Renoprotective Effects of Sildenafil in DOCA-Salt Hypertensive Rats

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Key Words
DOCA-salt • Hypertension • Glomerulosclerosis • Sildenafil • Renoprotection

Abstract
Background/Aims: Sildenafil, the first selective phosphodiesterase-5 (PDE5) inhibitor to be widely used for treating erectile dysfunction, has been investigated with regard to its cardio- and renoprotective effects in animal models. This study further investigated the renoprotective effects of sildenafil and their molecular mechanisms in deoxycorticosterone acetate (DOCA)-salt hypertensive (DSH) rats. Methods: DOCA strips (200 mg/kg) were implanted in rats 1 week after unilateral nephrectomy. These rats were fed on a control diet, with or without sildenafil (50 mg·kg⁻¹·day⁻¹), for 2 weeks. Systolic blood pressure (SBP) was measured by the tail cuff method, and the urinary albumin-to-creatinine ratio (ACR) was calculated. The extent of glomerulosclerosis and tubulointerstitial fibrosis was determined by Masson's trichrome stain. Renal expression of ED-1, transforming growth factor-β1 (TGF-β1), Bax, and Bcl-2 were determined by semiquantitative immunoblotting, polymerase chain reaction (PCR), and immunohistochemistry. TUNEL staining was used for detecting apoptotic cells. Results: The increased SBP in DSH rats was not attenuated by sildenafil treatment. The decreased creatinine clearance and increased ACR in DSH rats, compared with control animals, were attenuated by sildenafil treatment. Further, sildenafil treatment attenuated glomerulosclerosis and tubulointerstitial fibrosis in DSH rats and counteracted the increased expression of ED-1, TGF-β1, and Bax and the decreased expression of Bcl-2 in the kidneys of these rats. The increase in the number of apoptotic cells in DSH rats was attenuated by sildenafil treatment. Conclusion: Sildenafil effectively prevented the progression of renal injury in DSH rats via its anti-inflammatory, antifibrotic, and antiapoptotic effects.

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Introduction

The incidence of chronic kidney disease is increasing worldwide, resulting in the need to identify therapies capable of arresting or reducing disease progression. The pathogenesis of chronic kidney diseases is complex and involves hemodynamic and inflammatory processes that lead to glomerulosclerosis and tubulointerstitial fibrosis, with subsequent progression [1]. The current treatment for chronic nephropathies is limited to angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, but growing clinical and experimental evidence indicates sildenafil might play a therapeutic role. Recent studies have shown that, in addition to treating erectile dysfunction, sildenafil may be effective in preventing or decreasing tissue injury. Early treatment with sildenafil has been shown to ameliorate the progression of renal damage in the 5/6 nephrectomy model [2], and the drug has also been reported to provide cardioprotection against ischemic injury when infused at the onset of perfusion in rabbits [3]. Although it is difficult to determine whether its effects are dependent on the blood pressure-lowering effect of the sildenafil, emerging evidence indicates that the renoprotection provided by sildenafil is attributable to its anti-apoptosis and anti-inflammatory properties [4, 5].

The deoxycorticosterone acetate (DOCA)-salt hypertensive (DSH) rat is an established model of mineralocorticoid hypertension with renal dysfunction. Although mineralocorticoid administration is traditionally known to promote sodium retention, recent evidence indicates that it also causes oxidative stress and stimulates inflammation and fibrosis by activating transcription factors such as nuclear factor κB (NF-κB) and activating protein-1 (AP-1) [6, 7]. Accordingly, glomerulosclerosis, tubular fibrosis, and cardiac hypertrophy and fibrosis are commonly observed in DSH rats, along with the activation of renal transforming-growth factor-β1 (TGF-β1) expression [8].

The present study investigated the morphological and cellular alterations that occur in the kidneys of DSH rats, and the effects that sildenafil has on resultant renal dysfunction and progressive kidney disease.

Materials and Methods

Animals

The animal study was approved by the Ethics Committee of Chonnam National University Medical School. Male Sprague-Dawley rats weighing 180 to 200 g were used. DSH was induced by subcutaneous implantation of silicone rubber containing DOCA (200mg/kg) on 1 week after unilateral nephrectomy. Physiologic saline was supplied as a drinking water to all animals. Two weeks after DOCA implantation, DOCA-salt rats were randomly divided to receive control diet with or without sildenafil (viagra®, 50 mg/kg/day) for 2 weeks. Systolic blood pressure (SBP) was measured by tail cuff method (Power lab, ADI instrument, CA, USA). The rats were maintained individually in the metabolic cages for last 3 days to allow urine collections for the measurement of creatinine and microalbumin, which were used to calculate the albumin-to-creatinine ratio (ACR).

The rats were killed for semiquantitative immunoblotting and immunohistochemical studies on 4 weeks after DOCA implantation. Rats were anesthetized with isoflurane. Blood was collected from the inferior vena cava and analyzed for creatinine. The right kidney was rapidly removed, and processed for immunoblotting described below. Another series of experiment was done and the right kidney was fixed by retrograde perfusion for immunohistochemical studies.

Another series of experiment was done for the assay of real-time polymerase chain reaction (PCR). The rats were decapitated under a conscious state, and their kidneys were taken and kept at -70°C until assayed for the mRNA expression by real time-PCR.

mRNA Expression of inflammatory markers

The mRNA expression of transforming growth factor-β (TGF-β), monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) was determined by real time-PCR. cDNA was made
by reverse transcribing 5 μg of total RNA using oligo (dT) priming and superscript reverse transcriptase II (Invitrogen, Carlsbad, CA, USA). cDNA was quantified using Smart Cycler II System (Cepheid, Sunnyvale, CA, USA) and SYBR Green was used for detection. PCR was done using Rotor-Gene™ 3000 Detector System (Corbett Research, Mortlake, New South Wales, Australia). Primers were prepared as described previously [9]. Data from the reaction were collected and analyzed with the Corbett Research Software [10].

**Semiquantitative immunoblotting**

The dissected whole kidney were homogenized in ice-cold isolation solution containing 0.3 M sucrose, 25 mM imidazole, 1 mM ethylenediamine tetracetic acid, 8.5 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, with pH 7.2. The homogenates were centrifuged at 1,000 g for 15 min at 4°C to remove whole cells, nuclei and mitochondria. The total protein concentration was measured (Pierce BCA protein assay reagent kit, Pierce, Rockford, IL). All samples were adjusted with isolation solution to reach the same final protein concentrations and solubilized at 65°C for 15 min in SDS-containing sample buffer and then stored at −20°C. To confirm equal loading of protein, an initial gel was stained with Coomassie blue. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 9 or 12% polyacrylamide gels. The proteins were transferred by gel electrophoresis (Bio-Rad Mini Protein II, Bio-Rad, Hercules, CA, USA) onto nitrocellulose membranes (Hybond ECL RPN3032D, Amersham Pharmacia Biotech, Little Chalfont, UK). The blots were subsequently blocked with 5% milk in PBST (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 hour and incubated overnight at 4°C with primary antibodies (TGF β1, fibronectin; Santa Cruz Biotechnology, ED-1; serotec, α-smooth muscle actin (α-SMA); Sigma Chemical, Bax, Bcl-2; Cell Signaling Technology), followed by incubation with secondary anti-rabbit (P448, DAKO, Glostrup, Denmark) or anti-mouse (P447, DAKO, Glostrup, Denmark) horseradish peroxidase-conjugated antibodies. The labeling was visualized by an enhanced chemiluminescence system.

**Immunohistochemistry**

A perfusion needle was inserted into the abdominal aorta and the vena cava was cut to establish an outlet. Blood was flushed from the kidney with cold phosphate-buffered saline (PBS; pH 7.4) for 15 sec before switching to cold 3% paraformaldehyde in PBS (pH 7.4) for 3 min. The kidney was removed and cut into 2- to 3-mm-thick transverse sections and immersion fixed for additionally 1 hour, followed by three 10-min washes in PBS. The tissue was dehydrated in graded ethanol and incubated in xylene overnight. After the tissue was embedded in paraffin, 2-µm-thick sections were cut with a rotary microtome. Immunoperoxidase labeling was performed as previously described [11].

**Pathologic Examinations**

The extent of glomerulosclerosis (GS) was graded from 0 to 4 by a semiquantitative score: 0, normal; 1, mesangial expansion/sclerosis involving<25% of the tuft; 2, moderate GS (25 to 50%); 3, severe GS (50 to 75%); and 4, diffuse GS involving >75% of the glomerular tuft. Glomerulosclerosis index (GSI) for each rat was calculated as a mean value of all glomerular scores obtained [12]. Tubulointerstitial lesion indexes were determined using a semiquantitative scoring system [12]. Ten fields per kidney were examined, and lesions were graded from 0 to 3 (0, no change; 1, changes affecting<25% of the section; 2, changes affecting 25 to 50% of the section; and 3, changes affecting 50 to 100% of the section), according to the area with tubulointerstitial lesions (tubular atrophy, casts, interstitial inflammation, and fibrosis). The score index in each rat was expressed as a mean value of all scores obtained.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay**

The ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD) was used. The sections were dewaxed and treated with protease K, then incubated with equilibration buffer for 10 min, followed by incubation with working-strength TdT enzyme solution at 37°C for 2 hr. The reaction was terminated by incubation in working-strength stop/wash buffer for 30 min at 37°C. Sections were then incubated with antidigoxigenin peroxidase and then incubated with diaminobenzidine and 0.01% H₂O₂ for 5 min at room temperature. The sections were counterstained with hematoxylin and examined by light microscopy [13].

**Statistical analyses**

Results are expressed as mean ± SE. Multiple comparisons among the groups were made by one-way ANOVA and post hoc Tukey HSD test. Differences with values of p<0.05 were considered significant.
Results

Blood pressure and renal function

Table 1 summarizes the changes in body weight, SBP, and kidney and left ventricle weights in the control, DSH, and sildenafil-treated DOCA-salt rats (D+sildenafil). The DSH rats showed a 10% mortality rate, whereas all the control and D+sildenafil rats survived throughout the experiments. Compared with the control rats, SBP was markedly increased in the DSH rats and was not affected by sildenafil treatment. The DSH rats had increased kidney weights, compared with the control animals; the increase in kidney weight was prevented by sildenafil treatment. Left ventricle weights were increased, relative to the control animals, in both DSH and D+sildenafil rats.

Table 2 summarizes the changes in renal function among the 3 groups of animals. The DSH rats showed decreased creatinine clearance, resulting in increased plasma creatinine levels, compared with the control animals. Further, the DSH animals exhibited a significant increase in the fractional excretion of sodium into urine, suggesting adaptation to nephron loss. In addition, the albumin-to-creatinine ratio (ACR) was markedly increased in the DSH rats. Sildenafil treatment lowered plasma creatinine levels, increased creatinine clearance, and attenuated the ACR in the DSH animals.

Effect of sildenafil on pathological changes in DSH rats

Figure 1 shows the morphological changes observed in the kidneys among the 3 groups of animals. Hematoxylin and eosin staining indicated the presence of tubular casts, obstructions, and vessel dilatations in the DSH rats. The glomerulosclerosis index (GSI) attained values almost 3-fold higher in the DSH animals than in the control rats; treatment with sildenafil was associated with a less pronounced increase in GSI. In addition, interstitial expansion was also a prominent component of the renal injuries in the DSH rats, which was attenuated by sildenafil treatment.
Fig. 1. Hematoxylin and eosin (H & E) stain and Masson's trichrome (M-T) stain of the renal cortex. The extents of glomerulosclerosis and interstitial fibrosis were increased in the deoxycorticosterone acetate-salt rats (DSH), compared with the control animals. These effects were attenuated by sildenafil treatment.

*P < 0.05 compared with the control animals. *P < 0.05 compared with the DSH rats.

Fig. 2. (A) Semiquantitative immunoblotting of ED-1 in the kidney. Densitometric analysis showed that increased ED-1 expression in the DSH rats, compared with the control rats; the increase was counteracted by sildenafil treatment. *P < 0.05 compared with the control rats. *P < 0.05 compared with the DSH rats. (B) Immunoperoxidase microscopy of ED-1 in the renal cortex. Increased immunolabeling was evident in the DSH rats; the increase was prevented with sildenafil treatment. Magnification: ×200.
Immunohistochemistry staining and protein expression of ED-1

Compared with the control animals, the expression of ED-1 protein, a marker for mononuclear phagocytes, was significantly increased in the cortical glomerulus and interstitium of the DSH rats; this expression was reduced by sildenafil treatment (Figure 2).

mRNA expression of TGF-β1, MCP-1, and ICAM-1

Figure 3 shows the levels of mRNA expression for TGF-β1, MCP-1, and ICAM-1 in the kidney. Compared with the control animals, TGF-β1, MCP-1, and ICAM-1 mRNA were significantly increased in the kidneys of the DSH rats, and these expression levels returned to near normal levels with sildenafil treatment.

Immunohistochemical staining and expression of TGF-β1, fibronectin, and α-SMA

Figures 4 and 5 show the levels of expression of TGF-β1, fibronectin, and α-SMA among the various groups of animals. Increased expression of all 3 proteins was observed in the DSH rats, compared with the control animals. However, this increased expression was attenuated with sildenafil treatment.

Fig. 3. Expression of TGF-β1, MCP-1, and ICAM-1 in whole kidneys. Columns show real time polymerase chain reaction data. *\(P < 0.05\) compared with the control animals. #\(P < 0.05\) compared with the DSH rats.

Fig. 4. (A) Semiquantitative immunoblotting of TGF-β1 in the kidney. Densitometric analysis showed that increased TGF-β1 expression in the DSH rats is counteracted by sildenafil treatment. *\(P < 0.05\) compared with the control animals. #\(P < 0.05\) compared with the DSH rats. (B) Immunoperoxidase microscopy of TGF-β1 in the renal cortex. Increased immunolabeling is shown in the DSH rats; this increase is reversed by sildenafil treatment. Magnification: ×200.
Protein expression of Bax and Bcl-2 and TUNEL stain results

The DSH rats had increased Bax expression levels, but decreased Bcl-2 expression levels, compared with the control rats. As a result, the Bax/Bcl-2 ratio was markedly increased in the DSH rats (Figure 6). Interestingly, sildenafil treatment was also able to attenuate the increased Bax/Bcl-2 ratio in the D+sildenafil animals. Accordingly, the number of tubular epithelial cells containing TUNEL-positive nuclei increased in the kidneys of the DSH rats, whereas the effect was markedly attenuated in D+sildenafil animals (Figure 6).

Discussion

This study showed that sildenafil prevents progressive renal injury in DSH rats. As expected, the DSH rats showed systemic arterial hypertension, proteinuria, and impaired renal function. Sildenafil was shown to have renoprotective effects in these animals, attenuating renal inflammation, fibrosis, and apoptosis. The detection of inflammatory, profibrotic, and apoptotic gene induction and macrophage infiltration in the kidneys of the DSH rats supports the notion that inflammatory processes contribute to the progressive renal injury and fibrosis observed in this model.

Previously, inflammation was shown to be involved in the initiation and progression of glomerulosclerosis and tubulointerstitial fibrosis [14]. In the current study, sildenafil therapy reduced inflammatory marker expression. Consistent with this finding, previous reports have also indicated that sildenafil abrogates the renal expression of a number of inflammatory mediators in 5/6 nephrectomy rats, including anti-CD-5 and ED-1 [2], and in diabetic rats [5]. In addition, increased COX-2 expression has been reported in DSH rats, and COX-2 inhibitor treatment has been shown to decreased tubulointerstitial injury in these rats [15]. These findings suggest that macrophage infiltration and inflammation contribute to the renal injury associated with mineralocorticoid hypertension, and that sildenafil treatment reduces the inflammatory reaction and extent of renal injury.

TGF-β1, a multifunctional cytokine that induces the differentiation of fibroblasts to myofibroblasts, is implicated as a key inducer of fibrotic diseases, including diabetic nephropathy and hypertensive nephropathy [16, 17]. TGF-β1 induces fibrogenesis through smooth muscle cell proliferation, stimulation of extracellular matrix proteins, and inhibition of matrix degradation [18, 19]. In the present study, sildenafil was shown to downregulate the expression of fibronectin and α-SMA, an early marker of TGF-β1-induced smooth muscle cell proliferation. Thus, the results suggest that sildenafil may prevent progressive renal fibrosis.

An increase in the number of apoptotic cells in the kidney is also a hallmark of progressive renal disease, and increased apoptosis has also been identified as a component of the pathogenesis of progressive renal disease [2]. The increased number of apoptotic cells may reflect the increased number of cells associated with the on-going inflammatory reaction [20]. Apoptosis is associated with the upregulation of a proapoptotic protein (Bax), downregulation of an antiapoptotic protein (Bcl-2), and an increase in caspase-3 activity [21-23]. The current study showed significantly increased Bcl-2 levels in the sildenafil-treated DSH rats, compared with the DSH rats that were not treated with sildenafil. Furthermore, the number of TUNEL-positive cells was significantly lower in the kidneys of sildenafil-treated DSH rats than in the kidneys of the DSH rats. Previously, sildenafil has been shown to strongly protect against apoptosis through a nitric oxide signaling pathway [24], and the sildenafil-induced decrease in apoptosis in the current study suggests another potential renoprotective mechanism.
Fig. 5. (A) Semiquantitative immunoblotting of α-SMA in the kidney. Densitometric analysis showed that the expression of α-SMA was increased in the DSH rats, and this increase was counteracted by sildenafil treatment. *P < 0.05 compared with the control animals. *P < 0.05 compared with the DSH rats. (B) Immunoperoxidase microscopy of α-SMA in the renal cortex. Increased immunolabeling is shown in the DSH rats, which is reversed by sildenafil treatment. Magnification: ×200.
Fig. 6. (A) Increased Bax expression but decreased Bcl-2 expression was observed in the DSH rats, compared with the control animals. The markedly increased Bax/Bcl-2 ratio in the DSH rats was also attenuated by sildenafil treatment. (B) TUNEL staining indicated increased renal tubular apoptosis in the DSH rats; sildenafil treatment reduced the number of TUNEL-positive cells.

Conclusion

These findings suggest that sildenafil is effective in preventing the progression of renal injury in DSH rats. The mechanism through which this protection is mediated appears to be associated with anti-inflammatory, antifibrotic, and antiapoptotic effects. These processes appear to mediate a reduction of the overexpression of ED-1, a downregulation of TGF-β1, and a decreased Bax/Bcl-2 ratio in DSH rat kidneys.

Conflict of interests

The authors declare no conflict of interest.

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References

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