

# Calcium dobesilate potentiates endothelium-derived hyperpolarizing factor-mediated relaxation of human penile resistance arteries

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**1** We have evaluated the participation of endothelium-derived hyperpolarizing factor (EDHF) in the endothelium-dependent relaxation of isolated human penile resistance arteries (HPRA) and human corpus cavernosum (HCC) strips. In addition, the effect of the angioprotective agent, calcium dobesilate (DOBE), on the endothelium-dependent relaxation of these tissues was investigated.

**2** Combined inhibition of nitric oxide synthase (NOS) and cyclooxygenase (COX) nearly abolished the endothelium-dependent relaxation to acetylcholine (ACh) in HCC, while 60% relaxation of HPRA was observed under these conditions. Endothelium-dependent relaxation of HPRA resistant to NOS and COX inhibition was prevented by raising the extracellular concentration of K<sup>+</sup> (35 mM) or by blocking Ca<sub>v</sub><sup>2+</sup>-activated K<sup>+</sup> channels, with apamin (APA; 100 nM) and charybdotoxin (CTX; 100 nM), suggesting the involvement of EDHF in these responses.

**3** Endothelium-dependent relaxation to ACh was markedly enhanced by DOBE (10 μM) in HPRA but not in HCC. The potentiating effects of DOBE on ACh-induced responses in HPRA, remained after NOS and COX inhibition, were reduced by inhibition of cytochrome P450 oxygenase with miconazole (0.3 mM) and were abolished by high K<sup>+</sup> or a combination of APA and CTX.

**4** *In vivo*, DOBE (10 mg kg<sup>-1</sup> i.v.) significantly potentiated the erectile responses to cavernosal nerve stimulation in male rats.

**5** EDHF plays an important role in the endothelium-dependent relaxation of HPRA but not in HCC. DOBE significantly improves endothelium-dependent relaxation of HPRA mediated by EDHF and potentiates erectile responses *in vivo*. Thus, EDHF becomes a new therapeutic target for the treatment of erectile dysfunction (ED) and DOBE could be considered a candidate for oral therapy for ED.

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**Keywords:** Human corpus cavernosum; human penile resistance arteries; endothelium; endothelium-derived hyperpolarizing factor; calcium dobesilate

**Abbreviations:** APA, apamin; COX, cyclooxygenase; CTX, charybdotoxin; DOBE, calcium dobesilate; EDHF, endothelium-derived hyperpolarizing factor; HCC, human corpus cavernosum; HPRA, human penile resistance arteries; HPβCD, hydroxy-propyl-β-cyclodextrin; INDO, indomethacin; L-NNA, N<sup>G</sup>-nitro-L-arginine; NOS, nitric oxide synthase

## Introduction

The relaxation of arterial and trabecular penile smooth muscle is needed to achieve and maintain penile erection (Sáenz de Tejada *et al.*, 1991). Penile smooth muscle tone is under the control of relaxing and contractile mediators that are released from autonomic nerve terminals and the endothelia of corpus cavernosum lacunar spaces and penile arteries. The endothelium seems to play a crucial role in facilitating the erectile response. In fact, in aging and vascular diseases associated with endothelial dysfunction such as hypercholesterolemia, hypertension, and diabetes, a high prevalence of erectile dysfunction (ED) is observed (Martín-Morales *et al.*, 2001).

Several substances derived from the endothelium are involved in endothelium-dependent relaxation. Nitric oxide (NO) and prostacyclin (PGI<sub>2</sub>) were firstly identified as the main endothelial mediators participating in the local regulation of smooth muscle tone, but the existence of an additional unidentified endothelial factor that promotes smooth muscle hyperpolarization and is resistant to NO synthase (NOS) and cyclooxygenase (COX) inhibition has been clearly established (Garland *et al.*, 1995). The functional relevance of this endothelium-derived hyperpolarizing factor (EDHF) depends on the specific vascular bed, being more prevalent in small than in large arteries. EDHF has been reported to be an important contributor to vasodilation in human coronary arteries (Miura *et al.*, 1999) and resistance vessels (Coats *et al.*, 2001; Halcox *et al.*, 2001).

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Calcium dobesilate (DOBE) has been extensively used as an orally administered angioprotective agent, specially in the treatment of diabetic retinopathy (Berthet *et al.*, 1999). Although its mechanism of action is poorly understood, this compound has been shown to potentiate endothelium-dependent relaxation of rabbit aorta (Ruiz *et al.*, 1997) and aorta from diabetic rats and to improve endothelial function in diabetic patients (Berthet *et al.*, 1999; Tejerina *et al.*, 1999).

The aims of the present study were: firstly, to determine the possible contribution of EDHF to the regulation of human penile smooth muscle contractility. Secondly, to determine the effects of DOBE on endothelium-dependent relaxation of penile smooth muscle, as well as the effects of this agent on the erectile responses, *in vivo*, in a rat model.

## Methods

### Human penile tissue

Human corpus cavernosum (HCC) biopsies ( $n=50$ ) were obtained from men suffering from ED who gave informed consent at the time of penile prosthesis insertion. The causes of ED included failure of corporo-veno-occlusive mechanism, structural alterations, neurological dysfunction and vascular dysfunction. The study was approved by the local Ethics Committee. Tissues were maintained at 4–6°C in M-400 solution (composition per 100 ml: manitol, 4.19 g;  $\text{KH}_2\text{PO}_4$ , 0.205 g;  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , 0.97 g; KCl, 0.112 g;  $\text{NaHCO}_3$ , 0.084 g) until used, which was between 2 and 16 h from extraction. Penile resistance arteries and/or corpus cavernosum strips were obtained from these biopsies (Angulo *et al.*, 2002).

### Vascular reactivity of human penile resistance arteries (HPRA)

Human penile small arteries, helicine arteries (lumen diameter 150–400  $\mu\text{m}$ ), which are the terminal branches of deep penile arteries, were dissected by carefully removing the adhering trabecular tissue, and arterial ring segments (2 mm long) were subsequently mounted on two 40  $\mu\text{m}$  wires on microvascular double Halpern–Mulvany myographs (J.P. Trading, Aarhus, Denmark) for isometric tension recordings ( $n=79$  from 42 patients). The vessels were allowed to equilibrate for 30 min in physiological salt solution (PSS) of the following composition (mM): NaCl 119, KCl 4.6,  $\text{CaCl}_2$  1.5,  $\text{MgCl}_2$  1.2,  $\text{NaHCO}_3$  24.9, glucose 11,  $\text{KH}_2\text{PO}_4$  1.2, EDTA 0.027 at 37°C continuously bubbled with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  mixture to maintain a pH of 7.4. Passive tension and internal circumference of vascular segments, when relaxed *in situ* under a transmural pressure of 100 mmHg ( $L_{100}$ ), were determined. The arteries were then set to an internal circumference equivalent to 90% of  $L_{100}$ , at which the force development was close to maximal (Mulvany & Halpern, 1977). The preparations were then exposed to 125 mM  $\text{K}^+$  (KPSS, equimolar substitution of NaCl for KCl in PSS) and the contractile response was measured. The arteries were contracted with 1  $\mu\text{M}$  norepinephrine (80% of KPSS induced contraction approximately) and relaxation responses were evaluated by cumulative additions of compounds to the chambers. The arterial segments considered as lacking functional endothelium did not relax to 10  $\mu\text{M}$  acetylcholine.

### Experiments with HCC strips

Strips of corpus cavernosum tissue ( $3 \times 3 \times 7 \text{ mm}^3$ ) were immersed in 8 ml organ chambers containing PSS, maintained at 37°C and aerated with 5%  $\text{CO}_2$ /95%  $\text{O}_2$ , pH 7.4. ( $n=15$  from 12 patients). Each tissue strip was incrementally stretched to optimal isometric tension, as determined by maximal contractile response to 1  $\mu\text{M}$  phenylephrine (Kim *et al.*, 1991). Tissues were contracted with 0.5–3  $\mu\text{M}$  phenylephrine (80% of KPSS-induced contraction) and relaxation responses were evaluated by cumulative additions of compounds to the chambers.

### Evaluation of the effects of DOBE on endothelium-dependent relaxation of HCC and penile arteries

For evaluation of the effects of DOBE on endothelium-dependent relaxation, HPRA (from 36 patients) and corpus cavernosum (from 10 patients) were used. When a concentration–response curve to acetylcholine (ACh) was completed, tissues were washed and left to equilibrate. Then, DOBE (10  $\mu\text{M}$ ) was added and, after 30 min, tissues were recontracted and when a stable contraction was achieved, the responses to ACh were again evaluated. For evaluation of the effects of DOBE on EDHF-mediated responses, tissues that previously relaxed to ACh were treated with L-NNA (100  $\mu\text{M}$ ) and indomethacin (INDO) (5  $\mu\text{M}$ ) for 30 min and a concentration–response to ACh was evaluated. After a washout and equilibration period, tissues were cotreated with  $N^G$ -nitro-L-arginine (L-NNA), INDO and DOBE (10  $\mu\text{M}$ ) and the relaxant response to ACh was again evaluated. For evaluation of the effects of other treatments on the effects induced by DOBE on endothelium-dependent relaxation of human penile arteries, experiments were conducted in parallel. An arterial segment was treated with DOBE (10  $\mu\text{M}$ ) alone, while another arterial segment from the same patient was treated with DOBE plus potassium (35 mM), DOBE plus L-NNA (100  $\mu\text{M}$ ) plus INDO (5  $\mu\text{M}$ ) plus apamin (APA) (0.1  $\mu\text{M}$ ) plus charybdotoxin (0.1  $\mu\text{M}$ ) or DOBE plus L-NNA plus INDO plus miconazole (0.3 mM).

### Erectile responses to cavernosal nerve stimulation in anesthetized rats

Male Sprague–Dawley rats ( $n=8$ ) were anesthetized with ketamine (60 mg  $\text{kg}^{-1}$ ). The surgical procedure consisted of dissection and isolation of the right cavernous nerve through an abdominal midline incision and exposure of penile crura through a transverse perineal incision. Intracavernosal pressure (ICP) measurements were accomplished by insertion into the right crus of a 23-gauge needle connected to a disposable pressure transducer (Abbott, Sligo, Ireland) and a data-acquisition system (ADInstruments, Castle Hill, Australia). Left carotid artery and right external jugular vein were catheterized for constant blood pressure measurement and saline or drug infusion, respectively. Electrical stimulation was applied by a delicate platinum bipolar hook electrode connected to a stimulator and current amplifier (Cibertec, Madrid, Spain). Parameters of electrical stimulation consisted of pulses with a duration of 1 ms and 1.5 mA of current intensity for 1 min. Frequency–response curves were per-

formed by applying stimulation at 1, 3 and 10 Hz at 3 min intervals.

For evaluation of acute effects of DOBE on erectile responses, a control stimulation at 1, 3 and 10 Hz was performed and, after a stabilization period, DOBE ( $10 \text{ mg kg}^{-1}$ ) dissolved in 20% hydroxy-propyl- $\beta$ -cyclodextrin (HP $\beta$ CD) ( $n = 4$ ) or the vehicle alone ( $n = 4$ ) was intravenously (i.v.) administered. The stimulation was repeated at 60 min after the administration of DOBE or vehicle. The 1 h period from administration of DOBE for the evaluation of the erectile responses was chosen based on results obtained in pilot studies, testing responses at 30 and at 60 min post-treatment. Although there were effects at 30 min after administration, these were partial, while at 60 min, the effects were always present.

### Drugs and materials

Phenylephrine, norepinephrine (arterenol), ACh, INDO, L-NNA, APA, CTX and HP $\beta$ CD were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Miconazole was obtained from RBI (Natick, MA, U.S.A.). DOBE (calcium dihydroxy-2,5 benzenesulfonate, Doxium<sup>®</sup>) was provided by Dr Esteve Laboratories (Barcelona, Spain).

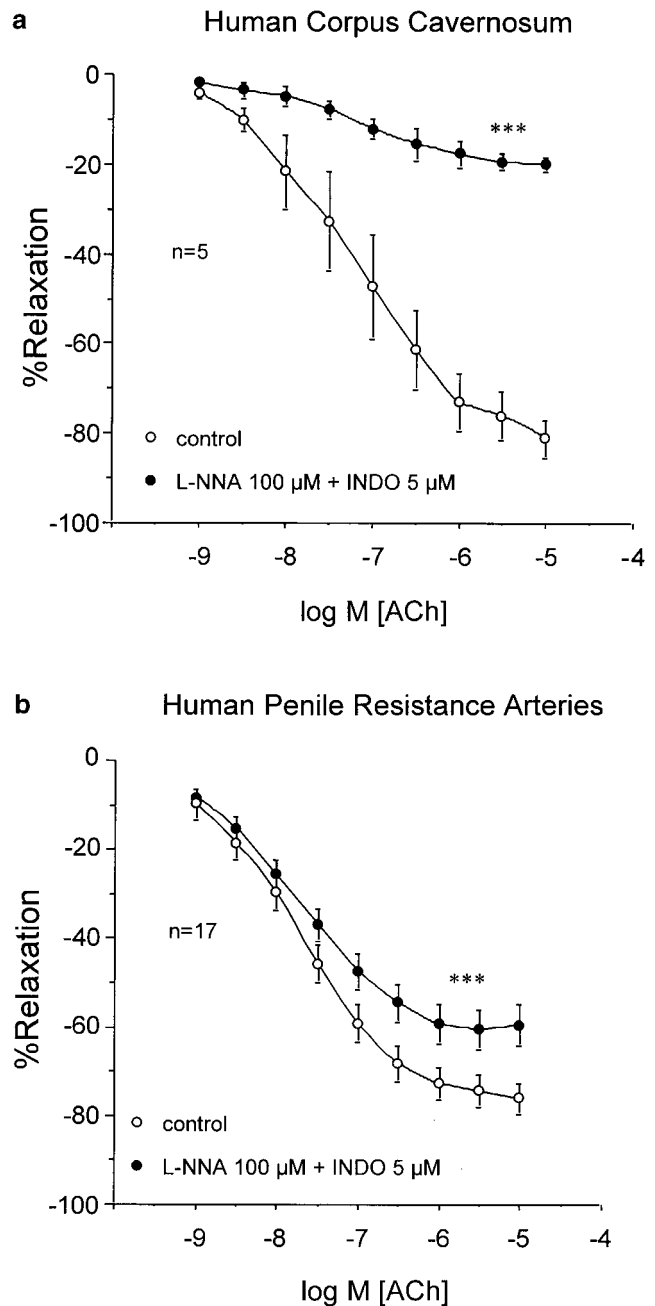
### Data analysis

Relaxation responses are expressed as percentage of total relaxation (loss in tone) induced by the addition of 0.1 mM papaverine HCl to the chambers at the end of the experiment. All data are expressed as mean  $\pm$  s.e.m. Complete concentration–response or frequency–response curves were obtained and compared by a two-factor analysis of variance (ANOVA) statistical test using StatView software for Apple computers. Erectile responses to rat cavernosal nerve stimulation were determined by measuring the peak of ICP increase normalized by mean arterial pressure (MAP) values, the duration of the response and the area under the curve (AUC) of the ICP increases normalized by MAP values. The complete frequency–response curves were compared by a two-factor ANOVA test.

## Results

### Role of EDHF in endothelium-dependent relaxation of HCC trabecular strips and penile resistance arteries

In strips of trabecular tissue ( $n = 5$ ), ACh; ( $1 \text{ nM}–10 \mu\text{M}$ ) produced concentration-dependent relaxation, which was nearly abolished after combined treatment with the NOS inhibitor, L-NNA ( $100 \mu\text{M}$ ) and the COX inhibitor, INDO ( $5 \mu\text{M}$ ) (Figure 1a). Conversely, in penile arteries ( $n = 17$ ), although the treatment with L-NNA ( $100 \mu\text{M}$ ) and INDO ( $5 \mu\text{M}$ ) significantly reduced ACh-induced relaxation, ACh produced approximately 60% relaxation of these arteries after NOS and COX inhibition (Figure 1b). The ACh-induced relaxations, in the presence of NOS and COX inhibition, were abolished by contracting the HPRA ( $n = 4$ ) with a high extracellular  $\text{K}^+$  concentration (35 mM) or by treating the arteries ( $n = 4$ ) with a combination of blockers of  $\text{Ca}^{2+}$ -

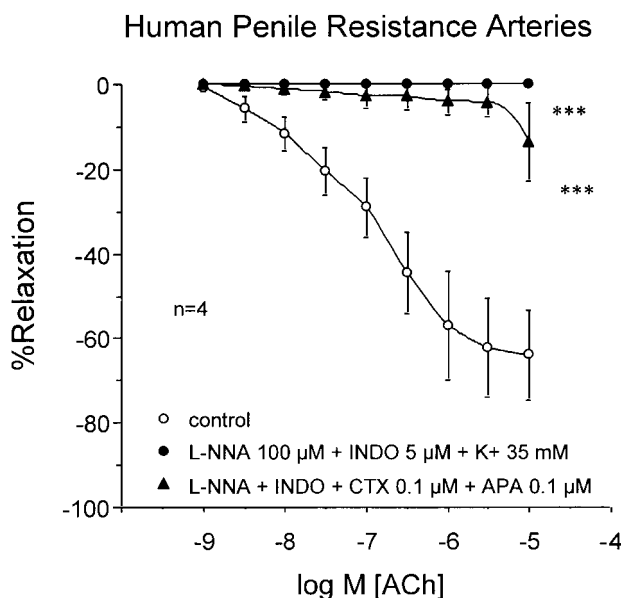


**Figure 1** Effects of combined inhibition of NOS and COX with L-NNA; ( $100 \mu\text{M}$ ) and INDO ( $5 \mu\text{M}$ ), respectively, on the endothelium-dependent relaxation induced by ACh ( $1 \text{ nM}–10 \mu\text{M}$ ) on phenylephrine-contracted HCC strips (a) and norepinephrine-contracted HPRA (b). Data are expressed as mean  $\pm$  s.e.m. of the percentage of total relaxation induced by 0.1 mM papaverine.  $n$  Indicates the number of patients from whom the tissues were collected for the experiments. \*\*\*Indicates  $P < 0.005$  vs control responses by a two-factor ANOVA test.

dependent  $\text{K}^+$ -channel blockers, APA; ( $100 \text{ nM}$ ) and CTX ( $100 \text{ nM}$ ) (Figure 2).

### Effects of DOBE on endothelium-dependent relaxation of human penile smooth muscle

Endothelium-dependent relaxation of HCC trabecular strips induced by ACh was not modified by treatment with DOBE



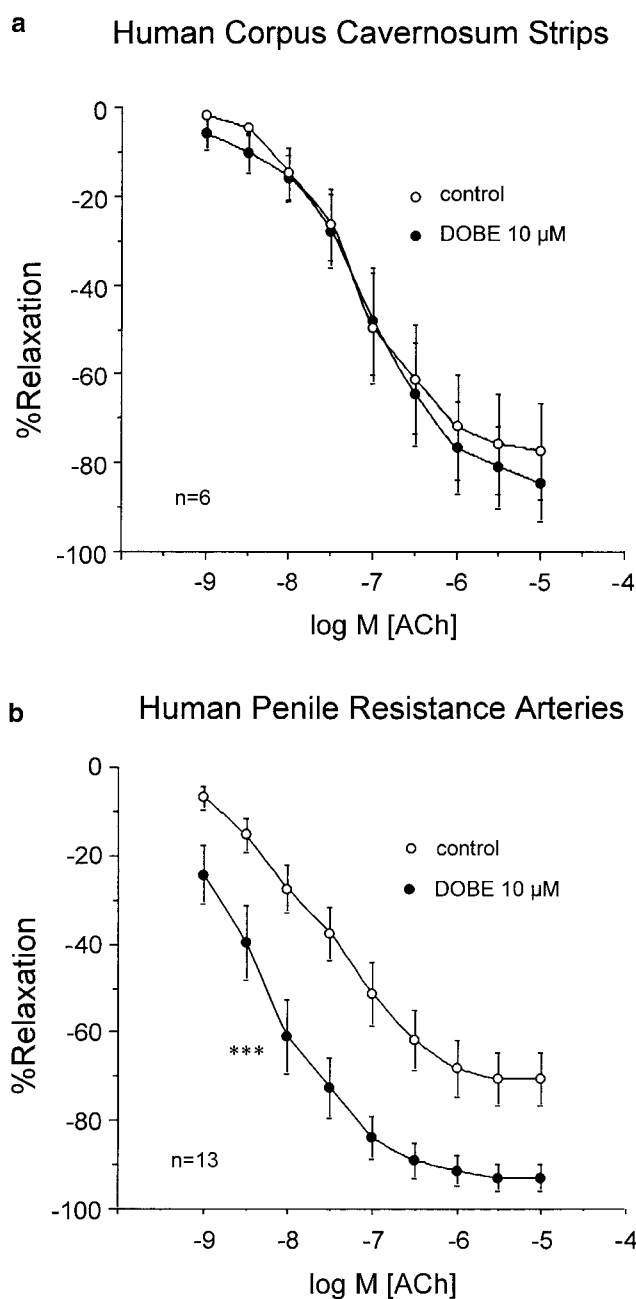
**Figure 2** Effects of high extracellular  $K^+$  concentration (35 mM) and  $Ca_2^+$ -activated  $K^+$ -channel blockade with APA; (100 nM) and CTX; (100 nM) in addition to combined inhibition of NOS and COX with L-NNA; (100  $\mu$ M) and INDO (5  $\mu$ M), respectively, on the endothelium-dependent relaxation induced by ACh (1 nM–10  $\mu$ M) on norepinephrine-contracted HPRA. Data are expressed as mean  $\pm$  s.e.m. of the percentage of total relaxation induced by 0.1 mM papaverine.  $n$  Indicates the number of patients from whom the tissues were collected for the experiments. \*\*\*Indicates  $P < 0.005$  vs control responses by a two-factor ANOVA test.

(10  $\mu$ M) in the presence ( $n = 4$ ) or absence ( $n = 6$ ) of combined NOS and COX inhibition (Figures 3a and 4a). On the contrary, DOBE (10  $\mu$ M) markedly potentiated ACh-induced relaxation of HPRA in the absence ( $n = 13$ ) (Figure 3b) or in the presence ( $n = 10$ ) (Figure 4b) of combined NOS and COX inhibition. Furthermore, in arteries treated with DOBE, combined treatment with L-NNA (100  $\mu$ M) and INDO (5  $\mu$ M) did not significantly affect ACh-induced relaxations (Figure 4c). However, when the arteries ( $n = 7$ ) were exposed to a high potassium concentration (35 mM), DOBE (10  $\mu$ M) had no effect on ACh-induced relaxations (Figure 5).

Exposure of HPRA ( $n = 5$ ) to a combination of APA (100 nM) and CTX (100 nM) that blocked ACh-induced relaxation resistant to NOS and COX inhibition, abolished the potentiating effects of DOBE (Figure 6). Finally, miconazole (0.3 mM) significantly reduced the potentiating effects of DOBE of ACh-induced relaxation of HPRA ( $n = 5$ ) resistant to NOS and COX inhibition (Figure 7).

#### Effects of DOBE on erectile responses in anesthetized rats

Electrical stimulation of the cavernosal nerve in anesthetized rats produced frequency-dependent ICP increases, which were not modified by the treatment with vehicle (20% HP $\beta$ CD) ( $n = 4$ ). I.v. administration of DOBE (10 mg kg $^{-1}$ ) ( $n = 4$ ) produced a rapid and transitory increase in systemic blood pressure (15–20 mmHg), which recovered to basal levels after 4–6 min. Treatment with DOBE significantly enhanced the erectile responses to cavernosal nerve stimulation (Figure 8). Statistically significant potentiating effects by DOBE were

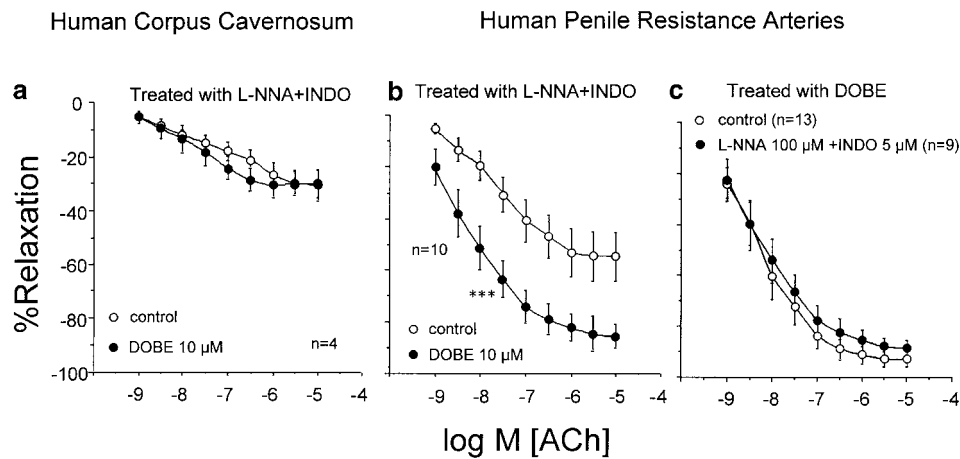


**Figure 3** Effects of the treatment with DOBE (10  $\mu$ M) on the endothelium-dependent relaxation induced by ACh (1 nM–10  $\mu$ M) on phenylephrine-contracted HCC strips (a) and norepinephrine-contracted HPRA (b). Data are expressed as mean  $\pm$  s.e.m. of the percentage of total relaxation induced by 0.1 mM papaverine.  $n$  Indicates the number of patients from whom the tissues were collected for the experiments. \*\*\*Indicates  $P < 0.005$  vs control responses by a two-factor ANOVA test.

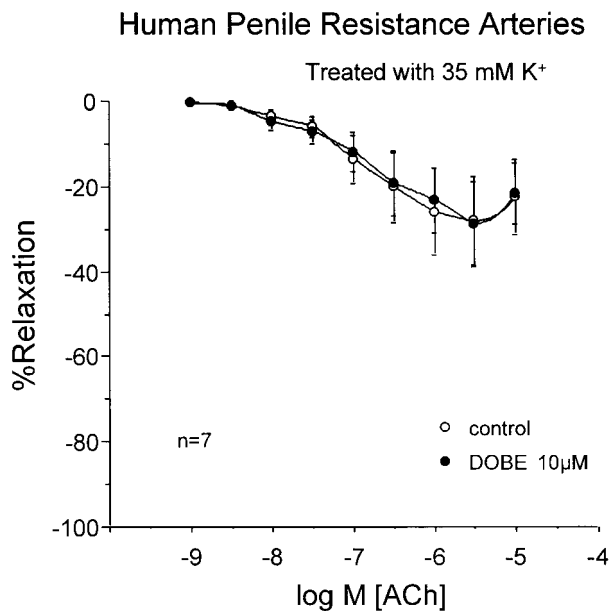
observed when erectile responses were expressed as the peak of ICP increase (Figure 8a), the duration of the response (Figure 8b) or the AUC of the ICP increase (Figure 8c).

## Discussion

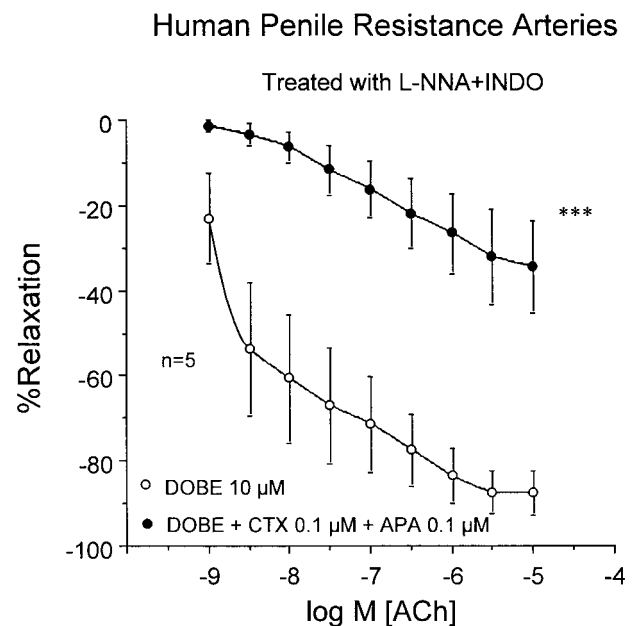
Preserved endothelial function seems to be fundamental for penile erection. Indeed, diseases in which endothelial dysfunc-



**Figure 4** Effects induced by DOBE (10 μM) on the endothelium-dependent relaxation induced by ACh (1 nM–10 μM) on phenylephrine-contracted HCC strips (a) and norepinephrine-contracted HPRA (b) after combined inhibition of NOS and COX with L-NNA (100 μM) and INDO (5 μM), respectively. (c) shows the comparison of ACh-induced responses in HPRA treated with DOBE (10 μM) in the presence or the absence of L-NNA (100 μM) plus INDO (5 μM). Data are expressed as mean ± s.e.m. of the percentage of total relaxation induced by 0.1 mM papaverine. *n* Indicates the number of patients from whom the tissues were collected for the experiments. \*\*\*Indicates *P* < 0.005 vs control curve by a two-factor ANOVA test.



**Figure 5** Effects induced by DOBE (10 μM) on the endothelium-dependent relaxation induced by ACh (1 nM–10 μM) on norepinephrine-contracted HPRA exposed to high extracellular K<sup>+</sup> concentration (35 mM). Data are expressed as mean ± s.e.m. of the percentage of total relaxation induced by 0.1 mM papaverine. *n* Indicates the number of patients from whom the tissues were collected for the experiments.

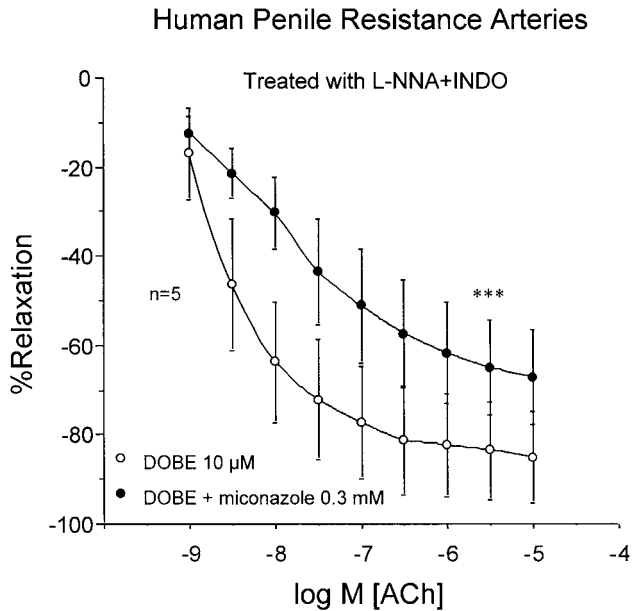


**Figure 6** Effects of Ca<sup>2+</sup>-activated K<sup>+</sup>-channel blockade with APA (100 nM) and CTX (100 nM) on the effects induced by DOBE (10 μM) on the endothelium-dependent relaxation induced by ACh (1 nM–10 μM) on norepinephrine-contracted HPRA in the presence of L-NNA (100 μM) plus INDO (5 μM). Data are expressed as mean ± s.e.m. of the percentage of total relaxation induced by 0.1 mM papaverine. *n* Indicates the number of patients from whom the tissues were collected for the experiments. \*\*\*Indicates *P* < 0.005 vs DOBE alone by a two-factor ANOVA test.

tion develops, such as in diabetes mellitus, hypertension, hypercholesterolemia or aging, are associated with a high prevalence of ED (Martin-Morales *et al.*, 2001). In animal models of these diseases, impaired endothelium-dependent relaxation of corpus cavernosum (Azadzi and Sáenz de Tejada (1991); Haas *et al.*, 1998; Keegan *et al.*, 2000) and reduced *in vivo* erectile responses (Azadzi *et al.*, 1996; Champion *et al.*, 1999; Escrig *et al.*, 2002) have been

demonstrated. Further evidence of the relevance of the endothelium in penile erection is that mice lacking the neuronal NO synthase (nNOS) gene have normal erectile function, possibly due to the overexpression of endothelial NOS (eNOS) (Burnett *et al.*, 1996), although the existence of splice variant genes or residual activity of the gene for nNOS

could also account for erectile function in these animals. In addition, the transfection in corpus cavernosum of aged rats of the eNOS gene reversed the ED observed in these animals (Champion *et al.*, 1999)

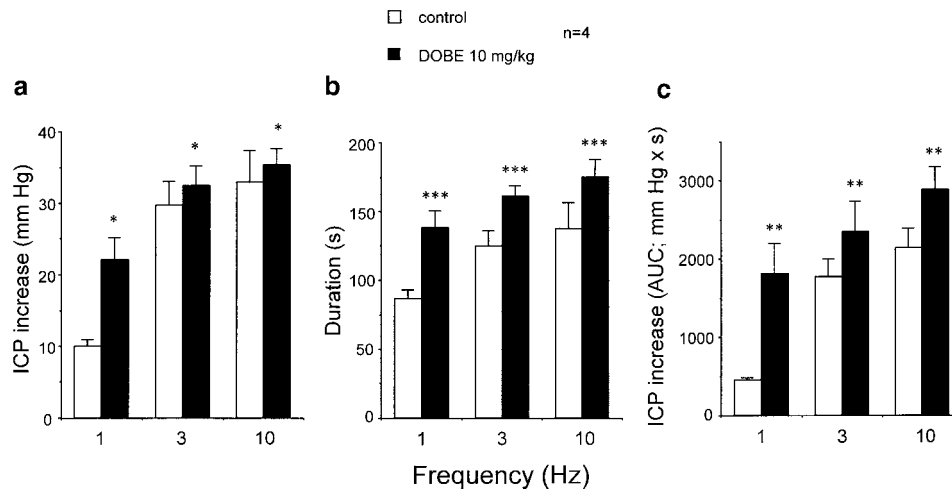


**Figure 7** Influence of cytochrome *P450* oxygenase inhibition with miconazole (0.3 mM) on the effects induced by DOBE (10  $\mu$ M) on the endothelium-dependent relaxation induced by ACh (1 nM–10  $\mu$ M) on norepinephrine-contracted HPRA in the presence of L-NNA (100  $\mu$ M) plus INDO (5  $\mu$ M). Data are expressed as mean  $\pm$  s.e.m. of the percentage of total relaxation induced by 0.1 mM papaverine. *n* Indicates the number of patients from whom the tissues were collected for the experiments. \*\*\*Indicates  $P < 0.005$  vs DOBE alone by a two-factors ANOVA test.

NO is a key mediator of endothelium-dependent relaxation (Moncada *et al.*, 1987), but it is not the only relaxant factor derived from the endothelium. It is well known that, in response to various stimuli, the human endothelium is able to produce prostacyclin (Osanai *et al.*, 2000; Yamasaki *et al.*, 2000), which, in turn, has the ability to relax a large number of vascular beds, including penile arteries (Angulo *et al.*, 2002). Furthermore, the endothelium-dependent relaxation of some human resistance arteries has a component resistant to the inhibition of NO and prostacyclin production, which is attributed to EDHF (Miura *et al.*, 1999; Coats *et al.*, 2001). In horse penile arteries, the existence of a hyperpolarizing factor contributing to endothelium-dependent dilation has been demonstrated (Prieto *et al.*, 1998). The results of the present study show that a factor resistant to NOS and COX inhibition, likely EDHF, participates in endothelium-dependent relaxation of HPRA but not in trabecular tissue. The involvement of EDHF in the endothelium-dependent relaxation of HPRA is strongly suggested by the fact that consistent relaxation to ACh remains after NOS and COX inhibition that is abolished by a high extracellular  $K^+$  concentration that prevents hyperpolarization of smooth muscle and the endothelium. Another characteristic feature of NOS and COX inhibition-resistant responses attributed to EDHF and present in HPRA is that relaxation is blocked by a combination of APA and CTX. These toxins most probably act on small ( $SK_{(Ca)}$ ), intermediate ( $IK_{(Ca)}$ ) and possibly large conductance ( $BK_{(Ca)}$ )  $Ca^{2+}$ -activated  $K^+$  channels ( $K_{(Ca)}$ ), localized in the endothelium (Busse *et al.*, 2002). Agonist-induced (e.g. ACh) hyperpolarization of the endothelium, apparently an essential step in the production/action of EDHF, is prevented by the combined action of APA (blocking  $SK_{(Ca)}$ ) and CTX (blocking  $IK_{(Ca)}$  and  $BK_{(Ca)}$ ).

Here we also report that DOBE is able to improve endothelium-dependent relaxation of HPRA, while not affecting these responses in corpus cavernosum tissue. This

#### Erectile responses to cavernosal nerve electrical stimulation



**Figure 8** Effects of i.v. administration of DOBE (10 mg  $kg^{-1}$ ) on erectile responses to cavernosal nerve electrical stimulation (CNES) in anesthetized male rats. Data are expressed as the mean  $\pm$  s.e.m. of the peak of ICP increase to CNES normalized by MAP values (a), the duration of the response to CNES (b) and the AUC (mmHg s) of ICP increase to CNES normalized by MAP values (c). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$  vs control frequency-response curve by a two-factors ANOVA test.

observation suggests that the mechanisms of endothelium-dependent relaxation differ in arterial and corpus cavernosum trabecular tissues. Functional differences between penile arterial and trabecular tissues have been reported affecting the receptor profile and hence the responses to vasoactive drugs (Hedlund & Andersson, 1985; Angulo *et al.*, 2002).

Dobesilate has been reported to reduce capillary permeability, to inhibit platelet aggregation and to reduce blood viscosity (Berthet *et al.*, 1999). In addition, DOBE is able to potentiate endothelium-dependent relaxation in the aorta of rabbit (Ruiz *et al.*, 1997) and diabetic rats (Tejerina *et al.*, 1999). DOBE has been reported to enhance endothelial NOS activity (Suschek *et al.*, 1997), but it is unlikely that this mechanism is involved in the effects of DOBE in human penile arteries, since DOBE was active despite NOS inhibition with L-NNA. In addition, it has been reported that DOBE has antioxidant capacity *in vitro* (Brunet *et al.*, 1998b) and *in vivo* (Brunet *et al.*, 1998a), which could be related to its inhibitory effects on aldose reductase activity (Schmidt & Michal, 1989). Since oxidative stress could affect endothelial function, this capacity of DOBE could be relevant to its effects on endothelium-dependent relaxation.

We propose that DOBE enhances the release, the availability or the action of EDHF. This proposal is based on three facts: first, DOBE is able to potentiate ACh-induced responses when NOS and COX are both inhibited; second, if the concentration of potassium ions is increased, precluding the hyperpolarization of the tissue, the potentiating effect of DOBE disappears; and third,  $K_{(Ca)}$  channels are required for the action of DOBE. This mechanism of action proposed for DOBE would explain the differential effects exerted by the compound on human penile arterial and corpus cavernosum trabecular tissues, because the participation of EDHF in endothelium-dependent relaxation of corpus cavernosum seems to be, at most, marginal.

The nature of EDHF remains unclear, although several candidates have been proposed. The ion potassium induces hyperpolarization and relaxation of smooth muscle and has been proposed as a putative EDHF in rat arteries (Edwards *et al.*, 1998; Dora & Garland, 2001). Hydrogen peroxide ( $H_2O_2$ ) has also been suggested to be an EDHF in human arteries (Matoba *et al.*, 2001). However, prevention of  $H_2O_2$  formation with catalase ( $1000\text{ U ml}^{-1}$ ) did not affect endothelium-dependent relaxation of HPRA resistant to NOS and COX inhibition and catalase did not modify the effect of DOBE in our preparations (data not shown). Finally, endothelial cytochrome P450 oxygenase (CYP) activity has been considered to be responsible for EDHF production in different human vascular beds (Miura *et al.*, 1999; Coats *et al.*, 2001; Halcox *et al.*, 2001). Epoxyeicosatrienoic acids (EET), derived from arachidonic acid through CYP activity, act as the EDHF in bovine coronary arteries (Campbell *et al.*, 1996). These compounds elicit hyperpolarization and relaxation of vascular smooth muscle with activation of  $K_{(Ca)}$  channels (Zhang *et al.*, 2001). In human penile arteries, the treatment with miconazole, which nonselectively inhibits the CYP activities (Rodrigues *et al.*, 1987; Ballard *et al.*, 1988), significantly attenuated the effect of DOBE on EDHF-mediated relaxations, suggesting that the effect of this agent is related to the enhancement of the production or the action of CYP derivatives. However, while miconazole significantly

reduced, it did not abolish the DOBE-induced enhancement of the responses attributed to EDHF in our preparations. This could be due to the existence of a pool of EETs associated with the membrane phospholipids that are released independently of CYP activity (Weintraub *et al.*, 1997). Nevertheless, we cannot exclude the coexistence of more than one factor, possibly of different chemical nature, acting as EDHF in human penile arteries. To our knowledge, DOBE is the first agent proposed to acutely potentiate EDHF-mediated relaxation of human arteries.  $\beta$ -naphthoflavone and the calcium antagonist, nifedipine, have been reported to potentiate EDHF-mediated responses by increasing expression of the CYP2C enzyme in bovine coronary arteries (Fisslthaler *et al.*, 2000). This is a mechanism that requires time; thus, it is unlikely that the acute effects of DOBE in human penile arteries were mediated by augmenting CYP2C expression.

Finally, the enhancement by DOBE of *in vivo* erectile responses in the rat confers additional relevance to the *in vitro* effects of this compound on human penile arteries. Potentiation of erectile responses by DOBE affected the amplitude and the duration of the response, and was specially marked at the lowest frequency used (1 Hz). This is not surprising since we are using healthy rats that are supposed to have normal erectile function, and the treatment with DOBE facilitates near to maximal responses at low frequencies, lowering the stimulation threshold. Similar finding has been reported with phosphodiesterase 5 inhibition (Gemalmaz *et al.*, 2001). Furthermore, the fact that an enhancement of penile arterial relaxation could improve erections provides the rationale to study the effects of therapeutic candidates for ED on penile arteries, and not only in corpus cavernosum tissue. It is important to note that the concentration of DOBE that we used for *in vitro* experiments is in the range of the plasma levels achieved after the oral dose of 500 mg ( $\sim 20\ \mu\text{M}$ ) (Benakis *et al.*, 1974), although higher doses of DOBE are commonly administered (Berthet *et al.*, 1999). In addition, the dose of DOBE used in the *in vivo* experiments is consistent with those used by other investigators in the rat model (Brunet *et al.*, 1998a).

In conclusion, EDHF plays an important role in the endothelium-dependent relaxation of HPRA. DOBE is able to clearly potentiate endothelium-dependent relaxation of HPRA likely by enhancing the production or the action of EDHF in these vessels. The effect of dobesilate results in enhanced erectile responses, as observed in the rat model. These observations make EDHF a possible therapeutic target for the treatment of ED, and DOBE a candidate for the treatment of ED. The use of DOBE as an oral therapeutic agent for the treatment of other diseases allows us to hypothesize its oral use for ED. Although the acute effects of DOBE observed in this study would lead us to speculate with an on-demand administration, further research is needed to support this speculation or to evaluate possible additional benefits of chronic administration.

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