

EFFECTS OF SPECIFIC ENDOTHELIN-1 RECEPTOR ANTAGONISTS ON PROLIFERATION AND FIBRONECTIN PRODUCTION OF GLOMERULAR MESANGIAL CELLS STIMULATED WITH ANGIOTENSIN II

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Angiotensin II (Ang-II) is a potent vasoactive hormone, which plays an important role in the pathogenesis of glomerulosclerosis. Ang-II activates many cytokine systems in the kidney. Recent studies indicate that Ang-II is closely related to the activation of the endothelin-1 (ET-1) system. The present study was designed to measure the [³H]-thymidine uptake and fibronectin production of cultured rat mesangial cells stimulated with Ang-II, and to evaluate the effects of specific ET-1 receptor antagonists, BQ123 (type A receptor antagonist) and IRL1038 (type B receptor antagonist) on the cells. ET-1 was measured by radioimmunoassay and fibronectin by Western blot analysis. The results were as follows: (1) Ang-II enhanced ET-1 production, [³H]-thymidine uptake, number of cells, and fibronectin production of mesangial cells; (2) all the baseline [³H]-thymidine uptake, number of cells, and fibronectin production of mesangial cells can be partly suppressed by BQ123, but not by IRL1038; (3) the increment of Ang-II-enhanced number of cells can be partly suppressed by BQ123, but not by IRL1038; and (4) the increment of Ang-II-enhanced fibronectin production can be partly suppressed by both BQ123 and IRL1038. Our results indicate that Ang-II is an active stimulant for the proliferation and fibronectin production of mesangial cells, and the effect is partly suppressed mainly by ET-1 type A receptor antagonists.

Key Words: angiotensin II, endothelin-1, fibronectin, mesangial cell
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Endothelin-1 (ET-1) is a potent vasoconstrictor peptide originally isolated and purified from the conditioned medium of cultured porcine aortic endothelial cells [1], and it has also been shown to process a wide spectrum of biological activities in kidneys [2]. Recent

studies indicate that ET-1 may also play an important role in glomerular diseases [3]. ET-1 induces mesangial cell proliferation and the production of extracellular matrix [4], both of which are important in the pathogenesis of glomerulosclerosis.

Angiotensin II (Ang-II), the main peptide of the renin-angiotensin system, is a renal growth factor, inducing hyperplasia/hypertrophy depending on the type of cell. Ang-II is also an important vasoactive peptide involved in the formation of glomerulosclerosis. All components of the renin-angiotensin system, including precursors and enzymes required for the formation and degradation of the biologically active

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forms of angiotensin, as well as different receptors, have been identified in the kidney [5,6]. Recent studies indicate that the effects of Ang-II may be mediated by ET-1, because the use of angiotensin-converting enzyme (ACE) inhibitor or angiotensin receptor antagonists blunts or inhibits the activation of the endothelin system [7]. However, Ang-II also activates other cytokine systems that may activate the glomerular cells consequently. Therefore, the importance of ET-1 in mediating the effect of Ang-II on glomerular cells remains unclear. Mesangial cell, the major intrinsic cell in the glomerulus, is one of the targets of Ang-II in various renal diseases. In this study, we measured the effects of specific ET-1 receptor antagonists, BQ123 (type A receptor antagonist) and IRL1038 (type B receptor antagonist) on the [^3H]-thymidine uptake and fibronectin production of cultured rat glomerular mesangial cells stimulated with Ang-II.

MATERIALS AND METHODS

Culture of mesangial cells

Mesangial cells were isolated and cultured from rat renal glomeruli according to the methods described previously [8]. Briefly, glomeruli were harvested from six to eight male Sprague–Dawley rats (150–200 g) by sieving the renal cortices, which were then digested with 0.25% trypsin and 0.05% collagenase. The digested glomeruli were incubated at 37°C in RPMI-1640 medium containing 20% fetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 µg/mL), and insulin (0.6 U/mL). After 2–3 weeks, mesangial cells appeared and were characterized. On phase contrast microscopy, the cells were stellate or spindle-shaped in appearance, and in postconfluent culture they piled and formed small nodules. The nodules were sensitive to mitomycin C, but not to aminonucleoside of puromycin, and they contracted in response to Ang-II. In addition, the cells failed to stain for Ia and leukocyte common antigen. The cells were passaged every 4–6 days. Cells were starved by incubating in a medium containing 0.5% FCS for 24 hours before the reagents were added. The animal experiment was approved by the animal committee of Kaohsiung Medical University.

Experimental protocol

Cultured rat glomerular mesangial cells were stimulated with Ang-II after obtaining a dose–response

curve [9]. Cells were also preincubated with BQ123, ET-1 type A receptor antagonist, or IRL1023, ET-1 type B receptor antagonist (both from Serva Company, Heidelberg, Germany) [10], or ET-1 antisense oligonucleotide for 1 hour before stimulation with Ang-II (Sigma Chemical Company, St Louis, MO, USA).

Thymidine uptake

Mesangial cells were brought to confluent density, trypsinized, and counted. The medium was aspirated and the cells were washed twice with Dulbecco's phosphate buffered saline (PBS) and treated with 1 mL of 0.2% trypsin plus 0.02% ethylene diamine tetraacetic acid (EDTA) solution for 10 minutes at 37°C. Cells were then resuspended in RPMI-1640 containing 20% FCS and were adjusted to a concentration of 2×10^4 cells/mL. An aliquot of 200 µL of cells was placed in each of the 96-well plates. The plates were cultured for 48 hours in a 5% CO₂ humidified atmosphere at 37°C, and the medium was then replaced with RPMI containing 0.5% FCS. After adding Ang-II for 3 hours, the plates were pulsed with 0.5 µCi of [^3H]-labeled thymidine per well for 18 hours [11]. The cells were harvested onto glass fiber filters and washed with distilled water with a semiautomatic cell harvester. The incorporated radioactivity was counted with a β-scintillation counter. All assays were performed in triplicate.

Determining the number of cells

Mesangial cells were cultured in six-well plates with RPMI-1640 medium containing 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin. After a 60–70% confluent, the medium was changed to RPMI-1640 containing 0.5% FCS, and cultured for another 3 days [11]. The medium was changed every 2 days, and the number of cells was counted on the 6th day. The cells were washed twice with PBS and treated with 0.1% trypsin 1 mL/well for counting.

Radioimmunoassay for ET-1

The ET-1-like immunoreactivity of supernatant was determined by a specific ET-1 radioimmunoassay (RIA) (Peninsula Laboratories Inc., Belmont, CA, USA) after extraction. The supernatant was applied to a Sep-Pak C₁₈ cartridge (Waters Associates, Milford, MA, USA) and eluted with 5 mL 60% acetonitrile in 0.1% trifluoroacetic acid. The eluate was lyophilized and reconstituted for RIA. The antibody

employed cross-reacted with ET-1 (100%), big ET-1 (17%), ET-2 (7%), and ET-3 (7%) and did not react with Ang-II, vasoactive intestinal peptide or α -atrial natriuretic peptide 1-28. The recovery rate of ET-1, extracted through a Sep-Pak C₁₈ column by adding radiolabeled ET-1 to the medium, was $61.2 \pm 1.2\%$. The sensitivity for ET-1 RIA was 0.4 pg/tube and the 50% intercept was 20 pg/tube. The intra- and inter-assay coefficients of variation were 9.7% and 10.5%, respectively, over a range of concentrations between 0.1 and 64 pg/tube [12].

Measurement of fibronectin

The mesangial cells were grown in a 24-well tissue culture plate. The medium of subconfluent cultures was switched to Ang-II stimulation. At the end of each experiment, the fibronectin content of the mesangial-conditioned tissue culture supernatant was measured. Cells were then exposed to 0.05% trypsin in 0.53 mM EDTA and counted in a hemocytometer. The primary antibody was a polyclonal rabbit anti-rat fibronectin antiserum (Calbiochem, CA, USA), and the secondary antibody was the peroxidase-conjugated goat anti-IgG (Calbiochem, San Diego, CA, USA). After incubation overnight at 4°C, the plates were washed with TTBS (1 M Tris, pH 7.6; 5 M NaCl; 0.1% Tween-20), stained with ECL system (Amersham, Buckinghamshire, England), and autoradiographed with Kodak X-OMAT-AR film [11].

Statistical analysis

Data are presented as mean \pm SEM. One-way ANOVA and unpaired *t* test were used to compare the difference between each pair.

RESULTS

Effects of Ang-II on ET-1 production

Both Ang-II (10^{-7} M) and 2% FCS enhanced ET-1 production ($p < 0.01$ for Ang-II, and $p < 0.001$ for 2% FCS, compared with the controls) (Figure 1).

Effects of ET-1 receptor antagonists on Ang-II-stimulated thymidine uptake

The baseline level of [³H]-thymidine uptake was significantly suppressed by BQ123 ($p < 0.001$) but not by IRL1038 (Figure 2). Ang-II (10^{-7} M) enhanced

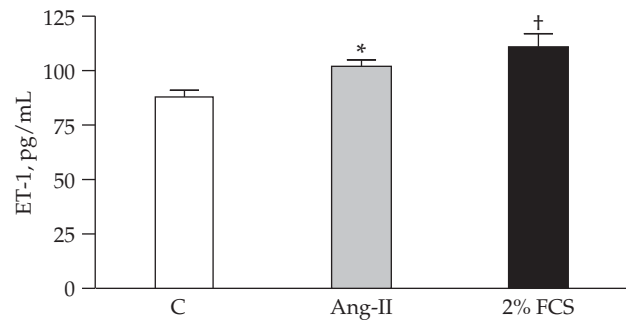


Figure 1. Effects of angiotensin II (Ang-II, 10^{-7} M) and 2% fetal calf serum (FCS) on the endothelin-1 (ET-1) production of mesangial cells. Data are presented as mean \pm SEM of all three independent experiments performed in triplicate. * $p < 0.01$, † $p < 0.001$ compared with the control (C) without stimulation.

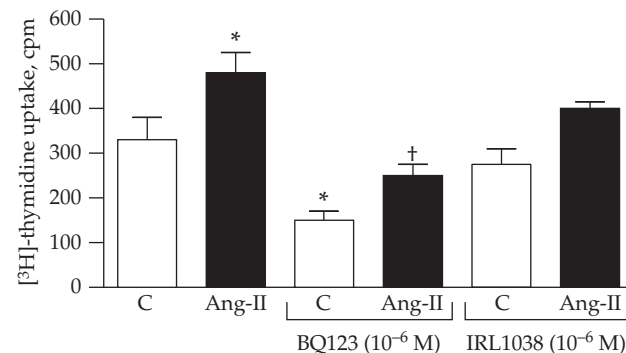


Figure 2. Effects of endothelin-1 (ET-1) receptor type A antagonist BQ123 (A, 10^{-6} M) and type B receptor antagonist IRL1038 (B, 10^{-6} M) on [³H]-thymidine uptake of glomerular mesangial cells at 24 hours. Cells were preincubated for 1 hour with either antagonist before stimulation with angiotensin II (Ang-II, 10^{-7} M). * $p < 0.001$ compared with the control (C) without adding ET-1 receptor antagonists; † $p < 0.001$ compared with the control (C) adding ET-1 receptor type A antagonist. Data are presented as mean \pm SEM of three independent experiments performed in quadruplicate.

the uptake of mesangial cells significantly ($p < 0.001$). The increment of Ang-II-enhanced [³H]-thymidine uptake was not suppressed by either BQ123 or IRL1038.

Effects of ET-1 receptor antagonists on Ang-II-stimulated number of cells

The baseline number of cells was significantly suppressed by BQ123 ($p < 0.001$) but not by IRL1038 (Figure 3). Ang-II (10^{-7} M) enhanced the number of cells significantly ($p < 0.001$), and the increment of Ang-II-enhanced number of cells was suppressed by BQ123 ($p < 0.01$) but not by IRL1038.

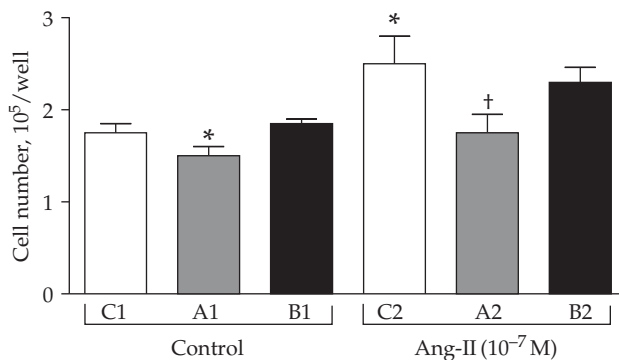


Figure 3. Effects of endothelin-1 receptor type A antagonist BQ123 (A, 10⁻⁶ M) and type B receptor antagonist IRL1038 (B, 10⁻⁶ M) on the number of glomerular mesangial cells on the 6th day. Cells were preincubated for 1 hour with either antagonist before stimulation with angiotensin II (Ang-II, 10⁻⁷ M), and the number of cells was counted after 6 days of culture. **p* < 0.001 compared with the control (C1) without any addition of drug; †*p* < 0.01 when comparing the increment of A2–A1 vs. C2–C1. Data are presented as mean ± SEM of three independent experiments performed in quadruplicate.

Effects of ET-1 receptor antagonists on Ang-II-stimulated production of fibronectin protein

The baseline production of fibronectin protein was significantly suppressed by BQ123 (*p* < 0.05) but not by IRL1038 (Figure 4). Ang-II (10⁻⁷ M) enhanced fibronectin production significantly (*p* < 0.01), and the increment of Ang-II-enhanced fibronectin production was suppressed by both BQ123 and IRL1038 (both *p* < 0.01).

DISCUSSION

Our study indicates that Ang-II is an active stimulant for the proliferation and fibronectin production of glomerular mesangial cells. Ang-II is known to be a critical factor for the progression of chronic renal diseases [13]. Ang-II contributes to the inflammatory process in glomerular disorders, facilitating the migration of mononuclear cells to the glomeruli, and ultimately participates in the fibrotic process. These inflammatory cells would, in turn, activate renal cells through the release of a wide range of growth factors, including Ang-II itself, and therefore contribute to the perpetuation of kidney damage [14]. There is *in vivo* evidence that injured glomeruli are also sensitive to local tissue actions of Ang-II, which promote proliferation and matrix accumulation within the glomerulus [15]. Blockade of Ang-II actions by ACE

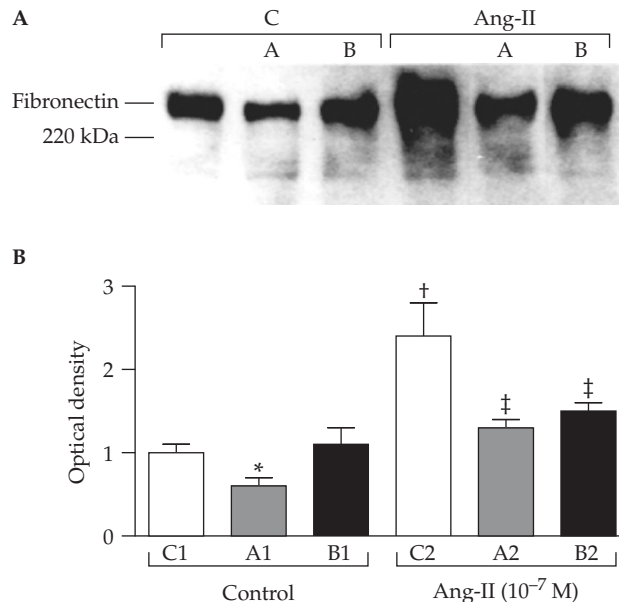


Figure 4. Effects of endothelin-1 receptor type A antagonist BQ123 (A, 10⁻⁶ M) and type B receptor antagonist IRL1038 (B, 10⁻⁶ M) on the fibronectin protein production of glomerular mesangial cells. Cells were preincubated for 1 hour with either antagonist before stimulation with angiotensin II (Ang-II, 10⁻⁷ M), and fibronectin was measured after 24 hours of culture. **p* < 0.05; †*p* < 0.01 compared to the baseline control without Ang-II stimulation (C1); ‡*p* < 0.01 when comparing the increment of A2–A1 vs. C2–C1 and B2–B1 vs. C2–C1, respectively. Data are presented as mean ± SEM of three independent experiments.

inhibitors and angiotensin type 1 antagonists prevents proteinuria, gene expression upregulation, fibrosis, as well as inflammatory cell infiltration.

Ang-II affects cellular behavior by binding to cell surface receptors [16]. Ang-II type 1 receptor antagonist has been found to ameliorate glomerulosclerosis and retard the progression of chronic renal diseases [17]. Jun-kinase mediates the proliferative effect of Ang-II in cultured human mesangial cells and thus represents a novel therapeutic target for the treatment of chronic renal diseases [18]. Ang-II also promotes the apoptosis of mesangial cells. This effect of Ang-II is mediated through downstream signaling involving transforming growth factor-β, phospholipase D, and calcium, contributing to the activation of NADPH oxidase and the generation of reactive oxygen species [19].

We have also demonstrated that the effects of Ang-II on mesangial cells are partly mediated by ET-1. ET-1 is another vasoactive peptide involved in glomerular diseases [3]. ET-1 was found to induce proliferation of mesangial cells and the production

of extracellular matrix [4], both of which are similar to the action of Ang-II and therefore may explain the possible mechanisms for the effect of Ang-II on mesangial cells.

The synthesis of extracellular protein in mesangial cells plays an important role in mesangial expansion and pathogenesis of glomerulosclerosis [20]. One of the major extracellular proteins is fibronectin. Fibronectin is a high-molecular-mass adhesive glycoprotein implicated in a wide variety of cellular properties, including cell adhesion, differentiation, proliferation, migration, and apoptosis [21]. It has been reported that the expression of fibronectin could be regulated by many molecules, such as transforming growth factor- β , cAMP, epidermal growth factor, platelet-derived growth factor, and interferon- γ . However, the mechanism responsible for production and accumulation of fibronectin remains poorly understood. We have demonstrated that Ang-II increases fibronectin production of mesangial cells, which is mediated by ET-1. Ang-II-induced fibronectin production can be suppressed by an RNA interference technique, which may be a new method for simultaneously inhibiting mesangial proliferation and extracellular matrix accumulation, and represent a novel therapeutic approach to glomerulosclerosis [22]. These findings may provide new insights into the mechanisms and the treatment of glomerular sclerosis associated with Ang-II.

In conclusion, we have demonstrated that Ang-II is an active stimulant for the proliferation and fibronectin production of mesangial cells, and the effect is partly suppressed by ET-1 type A receptor antagonists. These results support the importance of cross-effect between different cytokines in the regulation of vascular or renal function. ET-1, and particularly Ang-II, might constitute a combination in this regulation.

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內皮素-1 抗體拮抗劑對第二型血管收縮素所誘發之腎絲球間質細胞增生及纖維蛋白元產量之影響

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第二型血管收縮素 (以下簡稱 Ang-II) 是一種極強的血管收縮物質，與腎絲球硬化關係密切。最近的研究顯示 Ang-II 會影響內皮素-1 (以下簡稱 ET-1)，因此本研究之目的即探討 ET-1 抗體拮抗劑對 Ang-II 所誘發之腎絲球間質細胞增生及纖維蛋白元產量之影響。研究結果：(1) Ang-II 可誘發腎絲球間質細胞產製較多量之 ET-1，thymidine 攝取量，細胞數目，及纖維蛋白元產量；(2) 基礎 thymidine 攝取量，細胞數目，及纖維蛋白元產量均可受 ET-1 之 A 型抗體拮抗劑部分抑制，但不被 ET-1 之 B 型抗體拮抗劑抑制；(3) Ang-II 所增加之腎絲球間質細胞數目可受 ET-1 之 A 型抗體拮抗劑部分抑制，但不被 ET-1 之 B 型抗體拮抗劑抑制；(4) Ang-II 所增加之腎絲球間質細胞纖維蛋白元產量分別可受 ET-1 之 A 型及 B 型抗體拮抗劑部分抑制。本研究結果顯示 Ang-II 可明顯刺激腎絲球間質細胞之細胞增生及纖維蛋白元之產生，且此作用主要可受 ET-1 之 A 型抗體拮抗劑部分抑制。

關鍵詞：第二型血管收縮素，內皮素-1，纖維蛋白元，腎絲球間質細胞
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