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# Angiotensin II increases corpus cavernosal contractility and oxidative stress in partial bladder outlet obstructed rabbits: relevance to erectile dysfunction

**Hani Ertemi[a], David H. W. Lau[a], Dimitri P. Mikhailidis[a], Faiz H. Mumtaz[b] and Cecil S. Thompson[a*]**

[a]Departments of General Surgery & Clinical Biochemistry, Division of Interventional Science, University College London Medical School, Royal Free Campus, Pond Street, London NW3 2QG, UK.

[b]Department of Urology, Chase Farm Hospital, The Ridgeway, Enfield EN2 8JL, UK.

**Abstract**

***Introduction.*** We investigated the effect angiotensin II (Ang II), a corpus cavernosal smooth muscle (CCSM) constrictor peptide, has on tissue taken from rabbits following chronic partial bladder outlet obstruction (PBOO), since this model is characterised by an increase in corpus cavernosal collagen deposition and a marked reduction and impaired relaxation of CCSM cells.

***Aim.*** To determine the interaction between Ang II and nitric oxide (NO) and the development of oxidative stress (OS) in a rabbit model of chronic PBOO.

***Methods.*** Corpus cavernosal tissue was obtained from 12 sham-operated and 20 PBOO rabbits. Organ bath studies determined Ang II/NO interaction on CCSM function using losartan (AT1 receptor antagonist), sodium nitroprusside (SNP, NO donor), electrical field stimulation **(**EFS) and vardenafil (PDE5 inhibitor). The role of OS in the Ang II response was also determined using diphenyene iodonium chloride (DPI) the NAD(P)H oxidase inhibitor, which inhibits superoxide production and superoxide dismutase (SOD, the enzyme that accelerates the breakdown of superoxide).

***Main Outcome Measure***. Action of Ang II, AT1 receptor antagonist, as well as SOD and DPI on CCSM function.

***Results.*** Ang II caused a dose dependent contraction of CCSM strips that was enhanced in PBOO rabbits and inhibited by losartan, DPI and SOD. CCSM relaxation induced by SNP/EFS was impaired in this model and improved by vardenafil and losartan.

***Conclusions.*** These findings imply that the increased Ang II contractile response is a pathological consequence of PBOO and that AT1 receptor inhibition may be a therapeutic approach to treat ED associated with PBOO.

***Key Words.*** Angiotensin II; Losartan; Corpus Cavernosum Contraction; Partial Bladder Outlet Obstruction; Oxidative Stress; Benign prostatic hypertrophy/lower urinary tract symptoms

## Introduction

It is now recognised that benign prostatic hyperplasia (BPH) can cause partial bladder outlet obstruction (PBOO),[1] resulting in structural and functional changes to the bladder that can influence the storage and emptying of urine, with many patients developing detrusor overactivity.[2] The clinical consequences of PBOO associated with BPH[3], including urodynamics and structural changes in bladder pathophysiology can be reproduced in animal models, by tying a ligature around the proximal urethra at the base of the bladder neck. [4,5] An increase in bladder mass, as well as hypertrophy and hyperplasia of bladder smooth muscle, with thickening of the outer serosal layer have been reported in rabbits following this procedure. [5] Functional studies have also revealed a reduction in

electrical field stimulation (EFS) and chemical-induced bladder smooth muscle contraction, [6] supporting the clinical findings of impaired bladder function following PBOO.

There has been a continuing debate as to whether there is a link between BPH and erectile dysfunction (ED). Although some clinical studies suggest an association,[7,8] where sexual performance is related to the severity of BPH[9], a literature based study could not identify this link.[10] However, the use of the PBOO animal model has helped to investigate this association.[11,12] We were the first to show increased collagen deposition in the corpus cavernosum[13] and preliminary evidence of impaired corpus cavernosal smooth muscle (CCSM) relaxation in a chronic PBOO rabbit model,[14,15] . This has been substantiated by the findings that endothelium-dependent (ACh-mediated) and endothelium-independent (ATP/ sodium nitroprusside [SNP]-mediated) CCSM relaxation is impaired, with a marked reduction in smooth muscle cells in this model.[12] These findings imply that PBOO animals exhibit many of the features of ED and offer a test bed to determine its influence on other components of the erectile process, for example pro-contractile mechanisms, which terminate penile erection by keeping the smooth muscle of the penile arteries and trabeculae contracted.[16] This is of particular importance, since unlike most smooth muscle cells those of the corpus cavernosum spend the majority of the time contracted. [11]

Angiotensin II (Ang II), a bioactive octapeptide, is one such mediator of human CCSM contractility and tone. [17] This is supported by the finding that human corpus cavernosum produces and secretes physiological amounts of Ang II[18] and healthy men produce Ang II corpus cavernosal blood levels that are higher during penile detumescence compared with the tumescence phase.[19] Although the physiological actions of Ang II are mediated via AT1 and AT2 receptors, [20] in regards to the corpus cavernosum, human and rabbit studies have found activation of AT1 and not AT2 receptors elicit smooth muscle contraction, a response that can be blocked by AT1 receptor antagonists.[17,21] It is now clear that Ang II plays an important role in modulating the tone of human penile arteries and trabecular smooth muscle and that its regulation is governed by a balance with NO. [17] NO antagonises the vasoconstrictive and pro-atherosclerotic effect of Ang II, whereas Ang II decreases NO bioavailability by promoting oxidative stress (OS). [17,22] Ang II upregulates the production of superoxide ($O_2^-$ ; one of the reactive oxygen species elevated in OS), in endothelial and vascular smooth muscle, which is thought to directly contribute to Ang II-induced smooth muscle contraction.[23]

Here, we determined the effect Ang II, AT1 receptor inhibition and OS have on CCSM contractility, together with their modulation of NO-mediated relaxation in a chronic rabbit model of PBOO. The results from this study provide important information on the pathological role of Ang II in ED.

## Methods

### Induction of partial bladder outlet obstruction (PBOO)

Twenty 3kg adult male New Zealand White rabbits purchased from Highgate Farm (UK Home Office accredited source) underwent PBOO. Twelve age-matched, sham-operated rabbits formed the control group. All animal were fed ad libitum with SDS standard plain diet (SDS, Witham, UK) and allowed free access to water.

To create PBOO, each rabbit received a general anaesthetic (1-2% halothane in $O_2$) and was then placed on a heating pad regulated at $37^0$C. The abdomen was shaved and the operative site sterilised with betadine. A urinary balloon catheter (Foley, C.R.

Bard international Ltd, Crawley, UK) size 8 Fr gauge was inserted into the penile urethra and inflated inside the bladder. The bladder and proximal urethra was exposed following a lower midline laparotomy and cleared of fat and connective tissue. A 2-0 silk ligature was placed loosely around the catheterised proximal urethra at the base of the bladder neck. The bladder was then returned to the peritoneal cavity and the wound closed in layers. The laparotomy incision was closed with continuous stitching using 4-0 vicryl suture and the skin closed with subcuticular stitching using 4-0 vicryl suture. The catheter was then removed and the rabbit allowed to recover. Pain medication (buprenorphine, 0.1 mg/kg im, twice daily for 2 days) and antibiotics (enrofloxacin, 10 mg/kg im, twice daily for 5 days pre- and post-operatively) was administered to each rabbit. Sham-operated rabbits underwent the same surgical procedure without tying the ligature around the proximal urethra. All procedures were conducted under an approved Home Office Project Licence. After 8 weeks the sham-operated and PBOO rabbits were killed by cervical dislocation (using a method permitted by the Home Office). The penis was rapidly excised from each rabbit and placed in cold oxygenated Krebs solution at $4^0$C for the organ bath studies.

All rabbit penile tissue preparations were investigated on the same day of tissue acquisition.

**Tissue acquisition**

Fat and connective tissue was removed from the rabbit penis and the tunica albuginea opened. The corpus cavernosum was removed and cut into strips of approximately 1x3x1 mm. Tissue strips were taken from at least 3 animals for each experiment.

**Organ bath studies**

The tissue strips were mounted vertically in 10 ml organ baths, equipped with two parallel platinum electrodes for EFS. Tissues were bathed with Krebs solution and maintained at $37^0$C by a thermoregulated circuit and bubbled with a mixture of 95% $O_2$ / 5% $CO_2$. The Krebs solution was made up of NaCl 120 mM, NaHCO3 25.6 mM, KCl 4.7 mM, CaCl2 2.5 mM, $NaH_2PO4$ 1.2 mM and glucose 22 mM with a pH of 7.4. A 2g tension was applied to the suspended tissue strips and left for 1h to equilibrate (tension recorded on a Grass Polygraph, model 7D; Astro-med Grass, Slough, UK).

**Effects**

**Bladder weights:** The bladders were excised from sham-operated and PBOO rabbits after 8 weeks and weighed.

**Ang II.** The effect of Ang II ($10^{-8}$M – $10^{-5}$M) on CCSM function was investigated using tissue strips from sham-operated and PBOO rabbits.

**Ang II receptor antagonists.** After the effect of Ang II on tissue strips taken from sham-operated and PBOO rabbits were determined; the tissues were washed over a 30 min period and exposed to losartan ($10^{-5}$ M, AT1 antagonist) for 20 min before repeating the Ang II response. The effect of the vehicle (distilled water for 20 min) on the Ang II response was also determined.

**Oxidative Stress**

**DPI .** The effect diphenyene iodonium chloride made up in DMSO (DPI, $10^{-4}$M, NAD(P)H oxidase inhibitor, which inhibits $\dot{O}_2^-$

production) has on the Ang II ($10^{-6}$M) response from sham-operated and PBOO tissue strips was determined.

**SOD.** The effect superoxide dismutase (SOD, 200 IU/ml; the enzyme that accelerates the breakdown of $^{\bullet}O_2^-$) has on the Ang II ($10^{-6}$M) response from sham-operated and PBOO tissue strips was also determined.

**Electrical Field Stimulation**

**NANC neurotransmission.** In a series of experiments CCSM tissue from sham-operated and PBOO animals were exposed to guanethidine (5 x $10^{-6}$ M), atropine ($10^{-5}$ M) and indomethacin ($10^{-6}$ M), which were added to the bathing solution and left for 20 min to inhibit the adrenergic, cholinergic and cyclo-oxygenase pathways, respectively, leaving the NANC pathway intact. The tissue strips were then pre-contracted with PE followed by EFS of penile nerves with a Grass S88 (Astro-med Grass, Slough, UK) stimulator. The stimulator delivered single square waves (duration 0.4 ms; 20V) at a frequency of 8.0 Hz in 5 s trains. Losartan and DPI was then added and the EFS repeated.

We choose 8 Hz since this stimulation frequency is ideal to evaluate the effect of losartan on NANC neurotransmission. [17]

**Sodium Nitroprusside**

CCSM tissue strips from sham-operated and PBOO rabbits were pre-contracted with PE ($10^{-4}$M) and cumulative response curves were constructed for the NO donor SNP, $10^{-7}$ – 3x$10^{-6}$ M).

**Vardenafil.** After constructing the SNP cumulative response curve the strips were washed several times followed by the addition of vardenafil, ($10^{-8}$ M; PDE 5 inhibitor, a cGMP-specific phosphodiesterase type 5 inhibitor, which inhibits the hydrolysis of cGMP to 5'- GMP) to the organ bath for 20 min. The tissues were re-contracted with PE and cumulative response curves were again constructed for SNP. The stock solution of vardenafil ($10^{-3}$ M) was made up in acid water pH 4.5 and subsequently diluted in distilled water before adding to the organ bath. We found that the final dilution of acid had no effect on SNP-induced relaxation.

**Losartan.** In another series of experiments SNP cumulative response curves were again constructed using CCSM tissue from PBOO animals. The tissue was washed several times before the addition of losartan ($10^{-5}$ M) to the organ bath for 20 min and re-contracted with PE and a cumulative response curve constructed for SNP.

**Statistical Method**

Results were analysed and expressed as mean ± SEM using Graph Pad Prism 3.0 software. Ang II tissue responses were expressed as mg tension / mg tissue. EFS and SNP tissue responses were expressed as % relaxation of PE-induced tone. Comparisons of the Ang II dose response curves and SNP cumulative dose response curves were made using analysis of variance (2 way ANOVA, $p < 0.05$). Statistical analysis was determined using a Student's unpaired and paired t-test with statistical significance accepted at $p < 0.05$.

**Results**

**Bladder weights**

There was a significant increase in 8 weeks PBOO rabbit bladder weights when compared with sham-operated animals (sham-operated rabbits, 2.1 ± 0.1g vs PBOO rabbits, 23.2 ± 2.5g; n =13 $p<0.0001$ Student's unpaired t-test).

**Ang II and CCSM contraction**

The size and weight of cavernosal strips from sham-operated and PBOO rabbits were similar. Ang II caused a dose dependent contraction ($10^{-8}$M – $10^{-5}$M) of CCSM strips from sham-operated and PBOO rabbits, which was markedly increased in the PBOO group and reduced by losartan (Fig 1). The addition of the vehicle did not significantly influence the Ang II response in any experiment.

**Oxidative Stress**

**CCSM contraction.** DPI and SOD reduced the Ang II-induced contraction of CCSM strips from sham-operated and PBOO rabbits, Fig 2.

**CCSM relaxation.** EFS-induced CCSM relaxation of sham-operated and PBOO strips, was increased following adrenergic, cholinergic and cyclo-oxygenase inhibition and in the presence of losartan (Fig 3). In addition, EFS-induced CCSM relaxation of PBOO strips was increased by 18.4% following addition of the triple inhibitors (guanethidine, atropine and indomethacin), and in the presence of DPI.

**SNP and CCSM relaxation**

SNP-induced relaxation of CCSM strips taken from PBOO rabbits was impaired compared with sham-operated animals and improved by vardenafil (Fig 4) and losartan (Fig 5).

**Discussion**

This study shows, for the first time, a dose dependent enhancement of the Ang II-induced contraction of CCSM tissue taken from PBOO rabbits when compared with sham-operated controls. It also supports previous findings using human and rabbit corpus CCSM tissue, which revealed the Ang II–mediated contractile response is due to AT1 receptor activation[17,21] with the development of OS.[17]

Under physiological conditions Ang II-containing cells in the endothelium of arterioles, as well as the endothelium lining sinusoids and smooth muscle bundles of the corpus cavernosum, [17] secrete their Ang II content on adrenergic stimulation, keeping the smooth muscle of the penile arteries and trabeculae contracted; a scenario that has added significance, since the CCSM cells spend the majority of the time contracted during penile flaccidity/detumescence.

Previous *in vivo* experiments using anesthetised dogs have shown that intracavernosal injections of Ang II terminate spontaneous erections. In contrast, losartan induced penile erections by relaxing CCSM, affecting tone and contractility of vascular smooth muscle within the blood vessels embedded in the corporal bodies, as well as the corpus cavernosum itself.[18]

The present findings demonstrate that Ang II increases CCSM contraction as a pathological consequence of PBOO. We have used only one concentration of Ang II on each individual tissue strip, since multiple application of Ang II to isolated human arteries results in a marked desensitisation of the functional response (i.e. tachyphylaxis) [24]. This phenomenon may explain the large variation in the Ang II $10^{-5}$M error bar following PBOO (Fig 1).

Results from previous studies using PE as a mediator of CCSM contractility following PBOO in rabbits are inconclusive. Chang et al., [11] noted a 50% increase in CCSM contractile force after 2 weeks PBOO, due to an increase in smooth muscle bundles and cellular alterations in the contractile myosin-isoform composition. This increased contractility was not evident in the studies of Demir et al., [25] and Lin et al., [12] at a similar time point, possibly due to post-operative inflammation in the sham-operated group and a reduction in CCSM cells, respectively. Results from chronic PBOO studies have revealed a time-dependent change in the contractile response. Demir et al., [25] found an increase in CCSM contractility after 4 weeks PBOO, as the inflammatory response in the sham-operated group subsided. Whereas, Lin et al., [12] found the contractile response was reduced at 8 weeks, due to a reduction in CCSM content and an increase in collagen deposition.

In an attempt to shed more light on the changes in corpus cavernosal function following chronic PBOO, we conducted our experiments using Ang II a known physiological mediator of human and rabbit CCSM contraction. Our data suggests that the contractile capacity of each individual smooth muscle cell is increased in this model, even though the overall numbers are reduced due to collagen deposition[12], a finding that may have clinical relevance in the development of ED. This is in keeping with a previous study that showed PE elicited an increased contractile response of CCSM strips taken from men with ED, suggesting an increase in corporal vascular smooth muscle contractility may contribute to the pathophysiology of ED in older men. [26]

The PBOO-induced augmentation of the Ang II pathway could be due to an increase in Ang II release and/or AT1 receptor density, increased coupling efficiency of the agonist-receptor complex to the signal transduction machinery and/or increased amplification of second messenger formation subsequent to receptor activation. While the importance of each potential mechanism is uncertain, it is likely an increase in OS and excessive $^{\bullet}O_2^-$ production is involved. The role of OS in the Ang II-mediated contraction of human corpus cavernosum is known. [17] Ang II a potent stimulator of the smooth muscle enzyme NAD(P)H oxidase, stimulates the production of $^{\bullet}O_2^-$.[27] a mediator of Ang II-induced vasoconstriction.[28] We found the selective NAD(P)H oxidase inhibitor DPI significantly attenuated Ang II-induced CCSM contraction in sham-operated and PBOO rabbits. Although, the reduction in the Ang II response induced by the $^{\bullet}O_2^-$ scavenger SOD was not significant, this trend particularly following PBOO suggests that SOD, similar to DPI is capable of reducing OS. The effectiveness of these drugs is probably due to the abolishment of $^{\bullet}O_2^-$ generation. Similar observations have been reported using vascular tissue, [23,29] while pyrogallol a generator of $^{\bullet}O_2^-$ mimicked the Ang II enhancement.[29] The interaction of $\bullet O_2^-$ with NO decreases NO bioavailability by promoting OS and generating the potent oxidative peroxynitrite radical that reacts with proteins to produce tissue damaging nitrotyrosine, known to be elevated in PBOO. [30]

NANC neurotransmission is an important component of penile erection, since stimulating the cavernous nerve leads to smooth muscle relaxation. [31] This pathway is impaired following PBOO, [12] providing further evidence of the development of ED in this model. Losartan significantly increased the EFS-induced relaxation of corpus cavernosal strips taken from PBOO rabbits following cholinergic, prostaglandin and adrenergic inhibition. However, as Ang II release is via adrenergic stimulation, guanethidine should have blocked this pathway making losartan ineffective. The data implies that guanethidine does not fully inhibit the adrenergic pathway during EFS-induced relaxation, a point previously raised. [17]

Our findings also confirm that SNP-mediated relaxation of CCSM tissue is impaired in chronic PBOO rabbits as previously reported. [12,14] This reinforces the concept that this model demonstrates the salient features of ED. Vardenafil a member of a family of PDE-5 inhibitors used to treat ED[32-35], significantly improved the relaxation of CCSM strips taken from PBOO rabbits by

reducing the degradation of cGMP and enhancing CCSM relaxation, suggesting the functional response to NO is intact. Similarly, tadalafil another PDE-5 inhibitor increases the relaxant response to SNP of human vesicular-deferential arteries, which supply blood to the bladder and prostate. [36] Losartan also significantly improved the SNP-mediated relaxation of CCSM strips taken from PBOO rabbits, highlighting the interplay between Ang II and NO/cGMP, implying that an imbalance between these mediators is an important factor in the development of ED. Interestingly, an inverse correlation between Ang II responsiveness and endothelium-dependent relaxation has been demonstrated in isolated human arteries, which was related to the development of OS. [37]

The actions of losartan may have clinical importance in ED management. For, although PDE 5 inhibitors have become extremely effective oral agents for the treatment of ED, it has become increasingly apparent not all patients respond to this form of therapy, [38] moreover, some who initially respond develop tachyphylaxis or discontinue their use due to loss of efficacy. [39] Thus, the use of an Ang II antagonist, which reduce CCSM contractility, in conjunction with a PDE5 inhibitor may be beneficial for ED patients; not only by reducing the percentage of none responders but also the concentration of PDE 5 inhibitor required to maintain erection. This could explain, at least in part, why long-term losartan and PDE5 combination therapy has a beneficial effect on the structure and function of CCSM tissue taken from spontaneously hypertensive rats. [40]

In summary, Ang II causes a pathological enhancement of CCSM contraction from PBOO rabbits that was inhibited by losartan, probably due to a direct/indirect reduction in $^{\cdot}O_2^-$ production and OS. Losartan and vardenafil improved the impaired SNP/EFS-mediated relaxation, providing important evidence of the interplay between Ang II and NO/cGMP pathways in the regulation of CCSM tone. Elevated Ang II responsiveness, together with impaired relaxation of CCSM, may play a pivotal role in the development of ED. Further studies are required to determine the molecular events responsible for the cellular changes to CCSM in PBOO.

**Abbreviations**

| | |
|---|---|
| Ang II | angiotensin II |
| BPH | benign prostatic hyperplasia |
| CCSM | corpus cavernosal smooth muscle |
| CGMP | cyclic guanosine monophosphate |
| DPI | diphenyene iodonium chloride |
| EFS | electrical field stimulation |
| ED | erectile dysfunction |
| NAD(P)H | nicotinamide adenine dinucleotide phosphate |
| NO | nitric oxide |
| NANC | non-adrenergic non-cholinergic |
| OS | oxidative stress |
| SNP | sodium nitroprusside |
| SOD | superoxide dismutase |
| PBOO | partial bladder outlet obstruction |

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|---|---|
| PE | phenylephrine |
| PDE 5 | phosphodiesterase type 5 |
| $^{\bullet}O_2^-$ | superoxide |

**Corresponding Author:** Cecil S. Thompson PhD, Departments of Clinical Biochemistry & General Surgery, Division of Surgery & Interventional Science, University College London Medical School, Royal Free Campus, Pond Street, London NW3 2QG, United Kingdom Tel.+44 207 7940500 x 33440; FAX +442078302235

E.mail address: cecil.thompson@nhs.net; cecil.thompson@ucl.ac.uk

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**Figures & Legends**

**Fig 1**

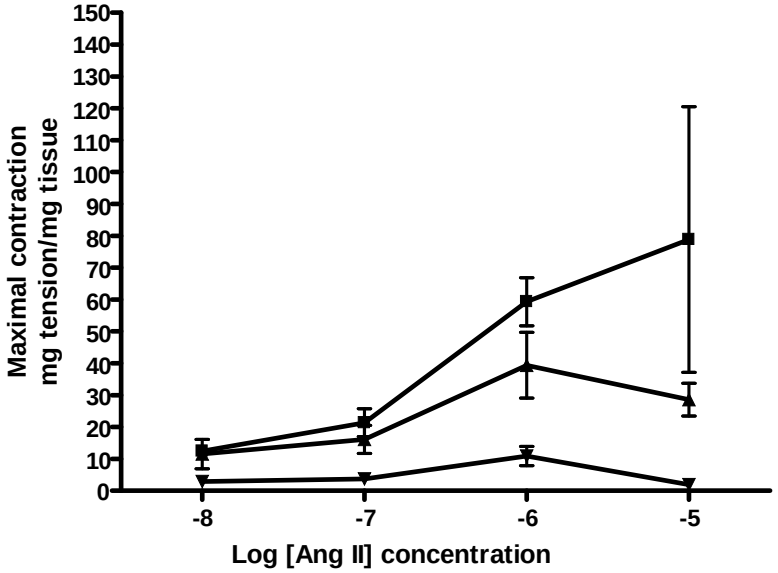


**Fig 2**

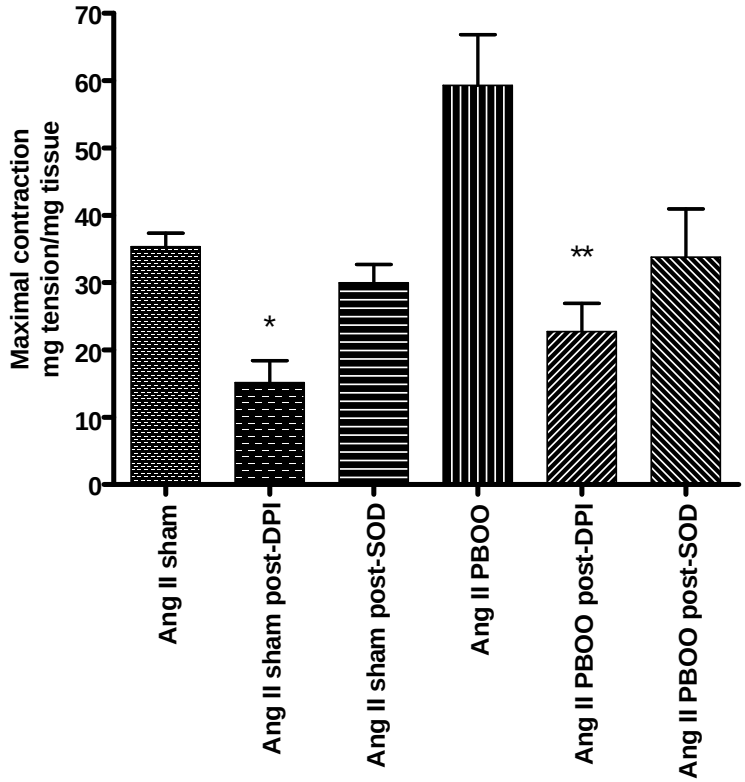

**Fig 3**

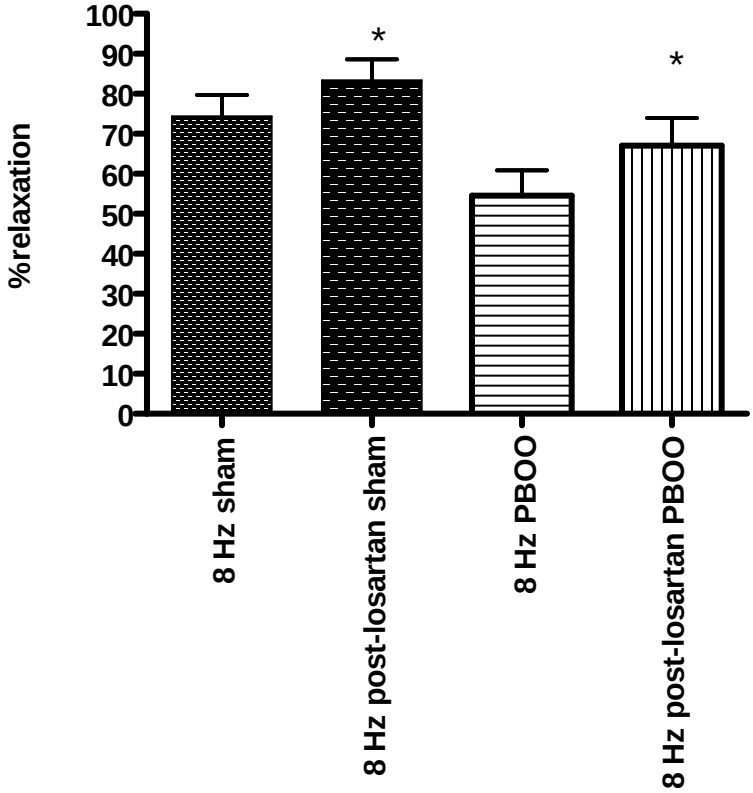
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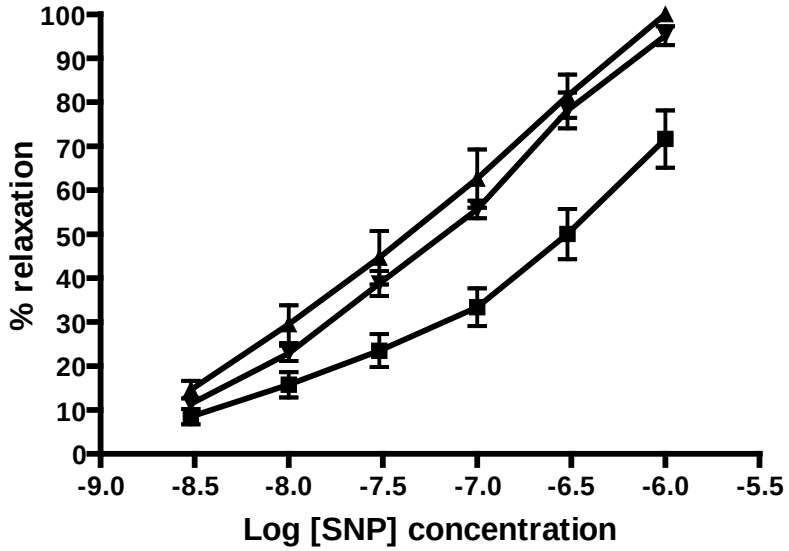
**Fig 4**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Log [SNP] concentration

**Fig 5**

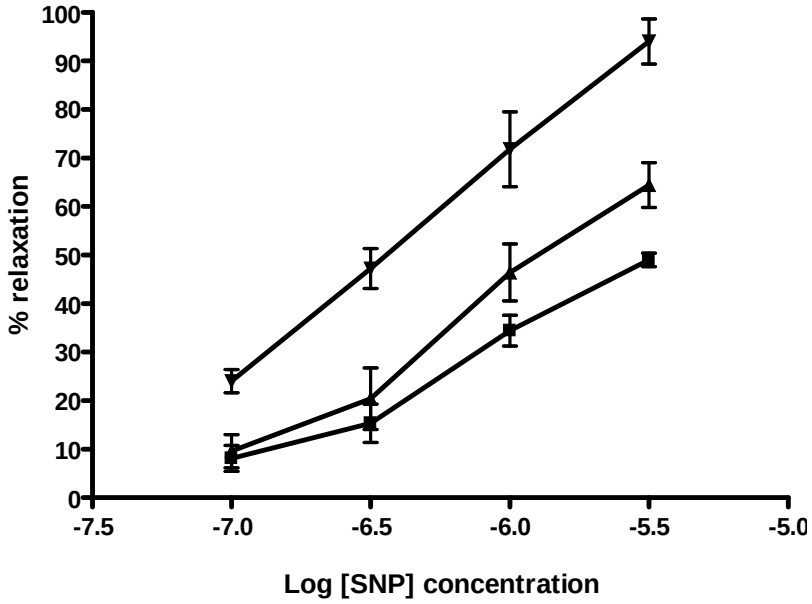


**Figure 1.** Ang II-induced contraction of CCSM strips taken from PBOO rabbits (■) was significantly (P<0.01) increased compared with sham-operated animals (▲). The PBOO-induced increase in CCSM contractility was significantly reduced post-losartan (▼), P <0.0001, n = at least 5 strips/concentration.

**Figure 2.** Ang II-induced contraction ($10^{-6}$ M) of corpus cavernosal strips taken from sham-operated and PBOO rabbits was significantly decreased post-DPI ($10^{-4}$ M), * p<0.03, ** p=0.001 and not significant post-SOD (200UI/ml), n = at least 5 strips, unpaired Student's t-test.

**Figure 3.** EFS-induced relaxation of corpus cavernosal strips taken from sham-operated and PBOO rabbits at 8 Hz (following the addition of guanethidine, atropine and indomethacin) was significantly increased post-losartan * p<0.02, n = at least 5 strips, paired Student's t-test.

**Figure 4.** SNP-induced relaxation of CCSM strips taken from PBOO (■) rabbits was significantly (P <0.0001) impaired compared with sham-operated (▲) animals and was significantly improved post-vardenafil (▼), P<0.0001, n = at least 6 strips.

**Figure 5.** SNP-induced relaxation of CCSM strips taken from PBOO (■) rabbits was significantly (P <0.0001) impaired compared with sham-operated (▼) animals and was significantly improved post-losartan (▲), P<0.01, n = at least 6 strips.

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# In vitro and in vivo Effects of Vardenafil (a PDE-5 Inhibitor) on Corpus Cavernosal Smooth Muscle Relaxation in Diabetic Rabbits

D.H.W. Lau[a] F.H. Mumtaz[b] D.P. Mikhailidis[a] C.S. Thompson[a]

[a]Departments of Clinical Biochemistry and Surgery, Royal Free Hospital and University College Medical School, University College London, Royal Free Campus, London, and [b]Department of Urology, Chase Farm Hospital, Enfield, UK

**Key Words**
Diabetic rabbits · Vardenafil · Impaired corpus cavernosal relaxation

**Abstract**
***Introduction:*** Diabetes mellitus is associated with impaired cavernosal smooth muscle relaxation (CSMR) and the development of erectile dysfunction (ED). Vardenafil, a phosphodiesterase type 5 inhibitor has been used to treat ED. The aim of this study was to assess the in vitro and in vivo effects of vardenafil on diabetic rabbit CSMR. ***Methods:*** Organ bath studies were used. ***Results:*** Sodium nitroprusside (SNP)- and electrical field stimulation (EFS)-induced CSMR in diabetic rabbits given the vehicle was significantly impaired when compared with controls. The in vitro addition of vardenafil significantly enhanced SNP-induced CSMR in diabetic animals given the vehicle. SNP-induced CSMR in diabetic animals given in vivo vardenafil was significantly increased when compared with the diabetic untreated group. The in vitro addition of vardenafil significantly enhanced SNP and EFS-induced CSMR in cavernosal tissue taken from diabetic animals given vardenafil in vivo. ***Conclusions:*** The present findings suggest that the combination of in vitro and in vivo vardenafil enhance diabetic CSMR, reinforcing the use of vardenafil for the treatment of diabetes-induced ED.



## Introduction

Penile erection is a haemodynamic process involving increased arterial inflow and restricted venous outflow, co-ordinated with corpus cavernosum smooth muscle relaxation (CSMR). Although this process is generally accepted to be under neuroregulatory control, biochemical mediators released locally from the cavernosal endothelium and/or smooth muscle also participates in initiating and maintaining an erection [1].

It is now well established that nitric oxide (NO) released by the endothelium of the arteries that supply the penis as well as the corpus cavernosum and non-adrenergic, non-cholinergic (NANC) neurotransmission mediate CSMR through the formation of cyclic guanosine monophosphate cGMP [2], resulting in penile erection [3–5].

Conversely, the erectile response is terminated when cGMP-specific phosphodiesterase catalyses the hydrolysis of cGMP to 5′-GMP, thus halting the cascade of reactions and leading to smooth muscle contraction and concomitant detumescence [6]. It is not unreasonable, therefore, that erectile dysfunction (ED) is often considered to be a situation where impaired CSMR has developed.

ED is defined as the persistent inability to attain and maintain an erection adequate to permit satisfactory sexual performance. It can affect up to 50% of men aged be-

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Dr. C.S. Thompson
Departments of Clinical Biochemistry and Surgery
Royal Free Hospital, Pond Street, London NW3 2QG (UK)
Tel. +44 207 794 0500 ext. 33440, Fax +44 207 830 2235
E-Mail cecil.thompson@royalfree.nhs.uk

tween 40 and 70 years [7]. Although not life-threatening, this common problem can significantly affect the quality of life as well as the psychological and social well-being of the sufferer.

The prevalence of ED in diabetic men is up to 70%, highlighting the magnitude of the problem in this patient group [8, 9]. Interestingly, neuronal and endothelium-dependent-mediated CSMR have been reported to be impaired in diabetic patients with ED [10], which suggests an adverse affect on the NANC drive.

In the past, pharmacological treatment of ED has been confined to intra-cavernosal [11–13] and transurethral [14] injections of drugs such as papaverine and prostaglandin $E_1$. More recently, the family of phosphodiesterase type 5 (PDE-5) inhibitors (sildenafil, tadalafil and vardenafil) has been increasingly used to treat ED [15–17]. They enhance NO-induced cGMP accumulation resulting in a significant relaxation of the corpus cavernosum [16, 18]. The effect of these PDE-5 inhibitors is dependent, at least in part, upon an intact NANC pathway.

We previously used organ bath studies to determine whether PDE-5 inhibition (using sildenafil) enhances sodium nitroprusside (SNP)-induced relaxation of in vitro corpus cavernosal smooth muscle strips taken from diabetic rabbits [18].

In the present study, we examine the effect in vitro exposure of vardenafil (added to the organ bath) and/or chronically fed vardenafil (4 weeks' in vivo treatment) has on CSMR.

## Method

*Induction of Diabetes*

Adult sexually mature male rabbits (n = 8) were injected intravenously with alloxan (65 mg/kg in a volume of 1 ml/kg), while 8 control animals were injected with the saline vehicle alone (1 ml/kg). Diabetic animals received 3 subcutaneous injections of 10 ml of 50% glucose, 4 h apart on the first day of alloxan treatment. A final glucose injection (10 ml of a 50% glucose solution) was administered on the morning (7.30 a.m.) of the second day. This procedure was carried out to counteract the hypoglycaemia caused by insulin release from necrosed pancreatic beta cells due to the acute action of alloxan.

Within 1 week of the alloxan injection, blood samples not exceeding more than 10% of the total blood volume were taken to confirm diabetes. Thereafter, blood samples not exceeding 15% of the total blood volume were taken at 4 and 6 months to monitor serum clinical biochemical variables.

*Experimental Animal Groups*

After 6 months, the control and diabetic animals were divided into 4 groups.

Group 1 (4 control rabbits) and group 2 (4 diabetic rabbits) were given vardenafil (3 mg/kg, Bayer Healthcare AG, Germany) made up in 120 ml HCl acid water pH 4.5 to drink each morning for 4 weeks, this was followed by HCl acid water pH 4.5 given ad libitum. Animals in group 3 (4 control rabbits) and group 4 (4 diabetic rabbits) received HCl acid water pH 4.5 to drink ad libitum, for 4 weeks. The vardenafil dose (3 mg/kg) chosen has previously been shown to be the minimum dose required to elicit a significant reduction in the urological changes following partial bladder outlet obstruction in the rat [19]. Rabbits in all four experimental groups readily drank the drug solution or acid water.

After taking a final blood sample at 7 months, the animals were killed by cervical dislocation and the penis was rapidly excised from each rabbit and placed in cold oxygenated Krebs solution at 4°C. Tissue preparations were investigated on the same day of acquisition. Epidermal tissue was removed and the tunica albuginea opened and the corpus cavernosum dissected out and cut into strips of approximately 1 × 3 mm. The size and weight for both control and diabetic cavernosal strips were similar.

*Organ Bath Studies*

The strips were mounted vertically in 10-ml organ baths, equipped with two parallel platinum electrodes for transmural electrical field stimulation (EFS). The tissues were bathed with Krebs solution at pH 7.4, maintained at 37°C by a thermoregulated circuit and bubbled with a mixture of 95% $O_2$-5% $CO_2$. An initial tension of 2 g was applied to the suspended tissue strips and the tension recorded on a Grass Polygraph (model 7D; Astro-med Grass, Slough, UK). All strips were equilibrated for at least 1 h. At the end of the equilibration period the strips were challenged with KCl (120 mM). After washing the tissue three times, guanethidine ($5 \times 10^{-6}$ M), atropine ($10^{-5}$ M) and indomethacin ($10^{-6}$ M) was added to the bathing solution and left for 20 min to inhibit the adrenergic, cholinergic and cyclo-oxygenase pathways, respectively, leaving the NANC pathway intact. Tissues were then precontracted with phenylephrine ($10^{-4}$ M). The EFS of penile nerves were performed with a Grass S88 (Astro-med Grass, Slough UK) stimulator. The stimulator delivered single square waves (duration 0.8 ms; 100 V) over a range of frequencies that gave an incremental increase in relaxation response (0.5–16 Hz) in 5 s trains at 2-min intervals. A series of relaxations in response to EFS in the absence and presence of vardenafil ($10^{-8}$ M) after a 20-min incubation period were recorded.

In other experiments, tissue strips were pre-contracted with phenylephrine ($10^{-4}$ M) and cumulative response curves were constructed for SNP ($3 \times 10^{-9}$–$10^{-6}$ M). The tissues were then washed several times over a 1-hour period. Vardenafil ($10^{-8}$ M) was then added to the organ bath and left for 20 min. The tissues were re-contracted with phenylepherine ($10^{-4}$ M) and cumulative response curves were again constructed for SNP.

The stock solution of vardenafil ($10^{-3}$ M) was made up in acid water pH 4.5 and subsequently diluted in distilled water, before adding to the organ bath. We found that the final dilution of acid had no effect on SNP-induced relaxation (results not included).

*Statistical Analysis*

Results were analysed using Graph Pad Prism 3.0. Isolated corpus cavernosal strips responses to SNP in the absence or presence of vardenafil are expressed as % relaxation of PE-induced tone. Results for the SNP (10–13 separate strips/experiment) and

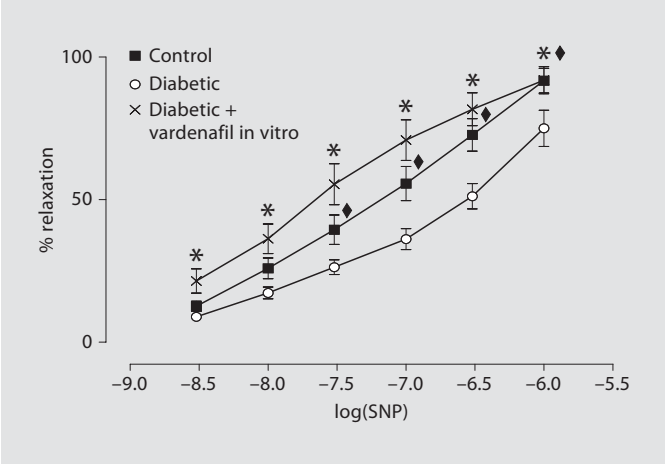


**Fig. 1.** SNP-induced relaxations of cavernosal strips taken from control (■) and diabetic rabbits (○) following vehicle treatment. ♦ Data points where there was a significant difference in SNP-induced relaxation between these two experimental groups (SNP (M): $3 \times 10^{-8}$, $p < 0.04$; $10^{-7}$, $p < 0.02$; $3 \times 10^{-7}$, $p < 0.009$; $10^{-6}$, $p < 0.04$; unpaired Student's t test). SNP-induced relaxations of cavernosal strips taken from diabetic rabbits following vehicle treatment in the absence (○) and presence (×) of in vitro vardenafil. * Data points where there was a significant difference in SNP-induced relaxation between these two experimental groups (SNP (M): $3 \times 10^{-9}$, $p < 0.007$; $10^{-8}$, $p < 0.0008$; $3 \times 10^{-8}$, $p < 0.0006$; $10^{-7}$, $p < 0.0001$; $3 \times 10^{-7}$, $p < 0.0001$; $3 \times 10^{-6}$, $p < 0.02$; paired Student's t test).

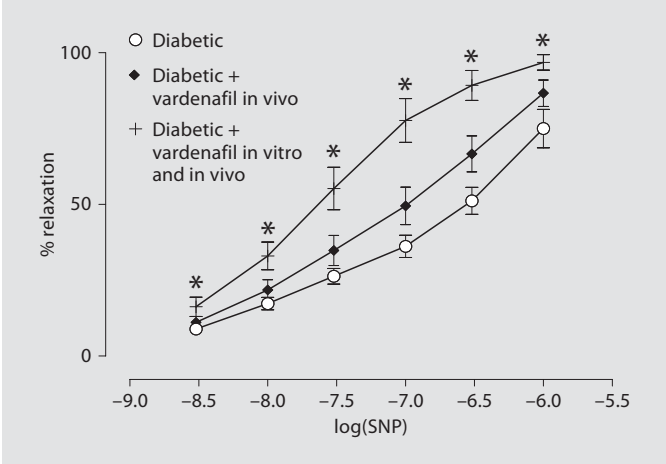


**Fig. 2.** SNP-induced relaxations of cavernosal strips taken from diabetic rabbits following in vivo vardenafil treatment in the absence (♦) and presence (+) of in vitro vardenafil. * Data points where there was a significant difference in SNP-induced relaxation between the two experimental groups (SNP (M): $3 \times 10^{-9}$, $p < 0.03$; $10^{-8}$, $p < 0.002$; $3 \times 10^{-8}$, $p < 0.002$; $10^{-7}$, $p < 0.0008$; $3 \times 10^{-7}$, $p < 0.002$; $3 \times 10^{-6}$, $p < 0.003$; paired Student's t test). Also presented is the SNP-induced relaxation of cavernosal strips taken from vehicle-treated diabetic (○) rabbits for comparison.

EFS (7–12 separate strips/experiment) were obtained from 4 animals in each experimental group. Comparisons of the cumulative dose response curves obtained were made using analysis of variance (2-way ANOVA) with statistical significance accepted at $p < 0.05$ (see 'Results'). The $EC_{50}$ value recorded expressed the concentration of SNP required to elicit 50% of the maximum relaxation of cavernosal strips (see 'Results'). *, ♦ denotes each data points where there was a significant difference in SNP-mediated relaxation or EFS responses between experimental groups (fig. 1–3). Statistical analysis of these data points have been determined using a Student's unpaired or paired t test with statistical significance accepted at $p < 0.05$ (see figure legends).

Results for clinical biochemical variables are expressed as mean ± SD and statistical comparison between control and diabetic animals were determined using the Student's unpaired t test, values were considered significant at $p < 0.05$.

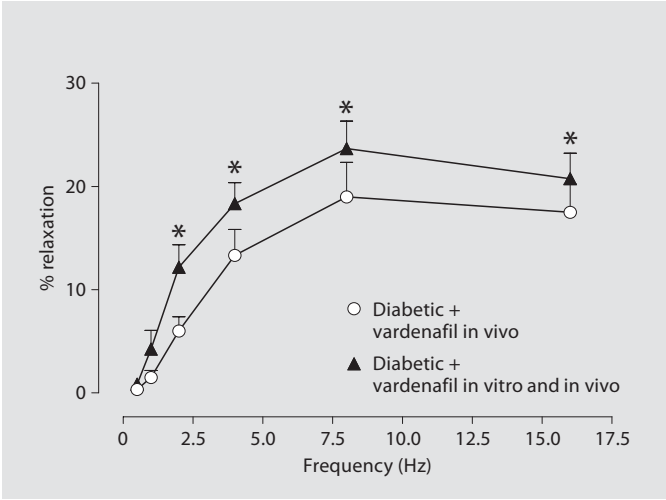


**Fig. 3.** EFS-induced relaxations of cavernosal strips taken from diabetic rabbits following in vivo vardenafil treatment in the absence (○) and presence (▲) of in vitro vardenafil. * Data points where there was a significant difference in EFS-induced relaxation between the two experimental groups (EFS (Hz): 2.0, $p < 0.007$; 4.0, $p < 0.001$; 8.0, $p < 0.005$; 16, $p < 0.02$; paired Student's t test).

## Results

Blood samples analysed after 6 months of diabetes revealed a significant increase in serum glucose, creatinine and urea concentrations with a significant fall in serum sodium concentration (table 1). The analysis of the 7-month blood sample collected after in vivo vardenafil or

**Table 1.** Serum clinical biochemical variables from 6-month control and diabetic rabbits

| Clinical biochemical variables | Control (n = 8) | Diabetic (n = 8) | p value (unpaired Student's t test) |
|---|---|---|---|
| Glucose, mM | 7.5 ± 0.4 | 26.2 ± 4.7 | <0.0001 |
| Sodium, mM | 143 ± 1.1 | 131 ± 3.5 | 0.0001 |
| Urea, mM | 5.2 ± 0.3 | 9.7 ± 2.0 | <0.0001 |
| Creatinine, μM | 79.0 ± 9.9 | 95.0 ± 8.9 | 0.0045 |

vehicle treatment for 4 weeks, showed similar results to the 6-month sample for serum glucose, urea and sodium concentrations (results not shown).

*SNP-Induced Cavernosal Smooth Muscle Relaxation*

(a) SNP-induced relaxation of diabetic cavernosal strips taken from animals given the vehicle was significantly (ANOVA; $p < 0.0001$) impaired when compared with controls (fig. 1). The $EC_{50}$ value which represents the concentration of SNP required to elicit 50% of the maximum relaxation was significantly increased following diabetes. Thus, the $EC_{50}$ value for the control strips was $7.9 \times 10^{-8}$ M and the diabetic strips $3 \times 10^{-7}$ M; $p < 0.01$. The in vitro addition of vardenafil ($10^{-8}$ M) significantly (ANOVA; $p < 0.0001$) enhanced the relaxation of strips taken from diabetic vehicle-treated animals (fig. 1). The $EC_{50}$ values in the absence and presence of vardenafil were $3 \times 10^{-7}$ M and $4.4 \times 10^{-8}$ M; $p < 0.0007$, respectively. Interestingly, the vardenafil-induced SNP relaxation of diabetic cavernosal strips was greater than that achieved by control strips (ANOVA; $p = 0.0013$) (fig. 1).

(b) The in vitro addition of vardenafil ($10^{-8}$ M) significantly (ANOVA; $p < 0.0001$: results not shown) enhanced the relaxation of strips taken from control vehicle-treated animals. The $EC_{50}$ values in the absence and presence of vardenafil were $7.9 \times 10^{-8}$ M and $1.2 \times 10^{-8}$ M; $p < 0.0003$, respectively.

(c) The cumulative response curve for cavernosal strips taken from diabetic animals given in vivo vardenafil showed a significant (ANOVA; $p = 0.0004$) increase in cavernosal relaxation when compared with untreated diabetic rabbits (fig. 2). The $EC_{50}$ value for the non-treated diabetic rabbits cavernosal strips was $3 \times 10^{-7}$ M and for diabetic in vivo vardenafil-treated rabbits $1.1 \times 10^{-7}$ M.

The in vitro addition of vardenafil ($10^{-8}$ M) significantly (ANOVA; $p < 0.0001$) enhanced the relaxation of strips taken from diabetic animals given in vivo vardenafil (fig. 2). The $EC_{50}$ value of strips taken from diabetic animals given in vivo vardenafil was $1.1 \times 10^{-7}$ M and $3 \times 10^{-8}$ M; $p < 0.02$, in the absence and presence of in vitro vardenafil, respectively. The cavernosal relaxation caused by the combination of in vitro and in vivo vardenafil was significantly (ANOVA; $p < 0.0001$) greater than in vivo but not in vitro vardenafil alone (fig. 2).

*Electrical Field Stimulation (EFS)*

(d) There was an impairment of EFS-induced relaxation of cavernosal strips taken from vehicle-treated diabetic animals compared with controls (ANOVA; $p = 0.0077$: results not shown).

(e) There was no evidence that in vitro or in vivo vardenafil alone enhanced EFS-induced relaxation of cavernosal strips taken from diabetic rabbits (results not shown).

In contrast, the in vitro addition of vardenafil significantly enhanced EFS-induced relaxation of strips taken from diabetic rabbits given in vivo vardenafil (ANOVA; $p = 0.0026$) (fig. 3).

## Discussion

Serum clinical biochemical analysis showed that diabetes was evident 1 week after the alloxan injection and was maintained for the duration of the study. Organ bath studies revealed that pre-contracted cavernosal strips taken from diabetic vehicle-treated rabbits exhibited impaired SNP-induced relaxation. Interestingly, diabetes-induced impairment of corpus cavernosal relaxation is known to have a deleterious effect on the erectile process [10] and probably explains why the incidence of ED is as high as 70% in diabetic men [8, 9] and these patients are some times referred to as a 'difficult-to-treat' ED group [20].

The release of NO by the endothelium of the arteries that supply the penis and the corpus cavernosum, as well as NANC neurotransmission, plays a crucial role in penile erection [3–5]. It is also recognized that a decrease in NO production is a contributing factor in the development of diabetic ED. For example, an impairment in mating and erectile reflexes, as well as a decrease in basal and stimulated NO levels in the corpora, together with a reduction in the intracavernosal pressure in response to cavernous nerve stimulation have been reported in diabetic rats [21]. This is supported by the finding that nitrergic relaxation responses in vitro and erectile responses to cavernous nerve stimulation in vivo were attenuated in a similar animal model [22]. The reduction in NO was associated with a fall in penile NOS activity, a phenom-

enon that has been observed in both type 1 and type 2 diabetic rats [23]. A reduction in cGMP/NO accumulation also caused the impairment of corpus cavernosal smooth muscle relaxation in diabetic rabbits [18]. Importantly, this impairment of smooth muscle relaxation was due to a decrease in NO bioavailability rather than an inherent inability of the corpus cavernosum to relax, since the sensitivity of corpus cavernosal tissue to exogenous NO was enhanced in diabetic rabbits [24]. The diabetes-induced reduction in NO could be due to a defect in NO synthesis and thus NANC neurotransmission or quenching of NO through the production of superoxide radicals and advanced glycation end products [25, 26].

In the present study the in vitro addition of vardenafil (a PDE-5 inhibitor) significantly improved the relaxation of corpus cavernosal strips taken from diabetic vehicle-treated rabbits (fig. 1). Vardenafil is a member of a family of PDE-5 inhibitors, which include sildenafil and tadalafil that reduce the degradation of cGMP thus enhancing cavernosal smooth muscle relaxation [16, 18], which has given them widespread use in the treatment of ED [15–17]. The present findings are consistent with our previous study, which found that sildenafil enhanced SNP-induced CSMR as well as the accumulation of cGMP in diabetic rabbits [18]. This is probably due to the dissociation of SNP in solution generating NO, which in turn activates guanylyl cyclase, the enzyme that causes the production of cGMP. One possibility is that diabetes impairs the activity of this enzyme, resulting in the reduction of cGMP formation.

The effect of in vitro vardenafil on corpus cavernosal strips taken from vehicle-treated diabetic animals was marked, since the $EC_{50}$ value showed a 10-fold decrease in the concentration of SNP required to elicit 50% of the maximum relaxation (fig. 1).

Moreover, the SNP-mediated relaxation of diabetic corpus cavernosal tissue following in vitro vardenafil was significantly greater than that achieved by SNP on control tissue, as shown by the $EC_{50}$ values and ANOVA analysis. Interestingly, in vitro vardenafil also significantly enhanced the relaxation of corpus cavernosal strips taken from vehicle-treated control animals (results not shown). These findings taken together confirm that vardenafil is a potent and highly selective PDE-5 inhibitor. This is supported by the finding that vardenafil is more potent and selective than sildenafil at inhibiting phosphodiesterase-5 [7]. It is also more effective than sildenafil in facilitating erections in anaesthetized rabbits [27] and relaxing pre-contracted bladder, prostate and urethral tissue taken from rats [19].

Diabetic rabbits receiving in vivo vardenafil showed an enhancement in SNP-induced relaxation of cavernosal strips compared with untreated diabetic animals. This enhanced cavernosal relaxation after in vivo vardenafil was augmented by in vitro vardenafil (fig. 2). The improvement of corpus cavernosal relaxation following the combination of in vitro and in vivo vardenafil provides further evidence of its potent and selective PDE-5 inhibitory capacity.

EFS-mediated corpus cavernosal relaxations were significantly impaired in vehicle-treated diabetic animals compared with controls (results not shown), which is in keeping with the development of diabetic ED.

We previously found that impaired EFS-mediated relaxation was evident after 6 months diabetes but not after 3 months. In contrast, impaired SNP-induced relaxation of cavernosal strips was evident after 3 months diabetes [18].

This discrepancy between the development of impaired SNP and EFS-induced diabetic cavernosal relaxation might reflect the time course of the deleterious effects of diabetes on penile function. It would suggest that disruption of endothelium function is probably an early event (impaired SNP-induced relaxations seen at 3 months), followed by the later impairment of the NANC pathway (impaired EFS-induced relaxations seen at 6 months). It is possible that at 6 months diabetic neuropathy may have developed. There is biochemical evidence to support the development of diabetic neuropathy in this model, since early signs of vascular neuropathy, characterised by a reduction in the neuronal content and release of noradrenaline by sympathetic nerves is evident in 6-week diabetic rabbits [28]. It is conceivable that the biochemical changes could in time cause some degree of neuropathy, leading to the impairment of the NANC pathway, contributing to the development of ED. Clinical evidence of possible diabetes-induced neuropathy is provided by the finding that impotence in diabetic men was secondary to a neuropathic change to cholinergic nerves in the corpus cavernosum [29]. It has also been reported that EFS-induced relaxations were impaired in corpus cavernosal tissue taken from diabetic patients with ED [10].

In the present study, EFS-induced relaxations were enhanced when vardenafil was added in vitro to cavernosal tissue taken from diabetic rabbits receiving in vivo vardenafil (fig. 3). This finding, together with the in vivo effect of vardenafil on SNP-induced diabetic corpus cavernosal relaxation (fig. 2), may have clinical significance.

Neuropraxia caused by nerve damage is thought to be a contributing factor for post-operative ED following

nerve-sparing radical prostatectomy, which can lead to poor corporeal oxygenation, facilitating corporeal fibrosis and veno-occlusive dysfunction [30].

Although PDE-5 inhibitors can be used to treat post-prostatectomy ED [31,32], their use as a prophylaxis has not been fully elucidated. It is thought, however, that taking these drugs at bedtime might facilitate nocturnal erections, giving some protection for corpus cavernosal baseline function [30].

Thus, in giving diabetic rabbits in vivo vardenafil we may be providing corpus cavernosal endothelium protection that is evident with/without in vitro vardenafil for SNP-mediated relaxations. On the other hand, the neuronal protection might be reduced because of the development of diabetic neuropathy, which might explain why EFS-mediated cavernosal relaxations were not enhanced by in vitro or in vivo vardenafil alone but only by the combination of both. This suggests that the combined treatment carried out in our experiment compensated for the impaired diabetic NANC drive.

The effectiveness of in vivo vardenafil was independent of glycaemic control since diabetic serum glucose concentration was similar before and after treatment. This is in agreement with clinical evidence that shows the dose-dependent benefit of vardenafil in diabetic ED [20] seems to occur regardless of the level of glycaemic control [9].

In conclusion, the present findings suggest that the functional response of the corpus cavernosal tissue of diabetic rabbits to NO is intact (i.e. an enhanced SNP-mediated relaxation of diabetic cavernosal tissue following in vitro and in vivo vardenafil treatment). In contrast, the failure of in vitro and in vivo vardenafil to elicit an increase in EFS-mediated relaxation suggests that there is a defect in the NANC drive, which is probably a reflection of diabetic neuropathy. The combination of in vitro and in vivo vardenafil compensated for the reduced NANC drive. These observations reinforce the use of vardenafil in the treatment of diabetic ED.


## Acknowledgement

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ORIGINAL ARTICLE

# Purinergic modulation of human corpus cavernosum relaxation

D. H. W. Lau,*† M. J. Metcalfe,† F. H. Mumtaz,‡ D. P. Mikhailidis*‡ and C. S. Thompson*†

Departments of *Clinical Biochemistry and †Surgery, Royal Free Hospital and University College Medical School, University College London, London, UK and ‡Department of Urology, Chase Farm Hospital, Middlesex, UK

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**Correspondence:**
C. S. Thompson, Department of Clinical Biochemistry, Royal Free Hospital, Pond Street, London, NW3 2QG, UK. E-mail: Cecil.Thompson@royalfree.nhs.uk

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## Summary

The activation of $P2Y_6$ receptors has been previously reported to cause vascular smooth muscle constriction and relaxation. The aim of our study was to determine the effect of $P2Y_6$ receptor subtype activation on human cavernosal function. Cavernosal tissue was obtained from 23 patients undergoing gender reassignment surgery. Immunohistochemistry (IHC) and Western blotting were used to determine the presence of $P2Y_6$ receptors in corpus cavernosal tissue. The effects of UDP (a selective $P2Y_6$ receptor agonist) before and after the addition of distilled water (control), cibacron blue 3GA (CB, a $P2Y_6$ receptor antagonist; $10^{-4}$ M) or *N*-nitro-L-arginine methyl esther (L-NAME, a NO synthase inhibitor; $10^{-4}$ M) were assessed on phenylephrine (PE; $10^{-4}$ M) pre-contracted cavernosal strips using organ baths. Electrical field stimulation (EFS; 0.5–32 Hz) was performed in the absence and presence of CB to determine neuronal-mediated $P2Y_6$ receptor responses. IHC and Western blotting revealed the presence of $P2Y_6$ receptors on cavernosal sections. UDP at $10^{-4}$ M and $10^{-3}$ M induced a 5% and 16% relaxation of the PE-mediated response (both $p < 0.0001$), respectively, which was significantly blocked by CB (48% reduction of the UDP $10^{-3}$ M response, $p < 0.002$) but not affected by L-NAME. EFS-induced relaxations of pre-contraction strips were not significantly altered by CB. We have found the presence of $P2Y_6$ receptors in human cavernosal tissues, that when activated induce cavernosal smooth muscle cell relaxation via non-neuronal and non-nitric oxide dependent mechanism. Further investigation is needed to establish whether $P2Y_6$ receptors play a physiological role in penile erection.

## Introduction

Purinergic signalling and purinergic neuro-transmission was first proposed over 30 years ago (Burnstock, 2002). Purine nucleotides are extra-cellular messengers, which act on either P1 or P2 receptors (Burnstock, 2002; Abbracchio *et al.*, 2003). P1 receptors are coupled to G-proteins and subdivided into $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ receptor subtypes. P2 receptors are subdivided into $P2X_{(1–7)}$ and $P2Y_{(1,2,4,6,11,12,13,14)}$ receptor subtypes. P2X receptors are ligand-gated ion channels whereas P2Y receptors are coupled to G-proteins. Activation of P1 and P2 receptors mediate many cellular functions including neurotransmission, cell proliferation and death (Burnstock, 2002). In the urinary tract purinergic signalling is implicated in the regulation of renin secretion and glomerular filtration in the kidney (Jackson, 2005) and afferent sensations such as pain and distension in the bladder and also neurogenic contraction of the bladder smooth muscle (Hashimoto & Kokubun, 1995; Burnstock, 2001). In penis, $P2Y_1$ receptor is expressed in endothelial cells which lines the lacunar space and blood vessels, but not expressed in corpus cavernosum smooth muscle cells and urethra (Obara *et al.*, 1998).

Previous studies provide evidence of the possible purinergic involvement in the erectile process. For example, adenosine has been shown to induce cavernosal smooth muscle (CSM) relaxation (Wu *et al.*, 1993; Chiang *et al.*, 1994; Mantelli *et al.*, 1995; Ragazzi *et al.*, 1996), increase cavernosal peak blood flow velocity (Filippi *et al.*, 2000)

 

and penile tumescence (Noto *et al.*, 2001). The adenosine-mediated relaxation of CSM was shown to act via $A_{2A}$ (pathway independent of nitric oxide, NO) (Mantelli *et al.*, 1995) and $A_{2B}$ (partially endothelium-dependent) (Chiang *et al.*, 1994) receptor subtypes.

Adenosine 5′-triphosphate (ATP) released neuronally (Burnstock, 2002) or possibly derived from the endothelium [based on the hypothesis for purinergic mechanosensory tranduction in tissues such as the bladder as described by Burnstock (Burnstock, 2002)], has also been shown to induce CSM relaxation (Tong *et al.*, 1992; Wu *et al.*, 1993; Levin *et al.*, 1994, 1995; Ragazzi *et al.*, 1996; Filippi *et al.*, 1999; Noto *et al.*, 2001). The effect of ATP on CSM-induced relaxation could be due, in part, to its metabolic breakdown to adenosine, which acts directly on the CSM $A_{2A}$ receptor subtype (Filippi *et al.*, 1999). Interestingly, ATP-mediated relaxation is more pronounced when the CSM has a high-basal tension (following pre-stimulation with phenylephrine (PE) (Wu *et al.*, 1993), akin to normal physiological basal tone). It may be that ATP forms part of a regulatory mechanism that maintains physiological CSM basal tone.

Although previous studies have indicated that the activation of P2Y receptors modulates CSM function (Shalev *et al.*, 1999; Staerman *et al.*, 2000), the activation of $P2Y_6$ receptors in vascular tissue has yielded conflicting results. Malmsjo *et al.* (2000) found that stimulation of $P2Y_{1,2,4}$ receptors led to relaxation of the rat-isolated mesenteric artery, while activation of $P2Y_6$ receptors caused vasoconstriction. In contrast, Guns *et al.* (2006) found that stimulation of $P2Y_6$ receptor by UDP led to relaxation of the mouse isolated thoracic aorta. This difference could reflect species variation. However, as the CSM is akin to a modified vascular tissue, it is important to determine whether $P2Y_6$ receptors are present in human CSM and if the activation of the receptor induces smooth muscle constriction or relaxation. Thus, the aim of this study was to establish the presence and functional response of $P2Y_6$ receptors in human cavernosal tissue. The results from this study may have important implications in the modulation of the erectile process.

## Material and methods

### Tissue

Human penile organs were obtained from 23 male patients undergoing gender reassignment surgery (male to female) at Charing Cross hospital, London, UK (mean age 30 years, range 23–57 years). Approval was obtained from the Riverside Ethics Committee, London, UK and all the patients gave their informed consent prior to surgery. All patients had no history of previous illness (including diabetes) and were not on any medication apart from oestrogen supplement for at least 2 years prior to surgery. Oestrogen therapy was discontinued 2 months prior to surgery.

### Immunohistochemistry

For tissue immunohistochemistry (IHC), the human cavernosal tissue sections were fixed in 4% neutral buffered formaldehyde for 24 h and then embedded in paraffin. After hematoxylin and eosin (H&E) staining the sections were examined to confirm tissue type (Fig. 1a). Paraffin sections (3 $\mu$m) of the cavernosal tissue sections were then deparaffinized and rehydrated, and for antigen retrieval, they were incubated with citrate buffer (10 mM, pH 6.0) and heated twice in a microwave oven at 750 watts for 5 min. The sections were incubated with primary polyclonal anti-$P2Y_6$ antibody (Alomone Laboratories, Jerusalem, Israel). The antibody was titrated prior to staining to obtain an optimal dilution producing crisp staining with minimum background. The sections were incubated with the primary antibody at a dilution of 1 : 200 for 24 h at 4 °C. After washing three times with posphate-buffered saline (PBS), the sections were incubated with biotinylated secondary antibody against goat IgG (Goat anti-rabbit immunoglobulin – biotinylated, Stratech Scientific Ltd, Suffolk, UK). The sections were kept for 45 min at room temperature and then washed three times with PBS. This was followed by the addition of the Avidin–Biotin complex for 45 min followed by a repeat series of washes with PBS.

DAB (3,3′-diaminobenzidin tetrahydrochloride reagent as included in the DAKO ChemMate$^{TM}$ kit, DAKO Ltd, Cambridgeshire, UK) was then added to the sections for approximately 10 min. The tissue sections were counter stained with haematoxylin, dehydrated, cleared and mounted in Pertex and viewed under light microscopy. Negative controls were performed by omitting the primary antibody in the steps described above. Positive controls were also performed on rat brain tissue, where it is known that $P2Y_6$ receptors are abundant.

### Western blotting

*Protein extraction*

Cavernosa tissues were snap frozen in liquid nitrogen and stored at −70 °C. Proteins were extracted from the samples using a ratio of 1 mL of lysis buffer (50 mM TRIS base and 50 mM NaCl; PH 7.4 with 1% w/v SDS) per 300 $\mu$g sample. Protease inhibitors were added (leupeptin 1 $\mu$/mL, chymostatin 10 $\mu$g/mL, bestatin 40 $\mu$/mL, pepstatin A 1 $\mu$/mL, *N*-α-p-tosyl-L-lysine chloromethyl ketone 50 $\mu$g/mL) to inhibit NOS proteolysis. Protein concentration was determined using a microplate DC Protein Assay Kit (Bio-Rad Laboratories, Hemel Hempstead, UK). The absorbance was read at 750 nm against a protein standard

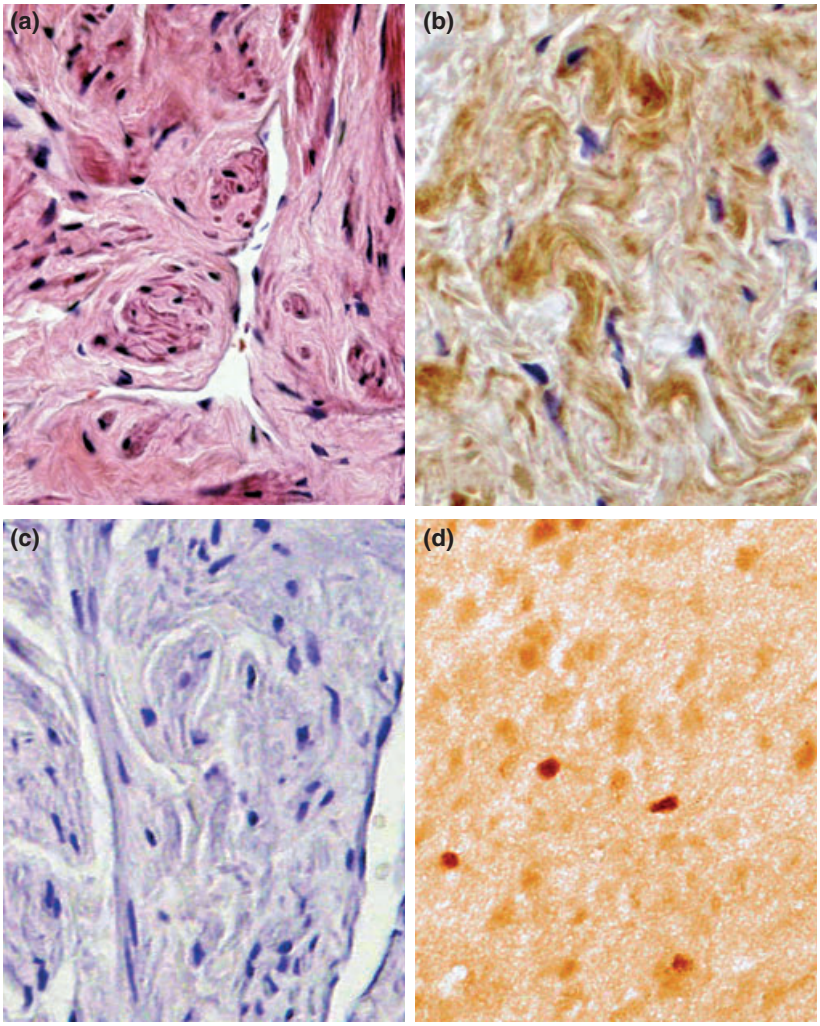


**Figure 1** Four cavernosal sections are shown; (a) stained with hematoxylin and eosin, (b) immunohistochemical staining with a $P2Y_6$ receptor antibody, (c) immunohistochemical staining following omission of the $P2Y_6$ receptor antibody (negative control) and (d) immunohistochemical staining with a $P2Y_6$ receptor antibody on sections of rat brain, which is known to have $P2Y_6$ receptors (positive control) ($n$ = 6).

curve generated using bovine serum albumin (0.2–10 mg/mL).

*Western blot analysis*

Protein was combined with an equal volume of loading buffer [0.5 M Tris–HCL (PH 6.8), 20% w/v glycerol 10% w/v SDS, 0.1 M DL-dithiothrietol and 0.05% w/v bromophenol blue] and denatured by boiling for 10 min. Samples were electrophoresed through a 7.5% w/v SDS-polyacrylamide gel and transferred to a 0.45 $\mu$m-nitrocellulose membrane (Schleicher, Dassel, Germany). Membranes were immersed in blocking solution containing 5% w/v non-fat dry milk before incubation with $P2Y_6$ receptor antibodies in TRIS – buffer saline with 0.15% w/v Tween-20 (TBS-T). After 2 h incubation at room temperature, membranes were washed for $1 \times 15$ min, followed by $3 \times 5$ min in TBS-T and then incubated for 1 h with goat anti-rabbit antisera conjugated with hourseradish peroxidase 1 : 13 000 with TBS-T. The protein was detected using an ECL$^{TM}$ – chemiluminescence detection Kit (Amersham, Little Chalfont, UK). Rat brain tissue lysates for $P2Y_6$ receptor was used as a positive control.

## Organ bath studies

Isolated human cavernosal tissues were cut into strips of approximately $2 \times 5$ mm length. The strips were mounted vertically in 10 mL organ baths containing Krebs solution. The Krebs solution (pH 7.2) had the following composition (mM): NaCl 133, KCl 4.7, $NaH_2PO_4$ 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and glucose 7.8. The tissue strips were maintained at 37±1 °C and bubbled with a mixture of 95% $O_2$ and 5% $CO_2$. An initial tension of 2 g was applied and the strips were allowed to equilibrate for 1 h before being challenged with KCl (124 M), which was repeated at the end of the experiment. Results were only accepted if both KCl responses varied in magnitude by <10%.

Initial experiments showed that UDP (a selective $P2Y_6$ receptor agonist) caused cavernosal relaxations.

 

Subsequently, all tissues were pre-contracted with PE $10^{-4}$ M to quantify UDP-induced relaxations at a concentration of $10^{-3}$ M and $10^{-4}$ M, ($n = 9$ for each concentration). In some experiments, cibacron blue 3GA (CB, a $P2Y_6$ receptor antagonist; $10^{-4}$ M, $n = 9$) or *N*-nitro-L-arginine methyl esther (L-NAME; a NO synthase inhibitor; $10^{-4}$ M, $n = 9$) was added to the strips and kept for 20 min prior to re-exposure to UDP $10^{-3}$ M.

In other experiments, strips were exposed to atropine $10^{-5}$ M, guanethidine $5 \times 10^{-6}$ M and indomethacin $10^{-6}$ M for 20 min to inhibit the parasympathetic, sympathetic and prostaglandin pathways, respectively. Electrical field stimulations (EFS) at 100 V and 0.1 ms (5 sec duration and a 2 min rest interval) at a frequency of 0.5–32 Hz were then performed on PE pre-contracted cavernosal strips before and after the addition of CB $10^{-4}$ M.

Control experiments were also performed examining the effect of distilled water (vehicle) on the UDP-induced relaxation on PE pre-contracted strips.

*Chemicals*

Phenylephrine, UDP, CB and KCl were supplied by Sigma Chemical Co. (Poole, UK).

*Statistical analysis*

Isolated cavernosal strips responses to UDP before and after pre-incubation with either CB, L-NAME or distilled water is expressed as mean values ± SEM, comparisons were made using Student paired *t*-test. Individual EFS stimulation points (expressed as mean values ± SEM) where compared before and after the addition of CB using Student paired *t*-test. Results from both series of experiments are expressed as a percentage of the PE response and significance was considered at $p < 0.05$.

The comparison of EFS curves was performed using ANOVA analysis, with statistical significance accepted with $p < 0.05$.

## Results

### Immunohistochemistry

Sequential tissue sections underwent one of two staining techniques: H&E staining identified cellular structure (nuclei and cytoplasm) (Fig. 1a), IHC of the $P2Y_6$ receptor antibody showed positive brown staining for the $P2Y_6$ receptor on the corpus CSM cells. No staining was demonstrated on endothelial cells of lacunar spaces and blood vessels (Fig. 1b), the negative control section showed no IHC staining following omission of the primary antibody (Fig. 1c). Positive control sections showed brown staining of the $P2Y_6$ receptor on rat brain cells (Fig. 1d).

### Western blotting

Western blotting demonstrated the presence of a band at 45 kDa, which corresponds to the $P2Y_6$ receptor protein in cavernosal tissue (Fig. 2).

### Organ bath studies

UDP caused a significant and sustained relaxation of PE pre-contracted tissue at both concentrations used ($10^{-4}$ M, 4.9 ± 0.8; $10^{-3}$ M, 15.7 ± 2.8, both $p < 0.0001$) when compared with controls. The UDP-mediated relaxation ($10^{-3}$ M) was significantly reduced by CB (8.2 ± 1.5, $p < 0.002$). Whilst in contrast, L-NAME had no significant effect on UDP-mediated relaxations ($10^{-3}$ M, 17.1 ± 4.4) (Fig. 3). EFS (0.5–32 Hz) demonstrated transient relaxations on PE pre-contracted tissue strips with maximal relaxation of 32.8 ± 4.9 at 8 Hz. EFS-induced relaxations were not significantly altered by pre-exposure to CB when the relaxation curves (Fig. 4) or individual points were compared.

## Discussion

We have shown for the first time, the presence of the $P2Y_6$ receptor subtype in human CSM using IHC and Western Blotting. The $P2Y_6$ receptor antibody used has been previously shown to act specifically on $P2Y_6$ receptors (Pinna *et al.*, 2005; Metcalfe *et al.*, 2007). In addition, organ bath studies revealed that activation of these receptors causes a significant receptor-mediated relaxation of human CSM. This was demonstrated by UDP ($10^{-4}$ M and $10^{-3}$ M) a compound known to act selectively on $P2Y_6$ receptor (Rubino *et al.*, 1999; Metcalfe *et al.*, 2007)

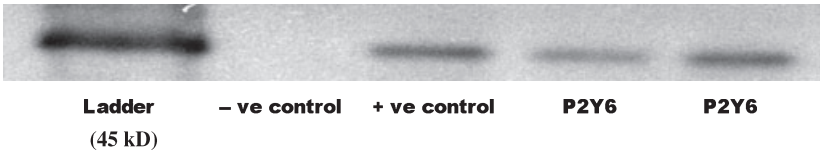


**Figure 2** Western blotting gel electrophoresis shows a positive control ($P2Y_6$ receptor protein of rat brain tissue lysates; denoted by +ve) and the presence of the 45 kDa protein of the $P2Y_6$ receptor in cavernosal muscle (denoted by $P2Y_6$; two samples are shown). The negative control was demonstrated by omitting the $P2Y_6$ receptor protein antibody (denoted by –ve). A kDa ladder for the protein is also shown ($n = 6$).

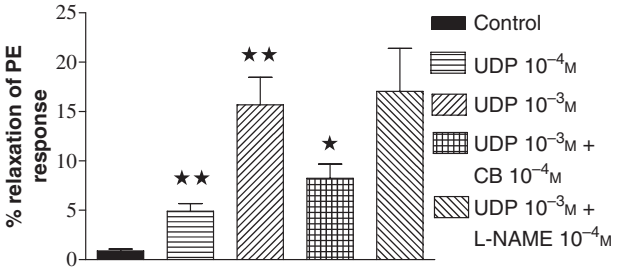


**Figure 3** Bar diagram represents the UDP (a $P2Y_6$ receptor agonist)-mediated relaxations ($10^{-4}$ M and $10^{-3}$ M) on phenylephrine (PE) pre-contracted human cavernosal strips. The UDP-mediated relaxations ($10^{-3}$ M) were also determined on the PE pre-contracted tissue strips in the presence of *N*-nitro-l-arginine methyl esther (l-NAME) $10^{-4}$ M or cibacron blue 3GA $10^{-4}$ M (CB, a $P2Y_6$ receptor antagonist). Control experiments were performed by the addition of distilled water ($DH_20$), the vehicle for l-NAME, UDP, PE and CB on the PE pre-contracted tissue strips. Relaxations are expressed as % of PE-mediated responses (mean ± standard error of means). Significance of result is denoted by $*p < 0.002$ or $**p < 0.0001$ ($n = 20$).

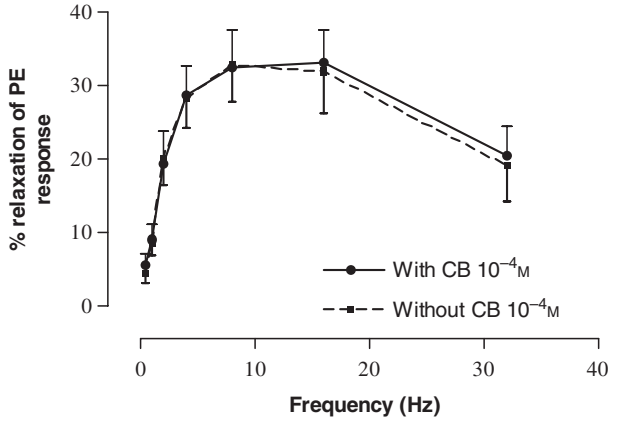


**Figure 4** Figure shows the electrical field stimulation (EFS)-induced relaxations in the absence and presence of cibacron blue 3GA $10^{-4}$ M (CB, a $P2Y_6$ receptor antagonist) ($n = 9$).

inducing a 5% and 16% relaxation of the PE-mediated response, respectively, which was significantly blocked by CB $10^{-4}$ M (48% reduction of the UDP $10^{-3}$ M response).

Our findings are in agreement with an earlier study, which showed that UDP causes vasorelaxation of the mouse thoracic aorta (Guns *et al.*, 2006). However, our study raises the question of whether the low % relaxation (5% and 16%) induced by UDP, albeit significant, has any physiological importance. No doubt more studies are required to answer this question. It may be that the activation of $P2Y_6$ receptors plays a role in 'fine tuning' the modulation of penile erection in what appears to be a complex process at the molecular level.

The notion that P2Y receptor activation induces endothelium-dependent relaxation of human CSM, via NO production is plausible (Staerman *et al.*, 2000) as NO plays a significant role in modulating human CSM relaxation and therefore erectile function (Burnett, 2004; Ghalayini, 2004; Gonzalez-Cadavid & Rajfer, 2004). Equally plausible is that the release of purines from the penile nerve or endothelium (possibly because of CSM stretching during tumescence) could stimulate the $P2Y_6$ receptors on the CSM or endothelium causing relaxation, a phenomenon that has been seen in the bladder (Sun & Chai, 2006). These are, however, unlikely scenarios as the addition of L-NAME, the nitric oxide synthase inhibitor, had no effect on UDP-induced relaxations of PE pre-contracted human CSM, ruling out the involvement of the nitrergenic pathway in this response. L-NAME is used widely to inhibit endogenous NO bioactivity of various organ systems (Reilly *et al.*, 1997; Takimoto *et al.*, 2005; Badn *et al.*, 2007; Wainwright *et al.*, 2007). In addition, CB, the $P2Y_6$ receptor antagonist, had no effect on EFS-induced relaxations of PE pre-contracted human CSM, ruling out the activation of a $P2Y_6$-mediated neuronal pathway.

The non-availability of normal human corpus cavernosum (HCC) is an unavoidable limitation of this study. In previous studies, HCC tissues were obtained from patients with Peyronie's disease, diabetes or undergoing penile prosthesis implants for erectile dysfunction (ED) (Mirone *et al.*, 2000), all these samples had some degree of pathology. Mirone *et al.* (2000) and Rees *et al.* (2001) proposed the use of HCC tissue obtained from patients undergoing gender reassignment surgery. However, these patients are normally on oestrogen for 2 years prior to withdrawal for 2 months before surgery. We, therefore, cannot exclude the effect of oestrogen on cavernosal tissue function. In fact, Adaikan & Srilatha (2003) showed that oestrogen causes pathophysiological changes in the erectile function of male rats. We did find, however, in a previous study, that one patient who had never received oestrogen therapy, had similar 5-HT-induced contractions to those patients on oestrogen (Lau *et al.*, 2006). In addition, many gender-reassigned patients who stop taking oestrogen, prior to surgery have 'normal' erections (indicated by the presence of early morning erections) based on clinical interviews, suggesting that the effect of oestrogen is reversible (Lau *et al.*, 2006).

Understanding the role the purinergic pathway might play in the erectile process is important, as this information would increase our knowledge of the mediators involved in the pathophysiology of erectile dysfunction. Erectile dysfunction is a known complication of diabetes mellitus (Sullivan *et al.*, 1999; Gur & Ozturk, 2000; Jackson, 2004) because of impaired relaxation of CSM (Gur

 

& Ozturk, 2000). Interestingly, adenosine and ATP are known to induce relaxation of CSM taken from diabetic (Gur & Ozturk, 2000). This would suggest that alteration in the purinergic system might be involved in the pathogenesis of diabetes-related erectile dysfunction.

Chiang *et al.* (1994) demonstrated that the combination of adenosine and prostaglandin (PGE1) is more effective than PGE1 alone in promoting erection in humans. Targeting the purinergic pathway (for example the $P2Y_6$ receptor) may form a novel therapeutic option in the treatment of erectile dysfunction, in combination with other erectogens such as $PDE_5$ inhibitors and $PGE_1$.

In conclusion, we have demonstrated that modulation of human CSM relaxation can be achieved by activation of the $P2Y_6$ receptor via non-neuronal and non-NO-dependent mechanisms, reinforcing the possible involvement of purinergic signalling in the erectile process. More work is required to determine the post-receptor mechanism(s) involved in $P2Y_6$ receptor-mediated relaxation, as well as, establishing whether purinergic modulation ($P2Y_6$ receptor activation) of human CSM plays a physiological role in penile erection.

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# The Effect of Vardenafil (a PDE Type 5 Inhibitor) on Renal Function in the Diabetic Rabbit: A Pilot Study

DAVID H. LAU, DIMITRI P. MIKHAILIDIS and CECIL S. THOMPSON

*Departments of Clinical Biochemistry and Surgery, Royal Free Hospital and University College Medical School, University College London, Hampstead Campus, Rowland Hill Street, London NW3 2PF, U.K.*

**Abstract**. *Background: Diabetic nephropathy is a common cause of impaired renal function. We investigated the effect vardenafil, a phosphodiesterase type 5 (PDE-5) inhibitor, has on renal function in the diabetic rabbit. Materials and Methods: Blood was taken at 4 and 6 months from control and alloxan-induced diabetic animals (n=8, in each group) and biochemical variables pertaining to renal function determined. A 7-month sample was also analysed after giving control and diabetic animals (n=4 in each group) either vardenafil (3 mg/kg) or vehicle to drink for 4 weeks. Spot urine total protein/ creatinine ratio (TP/C) was determined at 4 and 6 months. At 7 months a 24 h-urine sample was collected to measure TP/C and creatinine clearance (CrCl). Results: There was a significant increase in serum creatinine concentration after 6 months diabetes, which was significantly reduced by vardenafil. TP/C from diabetic rabbit spot urine samples at 6 months were significantly elevated compared to control animals, indicating the presence of proteinuria. Vardenafil treatment caused a normalisation of TP/C. Diabetic animals receiving vardenafil showed a significant improvement in CrCl when compared with diabetic animals given vehicle. Conclusion: These findings highlight a potential role for vardenafil in the treatment of diabetic nephropathy.*

Chronic kidney disease is increasing worldwide at an annual rate of 8%, with the prevalence higher in developing countries than in the developed world (1). Diabetic nephropathy is one of the most common causes of this problem (1). In fact it is the commonest cause of end stage renal failure in many countries and is associated not only with a high morbidity rate but also an increase in mortality (2-5). It can affect 20-30% of the diabetic population and presents in its earliest stage with an increased excretion of albumin (microalbuminuria) in the urine (4). There is also evidence of an increase in systemic and vascular markers of inflammation (6), with the progressive growth of the kidney (7). Accompanying these changes are abnormalities in the blood biochemical indices of renal function, which precede renal failure (8).

The primary treatment of diabetic nephropathy has focused on the integrated targeting of glycaemic and blood pressure control to reduce microalbuminuria (3, 9, 10).

Some patients, however, progress to end stage renal disease and require renal replacement therapy. It has been estimated that this treatment can cost as much as 40,000-50,000 Euros /patient each year (5). Not surprisingly, with the increasing number of diabetics on renal replacement therapy a financial strain is placed on health care systems (2). Thus, the need to develop new treatment strategies for diabetic nephropathy is obvious.

In the present study, we investigate the effect oral vardenafil, a phosphodiesterase type 5 (PDE-5) inhibitor, has on diabetic renal function.

## Materials and Methods

*Induction of diabetes.* Adult mature male rabbits (n=8), fed *ad libitum*, were injected intravenously with alloxan (65 mg/kg made up in 1 ml/kg, saline), while 8 control animals were injected with the saline vehicle alone (1 ml/kg), after a blood sample was taken. Diabetic animals received 3 subcutaneous injections of 10 ml of 50% glucose, 4 h apart on the first day of alloxan treatment. A final glucose injection (10 ml of a 50% glucose solution) was administered on the morning (7. 30 am) of the second day. This procedure was carried out to counteract the hypoglycaemia caused by insulin release from necrosed pancreatic beta cells due to the acute action of alloxan.

Within 1 week of the alloxan injection, blood samples not exceeding more than 10% of the total blood volume were taken to confirm diabetes. Thereafter, blood samples not exceeding 15% of the total blood volume were taken at 4 and 6 months to monitor serum biochemical variables that directly relate to renal function

*Correspondence to:* Dr. C.S. Thompson, Departments of Clinical Biochemistry and Surgery, Royal Free Hospital and University College Medical School, University College London, Hampstead Campus, Rowland Hill Street, London NW3 2PF, U.K. Tel: +44 207 794 0500 ex 33440, Fax: +44 207 830 2235, e-mail: cecil.thompson@ucl.ac.uk

*Key Words:* Diabetic rabbits, impaired renal function, vardenafil.

(urea, sodium, and creatinine), as well as, glucose and bicarbonate. Spot urine samples were also collected at 4 and 6 months from control and diabetic animals for the measurement of total protein and creatinine concentrations.

*Experimental animal groups.* After 6 months the control and diabetic animals were divided into 4 groups.

Group 1 (4 control rabbits) and Group 2 (4 diabetic rabbits) were given vardenafil (3 mg/kg, Bayer Healthcare AG, Germany) made up in 120 ml HCl acid water, pH 4.5, to drink each morning for 4 weeks, this was followed by HCl acid water, pH 4.5, given *ad libitum*. Animals in Group 3 (4 control rabbits) and Group 4 (4 diabetic rabbits) received HCl acid water, pH 4.5, to drink *ad libitum* for 4 weeks. The vardenafil dose used has been reported to be the minimum necessary to elicit urological changes following partial bladder outlet obstruction in the rat (11).

Rabbits in all four experimental groups readily drank the vardenafil solution or vehicle.

The final 7-month blood sample was taken after 4 weeks vardenafil or vehicle treatment.

All animals were placed in metabolic cages at 7 months to collect 24 h urine samples to measure total protein and creatinine concentrations, as well as to determine creatinine clearance (CrCl).

Kidney sections were also collected from control and diabetic vehicle-treated rabbits for transmission electron microscopy (TEM). Animals were weighed at the start and the end of the study.

*Statistical analysis.* For parametric analysis the results are expressed as mean±SD using a Student's unpaired or paired *t*-test, with statistical significance accepted at $p<0.05$. For nonparametric analysis, the results are expressed as median with range using the Mann Whitney unpaired test and the Wilcoxon paired test. Values from both tests were considered significant at $p<0.05$

## Results

The starting weights of control rabbits were less than the diabetic rabbits [control: 3.0 (2.8 -3.2kg); diabetic: 3.3 (3.2-3.4 kg; $p=0.002$) Mann Whitney test, n=8]. At the end of 7 months, the control animals were significantly ($p=0.008$; Wilcoxon test) heavier than their starting weights, while the diabetic animals showed no significant change from their starting weights, [control: 3.9 (3.6 - 4.3kg); diabetic: 4.0 (2.2-4.8 kg) n=8]. The final weight of the control animals was not significantly different from the final weight of the diabetic animals.

Serum glucose concentration was significantly elevated 1 week after the alloxan injection compared to vehicle-treated control animals (similar to serum glucose concentration in Table I). Acid water treatment did not induce metabolic acidosis, since there were no significant difference in serum bicarbonate concentration in control and diabetic animals before and after acid water or vardenafil treatment. For example, 6 month diabetic serum bicarbonate before vardenafil treatment was 26 mmol/l (20-28 mmol/l, n=4); 7 month diabetic serum bicarbonate after vardenafil treatment was 26 mmol/l (22-27 mmol/l, n=4).

Table I. *Serum biochemical variables from 6-month control and diabetic rabbits: evidence of renal impairment.*

| Biochemical variable | Control (n=8) mean±SD | Diabetes (n=8) mean±SD | *P*-value (unpaired Student's *t*-test) |
|---|---|---|---|
| Glucose (mmol/l) | 7.5±0.4 | 26.2±4.7 | <0.0001 |
| Sodium (mmol/l) | 143±1 | 131±4 | 0.0001 |
| Urea (mmol/l) | 5.2±0.3 | 9.7±2.0 | <0.0001 |
| Creatinine (μmol/l) | 79.0±10 | 95.0±9 | 0.0045 |

Blood samples analysed from 4-month (results not shown) and 6-month diabetic animals (Table I) revealed impaired renal function as there was a significant increase in serum creatinine and urea concentrations. There was also a significant increase in serum glucose concentration, with a significant fall in serum sodium concentration.

*Serum creatinine (μmol/l) concentration from diabetic rabbits before and after oral vardenafil treatment.* The diabetes-induced increase in serum creatinine concentration was significantly reduced by vardenafil (6-month diabetic creatinine: 97±13 μmol/l; n=4 *vs.* 7-month diabetic creatinine following vardenafil: 87±12 μmol/l; n=4, $p=0.015$; paired Student's *t*-test.

*Urinary total protein (g/l) / creatinine (mmol/l) ratio (TP/C) from control and diabetic rabbits with and without oral vardenafil treatment.* TP/C from diabetic rabbit urine samples at 6 months but not at 4 months (results not shown) was significantly elevated compared with controls, indicating the presence of proteinuria. Control TP/C: n=8, 0.0116 (0.0091-0.0191) *vs.* diabetic TP/C: n=8, 0.0227 (0.0096-0.1632), $p<0.038$ (Mann Whitney, unpaired test). Vardenafil treatment caused a normalisation of TP/C from diabetic animals (n=3) at 0.0143 (0.0118-0.0176).

*Creatinine clearance (CrCl) following 4 weeks' oral vardenafil or vehicle treatment from 7-month control and diabetic rabbits.* Control rabbits receiving vehicle had a CrCl of 9.3±3.3 ml/min, a value not statistically different from control animals receiving oral vardenafil (8.0±0.5 ml/min).

Diabetic vehicle-treated rabbits had a fall in CrCl compared with vehicle-treated controls, however, the difference was not statistically significant.

In contrast, diabetic animals receiving vardenafil showed a significant improvement in CrCl compared with diabetic animals given vehicle: diabetic vehicle-treated, 6.1±3.7 ml/min, n=4 *vs.* diabetic vardenafil-treated, 11.3±1.0 ml/min, n=4, $p=0.035$ (unpaired Student's *t*-test). This improvement in CrCl was not statistically different from

control animals receiving vehicle. Similar results were found even when CrCl was expressed per kg (results not shown).

*Transmission electron microscopy (TEM) comparison of kidney sections taken from vehicle-treated control and diabetic rabbits.* TEM revealed no significant evidence of morphological changes between control and diabetic rabbit kidney sections following vehicle treatment.

## Discussion

The serum biochemical data presented in this study demonstrate that significant renal impairment is evident 6 months following the induction of diabetes (a rise in serum creatinine and urea and a fall in sodium concentrations). We have previously reported similar findings. In addition, we found renal impairment starts much earlier than we report here (3 months after the induction of diabetes) (12). The effect of vardenafil (a PDE-5 inhibitor) on the erectile process and in particular its beneficial actions on patients with erectile dysfunction are well-documented (13,14).

Here, for the first time, we report that 4-week treatment with oral vardenafil significantly reduced the diabetic-induced increase in serum creatinine concentration. We also report that the diabetic rabbits had an elevated urinary TP/C [a test for proteinuria (15)], which was normalised by vardenafil. Taken together, these findings imply that vardenafil can reduce proteinuria and improve the renal status in diabetic nephropathy. It appears that this property is only evident when kidney function is impaired, since vardenafil had no effect on the renal function of control rabbits.

The early stages of diabetic nephropathy are characterised by an increase in glomerular hyperfiltration, which increases the glomerular filtration rate (GFR) and is believed to contribute to the progression of renal impairment (7, 16). As the nephropathy progresses, renal function deteriorates and a reduction in GFR becomes evident (17, 18).

In our study, we measured CrCl, an index of GFR (19), and found that control animals had similar CrCl values to the GFR values previously reported for control rabbits (20). We also found that diabetic vehicle-treated animals did not show evidence of glomerular hyperfiltration (elevated CrCl), when compared with vehicle-treated control animals. In fact, our data suggest that diabetic animals were moving into the phase when GFR starts to fall and before significant morphological changes become evident.

Interestingly, diabetic rabbits that received vardenafil showed a significant increase in CrCl, compared with diabetic vehicle-treated animals, providing further evidence of drug-induced improvement in renal function. Importantly, this increase did not induce glomerular hyperfiltration, since CrCl was not significantly greater than that obtained from control vehicle-treated animals. Nor was the increase related to differences in the final body weight of the animals in each group, since results were similar when CrCl was expressed per kg.

A possible mechanism for the action of vardenafil on diabetes-induced impaired renal function can be inferred from previous studies. It has been proposed that glomerular hyperfiltration is significantly dependent upon an increase in nitric oxide (NO) activity in the early phase of diabetic nephropathy (21). However, in the later phase when the GFR starts to fall a concomitant reduction in NO activity seems to occur. The diabetes-induced reduction in NO activity could be due to a defect in synthesis or quenching through the production of superoxide radicals and advanced glycosylation end products (22, 23). In the context of renal function an increase in NO/cGMP activity would cause renal vasodilation. Thus, vardenafil, a potent and highly selective PDE-5 inhibitor may be restoring GFR, reducing serum creatinine and urinary TP/C by enhancing NO-induced cGMP formation/accumulation, as with cavernosal tissue (24).

Cyclosporin A, a potent immunosuppressive agent, causes nephrotoxicity characterized by similar renal changes to those reported here, *i.e.* elevated serum creatinine levels and a decrease in CrCl (25, 26). FR226807 (Fujisawa Pharmaceutical, Japan) another PDE-5 inhibitor was found to improve cyclosporin A-induced nephrotoxicity in spontaneous hypertensive rats, as did sildenafil (25). This finding suggests that PDE-5 inhibitors have a beneficial effect on impaired renal function. These authors also suggested that the effect of FR226807 was probably due to an increase in cGMP content in the kidney, rather than *via* reducing blood pressure.

An important finding from that study was that cyclosporin A-induced pathological changes in renal morphological were improved by FR226807. Further work is required to determine whether vardenafil treatment can arrest or delay the known renal morphological changes that are associated with diabetic nephropathy.

Finally, the present study suggests a possible role for vardenafil in the treatment of diabetic nephropathy.

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# Serotonin Induces a Biphasic Response in Rabbit Cavernosal Smooth Muscle: Relevance to the Erectile Process

David H.W. Lau[a–c] Cecil S. Thompson[b, c] Faiz H. Mumtaz[c, d] Robert J. Morgan[a] Dimitri P. Mikhailidis[b, c]

Departments of [a]Urology, [b]Clinical Biochemistry, and [c]Surgery, Royal Free Hospital and University College Medical School, University College London, London, and [d]Department of Urology, Chase Farm Hospital, Enfield, UK

**Key Words**
Rabbit · 5-Hydroxytryptamine · Serotonin · Erectile dysfunction · Corpus cavernosum · Cavernosal tone

**Abstract**
***Introduction:*** Serotonin (5-hydroxytryptamine; 5-HT) can cause contraction in cavernosal smooth muscle. We further evaluated this effect of 5-HT. ***Methods:*** Organ bath studies were used. ***Results:*** 5-HT induced a sustained contraction occasionally accompanied by a transient relaxation (in 30% of rabbit cavernosal tissues) that preceded the contraction. Ondansetron and Y-25130 (both 5-$HT_3$ receptor antagonists) but not SB-269970 (a 5-$HT_7$ receptor antagonist) significantly inhibited or abolished this transient relaxation. Doxazosin (dox, an $\alpha_1$-receptor antagonist) and ketanserin (ketan, a 5-$HT_{2A}$ receptor antagonist) significantly inhibited or abolished the sustained contraction. The effects of dox on 5-HT-mediated contraction were concentration-dependent. ***Conclusions:*** Our findings further confirm that the peripheral serotonergic pathway may play a part in the erectile process via 5-$HT_{2A}$ receptor-mediated contractile and 5-$HT_3$ receptor-mediated relaxant activities. Our results also support the findings of human studies, which suggest that both ketan and dox may exert beneficial effects on the erectile process.


## Introduction

Erectile dysfunction (ED) is defined as the inability to achieve or maintain an erection sufficiently rigid to allow vaginal penetration for satisfactory sexual intercourse [1]. ED is estimated to affect about 1 in 10 adult males [2] and has a substantial impact on family life and employment [3].

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine transmitter, which was first discovered by Irvine Page and his colleagues in 1949. It is stored in both peripheral endocrine and neuronal cells. 5-HT may be involved in diseases, including depression and hypertension. The 5-HT receptors are a large and complex family of receptors. To date, seven classes (5-$HT_1$ to 5-$HT_7$) have been identified, based on structural and functional characteristics [4].

5-HT neurons are involved in the control of sexual behaviour in both humans and animals [5]. The paraventricular nucleus (brain) via descending serotoninergic raphe-spinal neurons is thought to play a role in penile erection [6]. In summary, central (brain) activation of the 5-$HT_{1A}$ receptor inhibits [7, 8], and activation of 5-$HT_{2A}$ and 5-$HT_{2C}$ receptor enhances erection [9–11]. Drugs that act in the brain via the 5-HT pathway may affect erectile function. 5-HT-specific reuptake inhibitors (e.g. paroxetine) increase the incidence of ED [12]. This is at-

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D.P. Mikhailidis, MD, FRCP, FRCPath
Department of Clinical Biochemistry, Royal Free Hospital, Pond Street
London NW3 2QG (UK)
Tel. +44 207 8302 258, Fax +44 207 8302 235
E-Mail mikhailidis@aol.com

**Table 1.** Effect on erection of activation of different 5-HT receptor subtypes (based on animal and human studies) and clinically used serotonergic agents (see the Introduction for references)

| Effect | Centrally sited 5-HT receptor subtype (species) | Peripherally sited 5-HT receptor subtype (species) | Effect of clinically used serotonergic drug |
|---|---|---|---|
| Tumescence/erection | 5-$HT_{2A}$ (rats)<br>5-$HT_{2C}$ (rats) | 5-$HT_4$ (rabbits, human) | Trazodone |
| Detumescence | 5-$HT_{1A}$ (rats) | 5-$HT_{1A}$ (rabbits, human)<br>5-$HT_{1B}$ (rabbits)<br>5-$HT_{2A}$ (rabbits, human) | Serotonin-specific reuptake inhibitors, e.g. paroxetine<br>Doxazosin |

tributed to inhibition of nitric oxide synthase (NOS) activity. Furthermore, trazodone, an antidepressant, can cause priapism via its major metabolite, metachlorophenylpiperazine (a neuronal 5-HT releaser) [13, 14].

At penile level, evidence has emerged of the role of the serotonergic pathway in the erectile process. Finberg and Vardi [15] demonstrated an in vivo 5-HT-mediated inhibitory action on penile erection in rats due to vasoconstriction of the cavernosal arteries. Also, Esen et al. [16] showed that the in vitro 5-HT-mediated contractile response in human penile veins was augmented in patients with veno-occlusive disease. The involvement of 5-$HT_{1A}$ [17, 18], 5-$HT_{1B}$ [18] and 5-$HT_{2A}$ receptors [17] in contracting cavernosal smooth muscle were shown in animal studies. Furthermore, 5-$HT_{1A}$, 5-$HT_{2A}$ and 5-$HT_4$ receptors were implicated in human erection [19–21]. Table 1 summarizes the effects on erection of activation of different 5-HT receptor subtypes and clinically used serotonergic agents.

Ketanserin (ketan, 5-$HT_{2A}$ receptor antagonist) and doxazosin (dox, an $\alpha_1$-blocker) can exert beneficial effects on ED. dox improved ED in combination with either sildenafil (silde) [22] or intracavernosal prostaglandin $E_1$ ($PGE_1$) therapy [23] when either silde or the cavernosal therapy alone had failed. Ketan combined with intracavernosal $PGE_1$ was effective in producing an erection sufficient for sexual intercourse in 76% of patients with ED when the $PGE_1$ therapy alone had failed (n = 45) [24]. Concomitant penile tumescence was noted [25] in a study which showed improved maximum urinary flow rates in patients with benign prostatic hyperplasia who were treated with ketan.

A significant increase in 5-HT levels in cavernous serum from flaccidity (113) to tumescence and rigidity and also the detumescence phase (123) in normal human subjects was reported [20]. There were less pronounced changes in 5-HT levels in the systemic circulation at all stages [20]. This variation in local 5-HT levels at different stages of erection may be important in ensuring detumescence. Therefore, 5-HT may play a physiological role in the control of penile flaccidity.

We further evaluated the role of 5-HT and the effects of dox and ketan on the erectile process (using normal rabbits).

## Materials and Methods

*Tissues*

Cavernosal tissues were obtained from 30 rabbits (New Zealand White); their weights were between 2.5 and 3 kg. Their diet was standardized (SDS, Whitham, UK) during a week of acclimatization. All animals used in these experiments were killed by cervical dislocation in accordance with Home Office (UK) permission. Their penises were immediately excised and kept in ice-cold Krebs solution. The Krebs solution was made up of NaCl 120 mM, $NaHCO_3$ 25.6 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $NaH_2PO_4$ 1.2 mM and glucose 11 mM with a pH of 7.4.

*Materials*

The following drugs and other materials were supplied by Sigma Chemical Co. (Poole, Dorset, UK): phenylephrine and 5-HT. The following chemicals were provided by Bachem Fine Chemicals (Switzerland): L-$N^G$-nitroarginine. Tocris Cookson Ltd, Bristol (UK), provided the following chemicals: SB-269970, ondansetron, Y-25130, corynanthine, yohimbine and ketanserin. Doxazosin was a gift from Pfizer Pharmaceuticals Group (UK).

*Organ Bath Studies*

Functional work was performed immediately on obtaining penile tissue. The tunica albuginea was opened to expose the cavernosal tissues. Once the cavernosal tissue was isolated, it was cut into approximately 3 × 4 × 5 mm strips. The tissues were dissected following the penile trabecular structure. The strips were strung up in a vertical organ bath system. Each bath chamber was filled with 10 ml of Krebs solution maintained at 37°C by a thermoregulated circuit and bubbled with a mixture of 95% $O_2$ and 5% $CO_2$. An initial tension of 2 g was applied and the strips were allowed to equilibrate for 1 h without any further mechanical manipulation.

**Table 2.** 5-HT-mediated contraction of rabbit cavernosal tissues before and after the addition of 100 μl of distilled water ($DH_2O$; n = 8) or ketanserin (ketan; n = 8) $10^{-5}$ M (results are presented as median (range) in mg tension/mg of tissue)

| | $DH_2O$ ($p > 0.1$) | Ketan $10^{-5}$ M ($p < 0.02$) |
|---|---|---|
| 5-HT $10^{-3}$ M before addition | 30.4 (4.9–107.8) | 64.5 (21.5–407.7) |
| 5-HT $10^{-3}$ M after addition | 28.5 (3.8–126.9) | 0 (0–8.3) |

**Table 3.** Description of agents used in our study apart from 5-HT

| Agent | Receptor/enzyme acted on |
|---|---|
| Phenylepherine (PE) | $\alpha_1$ |
| Ketanserin (ketan) | 5-$HT_{2A}$ |
| Doxazosin (dox) | $\alpha_1$, 5-$HT_{2A}$ |
| Ondansetron/Y-12530 | 5-$HT_3$ |
| SB-269970 | 5-$HT_7$ |
| Corynanthine | $\alpha_1$ |
| Yohimbine | $\alpha_2$ |
| L-$N^G$-nitroarginine (L-NAME) | NOS |

The mechanisms of 5-HT-mediated action via its different receptor subtypes as well as post $\alpha_1$-receptor actions involve activation of G-protein-coupled phospholipase C on the cavernosal membrane. The resulted activated phospholipase C converts phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol. $IP_3$ causes the release of calcium into the cytosol via its receptor binding on the endoplasmic reticulum. The overall outcome is the initiation of cavernosal contraction due to an increased intracellular concentration of calcium [4, 36]. L-$N^G$-nitroarginine (L-NAME) inhibits nitric oxide synthase (NOS), the enzyme which catalyses nitric oxide (NO) activity. NO activates soluble guanylate cyclase which leads to an elevation of intracellular cyclic guanosine monophosphate (cGMP) concentrations. The elevated cGMP levels activate G kinase. The exact mechanism by which elevated cGMP concentrations and activation of G kinase cause cavernosal relaxation is still not known. Nevertheless, the final common step is a reduction in intracellular calcium levels, which inhibits cavernosal contraction by preventing the calcium-dependent activation of myosin light chain kinase [37].

The cavernosal tissue strip was contracted at the beginning and end of every organ bath study with potassium chloride (KCl, 120 mM) to ensure tissue viability. The tissue was washed at least three times at the start of each experiment and left to recover for 15 min.

5-HT $10^{-3}$ M was added to the bath chamber to assess the response of cavernosal tissue strips to 5-HT. Accumulated dose-incremental 5-HT-mediated responses were not performed as we had previously shown tachyphylaxis of 5-HT with accumulating doses in human cavernosal strips. We demonstrated a 43.8% reduction of maximal/overall 5-HT contraction with accumulative doses ($5 \times 10^{-7}$ M, $3 \times 10^{-6}$ M, $10^{-5}$ M, $3 \times 10^{-5}$ M, $10^{-4}$ M and $10^{-3}$ M) of 5-HT 30 min following initial same accumulative doses of 5-HT followed by washout ×3 (*initial:* median: 11.88 mg/mg, min: 5.83 mg/mg, max: 25.65 mg/mg; *at 30 min:* median: 6.68 mg/mg, min: 3.65 mg/mg, max: 21.29 mg/mg; $p < 0.02$ Wilcoxon test, n = 7 each group). Similar 5-HT tachyphylaxis responses were also demonstrated by others [26–30].

However, a single-dosage exposure of rabbit cavernosal strips to 5-HT $10^{-3}$ M and subsequent same 5-HT re-exposure 30 min post-vehicle (distilled water) addition both gave similar 5-HT-mediated contractile responses with no significant difference (table 2). Thus, we adopted this single-dosage 5-HT addition in our study. The dose of $10^{-3}$ M was chosen as it was shown to give optimal results when assessing the responses of 5-HT with and without pre-exposure to its antagonists in our previous study [30].

5-$HT_3$ receptor antagonists $10^{-5}$ M (ondansetron (ondan) and Y-12530 (Y-3)), 5-$HT_7$ receptor antagonist $10^{-5}$ M (SB-269970, SB-7), L-NAME $10^{-4}$ M (a NOS inhibitor), corynanthine $10^{-5}$ M (coryn; $\alpha_1$-blocker), yohimbine $10^{-5}$ M (yohim; $\alpha_2$-blocker), dox $10^{-4}$ or $10^{-6}$ M (dox; $\alpha_1$-blocker), ketan $10^{-5}$ M (5-$HT_{2A}$ antagonist) or distilled water ($DH_2O$, as controls) was then added to the bath for 30 min followed by re-exposure of 5-HT to assess the effect of these compounds on the 5-HT-mediated response. All chemical agents used in this study are summarized in table 3.

Isometric responses of the tissue were amplified and recorded using a Chart 4 Windows programme. The tissue used in the organ bath studies was weighed and expressed as tension mg/mg of tissue by dividing the amount of contraction/relaxation occurring on exposure to an agent by the weight of the tissue.

*Statistical Analysis*

Statistical analysis software (Prism-Graph Pad Inc., USA) was used for statistical analysis. Comparisons were made using the two-tailed non-parametric paired (Wilcoxon) or unpaired (Mann-Whitney) tests. Significance was at $p < 0.05$.

## Results

Results are presented as median (range) of mg tension/mg of tissue.

*Pattern of 5-HT-Mediated Responses*

A biphasic 5-HT-mediated response of transient relaxation followed by sustained contraction was observed in 30% of the cavernosal strips used (38 strips of a total of 125) (fig. 1).

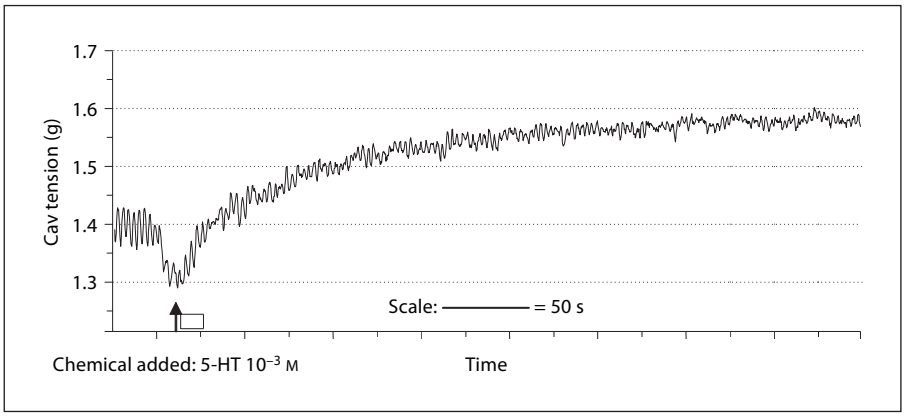


**Fig. 1.** A representative tracing showing the biphasic response following the addition of 5-HT to rabbit cavernosal (Cav) strips. The response was measured in grams and was characterized by a transient relaxation followed by a sustained contraction. The arrow shows the transient relaxation phase.

*5-HT-Mediated Contractions*

Consistent 5-HT-mediated ($10^{-3}$ M) contractions were demonstrated in the cavernosal strips. The median contraction (68.8 (4.9–407.7) mg/mg, n = 125) represented 34% of the phenylephrine (PE; $10^{-4}$ M)-mediated contraction (204.1 (18.8–784.4) mg/mg, n = 126) of all strips analysed.

*Establishing that the Vehicle Used (Distilled Water, $DH_2O$) to Dissolve the Test Compounds Did Not Affect 5-HT-Mediated Contractions*

Experiments with the vehicle (i.e. $DH_2O$) used to dissolve the substances evaluated in this study revealed a similar magnitude of 5-HT-mediated contraction before and after adding $DH_2O$ (table 2).

*Characterization of the 5-HT Receptor Subtype Responsible for the Contractile Phase*

The 5-HT-mediated contraction was inhibited by ketan ($10^{-5}$ M) by 100% (table 2). This response was also inhibited by dox ($10^{-4}$ M) by 87% and ($10^{-6}$ M) by 63% (table 4).

*Establishing the Non-Involvement of $\alpha_1$- and $\alpha_2$-Receptors in the 5-HT-Mediated Responses*

Both coryn ($10^{-5}$ M), an $\alpha_1$-blocker and yohim ($10^{-5}$ M) (yohim), an $\alpha_2$-blocker had no significant effect on 5-HT-mediated contractions (table 5).

*Characterization of the 5-HT Receptor Subtype Responsible for the Relaxation Phase*

The transient relaxation was inhibited by the 5-$HT_3$ antagonists, ondan $10^{-5}$ M and Y-25130 $10^{-5}$ M by 100% (table 6) but not the 5-$HT_7$ antagonist, SB-269970 $10^{-5}$ M (table 7).

**Table 4.** 5-HT-mediated contraction of rabbit cavernosal tissues before and after the addition of doxazosin $10^{-4}$ or $10^{-6}$ M (dox; n = 8 for each concentration). Results are presented as median (range) in mg tension/mg of tissue

| | Dox $10^{-4}$ M ($p < 0.01$) | Dox $10^{-6}$ M ($p < 0.02$) |
|---|---|---|
| 5-HT $10^{-3}$ M before addition | 34.6 (16.1–156.1) | 71.1 (17.9–100.9) |
| 5-HT $10^{-3}$ M after addition | 4.4 (0–136.2) | 26.1 (0–55.1) |

*Effect of a NOS Inhibitor L-NAME ($10^{-4}$ M) on 5-HT-Mediated Transient Relaxations*

L-NAME ($10^{-4}$ M) either diminished or abolished the 5-HT-induced transient relaxation phase (table 7).

*Establishing Tissue Viability at the Beginning and the End of the Experiments*

All cavernosal tissues used in this study showed a similar potassium chloride (120 mM)-induced contraction at the beginning and the end of the experiments (variability <10%). Those with variability in responses >10% were excluded from the study.

## Discussion

We demonstrate a new finding of a biphasic 5-HT-induced response (transient relaxation followed by sustained contraction) in cavernosal tissue obtained from healthy rabbits. Previously, Webber et al. [31] demonstrated the existence of a biphasic 5-HT-mediated re-

**Table 5.** 5-HT-mediated contraction of rabbit cavernosal tissues before and after the addition of corynanthine (coryn) $10^{-5}$ M or yohimbine (yohim) $10^{-5}$ M. Results are presented as median (range) in mg tension/mg of tissue

| Rabbits (n = 13) | | Rabbits (n = 9) | |
|---|---|---|---|
| 5-HT $10^{-3}$ M | 86.2 (18.7 to 201) | 5-HT $10^{-3}$ M | 83.5 (24.8–243.3) |
| Coryn $10^{-5}$ M and 5-HT $10^{-3}$ M | 89.5 (24.9 to 155) | Yohim $10^{-5}$ M and 5-HT $10^{-3}$ M | 80.7 (38.0–194.3) |

**Table 6.** Transient 5-HT-mediated relaxation of rabbit cavernosal tissues before and after the addition of ondansetron (ondan) $10^{-5}$ M or Y-12530 (Y-3) $10^{-5}$ M (5-$HT_3$ antagonists). Results are presented as median (range) in mg tension/mg of tissue (the minus sign denotes relaxation)

| Rabbits (n = 3) | | Rabbits (n = 4) | |
|---|---|---|---|
| 5-HT $10^{-3}$ M | –15.2 (–47.9 to –13.4) | 5-HT $10^{-3}$ M | –39.1 (–68.6 to –12.1) |
| Ondan $10^{-5}$ M and 5-HT $10^{-3}$ M | 0 (0 to 0) | Y-3 $10^{-5}$ M and 5-HT $10^{-3}$ M | 0 (0 to 0) |

**Table 7.** 5-HT-mediated transient relaxation of rabbit cavernosal tissues before and after the addition of SB-269970 $10^{-5}$ M or L-NAME $10^{-4}$ M. Results are presented as median (range) in mg tension/mg of tissue (the minus sign denotes relaxation)

| | SB-269970 $10^{-5}$ M ($p > 0.2$; n = 9) | L-NAME $10^{-4}$ M ($p < 0.02$; n = 5) |
|---|---|---|
| 5-HT $10^{-3}$ M before addition | 37.6 (13.6 to 109.6) | –39.5 (–84.4 to –11.6) |
| 5-HT $10^{-3}$ M after addition | 40.3 (16.8 to 113.1) | 0 (–25 to 0) |

sponse in the tracheal vasculature of sheep although they reported a contraction followed by relaxation. In our study, however, the transient relaxation only occurred in 30% of all the tissues analysed. This finding probably reflects biological variation in the rabbit population.

The biphasic response in the rabbit cavernosal tissue indicates that the relaxation has a rapid onset, is small in magnitude and short lasting when compared with the contractile response. It may be that the rapid-onset relaxation response modulates the activity of the predominant contractile activity so that the overall contractile response is gradual and thus, the detumescence process is more controlled.

The prime candidates in mediating the relaxation response are the 5-HT receptor subtypes 3 or 7. Kanada et al. [32] showed that a selective 5-$HT_3$ receptor agonist induced a dose-dependent relaxation of rat ileal circular muscle. The 5-$HT_7$ receptor also has an extensive vascular distribution and is responsible for the prominent, persistent vasodilator response to 5-HT in anaesthetized animals [4]. In our study, the relaxation response was mediated via the 5-$HT_3$ and not 5-$HT_7$ receptor subtype. The relaxant responses were inhibited by ondan and Y-25130. Ondan is an antiemetic agent which acts on the 5-$HT_3$ receptor subtype centrally. It would be of interest to evaluate its possible proerectile activity clinically via its action at the cavernosal smooth muscle as suggested by our findings.

The 5-$HT_3$ receptor-mediated transient relaxation may be NO-dependent since the relaxant responses were either diminished or abolished by L-NAME. Furthermore, studies have shown NO-mediated activity via the 5-$HT_3$ receptor (e.g. in the rat ileum) [32], neuronal cell lines [33] and rat spinal cord [34].

In our study, ketan inhibited the contractile response mediated by 5-HT on the corpus cavernosum suggesting the presence of 5-$HT_{2A}$ receptors which mediate smooth muscle contraction. Also, dox had a similar effect as ketan on the cavernosal smooth muscle. The latter finding suggests that dox has a 5-HT inhibitory action. This can also be inferred from previous studies of the 5-HT-mediated effects of dox in human platelets and rabbit bladder detrusor muscle [30, 35].

Our studies demonstrated that 5-HT $10^{-3}$ M does not act on $\alpha_1$- or $\alpha_2$-receptors as coryn and yohim respectively have no significant effect on the 5-HT-mediated contraction. Therefore, the antagonistic effect of ketan and dox on 5-HT-mediated contraction is via 5-HT and not α-receptors.

The cavernosal basal tone (CBT) is maintained by the interaction between contractile (including 5-HT) and relaxant (e.g. NO) pathways. The cavernosal smooth muscle, like other muscles, has a basal tone at rest. The CBT maintains the flaccidity of the penis. Tumescence occurs when the CBT is lowered to a 'trigger point' to allow adequate opening of the cavernosal lacunar spaces (complements the veno-occlusive mechanism) and hence the pooling of blood resulting in erection. Likewise, the reverse occurs during the detumescence process to return the 'relaxed' CBT to the normal physiological contracted (resting) state. Our findings suggest that erection may be enhanced by promoting relaxant pathways such as activation of 5-$HT_3$ receptor subtype and/or inhibiting the contractile pathways such as activation of 5-$HT_{2A}$ receptors.

In conclusion, our findings support the evidence that 5-HT plays a role in the erectile process via 5-$HT_{2A}$ receptor-mediated contractile action. More studies are needed to further clarify the role of 5-$HT_3$ receptor-mediated relaxant activity on the erectile process. 5-$HT_{2A}$ receptor antagonists (e.g. ketan) and possibly a 5-$HT_3$ receptor agonist (e.g. ondan) as well as dox may be beneficial in the treatment of ED as part of a multi-therapy regimen (when monotherapy fails) via 5-HT-dependent mechanisms (rather than exclusively via $\alpha_1$-adrenergic blockade as for dox).


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# Doxazosin and Serotonin (5-HT) Receptor (1A, 2A, and 4) Antagonists Inhibit 5-HT-Mediated Human Cavernosal Contraction

DAVID H. W. LAU,*†‡§ CECIL S. THOMPSON,†‡ JAMES F. BELLRINGER,|| PHILIP J. THOMAS,|| FAIZ H. MUMTAZ,‡§ ROBERT J. MORGAN,* AND DIMITRI P. MIKHAILIDIS†‡

*From the *Department of Urology, †the Department of Clinical Biochemistry, ‡and the Department of Surgery, Royal Free Hospital and University College Medical School, University College London, United Kingdom; the §Department of Urology, Chase Farm Hospital, Enfield, United Kingdom; and the ||Department of Urology, Charing Cross Hospital, London, United Kingdom.*

**ABSTRACT:** Penile erection results from the balance between relaxation and contractile mechanisms of the corpus cavernosum. Only a few studies suggest a role for endogenous contractile agents such as 5-hydroxytryptamine (5-HT). Our aim was to confirm the possible role of 5-HT in human erection. The effect of 5-HT on human cavernosal tissues, as well as those of doxazosin (shown previously to have 5-HT inhibitory action), ketanserin (5-HT(2A) receptor antagonist), NAN-190 (5-HT(1A) receptor antagonist), and SB 203186 (5-HT(4) receptor antagonist) on 5-HT-mediated effects, were assessed using the organ bath technique, including electrical field stimulation study (EFS). Results are presented as median (mg/mg = mg contraction/mg of tissue). Consistent 5-HT-mediated ($10^{-3}$ M) contractions were demonstrated (n = 18; 63 mg/mg). These contractions were inhibited with ketanserin by 90% (n = 8), NAN-190 by 68% (n = 12), and SB 203186 by 55% (n = 12). Doxazosin showed a similar 5-HT inhibitory action in a concentration-dependent manner ($10^{-4}$ M; 94% reduction; n = 8, $10^{-6}$ M; 68.3% reduction; n = 8). Our EFS studies indicated the presence of neuronally derived 5-HT and that a majority of the nonnoradrenogenic contraction (54%) was mediated via 5-HT(2A) receptors. These findings suggest that 5-HT may play a role in the human detumescence process via 5-HT(1A), 5-HT(2A), and 5-HT(4) receptors. Neuronally released 5-HT is probably an important contractile neurotransmitter in the erectile process. Doxazosin, ketanserin, and 5-HT(1A) and 5-HT(4) receptor antagonists may be useful as part of combination therapy used to treat erectile dysfunction.

Key words: 5-hydroxytryptamine, erectile dysfunction, corpus cavernosum, cavernosal tone.

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Erectile dysfunction (ED) is broadly defined as the inability to achieve or maintain an erection sufficiently rigid for satisfactory sexual intercourse (NIH Consensus Development Panel on Impotence, 1993). ED affects as many as 50% of men over the age of 40 years to some degree and has substantial impact on quality of family life (Carson, 2004).

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine transmitter found with its receptors both in the central and peripheral nervous system (CNS/PNS), as well as in a number of nonneuronal cells in the gut, cardiovascular system, and blood. 5-HT is one of the oldest neurotransmitters in evolution. It has been implicated in the etiology of numerous disease states, including depression, anxiety, hypertension, and irritable bowel syndrome. 5-HT receptors are divided into 7 distinct classes (5-HT(1) to 5-HT(7)) based on their structural and functional characteristics. These receptors are part of the G-protein-coupled receptor (GPCR) superfamily, with the exception of the 5-HT(3) receptor, which is a ligand-gated ion channel (Martin et al, 1994).

5-HT neuron participation in the control of sexual behaviour, both in humans and in animals, is well established (Hull et al, 2004). Specifically, Bancila et al (2002) demonstrated a possible role of the paraventricular nucleus (brain) in penile erection through the control of descending serotonergic raphe-spinal neurons. In general, central (brain) activation of the 5-HT(1A) receptor inhibits (Ahlenius et al, 1989; Rehman et al, 1999), and activation of 5-H(2A) and 5-HT(2C) receptor facilitates, erection (Steers et al, 1990; Bancila et al, 1999; Brotto et al, 2000). Central acting drugs that influence the 5-HT pathway can affect erectile function. For example, serotonin-specific reuptake inhibitors such as paroxetine can increase the incidence of ED due to inhibition of nitric oxide synthase (NOS) activity (Angulo et al, 2001). Also, trazodone, an antidepressant, which exerts its effect

Correspondence to: D. P. Mikhailidis MD, FRCP, FRCPath, Academic Head of Department, Department of Clinical Biochemistry, Royal Free Hospital, Pond Street, London NW3 2QG, United Kingdom (e-mail: mikhailidis@aol.com).

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via its major metabolite, metachlorophenylpiperazine (m-cpp, a neuronal 5-HT releaser) can cause priapism (Myrick et al, 1998; Rothman et al, 2002).

Peripherally, evidence has emerged of the involvement of the serotonergic pathway in the erectile process. Previous studies on penile vessels demonstrated an in vivo 5-HT-mediated inhibitory action on penile erection in rats due to vasoconstriction of the cavernosal arteries (Finberg et al, 1990). The in vitro 5-HT-mediated contractile response in human penile veins was augmented in patients with veno-occlusive disease (Esen et al, 1997). Animal studies indicated the involvement of 5-HT(1A) (Hayes et al, 2002; Furukawa et al, 2003), 5-HT(1B) (Hayes et al, 2002), and 5-HT(2A) receptors (Furukawa et al, 2003) in contracting cavernosal smooth muscle. In addition, Uckert et al (2003) had shown 5-HT(1A)–mediated contractile response (in vitro) in human corpus cavernosal strips. A human study by Hayes et al (1999) also suggested the presence of 5-HT(4) receptors in cavernosal muscle.

Doxazosin (an alpha-1–blocker shown to have 5-HT inhibitory action (Khan et al, 2000; Jagroop et al, 2001) and ketanserin (5-HT(2A) receptor antagonist) have been shown to have a beneficial action on ED. Doxazosin also acts on ED in combination with either sildenafil (de Rose et al, 2002) or intracavernosal prostaglandin E(1) therapy (Kaplan et al, 1998) when either sildenafil or the cavernosal therapy alone has failed. The combined intracavernosal injection therapy of ketanserin and prostaglandin E(1) was effective in producing an erection sufficient for sexual intercourse in 76% of patients with ED when the prostaglandin E(1) therapy alone had failed (n = 45; Mirone et al, 1996). Petersen et al (1985) noted concomitant penile tumescence in their study, which showed improved maximum urinary flow rates in patients with benign prostatic hyperplasia who were treated with ketanserin.

Since most functional (organ bath) studies were performed on animals, we aimed to further evaluate the involvement of 5-HT in the human erectile process via 5-HT(1A), 5-HT(2A), and 5-HT(4) receptors. We also evaluated whether doxazosin exhibits a protumescence effect.

## Materials and Methods

### Tissues

Human penile organs were obtained from patients undergoing gender reassignment surgery at Charing Cross Hospital, London, United Kingdom (15 patients, age range 23–57, mean age 30). Approval was obtained from the Riverside Ethics Committee, and all the patients gave their informed consent prior to surgery. Their penile organs were excised and immediately placed in Krebs solution and kept in an ice-containing box. The Krebs solution was made up of NaCl 120 mM, $NaHCO_3$ 25.6 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $NaH_2PO_4$ 1.2 mM, and glucose 22 mM with a pH of 7.4.

All patients underwent gender reassignment surgery and had no significant previous illness (including diabetes) and were not on any medication apart from estrogen for 2 years. However, the estrogen therapy was discontinued 2 months prior to surgery.

### Materials

The following drugs and other materials were supplied by Sigma Chemical Co. (Poole, Dorset, United Kingdom): atropine hydrochloride, guanethidine, indomethacin, and phenylephrine. Tetrodotoxin was provided by Bachem Fine Chemicals (Switzerland). Tocris Cookson Ltd, Bristol (United Kingdom), provided the following chemicals: corynanthine, yohimbine, NAN-190, SB 203186 and ketanserin. Doxazosin and 5-hydroxytryptamine were gifts from Pfizer (United Kingdom).

### Organ Bath Studies

*Tissue Preparation*—The tunica albuginea was opened to expose the cavernosal tissues. Once they were isolated, the cavernosal tissue was cut into $5 \times 5 \times 6$-mm strips. The tissues were dissected following the penile trabecular structure. The strips were strung up in vertical organ bath systems. Each bath chamber was filled with 10 ml of Krebs solution maintained at 37°C and continuously gassed with a mixture of 95% $O_2$ and 5% $CO_2$. An initial tension of 2 *g* was applied, and the strips were allowed to equilibrate for 1 hour without any further mechanical manipulation (Thompson et al, 2001).

*Establishment of 5-HT-Mediated Response*—Adding 5-HT $10^{-3}$ M to the bath chamber assessed the response of cavernosal tissue strips to 5-HT. Accumulated dose-incremental 5-HT-mediated responses were not performed, as we had previously demonstrated tachyphylaxis of 5-HT with accumulative doses in human cavernosal strips. Specifically, we showed 43.8% reduction of maximal/overall 5-HT contraction with accumulative doses ($5 \times 10^{-7}$ M, $3 \times 10^{-6}$ M, $10^{-5}$ M, $3 \times 10^{-5}$ M, $10^{-4}$ M, and $10^{-3}$ M) of 5-HT 30 minutes following initial same accumulative doses of 5-HT followed by washout × 3 (initial: median 11.88 mg/mg, minimum 5.83 mg/mg, maximum 25.65 mg/mg; at 30 minutes: median 6.68 mg/mg, minimum 3.65 mg/mg, maximum 21.29 mg/mg; $P < .02$ Wilcoxon test, n = 7 each group). Others had also shown similar 5-HT tachyphylaxis responses (Sicuteri, 1983; Javid et al, 1999; Whalen et al, 2000; Lopez-Tudanca et al, 2003). However, a single-dosage exposure of human cavernosal strips to 5-HT $10^{-3}$ M and subsequent same 5-HT reexposure 30 minutes after vehicle (distilled water) addition both gave similar 5-HT–mediated contractile responses, with no significant difference (Table 1). Thus, this single-dosage 5-HT addition was adopted in our study. The dose of $10^{-3}$ M was chosen because it was shown to give optimal results when assessing the responses of 5-HT with and without preexposure to its antagonists in our previous study (Khan et al, 2000).

*Characterization of 5-HT Receptor Subtype*—The effect of distilled water, NAN-190 ($10^{-5}$ M; 5-HT(1A) receptor antag-

Table 1. *5-HT-mediated contraction of human cavernosal tissues before (control) and after the addition of a chemical agent (antagonist or distilled water [vehicle]). Results are presented as median (range) in mg tension/mg of tissue. N denotes number of patients studied. Wilcoxon test is used for statistical analysis. Significance is described as P < .05*

| Control | Antagonist | Treatment | N | *P* value |
|---|---|---|---|---|
| 98.8 (8.9–177.5) | 92.0 (8.3–187.9) | Distilled water (vehicle) | 14 | > .1 |
| 53.7 (7.2–143.3) | 4.6 (0–32.3) | $10^{-5}$ M Ketanserin | 11 | < .02 |
| 107.6 (57.3–268.5) | 34.5 (3.6–75.6) | $10^{-5}$ M NAN-190 | 12 | < .01 |
| 107.8 (45.8–347.9) | 48.7 (10.6–203.3) | $10^{-5}$ M SB 203186 | 12 | < .01 |

onist), ketanserin ($10^{-5}$ M; 5-HT(2A) receptor antagonist), SB 203186 ($10^{-5}$ M; 5-HT(4) receptor antagonist), corynanthine ($10^{-5}$ M; alpha(1) receptor blocker), yohimbine ($10^{-5}$ M; alpha(2) receptor blocker) and doxazosin ($10^{-4}$ and $10^{-6}$ M; alpha(1) receptor blocker) on 5-HT-mediated responses were also assessed. This was carried out by adding the substance concerned including distilled water ($DH_2O$) (as controls) to the bath after initial exposure to 5-HT $10^{-3}$ M. The bath was then left for 30 min prior re-exposure to 5-HT $10^{-3}$ M.

*Electrical Field Stimulation (EFS) Studies to Assess Possible Neuronally Released 5-HT*—EFS studies were also carried out to assess the effect of ketanserin on the possible neuronally released 5-HT in cavernosal tissues. Each tissue strip was positioned between 2 metal rings connected to an electrical circuit and was also subjected to an applied tension of 2 g for 1 hour. Tissues were then exposed to atropine $10^{-5}$ M, guanethidine $5 \times 10^{-6}$ M, L-NAME $3 \times 10^{-4}$ M, and indomethacin $10^{-6}$ M (by adding the substances to the organ baths) to inhibit the parasympathetic, sympathetic, NO, and prostaglandin pathways, respectively. This treatment would enable the EFS studies to unmask any non-adrenergic-mediated contraction, which could include 5-HT–induced contraction. The tissues were left for 30 minutes following the addition of these substances. Electrical currents of increasing intensity (0.5, 1, 2, 5, 8, 16, and 32 Hz) were applied across the tissue strips. Each stimulus was applied for 5 seconds, with a rest interval of 2 minutes between each stimulus. Tissue strips with contractile responses were then exposed to ketanserin $10^{-5}$ M. After 30 minutes of exposure to ketanserin, the EFS (described above) were repeated in the tissues concerned to assess the possible neuronal 5-HT–mediated contractions. Tetrodotoxin $10^{-6}$ M, a neurotoxin, was used to determine the magnitude of contractions related to direct muscle stimulation as opposed to neuronal-mediated contraction. Tetrodotoxin was added to organ baths, and the tissue strips were exposed for 20 minutes before EFS was started. This was the last stage of each EFS study. We had previously shown that repeated EFS × 3 did not cause desensitization of the tissue (Calvert et al, 2001; Banks et al, 2006).

*Establishing Tissue Viability at the Beginning and the End of the Experiments*—All cavernosal tissues used in this study showed a similar potassium chloride (120 mM)-induced contraction at the beginning and the end of the experiments (variability < 10%). Those with variability in responses > 10% were excluded from the study.

*Measurement of Tissue Response*—Isometric responses of the tissue were amplified and recorded using a Chart 4 Windows program. The tissue used in the organ bath was weighed and this value recorded. The contractile/relaxant response of the tissue to a contractile, relaxant, or drug agent was reported in mg/mg (contraction/mg of tissue) by dividing the amount of contraction/relaxation occurring on exposure to an agent by the weight of the tissue concerned.

### Statistical Analysis

A statistical analysis software (PRISM, Graph Pad Inc., San Diego, Calif) was used for the statistical analysis of the human functional studies. Comparisons were made using the 2-tailed nonparametric paired (Wilcoxon) test.

## Results

Consistent 5-HT–mediated ($10^{-3}$ M) contractions from baseline recordings were demonstrated in human cavernosal tissues (n = 25, median 63 mg/mg, range 10.2–178.5 mg/mg).

Experiments with the vehicle (ie, distilled water) used to dissolve the substances evaluated in this study revealed a similar magnitude of 5-HT–mediated contraction before (median 98.8 mg/mg) and after (median 92.0 mg/mg, n = 14) adding $DH_2O$ ($P > .1$, Table 1). These contractions were inhibited by ketanserin by 91% (n = 11, Table 1), NAN-190 by 68% (n = 12, Table 1), and SB 203186 by 55% (n = 12, Table 1).

Doxazosin showed a similar 5-HT inhibitory action in a concentration-dependent manner ($10^{-4}$ M; 94% reduction; n = 8, $10^{-6}$ M; 84% reduction; n = 10, Table 2). The doxazosin response was not attributable to alpha blockade, since alpha-1 and 2 antagonists (corynanthine and yohimbine) had no significant effect on 5-HT–induced contractions (Table 2).

Optimal human cavernosal contractions of 6.3 mg/mg were observed at 32 Hz in the EFS studies where tissues were preexposed to indomethacin, guanethidine, atropine, and L-NAME (Figures 1 and 2). The subsequent addition of ketanserin led to abolition of 54% of the EFS-induced cavernosal contractions (Figure 1). Adding tetrodotoxin inhibited a further 34% of these reduced EFS-induced contractions (Figure 1).

Table 2. *5-HT–mediated contraction of human cavernosal tissues before (control) and after the addition of a chemical agent (antagonist or distilled water [vehicle]). Results are presented as median (range) in mg tension/mg of tissue. N denotes number of patients studied. Wilcoxon test is used for statistical analysis. Significance is described as P < .05*

| Control | Antagonist | Treatment | N | *P* value |
|---|---|---|---|---|
| 49.9 (7.8–179.5) | 8.0 (1.7–24.1) | $10^{-6}$ M Doxazosin | 10 | < .02 |
| 59.8 (10.1–84.6) | 3.4 (0–30.9) | $10^{-4}$ M Doxazosin | 8 | < .02 |
| 97.5 (25.3–205) | 99.4 (27.9–173) | $10^{-5}$ M Corynanthine | 13 | > .1 |
| 91.2 (26.6–230.1) | 93.7 (35.8–242.6) | $10^{-5}$ M Yohimbine | 9 | > .1 |

## Discussion

Our findings show for the first time that there is possibly preterminal neuronal storage of 5-HT in the human corpus cavernosum, which is released by EFS and acts on 5-HT(2A) receptors. This is shown following blockade of the effects of prostaglandin, neuronal- and endothelial-derived NO, sympathetic and parasympathetic pathways with indomethacin, L-NAME, guanethidine, and atropine, respectively, prior to EFS with and without ketanserin addition. The EFS-contractile responses in our study are nonnoradrenergic, as guanethidine leads to effective inhibition of noradrenaline release from sympathetic nerves. The neuronally released 5-HT acting on 5-HT(2A) receptors comprises 54% of the nonnoradrenergic (neuronal)-mediated human cavernosal contraction. Thus, neuronally released 5-HT is probably a contractile neurotransmitter in the erectile process in addition to noradrenaline (NA). This is in contrast to the findings of Uckert et al (2003). They concluded in their study that 5-HT did not contribute to neuronal derived function of the human corpus cavernosum (HCC). They added a 5-HT(1A) antagonist following EFS of precontracted cavernosal strips with phenylepherine. They showed a brief re-

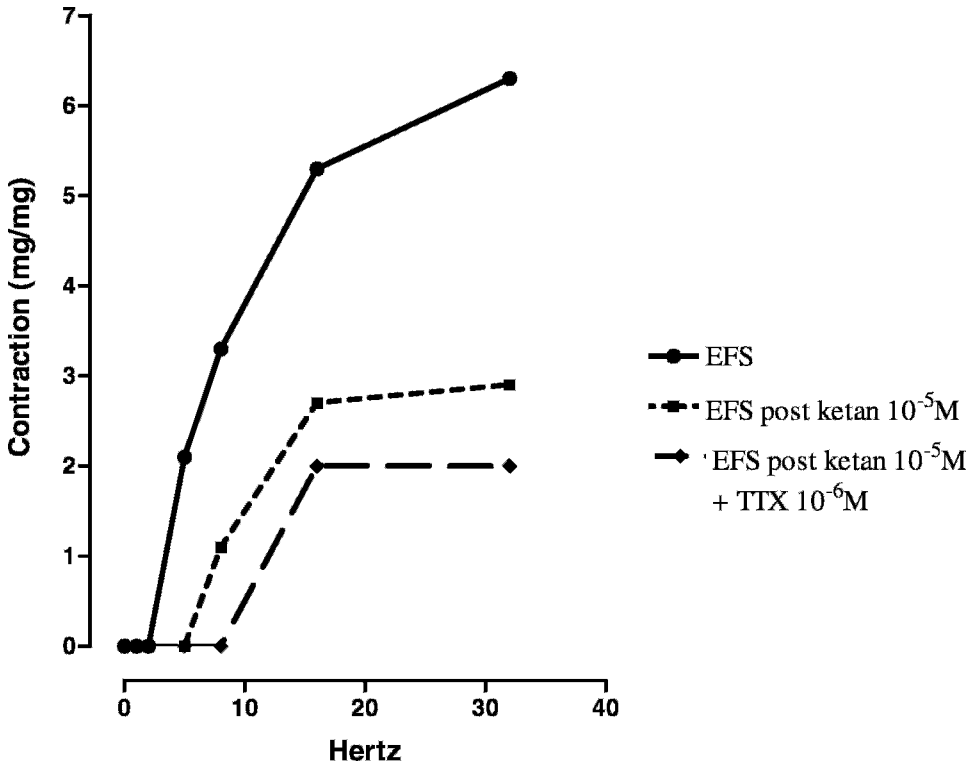


Figure 1. The effects of increasing frequencies of electrical field stimulation (median 6.3, range 3.0–89.7, control) on human corpus cavernosum (n = 8) pretreated with atropine $10^{-5}$ M, guanethidine 5 × $10^{-6}$ M, L-NAME 3 × $10^{-4}$ M, and indomethacin $10^{-6}$ M and the changes seen in EFS following the exposure to ketanserin (ketan) $10^{-5}$ M (median 2.9, range 1.3–5.4, *P* = .001 versus control) and then plus tetrodotoxin (TTX) $10^{-6}$ M (median 1.9, range 0.9–4.6, *P* = .001 versus control). Contractions are expressed as median and range values (mg tension/mg of tissue).

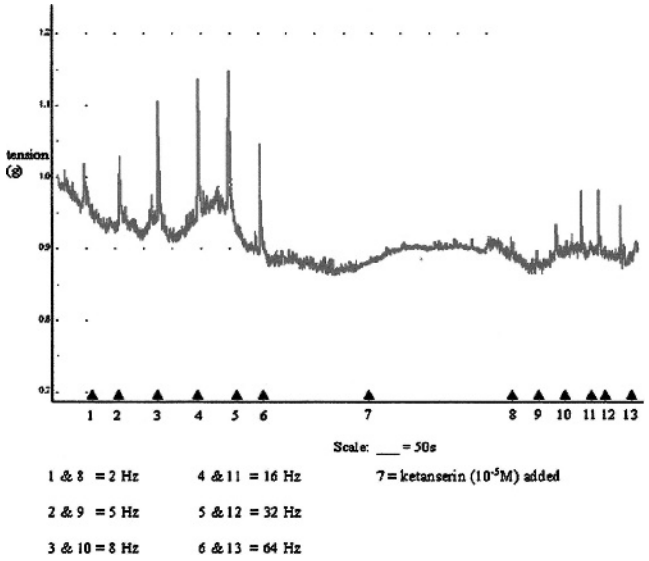


Figure 2. A representative tracing showing the contractile response following electrical field stimulation (EFS: 2, 5, 8, 16, 32, 64 Hz) of human cavernosal strips (preexposed to atropine $10^{-5}$ M, guanethidine $5 \times 10^{-6}$ M, L-NAME $3 \times 10^{-4}$ M, and indomethacin $10^{-6}$ M). Addition of ketanserin ($10^{-5}$ M), a 5-$HT_2$ antagonist, reduced the EFS-induced contractions. The response was measured in grams (g).

laxation response (attributed to neuronal nitric oxide release) with each EFS, which was not altered following subsequent addition of the 5-HT antagonist. If neuronally derived 5-HT acts on 5-HT(1A) receptors, this method did not guarantee effective blockage of the 5-HT(1A) receptors, as activation of the receptors by neuronally derived 5-HT would have occurred prior to the addition of the antagonist. It may also be that 5-HT(2A) (shown in our study) and not 5-HT(1A) receptors contribute to neuronal-derived 5-HT action on HCC.

Uckert et al (2003) also reported a significant increase in 5-HT levels in cavernous serum (mean ng/ml) from flaccidity (113) to tumescence and rigidity (140 and 141, respectively) and also the detumescence phase (123) in normal human subjects. There were less pronounced changes in 5-HT levels in the systemic circulation at all stages. This variation in local 5-HT levels in different stages of erection may be important in ensuring detumescence. It is possible that neuronally released 5-HT contributes to this variation. Therefore, 5-HT may have a physiological role in the control of penile flaccidity.

We show in our studies that 5-HT $10^{-3}$ M does not act on alpha-1 or alpha-2 receptors, as corynanthine and yohimbine, respectively, have no significant effect on the 5-HT-mediated contraction. Therefore, the antagonistic effects of ketanserin and doxazosin on 5-HT-mediated contraction are via 5-HT and not alpha-receptors.

We provide a new finding of the effect of a 5-HT(2A) receptor antagonist on HCC, suggesting possible anti-erectile role of the 5-HT(2A) receptor subtype. Furthermore, we support previous evidence (Mirone et al, 1996; De Rose et al, 2002) that doxazosin and ketanserin may be beneficial in the treatment of ED (findings presented at the 2nd International Consultation on Erectile and Sexual Dysfunction in Paris, 28th June–1 July 2003) as well as that the 5-HT(1A) receptor subtype might play a role in human detumescence (Uckert et al, 2003). Our previous and present studies have demonstrated that doxazosin had 5-HT inhibitory action not just in the human corpus cavernosum but also in rabbit bladder detrusor muscle (Khan et al, 2000) and human platelets (Jagroop et al, 2001). These suggest that doxazosin also acts on 5-HT receptors. Our studies set the precedent for future studies to evaluate the mechanisms of 5-HT-inhibitory actions by doxazosin.

The possible serotonergic-related action noted with doxazosin raises the question whether other alpha-blockers (e.g. alfuzosin or tamsulosin) exert a 5-HT-mediated effect. It is possible that similar bioprofile of serotonergic-induced action to that of doxazosin might account for the improvement in erection in men with lower urinary tract symptoms and concomitant sexual dysfunction treated with alfuzosin (van Moorselaar et al, 2005). Apart from erection, this possible blocking of 5-HT–mediated effect by alpha-blockers may also simultaneously improve bladder symptoms related to bladder outlet obstruction (Khan et al, 2005). Therefore, the beneficial effect of alpha-blockers on the bladder may not be exclusively mediated via alpha-receptor.

Our study suggests a contractile effect on HCC via 5-HT(4) receptors, which is in contrast to what was observed in rabbits (Furukawa et al, 2003), where a 5-HT(4) receptor antagonist potentiated 5-HT-mediated contraction. Therefore, 5-HT(4) receptor activation may contribute to cavernosal relaxation in rabbits. These findings indicate interspecies variability in 5-HT–mediated action via different receptor subtypes.

The potency of 5-HT receptor–mediated responses according to different receptor subtypes are in the order (% inhibition of 5-HT-mediated contraction by its respective antagonist): 5-HT(2A) 90% > 5-HT(1A) 68% > 5-HT(4) 55%. This order indicates the relative importance of each of the 3 receptors in affecting the 5-HT-mediated contraction, with the dominant receptor being 5-HT(2A). Therefore, 5-HT(2A) may play a greater part in the antitumescence process compared with 5-HT(1A) or 5-HT(4) receptor subtypes.

Erection depends on the balance of local contractile and relaxant forces in the corpus cavernosum (Cellek, 2000; Kim et al, 2000). Tumescence/erection is favored if the overall relaxant force dominates to lower cavernosal tone to a critical level and vice versa. Therefore, it is not inconceivable that by targeting the contractile pathway such as 5-HT as well as promoting a relaxant pathway (eg, with a phosphodiesterase-5 (PDE-5) inhibitor), the critical level will be achieved more readily in patients with ED. Our findings indicate that doxazosin and 5-HT(1A), 5-HT(2A) (such as ketanserin), and 5-HT(4) receptor antagonists may be useful as part of a multi-therapy regime, especially when a single therapy with a PDE-5 inhibitor fails.

Normal HCC is limited in its availability. In previous studies, HCC tissues were obtained from patients with Peyronie disease or diabetes or undergoing penile prosthesis implants for ED (Mirone et al, 2000). These samples are clearly pathological. Mirone et al (2000) and Rees et al (2001) proposed the use of HCC tissue obtained from patients undergoing gender reassignment surgery. These patients are normally on estrogen for 2 years prior to withdrawal 2 months before their surgery, as with the majority of patients involved in our study. We cannot exclude the effect of estrogen on the cavernosal tissue, as Adaikan et al (2003) showed that estrogen causes pathophysiological changes in erectile function in rats. However, in our study, 1 patient who refused estrogen therapy prior to surgery had similar 5-HT responses (with or without pre-exposure to its antagonists) to those on estrogen. Furthermore, those gender-reassigned patients previously on estrogen seem to have "normal" erections (indicated by the presence of early morning erections), based on clinical interviews post–estrogen withdrawal prior to surgery.

Future work should involve immunohistochemical studies using cavernosal tissue to further identify/confirm the 5-HT receptor subtype and distribution as well as their anatomical location (eg, nerve terminals and/or endothelium).

In conclusion, neuronally-released 5-HT may play a role in the human detumescence process. Doxazosin and 5-HT(1A), 5-HT(2A) (such as ketanserin) and 5-HT(4) receptor antagonists possess proerectile effects that may prove useful in the treatment of ED, possibly in combination with other therapy.

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