Valsartan attenuated oxidative stress, decreased MCP-1 and TGF-β₁ expression in glomerular mesangial and epithelial cells induced by high-glucose levels

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Summary

Our previous studies revealed that valsartan, an angiotensin II type I receptor blocker, exhibited renoprotective effects through decreasing urine protein excretion levels due to improving glomerular permeability in rats with diabetic nephropathy (DN). In this study, we sought to investigate the underlying mechanisms in perspectives of oxidative stress, transforming growth factor beta-1 (TGF-β₁) and monocyte chemoattractant protein-1 (MCP-1) expressions in glomerular mesangial cells (GMCs) and glomerular epithelial cells (GECs) since their roles are well-established in the development and progression of DN. High-glucose levels significantly increased oxidative stress in GMCs and GECs, as evidenced by enhanced generation of reactive reactive oxygen species (ROS), reduced levels of glutathione (GSH) and antioxidant enzyme superoxide dismutase (SOD), and increased production of malondialdehyde (MDA). Treatment with valsartan significantly restored the levels of those oxidative stress relevant molecules. Furthermore, valsartan obviously diminished the expression of proinflammatory cytokine MCP-1 in GMCs and GECs induced by high-glucose levels both at mRNA and protein levels, as determined by real-time PCR, immunocytochemistry, western blotting, and ELISA. In addition, the increased expressions of TGF-β₁ mRNA and protein induced by high-glucose level were also abrogated by valsartan treatment in GMCs, as evaluated by real-time PCR and ELISA. These results suggest that the renoprotective effects of valsartan may be related to its potential in decreasing oxidative stress and the expressions of MCP-1 and TGF-β₁ in GMCs and GECs.

Keywords: Diabetic nephropathy, valsartan, glomerular mesangial cells, glomerular epithelial cells, oxidative stress, MCP-1, TGF-β₁

1. Introduction

Diabetic nephropathy (DN) is the most common complication of diabetes mellitus, often leading to end-stage kidney disease and a high risk of mortality (1,2). It is characterized clinically by progressively increasing albuminuria and histopathologically by glomerular basement membrane (GBM) thickening and mesangial expansion due to accumulation of extracellular matrix (ECM) proteins (3). Functional changes in diabetic glomeruli, particularly in glomerular mesangial cells (GMCs) and glomerular epithelial cells (GECs) were demonstrated to exert critical roles in the development and progression of DN (4,5). On the one side, an enhancement of the production of ECM has been shown in GMCs under high-glucose conditions (6). On the other side, the damage of GECs which function as a fine filter contributing ultimate size-selectivity, permitting permeability to molecules smaller than albumin in the normal physiological state, leads to retraction of their foot processes and proteinuria (7). Thus, GMCs and GECs have been the focus in the field of research on DN.
Previous studies indicated that transforming growth factor beta (TGF-β), oxidative stress, and proinflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP-1), play important roles in progressive DN (8-11). The knowledge and control of these different mechanisms have become a fascinating therapeutic challenge, aimed to reduce the progression of DN. Although no curable therapy is yet available, an increasing number of reports indicate that blockade of the renin angiotensin system (RAS) is effective to delay the progression of DN and thereby to protect against end-stage renal failure. Treatment with angiotensin II (Ang II) type 1 receptor (AT1R) blockers (ARB), e.g. valsartan, by the current authors and other researchers, has been shown to have protective effects against the progression of DN through improving glomerular permeability and thus decreasing urine protein excretion levels (12,13). However, besides their well-documented efficiency, the underlying mechanisms remain to be elucidated.

In the present study, we first studied the effects of valsartan against oxidative stress in GMCs and GECs cultured in high-glucose conditions by evaluating levels of reactive oxygen species (ROS), glutathione (GSH), antioxidant enzyme superoxide dismutase (SOD), and malondialdehyde (MDA). Next, the expression of proinflammatory cytokine MCP-1 was measured in GMCs and GECs treated with or without valsartan. Additionally, the change of expression levels of TGF-β1 was determined in GMCs after treatment with valsartan.

2. Materials and Methods

2.1. Chemicals

Valsartan was purchased from Changzhou Kony Pharm Co., Ltd. (Changzhou, Jiangsu, China). Valsartan was dissolved in dimethyl sulfoxide (DMSO) before use. The final concentration of DMSO in the cell culture media is ≤ 3% (v/v).

2.2. Cell culture

Rat GMCs (HBZY-1) were purchased from Chinese Center for Typical Culture Collection (Wuhan, Hubei, China). GMCs were maintained in normal-glucose (5.6 mmol/L d-glucose) RPMI-1640 media supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) at 37°C in a humid atmosphere (5% CO2-95% air). To induce proliferation, cells were cultured on type I collagen-coated dishes in normal-glucose RPMI-1640 medium supplemented with 10% FCS and 10 U/mL murine interferon-γ (IFN-γ) at 33°C in a humid atmosphere (5% CO2-95% air). To induce differentiation, cells were maintained in normal-glucose RPMI-1640 with 10% FCS but without IFN-γ at 37°C for a period of two weeks without cell passage. Cells were identified as differentiated GECs by their arborized morphology and the presence of high levels of synaptopodin determined by an immunofluorescence assay (data not shown). To mimic the diabetic state, GMCs and differentiated GECs were pretreated in high-glucose (30 mmol/L d-glucose) RPMI-1640 medium with 10% FCS before the experiments.

For experiments, GMCs and GECs were distributed into three groups, respectively, and each group included two parallel samples: i) Normal group: cells were cultured in normal-glucose medium during the entire study; ii) Model group: cells pretreated with high-glucose medium were cultured in high-glucose medium without valsartan; iii) Valsartan group: cells pretreated with high-glucose medium were cultured in high-glucose medium with addition of 10^-6 molar/L valsartan. Each experiment was repeated four times.

2.3. Determination of ROS

ROS production was assessed using the fluorescent probe 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (DCDFH-DA) (Molecular Probes, Eugene, OR) (15). GMCs and GECs (1 × 10^4 per well) were seeded in 24-well plates, respectively. After cells were allowed to attach, the specified concentration of valsartan was added to the wells and incubated for 48 h. Cells were then rinsed twice with phosphate-buffered saline (PBS) and replaced with phenol red free RPMI-1640 containing 20 mM DCDFH-DA. After 60 min incubation, the fluorescence intensity was measured with a fluorescence microplate reader CytoFluor 2350 (Millipore, Bedford, MA, USA) with excitation and emission wavelengths of 502 and 530 nm, respectively.

2.4. Determination of SOD, GSH, and MDA levels

GMCs and GECs (1 × 10^4 per well) were seeded in 24-well plates, respectively. After cells were allowed to attach, the specified concentration of valsartan was added to the wells and incubated for 48 h. Then the cell culture supernatant was collected for determination of SOD, GSH, and MDA levels.

Total GSH content was determined spectrophotometrically using the method described by Akerboom and Sies (16). The assay mixture in 1 mL contained 200 μL cell culture supernatant, 730 μL 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 50 μL 0.5% NaHCO3, containing 4 mg/mL NADPH, and 20 μL 0.5% NaHCO3, containing 1.5 mg/mL 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich, USA). The reaction was started by adding 6
units of GSH reductase and lasted for 1 min at 25°C. Then the absorbance was measured at 412 nM using a Multilabel Plate Counter VICTOR31420 (Perkin-Elmer, Waltham, Massachusetts, USA).

SOD activity was determined using a commercially available SOD kit (RANSOD SD125, Randox, Antrim, UK). The method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical (produced in the incubation medium from the xanthine-xanthine oxidase reaction system), which is assayed spectrophotometrically at 505 nM using a Multilabel Plate Counter VICTOR31420 (Perkin-Elmer, Waltham, Massachusetts, USA). The inhibition of the produced chromogen is proportional to the activity of SOD present in the sample. Analysis was performed according to the manufacturer's recommended protocol.

The quantification of lipid peroxidation was estimated by determining malondialdehyde (MDA) reacting to thiobarbituric acid (TBA)-reactive substance following the method described by Ohkawa (17). Briefly, an aliquot of 200 μL of cell culture supernatant was mixed thoroughly with an aqueous solution of TBA and heated at 95°C for 30 min in a water bath. The suspension was then cooled to room temperature, centrifuged at 4,000 r/min for 10 min, and the pink colored supernatant was measured spectrophotometrically at 532 nM. Absorbance was determined using a Multilabel Plate Counter VICTOR31420 (Perkin-Elmer, Waltham, Massachusetts, USA). MDA concentration was calculated using the absorbance coefficient of MDA-TBA complex (absorbance coefficient = 1.56 × 10⁵ M⁻¹ cm⁻¹).

2.5. Real-time PCR

GMCs and GECs (5 × 10⁵ per well) were seeded in 6-well plates, respectively. After cells were allowed to attach, the specified concentration of valsartan was added to the wells and incubated for 48 h. Cells were then collected and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Two μg of total RNA was reverse-transcribed into cDNA by Super Script III first strand cDNA synthesis Kit (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The SYBR Green PCR Master Mix kits (Applied Biosystems, Foster City, CA, USA) were used according to the supplier's instructions for quantification of gene expression. Primer pairs used are as follows: for MCP-1, F: 5-GATCTCAGTGCAGAGGGCTCG-3 and R: 5-TGGTTGAGGCCTCCATCG-3; for GADPH, F: 5-TGCTTGTCCAGGTGGTCCAT-3; for GAPDH, F: 5-GGCTGTATTCCCCTCCCATCG-3 and R: 5-CAGTTGGTAACATGCCCATGT-3; for GADPH, F: 5-TGCCCTCAAGGTTGTCAGAA-3 and R: 5-AGATCCACAACGGATACATT-3. Thermal cycling conditions were as follows: 95°C for 2 min; 40 cycles of 10 s denaturation at 94°C, 10 s annealing at 54°C, and 20 s extension at 72°C; and 1 cycle of 5 min at 72°C. The calculation of the relative expression level of MCP-1 was conducted based on the cycle threshold (Ct) method. The relative mRNA levels of MCP-1 and TGF-β, were expressed as ratios compared with GAPDH and β-actin mRNA levels, respectively.

2.6. Immunocytochemistry

GMCs (5 × 10⁵ per well) were seeded in 6-well plates in which sterilized coverslips were pro-positioned. After cells were allowed to attach, the specified concentration of valsartan was added to the wells and incubated for 48 h. Coverslips were then taken out and washed twice with PBS. GMCs were fixed with 4% paraformaldehyde for 1 h at 4°C. After incubation with anti-MCP-1 (Boster, Wuhan, China) at 4°C, the cells were washed and treated with biotinylated anti-immunoglobulin, washed, reacted with avidin-conjugated horseradish peroxidase H complex, and incubated in diaminobenzidine and hydrogen peroxide. Cells were then rinsed in distilled water and counterstained with hematoxylin. Images were captured and the average grey scale was quantified by means of a computer-assisted image analyzer, Image Pro Plus 5.1 (Media Cybernetics, Inc., Bethesda, MD, USA).

2.7. ELISA

Cells (5 × 10⁵ per well) pretreated in high-glucose medium were seeded in 6-well plates. After cells were allowed to attach, the specified concentration of valsartan was added to the wells and incubated for 48 h. MCP-1 protein level in the culture supernatant was determined using commercially available ELISA MCP-1 and TGF-β₁ kits (Bionewtrans Pharmaceutical Biotechnology Co., Ltd., USA) according to the manufacturer's protocols. MCP-1 and TGF-β₁ protein levels were determined by comparing the samples to the standard curve generated by the kit.

2.8. Western blot

Western blotting was used to evaluate the expressions of MCP-1 in GECs. GECs (5 × 10⁵ per well) were seeded in 6-well plates. After cells were allowed to attach, the specified concentration of valsartan was added to the wells and incubated for 48 h. The cells were harvested and cell lysates (30 μg of protein per lane) were fractionated using 10% SDS-PAGE. Proteins were electro-transferred onto nitrocellulose
membranes and then the level of MCP-1 expression was detected using a rabbit polyclonal antibody to MCP-1 (Santa Cruz, USA). Blots were washed in 0.05% Tween-20/PBS and then incubated with horseradish peroxidase-conjugated secondary antibody. β-actin protein level served as a protein loading control. The bound antibodies were visualized using an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, USA) followed by exposure to X-ray film. The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany).

2.9. Statistical analysis

Data are expressed as mean ± S.D. One-way ANOVA followed by Dunnett's test was performed using SPSS/Win11.0 software (SPSS, Inc., Chicago, Illinois, USA); p < 0.05 was indicative of a significant difference.

3. Results

3.1. Valsartan decreased oxidative stress induced by high-glucose level

3.1.1. Valsartan decreased high-glucose level induced ROS production

Levels of ROS produced in GMCs and GECs were estimated using the fluorescent probe CDCFH-DA. Exposure of cultured GMCs to high-glucose conditions induced a significant increase in fluorescence intensity compared with exposure to normal-glucose conditions (p < 0.01, Figure 1A), suggesting a stimulatory effect of high glucose levels on free radical production. The fluorescence signal produced by GMCs cultured in high-glucose conditions was 1.60-fold of that produced by cells in normal-glucose conditions. After incubation of GMCs with valsartan at a concentration of 10⁻⁶ mol/L for 48 h, the fluorescence intensity was significantly decreased in GMCs (1.40-fold of that in normal group; p < 0.05), indicating that the ROS levels were significantly reduced.

Similar results were obtained in cultured GECs (Figure 1B). However, effects of valsartan in deceasing ROS levels in GECs were more potent than that in GMCs at the same concentration. An extremely significant difference in fluorescence intensity was showed between the model group (1.40-fold of that in normal group) and the valsartan group (1.20-fold of that in normal group) (p < 0.01).

3.1.2. Valsartan increased high-glucose level induced GSH reduction

GSH content in cultured supernatant of GMCs (Figure 2A) and GECs (Figure 2B) was measured by the enzymatic recycling method in which GSH was oxidized by DTNB and reduced by NADPH. In normal-glucose conditions, GSH concentrations in GMCs and GECs were determined at 1.67 and 1.07 nmol/L, respectively. They were significantly decreased to 0.82 and 0.42 nmol/L in GMCs and GECs cultured in high-glucose conditions, respectively (p < 0.01). After incubation of GMCs and GECs with valsartan at a concentration of 10⁻⁶ mol/L for 48h, GSH content was obviously increased to 1.12 nmol/L and 0.61nmol/L (p < 0.01 in GMCs, p < 0.05 in GECs).
3.1.3. Valsartan increased high-glucose level induced lessened SOD activity

SOD activity was evaluated by measuring the production of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical generated with the xanthine-xanthine oxidase reaction system. In normal-glucose conditions, SOD activities in GMCs (Figure 3A) and GECs (Figure 3B) were determined at 34.1 and 46.2 U/mL, respectively. SOD activities were significantly decreased to 23.5 and 35.4 U/mL in GMCs and GECs cultured in high-glucose conditions, respectively ($p < 0.01$). Incubation of GMCs and GECs with valsartan at a concentration of $10^{-6}$ mol/L for 48 h, SOD activities were obviously increased to 28.2 and 39.3 U/mL, respectively ($p < 0.01$ in GMCs, $p < 0.05$ in GECs).

3.1.4. Valsartan improved high-glucose level induced oxidative damage

MDA is produced by the hydrolysis of lipid hydroperoxides, which reacts with TBA to produce a complex that absorbs at 532 nM. In normal-glucose conditions, MDA levels in GMCs (Figure 4A) and GECs (Figure 4B) were determined at 2.32 and 2.10 mmol/L, respectively. They were significantly increased to 5.48 and 4.88 mmol/L in GMCs and GECs cultured in high-glucose conditions, respectively ($p < 0.01$). After incubation of GMCs and GECs with valsartan at a concentration of $10^{-6}$ mol/L for 48 h, MDA content was obviously decreased to 3.82 and 3.02 mmol/L, respectively ($p < 0.01$).

3.2. Valsartan diminished high-glucose induced MCP-1 expression

Since MCP-1 is a well-known proinflammatory cytokine and its expression was reported to be increased under diabetic conditions [11], we attempted to elucidate whether valsartan is able to decrease high-glucose levels induced by MCP-1 expression in GMCs and GECs both at the protein and mRNA levels.
MCP-1 protein expressed by GMCs was evaluated using an immunocytochemistry method (Figure 5A). Exposure of cultured GMCs to high-glucose levels induced a significant increase of MCP-1 signal compared with exposure to normal-glucose levels \((p < 0.01; \text{Figure 5B})\). Incubation of valsartan for 48 h with GMCs cultured in high-glucose condition resulted in a significant decrease of MCP-1 signal \((p < 0.05; \text{Figure 5B})\). The mRNA levels of MCP-1 in GMCs were determined using a real-time PCR method. As demonstrated in Figure 5C, the ratio of mRNA levels of MCP-1 and GAPDH was determined to be 0.47 in normal-glucose cultured cells and this value was dramatically augmented to 1.63 in high-glucose cultured cells \((p < 0.01)\). Valsartan significantly diminished MCP-1 mRNA expression induced by high-glucose levels, with a ratio of 1.14 \((p < 0.01)\).

In GECs, MCP-1 mRNA and protein expressions were evaluated using real-time PCR, ELISA and Western blotting methods. As shown in Figure 6A, the ratio of mRNA levels of MCP-1 and GAPDH was increased from 0.91 in the normal group to 4.85 in the model group \((p < 0.01)\). This value was remarkably decreased to 3.52 by addition of valsartan \((p < 0.05)\). In accordance with mRNA expression, similar profiles of extracellular (Figure 6B) and intracellular (Figures 6C and 6D) MCP-1 protein expression were found in GECs. The extracellular MCP-1 protein concentration was significantly increased from 220 ng/μL in the normal group to 484 ng/μL in the model group \((p < 0.01)\). MCP-1 concentration was obviously decreased in the valsartan treatment group with a value 385 ng/μL \((p < 0.05)\) (Figure 7B).

3.3. Valsartan diminished high glucose-induced TGF-β₁ expression

TGF-β₁ mRNA and protein expression in GMCs were determined using real-time PCR and ELISA methods. As shown in Figure 7A, high-glucose levels induced a significant increase in the ratio of mRNA levels of TGF-β₁ and β-actin, from 0.38 to 0.92 compared with the normal-glucose level \((p < 0.01)\). This ratio was significantly decreased to 0.80 after 48 h valsartan treatment \((p < 0.05)\). In accordance with the mRNA level, the extracellular TGF-β₁ protein concentration was significantly increased from 54.9 pg/mL in the normal group compared to 88.2 pg/mL in the model group \((p < 0.01)\), and decreased to 71.8 pg/mL due to valsartan treatment \((p < 0.05)\) (Figure 7B).

Figure 5. Effects of high-glucose levels and valsartan treatment on MCP-1 protein (A, B) and mRNA (C) expressions in GMCs. Abbreviations: N, normal group; M, model group; V, valsartan group. *, \(p < 0.05\); **, \(p < 0.01\); ###, \(p < 0.01\).
4. Discussion

In the present study, we investigated the mechanisms underlying the renoprotective effects of valsartan in GMCs and GECs in vitro. Valsartan significantly reduced the production of ROS, increased levels of antioxidant agent GSH and antioxidant enzyme SOD, thus decreasing cell oxidative damage in GMCs and GECs induced by high-glucose levels. Valsartan also obviously suppressed the expression of proinflammatory cytokine MCP-1 in GMCs and GECs both at the protein and mRNA levels, which were both dramatically increased when cells were cultured in high-glucose conditions. In addition, the increased TGF-β1 expression in GMCs induced by high-glucose levels was inhibited by valsartan treatment. These results suggest that the renoprotective effects of valsartan were possibly related with attenuating oxidative stress, decreasing the expression of MCP-1 and TGF-β1 in GMCs and GECs.

Among the factors that induce pathological injury of glomeruli in the background of the diabetic milieu, the role of oxidative stress is supported by the observations that antioxidants suppress high-glucose induced ECM protein synthesis in mesangial cells and prevent glomerular and renal hypertrophy, albuminuria, and glomerular expression of ECM in experimental diabetic animals (18-19). Previous studies indicated
that RAS contributes to increasing cell oxidative stress (20). Kidney cells such as GMCs and GECs are able to synthesize all of the components of RAS such as renin, the (pro)renin receptor, angiotensinogen, and Ang II receptors independently of the systemic RAS, thereby making the kidney capable of maintaining a high level of local Ang II (21). Hyperglycemia may activate the intrarenal RAS, leading to accumulation of Ang II and activation of AT1 receptor-mediated signaling pathway in the kidney (22-23). Portero-Otin and colleagues demonstrated the inhibition of RAS decreases renal protein oxidative damage in diabetic rats (24). These results are consistent with our results and may suggest that valsartan decreases oxidative stress and improves oxidative damage to GMCs and GECs cultured in high-glucose conditions through blocking AT1 receptor-mediated signal transduction.

Our results showed that high-glucose levels increased the production of ROS in GMCs and GECs. Studies indicated that ROS may act as integral signaling molecules in diabetic nephropathy and its activation of protein kinase C (PKC) and the subsequent mitogen-activated protein kinases (MAPKs) play a critical role in high-glucose induced renal injury (25-28). PKC activation increases the expression of TGF-β, which causes an increase in mesangial matrix deposition and GBM thickening and may promote GECs apoptosis or detachment (28-29). In addition, high-glucose could induce MCP-1 synthesis by a PKC-dependent pathway. Since MCP-1 is the strongest known chemotactic factor for monocytes, its over-production would result in increasing monocyte immigration and monocyte activity and exacerbating interstitial fibrosis, thus worsening renal function. In the current study, addition of valsartan decreased the production of ROS in GMCs and GECs induced by high-glucose levels. Thus, lessened activation of the PKC-MAPK pathway might contribute to down-regulation of TGF-β1 and MCP-1 expression in GMCs and GECs.

In conclusion, the current data demonstrated that valsartan efficiently decreased oxidative stress, TGF-β1 and MCP-1 expression in GMCs and GECs cultured in high-glucose conditions. These mechanisms might be related with the renoprotective effects of valsartan.

References


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