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ORIGINAL ARTICLE

Male Sexual Dysfunction

# Aldosterone induces inflammatory cytokines in penile corpus cavernosum by activating the NF- $\kappa$ B pathway

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Emerging evidence indicates that aldosterone and mineralocorticoid receptors (MRs) are associated with the pathogenesis of erectile dysfunction. However, the molecular mechanisms remain largely unknown. In this study, freshly isolated penile corpus cavernosum tissue from rats was treated with aldosterone, with or without MRs inhibitors. Nuclear factor (NF)-kappa B (NF- $\kappa$ B) activity was evaluated by real-time quantitative PCR, luciferase assay, and immunoblot. The results demonstrated that mRNA levels of the NF- $\kappa$ B target genes, including inhibitor of NF- $\kappa$ B alpha (*I $\kappa$ B- $\alpha$* ), *NF- $\kappa$ B1*, tumor necrosis factor-alpha (*TNF- $\alpha$* ), and interleukin 6 (*IL-6*), were higher after aldosterone treatment. Accordingly, phosphorylation of p65/RelA, I $\kappa$ B- $\alpha$ , and inhibitor of NF- $\kappa$ B kinase- $\beta$  was markedly increased by aldosterone. Furthermore, knockdown of MRs prevented activation of the NF- $\kappa$ B canonical pathway by aldosterone. Consistent with this finding, ectopic overexpression of MRs enhanced the transcriptional activation of NF- $\kappa$ B by aldosterone. More importantly, the MRs antagonist, spironolactone blocked aldosterone-mediated activation of the canonical NF- $\kappa$ B pathway. In conclusion, aldosterone has an inflammatory effect in the corpus cavernosum penis, inducing NF- $\kappa$ B activation via an MRs-dependent pathway, which may be prevented by selective MRs antagonists. These data reveal the possible role of aldosterone in erectile dysfunction as well as its potential as a novel pharmacologic target for treatment.

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**Keywords:** aldosterone; erectile dysfunction; NF- $\kappa$ B; receptors

## INTRODUCTION

Erectile dysfunction (ED) is a common sexual dysfunction, with estimating that it affects 22% of men over 40 years of age.<sup>1</sup> The incidence of ED is associated with diabetes, hypertension, aging, depression, and being overweight. The Massachusetts Male Aging Study reported that 52% men between 40 and 70 years old were affected by ED of varying severity.<sup>2</sup> Among men with chronic coronary artery disease, the incidence of ED was approximate 75%.<sup>3,4</sup> Although the etiology of ED is yet to be fully clarified, multiple signaling networks have been implicated in ED, including the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) pathway, generation of reactive oxygen species (ROS), renin/angiotensin system (RAS), RhoA/Rho-associated protein kinase pathway, and tumor necrosis factor-alpha (TNF- $\alpha$ ) pathway.<sup>5</sup>

Notably, the TNF- $\alpha$  pathway has direct effects on vascular function and plays a critical role in cardiovascular diseases (CVD) which shares several risk factors with ED. Accumulating evidence from clinical and experimental studies also suggested that TNF- $\alpha$  is implicated in ED.<sup>6</sup> For example, elevated plasma TNF- $\alpha$  levels have been linked to ED.<sup>7</sup> TNF- $\alpha$  infusion was found to impair the function of the corpora cavernosa in mice.<sup>8</sup> Binding of TNF- $\alpha$  to TNFR1 stimulated the expression of nuclear factor (NF)-kappa B (NF- $\kappa$ B) target genes, which are important for inflammatory responses and

cell survival.<sup>9</sup> In the canonical NF- $\kappa$ B pathway, TNF- $\alpha$  induces linear ubiquitination of inhibitor of kappaB kinase-gamma (IKK- $\gamma$ )/NEMO and phosphorylation of IKK- $\beta$ , with subsequently phosphorylation of inhibitor of NF- $\kappa$ B alpha (I $\kappa$ B- $\alpha$ ).<sup>10,11</sup> Phosphorylated I $\kappa$ B- $\alpha$  is ubiquitinated by beta-transducing repeat-containing protein ( $\beta$ -TRCP) and degraded by the 26S proteasome, resulting in releasing the p65 and p50 protein dimers for nuclear translocation.<sup>12</sup>

The hormone aldosterone, which was discovered over 50 years ago,<sup>13</sup> is responsible for maintaining the electrolyte balance by the regulation of Na<sup>+</sup> and K<sup>+</sup> in the distal tubules and collecting ducts of the kidney.<sup>13</sup> A retrospective study reported a 29% increase in aldosterone levels between control and severe ED groups.<sup>14</sup> In human penile corpus cavernosum tissue, aldosterone facilitated the noradrenaline-induced contraction in organ bath, but not NO-dependent relaxation.<sup>15</sup> mRNA expression of 11 beta-hydroxysteroid dehydrogenase type 2 (11 $\beta$ HSD2) and mineralocorticoid receptors (MRs), the receptor for aldosterone, have been proved to be existed in isolated human penile corpus cavernosum tissue.<sup>16</sup> It has been reported that aldosterone activates the NF- $\kappa$ B pathway in principal cells of the cortical collecting duct. However, the role of aldosterone in the corpus cavernosum of ED patients remains elusive. In the present study, we report that aldosterone stimulates the NF- $\kappa$ B transcriptional activity in penile corpus cavernosum tissue through MRs-dependent pathway.

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## MATERIALS AND METHODS

### Ethics approval

The study was conducted in accordance with ethical guidelines and was approved by the Ethics Committee of Huashan Hospital, Fudan University.

### Isolated corpus cavernosa tissues analysis

All experimental procedures were performed in accordance with the national experimental animal science guide for the care and use of laboratory animals. Sexually mature male Wistar-Kyoto rats (500–600 g body weight) were obtained from the Experimental Animal Center of Fudan University (Shanghai, China). Prior to experiments, rats were housed in a room with controlled temperature ( $23 \pm 1^\circ\text{C}$ ) and a 12 h light/12 h dark cycle. Access to water and food was *ad libitum*. Penis corpus cavernosum tissue was obtained from 16-week-old rats. After euthanasia, the penis of each rat was removed, and real-time PCR and immunoblot assays were performed, as described previously.<sup>17</sup> In each experimental group, isolated corpus cavernosa tissues from three rats were pooled to minimize the individual variation. Briefly, the whole penis was placed in cold Krebs-Henseleit buffer (NaCl: 118.4 nmol l<sup>-1</sup>, KCl: 4.7 nmol l<sup>-1</sup>, CaCl<sub>2</sub>: 2.5 nmol l<sup>-1</sup>, KH<sub>2</sub>PO<sub>4</sub>: 1.2 nmol l<sup>-1</sup>, NaHCO<sub>3</sub>: 25 nmol l<sup>-1</sup>, glucose: 11.7 nmol l<sup>-1</sup>). Then, the tunica albuginea, corpus spongiosum, and the urethra were anatomically separated and discarded. Longitudinal strips of corpus cavernosum were isolated and pulverized into cubes of approximately 1 mm<sup>3</sup>, which were placed in organ-bath chambers at 37°C (Radnoti Glass Technology Inc., Monrovia, CA, USA), followed by the addition of 10 ml of Krebs-Henseleit solution. The cubes were continuously bubbled with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>, and equilibrated for 30 min for further *in vitro* experiments. Three to four cubes were incubated in a single well, with or without the administration of aldosterone (10 nmol l<sup>-1</sup>–10 μmol l<sup>-1</sup>, Sigma Chemical Co., St. Louis, MO, USA) or recombinant human TNF-α protein (rhTNF-α or TNF-α, 20 ng ml<sup>-1</sup>, R and D system, Minneapolis, MN, USA), for prespecified time periods (1 h, 2 h, 3 h, 4 h, 5 h, and 6 h). With respect to co-administration, aldosterone and spironolactone (Sigma Chemical Co., St. Louis, MO, USA) were simultaneously added to the medium before harvest.

### Analysis of transcriptional activity by qRT-PCR

RNA was extracted using QIAGEN RN easy mini kit, according to manufacturer's instructions (Studio City, CA, USA). For each sample, 1 μg total RNA was reverse transcribed using the iScript Reverse Transcription Supermix (1708841, Bio-Rad Laboratories, Richmond, CA, USA). The generated cDNA template was mixed with primers for *IκB-α* (Forward: 5'-TGAAGGACGAGGAGTACGAGC-3'; Reverse: 5'-TGCAGGAACGAGTCTCCGT-3'), *NF-κB1* (Forward: 5'-ATGGCAGACGATGATCCCTAC-3'; Reverse: 5'-CGGAATCGAATCCCTCTGTT-3'), *IL-6* (Forward: 5'-CTGCAAGAGACTTCCATCCAG-3'; Reverse: 5'-AGTGGTATAGACAGGTCTGTTGG-3'), *TNF-α* (Forward: 5'-CAGGCGGTGCCTATGTCTC-3'; Reverse: 5'-CGATCACCCCGAAGTTCAGTAG-3') and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Forward: 5'-AGGTCGGTGTGAACGGATTTG-3'; Reverse: 5'-GGGGTCGTTGATGGCAACA-3'), as well as PowerUp SYBR Green Master Mix (A25778, Applied Biosystems, Foster City, CA, USA), and the real-time RT-PCR reaction was conducted with the CFX96 Touch Real-Time PCR Detection System. Data quantitation was performed using the comparative C<sub>T</sub> (ΔΔC<sub>T</sub>) method, with *GAPDH* gene expression as an endogenous reference.

### In vitro assay in HMVECs

The human microvascular endothelial cells (HMVECs) were a generous gift from Wei Lab.<sup>18</sup> The generation and identification of HMVECs were described in detail by Shao and Guo.<sup>19</sup> Briefly, HMVECs were cultured in endothelial growth medium containing 2% bovine serum and 0.2% bovine brain extract. The cells were grown at 37°C with 5% CO<sub>2</sub> and subcultured at 50%–80% confluence using 0.05% trypsin–0.02% Ethylenediaminetetraacetic acid (EDTA). Penile corpus cavernosum tissues and confluent HMVECs were treated with aldosterone (10<sup>-9</sup>–10<sup>-7</sup> mol l<sup>-1</sup>), which was dissolved in dimethylsulfoxide (DMSO; 10 nmol l<sup>-1</sup>), the vehicle control. In selected experiments, cells were co-incubated with spironolactone.

### Ectopic overexpression and endogenous knockdown

A Flag/Myc-tagged MRs plasmid was purchased from OriGene Technologies (Rockville, MD, USA). Cells were transfected using Lipofectamine (Life Technologies, Gaithersburg, MD, USA) in OptiMEM medium, according to the manufacturer's instructions. At 48 h posttransfection, transfected cells were subjected to immunoblot analysis to detect the efficacy of transfection. Lentiviral short hairpin RNAs (shRNAs) for human NR3C2 were purchased from GE Dharmacon (Chicago, IL, USA).

The following are DNA oligonucleotide sequences for the NR3C2-directed shRNA No. 1 (antisense, 5'-TTGTCTT GCTGAAGGCAAG-3'), shRNA No. 2 (antisense, 5'-AAGGCAA AGTTCTTCTGGG-3'). Lentiviral shRNA constructs against green fluorescent protein (GFP) were a generous gift from Dr. Yang Sun (Department of Dermatology, Qilu Hospital, Shandong University, China).

### Western blot analysis

Cells were lysed in EBC buffer (50 mmol l<sup>-1</sup> Tris [pH 7.5], 120 mmol l<sup>-1</sup> NaCl, 0.5% NP-40) supplemented with protease inhibitors and phosphatase inhibitors (phosphatase inhibitor cocktail set I and II, Calbiochem, La Jolla, CA, USA). Subcellular fractions of tissues were extracted by a Subcellular Proteome Extraction Kit (Merck Millipore, Darmstadt, Germany). Briefly, cells at 80% confluence in a 10-cm dish (5 × 10<sup>6</sup>–8 × 10<sup>6</sup> cells) were washed twice using cold phosphate-buffered saline solution (PBS) or wash buffer. The dish was overlaid with 1 ml of extraction buffer I, without disturbing cells. After incubation on ice for 10 min with gentle agitation, the supernatant was transferred to a clean tube as the cytoplasmic fraction. Remnant cellular material was added to 500 μl of extraction buffer III and incubated for 10 min at 4°C with gentle rotation. The samples were sonicated and centrifuged at 15 000 g for 10 min to obtain the supernatant as the nuclear fraction. Protein concentrations of the lysates were measured using the Bio-Rad protein assay reagent on a DU-800 UV/VIS spectrophotometer (Beckman Coulter, Brea, CA, USA). The lysates were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with the indicated antibodies. The detailed information of antibodies used in this study is listed in Table 1. The protocol for protein extraction and immunoblotting has been described in detail previously.<sup>20</sup>

### Luciferase assay

Luciferase assays were performed as described previously.<sup>21</sup> Briefly, cells grown in 6-well plates were transfected with an NF-κB firefly luciferase reporter plasmid 3X-κB-Luc (a gift from Dr. Hiroyuki Inuzuka) and a firefly luciferase reporter containing the IL-6 promoter (a gift of Dr. Hiroyuki Inuzuka) along with a pRL-CMV plasmid (Promega, Madison, WI, USA). After 24 h, cells were split into 96-well plates. After

**Table 1: A list of antibodies used in this study**

Source	Antibody	Description	Dilution
Cell signaling technology	p65/RelA	Transcription factor p65	1:1000
	I $\kappa$ B- $\alpha$	NF-kappa-B inhibitor alpha	1:2000
	IKK- $\alpha$	I $\kappa$ B kinase- $\alpha$	1:1000
	IKK- $\beta$	I $\kappa$ B kinase- $\beta$	1:1000
	p-p65 (Ser536)	Phosphorylated p65	1:1000
	p-I $\kappa$ B- $\alpha$ (Ser32)	Phosphorylated I $\kappa$ B- $\alpha$	1:2000
	p-IKK- $\alpha/\beta$ (Ser176/180)	Phosphorylated IKK- $\alpha/\beta$	1:1000
	NF- $\kappa$ B2	p100 and p52	1:1000
	Lamin B1	A component of nuclear lamina	1:2000
	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	1:2000
	Sigma	Vinculin	Actin filament (F-actin)-binding protein
Anti-flag		Rabbit polyclonal anti-flag antibody (F-2425)	1:1000
Anti-flag		Mouse monoclonal anti-flag antibody (F-3165)	1:10 000
NR3C2		Mineralocorticoid receptor/nuclear receptor subfamily 3, group C2	1:1000

NF: nuclear factor; IKK: inhibitor of kappa B kinase

stimulation with aldosterone, firefly luciferase activity was measured using the Dual Luciferase Assay System and normalized to renilla luciferase activity according to manufacturer's instructions (Promega, Madison, WI, USA).

#### Statistical analysis

Results are presented as mean  $\pm$  standard deviation from at least three independent experiments. Comparison between two groups was performed by one-way analysis of variance (ANOVA).  $P < 0.05$  was considered statistically significant.

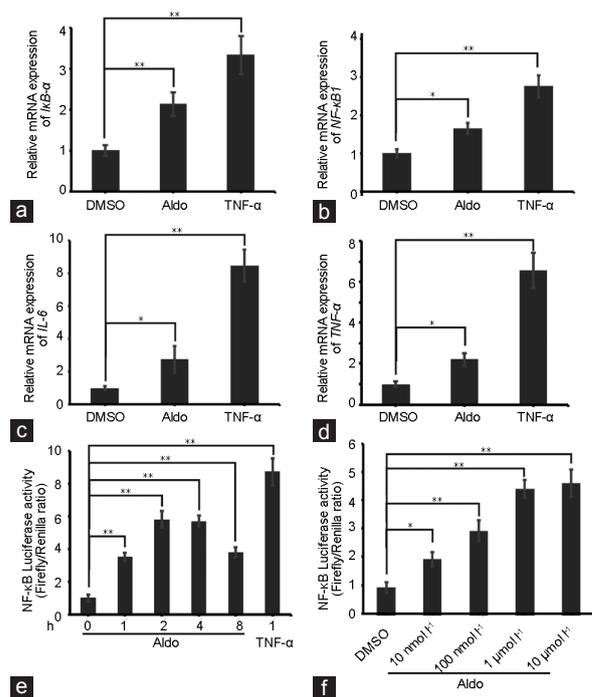
## RESULTS

### Activation of NF- $\kappa$ B transcription in freshly isolated corpus cavernosum

The freshly isolated penile corpus cavernosum tissues were treated with DMSO, aldosterone ( $1 \mu\text{mol l}^{-1}$ ) or TNF- $\alpha$  ( $20 \text{ ng ml}^{-1}$ ) for 1 h in Krebs solution, without bovine serum or antibiotics. qRT-PCR was applied to detect mRNA expression of the specified genes: *I $\kappa$ B- $\alpha$* , *NF- $\kappa$ B1*, *IL-6*, *TNF- $\alpha$* , and *GAPDH*. As shown in **Figure 1a–d**, relative mRNA expression of *I $\kappa$ B- $\alpha$* , *NF- $\kappa$ B1*, *IL-6*, and *TNF- $\alpha$*  was increased after aldosterone or TNF- $\alpha$  treatment compared with DMSO as a negative control ( $P < 0.05$ ). TNF- $\alpha$  was a well-recognized stimulator of the canonical NF- $\kappa$ B pathway and was used as a positive control. Although transcriptional activity of NF- $\kappa$ B by aldosterone was lower than that in the TNF- $\alpha$  group, the activating effect of aldosterone was statistically significant when compared with the negative control group ( $P < 0.05$ ). The optimal aldosterone concentration for its activation effect was confirmed and evaluated by luciferase assay in HMVECs. After co-transfection of NF- $\kappa$ B firefly luciferase reporter plasmid 3X- $\kappa$ B-Luc and a firefly luciferase reporter, luciferase assays showed that NF- $\kappa$ B transcriptional activity peaked at 2–4 h after aldosterone treatment (**Figure 1e**). Concentration-dependent assays indicated that the optimal concentration of aldosterone for maximum activation was approximately  $1\text{--}10 \mu\text{mol l}^{-1}$  (**Figure 1f**). In this study,  $1 \mu\text{mol l}^{-1}$  was selected as the concentration to amplify and visualize the effects of aldosterone on the corpus cavernosum and HMVECs, although  $1 \mu\text{mol l}^{-1}$  was hundreds of times higher than under physiological conditions.

### The canonical NF- $\kappa$ B pathway is activated by aldosterone

Protein readouts of NF- $\kappa$ B activation in the freshly isolated penile corpus cavernosum tissues were detected by immunoblot (**Figure 2a**). Phosphorylation of p65/RelA was increased at 1 h and 2 h after



**Figure 1:** Aldosterone (Aldo) stimulates nuclear factor (NF)-kappa B transcriptional activity in freshly isolated corpus cavernosum from rats. Relative mRNA levels of (a) inhibitor of NF- $\kappa$ B alpha (*I $\kappa$ B- $\alpha$* ), (b) *NF- $\kappa$ B1*, (c) interleukin 6 (*IL-6*), and (d) *TNF- $\alpha$*  were increased after aldosterone or tumor necrosis factor-alpha (TNF- $\alpha$ ,  $20 \text{ ng ml}^{-1}$ ) treatment compared with dimethylsulfoxide as a negative vehicle control. (e) Luciferase assays showed that maximum transcriptional activity of NF- $\kappa$ B occurred at 1–4 h after aldosterone treatment in human microvascular endothelial cells. (f) Concentration-dependent assays indicated that the optimal concentration of aldosterone for maximum effect was approximately  $1 \mu\text{mol l}^{-1}$ . DMSO: dimethylsulfoxide. Data are presented as the mean  $\pm$  standard deviation ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$  (one-way analysis of variance).

aldosterone stimulation. Concordant with this finding, I $\kappa$ B- $\alpha$  was phosphorylated at 1 h, with a marked decline from 1 h to 4 h, indicating that the NF- $\kappa$ B signaling pathway was active during this time. Phosphorylation of IKK- $\alpha/\beta$  was also altered by aldosterone treatment. Of importance, p100 and p52, which were key factors in the noncanonical NF- $\kappa$ B pathway, were not affected by aldosterone (**Figure 2a**). Consequently, phosphorylated p65/RelA

could undergo nuclear translocation and bind to the promoters of target genes. These molecular changes were also visualized in protein subcellular fractions analyzed by immunoblot (Figure 2b). In the nuclear fraction of isolated penile corpus cavernosum tissues, p65 protein was increased in the experimental groups compared with DMSO treatment, demonstrating that both TNF- $\alpha$  (20 ng ml<sup>-1</sup>) and aldosterone (1  $\mu$ mol l<sup>-1</sup>) induced the nuclear translocation of p65. Consistent with this finding, in the cytosolic fraction, phosphorylation of I $\kappa$ B- $\alpha$  increased dramatically and subsequently declined after aldosterone and TNF- $\alpha$  treatment (Figure 2b).

#### The activation of NF- $\kappa$ B signaling pathway is MRs dependent

To determine whether the activation of NF- $\kappa$ B signaling by aldosterone was MR dependent, experiments on the isolated penile corpus cavernosum were conducted with an MR antagonist, spironolactone. Compared with aldosterone stimulation, the combination of aldosterone and spironolactone restored the relative mRNA transcription of NF- $\kappa$ B target genes, including *I $\kappa$ B- $\alpha$* , *NF- $\kappa$ B1*, *IL-6*, and *TNF- $\alpha$*  (Figure 3a–3d). Interestingly, spironolactone treatment (0.5  $\mu$ mol l<sup>-1</sup> for 1 h) was sufficient to reverse the activating effect of aldosterone (1  $\mu$ mol l<sup>-1</sup>) on NF- $\kappa$ B transcriptional activity. The optimal concentration of spironolactone was further confirmed by luciferase assays in a concentration-dependent manner in HMVECs. Notably, 0.5  $\mu$ mol l<sup>-1</sup> was the least effective concentration to prevent the activation effect of aldosterone (Figure 3e). In HMVEC cells, a gain-of-function assay was performed by ectopic overexpression of a Flag/Myc-tagged MR protein. In MR-overexpressing cells, the transcriptional activation effect of aldosterone was amplified, based on luciferase assays (Figure 3f). To further confirm this mechanism at an endogenous level, shRNAs targeting the MR-encoding gene, nuclear receptor subfamily 3 group C member 2 (*NR3C2*), were packaged into a lentivirus to construct stable cell lines with MR knockdown. As shown in Figure 3g, approximately 80% of endogenous MRs were knocked down by the shRNA. In cell lines with stable MR knockdown, aldosterone failed to activate the transcriptional activity of the NF- $\kappa$ B complex, according to luciferase assays (Figure 3h).

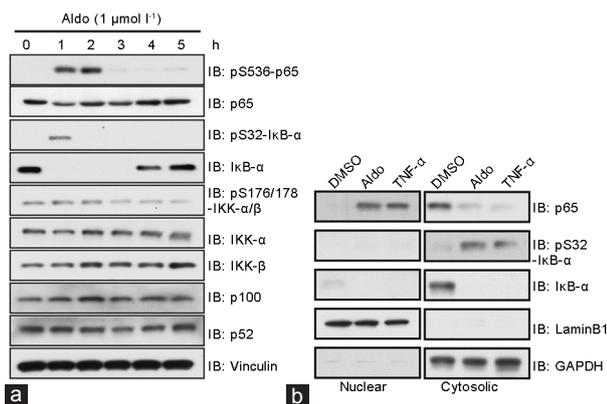
#### Spironolactone reverses the activation of NF- $\kappa$ B by aldosterone

To gain further insight into whether the MR-dependent effect of aldosterone was consistent in freshly isolated penile corpus cavernosum tissues, prevention of NF- $\kappa$ B transcriptional activation was examined by immunoblotting in a time course manner. Compared with previous assays using aldosterone treatment (Figure 2a), phosphorylation of p65 and I $\kappa$ B- $\alpha$  was delayed, and sharply attenuated, when spironolactone (0.5  $\mu$ mol l<sup>-1</sup>) and aldosterone (1  $\mu$ mol l<sup>-1</sup>) were added at the same time for indicated hours (Figure 4a). In isolated penile corpus cavernosum tissues, the time course of relative mRNA expression of NF- $\kappa$ B target genes was investigated to illustrate the dynamic effect of aldosterone and spironolactone on NF- $\kappa$ B signaling. As shown in Figure 4b, the increase in I $\kappa$ B- $\alpha$  mRNA induced by aldosterone was partially inhibited by spironolactone. Accordingly, spironolactone prevented the transcriptional activation of TNF- $\alpha$  (Figure 4c). Thus, in penile corpus cavernosum tissues, aldosterone activated NF- $\kappa$ B signaling, which was prevented by an MR antagonist, spironolactone. In both freshly isolated corpus cavernosum tissues and cultured HMVECs, NF- $\kappa$ B signaling was activated by aldosterone in an MR-dependent manner.

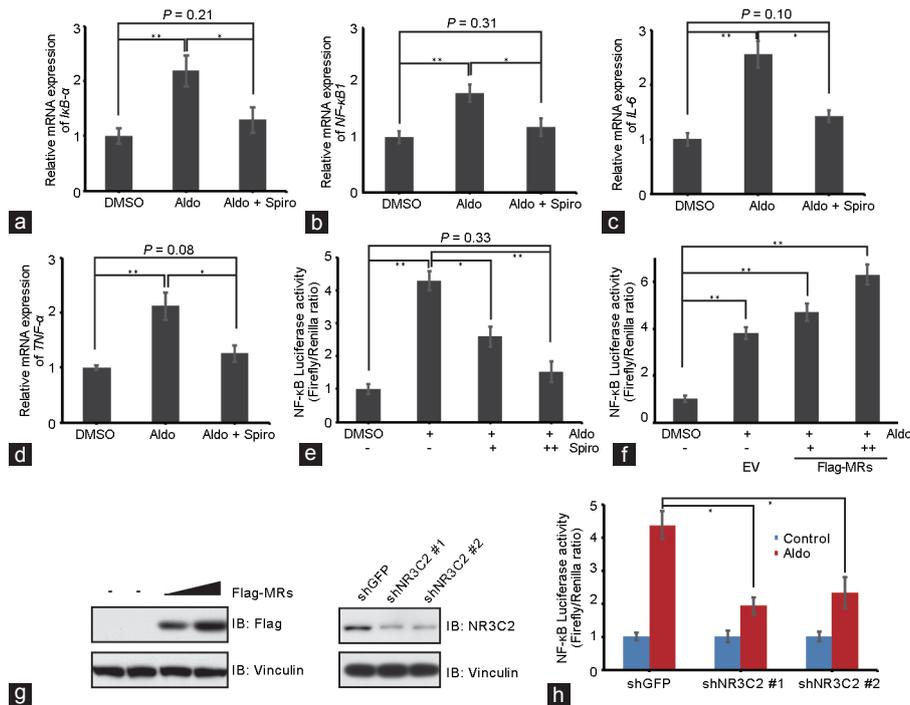
#### DISCUSSION

This study aimed to explore the mechanism of aldosterone in isolated penile corpus cavernosum tissues from rats. As a novel finding, the experiments showed that aldosterone activates transcriptional activity of the canonical NF- $\kappa$ B pathway in an MR-dependent manner in penile corpus cavernosum tissue and HMVECs. Specifically, transcription of NF- $\kappa$ B target genes, including *I $\kappa$ B- $\alpha$* , *NF- $\kappa$ B1*, *TNF- $\alpha$* , and *IL-6*, was enhanced by aldosterone treatment. Accordingly, phosphorylation of p65, I $\kappa$ B- $\alpha$ , and IKK- $\beta$  was also markedly increased. Furthermore, knockdown of MRs by lenti-NR3C2-shRNA prevented aldosterone-mediated activation of the canonical NF- $\kappa$ B pathway. In line with these findings, ectopic overexpression of MRs enhanced the transcriptional activation of NF- $\kappa$ B by aldosterone. More importantly, spironolactone inhibited the canonical NF- $\kappa$ B pathway activation induced by aldosterone.

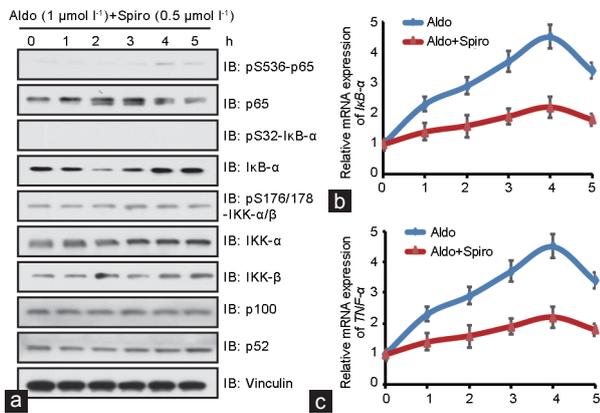
Two signaling pathways are involved in the functional NF- $\kappa$ B activity, the canonical and noncanonical pathways (also known as the classical and alternative pathways, respectively). Noncanonical NF- $\kappa$ B signaling, which is stimulated by CD40, lymphotoxin- $\beta$  receptor, or B-cell activating factor receptor, contributes to lymphoid organogenesis, B-cell maturation and osteoclast differentiation.<sup>22</sup> A critical signaling component of the noncanonical NF- $\kappa$ B pathway is the phosphorylation of IKK $\alpha$ , which induces phosphorylation-dependent ubiquitination and processing of p100.<sup>23</sup> In this study, immunoblotting of protein markers of the canonical pathway indicated that aldosterone elicited the canonical, but not noncanonical NF- $\kappa$ B signaling, in an MRs-dependent manner. Notably, canonical NF- $\kappa$ B signaling stimulated the production of inflammatory cytokines, such as TNF- $\alpha$  and IL-6, which promoted chemotaxis and differentiation of lymphocytes, causing pathogenic effects over time.<sup>24</sup> In relation to its influence on vascular disease, aldosterone was hypothesized to induce similar pathogenic effects in penile corpus cavernosum, including stress from reactive oxygen species, tissue remodeling, extracellular matrix degradation, and a self-perpetuating cycle of endothelial dysfunction and inflammation.<sup>25</sup> Here, HMVECs, derived from vascular endothelial cells, were selected for *in vitro* experiments due to the shared features and risk factors between ED and cardiovascular disease, which prompted initial evaluation of NF- $\kappa$ B signaling at an endothelial level.



**Figure 2:** The canonical nuclear factor (NF)-kappa B pathway is activated after aldosterone (Aldo) treatment. (a) Protein markers of NF- $\kappa$ B activation were detected by immunoblot analysis of freshly isolated penile corpus cavernosum tissues. (b) In the nuclear fraction of isolated penile corpus cavernosum tissues, p65 protein was increased in the experimental groups compared with dimethylsulfoxide vehicle control. In the cytosolic fraction, phosphorylation of inhibitor of NF- $\kappa$ B alpha (I $\kappa$ B- $\alpha$ ) displayed a sharp increase and subsequently declined after aldosterone or tumor necrosis factor-alpha (TNF- $\alpha$ , 20 ng ml<sup>-1</sup>) treatment. DMSO: dimethylsulfoxide; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.



**Figure 3:** Activation of nuclear factor (NF)-kappa B signaling pathway was mineralocorticoid receptors (MRs) dependent. Co-administration of aldosterone (Aldo) and spironolactone (Spiro) in human microvascular endothelial cells prevented the increase in relative mRNA levels of NF-κB target genes, including (a) inhibitor of NF-κB alpha (*IkB-α*), (b) *NF-κB1*, (c) interleukin 6 (*IL-6*) and (d) tumor necrosis factor-alpha (*TNF-α*). (e) Luciferase assays conducted in a concentration-dependent manner in HMVECs. 0.5 μmol l<sup>-1</sup> of spironolactone was the least effective concentration to prevent the activation effect of aldosterone. (f) The transcriptional activation effect of aldosterone was augmented, based on luciferase assays, when MRs were ectopically overexpressed in HMVECs. (g) Approximately 80% of endogenous MRs were knocked down by shRNA transfection in HMVECs. (h) In stable MR-knockdown HMVEC lines, aldosterone failed to activate the transcriptional activity of NF-κB, according to luciferase assays. Data are presented as the mean ± standard deviation (*n* = 3). \**P* < 0.05, \*\**P* < 0.01 (one-way analysis of variance). EV: empty vector; HMVECs: human microvascular endothelial cells; DMSO: dimethylsulfoxide; NR3C2: mineralocorticoid receptor/nuclear receptor subfamily 3, group C2; GFP: green fluorescent protein.



**Figure 4:** Reversion of aldosterone (Aldo)-induced nuclear factor (NF)-kappa B activation. (a) Phosphorylation of p65 and inhibitor of NF-κB alpha (*IkB-α*) was attenuated by co-administration of spironolactone (Spiro, 0.5 μmol l<sup>-1</sup>) and aldosterone (1 μmol l<sup>-1</sup>) in freshly isolated penile corpus cavernosum tissues. (b) Time courses obtained from quantitative real-time polymerase chain reaction showed that the aldosterone-mediated increase in *IkB-α* mRNA expression was dramatically inhibited by spironolactone in freshly isolated penile corpus cavernosum tissues. (c) Spironolactone prevented the aldosterone-mediated increase in tumor necrosis factor-alpha mRNA expression in freshly isolated penile corpus cavernosum tissues.

Importantly, an MR antagonist failed to completely reverse NF-κB activation by aldosterone. This may be explained by the MR-independent actions of aldosterone, in particular, direct activation of nicotinamide

adenine dinucleotide phosphate (NADPH) oxidase, which causes oxidative stress in endothelial cells, the heart or macrophages.<sup>26,27</sup> As a result, reactive oxygen species generated as a byproduct of aldosterone activity may promote the NF-κB signaling pathway.<sup>28</sup> However, based on data from this study, the MR-independent action of aldosterone on NF-κB signaling is not the dominant effect compared with the MR-dependent pathway. Therefore, MR antagonists may have potential to ameliorate injury to the penile corpus cavernosum tissues caused by aldosterone-MR-NF-κB pathway activation.

Taken together, the study results demonstrated that aldosterone activated the canonical NF-κB pathway in penile corpus cavernosum tissues and HMVECs in an MR-dependent manner. Increased expression of MRs led to elevation of NF-κB transcriptional activity induced by aldosterone, while MR knockdown prevented the activation. The MR antagonist spironolactone markedly suppressed activation of NF-κB signaling by aldosterone. These data may reveal a potential pharmacologic target for the treatment of ED.

#### AUTHOR CONTRIBUTIONS

FW, ZQX, and SHM performed the experiments, elaborated and analyzed the data. HWJ and QD analyzed and discussed the data. JMH and JQW contributed to the discussion of results. FW and ZQX designed the study, analyzed and discussed the results, and wrote the paper. All authors read and approved the final manuscript.

#### COMPETING INTERESTS

All authors declare no competing interests.

## ACKNOWLEDGMENTS

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