S1P1 Gene Transfection Improves Erectile Function in Spontaneously Hypertensive Rats

Zhichao Du, Jun Jiang, Bo Cheng, and Rui Jiang

OBJECTIVE To investigate the relationship between the upregulated expression of sphingosine-1-phosphate receptor 1 (S1P1) in the corpus cavernosum and erectile function in spontaneously hypertensive rats (SHRs).

METHODS Twelve-week-old healthy male Wistar-Kyoto rats (WKY) and SHR rats were randomly divided into 4 groups: WKY, SHR, WKY transfection, and SHR transfection ($n = 5$). A lentiviral vector carrying the S1P1 gene was injected into the corpus cavernosum penis of rats in the transfection groups (1×10^9 TU/mL, 20 μL). After 1 week, the maximum penile intracavernous pressure/mean arterial pressure (ICP_{max}/MAP), nitric oxide (NO) content, and the expression of eNOS, P-eNOS, ROCK1, ROCK2, and S1P1 in the corpus cavernosum penis of rats in each group were measured.

RESULTS The ICP_{max}/MAP value was significantly higher in the SHR transfection group than in the SHR group under 3-V and 5-V electrical stimulations ($P < .01$). The expression of S1P1 and P-eNOS proteins significantly increased ($P < .01$), while that of ROCK1 and ROCK2 proteins significantly decreased ($P < .01$) in the SHR transfected group compared with the SHR group. The NO content was significantly higher in the SHR transfection group than in the SHR group ($P < .01$).

CONCLUSION The upregulated expression of S1P1 in SHR corpus cavernosum penis may improve the SHR erectile function by upregulating the P-eNOS/eNOS ratio and inhibiting the RhoA/Rho kinase signaling pathway. UROLOGY 133: 249.e1–249.e7, 2019. © 2019 Elsevier Inc.

Erectile dysfunction (ED) is a condition in which the penis cannot reach or maintain an erection, thus leading to unsatisfactory sexual behavior.[1] Hypertension is closely related to ED. About 20%-25% of 45- to 50-year-old patients with hypertension also have ED.[2,3] The cause for hypertension with ED may be related to endothelial dysfunction in the corpus cavernosum, decreased eNOS activity and expression, and upregulation of RhoA/Rho kinase signaling pathway.[4] Phosphodiesterase type 5 inhibitors currently the first-line drug for ED, but it has an effective rate of only 60%-70% for treating hypertensive ED.[5,6] Therefore, new treatments need to be explored to improve efficacy.

Sphingosine-1-phosphate (S1P) is involved in the upstream regulation of eNOS and RhoA/Rho kinase.[7] S1P receptors are a class of G-protein-coupled receptors on cell membranes. They are divided into 5 subtypes, of which S1P1-3 is widely distributed in the corpus cavernosum.[4] In cultured endothelial cells, the knockout of S1P1 can result in no response of eNOS to S1P, suggesting that S1P1 plays a dominant role in the response of eNOS to S1P.[8] S1P1 is mainly expressed on the vascular endothelial cell membrane of the corpus cavernosum. It can upregulate the PI3K/Akt/eNOS signaling pathway after specific binding to S1P[9] and promote nitric oxide (NO) synthesis by the activation of eNOS, thereby inducing penile erection.[10]

Previous studies have confirmed that a decrease in the expression of S1P1 in the corpus cavernosum of spontaneously hypertensive rats (SHRs) can lead to ED by inhibiting the eNOS/NO/cGMP signaling pathway.[4] Nonetheless, whether upregulating the expression of S1P1 in the corpus cavernosum of SHR can improve the erectile function is still unclear. In this study, the expression of S1P1 in the vascular endothelial cells of the corpus cavernosum was upregulated by injecting a lentiviral vector carrying S1P1 gene into the SHR corpus cavernosum, to investigate its effect on the erectile function of SHR and the underlying mechanism, thereby providing a new target for treating hypertensive ED.

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MATERIALS AND METHODS

Animals and Grouping

Ten 12-week-old male Wistar-Kyoto (WKY) rats and 10 male SHRs of the same age were purchased from Dashuo Company, Chengdu, China [Number of the certificate: SCXK (Chuan) 2015-030]. They were divided into WKY, SHR, WKY transfection, and SHR transfection groups randomly. All procedures were carried out following the guidelines of the China Animal Care Committee.

Methods

Animal Model Establishment. According to the literature,[11,12] a lentiviral vector (virus titer, 1×10^9 TU/mL; 20 μLper rat)carrying S1P1 gene (JiKai Gene Company, GCPL0142868, China) was injected into the corpus cavernosum of the anesthetized rats in the transfection groups using a 26G needle. The bottom of the penis was wrapped with an elastic band prior to the injection, which was removed 3 minutes after the injection.

Determination of ICP_{max}/MAP and Serum Testosterone. At the end of 1 week from transfection, the right common carotid artery was successfully punctured using a 26G needle filled with heparinized saline to record the mean arterial pressure (MAP) changes in the common carotid artery. The posterior part of the bilateral lobes of the prostate was dissected to find star-shaped major pelvic ganglia, which were considered the electrical stimulation point. Then, a 24G needle filled with heparinized saline was inserted into the corpus cavernosum and connected to a pressure transducer to measure the intracavernous pressure (ICP). Different electrical stimulations were applied (intensities of 0 V, 3 V, and 5 V; amplitude of 5 milliseconds; stimulation frequency of 12 Hz; duration of 45 seconds; and stimulation interval of 3 minutes). After the measurement, 2 mL of blood was collected from the common carotid artery, and serum testosterone was measured by radioimmunoassay (Bayer Inc, Berlin, Germany). Subsequently, the rats were sacrificed by excessive anesthesia. After all extratunica tissues were removed, the corpus cavernosum from proximal to distal were divided into 4 parts, which were in orderly used for immunofluorescence staining, immunohistochemistry, Western blot analysis, and NO content determination, respectively.

Immunofluorescence Staining. The frozen sections of the corpus cavernosum were subjected to immunofluorescence treatment, and images were taken and analyzed using an inverted microscope (Olympus CK40, Tokyo, Japan). The expression of Green fluorescent protein (GFP) was quantified by counting, and the percentage of GFP-positive cells over the total number of cells was used as a transfection index.

Determination of the Expression of S1P1, P-eNOS, eNOS, ROCK1, and ROCK2 in the Corpus Cavernosum Using Immunohistochemistry. Referring to previous studies,[13,14] the frozen sections of the corpus cavernosum were added dropwise with rabbit antirat S1P1, P-eNOS, eNOS polyclonal antibody (1:50) (Abcam, Cambridge, UK), rabbit antirat ROCK1 polyclonal antibody (1:300), and donkey antirat ROCK2 polyclonal antibody (1:50) (Santa Cruz, CA). After incubation with the primary antibodies, horseradish peroxidase-labeled goat antirabbit immunoglobulin G (IgG) (1:100) and goat antidonkey IgG (1:100) (Beyotime Institute of Biotechnology, China) were added and developed. The integrated optical densities (IOD) were measured using Image-Pro Plus version 6.0 (Media Cybernetics Inc, MD).

Determination of the Expression of S1P1, P-eNOS, eNOS, ROCK1, and ROCK2 in the Corpus Cavernosum Using Western Blot Analysis. The protein was extracted from the corpus cavernosum tissue, and the protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). According to the method described in a previous study,[13,14] electrophoresis was sequentially performed at 60 V and 120 V, followed by transmembrane voltage at 100 V. Subsequently, rabbit antirat S1P1 (1:500), P-eNOS (1:500), eNOS (1:500) (Abcam), rabbit antirat ROCK1 (1:300) polyclonal antibody, and donkey antirat ROCK2 (1:50) (Santa Cruz) were separately added, and the samples were incubated overnight at 4°C. Subsequently, horseradish peroxidase-labeled goat antirabbit IgG (1:5000) and goat antidonkey IgG (1:4000) (Beyotime Institute of Biotechnology) were added. IOD was statistically analyzed using Quantity One 4.6 software, in which glyceraldehyde phosphate dehydrogenase (GAPDH; 1:1000 dilution; Bio-Rad, CA) was considered as an internal reference.

Determination of the NO Content in the Corpus Cavernosum. The measurement was carried out according to the instructions of the Nitric Oxide Kit (Nanjing JianCheng Institute of Bioengineering, China). NO content was calculated as follows: NO content (μmol/L) = (measured OD value − blank OD value)/(standard OD value − blank OD value) × (concentration of standard sample [20 μmol/L]/protein concentration of the sample).

Statistical Analysis. Statistical analysis was performed using SPSS19.0 software (SPSS Inc, IL). Data of immunofluorescence staining, ICP_{max}/MAP, immunohistochemistry, Western blot analysis, and NO content were expressed as mean ± SD, and the one-way analysis of variance, linear regression, and correlation analysis was performed. A P value <.05 was considered statistically significant.

RESULTS

ICP_{max}/MAP and Serum Testosterone

Serum testosterone did not show any significant difference among WKY (417.24 ± 22.21 ng/dL), SHR (409.95 ± 11.8 ng/dL), WKY transfection (428.82 ± 19.85 ng/dL), and SHR transfection (415.47 ± 11.5 ng/dL) groups (Supplement 1). With an electric stimulation of 3 V and 5 V, the ICP_{max}/MAP value significantly increased in the SHR transfection and WKY groups compared with the SHR group (both P <.01), while the ICP_{max}/MAP value had no significant change in the WKY transfection group compared with the WKY group (Fig. 1).

Fluorescence Staining of the Corpus Cavernosum

Detection by fluorescence microscopy showed that more than 50% of the cells (green fluorescence) were successfully transfected. The transfected cells were uniformly distributed with stronger fluorescence intensity, but were not morphologically changed compared with the untransfected cells. The semiquantitative IOD value showed that the proportion of GFP-positive cells was 86.15% ± 4.02% in the WKY transfection group and 89.39% ± 3.14% in the SHR transfection group (Fig. 2).

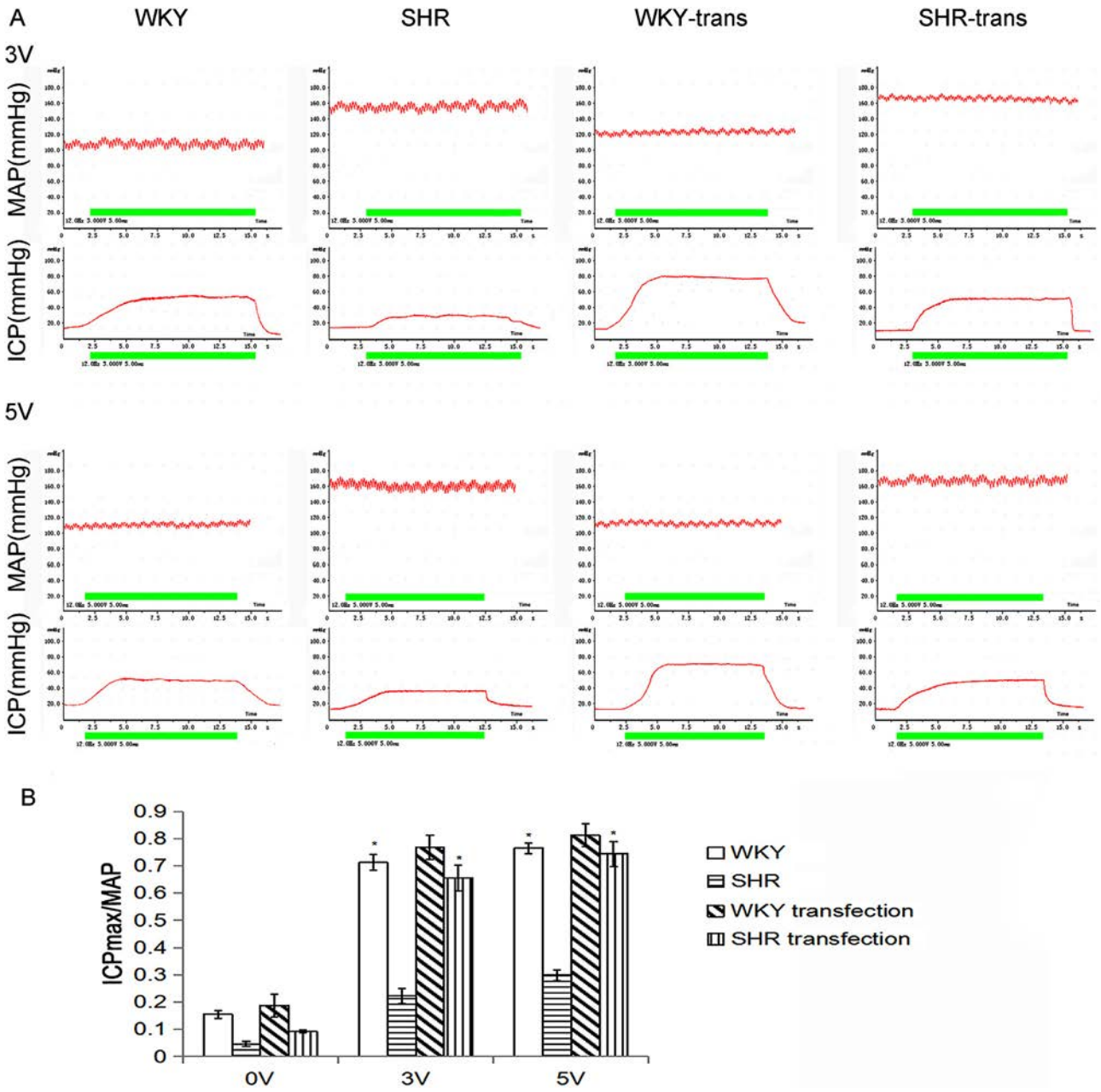

Figure 1. Erectile function was impaired in SHRs group and improved in SHR transfection group. (**A**) Representative original the curve of ICP_{max} and MAP in various groups with an electric stimulation of 3 V and 5 V. (**B**) With an electric stimulation of 3 V and 5 V, the ICP_{max}/MAP value significantly increased in the SHR transfection and WKY groups compared with the SHR group (both $*P < .01$), while the ICP_{max}/MAP value had no significant change in the WKY transfection group compared with the WKY group. Each bar referred to mean ± standard deviation ($n = 5$).

Immunohistochemical Determination of eNOS, P-eNOS, ROCK1, ROCK2, and S1P1 in the Corpus Cavernosum of Rats

The eNOS was mainly expressed in the vascular endothelial cells and blood vessel lumen of cavernous sinus, P-eNOS was mainly expressed in the vascular endothelial cells and cavernous sinus cavities, ROCK1 and ROCK2 were mainly expressed in the cytoplasm of cavernous vascular smooth muscle cells, and S1P1 was mainly expressed in the vascular endothelial cells, where the positive staining was brownish yellow (Fig. 3A). The expression of S1P1 and P-eNOS proteins significantly increased in the SHR transfection and WKY groups compared with the SHR group (both $P < .01$), and significantly increased in the WKY transfection group compared with the WKY group ($P < .01$). The expression of ROCK1 and ROCK2 proteins significantly decreased in the SHR transfection and WKY groups compared with the SHR group (both $P < .01$), and significantly reduced in the WKY transfection group compared with the WKY group ($P < .01$). eNOS did not show any significant difference among different groups (Fig. 3B).

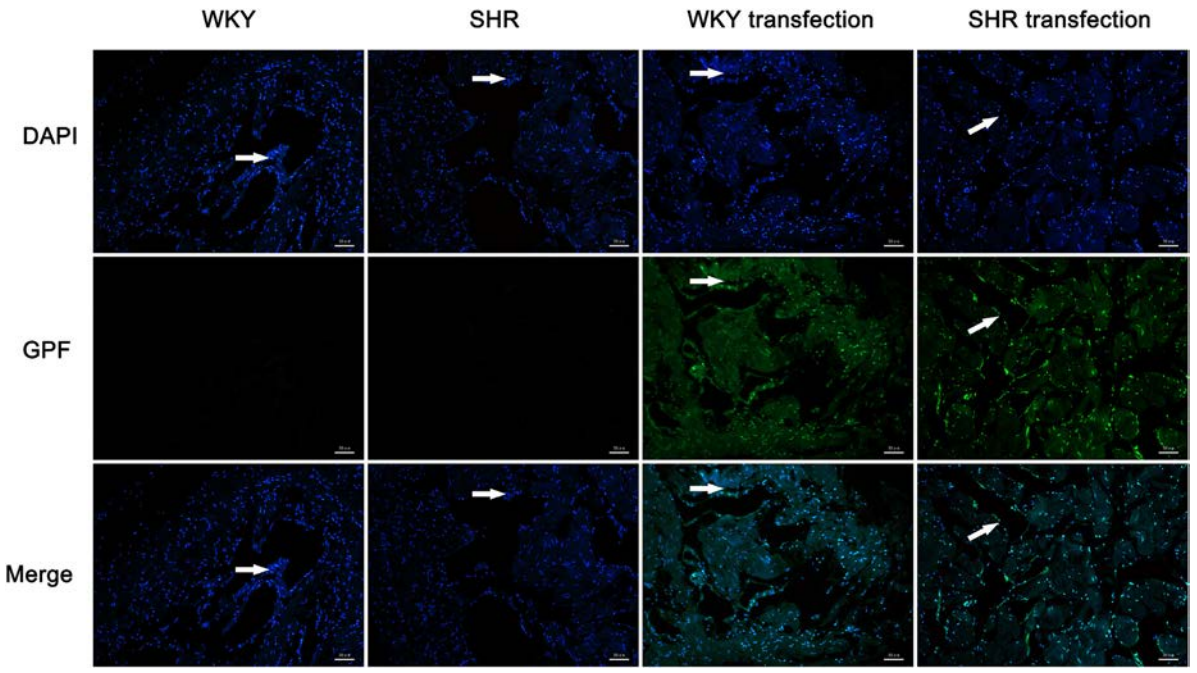

Figure 2. Fluorescent staining of the lentiviral vector carrying the S1P1 gene in the corpus cavernosum, where the green fluorescence referred to transfected cells that are mainly endothelial cells(white arrows). The transfection rate was 86.15% ± 4.02% for the WKY transfection group and 89.39% ± 3.14% for the SHR transfection group.

Expression of eNOS, P-eNOS, ROCK1, ROCK2, and S1P1 in the Corpus Cavernosum of Rats Detected Using Western Blot Analysis

The expression of S1P1 and P-eNOS proteins significantly increased in the SHR transfection and WKY groups compared with the SHR group (both P <.01), and significantly increased in the WKY transfection group compared with the WKY group (P <.01). The expression of ROCK1 and ROCK2 proteins significantly decreased in the SHR transfection and WKY groups compared with the SHR group (both P <.01), and significantly decreased in the WKY transfection group compared with the WKY group (P <.01). eNOS did not show any significant difference among different groups (Fig. 4A and B). The expression of P-eNOS/eNOS significantly increased in the SHR transfection (0.41 ± 0.04) and WKY groups (0.57 ± 0.02) compared with the SHR group (0.22 ± 0.03) (both P <.05), and significantly increased in the WKY transfection group (0.94 ± 0.04) compared with the WKY group (P <.05).

Correlation between S1P1 and NO content

The expression of S1P1 in the corpus cavernosum positively correlated with the NO content in each group, with a linear regression equation of $Y = -0.487 + 0.228X$, $r = 0.958$, P <.01 (Supplement 2 and 3).

DISCUSSION

S1P1 promotes NO production in the endothelial cells and mediates vasodilation.[15,16] It has been found using the Cre/Lox technology that S1P1 plays an important role in regulating vascular maturation associated with vascular dysplasia caused by endothelial tissue-specific S1P1 deletion.[17] When the β1 adrenergic receptor is overstimulated to cause heart failure, the expression of S1P1 in the cardiac intima reduces, while its restoration effectively alleviates heart failure.[18] In the process of treating pancreatic cancer, it was found that the tumor size significantly reduced and the expression of the anticancer gene PP2A was restored after the application of S1P1 antagonist FTY720.[19] These findings indicated that S1P1 played an important role in the body under physiological or pathological conditions.

Previous studies confirmed that the serum S1P level did not show a significant difference between SHR and WKY rats, while the expression of S1P1 in the corpus cavernosum decreased under high blood pressure, inhibiting the expression and activity of eNOS and decreasing NO, thereby leading to ED.[4] In this study, the lentiviral vector carrying S1P1 gene was injected into the corpus cavernosum of the rats, and the transfection rate was 86.15% ± 4.02% and 89.39% ± 3.14% in the WKY transfection and SHR transfection groups, respectively. The Western blot and immunohistochemical analyses prompted that the S1P1 protein content was significantly higher in the SHR transfection group than in the SHR group, and no significant difference in serum T value was found among different groups, indicating that the upregulation of S1P1 level was unlikely to affect the testosterone level. Meanwhile, no significant change in the eNOS level in the corpus cavernosum was found in the SHR transfection group, and the P-eNOS/eNOS was significantly higher than that in the SHR group, which was consistent with the findings that S1P1 and S1P binding

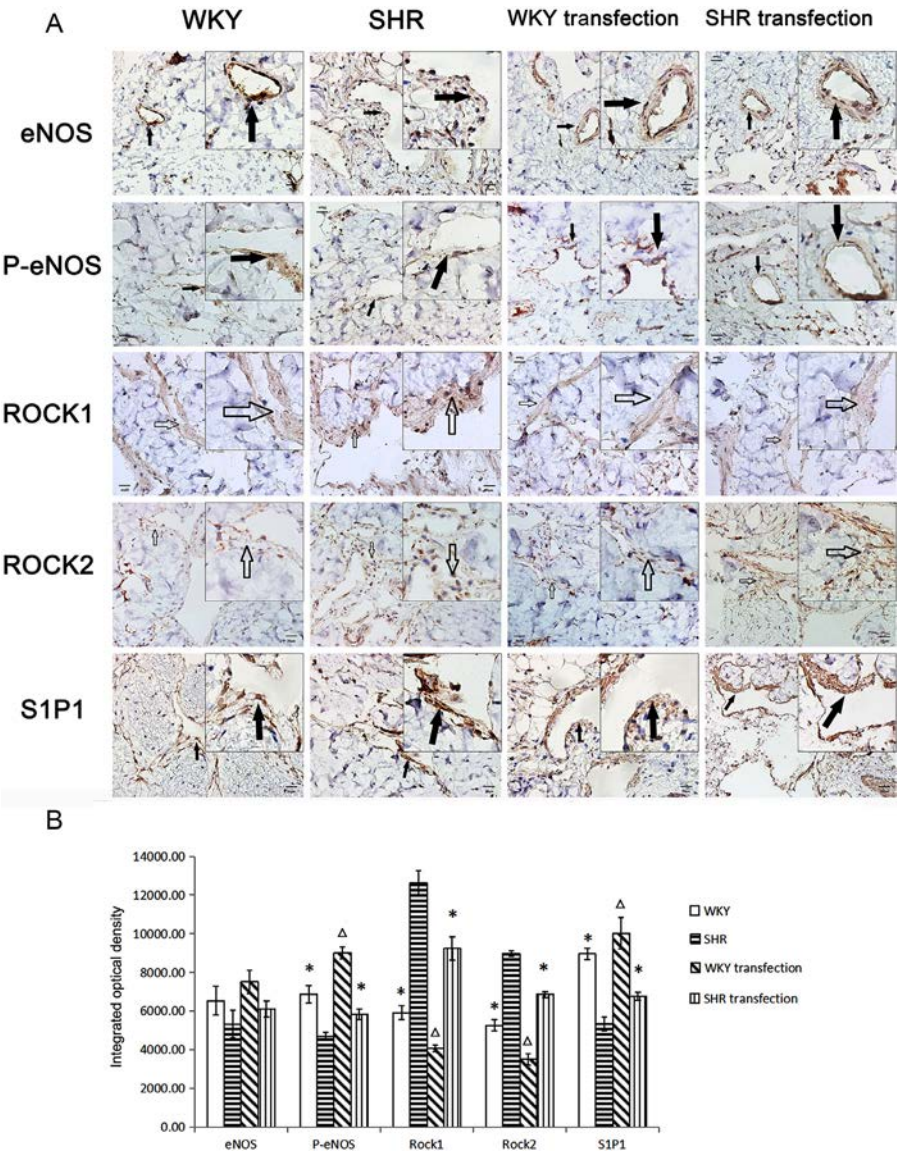

Figure 3. The expression and immunolocalization of eNOS, P-eNOS, ROCK1, ROCK2, and S1P1 in the corpus cavernosum of each group was detected by immunolocalization (×400 and ×800) (**A**) Immunolabeling of eNOS, P-eNOS, ROCK1, ROCK2, and S1P1 was brownish yellow. eNOS,P-eNOS, and S1P1 (solid arrows) are mainly expressed in the endothelial cells. Rock1 and Rock2 (empty arrows) are mainly distributed in the cytoplasts of smooth muscle cells. (**B**) The expression of S1P1 and P-eNOS proteins significantly increased in the SHR transfection and WKY groups compared with the SHR group (both $*P < .01$), and significantly increased in the WKY transfection group compared with the WKY group ($\triangle P < .01$). The expression of ROCK1 and ROCK2 proteins significantly decreased in the SHR transfection and WKY groups compared with the SHR group (both $*P < .01$), and significantly decreased in the WKY transfection group compared with the WKY group ($\triangle P < .01$).

activated eNOS in vascular endothelial cells and human cavernous endothelial cells in vitro by promoting eNOS phosphorylation.[20] Therefore, S1P1 could increase the NO content by increasing P-eNOS/eNOS after transfection, thereby increasing the ICP_{max}/MAP value and improving the erectile function.

However, the contraction of smooth muscle cells is mainly mediated by the RhoA/Rho kinase signaling pathway.[21] Hypertension can upregulate the RhoA/Rho kinase signaling pathway and inhibition of the relaxation of the smooth muscle in the corpus cavernosum.[22,23] Previous studies found that Rho kinase inhibitor (fasudil) could significantly improve SHR erectile function by inhibiting the RhoA/Rho kinase signaling pathway.[24] Importantly, the upregulation of the NO/cGMP signaling pathway also inhibits the RhoA/Rho kinase-mediated smooth muscle contraction.[25] In aortic smooth muscle cells of rats, cGMP analog bromide or nitroprusside can block the binding and activation of RhoA/Rho kinase to ROCK.[26,27] However, whether S1P1 directly regulates RhoA/Rho kinase has not been reported yet. Hence, the decrease in the expression of ROCK1/2 in SHR corpus cavernosum after S1P1 transfection may be the result of upregulation of NO/cGMP signaling pathway.

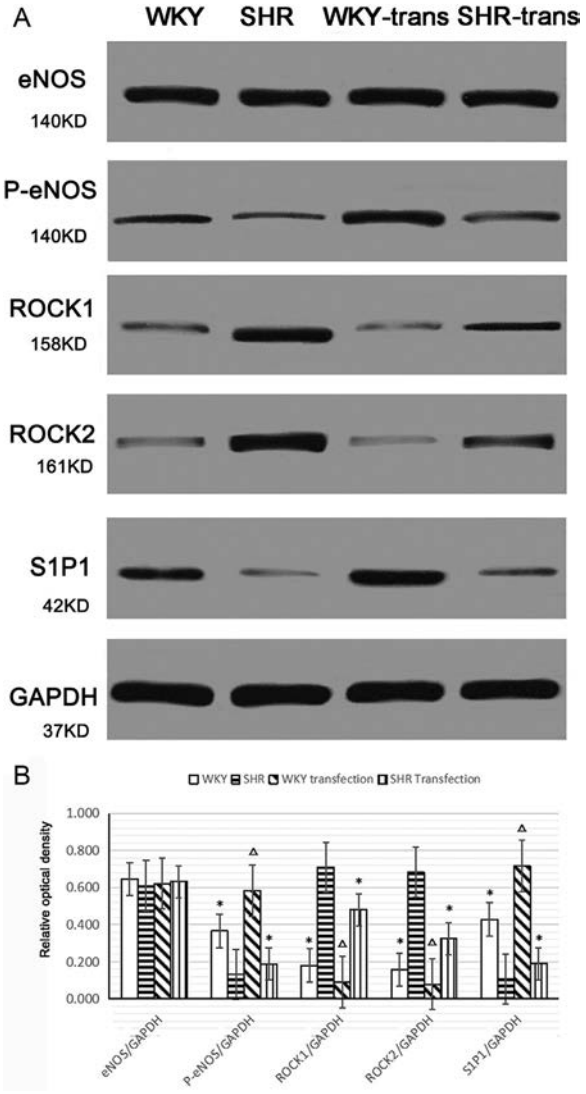

Figure 4. Expression of eNOS, P-eNOS, ROCK1, ROCK2, and S1P1 in the corpus cavernosum of rats. (**A**) Western blots of eNOS, P-eNOS, ROCK1, ROCK2, and S1P1 in different groups. (**B**) Expression of eNOS, P-eNOS, ROCK1, ROCK2, and S1P1 proteins in each group detected using histograms. The data were expressed as the ratio of the target protein to the internal reference (GAPDH). Each bar represented the mean ± standard deviation.

After applying electrical stimulation to the corpus cavernosum of SD rats, the ICP value increased with the increase in the NO concentration in the range of 1 to 4 μmol/gprot, while the ICP_{max} value was not significantly affected by the increase in the NO concentration when it reached the range of 4-10 μmol/gprot.[28,29] In this experiment, the NO concentration in the SHR and SHR transfection groups after 3 V and 5 V electrical stimulation was 2.42 ± 0.29 μmol/gprot and 3.26 ± 0.44 μmol/gprot, respectively. In this range of NO concentration, the ICP_{max} value was significantly higher in the SHR transfection group than in the SHR group. However, the NO concentration in the WKY and WKY transfection groups were 3.95 ± 0.1 μmol/gprot and 5.22 ± 0.18 μmol/gprot, respectively. In this range of NO concentration, although the 2 groups showed a significant difference ($P < .05$), the ICP_{max}/MAP values were not significantly altered.

In conclusion, lentiviral gene transfection upregulated the expression of S1P1 in the corpus cavernosum of SHR, which possibly promoted endogenous NO production by increasing the P-eNOS/eNOS ratio. At the same time, inhibited the RhoA/Rho kinase signaling pathway through NO/cGMP signaling pathway in the corpus cavernosum, thereby improving ED caused by hypertension. Therefore, upregulation of S1P1 expression in corpus cavernous tissue may be a new method for treating ED induced by hypertension in the future. The limitation of this study is that there is no study on the duration of upregulation of S1P1 and the improvement of erectile function after transfection. It needs further investigation to confirm and perfect the conclusions of this study.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.urology.2019.08.025.

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