Original Paper

Connexin 43 is Involved in Aldosterone-Induced Podocyte Injury

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Key Words

Connexin 43 • Podocytes • Aldosterone • ROS • Apoptosis

Abstract

Background/Aims: Connexin43 (Cx43) belongs to a family of transmembrane proteins that build cell-to-cell channels in gap junctions, which play an essential role in intercellular communication, cell proliferation, differentiation and apoptosis. The aim of this study is to clarify the effect of Cx43 in aldosterone (Aldo)-induced podocyte injury and explore the possible molecular mechanism behind this effect. **Methods:** Uninephrectomized Sprague-Dawley rats were given 1% NaCl (salt) in their water and an Aldo infusion (0.75 µg/h) for 28 days to induce podocyte injury. Podocytes were incubated in media containing either buffer or increasing concentrations of Aldo (10^{-9} - 10^{-7} M) for variable time periods. The podocytes were then examined and the mechanism of injury investigated using TUNEL assay, ELISA, immunofluorescence staining, western blot, RNA interference, and DCFDA fluorescence. **Results:** Here, we report that *in vivo* administration of Aldo caused greater numbers of TUNEL-positive podocytes, accompanied by increased Cx43 expression, but a reduction in WT1-positive podocytes. *In vitro* studies indicated that Aldo induces podocyte apoptosis in a dose- and time-dependent manner and is accompanied by increases in Cx43 expression, gap junction intercellular communication (GJIC), ROS production, and the Bax/Bcl-2 ratio. Silencing of Cx43 expression attenuates the increase of ROS production and the subsequent up-regulation of the Bax/Bcl-2 ratio, which partially inhibited the podocytes apoptosis. **Conclusions:** Our study provides preliminary evidence that upregulation of Cx43 expression is involved in Aldo-induced podocyte injury, and that Cx43 is a potentially relevant therapeutic target for the treatment of chronic kidney diseases (CKD).

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Introduction

Podocytes, derived from embryonic precursor mesenchymal cells, are considered terminally differentiated and highly specialized cells [1]. Over the past several decades, numerous studies have indicated that podocyte injury plays a role in the pathogenesis of various glomerular diseases [2].

Recently, accumulating evidence has suggested that aldosterone (Aldo), originally produced in the glomerulosa zone of the adrenal cortex, can also play an important role in the pathogenesis of podocyte injury [3-5]. In addition, a recent study demonstrated that Aldo stimulated oxidative stress is responsible for podocyte injury [6]. However, the exact molecular regulatory mechanism in Aldo-induced podocyte injury remains unclear.

Connexins (Cx) are a multigenic family of transmembrane proteins that form gap junctions (GJ), which allow small molecules (<1 kDa) and ions to flow from cell to cell. The process of material exchange between cells through GJ is referred to as gap junction intercellular communication (GJIC), and plays a critical role in tissue development, differentiation, and cell proliferation through regulation of signal transduction [7]. GJIC is critical to intercellular communication in many tissues [8], including the kidney [9]. Cxs are named according to their molecular weight (for example, Cx43 has a molecular weight of 43 kDa). To date, 20 different connexin isoforms have been found in humans [10]. In recent years, the important pathophysiological role of Cxs in the kidney has gained more and more attention from nephrologists [11, 12]. There are gap junctions between the podocytes, and these have been found to primarily contain Cx43 [13], although one study suggests that Cx45 may also be present in podocytes [14]. Growing evidence has indicated that an up-regulation in Cx43 expression is a marker of injury in podocytes [15, 16]. However, the role of Cx43 in Aldo induced podocytes injury is still not fully understood.

The aim of our study was to elucidate the effect of Cx43 in Aldo induced podocyte injury and to determine if regulation of Cx43 could protect podocytes from Aldo induced injury.

Materials and Methods

Materials

Aldo, N-acetyl-L-cysteine (NAC) and 20, 70-dichlorofluorescein diacetate (DCFDA) were purchased from Sigma (St. Louis, MO). Cell culture materials were purchased from Gibco BRL (Gaithersburg, MD, USA). β -Actin, Cx43, Bax, and Bcl-2 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). All other chemicals were of analytical grade.

Animals

Study protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Soochow University, China. In brief, 12 male Sprague-Dawley rats (5–6 weeks old, approximately 190 g) received a right uninephrectomy under light 3% isoflurane anesthesia. Two weeks after the surgery, rats weighing 260–290 g were randomly divided into two groups for 4 weeks. In the Aldo group (n=6), a subcutaneously osmotic minipump (model 2004; Alzet, Cupertino, CA) was implanted (at 0 weeks) under light 3% isoflurane anesthesia to infuse Aldo. No pump was implanted in animals of the control group (n=6). Both groups received 1% NaCl in their drinking water throughout the experimental period.

Systolic blood pressure (SBP) was measured in the conscious state by tail-cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan) at weeks 0 and 4 during the treatment period. Twenty-four-hour urine samples were collected starting after a 24-hour acclimatization period in metabolic cages. Urinary protein excretion was determined using enzyme-linked immunosorbent assay (EISA) kits from Exocell (Philadelphia, PA, USA). Urine and plasma creatinine levels were analyzed using an assay kit (Jiancheng, Nanjing, China).

Glomerular Isolation

Isolation of glomeruli was performed as previously reported [17]. Purity of the glomerular preparation was >95% as determined by light microscopy. Briefly, kidneys were firstly perfused with a mixture of

Dynabeads (diameter 4.5 μm) and iron powder (diameter 6 μm). Tissues were then rinsed on a 70 μm nylon mesh after magnetic treatment to remove small tubular fragments.

Podocyte Culture

The MPC5 conditionally immortalized mouse podocyte cell line was kindly provided by Dr. Yong Gu (Department of Nephrology, Huashan Hospital of Fudan University, Shanghai, China) and their cell lines were provided by Dr. Peter Mundel (Mount Sinai School of Medicine, New York, NY, USA) and were cultured as previously described [18]. Podocytes were maintained without interferon- γ at 37°C for 14 days before experimentation to induce differentiation. Differentiated podocytes were made quiescent in medium that contained 0.1% FBS for 24 h, then cells exposed to treatment for the indicated time periods.

Transient transfection of cells with siRNA

For knockdown experiments, podocyte cells were transiently transfected with siRNA specifically targeting Cx43 or a negative control siRNA using Lipofectamine PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were transfected with 20 nM Cx43 siRNA or control siRNA for 24 h before further treatment. Cellular protein was extracted and subjected to Western blot analysis for Cx43.

Apoptosis Studies

Analysis of podocyte apoptosis in vitro. The cytosolic DNA-histone complexes generated during apoptotic DNA fragmentation in treated podocytes were evaluated with a cell death detection ELISA kit (Cell Death Detection ELISA PLUS; Roche Applied Science, Indianapolis, IN, USA) and performed following the supplier's instructions.

Analysis of podocyte apoptosis in renal tissue. Apoptotic cells were detected on kidney section using a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Roche Molecular Biochemicals) following the manufacturer's protocol.

Immunofluorescence staining

Indirect immunofluorescence staining was performed according to an established procedure. Briefly, cryosections of 3 μm thickness were prepared. After being blocked with 3% BSA (bovine serum albumin) for 1 h, the sections were incubated with primary antibodies against Cx43 and Nephrin (sc-377246, Santa Cruz) in PBS containing 3% BSA overnight at 4°C. Sections were then washed in PBS and incubated in the dark with FITC and TRITC-conjugated secondary antibodies (Sigma-Aldrich) at a 1:200 dilution in PBS containing 3% BSA for 1 h. After being thoroughly washed with PBS, slides were mounted with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, H-1200) and viewed with a Nikon Eclipse 80i Epi-fluorescence microscope equipped with a digital camera (DS-Ri1, Nikon).

Localization of Cx43 in podocytes by immunofluorescence

Podocytes were treated with Aldo (10^{-7} M) for 24 h. After fixation with cold acetone for 10 min, the cells were blocked at room temperature with 5% BSA for 40 min. The cells were then incubated with 5% BSA (Negative control) or anti-Cx43 (Control and Aldo), respectively, followed by staining with TRITC-conjugated secondary antibodies. Finally, cells were double stained with DAPI and viewed with a Nikon Eclipse 80i Epi-fluorescence microscope equipped with a digital camera (DS-Ri1, Nikon).

DCFDA fluorescence detection of ROS

Podocyte ROS production was measured as previously described [19]. Relative fluorescence was detected using a fluorescence plate reader at the excitation/emission wavelengths of 485 and 535 nm, respectively.

Scrape Load Dye Transfer Technique (SLDT)

SLDT was used to assess GJIC activity [20]. Briefly, podocytes on coverslips at 90% confluence were rinsed three times with PBS containing 0.01% Ca^{2+} and Mg^{2+} . 1.5 mL of PBS containing 0.5% fluorescent dye (Lucifer yellow; Molecular Probes) was then added to the coverslips and a scalpel was used to scrape a cross through the monolayer. The cells were incubated for 3 minutes at room temperature and then rinsed again with PBS containing 0.01% Ca^{2+} and Mg^{2+} . The cells were fixed with 4% paraformaldehyde

Table 1. Biological parameters in control and Aldo-infused rats at 4 weeks. *p<0.05 compared to Control values

	Control	Aldo
Body weight (g)	464 ± 11	452 ± 9
Kidney weight/body weight ratio (mg/g)	5.8 ± 0.2	9.5 ± 0.6*
Urine volume (ml)	12 ± 3	45 ± 11*
Creatinine clearance (ml/min)	3.1 ± 0.4	2.9 ± 0.2
Albumin/creatinine (mg/mg)	1.4 ± 0.5	9.3 ± 2.4*
Urinary Aldo at end (mg/24 h)	0.04 ± 0.01	0.15 ± 0.03*
Blood pressure (mmHg)	140 ± 3	188 ± 5*

in PBS and photographed with a digital camera attached to a fluorescence microscope (Diaphot; Nikon, Tokyo, Japan). Only the cells labeled by fluorescent yellow dye in areas next to the scrape line (the cut cells) represent cell GJIC. The dye-coupled cells on either side of the scrape line were counted in at least ten random areas to evaluate the GJIC activity.

Western blot analysis

Podocytes harvested from plates and sieved glomeruli were lysed in SDS sample buffer containing 150 mM NaCl, 0.1% Triton X-100, 0.5% Deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 7.0), and 1 mM ethylenediaminetetraacetic acid (EDTA). Detection of protein expression by Western blot was carried out according to established protocols. The primary antibodies used were as follows: Cx43 (1:1000), Bax (1:1000), Bcl-2 (1:1000), and β -actin (1:1000). Densitometric analysis was performed using Quantity One Software (Bio-Rad).

Statistical Analyses

Data are expressed as the mean $\pm$ SD. Comparisons between groups were performed with one-way ANOVA followed by Dunnett's multiple comparison tests or Student's t-test. P<0.05 was considered statistically significant.

Results

Clinical characteristics and renal function

The kidney-to-body weight ratio, urine volume, urinary albumin/creatinine, ending urinary Aldo, and blood pressure were all significantly increased in the Aldo group compared to control rats. However, there were no significant differences in body weight and creatinine clearance between the two groups (Table 1).

Aldo induced podocytes injury in vivo

To determine whether podocyte apoptosis was increased following treatment with Aldo, the number of TUNEL-positive podocytes per glomerular cross-section was evaluated. Our results indicated that Aldo significantly increased podocyte apoptosis compared with the control group (P < 0.05) (Fig. 1A and B). Given the increase in TUNEL-positive cells in Aldo treated rats, immunostaining for Wilms's tumor protein (WT-1), a surrogate marker for podocyte number, was performed to determine if the decrease in WT-1-positive cells was secondary to apoptosis. Compared with the control group (15.6 $\pm$ 1.3), the number of podocytes was significantly decreased in Aldo infused rats (8.4 $\pm$ 0.9) (Fig. 1C and D). Taken together, these results indicate that Aldo induces podocyte injury *in vivo*.

Aldo increased podocytes Cx43 protein expression in vivo

Double-immunofluorescent staining assays and western blot were used to examine the expression of Cx43 in kidney tissues. Based on visual inspection of the co-localization of Cx43 with nephrin, our results demonstrate that the expression of Cx43 is significantly

Fig. 1. Podocyte injury in Aldo-infused rats. (A) The TUNEL assay in renal cortex sections of control and Aldo-infused rats. The red arrow indicates TUNEL-positive podocytes. (B) The bar graph shows the mean number of TUNEL-positive podocytes per glomerular cross-section. (C) Representative images of WT-1 immunostaining, a surrogate marker for podocyte number, in tissues from

Aldo-infused and control rats (D) Quantification of WT-1-positive cells per glomerulus. Values represent the means $\pm$ SEM (n=6 for each group). [#]P < 0.05 with respect to the control group.

Fig. 2. Cx43 protein expressions in Aldo-infused rats. (A) Representative images showing the expression and localization of Cx43 (green), nephrin (red), and DAPI (blue) by indirect immunofluorescent staining in paraffin embedded renal tissue sections. Magnification is 400X. (B) Western blot analysis of Cx43 protein levels from isolated glomeruli from control and Aldo-treated rat kidneys. The data are presented after normalization to β -actin expression and shown as mean $\pm$ SEM; n=3 per group; [#]P < 0.05 with respect to the control group.

increased in Aldo-infused rats, mainly due to their increases in podocytes, when compared to the control group ($P < 0.01$) (Fig. 2A and B).

Aldo Increased apoptosis in Cultured Mouse Podocytes

Compared with the control group, the addition of Aldo for 24 h increased podocyte apoptosis in a dose-dependent manner at concentrations ranging from 10^{-9} to 10^{-7} M ($P < 0.01$, Fig. 3A). Since the maximal induction was found at 10^{-7} M Aldo treatment, we therefore used 10^{-7} M in most of our cell experiments. As shown in Fig. 3B, 10^{-7} M Aldo induced apoptosis of podocytes in a time-dependent manner.

Aldo Increased Cx43 gene expression and GJIC in Cultured Mouse Podocytes

Cultured podocytes stimulated with Aldo up-regulated expression of Cx43 in a dose-dependent and time-dependent manner ($P < 0.01$, Fig. 4A and B). Immunofluorescent

Fig. 3. Dose- and time-dependent apoptosis and Cx43 expression induced by Aldo in cultured podocytes. (A) Podocytes were exposed to different concentrations (10^{-9} , 10^{-8} , and 10^{-7} M, respectively) of Aldo for 24 h or (B) 10^{-7} M Aldo for different time periods (12, 24, and 36 h, respectively). Quantitative analysis of podocyte apoptosis and data are expressed as mean $\pm$ SEM of three experiments. $^{\#}P < 0.05$ with respect to the control group.

Fig. 4. Aldo induced dose- and time-dependent Cx43 expression and GJIC in cultured podocytes. (A, B) Whole cell lysate was immunoblotted with a specific Cx43 antibody. The quantitative ratios are shown as relative optical densities of bands that are normalized to the expression of β -actin. The data are representative of three similar experiments and quantified as mean $\pm$ SEM. $^{\#}P < 0.05$ with respect to the control group. (C) Red fluorescence indicates localization of Cx43 and blue fluorescence indicates nuclei. A significant increase in Cx43 was observed after Aldo treatment. Magnification is 400X. (D) The effects of 10^{-7} M Aldo for 24 h on GJIC as measured by scrape loading dye transfer assay. The micrographs of Lucifer Yellow diffusion into the cellular monolayer after scrape loading are shown. Magnification is 100X. A bar graph shows a significant reduction in the number of dye-coupled cells in the control and Aldo treated cells. Data are presented as mean $\pm$ SEM. $^{\#}P < 0.05$ with respect to controls; $n = 10$.

A significant increase in Cx43 was observed after Aldo treatment. Magnification is 400X. (D) The effects of 10^{-7} M Aldo for 24 h on GJIC as measured by scrape loading dye transfer assay. The micrographs of Lucifer Yellow diffusion into the cellular monolayer after scrape loading are shown. Magnification is 100X. A bar graph shows a significant reduction in the number of dye-coupled cells in the control and Aldo treated cells. Data are presented as mean $\pm$ SEM. $^{\#}P < 0.05$ with respect to controls; $n = 10$.

staining also indicated that 10^{-7} M Aldo increased Cx43 expression (Fig. 4C). In addition, 10^{-7} M Aldo stimulation increased the GJIC in cultured podocytes (Fig. 4D).

Fig. 5. The Aldo-induced increase in ROS production and Bax/Bcl-2 ratio is responsible for podocyte apoptosis. (A) Podocytes were treated with Aldo (10^{-7} M) for 24 h and then exposed to dichlorodihydrofluorescein diacetate (DCFDA) for another 120 min. (B) Western blot analysis of the whole cell lysate, immunoblotted with Bcl-2 and Bax antibodies. Quantitative ratios are shown as relative optical densities of bands after normalization to β -actin expression. The data are representative of three similar experiments and quantified as mean $\pm$ SEM. [#] $P < 0.05$ with respect to the control group, ^{*} $P < 0.05$ with respect to the Aldo group. (C) NAC attenuated Aldo-induced podocyte apoptosis as determined by Cell Death Detection ELISA analysis. [#] $P < 0.05$ with respect to the control group, ^{*} $P < 0.05$ with respect to the Aldo group.

Increased ROS production and Bax/Bcl-2 ratio is involved in Aldo-induced Podocytes apoptosis

Since increased levels of ROS and the proteins of the Bcl-2 family are considered to be the major apoptotic signal transduction cascades associated with programmed cell death, we examined the production of ROS and the expression of Bcl-2 and Bax in cells treated with Aldo for 24 h (Fig. 5A). Compared with the control group, 10^{-7} M Aldo increased ROS generation (Fig. 5A) and the Bax/Bcl-2 ratio (Fig. 5B). However, treatment with NAC decreased the DCF fluorescence and Bax/Bcl-2 ratio, attenuating the Aldo-induced apoptosis (Fig. 5A, B, and C). Thus, increasing the ROS production and the ratio of Bax/Bcl-2 may contribute to the susceptibility of podocytes to Aldo-induced apoptosis ($P < 0.05$).

Silencing Cx43 expression inhibited the Aldo-induced apoptosis of podocytes by a reduction in ROS production and down-regulation of Bax/Bcl-2 ratio

To explore the role of Cx43 gene in Aldo-induced apoptosis of podocytes, we transfected podocytes with either siRNA-Cx43 or nonsense siRNA-con as a control. After 24 h, Cx43 expression was assessed by western blot analysis. Our results indicate that Cx43 protein levels were markedly lower in podocytes transfected with siRNA-Cx43 than those of control podocytes and podocytes transfected with siRNA-con (Fig. 6A). Moreover, Cx43 silencing inhibited the production of ROS, down-regulated the ratio of Bax/Bcl-2, and partially attenuated the Aldo-induced apoptosis of podocytes (Fig. 6 B, C and D).

Fig. 6. siRNA of Cx43 inhibited Aldo-induced apoptosis in podocytes. (A) Cx43 expression in infected podocytes. Podocytes were transfected with siRNA-Cx43 (20 nM) or siRNA-Con as the control. After infection for 24 h, podocytes were treated with Aldo (10^{-7} M) for another 24 h. Expression levels of Cx43 in the transfected cells were analyzed by western blot and shown as mean $\pm$ SEM of three separate experiments performed in triplicate; [#]P < 0.05 with respect to control or siRNA-Con. (B) Cx43 siRNA attenuated Aldo-induced podocyte apoptosis. (C) Podocytes in chamber slides were transfected with siRNA-Cx43 (20 nM) or siRNA-Con, then exposed to Aldo (100 nM) for 60 min in the presence of DCFDA. (D) BCL-2 and Bax protein levels among three different groups were examined by western blot. Quantitative analysis of the relative abundance of BCL-2 and Bax is shown in the bar graph and data are expressed as mean $\pm$ SEM of three experiments. [#]P < 0.05 with respect to control or siRNA-Con.

Discussion

In the present study, we investigated the role of Cx43 in Aldo-induced podocyte injury. *In vivo* studies demonstrated that Aldo infusion significantly increased SBP, the kidney/body weight ratio, and the urinary protein/creatinine ratio. Additionally, renal cortical sections of Aldo-infused rats showed a greater numbers of TUNEL-positive podocytes, reduced WT1-positive podocytes, and an increase in Cx43 expression. *In vitro* studies indicated that Aldo induced podocyte apoptosis in a dose- and time-dependent manner, accompanied by increases in Cx43 expression, ROS production, and the Bax/Bcl-2 ratio. Silencing Cx43 expression could attenuate podocyte apoptosis, which is mediated through the reduction of ROS production and the subsequent down-regulation of the Bax/Bcl-2 ratio.

Aldo, an important hormone, is associated with albuminuria [21], hypertension [22] and glomerulosclerosis [23]. Epidemiological studies have shown a beneficial effect of MR inhibitors on patient outcomes compared to placebo control [24, 25]. Aldo-induced apoptotic cell loss plays a critical role in the pathophysiology of kidney failure, and has recently attracted considerable attention from many kidney disease scholars. Aldo has been shown to cause tubular epithelial and mesangial cell apoptosis by affecting the mitochondrial

apoptotic pathway [26, 27]. Consistent with previous studies [28, 29], our results indicate that Aldo induced podocyte injury both *in vivo* and *in vitro* (Fig. 1 and 3).

Recently, growing evidence has shown that Cx43 might be involved in the process of podocyte injury in kidney pathology, including hypertension and diabetes nephropathy [13, 30]. Our *in vitro* and *in vivo* data has demonstrated that the treatment with Aldo could also elevate the expression of Cx43 in podocytes (Fig. 2 and 4A through C). In addition, GJIC was augmented in Aldo-induced podocytes (Fig. 4D), which may facilitate the spread of apoptotic signals. Interestingly, after silencing Cx43 expression, Aldo-induced apoptosis in cultured podocytes was partially attenuated, suggesting Cx43 was partially involved in Aldo-induced podocyte apoptosis and targeting of Cx43 could protect podocytes from Aldo-induced apoptosis (Fig. 6A and B).

Reactive oxygen species (ROS) also play a key role in the progression of renal injury [31, 32]. Nishiyama et al [33] demonstrated that renal injury was associated with increased renal cortical ROS levels in Aldo/salt hypertensive rats. Consistent with these previous studies [4], our data showed that Aldo increased the generation of ROS in cultured podocytes. As expected, pretreatment with NAC, a free radical scavenger, prevented the elevation of ROS levels and ameliorated renal injury (Fig. 5A and C). After silencing Cx43 expression, the Aldo-induced increased the generation of ROS by was partially inhibited, indicating that impairment of Cx43-coupling attenuates Aldo-induced podocyte apoptosis through inhibition of ROS generation (Fig. 6 B and C).

Aldo-induced increase of ROS production is thought to be the main reason for uncontrolled oxidative stress, which can lead to changes in expression of Bcl-2 and Bax (markers of mitochondrial dysfunction) [34]. An increase in the levels of pro-apoptotic proteins (Bax) and/or a decrease in the levels of apoptotic inhibitors (Bcl-2), is a key event in the induction of apoptosis [35-37]. Consistent with previous reports, in our study, we found that treatment of podocytes with Aldo resulted in a reduction in the levels of the anti-apoptotic protein Bcl-2 with a concomitant increase in the levels of the pro-apoptotic protein Bax when compared to the control podocytes (Fig. 5B). However, silencing Cx43 expression partially attenuated these changes, and may partially be responsible for the induction of apoptosis (Fig. 6 B and D).

Conclusion

In conclusion, our study provides evidence that Aldo-mediated upregulation of Cx43 expression, GJIC activity, and the following increase in ROS production and the Bax/Bcl-2 ratio is partially involved in podocyte injury. Cx43 might be a potential therapeutic target for the treatment of CKD.

Acknowledgments

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