Urologia Internationalis

Original Paper

Urol Int 2020;104:594–603
DOI: 10.1159/000507099

Received: November 25, 2019
Accepted: March 7, 2020
Published online: July 2, 2020

Salidroside Attenuates Hypoxia-Induced Expression of Connexin 43 in Corpus Cavernosum Smooth Muscle Cells

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Keywords
Connexin 43 · Hypoxia · Corpus cavernosum · Salidroside

Abstract
Introduction: Connexin 43 (Cx43) is the major component of gap junction in corpus cavernosum smooth muscle, which allows rapid intercellular communication. Cx43 coordinates corpus cavernosum smooth muscle cells and ensures erectile function. The role of hypoxia in Cx43 dysfunction resulting in erectile dysfunction has not been well studied, and salidroside has shown cell protective effects under hypoxia. ***Objective:*** We aimed to investigate the protective role of salidroside and the underlying mechanisms in hypoxia-induced dysfunction of Cx43. ***Methods:*** Corpus cavernosum smooth muscle cells prepared from young male Sprague-Dawley rats were pretreated with or without salidroside and exposed to hypoxic condition for 48 h. The cell viability, expression of hypoxia-inducible factor-1α (HIF-1α) and Cx43, and Ca^{2+} signals were investigated. ***Results:*** Pretreatment with salidroside attenuated loss of hypoxia-induced cell viability markedly and could downregulate the HIF-1α protein expression under hypoxia. Moreover, the expression of Cx43 was significantly increased by hypoxia but was decreased with salidroside pretreatment. The salidroside pretreated group exhibited enhanced release of intracellular Ca^{2+} in corpus cavernosum smooth muscle cells compared with the hypoxia group after stimulation. ***Conclusion:*** Salidroside has a protective effect against hypoxia-induced damage to corpus cavernosum smooth muscle cells.

Introduction

Erectile dysfunction (ED) is defined as the persistent inability to attain or maintain a penile erection sufficient for successful vaginal intercourse [1]. ED could affect physical and psychosocial health and may have great impact on quality of life of the patient and family. It is a common clinical disorder which mainly affects men older

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than 40 years of age. As the Massachusetts Male Aging Study report revealed that the overall prevalence of ED in men aged 40–70 years was 52% [2]. Further, it was predicted that the worldwide prevalence of ED will reach 322 million cases by the year 2025 [3, 4].

Evidence indicates that hypoxia is closely related to ED and that ED is frequent in patients with obstructive sleep apnea [5]. The penile oxygen saturation was significantly lower in men with ED than in normal men [6]. The sleep-related erection is an involuntary physiological phenomenon which occurs in healthy men at night, but be reduced severely in chronic hypoxic conditions [7]. In the flaccid state, penile hypoxia persists and the oxygen tension goes as low as 25–40 mm Hg. However, this situation is interrupted by the nocturnal penile tumescence that consistently reoxygenates the penis (PO_2 = 90–100 mm Hg) and may play a key role in the corpus cavernosum perfusion and oxygenation to represent an internal mechanism that protects the morphological integrity of the corpus cavernosum smooth muscle cells [8].

Penile erections are hemodynamic events attributed to penile artery dilatation and corpus cavernosum smooth muscle fiber relaxation caused by neurological, neurochemical, and endocrine mechanisms. Nitric oxide, released in response to sexual stimulus from the endothelium and the parasympathetic nerve terminals, is the primary neurotransmitter for maintaining a firm erection [9]. Nitric oxide and cyclic guanosine monophosphate act synergistically to reduce Ca^{2+} release, which subsequently contributes to the corpus cavernosum smooth muscle relaxation [10]. Current evidence shows that nitric oxide is not sufficient to maintain erection function, suggesting that additional processes participate in this process [11]. Gap junction is a kind of intercellular connection which is widely existed in mammals. It is the structural basis of intercellular information and material exchange. As early as 1997, Christ had proposed the theory of "syncytial tissue triad" that may explain the rapid intercellular communication and diversity in coordinating penile erection [12]. The major component of gap junction in corpus cavernosum smooth muscle is connexin 43 (Cx43), which allows intercellular communication between corpus cavernosum smooth muscle cells. It could play the role by coordinating corpus cavernosum smooth muscle cells and ensuring erection function. Therefore, Cx43 impairment may also be responsible for ED [11, 13].

The gap junction channels will open under specific physiological and pathological conditions, including ischemia [14, 15]. Studies have shown that hypoxia increases the expression and the open probability of Cx43 [16, 17]. Thus, whether hypoxia induces the dysfunction of Cx43 that consequently leads to ED is yet to be studied. Hence, we investigated the effects of hypoxia on Cx43 and the ability to release Ca^{2+} in corpus cavernosum smooth muscle cells. Furthermore, salidroside is a major biologically active ingredient extracted from *Rhodiola rosea* L and has diverse pharmacological effects, including anti-hypoxia properties. Studies have indicated that salidroside could protect cardiomyocytes against hypoxia-induced death via the HIF-1α pathway [18–20].

In our initial study, we discovered that salidroside could reduce the harmful effects in corpus cavernosum smooth muscle cells by hypoxia. In this study, we hypothesized that salidroside would improve Cx43 injury and enhance the ability to release intracellular Ca^{2+} in corpus cavernosum smooth muscle cells under hypoxia.

Material and Methods

Reagents

Salidroside (purity 99%, structure shown in Fig. 2a) was purchased from National Institutes for Food and Drug Control (Beijing, China, CAS No. 10338-51-9).

Corpus Cavernosum Smooth Muscle Cell Cultures and Hypoxia Treatment

Corpus cavernosum smooth muscle cells were prepared from young male Sprague-Dawley rats (6 weeks old) from SLRC Laboratory Animals (Shanghai, China) as previously described [21]. The experiments were conducted in accordance with Zhejiang Chinese Medical University Guidelines for Animal Care and Use. The cells were seeded onto 25-cm^2 cell culture flasks and cultured in Petri dishes (100 mm diameter) in Dulbecco's modified Eagle medium (DMED; Invitrogen, Grand Island, NY, USA) containing 20% fetal bovine serum (Invitrogen). When the cultures were about 70–80% confluent, the growth medium was removed, and the cells were washed twice in phosphate-buffered saline (PBS) and incubated in phenol red and serum-free medium containing 0.1% BSA (Amresco, Solon, OH, USA). After 24 h, the cells were maintained in a modular incubator chamber (StemCell, Vancouver, BC, Canada) filled with hypoxic gas (1% O_2, 5% CO_2, and balanced N_2), achieved as before [21]. The cells were treated under hypoxia for 48 h in the presence or absence of salidroside. The cells cultured as control were maintained for 48 h in normoxic atmosphere (95% air, 5% CO_2). At the end of each experiment, the cells were collected for protein extraction for further experiments.

MTT Assay

In this experiment, 7×10^3 cells per well were seeded into 96-well plates. The next day, the medium was changed, and the cells were treated with the medium containing salidroside (0, 0.5, 2, 8, 30, 120, and 500 μg/mL) for 48 h. Each group contained 8 identical samples. Cell viability was measured using the MTT assay at 490 nm by a microplate reader (Molecular Devices, Silicon Valley, CA, USA).

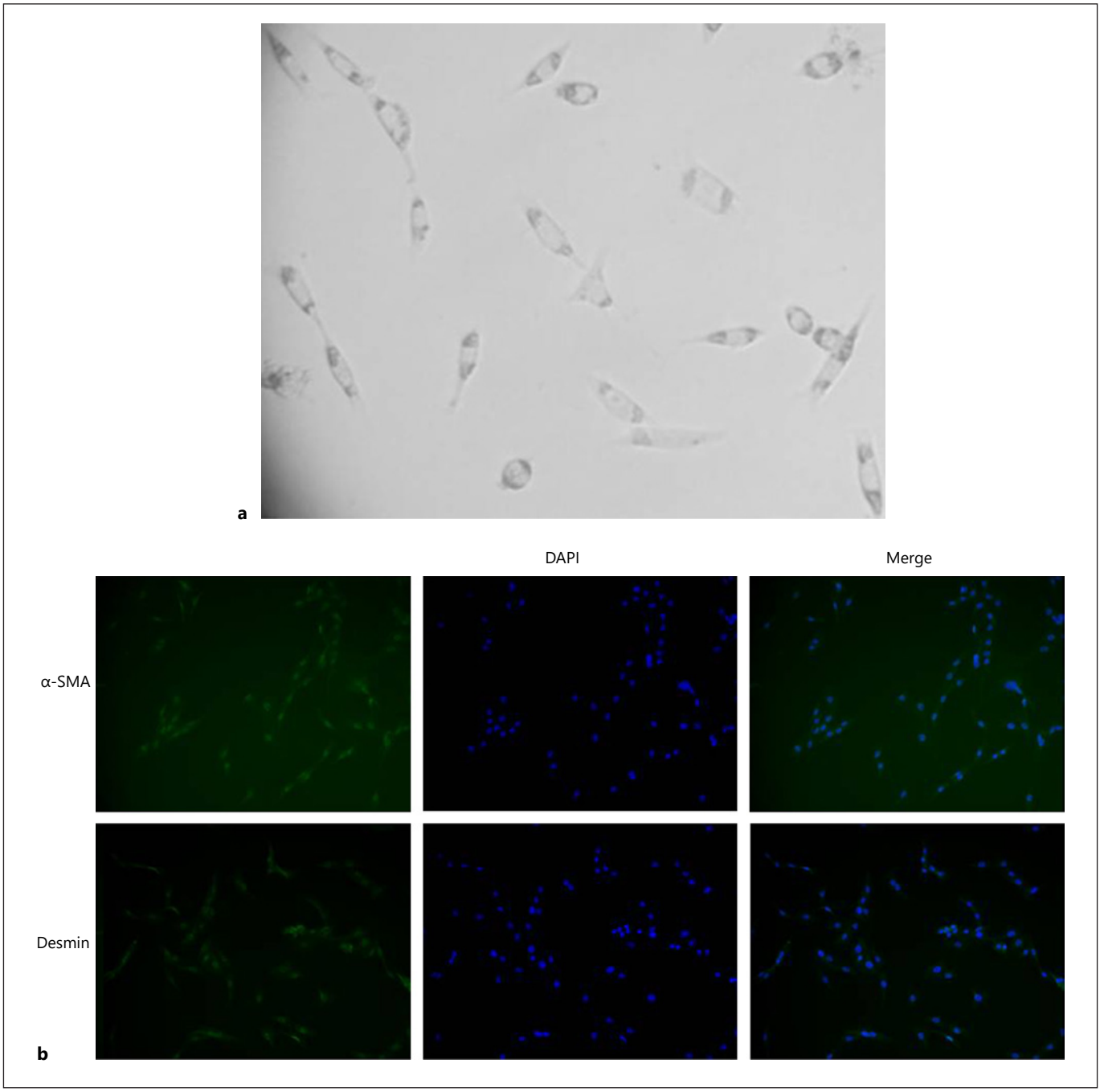

Fig. 1. Corpus cavernosum smooth muscle cells. **a** The shapes of corpus cavernosum smooth muscle cells. **b** Characterization of corpus cavernosum smooth muscle cells in vitro. Photos were taken by phase-contrast microscopy (magnification, ×100).

Immunofluorescent Staining

The cells cultured on sterile glass coverslips were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton X-100 in PBS for 15 min. Then, they were rinsed with PBS and blocked for 1 h in the blocking buffer containing 10% goat serum before incubated overnight at 4°C with the primary antibody α-SMA (1:200), desmin (1:200), HIF-1α (1:200), and Cx43 (1:200) (Abcam, Cambridge, MA, USA). After washing thoroughly, the cells were incubated with the FITC-conjugated secondary antibody at room temperature for 30 min. Fluorescent images were analyzed using a fluorescence inverted microscope (Nikon, Tokyo, Japan).

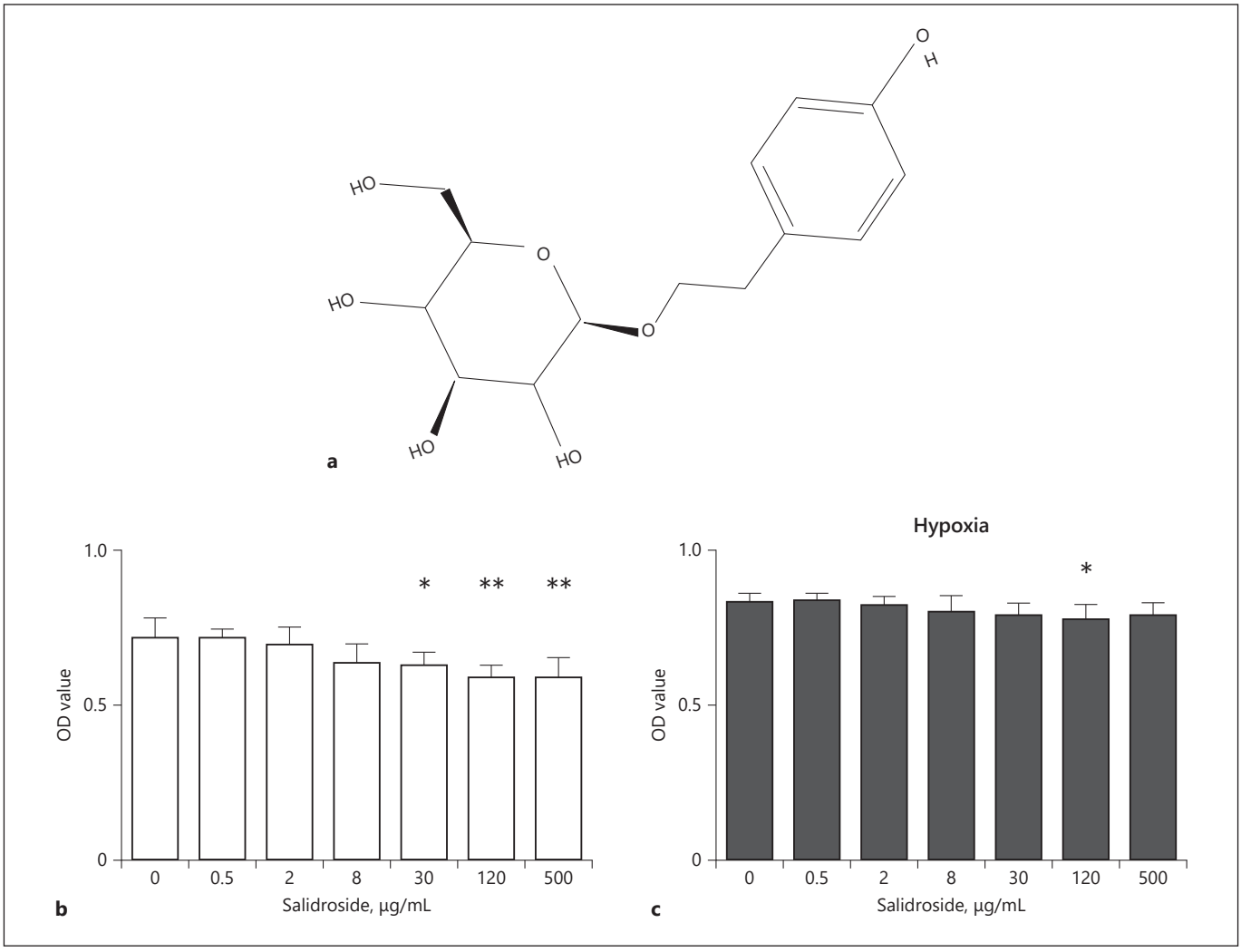

Fig. 2. The effect of salidroside on cell viability. **a** Chemical structure of salidroside. **b** The cells were added with different concentrations of salidroside (0, 0.5, 2, 8, 30, 120, and 500 μg/mL). Cell viability was decreased with 30 μg/mL of salidroside and decreased significantly with >120 μg/ml. **c** Cells were pretreated with various concentrations of salidroside (0, 0.5, 2, 8, 30, 120, and 500 μg/mL) and exposed to hypoxia for 48 h. Cell viability was decreased with 120 μg/mL of salidroside under hypoxia. Cell viability was determined with the MTT assay. All Data are shown as means ± SE ($n = 8$; $^*p < 0.05$, $^{**}p < 0.01$).

Western Blotting

The cells were seeded in 6-well plates and exposed to hypoxic condition for 48 h. Then, they were lysed in lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/mL leupeptin) on ice for 30 min and centrifuged at 8,000 *g* for 10 min at 4°C. Protein concentration was tested with a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of proteins (40 μg) were loaded on 5–10% SDS-PAGE gels and transferred to PVDF membranes (Bio-Rad). Blocking with 5% nonfat milk, the membranes were incubated with specific primary antibodies against HIF-1α (1:3,000), CX43 (1:3,000), and β-actin (1:3,000) (Abcam, Cambridge, MA, USA). Immunoblots were evaluated with the Odyssey Infrared Imaging System (Li-Cor).

Ca^{2+} Measurement

Intracellular Ca^{2+} signal measurements were performed with Fluo-3/AM according to the manufacturer's instructions. In brief, the cells were prepared into single cell suspension with trypsin (Gibco, Carlsbad, CA, USA). The cells were then gently rinsed with HBSS without Ca^{2+} and incubated for 30 min kept under dark in HBSS containing Fluo-3/AM (5 μmol/L) and pluronic F-127 (0.2%). After rinsing, the cells were suspended at the density of 1×10^6/mL. Norepinephrine (10 μmol/L) was added to stimulate intracellular Ca^{2+} release. The relative fluorescence units were detected by using a fluorescence microscope and multifunctional enzyme (Thermo Scientific, Waltham, MA, USA) at 525 nm.

Statistical Analysis

All the experiments were performed in triplicate, unless stated otherwise. All data are presented as means ± SEM and analyzed using Student's *t* test between 2 groups and one-way ANOVA followed by Bonferroni correction for the post hoc *t* test between multiple groups using SPSS 18.0 software (IBM, Chicago, IL, USA). A p value <0.05 was considered significant.

Results

Isolation of Corpus Cavernosum Smooth Muscle Cells

The primary corpus cavernosum smooth muscle cells initially appeared to have long spindle shapes (Fig. 1a). They maintained original morphology even in the fourth passage. The cells used for experiments were all within 4 generations. Immunofluorescent staining results showed that the cells used were strongly positive for the smooth muscle cell markers α-SMA and desmin (Fig. 1b).

Assessment of Cell Viability Treating with Salidroside

Salidroside was added into cell cultures in different concentrations under normoxic and hypoxic environments. Cell viability was assessed by the MTT assay 24 h later. Cell viability was decreased with 30 μg/mL salidroside under natural conditions and decreased obviously with >120 μg/mL salidroside (Fig. 2b). Cell viability was decreased with up to 120 μg/mL salidroside concentrations under hypoxia. However, there was no significant effect with 500 μg/mL salidroside in the hypoxic environment (Fig. 2c).

Salidroside Attenuates Hypoxia-Induced Expression of HIF-1α

It is well known that HIF-1α is activated when cells are exposed to hypoxia, which is an important pathway in lower oxygen tension. According to the western blotting and immunofluorescence results, HIF-1α was significantly upregulated in hypoxic corpus cavernosum smooth muscle cells, whereas it was mainly localized in the cytosol under natural conditions. Hypoxia induced the translocation of HIF-1α into the nuclei areas. Salidroside concentrations as low as 2 or 30 μg/mL decreased the HIF-1α expression in corpus cavernosum smooth muscle cells under hypoxic condition (Fig. 3a, b).

Salidroside Downregulated Expression of Cx43

As gap junction is an important determinant of intercellular connectivity, we analyzed the effects of hypoxia on Cx43 protein levels. The effect of hypoxia on cultured cell Cx43 protein distribution and quantity was determined by immunohistochemical staining and Western blot analysis. The cells exhibited a large increase in Cx43 staining on the cytomembrane when exposed to hypoxia. The Cx43 immunofluorescence signal was markedly decreased when treated with 30 μg/mL salidroside. Pretreating the cells with 30 μg/mL salidroside led to significant downregulation of the Cx43 protein. However, there was no significant decrease in the Cx43 protein with 2 μg/mL salidroside (Fig. 4a, b).

Salidroside Enhanced the Ca^{2+} Release in Cells with Hypoxic Pretreatment

To determine if hypoxia affects the Ca^{2+} signal in corpus cavernosum smooth muscle cells, changes in the Ca^{2+} signal were measured using the calcium ion fluorescent probe Fluo-3/AM. The results showed that Ca^{2+} fluorescent intensity in normoxia is stronger than in hypoxia. There is no difference between the cells in hypoxic condition with or without salidroside. After norepinephrine (NE) stimulation, the Ca^{2+} signal increased. However, relative fluorescence units illuminated that the cell Ca^{2+} signal in hypoxia was significantly lower than that in normal condition. Pretreatment with salidroside (30 μg/mL) under hypoxia could increase intracellular Ca^{2+} signals effectively after stimulation, but it was still lower than in normal conditions (Fig. 5).

Discussion

Salidroside is one of the dominant active components of *R. rosea* L and possesses diverse pharmacological effects [18]. In the present study, we have demonstrated that in the hypoxic environment, salidroside could attenuate hypoxia-induced expression of HIF-1α in corpus cavernosum smooth muscle cells. Salidroside could protect corpus cavernosum smooth muscle cells from viability, gap junction, and the ability of releasing calcium. To our knowledge, this report is the first to demonstrate salidroside's protective effect in corpus cavernosum smooth muscle cells' gap junction under hypoxia.

Fig. 3. Salidroside (2 μg/mL and 30 μg/mL) decreased HIF-1α expression under hypoxic condition. **a** Expression of HIF-1α was increased, which was detected by immunofluorescence. HIF-1α was decreased by salidroside. Photos were taken by phase-contrast microscopy (magnification, ×200). **b** Representative Western blot of HIF-1α. β-Actin was used as control. The blot shown was a representative of 3 independent experiments ($n = 3$; $^{\triangle\triangle}p < 0.01$, $^{**}p < 0.01$).

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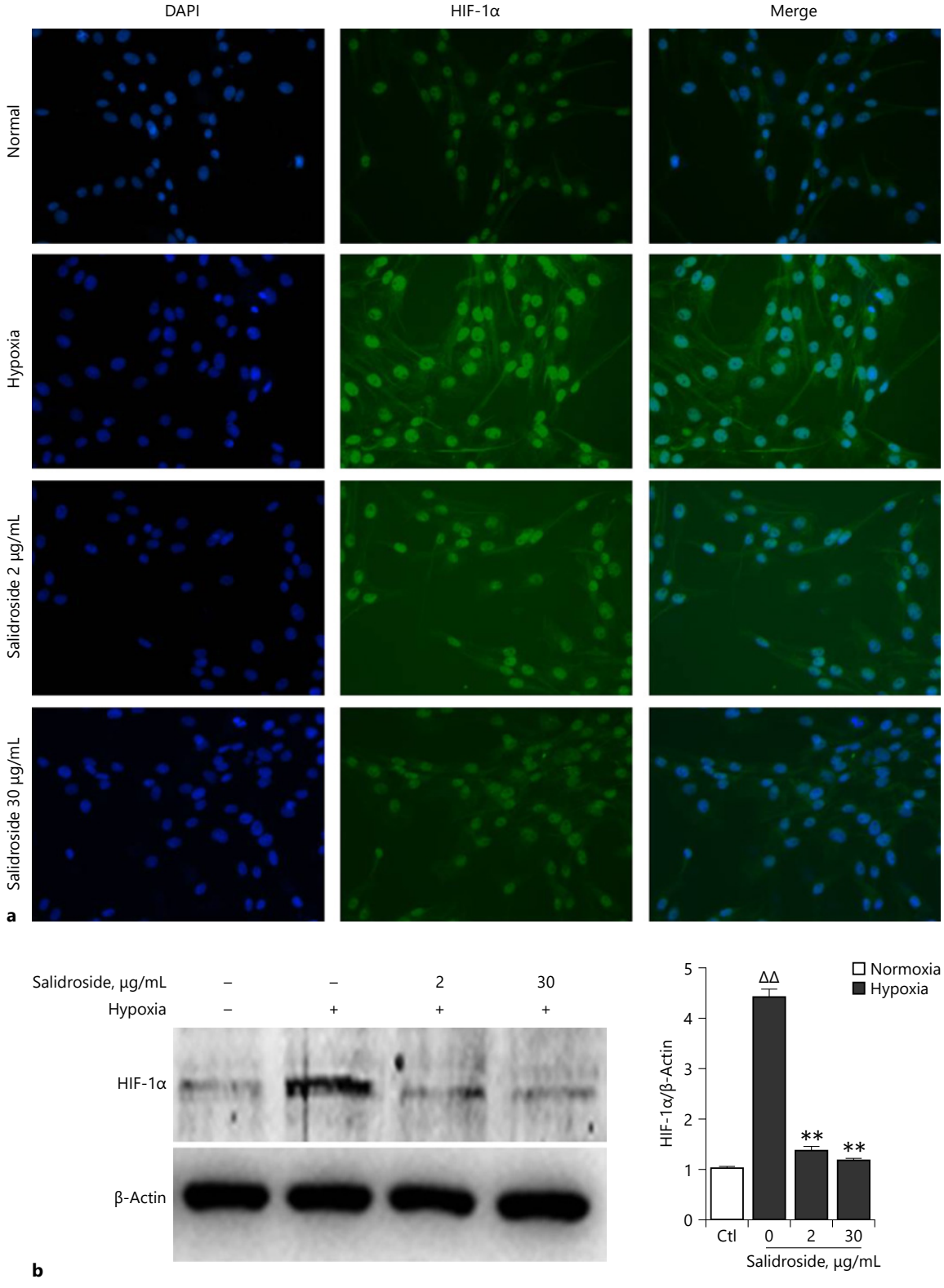

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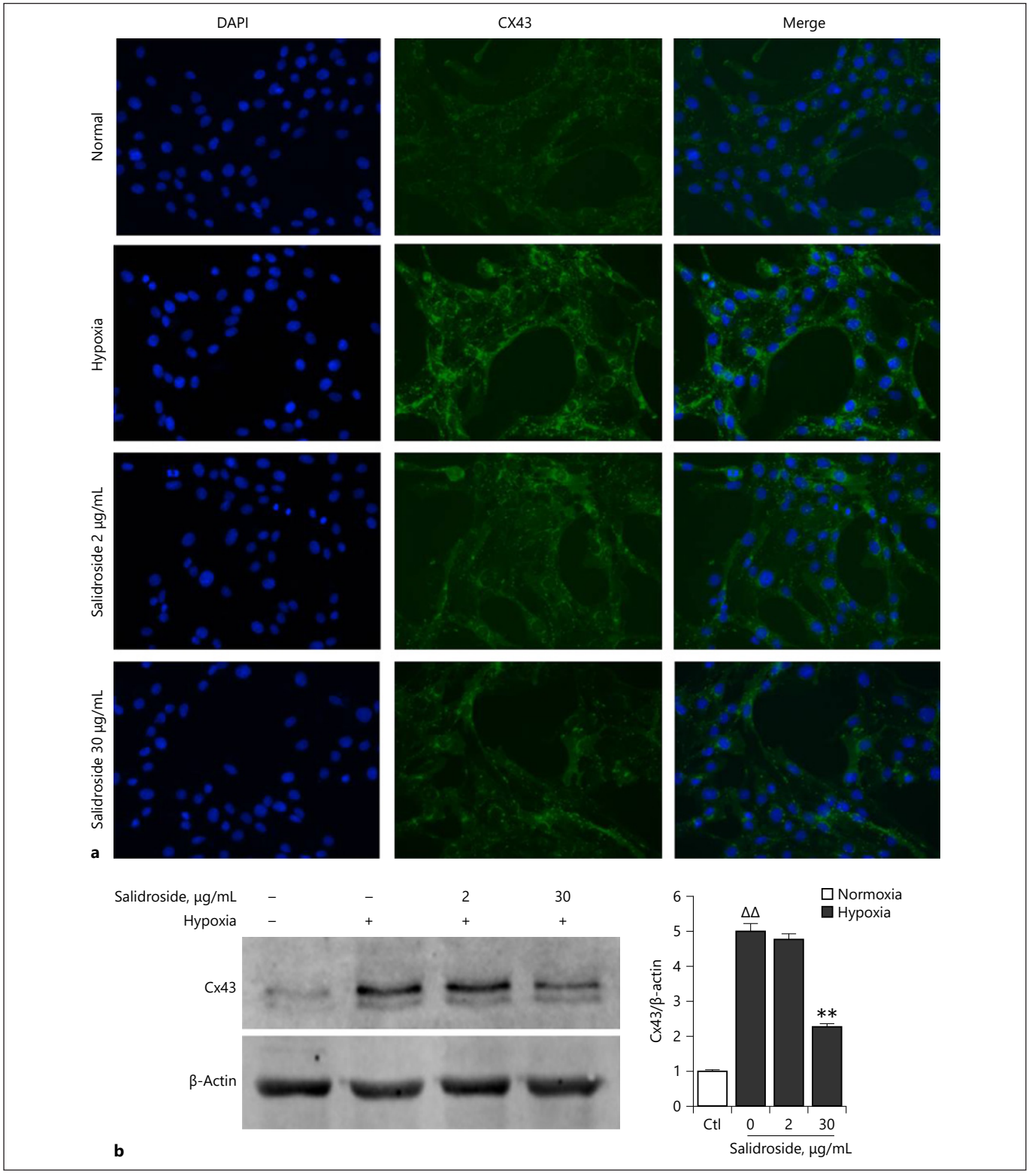

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Fig. 4. Salidroside (30 μg/mL) decreased Cx43 expression under hypoxic condition. **a** Expression of Cx43 was increased under hypoxia which was detected by immunofluorescence. Photos were taken by phase-contrast microscopy (magnification, ×200). **b** Representative Western blot of Cx43. β-Actin was used as control. The blot shown was a representative of 3 independent experiments ($n = 3$; $^{\Delta\Delta}p < 0.01$, $^{**}p < 0.01$).

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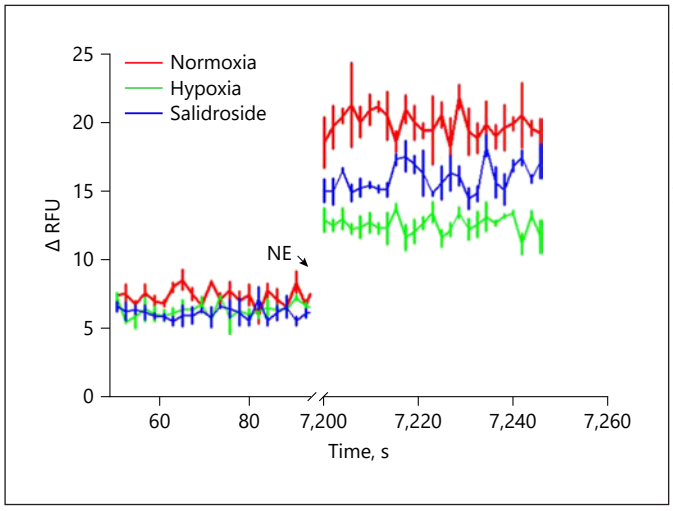

Fig. 5. Salidroside (30 μg/mL) enhanced Ca^{2+} release after stimulation in hypoxic pretreatment cells. There was no difference in initial Ca^{2+} signal in hypoxic condition with or without salidroside ($n = 3$; $^{**}p < 0.01$). NE, norepinephrine.

Oxygenation of the corpus cavernosum smooth muscle plays an important role in regulation of local mechanisms of erection. Numerous evidences show that the chronic state of corpus cavernosum hypoxemia is involved in the development and progression of ED [6]. HIF-1α is an important transcription factor involved in the hypoxia signal pathway and expressed consistently in hypoxic conditions [22]. We have previously reported that hypoxia induced the expression of HIF-1α in corpus cavernosum smooth muscle cells [21]. Our present study confirmed that hypoxia significantly increased the HIF-1α expression, and the latter was decreased after salidroside treatment. Further research proved that hypoxia could induce gap junction Cx43 remodeling in ventricular myocytes [23]. Hence, we investigated whether hypoxia modulates Cx43 in corpus cavernosum smooth muscle cells.

Penile erection is the result of a complex interaction of psychological, endocrine, vascular, and neurological systems. Finally, decrease of intracellular calcium leads to the relaxation of trabecular smooth muscle cells [9, 24], which are the ultimate main function cells in the penile erectile and comprises 42–50% of the cells in the corpus cavernosum [25]. It is well established that even the activation of the smallest fraction of corpus cavernosum smooth muscle cells could lead to penile erection. Gap junction, a specialized intercellular connection between animal cell types, is crucial for this process. It allows the passive intercellular diffusion of ions and small molecules such as amino acids, glucose, nucleotide, ATP, cAMP, IP3, and ions (Ca^{2+} and K^{+}) [26]. The gap junction Cx43 in corpus cavernosum allows intercellular communication between smooth muscle cells and establishes a syncytial cellular network allowing the passage of ions (Ca^{2+} and K^{+}) and signaling molecules (cAMP, cGMP, and IP3) by coordinating corpus cavernosum smooth muscle cells and ensuring penile erection. Thus gap junction Cx43 impairment could be responsible for some cases of ED [11, 13]. Our finding that hypoxia induced the Cx43 expression in corpus cavernosum smooth muscle cells suggests that Cx43 gap junction may play an important role in the physiological adaptation during hypoxia. The gap junction Cx43 expression and permeability properties were altered in corpus cavernosum smooth muscle cells of diabetic rats [27].

Calcium plays a key role in the relaxation of trabecular smooth muscle. Hypoxia could increase the open probability of Cx43 hemichannels [16, 17]. Although the Cx43 protein in corpus cavernosum smooth muscle cells was increased after hypoxia, the intracellular Ca^{2+} signal was obviously decreased. Interestingly, salidroside does not change the base line level of the Ca^{2+} signal in hypoxic condition but could enhances the Ca^{2+} release after stimulation. It suggests hypoxia reduced the ability of communication between corpus cavernosum smooth muscle cells. One study illustrated that ischemia caused dephosphorylation of Cx43 and accumulation of nonphosphorylated Cx43. Hypoxia reduced the Cx43 signal at the border area of the cells but increased the Cx43 signal at the non-border areas. In the study associated this with intercellular uncoupling during hypoxic conditions [15, 28], hypoxia leads to Cx43 dephosphorylation and gap junction uncoupling in cultured astrocytes and rat brain slices [29]. However, in our previous study we found that the expression of Cx43 in the corpus cavernosum was decreased in bilateral cavernous nerve resection rats after 12 weeks [30]. Consistent with this, an earlier research illuminated there was a negative correlation between age and Cx43 expression in the rat corpus cavernosum. Cx43 expression in diabetic corpus cavernosum was decreased significantly in the 2-month diabetic rats. However, it was slightly higher than that in control corpora at 4 months and there is no difference at 8 months [31]. Together, these in vivo findings implied diminished Cx43 in the rat corpus cavernosum with sub-type ED, which were different from our results in vitro. We considered that in vitro cultured corpus cavernosum smooth muscle cells were in an artificial environment which was not equal to the intricate body. Besides, hypoxia exposure in vitro just simulated an early

stage or acute process, while in vivo this was always situated in a later or chronic period of tissue hypoxia.

The above revealed Cx43 gap junctions in corpus cavernosum smooth muscle cells are attractive therapeutic targets for the treatment of ED [11]. In this study, we proved the salidroside could downregulate expression of Cx43 under hypoxic condition. Meanwhile, it enhanced Ca^{2+} release after stimulation in hypoxic pretreatment corpus cavernosum smooth muscle cells. We hypothesized that salidroside might be a good way to treat ED, but the exact mechanism is unclear. Further experiments should be done to test this hypothesis. Some researchers suggest that upregulation of Cx43 expression can lead to astrocytic death and decrease cell viability, while downregulation of Cx43 expression can conduce to astrocytic survival [32].

Overall, we tested the hypothesis that hypoxia causes dysfunction of Cx43 and discovered that the expression of the latter was increased significantly after 48 h of hypoxia. The Ca^{2+} concentration in corpus cavernosum smooth muscle cells under hypoxia was lower than that in normoxia. Further, salidroside could reverse these situations under hypoxia. There are still some limitations in our present study. Although in vitro models of hypoxic stress are meaningful and important cell culture tools, they cannot fully replicate the in vivo setting of corpus cavernosum hypoxia. Therefore, we will focus on the effects of salidroside on erectile function and Cx43 levels through an in vivo rat model with bilateral cavernous nerve injury in a further study.

Acknowledgement

This work was supported by the National Natural Science Foundation of China and the Natural Science Foundation of Zhejiang Province.

Statement of Ethics

The experiments were conducted in accordance with Zhejiang Chinese Medical University Guidelines for Animal Care and Use.

Disclosure Statement

The authors declare that they have no conflicts of interest.

Funding Sources

This work was funded by the National Natural Science Foundation of China (Nos. 81603620 and 81874400) and the Natural Science Foundation of Zhejiang Province (No. LQ19H040001).

Author Contributions

Jianfeng Zhao contributed significantly to analysis and manuscript preparation. Fan Zhao contributed to manuscript preparation. Miaoyong Ye, Ke Ma, and Wenjie Huang performed the experiments. Le Qian, Xiaojun Huang, and Huiyin Fu helped perform the analysis with constructive discussions. Bodong Lv approved the final version.

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