Colchicine Ameliorates High Glucose–Induced ICAM–1 and Fibronectin Expression in Renal Cells via Inhibiting Locally–Produced Angiotensin II

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Purpose: A previous study has demonstrated that colchicine abrogated intercellular adhesion molecule (ICAM)-1 and fibronectin expression in renal cells exposed to high glucose media, but the underlying mechanism was not clarified. This study was undertaken to elucidate whether it was attributed to the inhibitory effect of colchicine on locally-produced angiotensin II (AII) under diabetic conditions.

Methods: Rat mesangial cells and NRK-52E cells were cultured in media containing 5.6 mM glucose (NG), NG+10^{-7} M All (NG+AII), or 30 mM glucose (HG) with or without 10^{-8} M colchicine (Col) and/or 10^{-6} M L-158,809, an All type 1 receptor blocker (ARB). ICAM-1 and fibronectin mRNA and protein expressions were determined by real-time PCR (RT-PCR) and Western blot, respectively. All levels in conditioned media were determined by ELISA.

Results: AII levels in conditioned media were significantly higher in HG-stimulated mesangial cells and NRK-52E cells compared to NG cells (p<0.05). ICAM-1 and fibronectin mRNA and protein expression were significantly increased in renal cells exposed to HG media (p<0.05 or p<0.01), and these increases were significantly ameliorated by colchicine or ARB treatment (p<0.05). Colchicine and ARB also significantly attenuated All-induced ICAM-1 and fibronectin expression (p<0.05). However, there was no additive inhibitory effect of colchicine and ARB on the increases in ICAM-1 and fibronectin expression.

Conclusion: Colchicine abrogated increased ICAM-1 and fibronectin expression in renal cells under diabetic conditions, which is partly mediated by inhibiting HG-induced locally-produced All. These findings provide a new renoprotective mechanism of colchicine in diabetic nephropathy in addition to its impact on leukocyte functions.

Key Words: Colchicine, Diabetic nephropathy, Renin-angiotensin system, ICAM-1, Fibronectin

INTRODUCTION

Diabetic nephropathy is generally considered a non–immune renal disorder; however, the infiltration of inflammatory cells within glomeruli and tubulointerstitium can be commonly observed in both human diabetic patients and animal models of diabetes1–30. Monocytes/
macrophages are the principle inflammatory cells found in the diabetic kidney and accumulating evidence has suggested that monocytes/macrophages play a critical role in the development and progression of glomerular and tubulointerstitial lesions in diabetic nephropathy. Even though strict blood glucose control and tight blood pressure control and the use of renin-angiotensin system (RAS) blockers have been the gold standard for the management of diabetic patients, the administration of anti-inflammatory agents has been recently shown to reduce inflammatory cell infiltration and to prevent renal injury in experimental diabetic animals. Irradiation also had a beneficial effect on diabetic nephropathy via an anti-inflammatory mechanism. These findings suggest that an inflammatory process may also contribute to the pathogenesis of diabetic nephropathy and that drugs possessing anti-inflammatory action can be used for preventing nephropathy in diabetic patients.

Colchicine is an ‘old’ drug commonly used to relieve pain in acute gout and is known to inhibit the function and motility of granulocytes and other motile cells. In addition, colchicine was demonstrated to prevent experimental pulmonary and hepatic fibrosis. Moreover, recent studies showed that colchicine prevented renal injury in an animal model of chronic cyclosporine nephrotoxicity and in experimental diabetic rats via its anti-inflammatory action. Furthermore, we previously found that colchicine abrogated intercellular adhesion molecule (ICAM)-1 and fibronectin expression in renal cells exposed to high glucose medium. However, the underlying mechanism how colchicine ameliorates increased ICAM-1 and fibronectin expression under diabetic conditions was not clarified. Since a previous study suggested that colchicine may indirectly inhibit the action of AngII, we surmised that colchicine may inhibit locally-produced angiotensin II under diabetic conditions, resulting in reduced ICAM-1 and fibronectin expression. In this study, we investigated the effect of colchicine on the expression of ICAM-1 and fibronectin in high glucose– and angiotensin II (AngII)–stimulated mesangial cells and tubular epithelial cells. In addition, the effects of colchicine on high glucose–induced ICAM-1 and fibronectin expression were compared to those of L-158,809, an AngII type 1 receptor (AT1R) blocker (ARB).

**MATERIALS AND METHODS**

1. **Cell culture**

Primary culture of rat mesangial cells were done as previously described. Identification of mesangial cells was performed by their characteristic stellate appearance in culture and confirmed by immunofluorescent microscopy for the presence of actin, myosin, and Thy-1 antigen and the absence of factor VIII and cytokeratin (Synbiotics, San Diego, CA, USA). Mesangial cells and NRK-52E cells, immortalized rat tubular epithelial cells, were maintained in RPMI 1640 and DMEM medium, respectively, supplemented with 5% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, and 26 mM NaHCO₃, and were grown at 37°C in humidified 5% CO₂ in air. Subconfluent mesangial cells and NRK-52E cells were serum restricted for 24 hours, after which the medium was replaced by serum-free medium containing 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), NG+10⁻⁷ M AngII (NG+AngII), or 30 mM glucose (HG) with or without 10⁻⁸ M colchicine (Col) and/or 10⁻⁶ M L-158,809, an ARB. At 24 hours after the media change, cells were harvested and the conditioned culture media were collected.

2. **Measurement of AngII by ELISA**

AngII levels were determined in conditioned culture media using a commercial ELISA kit (Peninsula Laboratories, Belmont, CA, USA) by the avidin-streptavidin method, as previously described. Briefly, samples or standards were incubated with anti-AngII antibody and biotinylated AngII (B-AngII) in 96-well plates coated...
with Staphylococcus aureus Protein A. After incubation, the unbound B–AII was removed by washing, and the bound B–AII was determined by reaction of streptavidin–HRP in the wells using TMB (3,3’,5,5’-tetramethylbenzidine dihydrochloride) and H2O2 as a substrate. The reaction was terminated with 2 N HCl, and the color intensity in each well was read at 450 nm using an ELISA microtiter plate reader. The AII amount in each well was calculated from the standard curve and normalized with the total protein content, which had been previously determined by a modified Lowry method.

3. 125I–AII binding

The AII binding assays were performed as described by Becker and Harris18. Briefly, after the incubation of cells with 30 mM glucose for 24 hours, confluent monolayers of cells were washed twice with ice-cold phosphate-buffered saline (PBS) containing 0.1% albumin (PBS–A), further incubated in PBS–A supplemented with 125I–[Sar1, Ile8] AII (10–10–M) at 4°C for 4 hours, followed by three washes with the same ice-cold PBS–A. After solubilization in 0.5 N NaOH (1 mL), 900 μL of each sample were transferred into a scintillation tube and counted in a γ counter. Specific binding was determined by the equation (total binding – binding in the presence of 1 μM unlabeled AII). The radioactive counts in each sample normalized with the total protein content, which was previously determined by a modified Lowry method.

4. Total RNA extraction

Total RNA from renal cortical tissue was extracted as previously described10. Briefly, 100 μL of RNA STAT–60 reagent (Tel–Test, Inc., Friendswood, TX, USA) was added to the renal cortical tissues lysed by the procedure of freezing and thawing repeated three times. Another 700 μL of RNA STAT–60 reagent was then added and the mixture was vortexed and stored for 5 minutes at room temperature. Next, 160 μL of chloroform was added and the mixture was shaken vigorously for 30 seconds. After 3 minutes, the mixture was centrifuged at 12,000 × g for 15 minutes at 4°C and the upper aqueous phase containing the extracted RNA was transferred to a new tube. RNA was precipitated from the aqueous phase by adding 400 μL of isopropanol and then pelleted by centrifugation at 12,000 × g for 30 minutes at 4°C. The RNA precipitate was washed with 70% ice-cold ethanol, dried using a Speed Vac, and dissolved in DEPC-treated distilled water. RNA yield and quality were assessed based on spectrophotometric measurements at wavelengths of 260 and 280 nm. Total RNA from mesangial cells and NRK–52E cells was extracted in a similar way.

5. Reverse transcription

First strand cDNA was made by using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany). Two μg of total RNA extracted from renal cortex and cultured cells were reverse transcribed using 10 μM random hexanucleotide primer, 1 mM dNTP, 8 mM MgCl2, 30 mM KCl, 50 mM Tris–HCl, pH 8.5, 0.2 mM dithiothreitol, 25 U RNase inhibitor, and 40 U AMV reverse transcriptase. The mixture was incubated at 30°C for 10 minutes and 42°C for 1 hour followed by inactivation of the enzyme at 99°C for 5 minutes.

6. Real-time polymerase chain reaction (Real-time PCR)

The primers used for MCP–1, ICAM–1, fibronectin, and 18s amplification were as follows: ICAM–1 sense 5’–AGGTA TCCATCCATCCAC–3’, antisense 5’–GCCGAGGTTCTCGTCTTC–3’; fibronectin sense 5’–TGACAACGTCCGTAGACCTGG–3’, antisense 5’–TAC TGTTGTAGTTGGCCG–3’; and 18s sense 5’–AGTCCCTGCCCTTTGT ACACA–3’, antisense 5’–GATCGAGGGCCTCATAAAC–3’. cDNAs from 25
ng RNA of renal cortical tissue or cultured cells per reaction tube were used for amplification.

Using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), PCR was performed with a total volume of 20 μL in each well, containing 10 μL of SYBR Green® PCR Master Mix (Applied Biosystems), 5 μL of cDNA, and 5 pM sense and antisense primers. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. Each sample was run in triplicate in separate tubes to permit quantification of the gene normalized to the 18s rRNA. The PCR conditions were as follows: 35 cycles of denaturation at 94.5°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 minute. Initial heating at 95°C for 9 minutes and final extension at 72°C for 7 minutes were performed for all PCRs.

After real-time PCR, the temperature was increased from 60 to 95°C at a rate of 2°C/min to construct a melting curve. A control without cDNA was run in parallel with each assay. The cDNA content of each specimen was determined using a comparative CT method with 2^(-ΔΔCT). The results are given as relative expression of ICAM–1 and fibronectin normalized to the expression of the 18s housekeeping gene.

7. Western blot analysis

Renal cortical tissue and cultured cells harvested from plates were lysed in sodium dodecyl sulfate (SDS) sample buffer (2% sodium dodecyl sulfate, 10 mM Tris–HCl, pH 6.8, 10% [vol/vol] glycerol), treated with Laemmli sample buffer, heated at 100°C for 5 minutes, and electrophoresed in an 8% 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 × PBS, 0.1% Tween–20, and 8% nonfat milk) for 1 hour at room temperature, followed by an overnight incubation at 4°C in a 1:1,000 dilution of polyclonal antibodies to rat ICAM–1 (R&D systems, Minneapolis, MN, USA), fibronectin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or β–actin (Sigma, St. Louis, MO, USA). The membrane was then washed once for 15 minutes and twice for 5 minutes in 1 × PBS with 0.1% Tween–20. Next, the membrane was incubated in buffer A containing a 1:1,000 dilution of horseradish peroxidase–linked donkey anti–goat IgG (Amersham Life Science, Inc., Arlington Heights, IL, USA). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc.). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany).

8. 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. 11.0 (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.

RESULTS

1. Effect of colchicine on All concentrations

All levels in conditioned media were significantly higher in HG–stimulated mesangial cells (7.21±1.12 pg/μL) and NRK–52E cells (3.11±0.53 pg/μL) compared to NG cells (4.38±0.64 pg/μL, 1.45±0.23 pg/μL, respectively) (N=5) (p<0.05). However, the increases in All concentrations in HG cells were not significantly changed by colchicine (mesangial cells, 7.10±1.15 pg/μL; NRK–52E cells, 3.21±0.45 pg/μL) (N=5). On the other hand, mannitol had no effect on All concentra-
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tions in conditioned culture media.

2. Effect of colchicine on $^{125}$I-AII binding

$^{125}$I-AII–specific binding was significantly reduced in HG–treated mesangial cells and NRK–52E cells compared to NG cells, but colchicine had no significant effect on $^{125}$I–AII–specific binding in cells exposed to NG or HG medium (Fig. 1).

3. Effect of colchicine on high glucose- and AII–induced ICAM-1 expression

ICAM-1 mRNA expression assessed by real–time PCR was significantly induced in HG–stimulated mesangial cells (p<0.01) and NRK–52E cells (p<0.05). AII also significantly increased ICAM-1 mRNA expression in cultured mesangial cells (p<0.01) and NRK–52E cells (p<0.01) compared to NG cells. The ICAM–1 mRNA/18s rRNA ratios were 2.1– and 2.0–folds higher in HG–stimulated mesangial cells and tubular epithelial cells, respectively, and 2.2– and 2.3–folds higher in AII–stimulated mesangial cells and tubular epithelial cells, respectively. These increases in ICAM–1 mRNA expression in HG– and AII–stimulated cells were significantly abrogated by the administration of colchicine or L–158,809 (Fig. 2). The protein expression of ICAM–1 showed a si-

Fig. 1. $^{125}$I–AII–specific binding in mesangial cells (A) and NRK–52E cells (B) cultured in 5.6 mM glucose (NG), NG+10$^{-8}$ M colchicine (NG+Col), NG+24.4 mM mannitol (NG+M), 30 mM glucose (HG), or HG+10$^{-8}$ M colchicine (HG+Col) (N=5). $^{125}$I–AII–specific binding was significantly decreased in HG–stimulated mesangial cells and tubular epithelial cells compared to NG cells. However, colchicine had no significant effect on $^{125}$I–AII–specific binding in cells exposed to NG or HG medium. *p<0.05 vs. NG cells.

Fig. 2. ICAM–1 mRNA/18s rRNA ratios in mesangial cells (A) and NRK–52E cells (B) cultured in 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), 30 mM glucose (HG), or HG+10$^{-7}$ M AII (HG+AII) with or without 10$^{-8}$ M colchicine (Col) and/or 10$^{-6}$ M L–158,809 (ARB) (N=6). The ICAM–1 mRNA/18s rRNA ratios were 2.1– and 2.2–folds higher in HG– and AII–stimulated mesangial cells, respectively, and 2.0– and 2.3–folds higher in HG– and AII–treated tubular epithelial cells, respectively. These increases in ICAM–1 mRNA expression in HG– and AII–stimulated renal cells were significantly abrogated by the administration of Col or ARB. However, there was no additive inhibitory effect of Col and ARB on the increase in ICAM–1 mRNA expression in cells exposed to HG or AII. *p<0.01 vs. NG cells, †p<0.05 vs. HG– and AII–treated cells.
milar pattern to its mRNA expression (Fig. 3). On the other hand, there was no additive or synergistic effect of colchicine and L-158,809 on HG- and AII-induced ICAM-1 expression.

4. Effect of colchicine on high glucose- and AII-induced fibronectin expression

The fibronectin mRNA/18s rRNA ratios were significantly increased in HG-stimulated mesangial cells (p<0.01) and NRK-52E cells (p<0.05) relative to NG cells by 115.5% and 82.4%, respectively. In addition, there were significant increases in fibronectin mRNA expression in AII-stimulated mesangial cells (p<0.01) and tubular epithelial cells (p<0.01). These increases in fibronectin mRNA expression in HG- and AII-stimulated cells were significantly ameliorated with colchicine or L-158,809 treatment (Fig. 4). The expression of fibronectin protein was also significantly induced in HG- and AII-stimulated cells, and colchicine and L-158,809 significantly attenuated these increases in fibronectin protein expression (Fig. 5). On the other hand, there was no additive or synergistic effect of colchicine and L-158,809 on HG- and AII-induced fibronectin expression

DISCUSSION

We previously found that colchicine prevented renal injury in experimental diabetic nephropathy via inhibiting ICAM-1 and fibronectin expression\(^5\), but the underlying mechanism how it abrogated increased ICAM-1 and fibronectin expression under diabetic conditions has not clarified yet. In this study, it is demonstrated for the first time that colchicine ameliorates AII-induced ICAM-1 and fibronectin expression in cultured renal cells without any significant influence on binding of AII to its receptor, suggesting that the
The beneficial effect of colchicine may be mediated by inhibiting increased locally-produced AII under diabetic conditions.

Even though the diabetic milieu per se, hemodynamic changes, and local growth factors such as AII are considered mediators in the pathogenesis of diabetic nephropathy, the underlying pathways mediating these processes are still under investigation. Among these, numerous previous clinical and experimental studies on diabetic nephropathy have demonstrated that RAS blockades reduced proteinuria and the progression of renal lesions, which cannot be explained merely by their antihypertensive effect. These findings suggest that RAS inhibition may have direct effects on various renal cells. Indeed, mounting evidence has shown that the local RAS exists in various renal cells and is activated under diabetic conditions.

All RAS components were revealed to be present in proximal tubular cells, mesangial cells, and podocytes, and the expression of angiotensinogen, the substrate for AII, was increased in these cells under diabetic conditions. In addition, high glucose activated the local RAS leading to an increase in AII levels, known to induce ICAM-1, MCP-1, and fibronectin expression in mesangial cells and renal tubular cells. Taken together, activated local RAS under diabetic conditions seems to play an important role in the pathogenesis of diabetic nephropathy via facilitating inflammation and extracellular matrix (ECM) synthesis. In the present study, we also confirmed that AII levels were significantly higher in high glucose-conditioned media compared to NG media and that ARB significantly inhibited increased ICAM-1 and fibronectin expression in cultured mesangial cells and renal tubular cells. Colchicine is an alkaloid drug that has been used for many decades in acute gouty arthritis. Colchicine binds to tubulin molecules and inhibits their polymerization into microtubules, resulting in disruption of the mitotic spindles. Due to this basic property, colchicine is mainly considered to be an anti-mitotic drug. However, accumulating evidence has shown its additional effects on leukocytes and fibroblasts. Colchicine is known to alter leukocyte functions, such as chemotaxis, adhesion, and cytokine production. In addition, colchicine inhibits the release of fibronectin and collagen to the extracellular space, reduces collagen-
processing enzyme, stimulates tissue collagenase activity, and inhibits the proliferation of fibroblasts. Based on these anti-inflammatory and anti-fibrotic effects of colchicine, it has been used effectively to treat various diseases such as familial Mediterranean fever, primary biliary cirrhosis, and Behcet’s syndrome.

The beneficial effects of colchicine have also been demonstrated in several experimental kidney disease models, including severe crescentic glomerulonephritis, chronic cyclosporine nephrotoxicity, and diabetic nephropathy via anti-inflammatory, anti-fibrotic, and anti-apoptotic mechanisms. In addition, colchicine was revealed to inhibit the increases in ICAM-1 and fibronectin expression under diabetic conditions both in vivo and in vitro. In that study, however, the underlying mechanism how colchicine abrogated the increases in ICAM-1 and fibronectin expression under diabetic conditions was not elucidated, leaving us with another question: how does colchicine inhibit the up-regulation of these genes expression under diabetic conditions? Zhou et al demonstrated that AII-induced nuclear factor-κB in renal tubular cells was significantly attenuated by coadministration of losartan, a selective ARB, or colchicine, a selective cytoskeleton microtubule inhibitor known to block receptor-mediated endocytosis. Moreover, another recent study using the same cell line showed that intracellular accumulation of AII in response to extracellular AII, which was mediated by AT1R, was significantly ameliorated by not only losartan but also colchicine. These findings suggest extracellular AII may play a functional role through internalization via AT1R-mediated endocytosis and that colchicine may indirectly inhibit the action of AII. Taken together, we proposed that the inhibitory effect of colchicine on the expression of these genes may be attributed to the suppressed action of locally-produced AII under high glucose conditions.
in renal cells. This hypothesis is supported by our findings that AII concentrations are increased in high glucose–conditioned culture media and that colchicine attenuates high glucose– and AII–induced ICAM–1 and fibronectin expression. Furthermore, the results of this study showing that $^{125}$I–AII–specific binding was not changed by colchicine, indicating no significant direct effect of colchicine on the binding of AII to AT1R, and that there was no additive inhibitory effect of colchicine and ARB on ICAM–1 and fibronectin expression infer that colchicine and ARB may partly act on a common pathway under diabetic conditions.

In summary, colchicine abrogates increased ICAM–1 and fibronectin expression in renal cells under diabetic conditions, partly mediated by inhibiting high glucose–induced locally–produced AII. These findings provide a new renoprotective mechanism of colchicine in diabetic nephropathy in addition to its impact on leukocyte functions.

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