International Journal of Impotence Research (2002) 14, 295–307
 0955-9930/02 $25.00
www.nature.com/ijir

Endothelium-dependency of yohimbine-induced corpus cavernosum relaxation

S Filippi[1], M Luconi[1], S Granchi[1], A Natali[2], P Tozzi[3], G Forti[1], F Ledda[4] and M Maggi[1]*

[1]*Department of Clinical Physiopathology, Andrology Unit, University of Florence, Florence, Italy;* [2]*Department of Urology, University of Florence, Florence, Italy;* [3]*Azienda Ospedaliera Careggi, Florence, Italy; and* [4]*Department of Pharmacology, University of Florence, Florence, Italy*

Development and maintenance of penile erection requires the relaxation of the smooth muscle cells in the cavernous bodies and is essentially mediated by nitric oxide (NO). The penile flaccid state is conversely maintained by the alpha adrenergic neuroeffector system and by other vasoconstrictors, such as endothelin-1 (ET-1). In this study we examined the mechanisms involved in yohimbine-induced relaxation in human and rabbit corpora cavernosa (CC). We essentially found that yohimbine not only blocks contractions induced by adrenergic agonists, but also by non-adrenergic substances, such as ET-1. This effect was unrelated to antagonism at the level of ET receptors, because yohimbine did not affect ET-1-induced increase in intracellular calcium in isolated CC cells. Conversely, our data suggest that yohimbine counteracts ET-1-induced contractions by interfering with NO release from the endothelium. In fact, yohimbine-induced CC relaxation was inhibited by the mechanical removing of the endothelium and by blocking NO formation or signalling via guanylate cyclase and cGMP formation. Conversely, yohimbine activity was strongly increased by inhibiting cGMP degradation. In an experimental model of hypogonadism, performed on rabbits by chronic treatment with a long-lasting GnRH agonist, the relaxant yohimbine activity was also decreased, but completely restored by androgen supplementation. This effect was evident only in preparations in which the main source of NO was present (endothelium) or in which NO formation was not impaired by L-NAME. Our data indicate that the relaxant effect of yohimbine is both endothelium and androgen-dependent. This might justify the lack of efficacy of this drug in treatment of some form of organic erectile dysfunction.
International Journal of Impotence Research (2002) **14,** 295–307. doi:10.1038/sj.ijir.3900890

Keywords: yohimbine; corpus cavernosum; human; relaxation; nitric oxide; sildenafil

Introduction

Penile erection is a physiological event controlled by two opposite drives.[1,2] The first drive determines smooth muscle relaxation and an increase in cavernosal blood flow. The activity of this system is essentially mediated by the nitrergic network.[3] The second drive, operating for the majority of time, determines smooth muscle contraction and detumescence and it is essentially controlled by the sympathetic outflow. In the past few years, the physiological role of the nitrergic network has been extensively studied. Results from these studies culminated recently in the development of compounds that increase the effect of nitrergic transmission, such as sildenafil.[4] However, little attention has been dedicated to the role of sympathetic outflow that, as a component of the general reaction to stress, might be detrimental for penile erection and sexual activity. The lack of attention is quite surprising. Indeed, although the majority of patients with erectile dysfunction (ED) have an organic etiology, such a dysfunction can be further exacerbated by the co-existence of stress and anxiety.[1] Since at least a part of the negative effect of stress on penile erection is mediated by interactions between catecholamines and α-adrenoceptors present in the corpus cavernosum, the use of α-adrenoceptor antagonists seems to be the most rational approach to overcome this problem. Indeed, since the early 1980s, the intracavernous administration of α-adrenoceptor antagonists was a breakthrough in the oral treatment of ED.[5,6] Oral phentolamine was also promising, but the preparation never reached the market due to consistent side effects.[7] Up to now, yohimbine is the only world-wide available α-adrenoceptor antagonist for the treatment of ED. Yohimbine is a indole alkaloid obtained either from the bark of the tree *Pausinystalia yohimbe* or from

*Correspondence: M Maggi, Department of Clinical Physiopathology, University of Florence, V.le G. Pieraccini, 6, 50139 Florence, Italy.
E-mail: m.maggi@dfc.unifi.it
Received 10 June 2001; accepted 18 April 2002

the root of *Rauwolfia*, which has been known as an aphrodisiac compound since before the turn of the last century. Yohimbine was introduced in the treatment of erectile dysfunction more than 70 y ago.[8] A recent meta-analysis of all double-blind, randomised, placebo-controlled trials found a significant improvement in patients treated with yohimbine (odds ratio 3.85, confidence interval 2.22–6.67%).[9] Nevertheless, up to now, there is the general impression that yohimbine can be useful in patients with ED, but with limited benefit and that it should be probably reserved for patients with a psychogenic ED.[10–12] Reasons for the limited popularity of yohimbine might reside: (a) in its lack of patentability and commercial appeal; (b) in its unfavourable pharmacokinetic profile after oral administration; or (c) in its still unknown mechanism of action.[10,13]

Although several pharmacological properties of yohimbine have been described, the best documented activity of yohimbine was the antagonism at the α_2-adrenoceptor.[14] Current hypotheses on the beneficial mechanism of action of yohimbine on sexual activity mainly point to a central mechanism of action of the drug.[15–17] This suggestion is based chiefly upon results from animal studies showing the yohimbine increases sexual motivation even in sexually exhausted rats, due to its action on central α_2-adrenoceptors located in the locus coeruleus.[18–21] However this hypothesis is not completely convincing, because, when tested, yohimbine did not ameliorate sexual desire or thoughts in clinical studies.[15,22–23] In addition, the blockade of α_2-adrenoceptors is able not only to induce an enhancement of sympathetic outflow from the central nervous system but also an increase in catecholamine release from peripheral sympathetic nerve terminals.[24–25] The latter could be detrimental for penile erection because human corporal smooth muscle is endowed with postsynaptic α-adrenoceptors which predominantly belong to the α_1 subtype and mediate cellular contraction.[2,26–28] An alternative hypothesis is that yohimbine also acts at a peripheral level, blocking α-adrenoceptors. Indeed, recent evidences obtained by binding and functional studies suggest that not only the α_1 but also α_2-adrenoceptors mediating contractility are present in human and rabbit corpora cavernosa and that yohimbine might interact with both.[2,29–31] Hence, it is possible that the positive effect of yohimbine on penile erection is not only related to its central effect but also to a peripheral sympatholytic activity.[32] Accordingly, intracavernosal administration of 0.5 and 1 mg of yohimbine in anaesthetised rabbits induced a transient but consistent increase in intracavernosal pressure approaching maximal vasodilatation.[32] Finally, there is evidence that yohimbine might also relax corpora cavernosa (cc) with a non-adrenergic mechanism. In fact, in both rabbit and human CC, yohimbine antagonises the effect of a potent, non-adrenergic, contractor for erectile smooth muscle tissue, endothelin-1 (ET-1).[32] Also phentolamine, an α_1 and α_2-adrenoceptor antagonist for rabbit and human CC, blocks endothelin-1 (ET-1) induced contractility.[33] This non-adrenergic effect of phentolamine was dependent on the integrity of the endothelium and mediated by nitric oxide (NO) synthase activation.[33]

The aim of the present study was to investigate the biochemical and physiological mechanisms underlying the penile relaxant effect of yohimbine, using rabbit and human isolated preparations of erectile tissue. Experiments have been performed in the presence or absence of endothelium, utilizing pharmacological tools, which interfere with NO formation and action as well as after androgen ablation and substitution. This experimental model has been designed on the basis of recent evidence indicating that androgens act at the penile level regulating NO formation and α-adrenergic responsiveness.[34,35]

Materials and methods

Corpus cavernosum preparations

Rabbit corpora cavernosa were obtained from New Zealand White rabbits (weighing approximately 3 kg). The animals were killed by a lethal dose of pentobarbital; then the penis was removed and the corpora cavernosa were carefully dissected free from the tunica albuginea and cut into three to four strips ($0.2 \times 0.2 \times 0.7$ cm).

Human corpora cavernosa were obtained from impotent men at the time of penile prosthesis implantation ($n = 7$). After surgery, biopsy specimens of corpus cavernosum were immediately placed in cold Krebs solution and transported to the laboratory for *in vitro* experiments.

Human and rabbit strips were vertically mounted under 1.8 g resting tension in organ chambers containing 10 ml Krebs solution at 37°C, gassed with 95% O_2 and 5% CO_2 at pH 7.4. The solution had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 2.5, $CaCl_2$ 2.5, glucose 10. The preparations were allowed to equilibrate for at least 90 min, during this period the bath medium was replaced every 15 min. Changes in isometric tension were recorded on a chart polygraph. To evaluate the drug-induced vasorelaxant effect, the preparation was precontracted with concentrations of endothelin-1 (ET-1; 0.1–0.3 μM) or of Phe (100 μM) that were able to induce a vasoconstrictor response of a similar degree. The increase recorded in the presence of different concentrations of these agonists was expressed as percentage of maximal KCl

(80 mM) — induced response taken as 100%. The high potassium salt solution was made by equimolar substitution of sodium by potassium. The presence of functional endothelium was assessed by testing the vasodilator effect of acetylcholine (ACh); the preparations in which ACh (1 μM) reduced the tone by less than 40% were not used for the study. To remove endothelium, strips of corpus cavernosum were rubbed between the thumb and index finger for ~20 s.[36] The lack of a relaxation response to ACh in preconstricted preparations indicated that the procedure was successful.

Experiments were carried out in corporal smooth muscle pre-contracted with a concentration of ET-1 (0.1 μm) able to produce an increase in tension of about 1200 mg; this value was taken as 100% and the relaxant effect induced by different yohimbine concentrations was referred to this value. Drug cumulative concentrations were added, at 7 min intervals, to the bath in order to obtain a concentration–relaxant effect curve; a 15–30-min pre-treatment with selected antagonists and/or inhibitors was performed before repeating the concentration–response curve for yohimbine.

Cell cultures

Human corpora cavernosa (hCC) cells were prepared from corpus cavernosum samples obtained from two patients undergoing surgical correction for congenital curvature of the penis. Approval for the use of human material was given by the Local Ethical Committee. Briefly, tissues were mechanically dispersed and treated with 1 mg/ml bacterial collagenase for 15 min at 37°C. Fragments were then collected, washed in PBS and cultured in a mixture 1:1 (vol/vol) of Dulbecco's modified Eagle's medium and F-12 Ham (DMEM/F-12 1:1 Mix) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and enriched with a mixture of insulin/transferransferrin/selenium, in a fully humidified atmosphere of 95% air and 5% CO_2. Cells began to emerge within 24–48 h and were used within the second passage.

RT-PCR

Total RNA (500 ng) were retrotranscribed and then amplified using the Superscript One Step RT-PCR kit (Life Technologies, Milan, Italy). Specific oligonucleotidies primers for ET-1, ET_A and ET_B mRNA were purchased from Roche (Milan, Italy). The sequences of primers for ET-1 were: 5′-ATG GAT TAT TTG CTC ATG ATT TT-3′ (sense) and 5′-CAG TCT TTC TCC ATA ATG TCT TCA GC-3′ (antisense).[37] The sequences of primers for ET_A were: 5′-CCT TTT GAT CAC AAT GAC TTT-3′ (sense) and 5′-TTT GAT GTG GCA TTG AGC ATA CAG-3′ (antisense).[38] The sequences of primers for ET_B were: 5′-GGA CCC ATC GAG ATC AAG G-3′ (sense) and 5′-AGA ATC CTG CTG AGG TGA AGG-3′ (antisense).[39] The contamination of genomic DNA

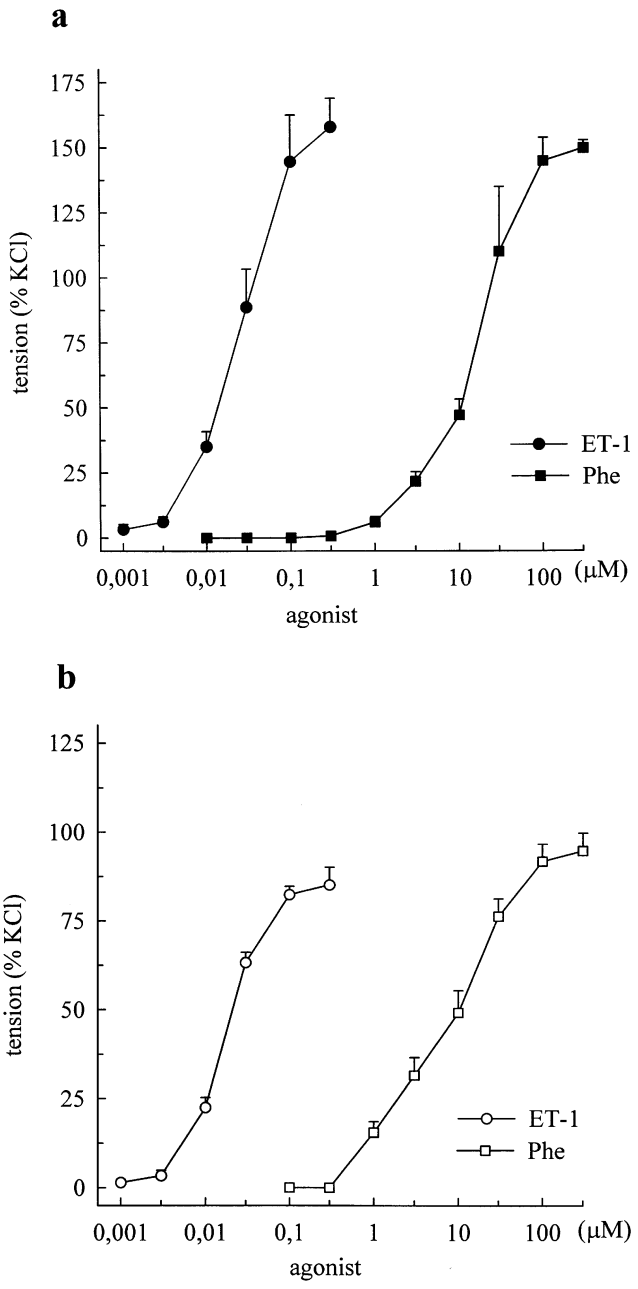

Figure 1 Effect of increasing concentrations of ET-1 (circles) and phenylephrine (Phe, squares) on the basal tone of rabbit (panel a, closed symbols, $n=8$ in six separate experiments) and human (panel b, open symbols, $n=6$ in five separate experiments) corpora cavernosa. The relative EC_{50} are reported in the text. Means ± s.e.m. Note that ET-1 is several-fold more potent than Phe in both rabbit and human preparations.

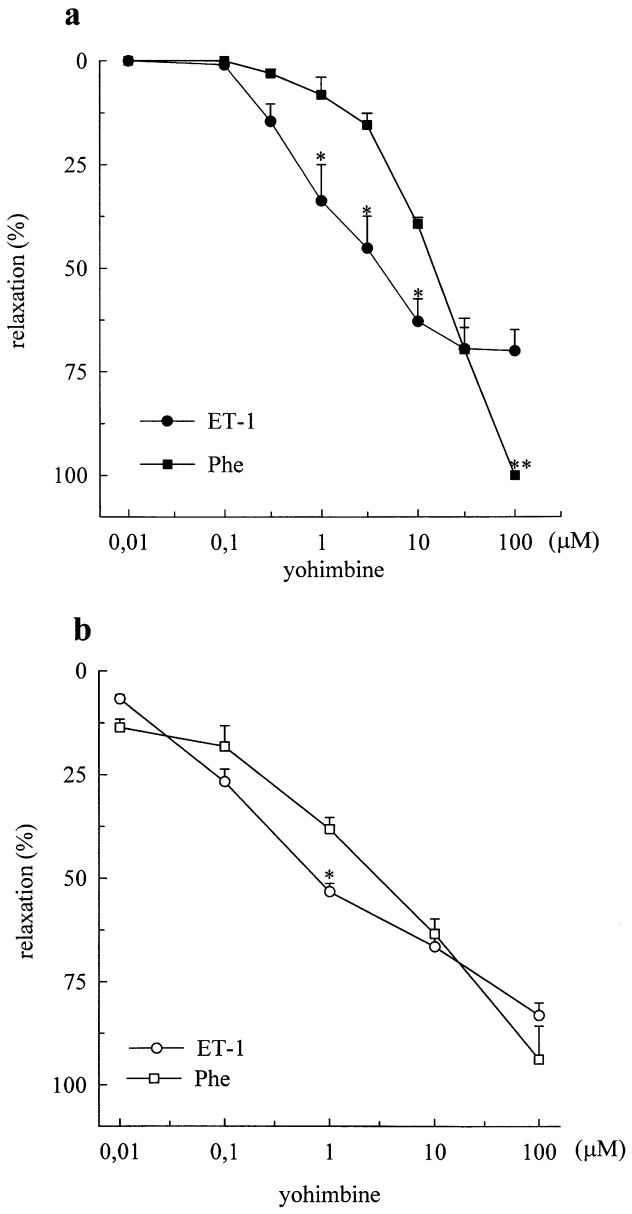

Figure 2 Relaxant effect of increasing concentrations of yohimbine in rabbit (panel a, closed symbols, $n=8$ in six separate experiments) and human (panel b, open symbols, $n=6$ in four separate experiments) corpora cavernosa pre-contracted with ET-1 (0.1 μM, circles) and phenylephrine (Phe, 100 μM, squares). The increase in tone induced by the two agonists before addition of yohimbine was taken as 100% response and the effect of the drug was evaluated as per cent inhibition of this response. Means ± s.e.m; $^*P<0.05$ *vs* the same concentration of yohimbine in preparations pre-contracted with the different agonist. Note that at low yohimbine concentrations (0.3 – 10 μM) was as potent as or even more potent in relaxing ET-1 than Phe pre-contracted strips.

was excluded by performing 35 cycles of amplification without retrotranscription. The integrity of total RNA was verified performing the RT-PCR for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. The sequences of primers for GAPDH were: 5′-CCA TGG AGA AGG CTG GGG-3′ (sense) and 5′-CAA AGT TGT CAT GGA TGA CC-3′ (antisense).[40]

Measurement of intracellular calcium concentration

For intracellular calcium measurements, cells from human adult corpora cavernosa were grown in F-12 Ham to 70 – 80% confluence on plastic coverslips (Aclar; Allied Engineering Plastic, Pottsville, PA). During the 24 h before the experiments, cells were maintained in serum-free medium. $[Ca^{2+}]_i$ was determined using the calcium-sensitive dye Fura-2/AM as described previously.[41] Briefly, cells were loaded with 4 μM Fura-2/AM for 45 min at 37°C, washed and incubated in Fura-2-free medium for another 20 min and finally resuspended in Krebs-Henseleit HEPES-KHH buffer (1.25 mM $CaCl_2$, 5.36 mM KCl, 0.81 mM $MgSO_4$, 130.62 mM NaCl, 5.55 mM, glucose, 8.60 mM HEPES sodium salt, 11.7 mM HEPES free acid, 0.1% BSA, pH 7.4). Coverslips were then mounted diagonally in a quartz cuvette so that the excitation and emission path were at a 45° angle to the coverslip and stimuli were added directly to the cuvette. Fluorescence was measured by a spectrofluorimeter (LS50B, Perkin-Elmer) using a single-wavelength excitation: emission/340 : 510 nm. Calibration was performed using ionomycin (8 μM) to obtain F_{max} followed by EGTA (10 mM, pH 10) to obtain F_{min}. Fluorescence measurements were converted to $[Ca^{2+}]_i$ according to Grynkiewicz assuming a dissociation constant of Fura-2 for calcium of 224 nM.[42]

Experimental hypogonadism and androgen replacement

The study was approved by the Local Ethical Committee for Investigations in Humans of the University of Florence. New Zealand White male rabbits (weighing approximately 3 kg, $n=12$) were divided into three groups. One group was kept intact (controls, $n=3$). The remaining groups of animals were treated with a single administration of 2.9 mg/kg of the long-acting GnRH analog triptorelin pamoate ($n=3$) or vehicle ($n=3$). After 15 days a subset of GnRH treated rabbits ($n=3$) were supplemented with a pharmacological dose of testosterone enanthate (T, 30 mg/kg weekly). After 2 months of triptorelin pamoate administration and after 1 week from the last supplementation of Testosterone, rabbits were killed and blood was taken from the heart for testosterone measurement.

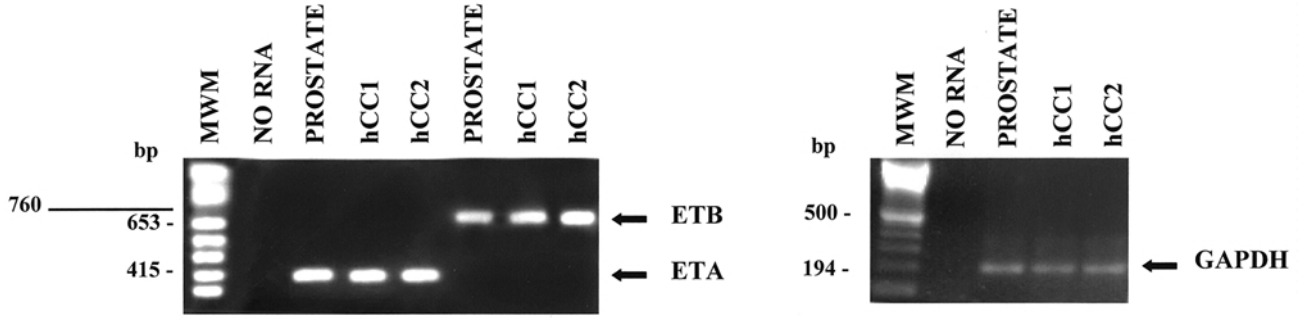

Figure 3 ET_A and ET_B gene expression in two preparation of hCC cell as detected by RT-PCR from total RNA using ET_A and ET_B specific primers (left panel). Human prostate total RNA was used as positive control. GAPDH mRNA amplification was performed to verify the integrity of the extracted total RNA (right panel). MWM = DNA molecular weight marker.

Measurement of testosterone

Plasma levels of testosterone were measured with an Automated Chemiluminescence System (Bayer Diagnostics, East Walpole, MA, USA), after appropriate extraction. Samples were mixed with four volumes of diethyl ether for 15 min, centrifuged for 5 min at 2000 rpm, and the aqueous phase was frozen in dry ice. The organic phase was recovered, evaporated to dryness under a nitrogen stream and reconstituted in the assay buffer.

Statistical analysis

Results are expressed as means ± s.e.m. for n experiments. Statistical analysis were performed with the Student's t-test for paired or unpaired data, with analysis of variance followed by least difference significance (LDS) test in order to evaluate the differences between groups, $P < 0.05$ was taken as significant. Half-maximal response effective concentration (EC_{50}) and half-maximal response inhibiting concentration (IC_{50}) values were calculated by Graph Pad program edited by HG Motulsky.[43]

Chemicals and solutions

Chemicals used included phenylephrine HCl, yohimbine HCl, ionomycin, acetylcholine, indomethacin, N^W-nitro-l-arginine-methyl-ester (L-NAME), DMEM, collagenase type IV, PBS, antibiotics, glutamine, BSA, reagents for intracellular calcium measurement, (Sigma, St Louis, MO); FBS (Unipath, Bedford, UK); 1H-[1,2,4] Oxadiazolo [4,3-a] quinoxalin-1-one (ODQ, Tocris, UK); Fura-2/AM (Calbiochem, La Jolla, CA); testosterone enanthate (Schering AG, Germany); endothelin-1, BQ123 and BQ788 (Sigma-RBI, USA); sildenafil (a gift from Dr Stief, Hannover, Germany), triptorelin pamoate (Ipsen, Milan, Italy); plastic ware for cell culture (Falcon, Oxnard, CA). Indomethacin and sildenafil were dissolved in ethanol; further dilutions were made in distilled water.

Results

Effect of exogenous different agonists on corpus cavernosum

The exposure of rabbit and human CC preparations to increasing concentrations of phenylephrine (Phe, 0.01 – 300 μM), a specific α_1-adrenoceptor agonist, and endothelin-1 (ET-1, 0.001 – 0.3 μM) induced a dose-dependent contractile response characterised by similar E_{max} but different EC_{50}. Indeed, the EC_{50} for Phe was 9.00 ± 0.06 μM and for ET-1 was 30.00 ± 0.02 nM in rabbit CC (Figure 1a, $n = 8$ in six different animals). In human CC, the EC_{50} for Phe was 8.00 ± 0.04 μM and for ET-1 it was 20.00 ± 0.04 nM (Figure 1b, $n = 6$ in five patients).

Effect of yohimbine on pre-contracted corpus cavernosum

As shown in Figure 2a, increasing concentrations of yohimbine relaxes rabbit Phe (100 μM) or ET-1 (0.1 μM) pre-contracted preparations almost completely, with $IC_{50} = 14 \pm 0.04$ μM and 2.10 ± 0.03 μM, respectively ($n = 8$, obtained in six animals). Although yohimbine, at lower concentrations, was even more potent in ET-1 than Phe pre-contracted rabbit strips, the maximal yohimbine-induced relaxation was lower in strips pre-contracted with ET-1 than with Phe ($E_{max} = 70.00 \pm 5.1\%$, *vs* 100.00 ± 0%, $P < 0.01$). Similar results were obtained in human CC (Figure 2b). The relative IC_{50} were 2 ± 0.15 μM for Phe and 0.70 ± 0.13 μM for ET-1. Maximum relaxation obtained with 100 μM

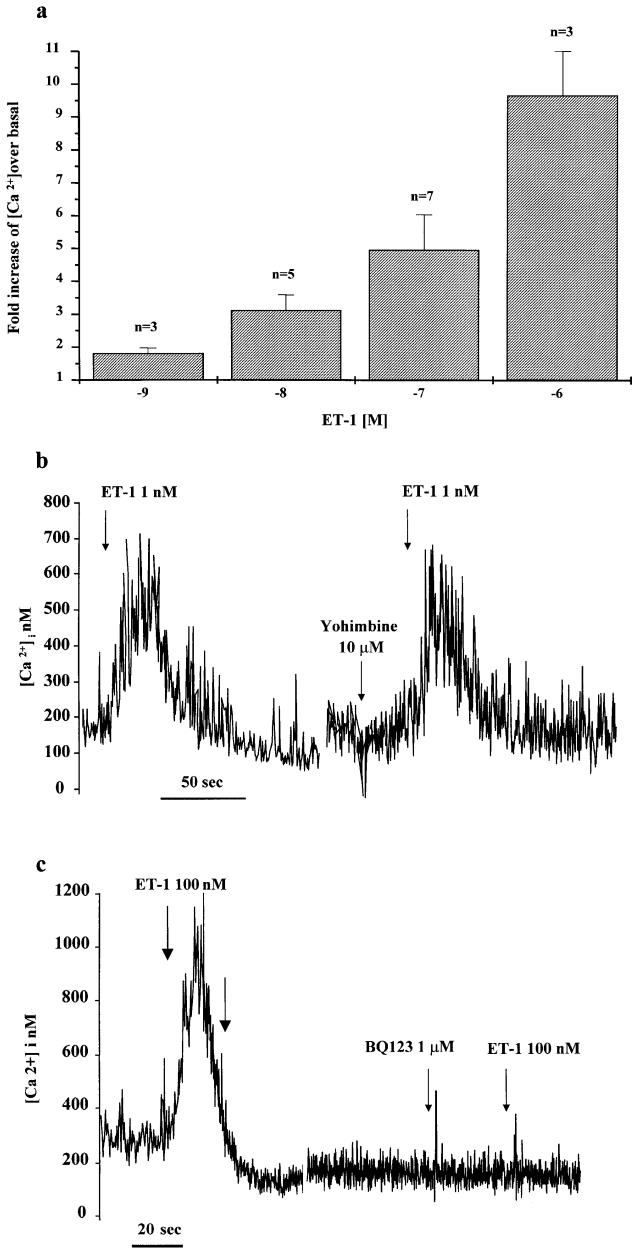

Figure 4 Effect of increasing concentrations of ET-1 on intracellular calcium concentration in Fura 2-loaded hCC cells (panel a). Results are expressed as means ± s.e.m of intracellular calcium fold increase over basal in the number of indicated experiments. Effect of yohimbine and BQ123 on intracellular calcium concentrations increase in response to Endothelin-1 (ET-1) in Fura 2-loaded hCC cells. The addition of ET-1 (1 nM, panel b) and 100 nM (panel c) induced a rapid calcium wave in hCC cells characterized by a first rapid phase followed by a longer sustained one (plateau phase). A previous administration of yohimbine (10 μM) did not affect the calcium responsiveness to ET-1 (1 nM, panel b), while BQ123 (1 μM) totally blunted ET-1 induced calcium waves, even if ET-1 was employed at high concentrations (100 nM, panel c). Results are representative of four different experiments in two separate preparations of hCC.

yohimbine was 93.9 ± 8.00% for Phe and 83.30 ± 3.00% for ET-1 (Figure 2b, $n = 6$, obtained in four patients).

Effect of yohimbine on isolated human corpus cavernosum cells

The relaxant effect of yohimbine on Phe precontracted CC strips is not surprising because it is well known that it antagonises α-adrenoceptor signalling, acting at the receptor level.[2] However, it is unclear how yohimbine does interfere with ET-1 signalling. We first hypothesised that yohimbine directly interacts with ET-1 receptors present in the CC. We tested this hypothesis in two preparations of human CC (hCC) stromal cells. As shown in Figure 3, these cells express genes for both classes of ET-1 receptors (ET_A and ET_B), as determined by RT-PCR. Since one of the first intracellular events observable after ET-1 administration is an increase in intracellular calcium concentration $[Ca^{2+}]_i$, we tested the effect of yohimbine on $[Ca^{2+}]_i$ increase stimulate by ET-1. In CC stromal cells, ET-1 induced a dose-dependent increase in $[Ca^{2+}]_i$ with an $IC_{50} = 0.13 \pm 0.3$ μM (Figure 4a). Yohimbine (10 μM) did not affect basal $[Ca^{2+}]_i$ and responsiveness to either low (1 nM, Figure 4b) or elevated (100 nM, not shown) concentrations of ET-1. Conversely the ET_A receptor antagonist BQ123 (1 μM), but not ET_B antagonist BQ788 (not shown), completely blunted the effect of the concentration of ET-1 tested (100 nM, Figure 4c). This indicates that in hCC stromal cells ET-1 increases $[Ca^{2+}]_i$ concentration acting through the ET_A receptors and that yohimbine does not interact with them.

Effect of yohimbine on endothelium-deprived corpus cavernosum and on the NO pathway

To examine whether the presence of endothelium was essential for the functional antagonism of yohimbine on ET-1-induced CC contractility we repeated experiments in endothelium-deprived rabbit CC. We found that without endothelium, the inhibitory dose–response curve for yohimbine was reduced and significantly shifted to the right (Figure 5a and b, middle panel, $n = 6$, obtained in five animals). Similar results were obtained in intact rabbit CC strips by inhibiting the nitric oxide synthase with the reversible nitric oxide synthase inhibitor, N^W-nitro-l-arginine-methyl-ester (L-NAME). Indeed, in the presence of L-NAME (100 μM, Figure 5a and b, lower panel) the yohimbine-induced CC relaxation was also reduced and shifted to the right ($n = 7$, obtained in six animals). Similar results were obtained with 1 mM L-NAME. Conversely, the relaxant response to yohimbine was not influenced by a pre-treatment of the preparations with the cyclo-oxygenase inhibitor indomethacin (3 μM, data not shown). It is noteworthy that the concentrations of L-NAME and indomethacin used

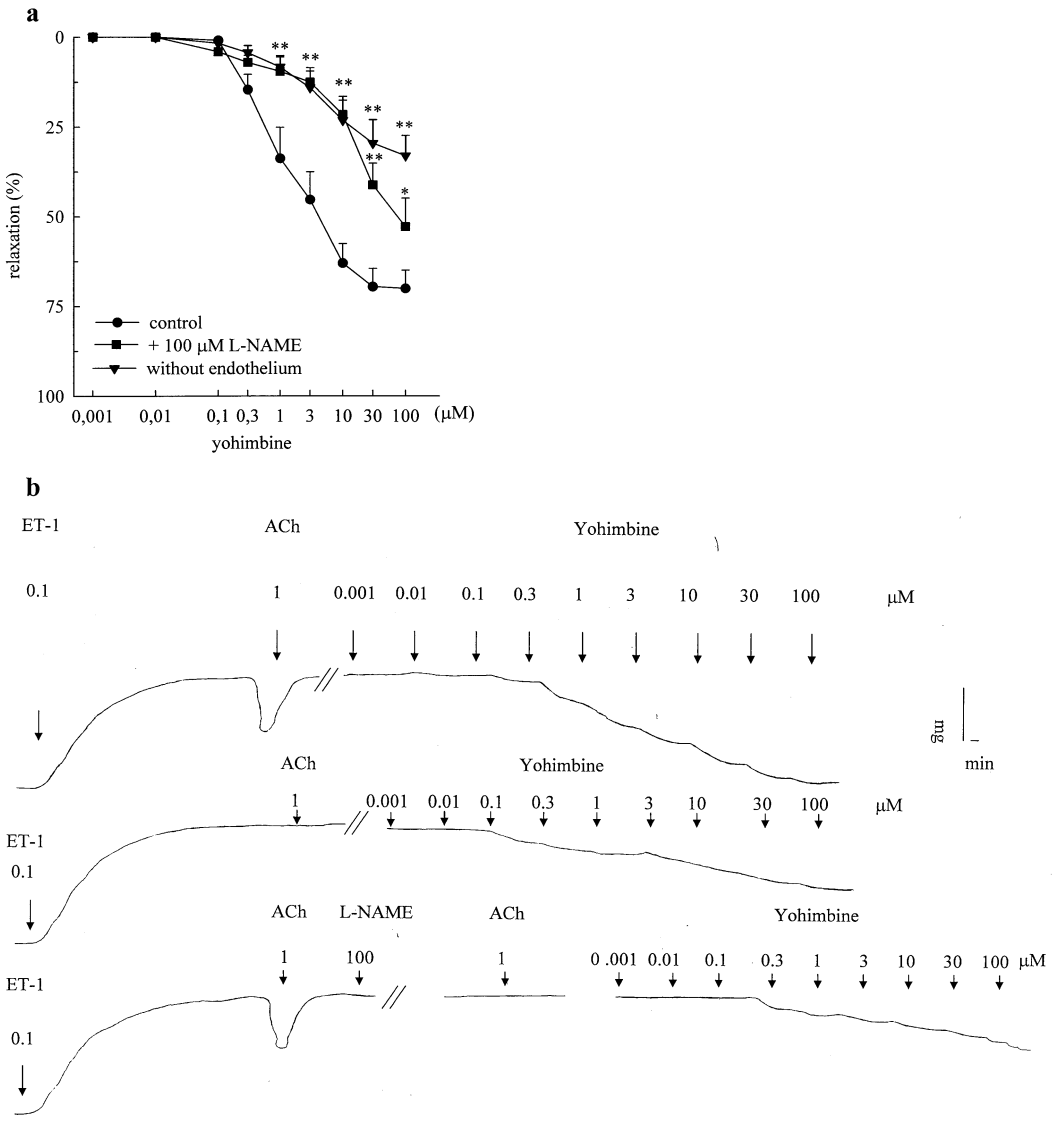

Figure 5 (a) Relaxant effect of increasing concentrations of yohimbine in rabbit corpora cavernosa pre-contracted by ET-1 (0.1 μM) in the presence (closed circles, $n=10$ in eight separate experiments) or not (closed triangles, $n=6$ in five separate experiments) of an intact endothelium or after L-NAME pre-treatment (100 μM, closed squares, $n=7$ in six separate experiments). The increase in tone induced by ET-1 before addition of yohimbine was taken as 100% response and the effect of the drug was evaluated as per cent inhibition of this response. Means ± s.e.m. $^*P<0.05$ and $^{**}P<0.01$ *vs* control curve (closed circles). (b) Typical experiment showing the relaxant effect induced by increasing concentrations of yohimbine in rabbit corpus cavernosum preparations pre-contracted with ET-1 (0.1 μM) in the presence (upper tracing) or not (middle tracing) of an intact endothelium or after L-NAME (100 μM) pre-treatment (lower tracing). The effect of ACh (1 μM; control for a functional endothelium) is shown in each tracing. Arrows indicate the addition of the substances.

in these experiments were not able to significantly change the baseline tone of the preparations.

In order to test whether or not the relaxation induced by yohimbine was due to NO production followed by soluble guanylate cyclase activation, experiments were carried out in the presence of either the potent and selective guanylate cyclase inhibitor ODQ or the cGMP-specific PDE_5 inhibitor, sildenafil.[4,44] Inhibition of soluble guanylyl cyclase by addition of ODQ (1 μM) reduced, while sildenafil (0.3 μM) strongly increased, the yohimbine-induced relaxation in ET-1 pre-contracted rabbit (Figure 6a and b) and human (Figure 7a and b) CC preparations. Indeed, in the presence of the guanylate cyclase inhibitor, the dose–response curve for yohimbine was substantially shifted to the right and the maximal relaxation obtained (100 μM) was 35.00 ± 5.50% in rabbit strips and just 11.10 ± 4.60% in human strips as compared to their relative controls ($n=9$, obtained in seven animals; and $n=6$, obtained in three patients, respectively). On the contrary, the relaxant response to yohimbine was significantly enhanced and shifted to the left by 0.3 μM sildenafil in both rabbit (Figure 6a, $n=7$, obtained in five animals) and human preparations (Figure 7a, $n=4$, obtained in three patients).

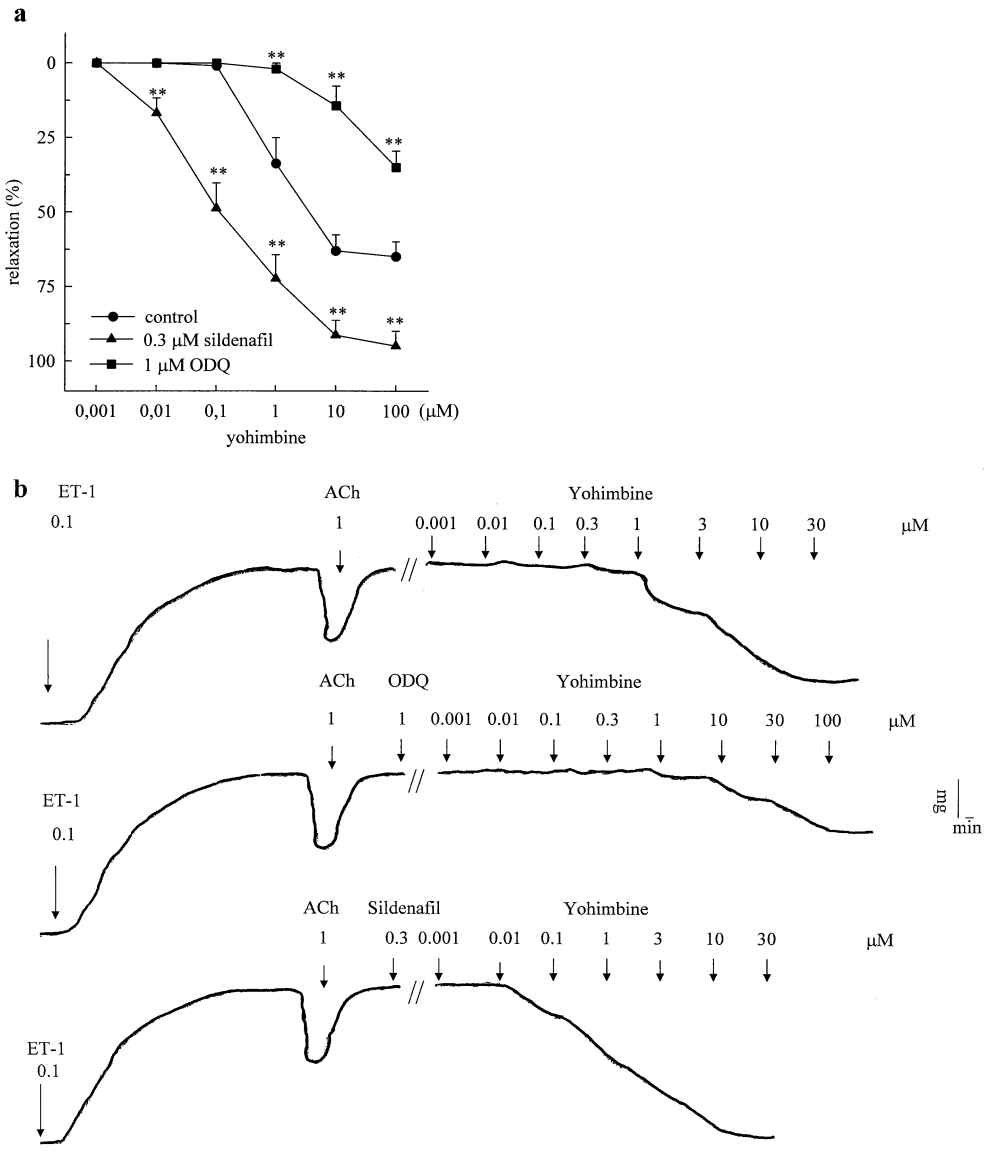

Figure 6 (a) Relaxant effect of increasing concentrations of yohimbine in rabbit corpora cavernosa pre-contracted by ET-1 (0.1 μM) alone (closed circles, $n=10$ in eight separate experiments) or in presence of ODQ (1 μM, closed squares, $n=9$ in seven separate experiments) and sildenafil (0.3 μM, closed triangles, $n=7$ in five experiments). ODQ significantly shifted to the right, and sildenafil to the left, yohimbine curves. The increase in tone induced by ET-1 before addition of yohimbine was taken as 100% response and the effect of the drug was evaluated as per cent inhibition of this response. Means ± s.e.m. $*P<0.05$ and $**P<0.01$ *vs* control curve (closed circles). (b) Typical experiment showing the relaxant effect induced by increasing concentrations of yohimbine in rabbit corpus cavernosum preparations pre-contracted by ET-1 (0.1 μM) alone (upper tracing) and in the presence (middle tracing) of ODQ (1 μM) or sildenafil (0.3 μM, lower tracing). The effect of Ach (1 μM; control for a functional endothelium) is shown in each tracing. Arrows indicate the addition of the substances.

Effect of ET_B antagonist on the relaxant response to yohimbine on corpus cavernosum

In order to test whether or not the relaxation induced by yohimbine was due to an ET_B-mediated NO production, experiments were carried out in the presence of the potent and selective ET_B receptor antagonist, BQ788. The dose–response curve for yohimbine was unaffected by 1 μM BQ788 either in rabbit (Figure 8a $n=6$, obtained in four animals) or human preparations (Figure 8b, $n=4$, obtained in three patients). This indicates that ET_B receptors are not involved in the yohimbine-induced relaxation.

Effect of androgen deprivation on yohimbine-induced relaxation of rabbit corpus cavernous

A single administration of the long-acting GnRH analog triptorelin palmate (2.9 mg/kg) induces

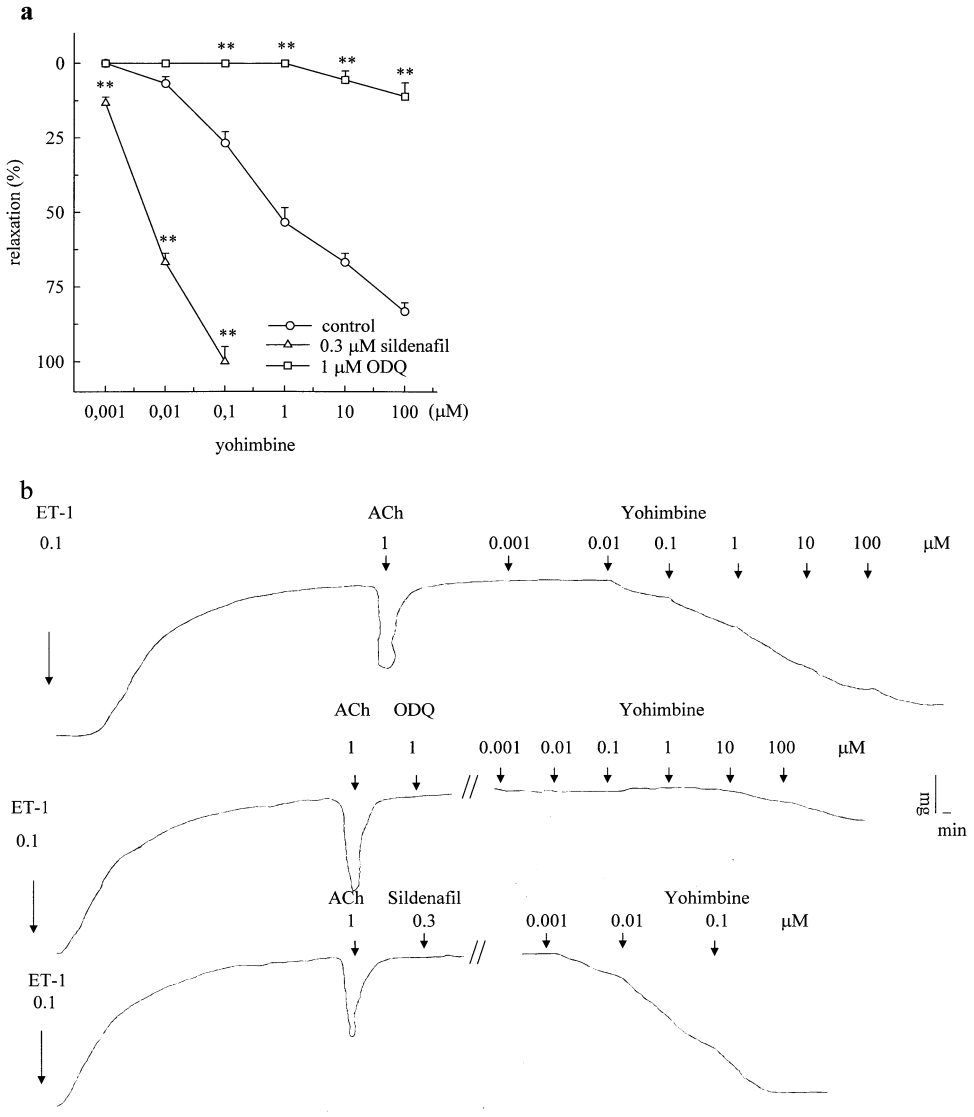

Figure 7 (a) Relaxant effect of increasing concentrations of yohimbine in human corpora cavernosa pre-contracted by ET-1 (0.1 μM) alone (open circles, $n=6$ in three separate experiments) or in presence of ODQ (1 μM, open squares, $n=6$ in three separate experiments) and sildenafil (0, 3 μM, open triangles, $n=4$ in three separate experiments). ODQ significantly shifted to the right and sildenafil to the left, yohimbine curves. The increase in tone induced by ET-1 before addition of yohimbine was taken as 100% response and the effect of the drug was evaluated as per cent inhibition of this response. Means ± s.e.m. $^{**}P<0.01$ *vs* control curve (open circles). (b) Typical experiment showing the relaxant effect induced by increasing concentrations of yohimbine in human corpus cavernosum preparations pre-contracted with ET-1 (0.1 μM) alone (upper tracing) and in the presence (middle tracing) of ODQ (1 μM) or sildenafil (0.3 μM, lower tracing). The effect of ACh (1 μM; control for a functional endothelium) is shown in each tracing. Arrows indicate the addition of the substances.

hypogonadism in the treated rabbits. Indeed, 2 months after GnRH administration we observed a significant decrease ($P<0.01$) in testosterone plasma levels, when compared to untreated or vehicle-treated rabbits. Weekly administration of testosterone enanthate (T, 30 mg/kg weekly, $n=3$) to hypogonadal rabbits restored testosterone plasma levels to values not statistically different from controls (Table 1). According to a previous study, chronic hypogonadism reduces the responsiveness to Phe, while testosterone administration restores it.[45] In untreated or vehicle-treated rabbits the percentage of maximal (KCl-induced) contraction obtained with Phe (100 μM) was 157.90 ± 12.70% and 150.20 ± 12.80%, respectively. Responsiveness to Phe was significantly reduced in the hypogonadal rabbits and completely restored in the T-treated hypogonadal rabbits (Table 2). The relaxant effect of yohimbine on ET-1 pre-contracted corpora cavernosa was also different, according to the different androgen exposure (Figure 9a). Hypogonadism significantly ($P<0.05$) shifted the yohimbine dose–response curve to the right ($IC_{50}=8.00\pm0.04$ μM, $n=4$, obtained in three animals), when compared to

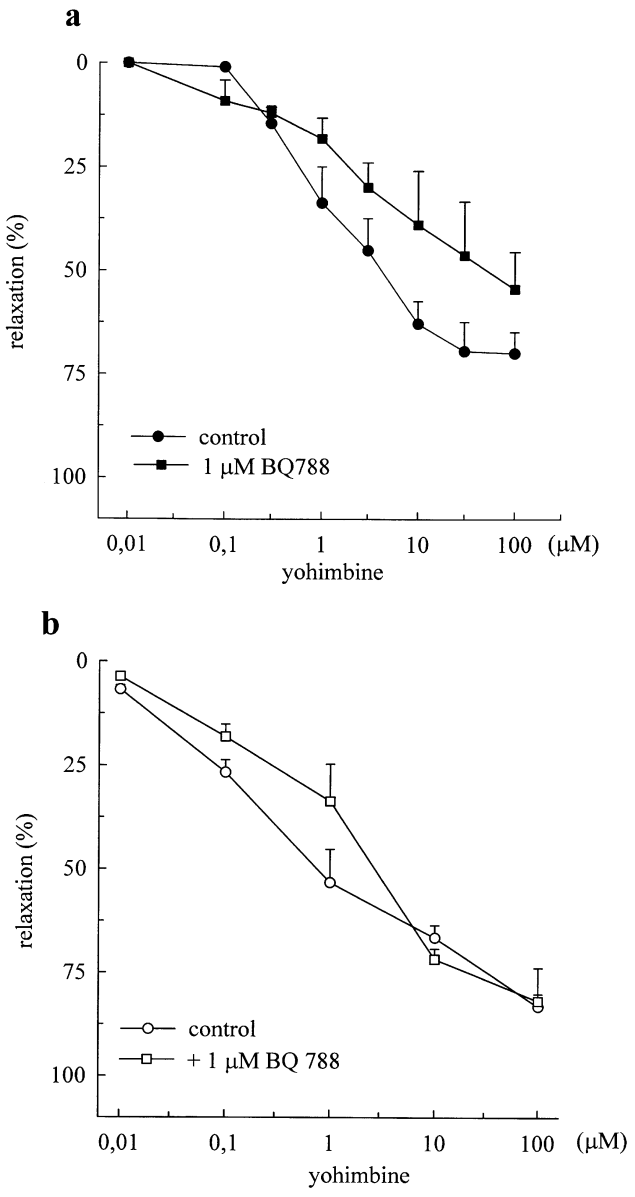

Figure 8 Relaxant effect of increasing concentrations of yohimbine in rabbit (panel a, closed symbols, $n=6$ in four separate experiments) and human (panel b, open symbols, $n=4$ in three separate experiments) corpora cavernosa pre-contracted by ET-1 (0.1 μM) alone (circles) or in presence of BQ788 (1 μM, squares) The increase in tone induced by ET-1 before addition of yohimbine was taken as 100% response and the effect of the drug was evaluated as per cent inhibition of this response. Means ± s.e.m.

dose–response curves obtained in control ($IC_{50}=1.20\pm0.04$ μM, $n=4$, obtained in three animals) or vehicle-treated ($IC_{50}=1.50\pm0.07$ μM, $n=4$, obtained in three animals) rabbits. T supplementation to hypogonadal rabbit not only restored the normal sensitivity of corpora cavernosa to the relaxant effect of yohimbine ($IC_{50}=2.00\pm0.05$ μM, $n=4$, obtained in three animals), but even increased its maximal effect. Indeed 100 μM of yohimbine induced a 75.00 ± 1.50% of relaxation in control and 73.60 ± 8.00% in vehicle-treated rabbits, while in T-treated hypogonadal rabbits the relaxation was almost maximal (96.00 ± 3.00%). All these differences were not present in endothelium-deprived corpora cavernosa or in L-NAME (100 μM) pre-treated preparations (Figure 9b and 9c, $n=4$, obtained in three animals).

Table 1 Testosterone plasma level in untreated and testosterone-replaced hypogonadal rabbits

(a) Control ($n=3$)	4.54 ± 0.90 nmol/l
(b) Sham-operated ($n=3$)	5.73 ± 1.40 nmol/l
(c) GnRH analog ($n=3$)	0.60 ± 0.10 nmol/l**
(d) GnRH analog plus testosterone enanthate ($n=3$)	2.30 ± 0.40 nmol/l

Blood for testosterone measurement was drawn after 2 months from a single administration of the long-acting GnRH analog triptorelin pamoate (2.9 mg/kg day 1, groups c and d) or vehicle (group b) and after one week from the last injection of testosterone enanathate (30 mg/kg per week from day 14, group (d). Triptorelin pamoate significantly reduced T plasma levels in group (c). Weekly injection with the long-lasting T ester (d), restored T plasma levels to values not significantly different from untreated (a) or vehicle-treated rabbits (b). $^{**}P<0.01$ versus (a), (b) and (d). n = number of animals.

Table 2 Phe response (% KCl response) in untreated and testosterone-replaced hypogonadal rabbits

(a) Control ($n=5$)	157.90 ± 12.70%
(b) Sham-operated ($n=5$)	150.20 ± 12.80%
(c) GnRH analog ($n=4$)	100.50 ± 15.30%*
(d) GnRH analog plus testosterone enanthate ($n=4$)	205.10 ± 20.80%

Experiments were performed after 2 months from a single administration of the long-acting GnRH analog triptorelin pamoate (2.9 mg/kg day 1, groups c and d) or vehicle (group b) and after one week from the last injection of testosterone enanthate (30 mg/kg per week from day 14, group d). Contractility studies were performed as described in Materials and methods. Values represents the mean ± s.e.m of phenylephrine (Phe, 100 μM)-induced contractility, expressed as percentage of maximal KCl (80 mM)-induced response. Triptorelin pamoate significantly reduced Phe responsiveness in group (c). Weekly injection with the long-lasting T ester (d), restored Phe response to values not significantly different from untreated (a) or vehicle-treated rabbits (b). $^{*}P<0.05$ versus (a), (b) and (d).

Discussion

In this study we demonstrated for the first time that micromolar concentrations of yohimbine relaxes rabbit and human corpora cavernosa in an endothelium-dependent and androgen-dependent way.

It is important to note that the concentrations of yohimbine able to relax human corpora cavernosa '*in vitro*' are rather similar to those measured '*in vivo*' in the plasma of human subjects assuming therapeutic doses of the yohimbine tablets.[2,46]

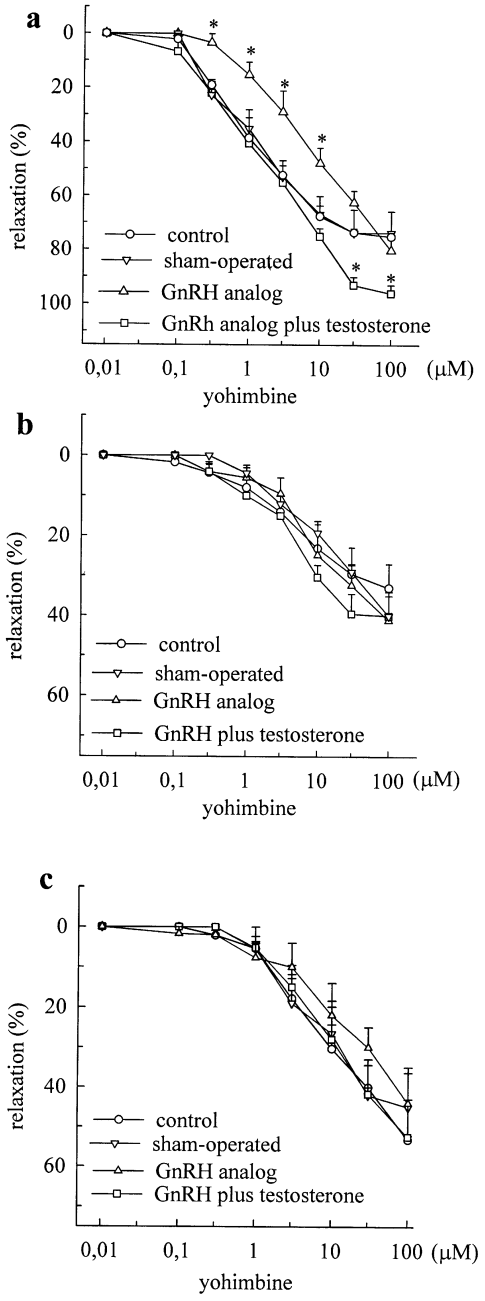

Figure 9 Relaxant effect of increasing concentrations of yohimbine in untreated (open circles), vehicle-treated (open down triangles) and hypogonadal rabbit with (open squares) or without (open triangles) weekly administration of testosterone enanthate in corpora cavernosa pre-contracted by ET-1 (0.1 μM, $n=4$ in three separate experiments). Preparations with an intact endothelium are shown in panel (a), while preparations without endothelium are in panel (b). Panel (c) shows results obtained in intact preparations pre-treated with 100 μM L-NAME. Hypogonadism induced a lower responsiveness to yohimbine only in preparation not treated with L-NAME and with an intact endothelium. In these preparations, responsiveness to yohimbine was completely restored and even increased in testosterone enanthate-treated rabbits. The increase in tone induced by ET-1 before addition of yohimbine was taken as 100% response and the effect of the drug was evaluated as per cent inhibition of this response. Means ± s.e.m. $*P<0.05$ and $**P<0.01$ *vs* control curve (closed circles).

Hence, it is possible that the relaxant effect we described might at least partially underlie the therapeutic action of yohimbine in erectile dysfunction. Up to now, there is a general agreement on the positive effect of yohimbine on ED, however its clinical use is currently indicated only for the management of the milder, non-organic form of the disease.[9–12] This assumption is essentially based on negative clinical trials employing yohimbine in patients with organic ED.[47,48] Our experimental results partially justify this assumption. In fact, we found that the relaxant effect of yohimbine is strongly decreased by a functional or mechanical disruption of the endothelium or by an inadequate androgenization. In these experimental conditions the relaxant dose–response curves for yohimbine are consistently shifted to the right or even abolished. Therefore it is possible that in pathological conditions characterised by a low testicular production of testosterone (hypogonadism) or by an impaired endothelial function (diabetes, hypertension and atherosclerosis) the concentrations of yohimbine needed to relax the smooth muscle cells of the erectile tissue are several fold higher than those achieved after oral intake of customary doses of the medication.

The relaxant effect of yohimbine in rabbit and human corpora cavernosa is not apparently due to antagonism at the α_2 adrenoceptor only. In fact yohimbine also blocks the contractions induced by non-adrenergic compounds as ET-1.[29–32] Since yohimbine does not interfere with ET-1-induced $[Ca^{2+}]_i$ increase in isolated corpora cavernosa cells, it seems unlikely that it acts as an ET-1 antagonist at receptor level. Hence, it is possible that yohimbine releases other relaxant factors able to counteract the effect of contractile agents such as ET-1. In this study we provided evidence that one of these factors corresponds to NO. In fact, removing the main source of NO (endothelium), decreasing NO formation (L-NAME) or action (ODQ), strongly impaired yohimbine induced relaxation of ET-1 pre-contracted corpora cavernosa. In addition, blocking degradation of cGMP by sildenafil (and therefore increasing NO signalling) substantially amplified yohimbine activity. All these findings seem to suggest an involvement of NO in yohimbine-induced relaxation. Finding that sildenafil strongly increases the yohimbine-induced corpora cavernosa relaxation might have clinical implications, suggesting the combined use of the two drugs in patients affected by ED. As far as we know, no clinical trials have been reported until now using yohimbine and sildenafil, although a combination of yohimbine with agents that enhance the release of NO should enhance its therapeutic effect on ED.[49]

The aforementioned hypothesis, that yohimbine allows penile erection not only by blocking α-adrenoceptors, but also by releasing NO from the endothelium, is also in agreement with

experimental results obtained in hypogonadal rabbits. We found that a chronic low androgen exposure decreased the responsiveness of corpora cavernosa to yohimbine and that this was completely counteracted by an adequate substitutive treatment with testosterone. Androgens have long been known to have a major stimulatory influence on male sexual behaviour and recently it has been demonstrated that they are essential in the maintenance of NO-mediated erectile activity.[35] In particular, androgens stimulate neuronal and endothelial NOS and increase the amount of NO produced by corpus cavernosum and penile arteries during erection.[35,50–52] Therefore, it is possible to speculate that the lower responsiveness of corpora cavernosa to yohimbine in hypogonadal rabbits is due to a lower formation of NO, and that the androgen substitution, repristinating NO activity, allows a normal NO-dependent yohimbine relaxant activity. In fact, we found no effect of androgen deprivation or substitution on yohimbine activity in corpora cavernosa in which the main source of NO, the endothelium, was removed or NO formation was impaired.

In conclusion, our study indicates that, beside its central effect, yohimbine may act directly at the level of corpora cavernosa facilitating their relaxation (and therefore penile erection) by a dual mechanism: blocking adrenergic receptors and releasing relaxing substances as NO. The lower relaxant effect of yohimbine in corpora cavernosa characterized by a damaged endothelium or by an inadequate androgenization might justify the lower efficacy of the drug in patients with some type of organic ED.

Acknowledgements

We thank Dr Clara Crescioli (Department of Clinical Physiopathology, Endocrinology Unit, University of Florence) for cell cultures, Dr Elisabetta Baldi (Department of Clinical Physiopathology, Andrology Unit, University of Florence) for technical assistance in calcium measurement and Dr Monica Muratori (Department of Clinical Physiopathology, Andrology Unit, University of Florence) for technical assistance in testosterone measurements.

References

1 Andersson KE, Wagner G. Physiology of penile erection. *Pharmacol Review* 1995; **75**: 191–236.
2 Maggi M *et al*. Erectile dysfunction: from biochemical pharmacology to advances in medical therapy. *Eur J Endocrinol* 2000; **143**: 143–154.
3 Ignarro LJ *et al*. Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem Biophys Res Comm* 1990; **170**: 843–850.
4 Boolell M, Gepi-Attee S, Gingell JC, Allen MJ. Sildenafil, a novel effective oral therapy for male erectile dysfunction. *Br J Urol* 1996; **78**: 257–261.
5 Traish AM, Kim NN, Goldstein I, Moreland RB. Alpha-adrenergic receptors in the penis: identification, characterization, and physiological function. *J Andr* 1999; **20**: 671–682.
6 Langez SZ *et al*. Role of alpha1-adrenoceptors in the future treatment of urological diseases. *Eur Urol* 1999; **36**: 66–67.
7 Becker AJ *et al*. Pharmacological therapy of erectile dysfunction. *Der Urol Ausg A* 1998; **37**: 503–508.
8 Hunner M. A practical treatise on disorders of the sexual function of the male and female. FA Davis: Philadelphia, 1926.
9 Webb DJ, Freestone S, Allen MJ, Muirhead GJ. Sildenafil citrate and blood-pressure-lowering drugs: result of drug interaction studies with an organic nitrate and a calcium antagonist. *Am J Cardiol* 1999; **83**: 21C–28C.
10 Morales A. Yohimbine in erectile dysfunction: the facts. *Int J Impot Res* 2000; **12**: S70–S74.
11 Bevilacqua K, Barad D, Youchah J, Witt B. Is affect associated with infertility treatment outcome? *Fertil Steril* 2000; **73**: 648–649.
12 Lue TF. Erectile dysfunction. *New Engl J Med* 2000; **342**: 1802–1813.
13 Ernst E, Pittler MH. Yohimbine for erectile dysfunction: a systematic review and meta-analysis of randomized clinical trials. *J Urol* 1998; **159**: 433–436.
14 Goldberg MR, Robertson D. Yohimbine: a pharmacological probe for study of the α_2 adrenoreceptor. *Pharmacol Rev* 1983; **35**: 143–180.
15 Riley AJ, Goodman RE, Kellet JM, Orr R. Double blind trial of yohimbine hydrochloride in the treatment of erection inadequacy. *J Sex Marital Ther* 1989; **4**: 17–26.
16 Sonda LP, Mazo R, Chancellor MB. The role of yohimbine for the treatment of erectile impotence. *J Sex Marital Ther* 1990; **1**: 15–21.
17 Riley AJ. Local pharmacotherapy for impotence. *Br J Clin Pract* 1993; **47**: 118–119.
18 Clark J, Smith E, Davidson J. Enhancement of sexual motivation in male rats by yohimbine. *Science* 1984; **225**: 847–848.
19 Sala M *et al*. Central effect of yohimbine on sexual behaviour in the rat. *Physiol Behav* 1990; **47**: 165–173.
20 Rodriguez-Manzo G. Yohimbine interacts with the dopaminergic system to reverse sexual satiation: further evidence for a role of sexual motivation in sexual exhaustion. *Eur J Pharm* 1999; **372**: 1–8.
21 Quintin L *et al*. Catecholamine metabolism in the rat locus coeruleus as studied by *in vivo* differential pulse voltammetry. III. Evidence for the existence of an alpha 2-adrenergic tonic inhibition in behaving rats. *Brain Res* 1986; **375**: 235–245.
22 Vogt HJ. Double-blind, placebo-controlled safety and efficacy trial with yohimbine hydrochloride in the treatment of nonorganic erectile dysfunction. *Int J Impot Res* 1997; **9**: 155–161.
23 Rowland DL, Kallan K, Slob AK. Yohimbine, erectile capacity, and sexual response in men. *Arch Sex Behav* 1997; **26**: 49–62.
24 Langer S. Presynaptic regulation of catecholamine release. *Biochem Pharmacol* 1974; **23**: 1793–1800.
25 Starke K, Taube HD, Browski E. Presynaptic receptor systems in catecholaminergic transmission. *Biochem Pharmacol* 1977; **26**: 259–268.
26 Hedlund H, Andersson KE. Comparison of the responses to drugs acting on adrenoreceptors and muscarinic receptors in human isolated corpus cavernosum and cavernous artery. *J Aut Pharm* 1985; **5**: 81–88.
27 Saenz De Tejada I *et al*. Regulation of adrenergic activity in penile corpus cavernosum. *J Urol* 1989; **142**: 1117–1121.
28 Christ GJ, Maayani S, Valcic M, Melman A. Pharmacological studies of human erectile tissue: characteristics of spontaneous contractions and alterations in α-adrenoceptor responsiveness with age and disease in isolated tissues. *Br J Pharmacol* 1990; **101**: 375–381.

29 Traish AM, Moreland RB, Huang YH, Goldstein I. Expression of functional alpha2-adrenergic receptor subtypes in human corpus cavernosum and in cultured trabecular smooth muscle cells. *Recept Signal Transduct* 1997; **7**: 55–67.
30 Gupta S *et al*. The expression of functional postsynaptic α_2-adrenoceptors in the corpus cavernosum smooth muscle. *Br J Pharmacol* 1998; **123**: 1237–1245.
31 Steers WD, Mcconnell J, Benson GS. Some pharmacological effects of yohimbine on human and rabbit penis. *J Urol* 1984; **131**: 799–802.
32 Saenz De Tejada I *et al*. Design and evaluation of nitrosylated α-adrenergic receptor antagonists as potential agents for the treatment of impotence. *J Pharmacol Exp Ther* 1999; **290**: 121–128.
33 Traish A *et al*. Phentolamine mesylate relaxes penile corpus cavernosum tissue by adrenergic and non-adrenergic mechanisms. *Int J Impot Res* 1998; **10**: 215–223.
34 Mills TM, Stopper VS, Reilly CM. Sites of androgenic regulation of cavernosal blood pressure during penile erection in the rat. *Int J Impot Res* 1996; **8**: 29–34.
35 Reilly CM, Zamorano P, Stopper VS, Mills TM. Androgenic regulation of NO availability in rat penile erection. *J Androl* 1997; **18**: 110–115.
36 Saenz De Tejada I *et al*. Cholinergic neurotrasmission in human corpus cavernosum. I. Responses of isolated tissue. *Am J Physiol* 1988; **254**: H459–467.
37 Itoh Y *et al*. Cloning and sequence analysis of cDNA enconding the precursor of a human endothelium-derived vasoconstrictor peptide, endothelin: identity of human and porcine endothelin. *FEBS Lett* 1988; **231**: 440–444.
38 Hosoda K *et al*. Cloning and expression of human endothelin-1 receptor cDNA. *FEBS Lett* 1991; **287**: 23–26.
39 Ogawa Y *et al*. Molecular cloning of a non-isopeptide selective human endothelin receptor. *Biochem Biophys Res Commun* 1991; **178**: 248–255.
40 Ercolani L, Florence B, Denaro M, Alexander M. Isolation and complete sequence of a functional human glyceraldehydes-3-phosphate dehydrogenase gene. *J Biol Chem* 1988; **263**: 15335–15341.
41 Maggi M *et al*. Platelet-activating factor mediates an autocrine proliferative loop in the endometrial adenocarcinoma cell line HEC-1A. *Cancer Res* 1994; **54**: 4777–4784.
42 Grynkiewicz G, Poenie M, Tsien RY. A generation of a Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; **260**: 3440–3450.
43 Motulsky HJ, Ransnas LA. Fitting curves to data using nonlinear regression: a practical and nonmathematical review. *FASEB J* 1987; **1**: 365–374.
44 Garthwaite J *et al*. Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol Pharmacol* 1995; **48**: 184–188.
45 Traish AM *et al*. Effects of castration and androgen replacement on erectile function in a rabbit model. *Endocrinology* 1999; **140**: 1861–1868.
46 Sturgill MG *et al*. Yohimbine elimination in normal volunteers is characterized by both one-and two-compartment behavior. *J Cardiovasc Pharmacol* 1997; **29**: 697–703.
47 Kunelius P, Hakkinen J, Lukkarinen O. Is high-dose yohimbine hydrochloride effective in the treatment of mixed-type impotence? A prospective, randomized, controlled double-blind crossover study. *Urology* 1997; **49**: 441–444.
48 Teloken C. Therapeutics effects of high dose yohimbine hydrochloride on organic erectile dysfunction. *J Urol* 1998; **159**: 122–124.
49 Tam SW, Worcel M, Wyllie M. Yohimbine: a clinical review. *Pharmacol Ther* 2001; **91**: 215–243.
50 Chamness SL *et al*. The effect of androgen on nitric oxide synthase in the male reproductive tract of the rat. *Fertil Steril* 1995; **63**: 1101–1107.
51 Marin R, Escrig A, Abreu P, Mas M. Androgen-dependent nitric oxide release in rat penis correlates with levels of constitutive nitric oxide synthase isoenzymes. *Biol Reprod* 1999; **61**: 1012–1016.
52 Lugg JA, Gonzalez-Cadavid NF, Rajfer J. The role of nitric oxide in erectile function. *J Androl* 1995; **16**: 2–4.