ERECTILE DYSFUNCTION

Hypoxia-Induced Phenotypic Transformation of Corpus Cavernosum Smooth Muscle Cells After Cavernous Nerve Crush Injury by Down-Regulating P38 Mitogen-Activated Protein Kinase Expression

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ABSTRACT

Introduction: Cavernosal nerve (CN) injury is commonly caused by radical prostatectomy surgery, and it might directly lead to erectile dysfunction (ED). Currently, the role of mitogen-activated protein kinase (MAPK) family proteins in phenotypic transformation of corpus cavernosum smooth muscle cell (CCSMC) after CNs injury is poorly understood.

Aim: To investigate the role of p38 MAPK in hypoxia-induced phenotypic transformation of CCSMCs after CN injury.

Methods: In total, 20 Sprague–Dawley rats (male and 8 weeks of age) were randomly divided into 2 groups, including a sham group and CNCI group. In the sham group, rats were sham-operated by identifying 2 CNs without causing direct damage to the CNs. In the CNCI group, rats were subjected to bilateral CN crush injury. CCSMCs were isolated from the normal corpus cavernosum tissues of the Sprague–Dawley rat and then cultured in 21% or 1% O_2 concentration context for 48 hours.

Main Outcome Measures: Intracavernous pressure/mean arterial pressure were analyzed to measure erectile response. The impact of hypoxia on penile pathology, as well as the expression of extracellular signal-regulated kinases, the c-Jun NH2-terminal kinase, and p38 MAPK, were analyzed.

Results: Compared with the sham group, the intracavernous pressure/mean arterial pressure rate and α-smooth muscle actin expression of CNCI group were decreased significantly ($P = .0001$; $P = .016$, respectively), but vimentin expression was significantly increased ($P = .023$). Phosphorylated p38 level in CNCI group was decreased significantly ($P = .017$; sham: 0.17 ± 0.005; CNCI: 0.14 ± 0.02). The CCSMCs in the normoxia group were long fusiform, whereas the morphology of CCSMCs in the hypoxia group became hypertrophic. After hypoxia for 48 hours, the expression of α-smooth muscle actin and phosphorylated p38 MAPK was decreased significantly ($P = .01$; $P = .024$, normoxia: 0.66 ± 0.18, hypoxia: 0.26 ± 0.08, respectively), and the expression of hypoxia-inducible factor-1α and collagen I was increased significantly in hypoxia group ($P = .04$; $P = .012$, respectively).

Conclusions: Hypoxia induced the phenotypic transformation of CCSMCs after CNCI might be associated with the downregulation of phosphorylated p38 MAPK. **Chen S, Huang X, Kong X, et al. Hypoxia-Induced Phenotypic Transformation of Corpus Cavernosum Smooth Muscle Cells After Cavernous Nerve Crush Injury by Down-Regulating p38 Mitogen-Activated Protein Kinase Expression. Sex Med 2019;7:433–440**

Key Words: Erectile Dysfunction; Neurogenic; Phenotypic Transformation; Mitogen-Activated Protein Kinase (MAPK); p38 MAPK; Hypoxia

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INTRODUCTION

Erectile dysfunction (ED) is one of the most common diseases in men, which usually exhibits the inability of a man achieving or maintaining an erection enough for satisfactory intercourse with his partner. ED was considered as an entirely psychogenic disorder in previous studies until increasing evidence indicated that greater than 80% of patients have an organic etiology, and the organic etiology can be classified as nonendocrine and endocrine.[1] Neurogenic ED is the main cause of nonendocrine types, which can be caused by medical diseases (like multiple sclerosis, Parkinson disease, and diabetes), surgical diseases (like spinal cord injury, lumbar disc disease, traumatic brain injury), and radical pelvic surgery (like radical prostatectomy, radical cystectomy, abdominoperineal resection).[1]

In particular, neurogenic ED is mainly caused by cavernous nerve (CN) injury during radical prostatectomy surgery (RP),[2] and it eventually leads a change in penis structure. Corpora cavernosum smooth muscle cells (CCSMCs) account for 42−50% of the corpora cavernosum, which plays an important role in erectile response.[3] The penis has a special configuration similar to vascular tissue.[4] Similar pathologic features can be found between the vascular system and penis, such as phenotypic transformation.

Phenotypic transformation is defined as the state of smooth muscle cells (SMCs) converted from contractile to synthetic, and it plays a crucial role in vascular disease[5] and ED. Phenotypic transformation of CCSMCs occurs in diabetic rats with ED, rats with CN injury, and rats with hyperlipidemia-associated ED.[6−8] However, the function of phenotypic transformation of CCSMCs remains unclear.

Currently, many treatments are suggested for the rehabilitation of erectile function, including daily dosing of phosphodiesterase type 5 inhibitors, intracavernosal injection therapy, or the use of vacuum erection devices.[9] However, the treatment of neurogenic ED is still limited. Therefore, phenotypic transformation of CCSMCs may be a new potential target of neurogenic ED.

Mitogen-activated protein kinases (MAPKs) highly participate in embryonic development, as well as proliferation, differentiation, death, and apoptosis of the cell,[10,11] including extracellular signal-regulated kinases (ERK), the c-Jun NH_2-terminal kinase (JNK), and p38 MAPK. Activation of JNK and p38 MAPK signaling pathway can promote the apoptosis in the early stage of the corpus cavernosum injury.[12,13] Furthermore, p38 MAPK signaling pathway can induce vascular smooth muscle cell (VSMC) phenotypic transformation.[14−16] However, the effect of the MAPK pathway on phenotypic transformation of CCSMCs after CN injury is still poorly understood. In this study, we aimed to clarify the correlation between MAPK family and phenotypic transformation of CCSMCs after CNCI.

MATERIALS AND METHODS

Animals

Animal ethics approval for this study was approved by the Committee on the Ethics of Animal Experiments of Zhejiang Chinese Medical University. All rats were kept under the specific-pathogen-free conditions in the animal facility of Zhejiang Chinese Medical University. A total of 20 Sprague−Dawley male rats (male and 8 weeks of age) with a body weight of 330−380 g (SIPPER-BK Laboratory Animals, Shanghai, China) were randomly divided into 2 groups and treated as follows. In the sham group, rats (n = 10) were sham-operated by identifying 2 CNs without causing direct damage to the CNs. In the CNCI group, rats (n = 10) underwent a bilateral cavernous nerve crush injury (CNCI) with the use of a microsurgical vascular clamp to the CN 2−3 mm distal to the major pelvic ganglion for two 60-second periods.[17]

Measurement of Erectile Function

At the end of 4 weeks after surgery, intracavernous pressure (ICP) and mean arterial pressure were measured to evaluate erectile function as described.[18] Then, penile tissues were harvested for further histologic studies after euthanasia.

Cell Culture and Hypoxia

CCSMCs were isolated from the penis tissues of male rats (6 weeks of age) as described[19,20] and cultured by using a tissue explant−adherent method as described.[21,22] CCSMCs, which were from the passage of 3 cells, were identified by the expression of α smooth muscle actin (α-SMA) and desmin. The hypoxic group was cultured in a modular incubator chamber (StemCell, Vancouver, BC, Canada) filled with hypoxia gas (1% O_2, 5% CO_2, and 94% N_2) and set at 37°C. The normoxic group was incubated in the same conditions as the hypoxic group except for the gas (air containing 5% CO_2). After 48 hours, the morphologic features of CCSMCs cultured in normoxia or hypoxia were observed in an inverted microscope. Then, CCSMCs were harvested for further studies.

Staining and Immunohistochemistry

Hematoxylin−eosin staining was performed after the tissues were embedded in paraffin and sectioned at 4 mm. The expression of α-SMA, vimentin, ERK, JNK, and p38 in corpus cavernous was examined with immunohistochemical staining as described.[7] The primary antibodies are presented in the Supplemental Materials (eTable 1). Images were captured by a LEICA DM 2500 microscope (LEICA, Wetzlar, Germany) under 200× magnification.

Western Blot Analysis

Western blot analyses were performed as described.[20] The primary antibodies are presented in Supplemental Materials (eTable 1). Immunoreactivity proteins were visualized with the ChemiDoc touch Imaging System (Bio-Rad, Hercules, CA, USA) and normalized to the β-actin expression level (internal control), then semiquantitative analysis of immunoreactive proteins was performed by Image J software (National Institutes of Health, Bethesda, MD, USA).

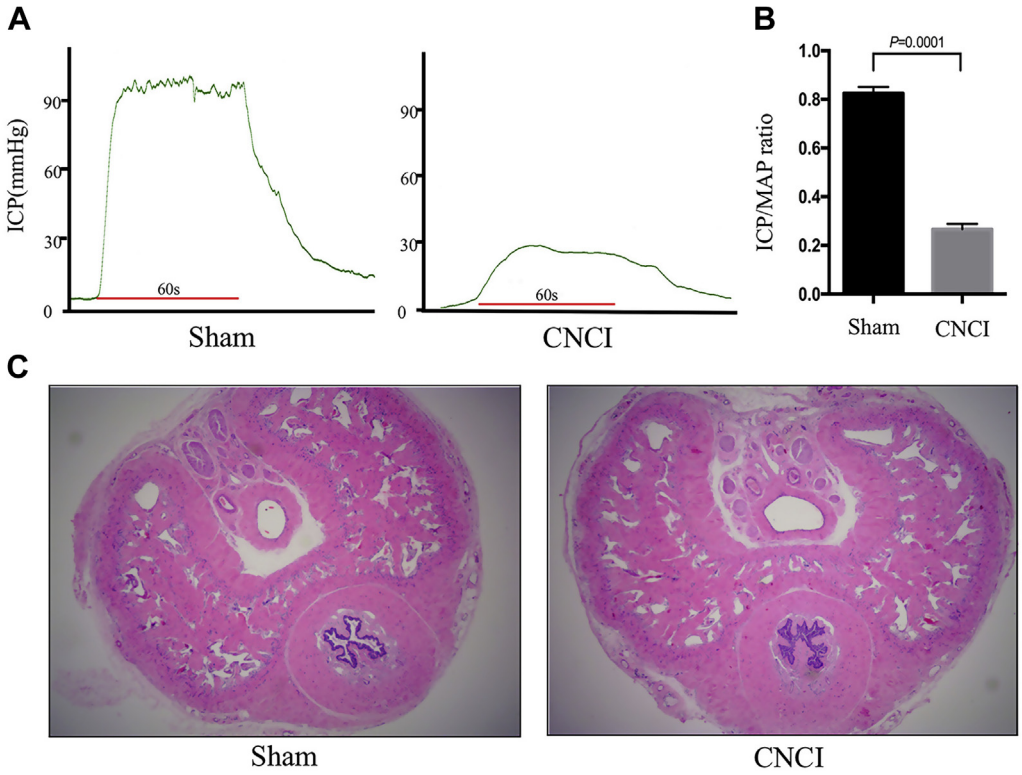

Figure 1. Effect of bilateral cavernous nerve injury on erectile response and corpora cavernosa morphologic alteration. Panel A shows the values of ICP in both groups. Panel B shows the ICP/MAP ratio in both groups. Panel C shows representative hematoxylin–eosin staining photomicrographs in both groups (magnification ×40). Statistical analysis by independent *t* test. CNCI = cavernous nerve crush injury; ICP = intracavernous pressure; MAP = mean arterial pressure.

Immunofluorescence

Parffin-embedded sections of penile tissue were incubated overnight with primary antibodies (eTable 1). After washing in phosphate-buffered saline, the sections were incubated with 2 secondary antibodies (eTable 1) in 1% bovine serum albumin at room temperature for 1 hour. Relevant antibodies were presented in images captured by a LEICA DM 2500 microscope (LEICA) under the 100× magnification.

Quantitative Real-Time Polymerase Chain Reaction

Tissues from each group were collected and lysed according to the TRIzol instructions, and total RNA was extracted. The rat α-SMA, vimentin, ERK, JNK, p38, and β-actin sequences reported in the National Center for Biotechnology Information gene database were designed using Oligo 6.71 primer analysis software, and then were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The primers sequence are presented in Supplemental Materials (eTable 2). The cDNA was synthesized from 1 μg of RNA by applying to the Bio-Rad iScript cDNA synthesis kit (Bio-Rad). Quantitative polymerase chain reaction was performed on the MJ Opticon (Bio-Rad) system using iTaq Universal SYBR Green Supermix (Bio-Rad). The relative expression of mRNA was calculated as: target gene = value of target gene / value of β-actin.

Statistics

Differences between 2 groups were analyzed by the independent *t* test. All variables were reported as mean ± standard deviation. A 2-sided *P*-value <.05 was considered statistically significant. SPSS 23.0 software (IBM Corp., Armonk, NY, USA) was used for all statistical analyses.

RESULTS

CN Injury Significantly Decreased Erectile Response

At the end of the fourth week, the CNCI group saw a significant decline in ICP compared with that of the sham group (Figure 1A). In addition, the ICP/mean arterial pressure rate of CNCI group was significantly lower than the sham group ($P = .0001$; sham: 0.83 ± 0.03; CNCI: 0.27 ± 0.02) (Figure 1B). Hematoxylin–eosin staining showed that the penile morphology of CNCI group was similar to the sham group when viewed on a microscope (Figure 1C).

Marker Protein Expression of Phenotypic Transformation After CN Injury

Immunohistochemical staining results revealed that the expression of α-SMA and vimentin in the CNCI group was decreased and increased, respectively, as compared with that in the sham group (Figure 2A). Subsequently, the results of western

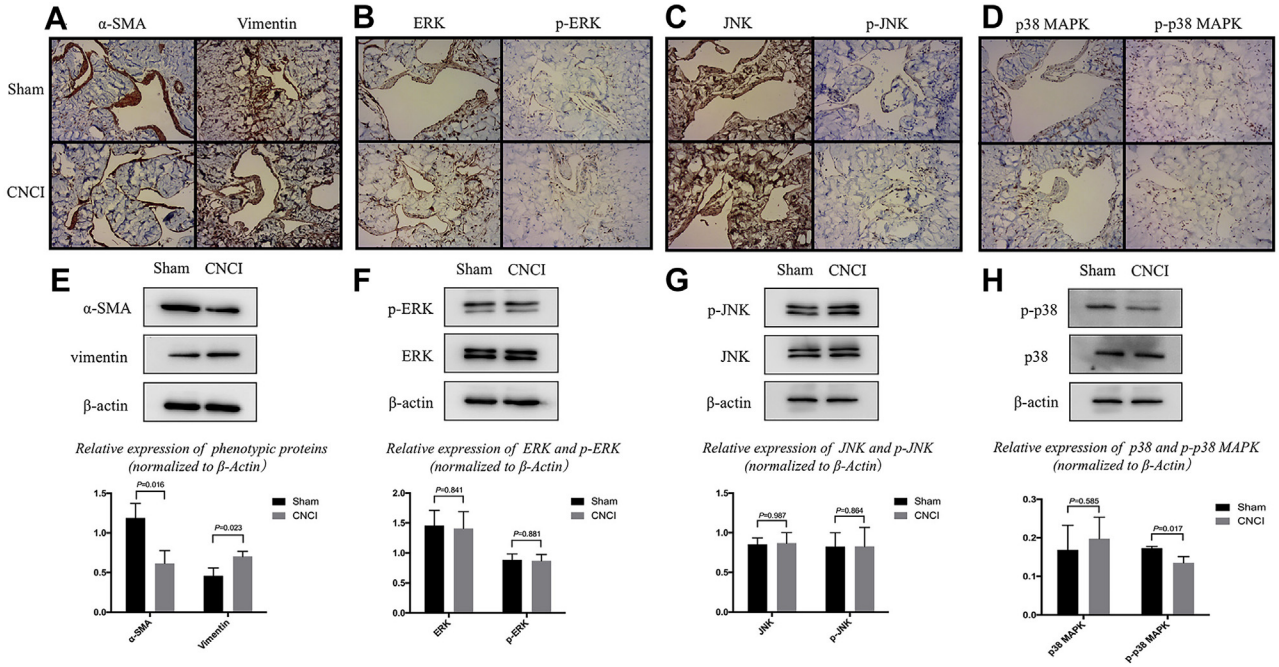

Figure 2. Expression of proteins of phenotypic transformation and MAPK by immunohistochemistry and Western blot. Panel A shows the results of marker proteins of phenotypic transformation performed by immunohistochemical staining in both groups. Compared with the sham group, weak expression of α-SMA and strong expression of vimentin were detected in cytoplasm in the CNCI group. Panels B-D shows the results of MAPK presented by immunohistochemical staining in both groups. The site of expression of all was the nucleus. Only the expression of p-p38 MAPK in the sham group was stronger than that in CNCI group. In Panel E, compared with the sham group, the expression of α-SMA was decreased significantly and vimentin increased significantly in the CNCI group (mean ± standard deviation). In Panels F-H, compared with the sham group, the expression of p-p38 was significantly decreased in the CNCI group, and for the others there was no statistical significance between both groups. (mean ± standard deviation). The data were normalized by β-actin expression. α-SMA = α-smooth muscle actin; CNCI = cavernous nerve crush injury; ERK = extracellular signal-regulated kinases; JNK = the c-Jun NH_2-terminal kinase; MAPK = mitogen-activated protein kinase; p-ERK = phosphorylated ERK, p-JNK = phosphorylated JNK, p-p38 MAPK = phosphorylated p38 mitogen-activated protein kinase (magnification ×200). Statistically significant (independent *t* test): α-SMA ($P = .016$); vimentin ($P = .023$); p-p38 MAPK ($P = .017$).

blot analysis also indicated that the expression of α-SMA and vimentin was significantly downregulated ($P = .016$; sham: 1.19 ± 0.19; CNCI: 0.61 ± 0.16) and upregulated ($P = .023$; sham: 0.46 ± 0.1; CNCI: 0.7 ± 0.06), respectively (Figure 2E). However, the mRNA levels of α-SMA and vimentin did not reach statistical significance (Figure 3A).

Significantly Decreased Phosphorylated p38 Expression After CN injury

Immunohistochemical staining and western bolt were applied to investigate the expression of the MAPK family, containing p38 MAPK, phosphorylated p38 MAPK (p-p38 MAPK), JNK, phosphorylated-JNK, ERK, and phosphorylated-ERK. Only the expression of p-p38 MAPK was decreased dramatically in the CNCI group ($P = .017$; sham: 0.17 ± 0.005; CNCI: 0.14 ± 0.02) (Figure 2B-D, Figure 2F-H). However, the mRNA levels of p38 did not reach statistical significance (Figure 3B). Immunofluorescence staining showed that the expression of phosphorylated p38 was decreased in similar locations of the corpus cavernosum, whereas the expression of vimentin was increased (Figure 4, white arrows).

Hypoxia-Inducible Factor 1-α (HIF-1α) Expression In Vivo

The expression of HIF-1α at protein level was significantly increased in the CNCI group compared with that of the sham group ($P = .047$; sham: 0.67 ± 0.19; CNCI: 1.06 ± 0.14) (Figure 3C and D).

CCSMC Identification and the Morphologic Change of CCSMCs After 48 Hours

SMCs were isolated according to the positive expression of α-SMA and desmin by immunofluorescence staining (Figure 5A) as in a previous study.[23] The primary CCSMCs appeared to be spindle shaped initially and then tended to become hypertrophic after hypoxia for 48 hours (Figure 5B).

Phenotypic Transformation and Phosphorylated p38 In Vitro

The expression of HIF-1α and collagen I at protein level was increased significantly ($P = .04$; normoxia: 2.81 ± 0.38; hypoxia: 4.24 ± 0.15; $P = .012$, normoxia: 0.29 ± 0.04, hypoxia: 0.49 ± 0.07, respectively), but the expression of α-SMA and

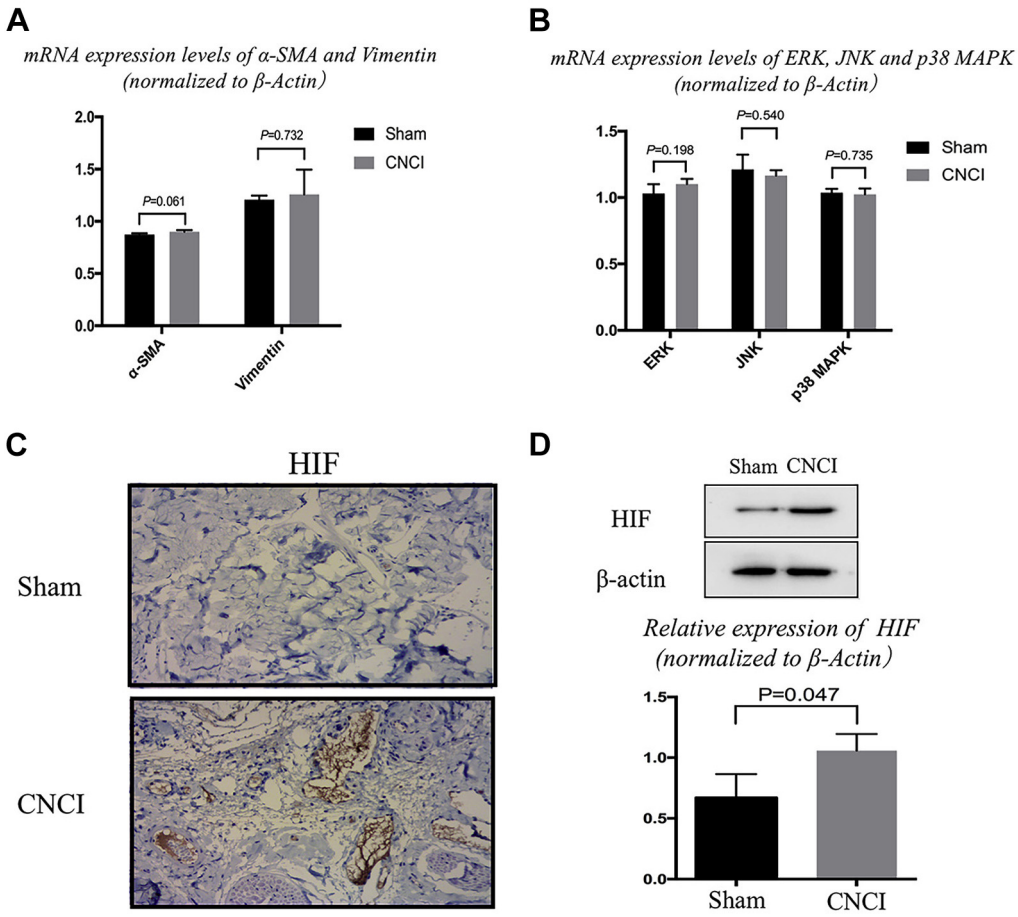

Figure 3. Expression of HIF-1α in cavernous tissue. In Panels A and B, there were no significant differences in α-SMA, vimentin, ERK, JNK, and p38 between both groups. In Panel C, the result of HIF-1α was presented by immunohistochemical staining in both groups. Strong expression of HIF-1α was detected in CNCI group. In Panel D, the results of western blot show that HIF-1α increased significantly in CNCI groups compared with sham group (mean ± standard deviation) (magnification ×200). Statistically significant (independent *t* test): HIF-1α ($P = .047$). α-SMA = α-smooth muscle actin; CNCI = cavernous nerve crush injury; ERK, extracellular signal-regulated kinase; HIF-1α = hypoxia-inducible factor-1α; JNK = c-Jun NH_2-terminal kinase.

phosphorylated p38 MAPK at protein level was obviously decreased ($P = .01$, normoxia: 0.47 ± 0.02; hypoxia: 0.35 ± 0.38; $P = .024$, normoxia: 0.66 ± 0.18, hypoxia: 0.26 ± 0.08, respectively). However, the expression of p38 MAPK was not statistically significant ($P = .808$; normoxia: 1.05 ± 0.47; hypoxia: 0.96 ± 0.37) (Figure 5C).

DISCUSSION

Phenotypic transformation is highly involved in the pathologic development of VSMCs.[24] Many vascular diseases, such as atherosclerosis and restenosis, are closely associated with phenotypic changes from the contractile to synthetic phenotype for VSMCs.[25,26] Furthermore, α-SMA, SM myosin heavy chain, vimentin, and collagen I have been found as phenotypic markers for VSMCs.[27–29] Also, corpus cavernosum is considered as a specific vascular tissue with phenotypic transformation.[4] He et al[30] have suggested that erectile function significantly improves in diabetic rats with ED by transforming CCSMCs from synthetic to the contractile phenotype. In addition, the phenotypic transformation of CCSMCs also occurs in rats with CN injury.[7,21]

The occurrence of ED after RP is closely related to neurogenic factors, especially CN injury. CNCI is widely applied in animal experiments to imitate the injury that often occurs after RP. Previous studies have reported that the 1-, and 4-, and 12-week intervals represent the acute, subacute, and chronic phases following CN injury, respectively.[31,32] Yang et al[7] have shown that phenotypic transformation occurs in CCSMCs at 12 weeks after CN injury. In our study, downregulated α-SMA and upregulated vimentin indicated the phenotypic transformation of CCSMCs can occur at the fourth week after CN injury. Hence, we speculated that phenotypic transformation of CCSMCs after CN injury is the main cause of pathologic development.

MAPKs can regulate various basic life activities of cells through multifunctional signals, including embryonic development, cell proliferation, cell differentiation, and cell death–related pathways.[10] In mammals, ERK, JNK, and p38 are the main MAPK proteins.[11] Furthermore, vascular studies

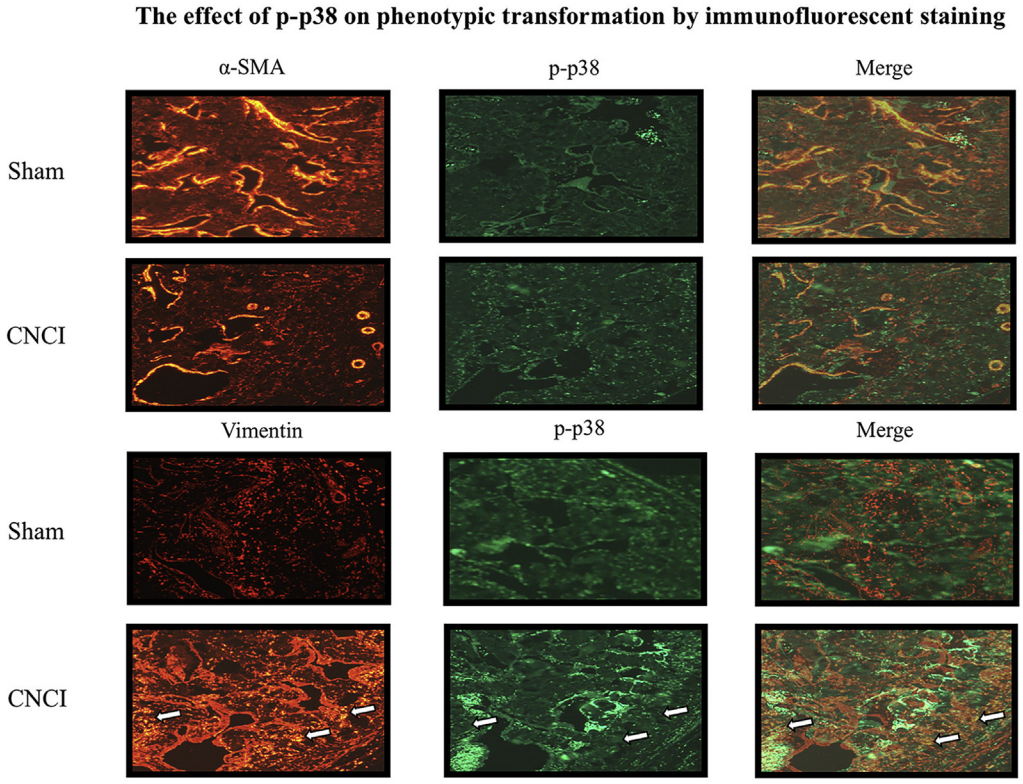

Figure 4. The effect of p-p38 on phenotypic transformation by immunofluorescent staining. Representative expressions of α-SMA, vimentin, and p-p38 MAPK are presented. White arrows represent smooth muscle cells in the corpus cavernosum (magnification $\times$100). α-SMA = α-smooth muscle actin; CNCI = cavernous nerve crush injury; p-p38 MAPK = phosphorylated p38 mitogen-activated protein kinase.

show that phenotypic transformation of VSMCs is highly associated with p38 MAPK and ERK.[14–16] In our study, expression of p-p38 MAPK decreased significantly in CNCI with upregulated vimentin and downregulated α-SMA. It was likely that p38 MAPK also participated in the occurrence of phenotypic transformation of CCSMC after CNCI.

We also found that HIF-1α was increased significantly at the fourth week after CNCI, and it indicated that hypoxia was already present in the corpus cavernosum at the fourth week after CNCI. In vitro, the expression of p-p38 MAPK also decreased significantly in isolated CCSMCs with the downregulated α-SMA and the upregulated collagen I after acute hypoxia for 48 hours. Therefore, we speculated that hypoxia could induce the phenotypic transformation of CCSMCs after CNCI and that it might be closely associated with the downregulated p-p38 MAPK.

However, based on the current studies,[11] we found that p38 MAPK usually is activated by environmental stresses and inflammatory cytokines. For example, phosphorylated p38 is upregulated by various pathogenic factors such as angiotensin II and streptozotocin.[33–35] However, our results were inconsistent with these studies. Downregulation of the p38 activity might promote the production of synthetic SM cells. Therefore, the p38 MAPK signaling pathway might play a critical role in ED after CNCI. However, the potential effect of p38 still needs to be further studied.

There were some limitations in our study. First, we did not use a blocking or activating method to determine the causal relationship between dysregulated p38 and phenotypic transformation of cavernosal SM in vivo and vitro. However, we identified that p38 rather than ERK or JNK might involve phenotypic transformation of cavernosal tissue following partial CN damages. Second, our experiments were performed at only one time point (4 weeks) after CN injury. However, the selection of time point based on some previous studies.[7,9]

CONCLUSIONS

In summary, hypoxia can induce the phenotypic transformation of CCSMCs after CNCI, and the occurrence of phenotypic transformation is likely associated with the downregulated p-p38 MAPK. Moreover, p38 MAPK might be a potential target for the treatment of ED by reversing the phenotypic transformation of CCSMC. Finally, the causal relationship between p38 MAPK signaling pathway and phenotypic transformation still needs to be further clarified.

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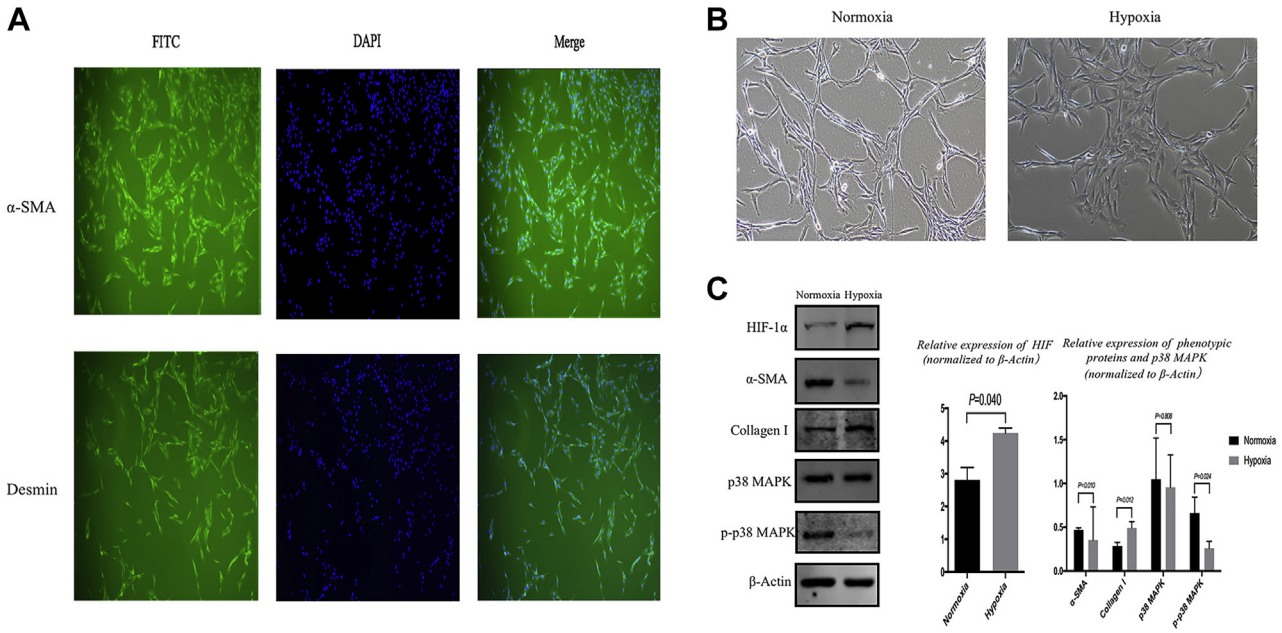

Figure 5. Phenotypic transformation and p-p38 in CCSMCs. Panel A shows the results of CCSMC identification presented by immunofluorescent staining in the 2 groups. Panel B shows the morphologic change of CCSMCs. In Panel C, compared with the normoxia group, α-SMA and p-p38 were decreased significantly in the hypoxia group, and collagen I and HIF-1α were increased significantly in the hypoxia group (mean ± standard deviation) (magnification ×100). Statistically significant (independent t test): α-SMA ($P = .01$); collagen I ($P = .012$); p-p38 MAPK ($P = .024$); HIF-1α ($P = .04$). α-SMA = α-smooth muscle actin; CCSMC = corpora cavernosum smooth muscle cell; HIF-1α = hypoxia-inducible factor-1α; MAPK = mitogen-activated protein kinase; p-p38 MAPK = phosphorylated p38 mitogen-activated protein kinase.

Conflict of Interest: The authors report no conflicts of interest.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.esxm.2019.08.005.