Original Paper

Beyond Gap Junction Channel Function: the Expression of Cx43 Contributes to Aldosterone-Induced Mesangial Cell Proliferation via the ERK1/2 and PKC Pathways

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Key Words

Connexin 43 • Gap junction • Mesangial cells • Proliferation

Abstract

Aims: This study aimed to explore the precise mechanism and signaling pathways of mesangial cell (MC) proliferation from a new point of view considering Connexin 43 (Cx43). **Methods:** MC proliferation was measured by the incorporation of 3H-thymidine (3H-TdR). Cx43 was over-expressed in MC cells using lipofectamine 2000, and the expression level was tested with reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses. The gap junction channel function was explored by Lucifer Yellow scrape loading and dye transfer (SLDT), and the intracellular calcium concentrations ($[Ca^{2+}]_i$) were characterized by confocal microscopy on cells loaded with Fura-3/AM. **Results:** There was an inverse correlation between Cx43 expression and MC proliferation ($P < 0.05$). SLDT studies revealed that there was no difference in the gap junction channel function between the normal and Aldosterone (Aldo)-stimulated groups ($P > 0.05$). Our data also showed that the mineralocorticoid receptor (MR) antagonist spironolactone, ERK1/2 inhibitor PD98059 and PKC inhibitor GF109203X could attenuate the down-regulation of Cx43 expression in Aldo-induced MC proliferation; however, the PI3K inhibitor LY294002 could block MC proliferation without affecting Cx43 expression at either the mRNA or protein level. In addition, Aldo promoted MC proliferation in parallel with increasing $[Ca^{2+}]_i$ ($P < 0.05$), suggesting that the classical PKC pathway might be activated. **Conclusions:** Our study provides preliminary evidence that Cx43 is an important regulator of Aldo-promoted MC proliferation. Furthermore, reduced Cx43 expression promoted MC proliferation independent of the gap junction channel function, and this process might be mediated through the ERK1/2- and PKC-dependent pathways.

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Introduction

The renin-angiotensin-aldosterone system (RAAS) plays a critical role in the progression and prognosis of chronic kidney disease (CKD), leading to inflammation, fibrosis, proteinuria, hypertension and metabolic syndrome [1-5]. Several studies have revealed that the renal mesangial cell (MC) is a target local of aldosterone (Aldo) action, where Aldo can induce glomerular injury characterized by mesangial matrix expansion and cell overgrowth through mineralocorticoid receptor (MR) activation [6-8], ROS generation, epithelial growth factor receptor (EGFR) phosphorylation, cell cycle-regulatory protein expression, and multiple signaling pathways [8-10]. However, the actual molecular mechanism responsible for Aldo-induced MC proliferation is far from clear.

Considering the presence of connexins within the kidney and their functional role in growth regulation [8, 11-13], analysis of connexins provides a new viewpoint for investigating CKD. Connexins are a multi-gene family of transmembrane proteins with ~20 isoforms. Structurally, six connexins have two hemichannels and then form a gap junction channel. The channel permits the intercellular passage of small molecules (generally less than 1000 Da), such as inorganic ions (e.g., Na⁺ and K⁺), second messengers (e.g., Ca²⁺, IP₃, and cAMP), hormones, and other metabolites, enabling the neighboring cells to achieve gap junction intercellular communication (GJIC). Connexins and GJIC play crucial roles in the regulation of embryonic development, maintenance of tissue/organ homeostasis, cellular metabolism, morphogenesis, and growth control [14-16]. Of note, Cx43 is the most abundant connexin, and it is the most closely related to cell growth. The Cx43 lifecycle begins with gene expression, which is followed by oligomerization into hexameric channels to effect intercellular coupling. Accordingly, Cx43 has dual functions in cell growth regulation, a well-accepted role in forming a gap junction channel (termed as channel-dependent mechanisms) and a direct action on growth (termed as channel-independent mechanisms).

The purpose of this study is to explore the molecular mechanism of Aldo-induced MC proliferation targeted at Cx43 expression and/or its gap junction channel function.

Materials and Methods

The rat mesangial cell line HBZY-1 was obtained from the Chinese Center for Type Culture Collection (Wuhan, China). Cell culture materials were purchased from Gibco BRL (Gaithersburg, MD, USA). Aldo, Spironolactone (Spi), Lucifer Yellow (LY), GF109203X (PKC inhibitor), and Fura-3/AM were from Sigma Chemical Co. (St. Louis, MO, USA). PD98059 (ERK1/2 inhibitor) and LY294002 (PI3K inhibitor) were purchased from Calbiochem (Cambridge, MA, USA). Antibodies against Cx43, β -actin and all secondary horseradish peroxidase-conjugated antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Immobilon PVDF membrane was obtained from Millipore (Bedford, MA, USA). All other chemicals are analytical grade.

Cell culture and transfection

The rat MCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C in an atmosphere containing 5% CO₂ before being used in the different experiments. For passage, confluent cells were first washed with PBS and then removed with 0.25% trypsin / 0.5 mM EDTA in PBS and plated in DMEM. MCs were used for experiments at passages 3 to 16.

Plasmid transfections were performed with the lipofectamine™ 2000 (lipo 2000) transfection reagent according to the manufacturer's instructions (Invitrogen). Briefly, one day before transfection, 2 × 10⁵ cells were plated in 500 μ l of growth medium without fetal serum and antibiotics. The rat wild-type cd43 in the pcDNA vector was gently mixed with lipo 2000 at a ratio of 1:2.5. The appropriate amount of complexes was added to a well containing cells and medium, and the medium was replaced after 4-6 h; cells were incubated for 48 h prior to testing for transgene expression.

MC proliferation assay

The incorporation of 3H-thymidine (3H-TdR) was used to measure MC proliferation. Cells were seeded into 96-well plates (5×10^3 /well) and grown for 24 h in 10% FCS-DMEM; they were then preincubated in 0.5% FCS-DMEM for another 24 h. To assess the concentration effect of Aldo, MCs were stimulated by Aldo in concentrations ranging from 0.1 nM to 100 nM ($n=6$ for each) for 24 h. To explore the underlying mechanisms of MC proliferation, the MR antagonist spironolactone (Spi, 10 nM), ERK1/2 inhibitor PD98059 (10 μ M), PI3K inhibitor LY294002 (10 μ M) or PKC inhibitor GF109203X (10 μ M) was administered with or without 100 nM Aldo for 24 h. As previously described by Huang [6], cells were pulsed with 1 μ Ci/mL 3H-TdR during the final 5 h, harvested, and counted on a scintillation counter. The level of 3H-TdR incorporation is represented by Cpm, and the final results are expressed as the percentage of the control group.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

To evaluate the expression level of Cx43 mRNA, we adopted a semiquantitative RT-PCR technology. Total RNA (1 μ g) extracted from MC with TRIZOL (Gibco BRL, Gaithersburg, MD) was reverse-transcribed using Superscript reverse transcriptase (Invitrogen) to yield the respective cDNA. The RNA concentrations were determined by measuring the A260 / A280 absorption. The efficiency of RT-PCR was controlled by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification. Each sample mixture contained standard PCR buffer, 2.5 mM dNTP, 2 U Taq polymerase, and 5 pM of each of the following primers: GAPDH, 5'-CAAGTTCAACGGCACAGTCAA-3' and 5'-TGGTGAAGACGCCAGTAGACTC-3', and cx43, 5'-TCTGCCTTTCGCTGTAACACT-3' and 5'-GGGCACAGACGAATATGAT-3'. The PCR cycles consisted of 30 cycles of a step at 95°C for 1 min, a denaturation step at 94°C for 30 s, an annealing step at 57.5°C and an extension step at 72°C for 30 s, followed by a final extension at 72°C for 7 min. The expected PCR product sizes were 149 bp (for GAPDH) and 117 bp (for cx43). The products were subjected to computer-assisted densitometry after electrophoresis on a 1.5% agarose gel and staining with ethidium bromide.

Western blotting

After the treatment with different compounds for the indicated time, the cells were harvested and washed with ice-cold phosphate buffer. Total cellular proteins were extracted by lysing cells with 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), and nuclear proteins were obtained using previously described methods. The protein concentrations were determined with the BCA method (Beyotime Institute of Biotechnology, China). Thirty micrograms of protein extract was loaded onto each lane, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose membranes. After being blocked with 5% w/v bovine serum albumin (BSA), 1x Tris-buffered saline (TBS) (pH 7.6), and 0.1% Tween-20 at room temperature, the membranes were incubated at 4 °C overnight with primary antibodies for total Cx43 (1:1000) and β -actin (1:3000). After being incubated with the respective second antibody, immune complexes were detected by ECL Western blotting reagents. The detected proteins were normalized to β -actin as appropriate.

Measurement of the gap junction channel function

The gap junction channel function was detected by SLDT. In brief, confluent MCs in 35 mm dishes were starved for 24 h in 0.5% FCS-DMEM. To detect the dose-dependent GJIC function of Aldo, MCs were incubated with Aldo at a concentration of 0.1, 1, 10 or 100 nM for 24 h. After Aldo treatment, the cells were rinsed three times with phosphate-buffered saline (PBS), and 1 ml of 0.05% Lucifer Yellow (0.05% V/V dissolved in PBS) was then added to the cell cultures and scrape loaded with several scrapes using a steel surgical blade. The dye solution was left on the cell cultures for 3 min and then discarded. The cell cultures were carefully rinsed three times with PBS to remove detached cells and background fluorescence. An inverted fluorescence microscope (Olympus, Japan) was employed to record the migration of the Lucifer Yellow dye from the edge cells of the scrape. An average value of 9 measurements for each treatment (3 measurements per dish) was regarded as the migration of dye in the cell cultures. The percentage of migration of dye in the cell cultures exposed to target compounds to the migration of dye traveling in the vehicle control was recorded to evaluate the function of GJIC.

Measurement of the Intracellular calcium concentrations ($[Ca^{2+}]_i$)

The intracellular calcium concentrations ($[Ca^{2+}]_i$) were examined with laser scanning confocal microscopy after being loaded with Fura-3/AM. Cells were seeded into 6-well plates (4×10^5 /well) with a glass bottom after being treated with Aldo at a concentration of 0.1, 1, 10 or 100 nM for 24 h. Cells were harvested and washed with PBS three times, treated with 5 μ M Fura-3/AM (dissolved in DMSO) and then incubated at 37 °C for 30 min. Afterwards, cells were washed with PBS another three times to remove the unloaded Fura-3/AM, and they were immediately observed using laser scanning confocal microscopy (LSM710, Germany) with an excitation wavelength of 488 nm and emission wavelength of 525 nm. The gray value of the fluorescence represents the $[Ca^{2+}]_i$, which was directly obtained from the computer connected to the LSM analysis system. The final results are presented as the fluorescence gray value.

Statistical analysis

SPSS17.0 statistical software was used for the statistical analysis. All values are expressed as the mean $\pm$ S.D. The differences were tested using analysis of variance (ANOVA). A P-value of less than 0.05 was considered statistically significant.

Results

Effects of Aldo on MC Proliferation and DNA synthesis rate

The incorporation of 3H-TdR showed that Aldo increased MC proliferation in a dose-dependent manner, ranging from 0.1 nM to 100 nM ($P < 0.05$, $n = 6$) (Fig.1.A). Additionally, 100 nM Aldo achieved its desired effect of promoting MC proliferation. Next, the MR antagonist spironolactone (Spi, 10 nM), ERK1/2 inhibitor PD98059 (10 μ M), PI3K inhibitor LY294002 (10 μ M) or PKC inhibitor GF109203X (10 μ M) was administered concomitantly with 100 nM Aldo for 24 h. The data showed that MC proliferation was inhibited to various degrees ($P < 0.01$, $n = 6$) (Fig.1.B). However, exposing MCs to an individual inhibitor alone for 24 h did not affect MC growth compared to the control group ($P > 0.05$, $n = 6$) (Fig.1.B).

Fig. 1. A) Effects of Aldo on MC proliferation by 3H-thymidine (3H-TdR) incorporation. B). Involvement of MR, ERK1/2, PI3K and PKC in Aldo-induced MC proliferation. Spironolactone (10 nM), PD98059 (10 μ M), LY294002 (10 μ M) or GF109203X (10 μ M) was administered with or without 100 nM Aldo for 24 h in cultured MC. "Control" refers to cells growing in DMEM containing 10% FCS without Aldo intervention. Values are given as the mean $\pm$ S.D, and $p < 0.05$ is considered statistically significant by ANOVA ($n = 6$, * $p < 0.05$, ** $p < 0.01$ vs. control, # $p < 0.05$, ## $p < 0.01$ vs. 100 nM Aldo group).

Fig. 2. A and B) Effects of Aldo on the protein expression of Cx43 by Western blot analysis. The rat MCs were treated with Aldo at a concentration of 0.1, 1, 10 or 100 nM for 24 h. Total cellular protein was extracted. Thirty micrograms of protein was loaded for Western blot analysis of total Cx43 and β -actin. C and D) Effects of Aldo on Cx43 mRNA by RT-PCR. The rat MCs were treated with Aldo at a concentration of 0.1, 1, 10 or 100 nM for 24 h. Total RNA was extracted from cultured MCs and analyzed by RT-PCR as described in the Materials and Methods for Cx43 and GAPDH. "Control" refers to cells growing in DMEM containing 10% FCS without Aldo intervention. Values are given as the mean $\pm$ S.D. and $p < 0.05$ is considered statistically significant by ANOVA ($n = 3$, * $p < 0.05$, ** $p < 0.01$ vs. control).

Effects of Aldo on the Expression of Cx43

Cultured MCs were stimulated for 24 h with Aldo in increasing concentrations of 0.1, 1, 10 nM and 100 nM, and Aldo significantly decreased the level of total Cx43 in a dose-dependent manner as follows: 0.1 nM Aldo: 0.91 ± 0.02 ($P < 0.05$, $n = 3$), 1 nM Aldo: 0.79 ± 0.02 ($P < 0.01$, $n = 3$), 10 nM Aldo: 0.67 ± 0.03 ($P < 0.01$, $n = 3$), and 100 nM Aldo: 0.43 ± 0.02 ($P < 0.01$, $n = 3$), compared to β -actin expression, which did not change with the treatment (Fig. 2.A and B).

To determine whether this down-regulation of the protein level of Cx43 expression is due to regulation of transcription rather than due to Cx43 protein degradation or some other mechanism, semi-quantitative RT-PCR studies were performed. Cx43 mRNA relative to the housekeeping gene GAPDH reduced gradually with concentrations as follows: 0.1 nM Aldo: 0.90 ± 0.02 ($P < 0.01$, $n = 3$), 1 nM Aldo: 0.75 ± 0.04 ($P < 0.01$, $n = 3$), 10 nM Aldo: 0.61 ± 0.02 ($P < 0.01$, $n = 3$), and 100 nM Aldo: 0.32 ± 0.03 ($P < 0.01$, $n = 3$) (Fig. 2.C and D).

Effects of Aldo on MC Proliferation with elevated Cx43 expression

The data presented in Fig. 3 indicate that the rat wild-type Cx43 vector increased the expression of the total Cx43 protein ($P < 0.01$, $n = 3$). To further assess the role of Cx43 in Aldo-induced MC proliferation, cell proliferation was analyzed following treatment with different concentrations of Aldosterone in the presence or absence of Cx43 over-expression for 24 h. Under elevating Cx43 protein expression level by plasmid transfection, Aldo-induced MC proliferation was decreased as follows (Fig. 4): control group: 1.00 ± 0.56 ; 0.1 nM Aldo: 1.16 ± 0.69 , 0.1 nM Aldo + Cx43 over-expression: 0.85 ± 0.06 ($P < 0.01$, $n = 6$); 1 nM Aldo: 1.33 ± 0.53 , 1 nM Aldo + Cx43 over-expression: 0.98 ± 0.07 ($P < 0.01$, $n = 6$); 10 nM Aldo: 1.57 ± 0.53 , 10 nM Aldo + Cx43 over-expression: 1.47 ± 0.09 ($P < 0.01$, $n = 6$); and 100 nM Aldo: 1.98 ± 0.87 ($P < 0.01$, $n = 6$), 100 nM Aldo + Cx43 over-expression: 1.92 ± 0.12 ($P > 0.05$, $n = 6$). From two aspects of positive and negative, the data obtained Fig. 2 and Fig. 4 suggested that

Fig. 3. A) Confirmation of the Cx43 protein expression level after plasmid transfection by Western blot analysis. The rat MCs were transfected with the Cx43 plasmid, and then total cellular protein was extracted. Thirty micrograms of protein was loaded for Western blot analysis of total Cx43 and β -actin. B) Quantitative analysis of the mRNA expression level of Cx43. "Control" refers to cells growing in DMEM containing 10% FCS without Aldo intervention. The values are given as the mean $\pm$ S.D., and $p < 0.05$ is considered statistically significant by ANOVA ($n = 3$, * $p < 0.05$, ** $p < 0.01$ vs. control).

Cx43 expression as a 'negative' regulator contributed to aldosterone-induced mesangial cell proliferation.

Effects of Aldo on the gap junction channel function

To further explore how Cx43 affects MC proliferation, we next performed SLDT. As shown in Fig. 5, none of concentrations of Aldo, ranging from 0.1 nM to 100 nM, had any notable effect on GJIC marked by the diffusion length of Lucifer Yellow dye at the following levels ($p > 0.05$, $n = 9$): control, $100.11 \pm 2.95\%$; 0.1 nM Aldo, $97.89 \pm 2.20\%$; 1 nM Aldo, $98.11 \pm 4.17\%$; 10 nM Aldo, $96.56 \pm 4.56\%$; and 100 nM Aldo, $98.00 \pm 4.47\%$ ($p > 0.05$, $n = 9$), indicating that Cx43 might contribute to one of the critical mechanisms for MC proliferation that is independent of gap junction channel function.

Involvement of MR, ERK1/2, PI3K and PKC kinase in Cx43 expression in Aldosterone-induced MC Proliferation

The MR antagonist spironolactone, ERK1/2 inhibitor PD98059 and PKC inhibitor GF109203X (instead of the PI3K inhibitor LY294002) could markedly reverse the down-regulation of the Cx43 expression at the gene level as follows (Fig. 6): Spi 0.92 ± 0.02 ($P > 0.05$ vs. control, $p < 0.01$ vs. 100 nM Aldo, $n = 3$), PD 0.78 ± 0.03 ($P < 0.01$ vs. control, $p < 0.01$ vs. 100 nM Aldo, $n = 3$), LY 0.28 ± 0.03 ($P < 0.01$ vs. control, $p > 0.05$ vs. 100 nM Aldo, $n = 3$), GF 0.67 ± 0.04 ($P < 0.01$ vs. control, $p < 0.01$ vs. 100 nM Aldo, $n = 3$), which is in parallel with the protein levels as follows (Fig. 7): Spi 0.88 ± 0.03 ($P < 0.05$ vs. control, $p < 0.01$ vs. 100 nM Aldo, $n = 3$), PD 0.69 ± 0.05 ($P < 0.01$ vs. control, $p < 0.01$ vs. 100 nM Aldo, $n = 3$), LY 0.33 ± 0.04 ($P < 0.01$ vs. control, $p > 0.05$ vs. 100 nM Aldo, $n = 3$), and GF 0.54 ± 0.03 ($P < 0.01$ vs. control, $p < 0.01$ vs. 100 nM Aldo, $n = 3$). Similarly, the inhibitor itself did not exert a substantial effect on Cx43 expression compared to the control group ($p > 0.05$, $n = 3$).

Fig. 4. Effects of Aldo on MC proliferation under elevated Cx43 protein. The rat MCs were treated with Aldo at a concentration of 0.1, 1, 10 or 100 nM in the presence or absence of Cx43 over-expression for 24 h. "Control" refers to cells growing in DMEM containing 10% FCS without Aldo intervention. The values are given as the mean±S.D, and $p<0.05$ is considered statistically significant by ANOVA ($n=6$).

Fig. 5. A) Effects of Aldo on the gap junction channel function using SLDT. Magnification, $\times 100$. The rat MCs were treated with Aldo at a concentration of 0.1, 1, 10 or 100 nM for 24 h. Then, SLDT was performed as described in the Materials and Methods. B). Quantitative analysis of the function of GJIC in Aldo-induced MC proliferation. "Control" refers to cells growing in DMEM containing 10% FCS without Aldo intervention. Values are given as the mean±S.D, and $p<0.05$ is considered statistically significant by ANOVA ($n=9$, * $p<0.05$, ** $p<0.01$ vs. control, # $p<0.05$, ## $p<0.01$ vs. 100 nM Aldo group).

Effects of Aldo on the Intracellular calcium concentrations ($[Ca^{2+}]_i$)

As shown in Fig. 8. A and B, Aldo significantly decreased the level of $[Ca^{2+}]_i$ in a dose-dependent manner as follows: control group: 21.62 ± 3.31 , 0.1 nM Aldo: 29.92 ± 3.24 ($P<0.05$, $n=3$), 1 nM Aldo: 37.20 ± 3.62 ($P<0.01$, $n=3$), 10 nM Aldo: 57.60 ± 3.16 ($P<0.01$, $n=3$), and 100 nM Aldo: 74.80 ± 2.85 ($P<0.01$, $n=3$), suggesting that the classical PKC pathway might be activated.

Fig. 6. Involvement of MR, ERK1/2, PI3K, and PKC kinase in Cx43 mRNA expression by RT-PCR. Spironolactone (10 nM), PD98059 (10 μM), LY294002 (10 μM) or GF109203X (10 μM) was administered concomitantly with 100 nM Aldo for 24 h to cultured MCs. Total RNA was extracted from cultured MCs and analyzed by RT-PCR as described in the Materials and Methods for Cx43 and GAPDH. "Control" refers to cells growing in DMEM containing 10% FCS without Aldo intervention. Values are given as the mean ± S.D., and $p < 0.05$ is considered statistically significant by ANOVA ($n = 3$, * $p < 0.05$, ** $p < 0.01$ vs. control, # $p < 0.05$, ## $p < 0.01$ vs. 100 nM Aldo group).

Discussion

Over the past several decades, various biological mechanisms have been demonstrated [8-10]; however, the mechanism by which Aldo regulates MC growth is far from clear. Recently, the role of connexins as 'players' in the field of growth regulation, not only in many types of cancer [17, 18] but also in normal cells [19, 20], has gained increasing attention. As the most abundant and extensively studied connexin, Cx43 has dual functions in controlling cell growth, dependent on or independent of the gap-junctional communication capacity [21, 22]. In addition, it should be noted that Cx43 has a half-life in the range of 1–3 h in cultured cells or in tissues [23-25]. Therefore, it seems reasonable that stimulation by Aldo for 24 h would allow for a several-fold turnover in Cx43 protein. Additionally, this time

Fig. 7. Involvement of MR, ERK1/2, PI3K, and PKC kinase in total Cx43 protein expression by Western blot analysis. Spironolactone (10 nM), PD98059 (10 μM), LY294002 (10 μM) or GF109203X (10 μM) was administered concomitantly with 100 nM Aldo for 24 h to cultured MCs. Total cellular protein was extracted. Thirty micrograms of protein was loaded for Western blot analysis of total Cx43 and β-actin. "Control" refers to cells growing in DMEM containing 10% FCS without Aldo intervention. Values are given as the mean±S.D, and $p < 0.05$ is considered statistically significant by ANOVA ($n = 3$, * $p < 0.05$, ** $p < 0.01$ vs. control, # $p < 0.05$, ## $p < 0.01$ vs. 100 nM Aldo group).

schedule might simulate a long-term or 'chronic' response to Aldo, which can be expected in mesangial proliferative renal disease.

Our data showed that Aldo treatment for 24 h dose-dependently repressed Cx43 expression and promoted cell proliferation in MCs (Fig. 1.2), suggesting a potential role of Cx43 in the negative regulation of MC proliferation. As previously reported, the lack of Cx43 led to enhanced proliferation of fibroblast-like cells from Cx43-null mice [26]. Moreover, in primary cultures, silencing Cx43 enhanced the rate of astrocyte proliferation [27], which is characterized by the upregulation of cyclins D1 and D3 [28]. By contrast, restoration of Cx43 in glioma cells decreased the rate of proliferation [29, 30]. Nevertheless, Cheng et al. have reported that the up-regulation of Cx43 expression is necessary for basic fibroblast growth factor (bFGF) to maintain neural progenitor cells (NPCs) in a proliferative and self-renewal

Fig. 8. A) Effects of Aldo on intracellular calcium concentration ($[Ca^{2+}]_i$) using laser scanning confocal microscopy after loading by Fura-3/AM. Magnification, 10x. The rat MCs were treated with Aldo at a concentration of 0.1, 1, 10 or 100 nM for 24 h. Then, $[Ca^{2+}]_i$ was examined as described in the Materials and Methods section. B). Quantitative analysis of the fluorescence gray value ($[Ca^{2+}]_i$ concentrations in Aldo-induced MC proliferation). "Control" refers to cells growing in DMEM containing 10% FCS without Aldo intervention. Values are given as the mean $\pm$ S.D, and $p < 0.05$ is considered statistically significant by ANOVA ($n = 3$, * $p < 0.05$, ** $p < 0.01$ vs. control).

state [31]. In addition, the decreased proliferation in response to the reduction of the Cx43 expression levels has been observed in primary arterial smooth muscle cells (SMC) [32]. Unlike in our study, Yao et al. reported that the short-term (within 2 h) or long-term (48 h) exposure of MCs to 30 ng/ml PDGF-BB did not alter the level of total Cx43 protein expression [33]. Taken together, this discrepancy between up- or down-regulation of Cx43 expression and cell proliferation may be attributed to cell-, tissue- or connexin-type specificity, different culture conditions, different time courses of stimulator addition, and different concentration-related responses as well as reflect the many facets of connexin function and regulation. Thus, there was insufficient evidence to conclude that Aldo-induced MC proliferation was mediated by down-regulating Cx43 expression. To verify this viewpoint, cell proliferation was re-analyzed by treatment with Aldo under the condition of elevated Cx43 expression. Then, Cx43 over-expression decreased cell proliferation (Fig. 4). Taken together, these results indicated that Aldo promoted MC proliferation by directly down-regulating Cx43 expression at the transcriptional and translational levels.

In addition to the expression of Cx43 contributing to MC proliferation, another point to address was whether Cx43 was also capable of inducing changes in the gap junction channel function, which was analyzed by SLDT [34]. In the present study, there was no difference in the length of dye between the four groups exposed to Aldo at different concentrations and the control group (Fig. 5). Therefore, our data might support the hypothesis that Cx43-mediated MC proliferation may be independent of its well-accepted role in forming a gap junction channel; it could instead act via connexin-dependent mechanisms. In addition to the effects on gene and protein expression that have been confirmed above, connexin-dependent mechanisms was involved in Cx43 localization, its interactions with growth regulating partners (e.g., β -catenin, ZO-1 and CCN proteins), and its phosphorylation at specific sites [35]. Unfortunately, the present data do not allow for a firm conclusion about the precise molecular mechanism by which Cx43 affects cell proliferation, meriting further investigation.

In view of two confirmed pathways (MAPK and PI3K pathways) involved in Aldo-induced MC proliferation [6, 7], we sought to determine whether the similar signaling mechanisms contribute to Aldo-induced Cx43 reduction. Fortunately, we found that Cx43 reduction was significantly reversed for both the mRNA and protein levels by treatment with the MR antagonist spironolactone (Fig. 6.A and 7.A) and ERK1/2 inhibitor PD98059 (Fig. 6.B and 7.B). Nevertheless, the PI3K inhibitor LY294002 affected neither the mRNA nor protein level of Cx43 (Fig. 6.C and 7.C), but it blocked Aldo-induced cell proliferation (Fig. 1.B). Therefore,

our results seemed to indicate the hypothesis that Cx43 expression in Aldo-induced MC proliferation might not be regulated, at least at the transcriptional and translational levels, via the PI3K pathway. Instead, it may possibly be mediated through activation of the PI3K pathway and then induction of other downstream molecules. Just as previously reported, in human MCs, Aldo activated PI3K, subsequently enhanced Akt, mTOR and p70S6 phosphorylation, and finally promoted cell proliferation [6]. In addition, in MCs, the effect of PDGF-BB on Cx43 expression is mediated by PI3K-dependent pathways, considering that the PI3K inhibitor LY294002 prevented the activation of ERK and phosphorylation of Cx43 [14].

PKC is another important pathway implicated in regulating a broad spectrum of cellular functions, including growth, proliferation, differentiation, and carcinogenesis. The PKC isoforms belong to three groups based on calcium dependency and activators. Classical PKCs (including the α , β 1, β 2, and γ isoforms) are calcium-dependent, novel PKCs (including the δ , ϵ , η , and θ isoforms), and atypical PKCs (including the ξ , λ , and τ isoforms) are calcium independent. Several studies have shown that the PKC family, mainly classical PKCs, are implicated in Cx43 phosphorylation at multiple serines (S365, S368, S369, S372, and S373) [36, 37], expression [38] and GJIC regulation [39-41]. We found, for the first time, that inhibition of PKC pathways by GF109203X could also block MC proliferation (Fig. 1.B) and reverse down-regulation of Cx43 expression (Fig. 6.D and 7.D), which matched the effects of the inhibition of the MR, ERK1/2, and PI3K pathways. In addition, Aldo promoted MC proliferation, which was in parallel with increasing $[Ca^{2+}]_i$ in a dose-dependent manner (Fig. 8. A and B). Combined with both aspects, it would ultimately be more plausible, not less, that the PKC pathway might be involved in Aldo-induced MC proliferation.

Conclusions

Taken together, our data support the hypothesis that Cx43 is an important regulator of Aldo-promoted MC proliferation. Furthermore, this is related to down-regulation of the transcriptional and translational levels and is not likely due to changes in the gap junction channel function. This process may be MR-mediated and dependent on the ERK1/2 and PKC pathways without being affected by the PI3K-dependent pathways. Furthermore, the involvement of the ERK1/2 and PKC pathways in Cx43 expression also acts as a good example of the close coordination of molecular events that is of great relevance in cell growth control. However, further investigation is needed to determine whether the *in vitro* results could be extended to conditions *in vivo*, how the signaling pathways were activated, and how Cx43 affects cell proliferation through additional mechanisms. Here, connexins provide us a new option for exploring the molecular mechanism of Aldo-induced MC proliferation, which may also serve as a potential therapeutic approach for mesangial proliferative renal disease.

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Disclosure Statement

The authors have declared no conflict of interest

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