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Differential regulation of angiotensin II-induced extracellular signal regulated kinase-1/2 and -5 in progressive glomerulonephritis

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Paper Title

Differential regulation of angiotensin II-induced extracellular signal regulated kinase-1/2 and -5 in progressive glomerulonephritis

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Short Title

Role of ERK1/2 and ERK5 in glomerulonephritis

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Abstract

Aim: Extracellular signal regulated kinase (ERK)1/2 and ERK5 are key kinases of the signaling pathways involved in various cellular responses to kidney injury; however, the mechanistic links between those kinase and renin-angiotensin system (RAS) activation in glomerulonephritis (GN) have not been fully elucidated. In this study, we sought to clarify the potential roles of ERK1/2 and ERK5 via RAS activation in the pathogenesis of GN.

Methods: A rat model of progressive GN was induced by anti-glomerular basement membrane (GBM) injection and the signal transduction pathway in angiotensin II (Ang II)-induced glomerular pathologic alterations were investigated in primary cultured mesangial cells (MCs). Results: Rats developed typical cellular crescents in glomeruli on day 7 that progressed to severe fibrocellular crescents and glomerulosclerosis on day 28. Strong expression of phospho-ERK1/2 was observed on day 7 and phospho-ERK5 expression was markedly increased on day 28 of GN. An angiotensin II type 1 receptor blocker (ARB) suppressed those augmentations. Moreover, ARB treatment attenuated the increases in macrophage infiltration and PCNA-positive cells observed on day 7 in GN rats, as well as the increase in collagen type 1 expression on day 28. Consistently, MCs stimulated by Ang II showed significant increases in proliferation and the expression of MCP-1 and collagen type 1. Interestingly, while the ERK1/2 inhibitor PD98059 abolished the elevations in MCP-1 expression and cell proliferation, the ERK5 inhibitor BIX02189 abrogated the elevation in collagen type 1 expression.

Conclusion: Altogether, these data suggest that ERK1/2 regulates acute inflammatory reactions, while ERK5 promotes the development of RAS-induced chronic glomerular fibrosis activation in GN.

Key words:

Extracellular signal-regulated kinase, glomerulonephritis, renin-angiotensin system, macrophage infiltration, fibrosis

Introduction

The renin angiotensin system (RAS) is essential for the homeostatic regulation of fluid volume, blood pressure, and sodium levels throughout the body. [1] While angiotensin II (Ang II) is the most biologically active peptide, local/tissue RAS in specific tissues has recently garnered considerable interest. [2] Local RAS activation in the kidney has several pathophysiological functions such as renal cell growth and in the development of glomerulosclerosis that occurs during the development of renal fibrosis. [3] Indeed, previous studies have shown that RAS blockade has beneficial effects in rats and humans with various renal diseases, which are often significantly greater than their suppressive effects on blood pressure. [4,5] Chronic glomerulonephritis (GN) is an aggressive disease characterized by substantial renal damage that ultimately results in end-stage renal disease. Renal Ang II, whose production is enhanced in chronic GN, which can lead to increased intraglomerular pressure, glomerular cell hypertrophy, and extracellular matrix (ECM) accumulation. [6,7] Ang II antagonists or synthesis inhibitors markedly decelerate, and can even prevent, renal deterioration in renal disease. [6,8-10] However, it remains unknown whether this effect is due to the relatively short-term nature and small sample size of these studies, or an indication that factors other than Ang II play an important role in the progression of GN.

The mitogen activated protein kinase (MAPK) cascade is an intracellular signaling pathway involved in the regulation of cell proliferation, survival differentiation, and migration, in response to extracellular stimuli, such as growth factors and environmental stress. [11] At least four MAPK pathways, specifically the ERK1/2, ERK5, p38, and c-Jun N-terminal kinase (JNK) cascades, display the classical MAPK triple kinase phospho-relay signaling architecture, whereby a MAPK kinase kinase (MAPKKK) phosphorylates and activates a MAPK kinase (MAPKK), which, in turn, phosphorylates and activates the effector MAPK itself. [11] Although ERK1/2 and ERK5 both contain a TEY dual phosphorylation motif, each

includes features distinct from other MAPK family members, suggesting that the ERK5 and the ERK1/2 pathways may differ in their method of activation and function.

It was recently reported that ERK1/2 activation occurs in the rat Thy-1 model of mesangioproliferative nephritis, and that ERK1/2 pathway inhibition results in a significant reduction in mesangial cell (MC) proliferation in this model. [12] Accordingly, ERK1/2 activation in human glomerulopathies is associated with cell proliferation, histologic lesions, and renal dysfunction. [13] In comparison, ERK5-mediated MC growth is involved in the pathogenesis of diabetic nephropathy. [14] Moreover, we reported that enhanced ERK5 phosphorylation appeared to associated with increased MC viability and pathological ECM accumulation in GN. [15] Although extensive research has been conducted on the role of MAPK cascade in GN, [16] the mechanism of ERK1/2 and ERK5 regulation and its involvement in RAS activation remains uncharacterized. In this study, we investigated the roles of ERK1/2 and ERK5 signaling pathway and their interactions with Ang II in GN pathogenesis, and observed that ERK1/2 and ERK5 activation are differentially regulated during the course of GN.

Material and Methods

Antibodies and reagents

Polyclonal rabbit antibodies to phospho-ERK1/2, phospho-ERK5, and total ERK5 were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibodies to total-ERK1/2, ED-1, PCNA, and collagen type 1 were purchased from Santa Cruz Biotechnology (Dallas, TX), Serotech (Oxford, UK), Sigma-Aldrich (St. Louis, MO), and Chemicon International (Temecula, CA), respectively. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L; Bio-Rad, Hercules, CA) and HRP-conjugated horse anti-rabbit IgG (H+L; Cell Signaling Technology) were used for western blot analysis.

A 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) assay kit was purchased from Dojindo (Kumamoto, Japan).

Animal preparation

The Institutional Animal Care and Use Committer of the University of Tokushima Graduate School approved all procedures and protocols used in this study. Progressive GN was induced in 7-week-old male WKY rats by a single intravenous injection of rabbit anti-rat glomerular basement membrane (GBM) antiserum (0.2 mg/100g body weight) via the tail vein.[17] Nephritic rats were divided into two groups (n = 36) and given either vehicle or 70 mg/L candesartan (Ang II type 1 receptor blocker (ARB); Takeda Chemical Industries, Osaka, Japan) daily in drinking water. The therapeutic regimen that was used in our study was pharmacologically valid and efficient according to the manufacturer's information, and the dose was high enough (10 mg/kg per day) to inhibit Ang II receptor binding to kidney tissue.[17] Candesartan was started on the same day as disease induction. Age-matched male WKY rats (n = 18) without nephritis were used as controls. Rats were sacrificed on post-induction days 7 and 28, and the kidneys excised histologic analysis.

Kidney histology and immunohistochemistry

Kidneys were immediately fixed in 10% buffered formalin, paraffin embedded, sectioned (3-μm), and stained with periodic acid-Schiff reagent. Glomerular crescent formation was assessed blindly in >50 glomeruli per rat. Glomeruli were considered to exhibit crescent formation when at least 2 layers of cells were observed in Bowman's space and used to calculate the ratio of crescent-positive to total glomeruli. Glomerulosclerosis was defined as glomeruli that exhibited adhesion of the capillary tuft to Bowman's capsule, capillary obliteration, and mesangial expansion. The severity of sclerosis for each glomerulus was

graded semiquantitatively as follows: score 0, no lesions; score 1, less than 25% of the glomerular area affected; score 2, 25% to 50% affected; score 3, 50% to 75% affected; and score 4, 75% to 100% affected. The mean score was calculated as the glomerulosclerosis score. Immunohistochemistry for phospho-ERK1/2, phospho-ERK5, ED-1, or PCNA was performed by incubating 3-μm sections with primary antibodies overnight at 4°C. The sections were then rinsed and incubated with either biotinylated anti-rabbit IgG or anti-mouse IgG (Vector Labs, Burlingame, CA). After second rinsing, avidin-biotin-peroxidase complex (ABC Elite; Vector Labs) and 3,3'-diaminobenzidine (Dojindo) were added sequentially to each section, which were then counterstained with Mayer's hematoxylin (Wako, Tokyo, Japan), dehydrated, and cover-slipped. Frozen sections (3-μm) were fixed in acetone incubated with a goat anti-collagen type 1 antibody overnight at 4°C, and then incubated with a FITC-conjugated donkey anti-goat IgG antibody. The fractions of immuno-reactive areas (brown or green) were measured using the EIS-Elements software (Nikon Corporation, Tokyo, Japan). For each rat, 20 glomeruli were examined and the mean percentages of the affected lesions were calculated. In double-staining experiments, sections were stained with anti-phospho-ERK1/2 or anti-phospho-ERK5 antibody and further incubated with antibodies for PCNA or ED-1, respectively, and then with FITC-labeled anti-mouse IgG and Rhodamine-labeled anti-rabbit IgG (Jackson ImmunoResearch Laboratories, PA).

Rat mesangial cell culture

Cultured rat MCs were established from rat kidney by multiple sieving techniques and placed under an inverted tissue culture microscope with phase-contrast optics. [15] MCs were serum-starved for 48 h in a serum-free RPMI 1640 medium (Sigma) before stimulation with Ang II in the presence of absence of reagents.

Transfection

For small interfering RNA (siRNA) assays, MCs were transiently transfected with MAP kinase kinase (MEK)1/2 or MEK5-specific MISSION siRNA designed by Sigma using Lipofectamine RNAiMAX (Invitrogen, Calsbad, CA). After incubation in a low-serum-containing medium (5% FBS), the serum concentrate was adjusted to that of a complete medium and the cells were cultured. A non-silencing (Sigma) that does not target any known mammalian genes was used as a negative control.

Quantitative real-time RT-PCR.

Total RNA was extracted using a commercially available kit (Qiagen, Valencia, CA). Total RNA (1 μg) was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays (Applied Biosystems) were used for the PCR reactions on a StepOne Plus Real-time PCR System (Applied Biosystems). TaqMan Gene Expression Assays were purchased for the following rat genes: monocyte chemotactic protein (MCP)-1 (Rn00580555_m1), collagen type 1 (Rn01463848_m1) and GAPDH (Rn01775763_g1).

Western blot analyses

MCs were treated with lysis buffer (Cell Signaling Technology, Inc., Beverly, MA) and cells were scraped off the dish. The samples were centrifuged and the protein concentrations were determined using Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Protein samples (20 μg) were separated by SDS-PAGE using 12.5% gel and transferred to PVDF membranes. The membranes were probed with either rabbit anti-phospho-ERK1/2 or

rabbit anti-phospho-ERK5 antibody, and then incubated with the appropriate HRP-conjugated anti-rabbit secondary antibody. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). Blots were appropriately reprobed with either a mouse anti-total ERK1/2 or a rabbit anti-total ERK5 antibody as a lording control. Densitometric analysis was performed using an LKB UltroScan XL apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Cell proliferation assay

Cell proliferation ability was evaluated by the WST-8 assay according to the manufacturer's protocol. Briefly, cultured cells were grown to subconfluency in serum-containing medium, starved for 24 h, and then cultured for an additional 24h with or without treatment prior to the addition of WST-8 reagent. After incubation for 4 h, sample absorbance at 450 nm was measured with a microplate reader. [15]

Statistical analysis.

Statistical analysis was performed using a one-way factorial ANOVA with the post-hoc Scheffe's F testing. All data are presented as the means +/- SD. Probability values <0.05 were considered statistically significant.

Results

ARB treatment inhibits glomerulosclerosis and fibrocellular crescent formation

We first examined the effects of ARB treatment on renal histology by using periodic acid-Schiff-stained sections on days 7 and 28 post-GN induction (Figure 1A). Glomerular crescent formation was the most prominent change in vehicle-treated GN, which was present in more than 60% of glomeruli on day 7 and often accompanied by severe necrotizing lesions

and/or glomerulosclerosis (Figure 1B). No notable differences in glomerulosclerosis were observed between ARB- and vehicle-treated GN rats on day 7 (Figure 1A and C). However, severe glomerulosclerosis and fibrocellular crescents and were found in vehicle-treated GN rats on day 28, whereas ARB significantly attenuated this progression in treated counterparts (Figure 1A-C). Moreover, the vehicle-treated GN rats developed progressive proteinuria, which was suppressed in those treated with ARB (Figure 1E). Correspondingly, ARB-treated GN rats exhibit significant reductions in systolic blood pressure, while vehicle-treated GN rats showed elevations when compared to control rats from day 7 to day 28 of GN (Figure 1F).

Glomerular expression of phospho-ERK1/2 and phospho-ERK5

ERK1/2 phosphorylation was only rarely detected in the glomeruli of normal rats (Figure 2A and B). Interestingly, glomerular ERK1/2 phosphorylation was detected on day 7 in vehicle-treated GN rats, which decreased slightly by day 28 (Figure 2A and B). Treatments with ARB significantly attenuated glomerular phospho-ERK1/2 in GN rats (Figure 2A and B). Comparatively, glomerular phospho-ERK5 expression progressively increased throughout the time course (Figure 2B and D). Moreover, ARB treatment significantly inhibited glomerular ERK5 phosphorylation on day 28 (Figure 2B and D). Furthermore, enhanced collagen type 1 expression was observed on day 7 and dramatically increased by day 28 in vehicle-treated nephritic rats (Figure 2E and F). Interestingly, ARB treatment had no effect on collagen staining on day 7, but significantly decreased this on day 28 in nephritic rats (Figure 2E and F).

Glomerular PCNA expression, macrophage infiltration, and collagen expression

The number of PCNA-positive cells in nephritic glomeruli was markedly increased in

vehicle-treated GN rats when compared to the ARB-treated GN and non-GN control rats on day 7; however, no differences were observed between groups on day 28 (Figure 3A and B). Consistently, significantly more glomerular ED-1 positive macrophages were observed in the kidneys of vehicle-treated GN rats compared with the non-GN rats on day 7, whereas this was notably lower in ARB-treated GN rats, albeit not to the point of normal controls (Figure 3C and D). To investigate the localization of phospho-ERK1/2 and phospho-ERK5 in nephritic glomeruli, double immunofluorescence staining was performed. Phospho-ERK1/2 co-localized with PCNA-positive cells and ED-1 positive macrophages, on the other hand, positive but very weak phospho-ERK5 staining was observed in those cells (Figure 3E and F).

Effects of Ang II on cell proliferation, and the mRNA expression of MCP-1 and collagen type 1 in cultured mesangial cells

To determine the role of ERK in GN pathogenesis, we examined the endogenous function of ERK1/2 or ERK5 in cultured MCs with selective pharmacological antagonists, PD98059 or BIX02189, respectively. Optimization of the effective concentrations was carried out to inhibit the phosphorylation, selectively. As shown in Figure 4A, treatment with 10 μM PD98059 specifically inhibited ERK1/2 phosphorylation, but had no effect on ERK5 phosphorylation. On the other hand, BIX02189 inhibited ERK5 phosphorylation independent of ERK1/2 when used at 10 μM (Figure 4B). To confirm the selectivity of each antagonist, we monitored the inhibition of ERK1/2 or ERK5 phosphorylation in cells treated with siRNAs targeting for MEK1/2 or MEK5 by Western blot analysis (Figure 4C and D). We next examined the effect of these inhibitors on the proliferation of MCs stimulated with Ang II. Notably, these analyses revealed that treatment with ARB or PD98059 treatment completely suppressed Ang II-induced cell proliferation, whereas BIX02189 only moderately reduced this effect (Figure 5A). Following incubation of quiescent cultured MCs with Ang

II, quantitative real-time PCR (qRT-PCR) revealed increases in MCP-1 and collagen type 1 mRNA expression (Figure 5B and C) and ARB treatment reduced those augmentations (Figure 5B and C). Interestingly, PD98059 completely prevented this induction of MCP-1 mRNA, but did not inhibit that of collagen type 1 (Figure 5B); whereas BIX02189 had the opposite effect by inhibiting collagen type 1 mRNA levels, with only a modest effect on MCP-1 (Figure 5C).

Discussion

It was previously reported that the MAPK pathway plays a crucial role in the development of renal diseases. [16,18] Bokemeyer et al. demonstrated that glomerular ERK1/2 activation peaked on day 6, where it likely served as a mediator of the proliferative response in mesangioproliferative GN. [12] We also found in a previous study that phospho-ERK5 expression peaked during the phase of massive ECM accumulation in a rat progressive model of mesangioproliferative GN. [15] In the present study, we showed that a marked induction of ERK1/2 phosphorylation was detected on day 7 during the early phase of acute inflammation-induced cellular proliferation, whereas elevated phospho-ERK5 expression was observed on day 28 in the later phase of ECM accumulation in a progressive anti-GBM rat model of GN. Together, these studies support the possibility that ERK1/2 signaling is important for acute inflammation-induced macrophage, while ERK5 controls the development of chronic glomerular fibrosis in GN.

Glomerular crescents are defined as the presence of >2 layers of cells in the Bowman's space. Monocyte/macrophages and parietal epithelial cells are principal mediators of crescent formation, which is a marker of severe glomerular injury. [19] The progression or resolution of crescents may depend upon the integrity of the Bowman capsule and the crescents' resulting cellular composition. In addition, the progression of crescents to the

fibrous stage is commonly observed in ruptured capsules, as well as when fibroblasts and macrophages dominate the Bowman's space, and it usually correlates with irreversible glomerular sclerosis. [19] RAS is known to play a critical role in the progression of renal disease, [20] and is a major mediator of progressive renal injury in GN. [18] Our previous studies have shown that RAS blockades have beneficial effects in various GN models [18,21], which are often considerably more significant than their suppressive effects on blood pressure. In the present study, we demonstrated that ARB treatment attenuated glomerular macrophage infiltration and crescent formation on day 7 in anti-GBM GN rats and hindered the progression of glomerular fibrosis and collagen type 1 expression on day 28. From these findings, we believe that suppressing RAS activation is an effective means to attenuate the progression of GN.

ERK1/2 regulates cell cycle progression, proliferation, cytokinesis, transcription, differentiation, senescence, cell death, migration, and cell adhesion. [22] Owing to its role in cellular biology, ERK1/2 plays a prominent role in most physiological settings by influencing the immune system and participating in the cellular response to various hormones and growth factors. [23] The present study indicated that glomerular ERK1/2 phosphorylation parallels ED-1 positive macrophage infiltration and the presence of PCNA-positive cells in the progressive anti-GBM rat model of GN. Furthermore, we found that Ang II modestly but significantly enhanced the expression of MCP-1 that is the key molecule in macrophage chemotaxis and activation, and cell proliferation in cultured MCs. Notably, these changes were significantly abrogated in MCs treated with PD98059, suggesting a possible role of ERK1/2 expression in the active phase of glomerular inflammation.

ERK5 was originally shown to be activated by stress stimuli or serum. [24,25] Ang II and other stimulators of ERK1/2 can also increase ERK5 activity [24], and similarities exist between the activation modes and functions of these two kinases. However, recent studies have also

identified some distinctive features of the ERK1/2 and ERK5 pathways. [26,27] In the present study, phospho-ERK5 was dramatically increased in severely diseased glomeruli such as fibrocellular crescents and glomerulosclerosis on day 28 in the progressive anti-GBM rat model of GN, and collagen type 1 was strongly expressed in those glomeruli. Furthermore, we found that Ang II stimulation led to modest significant increases in collagen type 1 mRNA expression in cultured MCs, which was inhibited in MCs treated with BIX02189. Those results suggest that MC ERK5 phosphorylation by Ang II enhances fibrous changes associated with collagen accumulation during GN progression.

In conclusion, the present study has revealed that ERK1/2 and ERK5 phosphorylation are markedly increased in glomeruli of anti-GBM rat model of GN. Importantly, glomerular ERK1/2 activation appears to play an important role in acute inflammation-induced cell proliferation and macrophage infiltration during the development of GN, whereas ERK5 phosphorylation induced by Ang II is likely associated with the pathological fibrous changes in glomerular crescent that coincides with progressive disease. Thus, it might be that ERK1/2 and ERK5 signaling pathway and their interactions with Ang II are differentially regulated throughout the course of GN. Therefore, we propose that the controlled regulation of glomerular ERK1/2 and ERK5 activation could provide a basis for the development of an effective therapeutic strategy to preclude the progression of GN.

Acknowledgments

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Conflict of Interest Statement

None to disclose

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Figure Legend

Figure 1. Effect of ARB on crescent formation in the anti-GBM rat model of GN on days 7 and 28. (A) Periodic acid-Schiff-stained sections. Original magnification ×400. (B) Percentage of glomeruli with crescent formation. (C) Quantitative analysis of the crescent score. (D) Urinary protein excretion. (E) Systolic blood pressure. The nephritic rats showed severe crescent formation and glomerulosclerosis and treatment with ARB attenuated those pathologic alterations on day 28 but not on day 7 of nephritic rats. Data are mean ± SD. *P < 0.05 and **P < 0.01 between groups as indicated. N.S., not significant.

Figure 2. Effect of ARB on phospho-ERK1/2 and ERK5 expression in nephritic rat glomeruli on days 7 and 28. (A) Representative images of phsopho-ERK1/2. (B) Semiquantitative assessment of phospho-ERK1/2. (C) Representative images of phospho-ERK5. (D) Semiquantitative assessment of phospho-ERK5. Glomerular phospho-ERK1/2 expression was enhanced in vehicle treated nephritic rats and treatment with ARB significantly suppressed on day 7 (A and B). In contrast, glomerular phospho-ERK5 progressively enhanced in vehicle treated nephritic rats and treatment with ARB significantly suppressed on day 7 and 28 (C and D). Data are mean ± SD. *P < 0.05 and **P < 0.01 between groups as indicated. N.S., not significant.

Figure 3. Effect of ARB on PCNA positive cells, ED-1 positive macrophage infiltration and collagen type 1 expression on days 7 and 28 and immunofluorescence staining of glomeruli on day 7 in the anti-GBM rat model of GN. (A) Representative images of PCNA-positive cells in the glomeruli. (B) Semiquantitative assessment of PCNA positive cells. (C) Representative images of ED-1-positive macrophage infiltration into the glomeruli. (D) Semiquantitative assessment of ED-1 positive cells. Data are mean ± SD. *P < 0.05 and

**P < 0.01 between groups as indicated. N.S., not significant. (E) Double staining for PCNA (green) and phospho-ERK1/2 or phospho-ERK5 (red). Strong immuno-reactivity for phospho-ERK1/2 was observed on PCNA positive cells (yellow; white arrow), while staining for phospho-ERK5 was hardly seen on PCNA positive cells. (F) Double staining for ED-1-positive macrophage (green) and phospho-ERK1/2 or phospho-ERK5 (red). Strong immuno-reactivity for phospho-ERK1/2 was observed on ED-1-positive macrophage (yellow; white arrow), while staining for phosphor-ERK5 was not observed on ED-1-positive macrophage.

Figure 4. Optimization of the PD98059 or BIX02189 effective concentration in cultured MCs and MEK1/2 or MEK5 siRNA-transfected MCs. Inhibition of (A) ERK1/2 phosphorylation and (B) ERK5 phosphorylation by PD98059 (PD) or BIX02189 (BIX) in cultured MCs. Inhibition of (C) ERK1/2 phosphorylation and (D) ERK5 phosphorylation in MEK1/2 or MEK5 siRNA-transfected MCs. Data are mean ± SD. *P < 0.05 and **P < 0.01 between group indicated. N.S., not significant. n=4 per group.

Figure 5. Effect of Ang II on MC proliferation and Analysis of Ang II-induced MCP-1 and collagen type 1 mRNA expression in cultured MCs. (A) Cell proliferation in cultured MCs following pretreatment with ARB, or PD98059, and/or BIX02189. n=6 per group. (B) Pretreatment with ARB or PD98059 abolished the elevated levels of MCP-1 stimulated by Ang II. n=3 per group. (C) Pretreatment with ARB or BIX02189 abolished the elevated levels of collagen type 1 stimulated by Ang II. n=3 per group. Data are mean ± SD. *P < 0.05 and **P < 0.01 between groups as indicated. N.S., not significant.

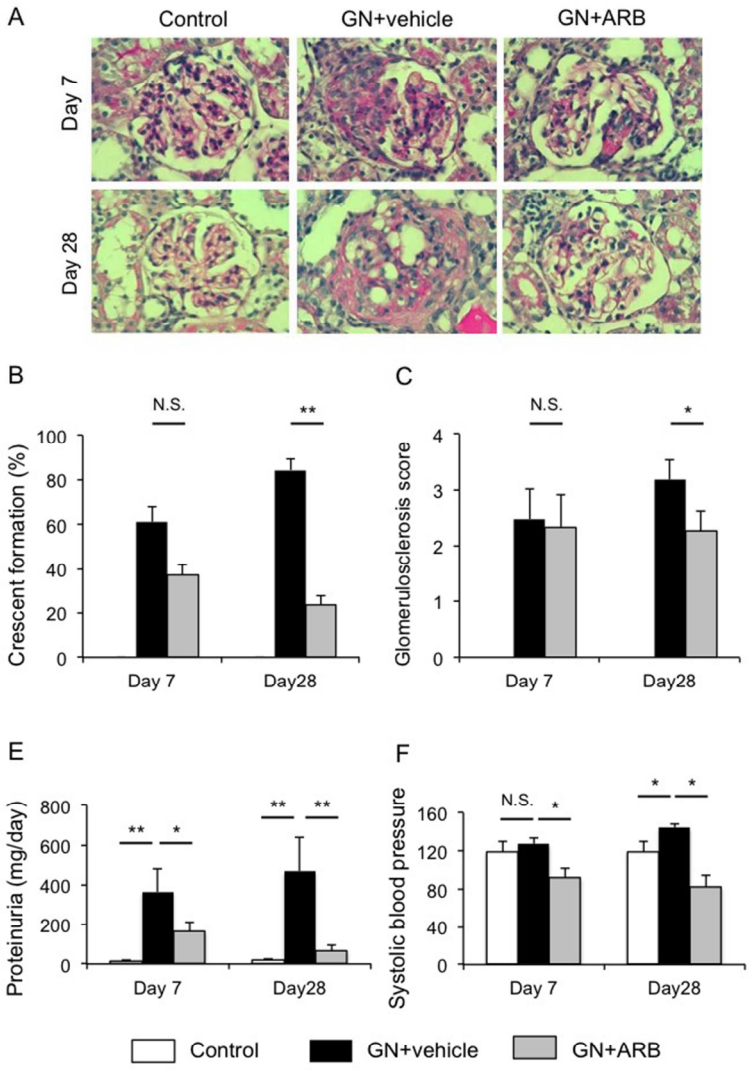

Figure 1

Figure 1
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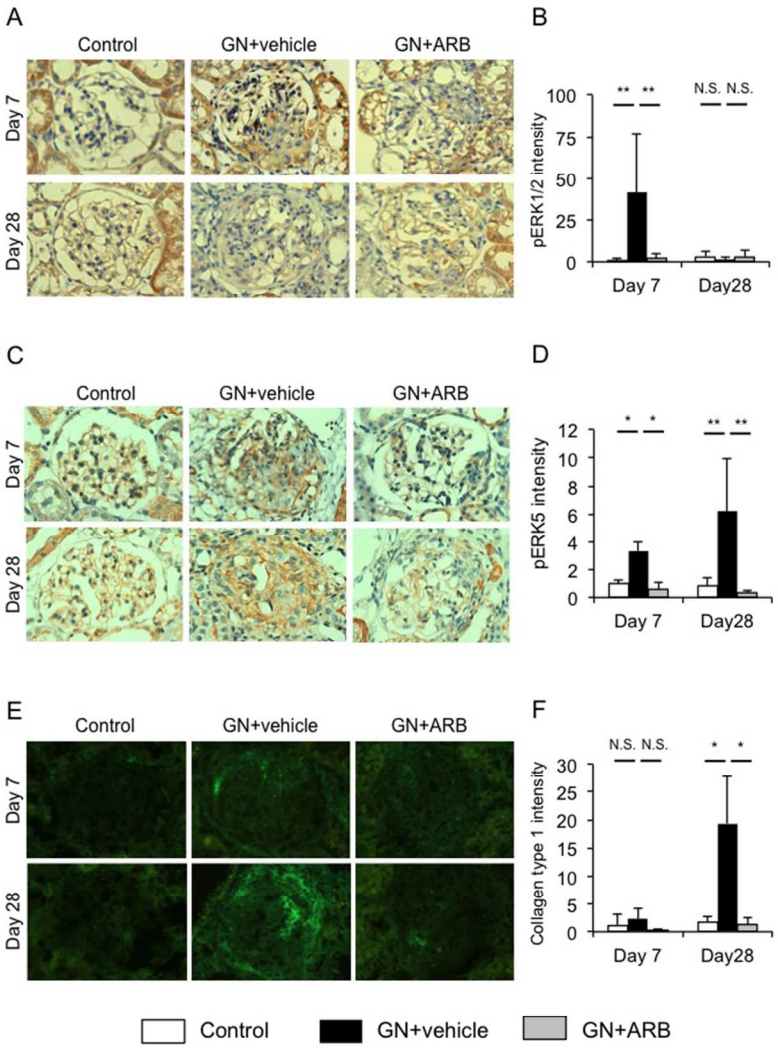

Figure 2

Figure 2
254x366mm (300 x 300 DPI)

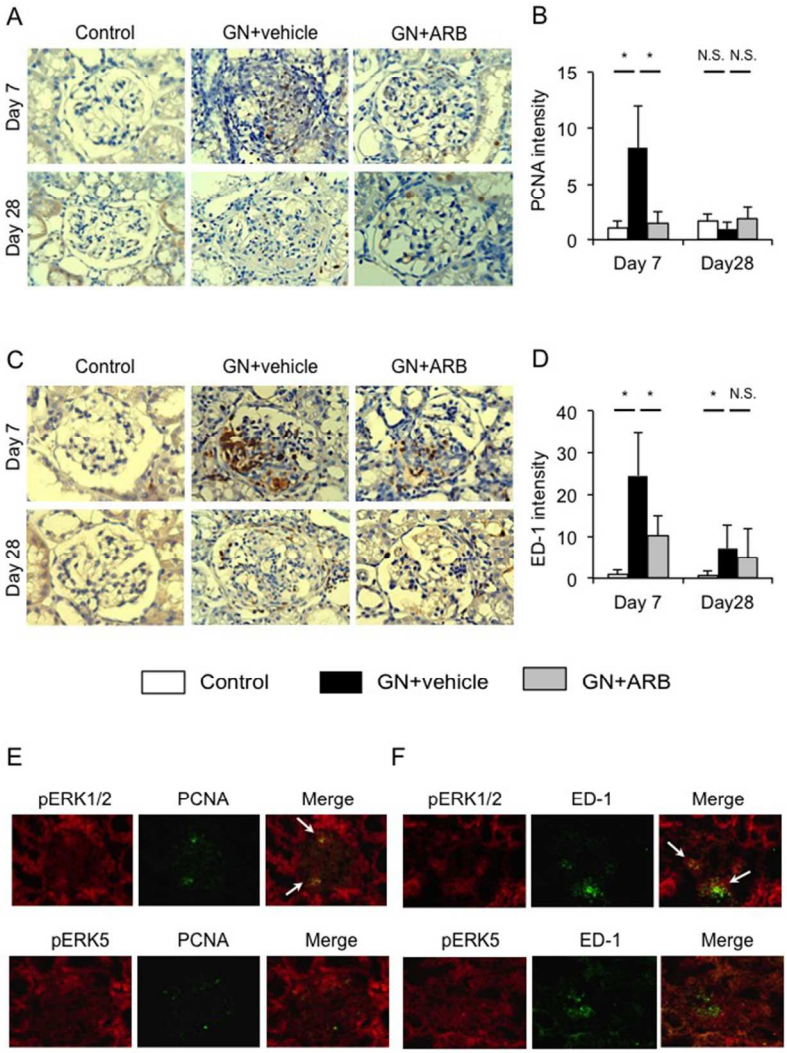

Figure 3

Figure 3
254x366mm (300 x 300 DPI)

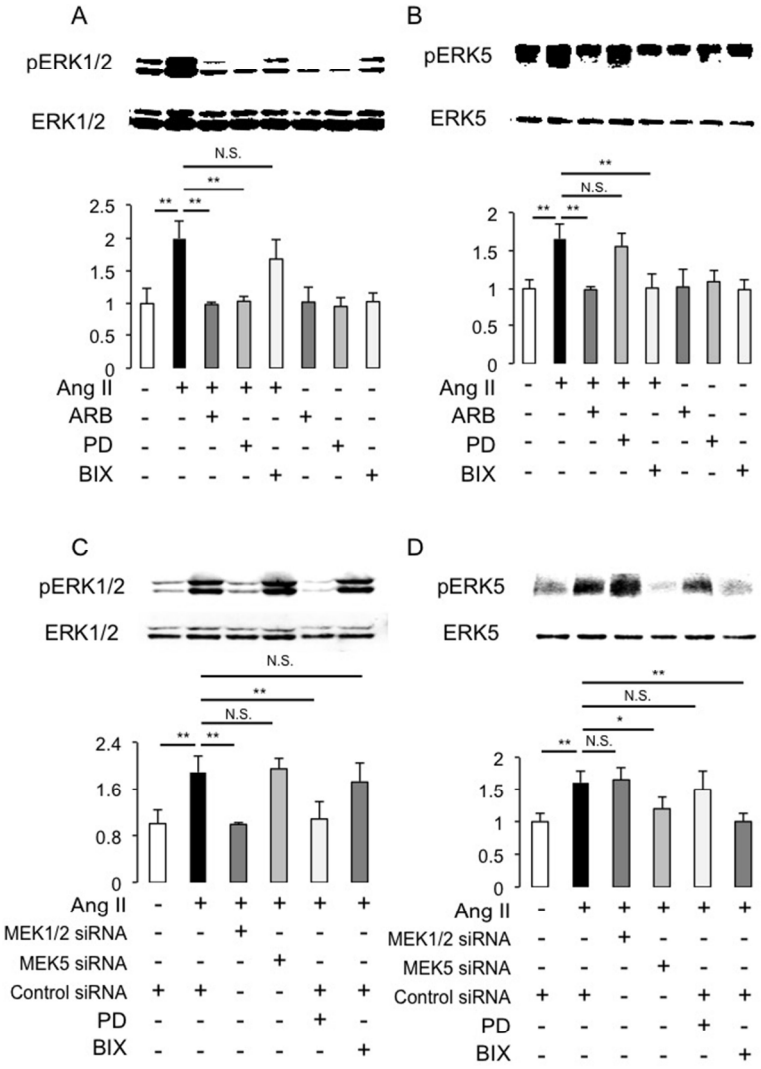

Figure 4

Figure 4
254x366mm (300 x 300 DPI)

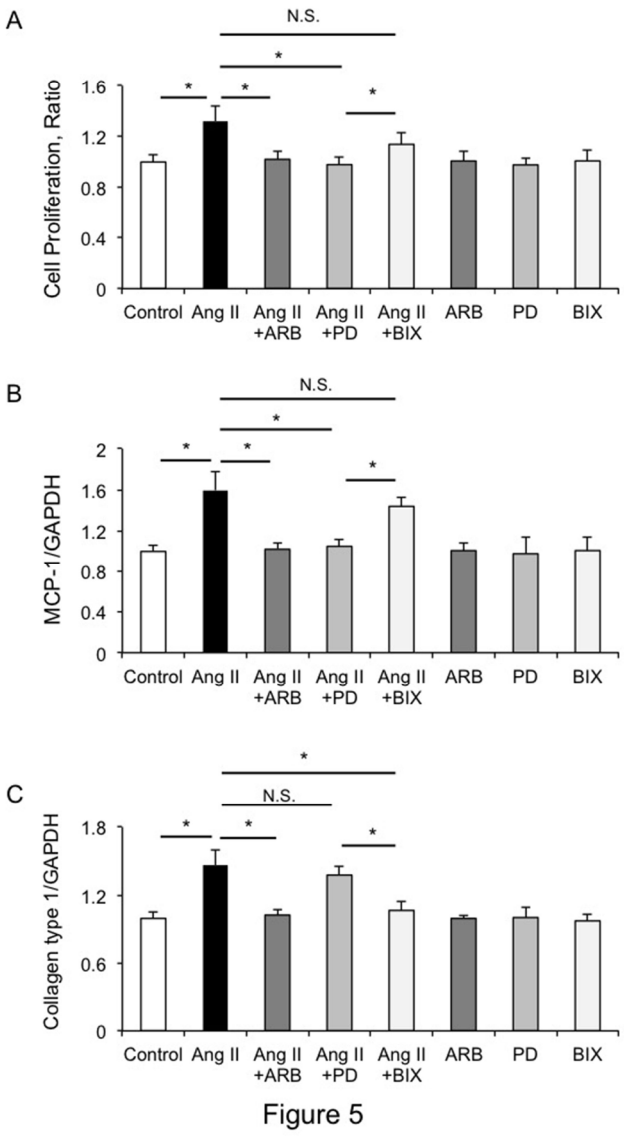

Figure 5

Figure 5
254x366mm (300 x 300 DPI)