Vasoactive intestinal polypeptide, an erectile neurotransmitter, improves erectile function more significantly in castrated rats than in normal rats

Min-Guang Zhang¹, Zhou-Jun Shen¹*, Cun-Ming Zhang¹, Wei Wu², Ping-Jin Gao³, Shan-Wen Chen⁴, Wen-Long Zhou¹

¹Department of Urology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, ²Department of General Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, ³Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences & Shanghai Jiao Tong University School of Medicine, Institute of Health Sciences, ⁴Department of Urology, ¹st Affiliated Hospital, School of Medicine, Zhejiang University, *Correspondence Author
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OBJECTIVE

Androgen is essential for physiological erection. Vasoactive intestinal polypeptide (VIP) is an important erectile neurotransmitter. While previous studies demonstrated that VIP expression in the penis was androgen-independent, it remains controversial whether androgen has any effect on VIP-mediated erection. The present study aims to investigate the regulatory role of androgen in VIP-mediated erectile effect.

MATERIALS AND METHODS

Male SD rats were divided into a control group, a castration group, and a castration-with-testosterone-replacement group. Four weeks later, each group was subdivided into low and high-dose VIP subgroups and subjected to intracavernous injection of 0.5 and 2 μg VIP, respectively. Erectile function was tested by recording intracavernosal pressure (ICP) and mean arterial blood pressure (MAP) before and after VIP injection. The expressions of the VIP-receptor (VPAC2), G-protein stimulatory and inhibitory alpha subunits (Gs-α, Gi-α), and PDE3A in rat corpus cavernosum (CC) was qualified by real-time PCR and Western blot analysis.

RESULTS

Castration reduced erectile function while testosterone restored it. VIP improved erectile function in a dose-dependent manner. High-dose VIP significantly enhanced erectile function in castrated rats and there was no difference of ICP/MAP among three groups after injection of high-dose VIP. Low-dose VIP also resulted in a higher improvement of erectile function in castrated rats, although the ICP/MAP was lower in these rats than in the other two groups. VPAC2 and Gs-α were up-regulated while Gi-α and PDE3A were down-regulated in CC of castrated rats.

CONCLUSIONS

VIP improves erectile function much more significantly in hypogonadal condition, mainly due to the higher expression of VPAC2, Gs-α, and lower expression of Gi-α and PDE3A in CC of castrated rats. Androgen may negatively regulate the erectile effect of VIP.

KEYWORDS

androgen, vasoactive intestinal polypeptide, erectile function, castration, signaling pathway

INTRODUCTION

Androgen plays an essential role in physiological erection and is critical for the development and growth of erectile organs. The NO/cGMP pathway, the principle erectile signalling pathway, has been proven to be strongly androgen-dependent, both in the expression and activity of nitric oxide synthase (NOS) and in the degradation of cGMP [1–3]. Phosphodiesterase type 5 (PDE5) inhibitors, the most predominant and effective drugs for erectile dysfunction (ED), have also been shown to be positively regulated by androgen [4]. As penile erections are present in infants and hypogonadal males [5] and 20–45% of medically or surgically castrated patients retain normal erections [6], however, it is clear that the relationship between androgen and erection is complicated. It also evokes the hypothesis that there may be an erectile signalling pathway differing from the NO/cGMP pathway which plays a major role in hypogonadism.

Vasoactive intestinal peptide (VIP), a naturally occurring 28-amino acid neurotransmitter, was first isolated from the small intestines of pigs and found to have the potency of vascular dilation [7]. It was also found to be present in the male genital tract [8,9], acting as an important erectile neurotransmitter that regulates the relaxation of the smooth muscle cells of the corpus cavernosum (CC) via a signalling pathway different from the NO/cGMP pathway [10]. By binding and activating the VPAC2 receptor, a G-protein coupled receptor, VIP participates in the erectile process via the activation of the adenyl cyclase (AC)/cAMP pathway. It was found that VIPergic nerves were depleted in
The corpus cavernosum of men with ED and diabetes-related ED [11]. We have previously reported that gene transfer of VIP into the cavernosum improved erectile response in diabetic rats [12]. Clinical studies also showed that Invicorp® (Plethora Solutions, London, UK), a combination of VIP 25 μg and phentolamine mesylate 1 or 2 mg for intracavernosal injection (ICI), was effective in more than 80% of patients with ED, including those who were non-responsive to other therapies [13]. However, in normal men, ICI of VIP alone did not induce erection [14], indicating that VIP improves erectile response more significantly in pathological conditions than in normal conditions.

Our previous study revealed that the expression of VIP at the mRNA level was androgen-independent in rat CC [15]. Another study also found that the protein expression of VIP was androgen-independent in the penis [16]. However, up to now the relationship between androgen and VIP and the effect of VIP on erection is unclear, and the hypothesis that androgen has an effect on VIP-mediated erection remains controversial.

The aim of the present study was to investigate the regulatory role of androgen in VIP-mediated erection and elucidate the underlying molecular mechanisms by examining the VIP-mediated signalling pathway.

MATERIALS AND METHODS

In all, 60 adult male Sprague-Dawley rats, weighing 250–300 g, were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. The rats were randomly divided into three groups: control; surgical castration; and castration-with-testosterone (T)-replacement (castrated but given testosterone undecanoate 100 mg/kg per month by s.c. injection). After 4 weeks, each group was subdivided into low-dose and high-dose VIP subgroups by ICI of 0.5 μg and 2 μg VIP, respectively. After evaluation of erectile function, a blood sample was collected through an abdominal aorta cannula. The rats were killed by injecting air into the left carotid artery and the connective tissues were removed and samples were frozen in liquid nitrogen and stored at −80°C until further analysis. The study was approved by the Animal Experiment Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine.

Erectile function was evaluated as previously reported [17]. Briefly, systemic mean arterial pressure (MAP) was continuously monitored via a PE-10 cannula placed in the left carotid artery. Each 22-gauge needle was inserted in the right and left penile crus for recording intracavernosal pressure (ICP) and drug administration. Both PE-10 tubing and the needle were connected to PE-50 tubing which was filled with heparin (250 IU/mL). ICP and MAP were recorded via pressure transducers connected to an MPA2000 recorder (Shanghai Alcott Biotech. Co., Ltd., Shanghai, China). The cavernous nerve was exposed at the right side of the prostate and mounted on a bipolar platinum hook electrode connected to a JL-B electrical stimulator (Shanghai Jialong Teaching Instrument Manufacturer, Shanghai, China). Electrical stimulation (ES) parameters were 5 V, 25 Hz with a square-wave duration of 2 ms for 30 to 60 s, with a 5-min interval between subsequent stimulations. The erectile response elicited by electric stimulation or ICI of VIP was quantified by calculating maximal ICP/MAP. The ratio of ICP/MAP elicited by electric stimulation of the cavernous nerve before ICI of VIP, called the ‘pRatio’, represented basal erectile function. ICI of 0.5 μg VIP decreased MAP by 20% and MAP was elevated to normal levels within 2 min. Electric stimulation of the cavernous nerve was performed at 5, 10 and 15 min after ICI of 0.5 μg VIP and the maximal ratio of ICP/MAP, called the ‘pRatio’, represented erectile function after ICI of 0.5 μg VIP. ICI of 2 μg VIP decreased MAP by 30–40% and MAP was elevated to normal levels within 3–5 min. A spontaneous increase of ICP was found within 5 min after injection and reached its peak about 20 min later. ES of the cavernous nerve, therefore, was not performed in these subgroups and the pRatio represented erectile function after ICI of 2 μg VIP. We then took pRatio/bRatio as the indicator of the in vivo responsiveness to VIP.

Serum testosterone level was determined by ELISA using a commercial kit (Westang Biotech Co., Ltd., Shanghai, China) and an ELISA plate reader (Multiskan Ex Primary EIA V. 2.3, Thermo, Vantaa, Finland).

Real-time quantitative reverse transcriptase (RT)-PCR was performed as previously described [2]. For each sample 500 ng of total RNA were reverse transcribed to cDNA in a final volume of 10 μl using the PrimeScript RT reagent Kit (Takara, Kyoto, Japan). Real-time quantitative RT-PCR was performed using an ABI 7500 FAST fluorescent quantitation system with the following thermal cycler conditions: 1 cycle of pre-incubation at 95 °C for 10 s, 40 cycles of amplification at 95 °C for 5 s and 60 °C for 34 s. Each measurement was carried out in triplicate. The PCR mixture (20 μl final volume) consisted of 2x the final concentration of SYBR Green Realtime PCR Master Mix (Takara), Beta-actin was chosen as a reference gene. The primer sequences are shown below. The results were analysed using the comparative Ct method according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA).

Frozen CC samples were lysed as described previously [2]. Ten μg of protein extracts were separated on 10% denaturing SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes, which were probed with monoclonal anti-VPAC2 antibodies (Abcam, Cambridge, UK, 1:1000), anti-PDE3A antibodies (Abcam, 1:1000), anti-Gs-α antibodies (Cell Signaling Technology, Danvers, MA, USA, 1:1000) and anti-beta-actin (Sigma, St Louis, MO, USA, 1:8000), followed by peroxidase-conjugated IgG (1:3000) incubation and final detection by electrochemiluminescence (ECL) plus reagents (GE Healthcare, Little Chalfont, UK). Densitometry was used to measure the expression of VPAC2, Gi-α and PDE3A relative to beta-actin with Bio-Rad Gel doc. Software (Bio-Rad Laboratories, Hercules, CA, USA).

Data are expressed as mean ± SD. Comparisons between two groups were carried out using an unpaired t-test, among more than two groups using a one-way ANOVA and least significant difference (LSD) test in case of homogeneity of variance, and Dunnett’s T3-test otherwise. Two-tailed P values < 0.05 were defined as significant. Data management and statistical calculations were conducted using the SPSS software for Windows version 16.0 (SPSS, Inc., Chicago, IL, USA).
RESULTS

The castration group had the lowest serum testosterone concentration (ng/mL) compared with the control group and castration-with-T-replacement group (0.467 ± 0.124 vs 1.437 ± 0.484 and 0.994 ± 0.473; \(P = 0.000\) and \(P = 0.001\), respectively). T replacement recovered T concentration but the concentration was still lower than that of the control group \((P = 0.032)\).

As shown in Table 1, castration significantly reduced the weight, baseline ICP, and bRatio of the rats in this group compared with the control rats, and T replacement counteracted these effects. There was no difference in MAP between the control and castration groups, but T replacement after castration elevated MAP by 10–15 mmHg \(P = 0.007\) and \(P = 0.000\), respectively).

Intracavernous injection of VIP has been shown to induce erection in rats, but the doses of VIP ranged from \(10(-8)\) to \(4 \times 10(-5)\)M \[18,19\]. Therefore, we needed to determine the proper dose of VIP and examine the erectile response to different doses of VIP \((0.05–10 \mu g)\). As shown in Fig. 1, VIP induced a dose-dependent increase in ICP/MAP elicited by ES of the cavernous nerve 5–15 min after injection. Moreover, the induced increase became statistically significant when VIP was \(\geq 0.5 \mu g\). Meanwhile, 2 \(\mu g\) of VIP induced a spontaneous increase in ICP/MAP in all rats, which started \(\approx 5\) min after injection, reached its peak \(\approx 20\) min after injection, and decreased gradually over the next 10 min. We
therefore took 0.5 μg and 2 μg as the low and high dose of VIP, respectively.

Next we compared the ICP/MAP before and after VIP injection to evaluate the in vivo responsiveness of rats to VIP. As shown in Tables 2 and 3 and Fig. 2, the in vivo responsiveness (pRatio/bRatio) was significantly higher in the castration group, both in low-dose VIP (castration group vs control group and castration-with-T-replacement group, 1.54 ± 0.27 vs 1.06 ± 0.14 and 1.06 ± 0.16, P = 0.001 and P = 0.001, respectively) and high-dose VIP subgroups (castration group vs control group and castration-with-T-replacement group, 2.05 ± 0.36 vs 1.19 ± 0.14 and 1.12 ± 0.16, P = 0.000 and P = 0.000, respectively). Although in the low-dose VIP subgroups, the pRatio was lower in the castration group than in other groups (castration group vs control group and castration-with-T-replacement group, 55.6 ± 9.8 vs 73.0 ± 9.4 and 68.5 ± 10.6, P = 0.008 and P = 0.038, respectively); in the high-dose VIP subgroups, there was no difference in pRatio among the three groups (castration group vs control group and castration-with-T-replacement group, 73.7 ± 12.9 vs 81.6 ± 9.6 and 72.2 ± 10.3, P = 0.336).

As VPAC2, Gs-α, Gi-α, and PDE3A are components of the VIP/cAMP erectile pathway that mediates VIP response, we next examined their expression in differently treated rats. Compared with the control, the mRNA level of VPAC2 was higher in the castration and castration-with-T-replacement groups (control group vs castration and castration-with-T-replacement groups, 1.039 vs 2.854 and 2.138, P = 0.000 and P = 0.000, respectively; Fig. 3, panel a). Moreover, the mRNA level of VPAC2 was higher in the castration group than in the castration-with-T-replacement group (P = 0.009). This differential pattern of VPAC2 expression was also revealed at the protein level by Western blot analysis (Fig. 4, panels a and b).

We further found that castration resulted in increased expression of Gs-α at the mRNA level (castration group vs control and castration-with-T-replacement groups, 1.388 vs 1.013 and 0.868, P = 0.001 and P = 0.000, respectively) (Fig. 3, panel b). Protein expression of Gs-α was not examined because the anti-Gs-α antibody was

### TABLE 3 Spontaneous erectile responses to ICI of 2 μg VIP in the experimental rat groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Before ICI</th>
<th>Spontaneous erectile response to ICI of 2 μg VIP</th>
<th></th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>bRatio, %</td>
<td>ICPmax, mmHg</td>
<td>MAPmax, mmHg</td>
<td>pRatio, %</td>
<td>pR/bR</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>68.6 ± 9.3*</td>
<td>102.6 ± 22.2</td>
<td>124.5 ± 15.6</td>
<td>81.6 ± 9.6</td>
<td>1.19 ± 0.14*</td>
<td></td>
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<tr>
<td>Castration</td>
<td>36.0 ± 10.5</td>
<td>76.0 ± 27.8</td>
<td>101.5 ± 27.2</td>
<td>73.7 ± 12.9</td>
<td>2.05 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>Castration+T</td>
<td>64.6 ± 10.4*</td>
<td>91.1 ± 18.8</td>
<td>125.8 ± 14.8</td>
<td>72.2 ± 10.3</td>
<td>1.12 ± 0.16*</td>
<td></td>
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</tbody>
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Values are expressed as mean ± SD; ICPmax, maximal ICP after ICI of 2 μg VIP; MAPmax, MAP corresponding to ICPmax after ICI of 2 μg VIP. One-way ANOVA, LSD test: *P < 0.01 compared with castration group.
FIG. 3. mRNA expressions of VPAC2, Gs-α, Gi-α and PDE3A in corpus cavernosum of experimental rats. Panel a, b, c, d: mRNA levels for VPAC2, Gs-α, Gi-α and PDE3A gene in penile tissues were quantitated by real-time RT-PCR. Values were calculated according to the comparative Ct method and using beta-actin as reference gene. One-way ANOVA, LSD test. **P < 0.01 compared with castration group.

unavailable. We also found that castration reduced the expression of Gi-α at the mRNA level, while T replacement significantly increased its expression (castration group vs control and castration-with-T-replacement groups, 0.431 vs 1.028 and 3.140, \( P = 0.000 \) and \( P = 0.004 \), respectively) (Fig. 3, panel c). A similar pattern of Gi-α expression at the protein level was revealed by Western blot analysis (Fig. 4, panels c and d). Castration also resulted in decreased expression of PDE3A at the mRNA level (castration group vs control and castration-with-T-replacement groups, 0.324 vs 1.034 and 1.512, \( P = 0.000 \) and \( P = 0.004 \), respectively) (Fig. 3, panel d) and protein level (Fig. 4, panels e and f).

DISCUSSION

In the present study, we showed that VIP significantly improved erectile function in castrated rats. Furthermore, we found that the levels of key components of the VIP/cAMP erectile pathway, VPAC2, Gs-α, Gi-α, and PDE3A, were related to serum T concentration in rat CC; levels of VPAC2 and Gs-α were higher while those of Gi-α and PDE3A were lower in castrated rats, suggesting that the changes in expression pattern may mediate the enhanced effect of VIP on erection.

Androgen plays a critical role in physiological erection and is essential for the development, growth, and maintenance of penile-tissue function [1]. The NO/cGMP pathway, the principle erectile pathway, has been shown to be highly dependent on androgen [2,4,20]. However, physiological erection is a complicated neurovascular process regulated by multiple factors. It has been confirmed that there was no correlation between total T levels and ED, or with the severity of ED [21,22]. Although T-replacement therapy (TRT) has attracted increasing attention and has been used in prostate patients with ED after radical prostatectomy [23], meta-analysis has revealed that the efficacy of hormonal treatments in men with ED was inconclusive [24], and the American College of Physicians does not recommend routine use of hormonal blood tests or TRT for patients with ED [25]. It is therefore worth investigating the relationship between androgen and other erectile signalling pathways.

Vasoactive intestinal peptide is an important erectile neurotransmitter which was first isolated from the small intestines of pigs and found to have the potency of vascular dilation [7]. The injection of VIP induces erection in several animal species, including humans [26,27]. The VIP-induced vasodilation, which results in tumescence, is mediated by cAMP upon the activation of AC after the binding of VIP to its receptor [10]. Interestingly several studies, as well as the present study, have shown that VIP expression in CC is androgen-independent, both in rats and humans [15,16].

In the present study, we aimed to investigate further the relationship between androgen and the effect of VIP on erection. By surgical castration and T replacement, we established rat models with different T concentrations and basal erectile function (Table 1). To evaluate the in vivo erectile responsiveness to VIP, we used the ratio of post-ICI ICP/MAP to baseline-ICI/MAP (pRatio/bRatio) as the indicator of the in vivo erectile responsiveness to VIP and found that this responsiveness was significantly higher in the castration group than in the other two groups, both in low-dose and high-dose VIP. Furthermore, high-dose VIP restored the reduced erectile function in castrated rats to the same level as that in the control and castrated-with-T-replacement rats. This suggests that the effect of VIP on erection is independent of androgen, at least in rats.

Based on a previous report that ICI of 25 μg VIP was safe and effective in the treatment of ED and that low doses of VIP (up to 1.2 μg) could not induce useful erection in impotent men [13], we tested the effective dose of VIP in rats in the dose range from 0.05 μg to 10 μg. The dose of VIP used in the present study was consistent with (0.5 μg), or higher than (2 μg), that used in the human body according to body weight, because of species differences.

As an important erectile neurotransmitter, VIP regulates the relaxation of cavernous smooth muscle cells through the VIP/VPAC2/AC/cAMP/protein kinase A erectile signalling pathway. Binding of VIP to the VPAC2 receptor activates AC, promoting the production of cAMP and activation of cAMP-dependent protein kinase A, which results in the relaxation of smooth muscle cells. Gs-α and Gi-α are two distinct G-proteins which mediate the stimulation and inhibition of AC and thus are involved in the regulation of cavernous smooth muscle tone [28]. cAMP is further regulated by its degradation by cAMP-
binding PDEs (PDE3A and PDE4), and PDE3A is highly expressed in CC [29–32]. Thus, the relaxation effect of VIP on cavernous smooth muscle depends on VPAC2, G-proteins, and PDE3A.

To explain why VIP improves erectile function more significantly in castrated rats than in normal rats, we examined the expression of VPAC2, Gs-α, Gi-α, and PDE3A in CC by quantitative real-time PCR and Western blot. As shown in Fig. 3, VPAC2 and Gs-α were upregulated, while Gi-α and PDE3A were downregulated in castrated rats, and T replacement reversed the changes in expression. Overexpression of VPAC2 is consistent with higher sensitivity to VIP in castrated rats, while changed expression of Gi-α and Gi-α proteins is associated with reduction of cavernous smooth muscle tone. The reduced expression of PDE3A may decrease the degradation of cAMP, thus indirectly enhancing the effect of VIP. Taken together, we speculate that the reason that the effect of VIP on erection was more significant in castrated rats could be because of higher expression of VPAC2 and Gi-α, as well as lower expression of Gi-α and PDE3A in CC of these rats.

To our knowledge, the present study establishes for the first time that androgens may negatively regulate an important erectile pathway, the VIP/cAMP pathway, to modulate erectile response. Therefore, we propose that in the process of penile erection, the VIP/cAMP signalling pathway serves as a complementary mechanism to the main NO/cGMP pathway, and VIP could play a dominant role in erection in hypogonadal conditions when the NO/cGMP pathway is inhibited by androgen deprivation. Our findings may have important clinical significance for patients with prostate cancer who have ED and are unresponsive to PDE5 inhibitors because of androgen deficiency, and who are unsuitable for androgen replacement. Our study may also be helpful for the treatment of late onset hypogonadal-related erectile dysfunction.

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CONFLICT OF INTEREST

None declared.

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Correspondence: Zhou-Jun Shen, Department of Urology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 2nd Ruijin Road, Shanghai, 200025, PR China. Tel: +86 21 6431 5370; Fax: +86 21 6431 5370. e-mail: shenzj6@sina.com, zhangmg1970@hotmail.com

Abbreviations: NOS, nitric oxide synthase; PDE5, phosphodiesterase type 5; ED, erectile dysfunction; VIP, vasoactive intestinal peptide; CC, corpus cavernosum; AC, adenylyl cyclase; IC, intracavernosal injection; MAP, mean arterial pressure; ICP, intracavernosal pressure; ES, electrical stimulation; RT, reverse transcriptase.