided into 4 experimental groups: sham operation with diluent; sham operation with sRAGE (4 μg/kg); UUO operation with diluent; and UUO operation with sRAGE. Each group consisted of 6 rats. The animals were treated intraperitoneally with either diluent or sRAGE at 1 hr before and every 48 hr after sham or UUO surgery. Rats were sacrificed at 10 days after operation, and the left kidneys were removed for histological evaluation and molecular biological analysis.

3. Generation of renal tubulointerstitial fibrosis animal model by UUO

UUO rats were generated as previously described.[25,26] Briefly, SD rats were anesthetized with sodium pentobarbital, and after then a left flank incision was performed and the left ureter was exposed, ligated with 6-0 silk sutures at two points, and cut between two ligatures. Lastly, the peritoneal membrane and skin

were sutured. Sham operation was performed as control by following all steps of the UUO procedure except for ligation and cut of the ureter.

4. Western blot analysis

For western blot, cultured cells and the homogenized whole kidney were lysed in sodium dodecyl sulfate (SDS) sample buffer [2% SDS, 10 mM Tris-HCl, pH 6.8, 10% (vol/vol) glycerol], treated with Laemmli sample buffer, heated at 100°C for 5 min, and electrophoresed in an 8%-12% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA), and the membrane was then incubated in blocking buffer A (1 x PBS, 0.1% Tween-20, and 8% nonfat milk) at room temperature for 1 hr, followed by an overnight incubation at 4°C in a 1:1000 dilution of primary antibodies to RAGE (Abcam, Cambridge, UK), HMGB1 (Cell Signaling, Inc., Beverly, MA, USA), fibronectin (DAKO, Glostrup, Denmark), type I collagen (Southern Biotech, Birmingham, AL, USA), α-smooth muscle actin (α-SMA), connective tissue growth factor (CTGF) (Abcam), ICAM-1 (R&D systems, Minneapolis, MN, USA), phospho-ERK/ERK, phospho-p38/p38, phospho-JNK/JNK, MyD88, phospho-NF-κB/NF-κB (Cell Signaling, Inc.), or β-actin (Sigma-Aldrich Corp., St. Louis, MO, USA). The membrane was then washed once for 15 min and twice for 5 min in 1 x PBS with 0.1% Tween-20. Next, the membrane was incubated in buffer A containing a 1:1000 dilution of horseradish peroxidase-linked goat anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc., Arlington Heights, IL, USA). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany). Especially for NF-κB protein expression, western blot was performed with nuclear and cytosolic fractions of cultured cells harvested from plates, which were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermoscientific, Waltham, MA, USA) according to the manufacturer's protocol.

5. Immunohistochemistry and Masson's trichrome staining

Slices of the left kidney were fixed in 10% neutral-buffered formalin, processed in the standard manner, and 5 μm-thick sections of paraffin-embedded tissues were utilized for immunohistochemical (IHC) staining. Slides were deparaffinized, hydrated in ethyl alcohol, and washed in tap water. Antigen retrieval was carried out in 10 mM sodium citrate buffer for 20 min using a Black & Decker vegetable steamer. Primary antibodies for RAGE, HMGB1, fibronectin, type I collagen, and ED-1 (Chemicon International, Inc., Billerica, MA, USA) were diluted to the appropriate concentrations with 2% casein in bovine serum albumin and then add to the slides, followed by an overnight incubation at 4°C. After washing, a secondary antibody was added for 20 min, and the slides were washed and incubated with a tertiary rabbit-PAP complex (DAKO) for 20 min. Deaminobenzidine was added for 2 min and the slides were counterstained with hematoxylin. For Masson's trichrome staining, 5 μm-thick sections of paraffin-embedded tissues were deparaffinized, hydrated in ethyl alcohol, washed in tap water, and re-fixed in Bouin's solution at 56°C for 1 hr. After washing in running tap water for 10 min and staining with Weigert's iron hematoxylin working solution for 10 min, the sections were stained with Biebrich scarlet-acid fuchsin solution for 15 min, followed by a wash for 10 min. Next, the slides were then differentiated in phosphomolybdic-phosphotungstic acid solution for 15 min, transferred to aniline blue solution and stained for 10 min, and after then reacted with 1% acetic acid solution for 5 min. A semi-quantitative score of staining intensity was determined by examining at least 20 tubulointerstitial fields under x 400 magnification. The positive-staining intensity of 1+ to 4+ compared to a negative control (score = 0), in which the IHC staining was performed without the primary antibodies, were defined by two investigators in a blinded fashion, based on the lightness and darkness of the brownish color using a digital image analyzer (MetaMorph version 4.6r5, Univeral Imaging, Downingtown, PA). The staining score was obtained by multiplying the intensity of staining by the percentage of tubulointerstitium staining for that intensity; these numbers were then added for each experimental animal to give the staining score [= Σ (intensity of

staining) x (% of tubulointerstitium with that intensity)]. The number of ED-1 positive cells was counted in at least 20 fields of the tubulointerstitium/section under x 400 magnification.

6. Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using the statistical package SPSS for Windows Ver. 20.0 (SPSS, Inc., Chicago, IL, USA). Results were analyzed using the Kruskal-Wallis non-parametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were further confirmed by the Mann-Whitney *U*-test. *P*-values less than 0.05 were considered to be statistically significant.

III. RESULTS

1. sRAGE abrogates the protein expression of fibronectin, type I collagen, α-SMA, and CTGF in HMGB1-stimulated NRK-52E cells

To examine the effect of sRAGE on HMGB1-induced renal tubular epithelial cell injury, I first determined the changes in fibronectin, type I collagen, α-SMA, and CTGF protein expression in cultured NRK-52E cells. The administration of 10 μg/ml HMGB1 significantly increased the protein expression of fibronectin, type I collagen, α-SMA, and CTGF in cultured NRK-52E cells ($P < 0.05$ or 0.01), and these increases were significantly abrogated by sRAGE treatment ($P < 0.05$ or 0.01) (Fig. 1).

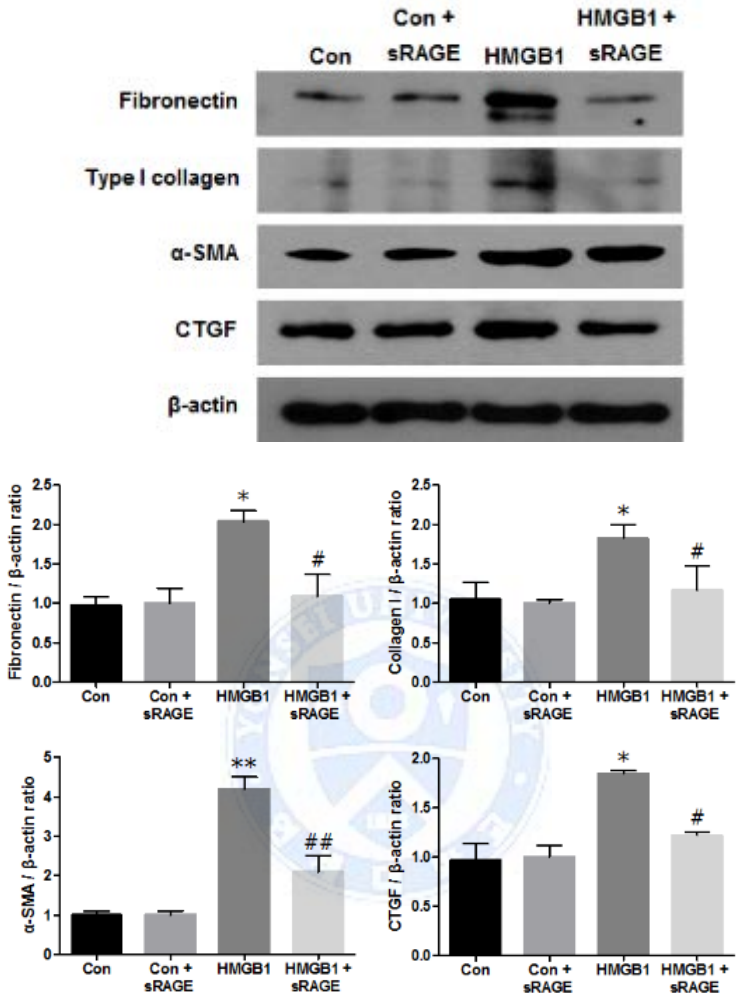


Figure 1. A representative western blot of fibronectin, type I collagen, α-SMA, and CTGF protein in control (Con), Con+sRAGE, HMGB1 (10 μg/ml), and HMGB1+sRAGE (1 μg/ml) groups (Representative of six blots)**.** Compared to Con cells, there were 2.1-, 1.9-, 4.2-, and 1.8-fold increases in fibronectin, type I collagen, α-SMA, and CTGF protein expression, respectively, in HMBG1-stimulated NRK-52E cells, and these changes were significantly abrogated by sRAGE treatment. *; $P < 0.05$ vs. Con and Con+sRAGE groups, #; $P < 0.05$ vs. HMGB1 group, **; $P < 0.01$ vs. Con and Con+sRAGE groups, ##; $P < 0.01$ vs. HMGB1 group.

2. sRAGE ameliorates MAP kinase activation in HMGB1-stimulated NRK-52E cells

To clarify the effect of sRAGE on HMGB1-activated MAP kinase, I next examined the changes in RAGE, phospho-ERK/ERK, phospho-p38/p38, phospho-JNK/JNK, and MyD88 protein expression in cultured NRK-52E cells. Compared to control cells, the protein expression of RAGE, phospho-ERK, phospho-p38, phospho-JNK, and MyD88 were significantly increased in HMGB1-stimulated NRK-52E cells ($P < 0.05$), and sRAGE significantly ameliorated these increases ($P < 0.05$) (Fig. 2).

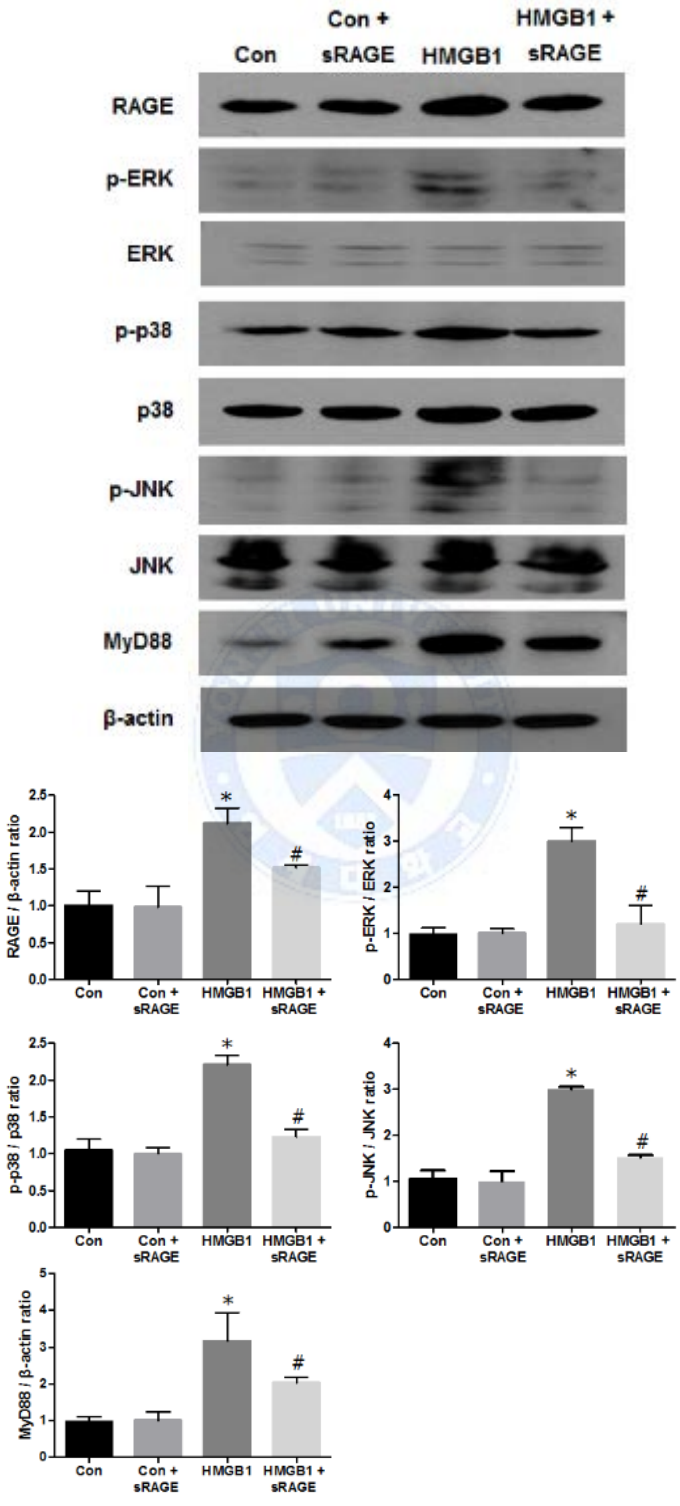

Con
Con + sRAGE
HMGB1
HMGB1 + sRAGE
RAGE
p-ERK
ERK
p-p38
p38
p-JNK
JNK
MyD88
β-actin
RAGE / β-actin ratio
p-ERK / ERK ratio
p-p38 / p38 ratio
p-JNK / JNK ratio
MyD88 / β-actin ratio
*
#

Figure 2. A representative western blot of RAGE, phospho-ERK/ERK, phospho-p38/p38, phospho-JNK/JNK, and MyD88 protein in control (Con), Con+sRAGE, HMGB1 (10 μg/ml), and HMGB1+sRAGE (1 μg/ml) groups (Representative of six blots)**.** Compared to Con cells, there were 2.2-, 3.0-, 2.2-, 3.0-, and 3.1-fold increases in RAGE, phospho-ERK/ERK, phospho-p38/p38, phospho-JNK/JNK, and MyD88 protein expression, respectively, in HMBG1-stimulated NRK-52E cells, and sRAGE significantly ameliorated these increases. *; $P < 0.05$ vs. Con and Con+sRAGE groups, #; $P < 0.05$ vs. HMGB1 group.

3. sRAGE attenuates NF-κB activation in HMGB1-stimulated NRK-52E cells

Since HMGB1 was known to activate NF-κB, nuclear translocation of NF-κB was elucidated to determine the effects of sRAGE on HMGB1-induced NF-κB activation by using the changes in NF-κB protein expression in both cytosolic and nuclear fractions. NF-κB protein expression in the cytosolic fraction was significantly decreased at 12 hr after the administration of HMGB1 ($P < 0.05$), and this decrease was significantly attenuated by sRAGE treatment ($P < 0.05$). In contrast, NF-κB protein in the nuclear fraction was significantly increased in HMGB1-stimulated NRK-52E cells ($P < 0.05$), and sRAGE significantly inhibited this increase ($P < 0.05$) (Fig. 3).

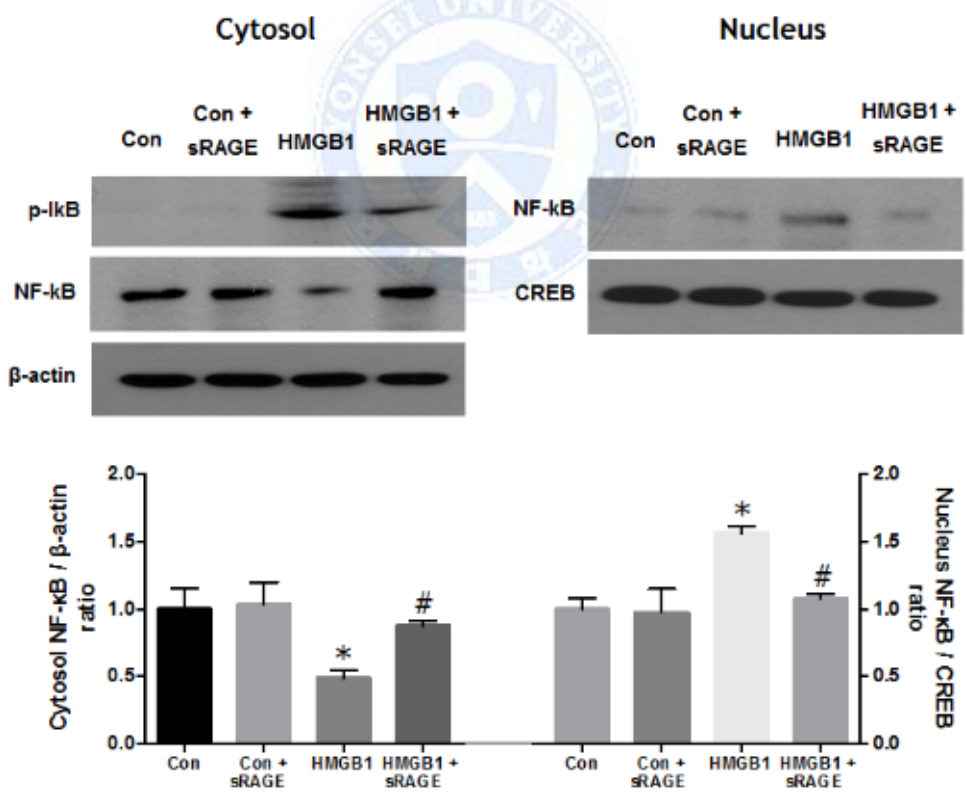


Figure 3. A representative western blot of NF-κB protein in control (Con), Con+sRAGE, HMGB1 (10 μg/ml), and HMGB1+sRAGE (1 μg/ml) groups (Representative of six blots)**.** Compared to Con cells, there was a significant decrease in NF-κB protein expression in the cytosolic fraction by 51.2% and a significant 1.6-fold increase in the nuclear fraction of HMBG1-stimulated NRK-52E cells, and these changes were significantly attenuated by sRAGE treatment. *; $P < 0.05$ vs. Con and Con+sRAGE groups, #; $P < 0.05$ vs. HMGB1 group.

4. sRAGE abrogates RAGE and HMGB1 protein expression in UUO rats

Compared to sham-operated rats, HMGB1 and RAGE protein expression in the left kidney were significantly increased in UUO rats treated with diluent ($P < 0.001$), and administration of sRAGE significantly inhibited these increases in UUO rats ($P < 0.05$) (Fig. 4). IHC staining for HMGB1 and RAGE protein also supported the western blot findings. The significant increases in IHC staining scores for HMGB1 and RAGE within the tubulointerstitium of UUO rats were significantly abrogated in the UUO rats treated with sRAGE ($P < 0.05$ or 0.01) (Fig. 5A, 5B, and 5C).

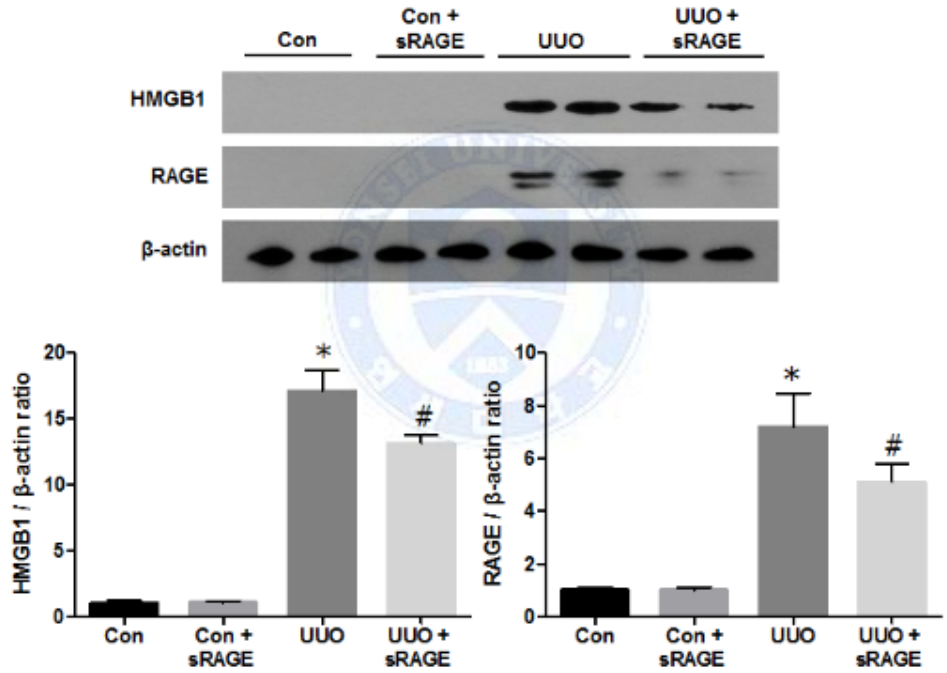
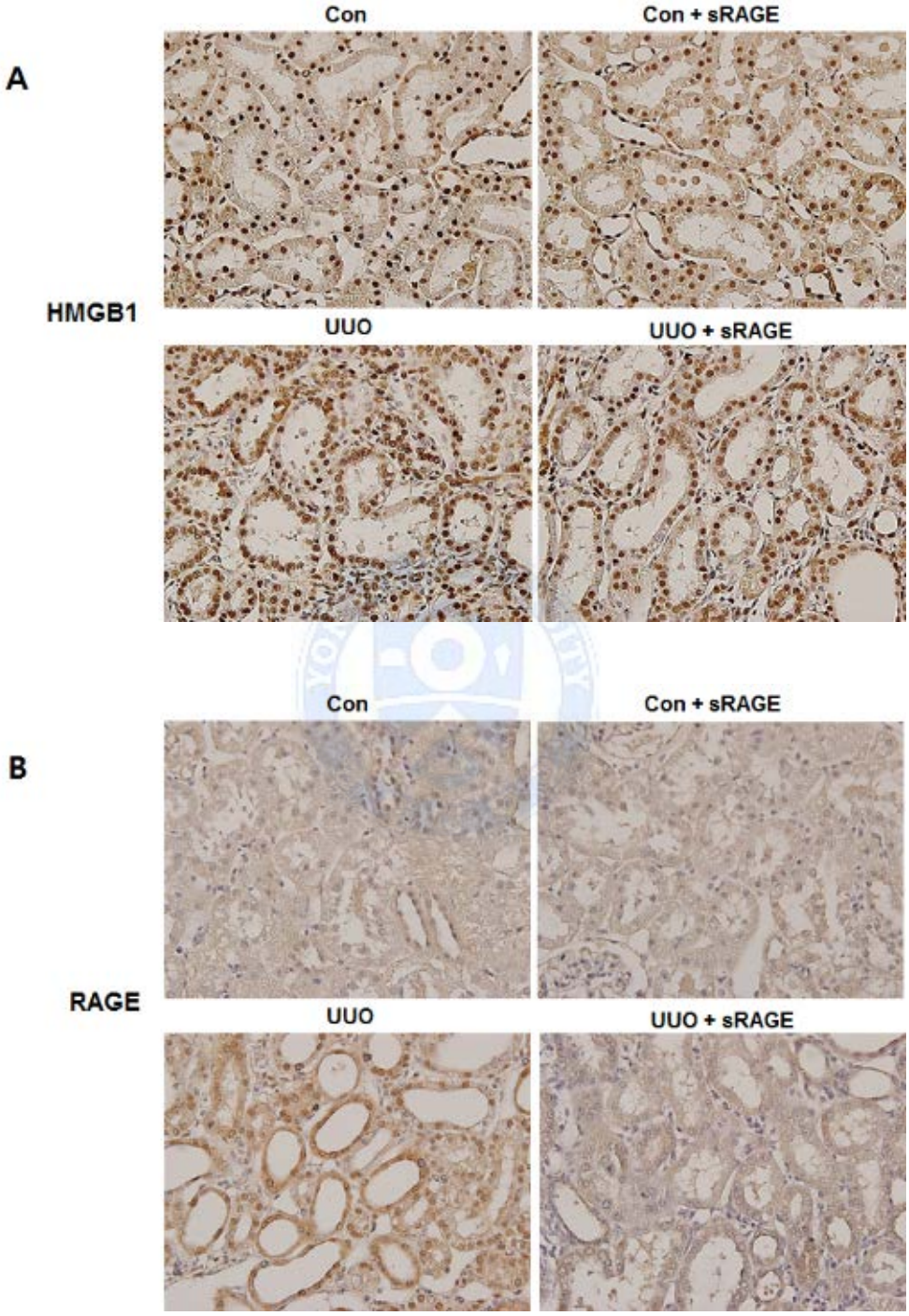


Figure 4. A representative western blot of HMGB1 and RAGE protein in the left kidney of sham-operated (Con), Con+sRAGE, UUO, and UUO+sRAGE rats (Representative of five blots)**.** Compared to Con rats, there were 17.4- and 7.2-fold increases in HMGB1 and RAGE protein expression, respectively, in the left kidney of UUO rats treated with diluent (UUO), and administration of sRAGE significantly inhibited these increases in UUO rats. *; $P < 0.001$ vs. Con and Con+sRAGE groups, #; $P < 0.05$ vs. UUO group.


A
Con
Con + sRAGE
HMGB1
UUO
UUO + sRAGE
B
Con
Con + sRAGE
RAGE
UUO
UUO + sRAGE

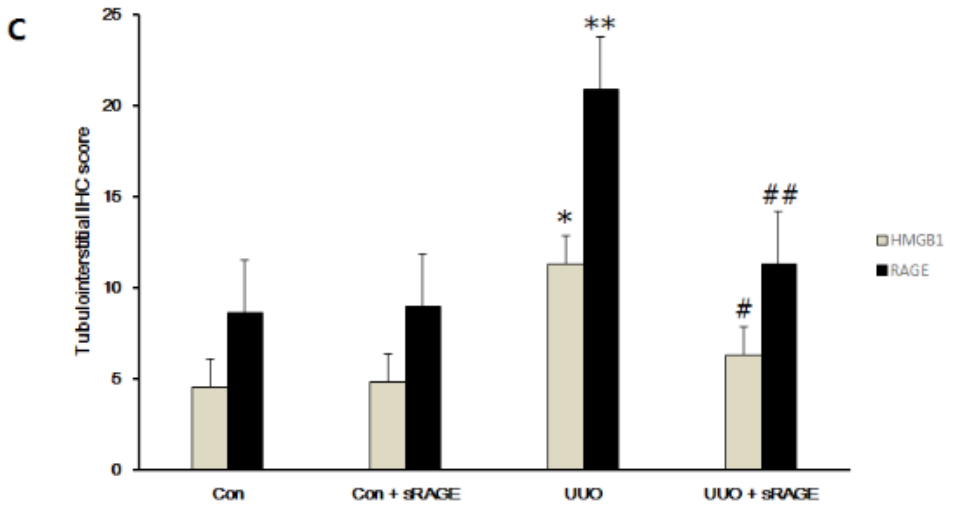


Figure 5. Immunohistochemical staining for HMGB1 and RAGE with the left kidney tissues of sham-operated (Con), Con+sRAGE, UUO, and UUO+sRAGE rats. Compared to Con and Con+sRAGE rats, tubulointerstitial HMGB1 (A) and RAGE (B) protein expression were significantly increased in UUO rats treated with diluent, and these increases were significantly abrogated by sRAGE treatment. (C) The significant increases in IHC staining scores for HMGB1 and RAGE within the tubulointerstitium of UUO rats were significantly mitigated in the UUO rats treated with sRAGE. *; $P < 0.05$ vs. Con and Con+sRAGE groups, **; $P < 0.01$ vs. Con and Con+sRAGE groups, #; $P < 0.05$ vs. UUO group, ##; $P < 0.01$ vs. UUO group.

5. sRAGE ameliorates tubulointerstitial fibrosis and macrophage accumulation in UUO rats

To examine the effect of sRAGE on UUO-induced tubulointerstitial fibrosis and macrophage accumulation, the changes in fibronectin, type I collagen, α-SMA, and ED-1 protein expression were determined by western bot and/or IHC. Masson's trichrome staining was also performed. Compared to sham-operated rats, fibronectin, type I collagen, and α-SMA protein expression were significantly increased in the left kidney of UUO rats ($P < 0.05$ or 0.01), and these increases were significantly ameliorated by sRAGE treatment ($P < 0.05$) (Fig. 6). IHC staining for fibronectin and type I collagen protein also confirmed the western blot findings. The significant increases in IHC staining scores for fibronectin and type I collagen within the tubulointerstitium of UUO rats were significantly inhibited by the administration of sRAGE ($P < 0.01$) (Fig. 7A, 7B, and 7E). Moreover, the number of macrophages within the tubulointerstitium, assessed by IHC staining with ED-1 antibody, was significantly higher in the UUO group compared to the control group, and sRAGE treatment significantly abrogated the increase in the number of ED-1-positive cells in UUO rats ($P < 0.05$) (Fig. 7C). Furthermore, Masson's trichrome staining revealed that renal tubulointerstitial fibrosis was significantly severer in UUO rats relative to control rats, and this excessive fibrosis was significantly mitigated in sRAGE-treated UUO rats (Fig. 7D).

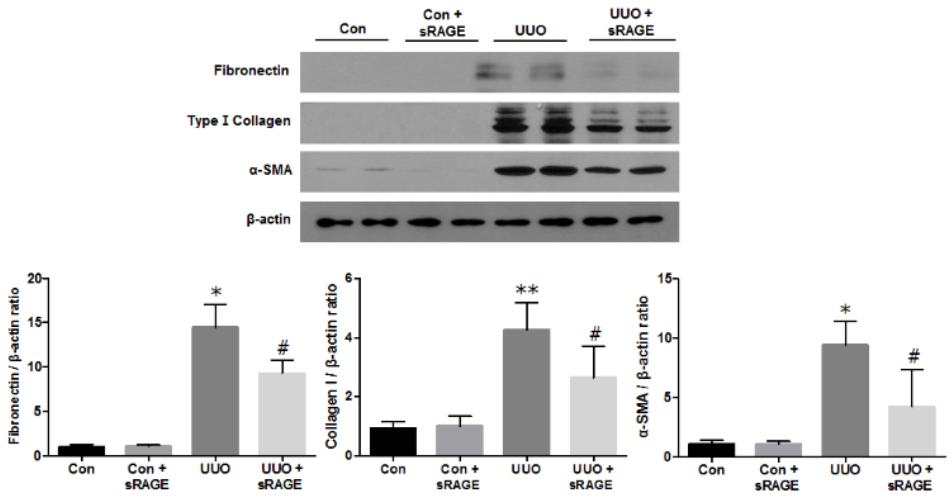


Figure 6. A representative western blot of fibronectin, type I collagen, and α-SMA protein in the left kidney of sham-operated (Con), Con+sRAGE, UUO, and UUO+sRAGE rats (Representative of five blots)**.** Compared to Con rats, there were 14.8-, 4.2-, and 9.4-fold increases in fibronectin, type I collagen, and α-SMA protein expression, respectively, in UUO rats, and these increases were significantly ameliorated by sRAGE treatment. *; $P < 0.01$ vs. Con and Con+sRAGE groups, **; $P < 0.05$ vs. Con and Con+sRAGE groups, #; $P < 0.05$ vs. UUO group.

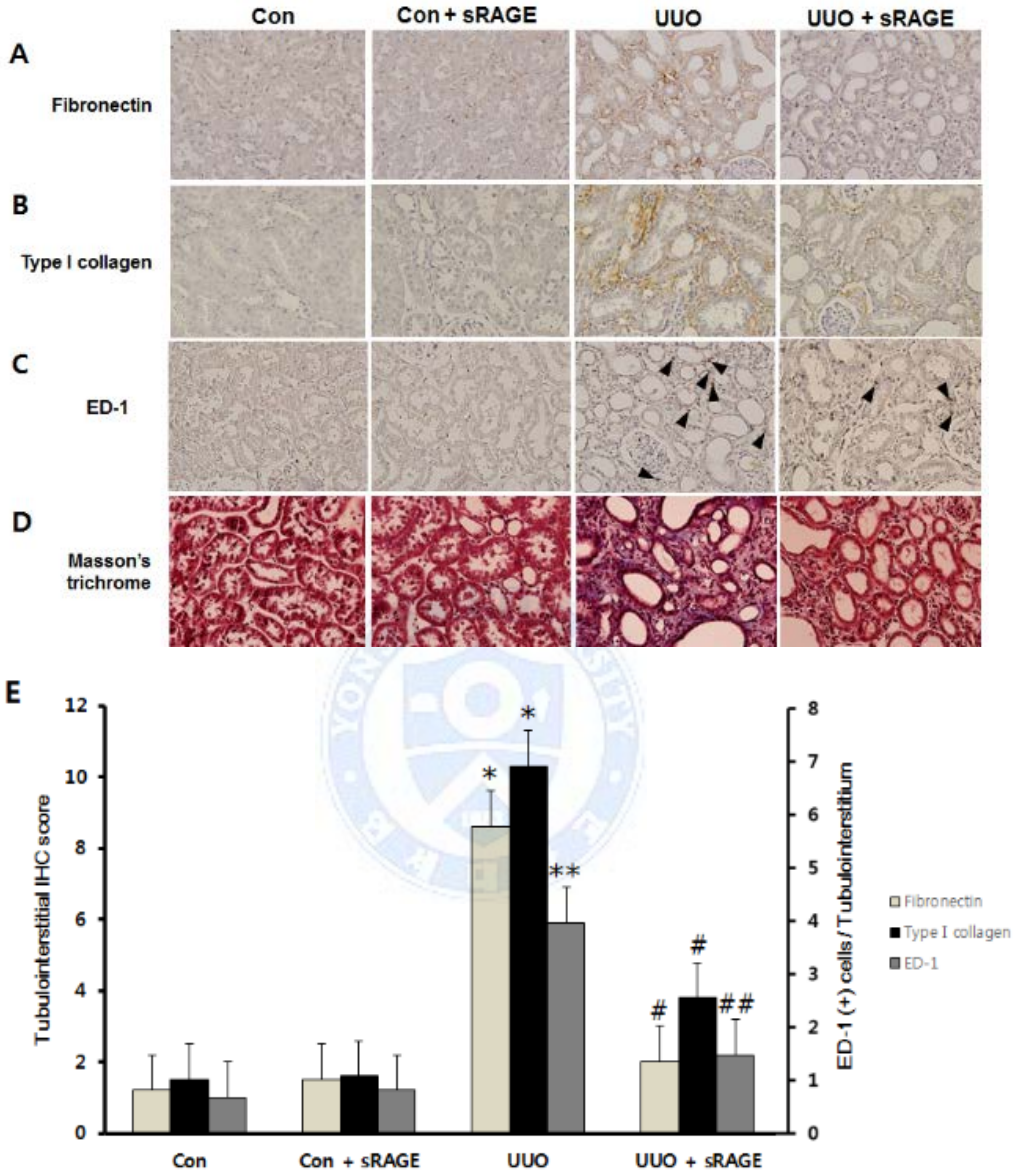
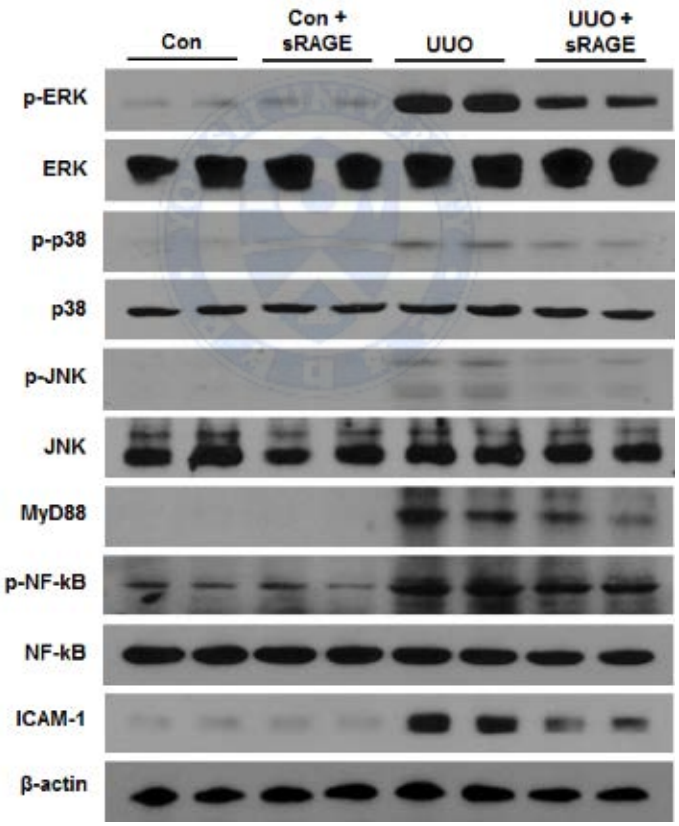

Con
Con + sRAGE
UUO
UUO + sRAGE
A
Fibronectin
B
Type I collagen
C
ED-1
D
Masson's trichrome
E
Tubulointerstitial IHC score
ED-1 (+) cells / Tubulointerstitium
Fibronectin
Type I collagen
ED-1
Con
Con + sRAGE
UUO
UUO + sRAGE

Figure 7. Immunohistochemical staining for fibronectin, type I collagen, and ED-1, and Masson's trichrome staining with the left kidney tissues of sham-operated (Con), Con+sRAGE, UUO, and UUO+sRAGE rats. Compared to Con and Con+sRAGE rats, the protein expression of fibronectin (A) and type I collagen (B) within the tubulointerstitium were significantly increased in UUO rats, and sRAGE treatment significantly ameliorated these increases in UUO rats. (C) The significant increase in the number of ED-1-positive cells (arrowhead) within the tubulointerstitium of UUO rats was significantly inhibited by the administration of sRAGE. (D) Masson's trichrome staining revealed that tubulointerstitial fibrosis was significantly severer in UUO rats relative to Con and Con+sRAGE rats, and this excessive fibrosis was significantly mitigated in sRAGE-treated UUO rats. *; $P < 0.01$ vs. Con and Con+sRAGE groups, **; $P < 0.05$ vs. Con and Con+sRAGE groups, #; $P < 0.01$ vs. UUO group, ##; $P < 0.05$ vs. UUO group.

6. sRAGE attenuates the activation of the MAP kinases and NF-κB in UUO rats

Finally, the impact of sRAGE on the MAP kinase pathways and NF-κB was explored in UUO rats. As seen in Figure 8, phospho-ERK, phospho-p38, phospho-JNK, MyD88, phospho-NF-κB, and ICAM-1 protein expression were significantly increased in the left kidney of UUO rats compared to sham-operated rats ($P < 0.05$ or 0.01), and these increases were significantly attenuated by sRAGE treatment ($P < 0.05$).



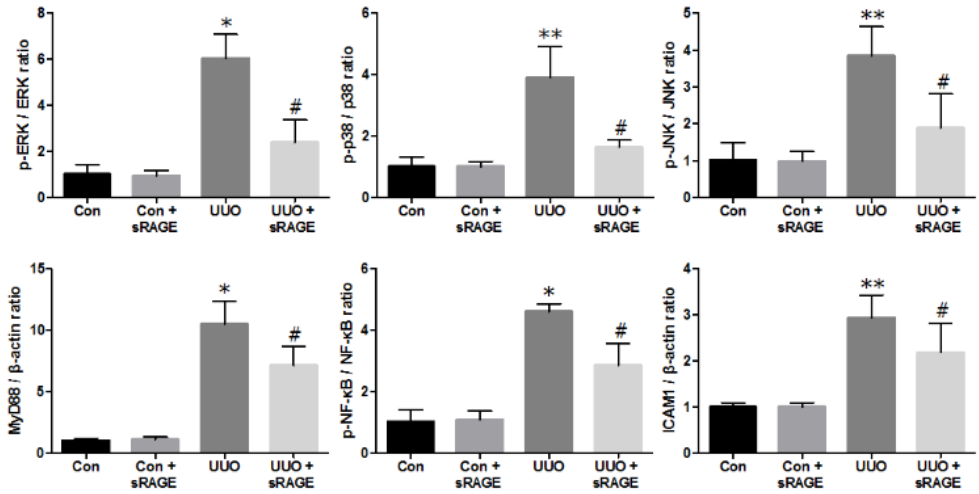


Figure 8. A representative western blot of phospho-ERK/ERK, phospho-p38/p38, phospho-JNK/JNK, MyD88, phospho-NF-κB/NF-κB, and ICAM-1 protein in the left kidney of sham-operated (Con), Con+sRAGE, UUO, and UUO+sRAGE rats (Representative of five blots)**.** Compared to Con rats, there were 6.0-, 3.9-, 3.8-, 10.8-, 4.7-, and 3.0-fold increases in phospho-ERK/ERK, phospho-p38/p38, phospho-JNK/JNK, MyD88, phospho-NF-κB/NF-κB, and ICAM-1 protein expression, respectively, in UUO rats, and these increases were significantly attenuated by sRAGE treatment. *; $P < 0.01$ vs. Con and Con+sRAGE groups, #; $P < 0.05$ vs. UUO group, **; $P < 0.05$ vs. Con and Con+sRAGE groups.

IV. DISCUSSION

The results of present study showed that the RAGE expression was up-regulated in HMGB1-treated NRK-52E cells and the kidney of UUO rats. In addition, the protein expression of fibrosis-related molecules such as fibronectin, type I collagen, and α-SMA were significantly increased in HMGB1-stimulated NRK-52E cells and in the kidney of UUO rats, and these changes were significantly abrogated by sRAGE treatment, suggesting that sRAGE may be a potential therapeutic approach to various kidney diseases associated with renal fibrosis.

Renal tubulointerstitial fibrosis is considered a common final pathway by which kidney diseases with various etiologies progress to end-stage renal failure.[1,2,19] Therefore, it is of great importance to identify factors that are involved in the development and progression of renal tubulointerstitial fibrosis during renal injury. Recently, a number of studies have suggested that RAGE plays an important role in the pathogenesis of inflammatory and/or fibrotic diseases, but the role of RAGE and the consequences of its inhibition are not fully explored in terms of renal tubulointerstitial fibrosis.[9,10,15,27,28] RAGE is a multi-ligand transmembrane receptor, which interacts with various proteins including AGEs.[7,9,10] It is mainly expressed in vascular smooth muscle cells and endothelial cells, mononuclear phagocytes, neurons, and lung alveolar epithelial cells.[6,9,10] In the kidney, RAGE has also been identified on the surface of glomerular endothelial cells, podocytes, mesangial cells, and tubular epithelial cells.[6,9,10] The interaction of RAGE and its ligands activates a series of intracellular signaling pathways, including the Janus kinase/signal transducer and activator of transcription (JAK/STAT), nuclear factor of activated T-cells 1 (NFAT1), and MAP kinase/NF-κB pathways.[27,29] The subsequent cellular responses involve several types of events such as proinflammatory, profibrotic, procoagulant, angiogenic, and/or even repair processes, depending on the ligand, environment, and developmental stage.[6,15] A number of previous studies demonstrated that engagement of RAGE with various ligands was associated with

the pathogenesis of several fibrotic diseases in diverse organs including hepatic fibrosis, idiopathic pulmonary fibrosis, myocardial infarction, and diabetic cardiomyopathy,[28,30-34] and the blockade of RAGE-ligands axis protected these organs against fibrosis.[28,31,32,34]

Meanwhile, HMGB1 is a nuclear DNA-binding protein that resides inside the nucleus and can be released to the extracellular space from injured cells and macrophages.[35-38] Once extracelluar HMGB1 binds to its functional receptors, RAGE or toll-like receptors, a series of reactions can be triggered.[39-42] Recently, it has been founded that HMGB1 is involved in renal fibrosis through a RAGE-dependent pathway.[11,13,43] In this study, RAGE and HMGB1 expression were significantly increased in the kidney of UUO rats along with enhanced expression of fibronectin, type I collagen, α-SMA, and CTGF. The protein expression of not only RAGE but also these fibrosis-related molecules were also significantly increased in HMBG1-stimulated NRK-52E cells. Based on these findings, I speculated that tubular cell injury induced by UUO operation might lead to an increase in HMGB1 expression and that enhanced HMGB1 in the kidney of UUO rats and exogenous HMGB1 on cultured tubular cells could bind to RAGE, and this RAGE-HMGB1 interaction could further induce RAGE expression through a positive feedback loop. In consistent with this surmise, the results of previous studies revealed that binding of various ligands such as HMGB1 to RAGE resulted in a consequent upregulation of RAGE, and thus the effects of RAGE and its ligands could be amplified in various diseases, including vascular disease, diabetes, cancer, hepatic fibrosis, and neurodegenerative disease.[27,33,34]

Since sRAGE is known to act as a decoy by binding to AGEs, it can inhibit RAGE activation.[9,10,27] In fact, the beneficial effects of sRAGE have been shown in various experimental disease models. Intraperitoneal administration of sRAGE attenuated albuminuria, glomerulosclerosis, and glomerular basement membrane thickening in db/db mice.[44] Moreover, sRAGE treatment significantly abrogated

proteinuria and histological renal damage along with reduced inflammation in lupus-prone mice.[17] Lee et al. also demonstrated that angiotensin II-mediated atherosclerosis was significantly mitigated by sRAGE in apolipoprotein E-deficient mice.[16] However, the therapeutic effect of sRAGE on renal tubulointerstitial fibrosis has never been explored. In the current study, I found for the first time that increased protein expression of fibrosis-related molecules in the kidney of UUO rats and HMGB1-stimulated renal tubular cells were significantly ameliorated by sRAGE, indicating sRAGE can be a potential therapeutic agent to prevent renal tubulointerstitial fibrosis in numerous renal diseases.

Signaling of RAGE leads to the activation of MyD88 and the MAP kinases, which promote cytokine production and operate as a key cell signaling pathway of fibrosis and apoptosis.[27,45,46] The results of present study showed that MyD88 protein expression and phosphorylated protein levels of ERK, p38, and JNK were significantly increased in the kidney of UUO rats and NRK-52E cells exposed to HMGB1, and these increases were significantly attenuated by sRAGE treatment. Taken together with the changes in fibrosis-related molecules, it was inferred that the induction of MyD88 and activation of ERK, p38, and JNK played a pivotal role in the pathogenesis of renal tubulointerstitial fibrosis via RAGE-dependent signaling and the beneficial effect of sRAGE on fibrosis was partly attributed to its inhibitory impact on these MAP kinase pathways.

Another important downstream signaling through RAGE is NF-κB signaling pathway, which is known to lead renal damage by inducing the transcription of inflammation-associated genes,[43] including ICAM-1, which is a cell surface glycoprotein and plays a major role in the infiltration of macrophages and monocytes.[47] Once the macrophages/monocytes are infiltrated and activated, they release lysosomal enzymes, nitric oxide, reactive oxygen species, tumor necrosis factor-α, interleukin-1, and TGF-β, and consequently promote renal injury.[48,49] In UUO animals, leukocyte recruitment mediated by adhesion molecules and

chemokines is critical in the development of obstructive nephropathy, resulting in interstitial inflammation and renal fibrosis.[50,51] On the other hand, recent studies revealed that RAGE is also implicated in the inflammation process by mediating leukocyte recruitment.[15,47] The findings of my study demonstrated that the expression of phospho-NF-κB and ICAM-1 along with fibrosis-related molecules were significantly increased in the kidney of UUO rats, and these changes were abrogated by sRAGE treatment. Furthermore, administration of sRAGE significantly ameliorated tubulointerstitial macrophage infiltration and tubulointerstitial fibrosis in the kidney of UUO rats, suggesting that renal tubulointerstitial fibrosis may be attributed to the induction of ICAM-1 and consequent macrophage/monocyte infiltration through the NF-κB-dependent pathway, and sRAGE attenuated renal injury via its anti-inflammatory effect in UUO-induced tubulointerstitial fibrosis model. *In vitro,* however, the increases in the protein expression of fibrosis-related molecules in HMGB1-stimulated tubular cells were also significantly mitigated by RAGE inhibition. Collectively, the impact of sRAGE on renal tubulointerstitial fibrosis seems to be mediated by not only inhibiting macrophage/monocyte infiltration but also direct beneficial effects on renal tubular cells independently of inflammation (Fig. 9).

In summary, I showed for the first time that sRAGE abrogated renal tubulointerstitial fibrosis both *in vitro* and *in vivo* by inhibiting the MAP kinases and NF-κB activation, suggesting that RAGE might be an important mediator in the pathogenesis of renal tubulointerstitial fibrosis. Therefore, sRAGE can be considered a potential therapeutic candidate to various kidney diseases associated with renal tubulointerstitial fibrosis.

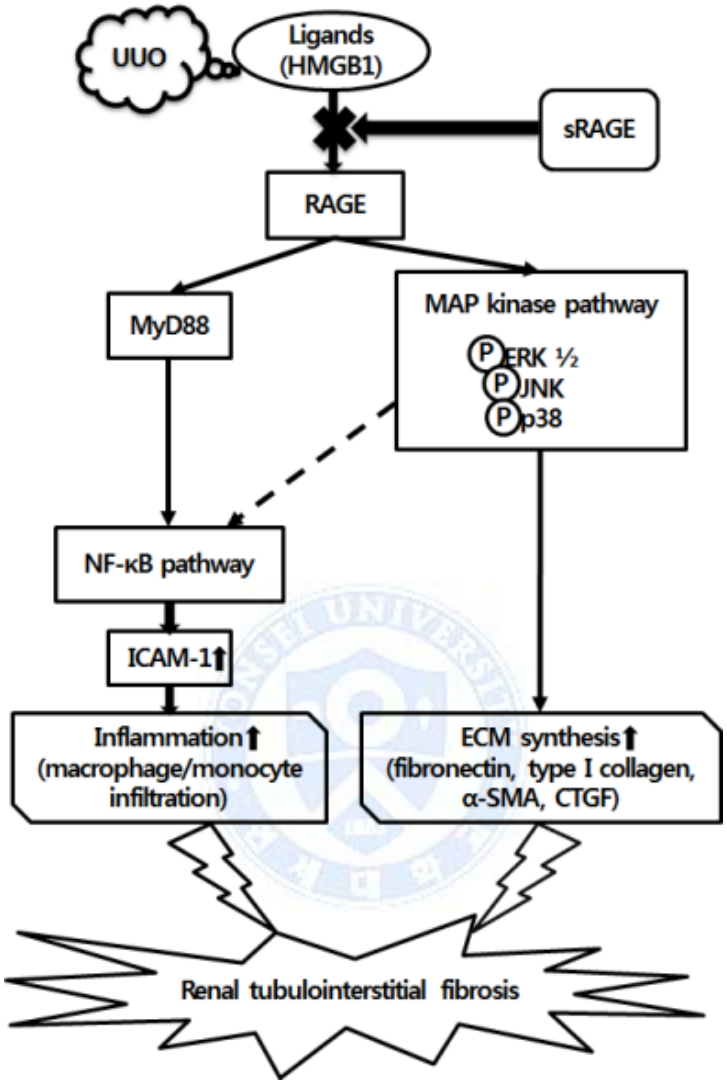


Figure 9. Proposed protective role of sRAGE in renal tubulointerstitial fibrosis. sRAGE may play a renoprotective role in renal tubulointerstitial fibrosis through a direct anti-fibrotic impact on renal tubular cells and an indirect effect mediated by anti-inflammation through the MAP kinases and NF-κB signaling pathways.

V. CONCLUSION

To clarify the role of RAGE and the consequences of its inhibition by sRAGE in renal tubulointersititial fibrosis, I investigated the therapeutic effect of sRAGE on HMGB1-induced tubular epithelial cell injury *in vitro* and unilateral ureteral obstruction (UUO)-induced renal fibrosis *in vivo*.

1. The administration of HMGB1 significantly induced the protein expression of fibronectin, type I collagen, α-SMA, and CTGF in cultured NRK-52E cells, and these increases were significantly abrogated by sRAGE treatment.
2. The protein expression of RAGE, phospho-ERK, phospho-p38, phospho-JNK, and MyD88 were significantly increased in cultured NRK-52E cells exposed to HMGB1, and administration of sRAGE significantly ameliorated these increases.
3. NF-κB protein expression in the cytosolic fraction was significantly decreased, while its expression was significantly increased in the nuclear fraction in HMGB1-stimulated NRK-52E cells, and these changes were significantly attenuated by sRAGE treatment.
4. HMGB1 and RAGE protein expression in the left kidney, assessed by western blot and IHC staining, were significantly increased in UUO rats compared to sham-operated rats, and sRAGE significantly abrogated these increases in UUO rats.
5. Compared to control rats, the protein expression of fibronectin, type I collagen, and α-SMA in the left kidney, assessed by western blot and IHC staining, were significantly increased in UUO rats, and these increases were significantly ameliorated by sRAGE administration in UUO rats.
6. The number of infiltrated macrophages within the tubulointerstitium was significantly higher in the left kidney of UUO rats compared to sham-operated rats, and sRAGE treatment significantly attenuated macrophage infiltration in the left kidney of UUO rats.
7. Masson's trichrome staining revealed that tubulointerstitial fibrosis was significantly severer in the left kidney of UUO rats compared to control rats, and

this excessive fibrosis was significantly abrogated in sRAGE-treated UUO rats.

8. Compared to sham-operated rats, phospho-ERK, phospho-p38, phospho-JNK, MyD88, phospho-NF-κB, and ICAM-1 protein expression were significantly increased in the left kidney of UUO rats, and sRAGE significantly ameliorated these increases in UUO rats.

In conclusion, I demonstrated that sRAGE played a renoprotective role in UUO-induced renal fibrosis through a direct anti-fibrotic impact on renal tubular cells and an indirect effect mediated by anti-inflammation. These data suggest that RAGE modulation by sRAGE may be a potential therapeutic approach to various kidney diseases associated with renal tubulointerstitial fibrosis.

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ABSTRACT (IN KOREAN)

일측성 요관 폐쇄로 유발된 신섬유화 모델에서 soluble receptor for advanced glycation end-products의 신보호 효과

<지도교수 강 신 욱>

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김 찬 호

배경: 세뇨관-간질성 섬유화는 만성 신질환의 전형적인 병리학적 소견으로, 만성 신질환의 진행과 밀접한 연관이 있다. 최근의 연구들에 의하면 high mobility group protein box1 (HMGB1)과 그 수용체 중 하나인 receptor for advanced glycation end-products (RAGE)가 세뇨관-간질성 섬유화의 병태생리와 관련되어 있는 것으로 보고되고 있다. Soluble RAGE (sRAGE)는 RAGE와 동일한 리간드 결합 특이성을 지니고 있기 때문에 RAGE와 결합하는 각종 리간드와 경쟁적으로 결합하여 세포막으로부터 시작되는 RAGE 신호전달체계의 활성화를 억제하는 유인체 역할을 할 수 있는 것으로 알려져 있다. 본 연구에서는 HMGB1 자극으로 인한 신세뇨관 세포의 손상 및 일측성 요관 폐쇄로 유발된 신섬유화 모델에서 sRAGE의 신보호 효과를 규명하고자 하였다.

방법: 생체 외 실험으로는 신세뇨관 세포를 대조군과 HMGB1 투여군 (10 μg/ml)으로 나누어 배양하였으며, sRAGE (1 μg/ml)를 동시에 처치한 실험도 하였다. 생체 내 실험은 Sprague-Dawley 백서 24 마리를 4 그룹 (그룹당 6마리)으로 나누어 진행하였다: sham 수술 및 위약 투여군 (대조군); sham 수술 및 sRAGE 투여군; 일측성 요관 (좌측) 폐쇄 수술 및 위약 투여군; 그리고 일측성 요관 폐쇄 수술 및 sRAGE 투여군. 모든 투약은 수술 전 1시간 그리고 수술 후 48시간 마다 복강 내로 주사로 시행하였다. 수술 10일 후 동물을

희생시켜 좌측 신장을 적출하였다. 섬유화-관련 단백, mitogen-activated protein (MAP) kinase, 그리고 NF-κB를 포함한 RAGE 신호전달체계와 연관된 단백의 발현은 western blot와 면역 조직화학적 염색을 이용하여 분석하였으며, 신섬유화 정도는 Masson's trichrome 염색으로 관찰하였다. 또한, 대식세포 침윤은 ED-1에 대한 항체를 이용한 면역 조직화학적 염색으로 확인하였다.

결과: HMGB1으로 자극한 신세뇨관 세포에서 fibronectin, type I collagen, α-smooth muscle actin (α-SMA), 그리고 connective tissue growth factor의 단백 발현은 대조군에 비하여 의미있게 증가되었으며, 이러한 증가는 sRAGE 처치로 의의있게 억제되었다. MAP kinases와 NF-κB도 HMGB1 자극에 의하여 활성화되었으며, sRAGE에 의하여 유의하게 억제되었다. 일측성 요관 폐쇄 수술 및 위약을 투여 받은 백서의 좌측 신장 내 RAGE와 HMGB1의 단백 발현이 대조군에 비하여 의미있게 증가되었으며, 섬유화-관련 단백인 fibronectin, type I collagen, 그리고 α-SMA의 단백 발현도 의의있게 증가되었다. 이러한 증가는 sRAGE의 투여에 의하여 유의하게 억제되었다. Masson's trichrome 염색 및 ED-1 염색으로 확인한 일측성 요관 폐쇄 및 위약 투여군에서의 좌측 신섬유화 및 신장 내 대식세포의 침윤도 sRAGE의 투여에 의하여 의미있게 억제되었다. 또한, sRAGE는 요관 폐쇄된 좌측 신장 내 각종 MAP kinases의 활성과 NF-κB의 발현을 의의있게 억제하였다.

결론: 이상의 결과를 종합하여 볼 때, sRAGE가 신섬유화 모델에서 RAGE를 억제함으로써 직간접적으로 세뇨관 세포에서의 섬유화 과정을 억제함을 알 수 있었으며, 향후 신섬유화와 관련된 다양한 신질환의 잠재적인 치료 방법으로 고려할 수 있을 것으로 생각된다.

핵심되는 말: receptor for advanced glycation end-products (RAGE), soluble RAGE, 일측성 요관 폐쇄, 신섬유화