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Activation and potentiation of the NO/cGMP pathway by N^G-hydroxyl-L-arginine in rabbit corpus cavernosum under normoxic and hypoxic conditions and ageing

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1 When nitric oxide synthase (NOS) produces NO from N^G-hydroxy-L-arginine (OH-arginine) instead of L-arginine, the total requirement of molecular oxygen and NADPH to form NO is reduced. The aim of this work was to evaluate the effects of OH-arginine on the contractility of rabbit corpus cavernosum (RCC) and to compare the capacities of L-arginine and OH-arginine to enhance NO-mediated responses under normoxic and hypoxic conditions and in ageing, as models of defective NO production.

2 OH-arginine, but not L-arginine, was able to relax phenylephrine-contracted rabbit trabecular smooth muscle. OH-arginine-induced relaxation was inhibited by the NOS-inhibitor, L-NNA (300 μM), and by the guanylyl cyclase inhibitor, ODQ (20 μM), while it was not affected by the cytochrome P450 oxygenase inhibitor, miconazole (0.1 mM). Administration of OH-arginine, but not L-arginine, produced a significant increment of cGMP accumulation in RCC tissue.

3 Relaxation elicited by OH-arginine (300 μM) was still observed at low oxygen tension. The increase of cGMP levels induced by ACh (30 μM) in RCC was significantly enhanced by addition of OH-arginine (300 μM) in normoxic conditions, as well as under hypoxia, while L-arginine did not alter the effects of ACh on cGMP accumulation.

4 Endothelium-dependent and nitrergic nerve-mediated relaxations were both significantly reduced in RCC from aged animals (>20-months-old) when compared with young adult rabbits (5-months-old). Treatment with OH-arginine (300 μM) significantly potentiated endothelium-dependent and neurogenic relaxation in corpus cavernosum from aged rabbits, while L-arginine (300 μM) did not have significant effects.

5 Results show that OH-arginine promotes NO-mediated relaxation of RCC and potentiates the NO-mediated responses induced by stimulation of endogenous NO generation in hypoxic and aged tissues. We propose that the use of OH-arginine could be of interest in the treatment of erectile dysfunction, at least in those secondary to defective NO production.
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Keywords: Nitric oxide; N^G-hydroxy-L-arginine; corpus cavernosum; hypoxia; ageing

Abbreviations: ACh, acetylcholine; cGMP, cyclic guanosin-monophosphate; NOS, nitric oxide synthase, OH-arginine, N^G-hydroxy-L-arginine

Introduction

Nitric oxide (NO) plays a fundamental role in the relaxation of human penile smooth muscle (Kim *et al*., 1991; Rajfer *et al*., 1992) and penile erection (Burnett *et al*., 1992; Trigo-Rocha *et al*., 1993). Nitric oxide is released by nitrergic nerves within the trabecular and penile arterial tissues as well as the endothelia that line the lacunar spaces and the intima of penile arteries (Kim *et al*., 1991), exerting its relaxing action on corpus cavernosum and penile arteries by activating smooth muscle soluble guanylyl cyclase and increasing intracellular concentration of cGMP (Kim *et al*., 1991; Burnett, 1995).

The enzymatic activity responsible for NO generation is NO synthase, which uses L-arginine as substrate, promoting its oxidation, with NADPH and oxygen consumption, and yielding L-citrulline and NO (Palmer & Moncada, 1989). Although, theoretically, L-arginine concentration is not a limiting step in NO formation, administration of high doses of L-arginine has been shown to improve NO-mediated relaxations in diseases involving impairment of endothelial relaxations, such as diabetes (Pieper & Dondlinger, 1997; Angulo *et al*., 1998). This fact supports the rationale for the utilization of NO synthesis precursors for the treatment of erectile dysfunction. Long-term oral administration of L-arginine was reported to enhance endothelium-dependent relaxation of corpus cavernosum from diabetic rabbits (Yildirim *et al*., 1999) and to improve erectile responses in aged rats (Moody *et al*., 1997). Indeed, L-arginine has been tested for the treatment of impotence, obtaining discretely encouraging results (Zorgniotti & Lizza, 1994). An intermediate product in the synthesis of NO from L-arginine is

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N^G-hydroxy-L-arginine (OH-arginine) which carries an atom of oxygen bonded to the guanidine group of L-arginine, forming a hydroxyl group (Figure 1). When starting from OH-arginine, NOS requires a lesser amount of oxygen and NADPH to produce NO. Thus, NO generation from OH-arginine could be faster and more effective under low oxygen tension conditions. This fact is very relevant with respect to erection, since low oxygen tension is present in corpus cavernosum in the flaccid state and the activation of NOS for penile smooth muscle relaxation requires the presence of oxygen.

The aims of this work were to analyse the effects of OH-arginine on contractility of rabbit trabecular smooth muscle and to evaluate the ability of L-arginine and OH-arginine to improve the NO-mediated responses in this tissue under normoxic and hypoxic conditions and in corpus cavernosum from aged rabbits.

Methods

Animals

Studies were performed in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by National Institutes of Health. Male New Zealand White rabbits (Panlab, Navarra, Spain) were used in this study. Rabbits more than 20 months old were used for ageing studies. Five-month-old rabbits were used for comparison with aged animals and for the rest of experiments.

Rabbit corpus cavernosum tissues

Rabbits were euthanized with an overdose of pentobarbital (60 mg kg^{-1}) and immediately exanguinated. The entire penis was then removed from the animal. Rabbit corpus cavernosum tissue was dissected free from the surrounding tunica albuginea, cut into tissue strips ($3 \times 3 \times 7$ mm) and used for organ chamber contractility studies (Kim *et al*., 1991).

Organ chamber studies

Strips of corpus cavernosum tissue were immersed in 10 ml organ chambers containing physiological salt solution, maintained at 37°C and aerated with 5% CO_2/95% O_2, pH 7.4 in the presence of 5 μM indomethacin. Each tissue strip was incrementally stretched to optimal isometric tension, as determined by maximal contractile response to 1 μM phenylephrine (Kim *et al*., 1991). For the relaxation studies, tissues were contracted with 0.5 μM phenylephrine and relaxation responses were evaluated by cumulative additions of compounds to the chambers. 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 $\pm$ standard error of the mean. When stated, tissues were submitted to different percentages of oxygen by exchanging the oxygen by nitrogen in the air mixture for 60 min before and during the experimental protocol. The pO_2 values in the PSS from the different gas mixtures were (mean $\pm$ s.e.m.): 140 ± 2 mmHg; 55 ± 7 mmHg; 36 ± 2 mmHg; 19 ± 3 mmHg for 20; 5; 2 and 1% O_2 respectively.

Measurement of cyclic nucleotides in rabbit corpus cavernosum tissue

Corpus cavernosum strips were immersed in 8 ml organ chambers containing PSS, maintained at 37°C and aerated with 5% CO_2/95% O_2 pH of 7.4. Each tissue strip was incrementally stretched to optimal isometric tension, as determined by maximal contractile response to 1 μM phenylephrine. After an equilibration period, tissues were submitted for 60 min to air mixtures with 5% CO_2/20% O_2/75% N_2 (pO_2 140 ± 2 mmHg) or 5% CO_2/1% O_2/94% N_2 (pO_2 19 ± 3 mmHg). Then each tissue was given 0.5 μM phenylephrine, 30 μM zaprinast and 100 μM 3-isobutyl-1-

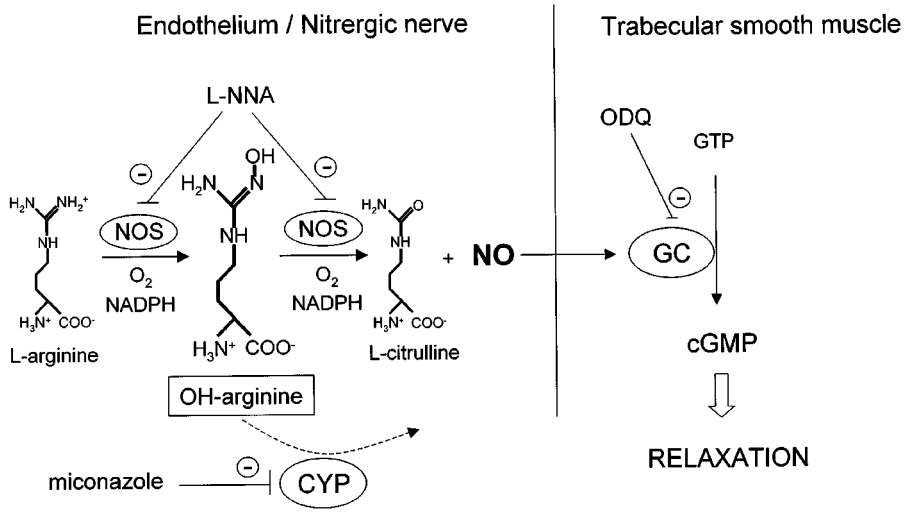

Figure 1 Schematic representation of the metabolic pathway of endothelial or neuronal production of nitric oxide (NO), which stimulates guanylyl cyclase (GC) promoting cGMP generation and relaxation of trabecular smooth muscle. The sites of action of the NO synthase (NOS) inhibitor (N^G-nitro-L-arginine (L-NNA), the cytochrome P450 oxygenase (CYP) inhibitor, miconazole, and the GC inhibitor, ODQ, are shown.

methylxanthine (IBMX) and allowed to incubate for 15 min, after which time tissues were treated with drug or vehicle. Tissues were allowed to incubate for another 5 min then immediately frozen in liquid nitrogen and stored at −80°C until extraction for cyclic nucleotide assay. Tissues were extracted by homogenization in 6% trichloroacetic acid followed by ether (H_2O-saturated) extraction and lyophilization. Cyclic nucleotides were determined by ELISA using a kit from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.).

Protein determinations

Proteins were determined using the Bio-Rad Protein Assay Kit microtiter plate assay procedure (Bio-Rad, Hercules, CA, U.S.A.) with bovine serum albumin as standard.

Drugs

Acetylcholine chloride, phenylephrine, L-arginine, N^G-nitro-L-arginine (L-NNA), miconazole, indomethacin and zaprinast, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). IBMX was obtained from RBI (Natick, MA, U.S.A.). N^G-hydroxy-L-arginine and 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ) were obtained from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.).

Statistical analysis

The complete concentration-response or frequency-response curves were compared by a two-factor analysis of variance (ANOVA) using StatView software for Apple computers. Data on relaxation using a unique concentration and data on cGMP accumulation were compared by one-way ANOVA followed by a Student–Newman–Keuls test using GraphPad InStat software.

Results

Effects of OH-arginine on contracted rabbit corpus cavernosum strips

OH-arginine produced consistent concentration-dependent relaxation of the rabbit corpus cavernosum strips contracted with phenylephrine. At the same concentrations, L-arginine did not induce significant relaxant responses (Figure 2). The treatment of the strips with the cytochrome P450 oxygenases inhibitor, miconazole (0.1 mM) did not significantly modify the relaxation of rabbit cavernosal strips induced by OH-arginine (Figure 3A). Conversely, preincubation of strips with the NOS inhibitor, N^G-nitro-L-arginine (L-NNA, 0.1 mM) or the inhibitor of guanylyl cyclase, ODQ (0.02 mM), produced a marked reduction of OH-arginine-induced responses (Figure 3B).

Effects of OH-arginine on cGMP accumulation in rabbit corpus cavernosum strips

Addition of 300 μM concentration of L-arginine did not significantly modify the cGMP content in rabbit corpus cavernosum, while OH-arginine, at the same concentration

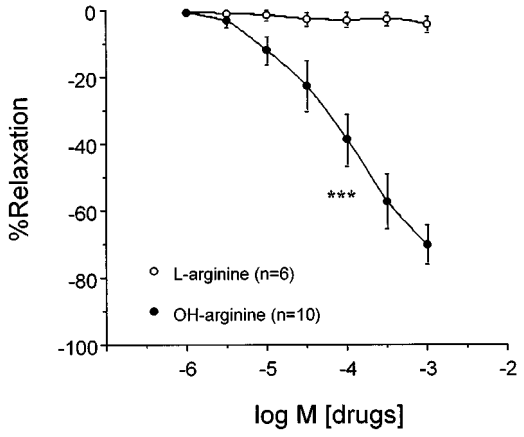

Figure 2 Relaxant effects of L-arginine and N^G-hydroxy-L-arginine (OH-arginine) (1 μM to 1 mM) in rabbit corpus cavernosum strips contracted with phenylephrine. Data are expressed as mean ± s.e.mean of the percentage of total relaxation induced by 0.1 mM papaverine. *n* indicates the number of strips used, each strip from a different animal. *Indicates $P<0.05$ vs L-arginine.

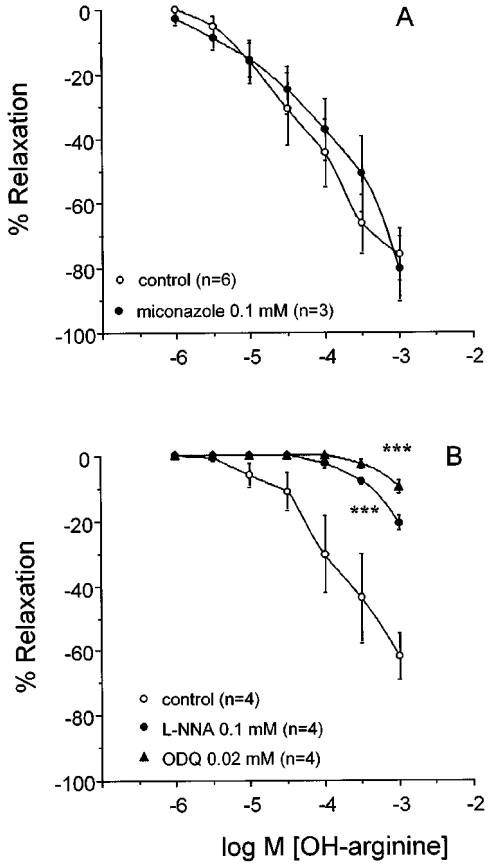

Figure 3 Effects of cytochrome P450 oxidase inhibition with miconazole (0.1 mM) (A) and NO synthase and guanylate cyclase inhibition with N^G-nitro-L-arginine (L-NNA; 0.1 mM) and ODQ (0.02 mM), respectively (B) on relaxations induced by N^G-hydroxy-L-arginine (OH-arginine) in rabbit corpus cavernosum strips contracted with phenylephrine. Data are expressed as mean ± s.e.mean of the percentage of total relaxation induced by 0.1 mM papaverine. *n* indicates the number of strips used, each strip from a different animal. ***Indicates $P<0.005$ vs control.

(300 μM), produced a significant increase of cGMP content in this tissue (Figure 4).

Influence of oxygen tension on relaxation to OH-arginine

Under normoxic conditions (20% O_2), exposure of phenylephrine-contracted rabbit corpus cavernosum strips to OH-arginine (300 μM) produced a consistent relaxation which was significantly larger than the poor relaxation induced by L-arginine at the same concentration (300 μM). Relaxation to OH-arginine was not significantly modified after reducing the oxygen concentration up to pO_2 55 ± 7 mmHg (5% O_2 mixture), 36 ± 2 mmHg (2% O_2 mixture) or even 19 ± 3 mmHg (1% O_2 mixture). The relaxation induced by OH-arginine at each of these low oxygen concentrations was significantly larger than that exerted by L-arginine in normoxic conditions (Figure 5).

Effects of OH-arginine on ACh-induced increase of cGMP levels under normoxic and hypoxic conditions

The cGMP content in rabbit corpus cavernosum at normoxic conditions (20% O_2) was significantly increased after exposure to 30 μM ACh. This increase was not significantly affected by co-incubation with L-arginine (300 μM), but the effect of ACh on cGMP levels was significantly potentiated by the treatment with OH-arginine (300 μM) by 46% (Figure 6A). When the tissues were submitted to low oxygen tension (1% O_2), the cGMP content after ACh (30 μM) addition was reduced with respect to normoxia, but the treatment with OH-arginine (300 μM) maintained the ability to significantly potentiate the increase of cGMP induced by ACh under hypoxic conditions by 40%. L-arginine had no significant effects on cGMP content under hypoxic conditions (Figure 6B).

Effects of ageing on neurogenic and endothelium-dependent relaxation of rabbit corpus cavernosum

Endothelium-dependent relaxations induced by ACh (1 nM to 10 μM) in corpus cavernosum from aged rabbits (>20-

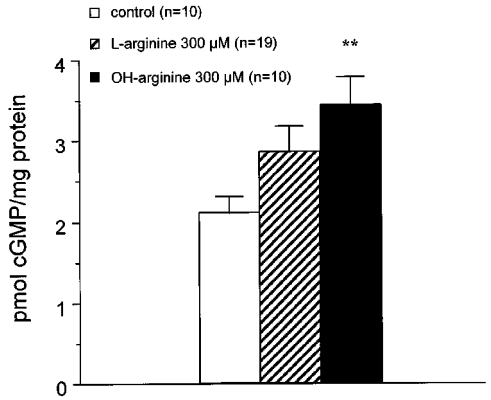

Figure 4 Effects of L-arginine and N^G-hydroxy-L-arginine (OH-arginine) on cGMP accumulation in rabbit corpus cavernosum. Data are expressed as mean$\pm$s.e.mean of pmoles of cGMP per mg of tissue protein. *n* indicates the number of strips used, each strip from a different animal. **Indicates $P<0.01$ vs control.

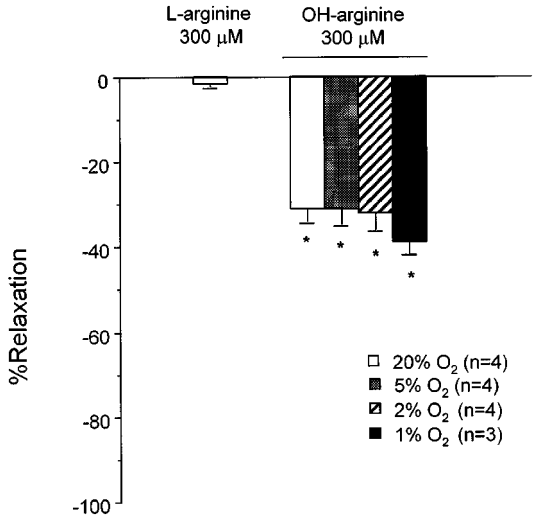

Figure 5 Relaxant effects of L-arginine (300 μM) and N^G-hydroxy-L-arginine (OH-arginine, 300 μM) in rabbit corpus cavernosum strips contracted with phenylephrine under different oxygen concentrations (20, 5, 2 and 1%). Data are expressed as mean$\pm$s.e.mean of the percentage of total relaxation induced by 0.1 mM papaverine. *n* indicates the number of strips used, each strip from a different animal. *Indiates $P<0.05$ vs L-arginine.

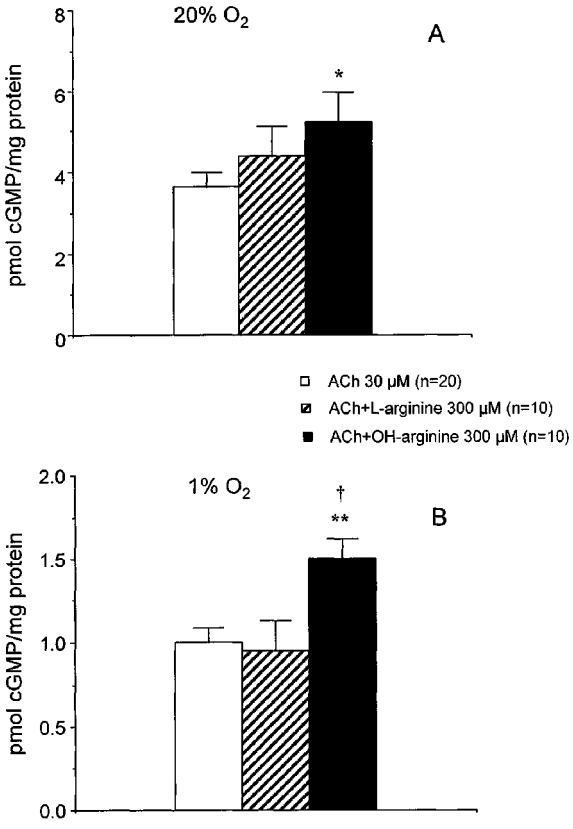

Figure 6 Effects of L-arginine and N^G-hydroxy-L-arginine (OH-arginine) on the increase of cGMP levels after exposure of rabbit corpus cavernosum to acetylcholine (ACh) under normoxic (A) or hypoxic (B) conditions. Data are expressed as mean$\pm$s.e.mean of pmoles of cGMP per mg of tissue protein. *n* indicates the number of strips used, each strip from a different animal. *Indicates $P<0.05$, **$P<0.01$ vs ACh alone.

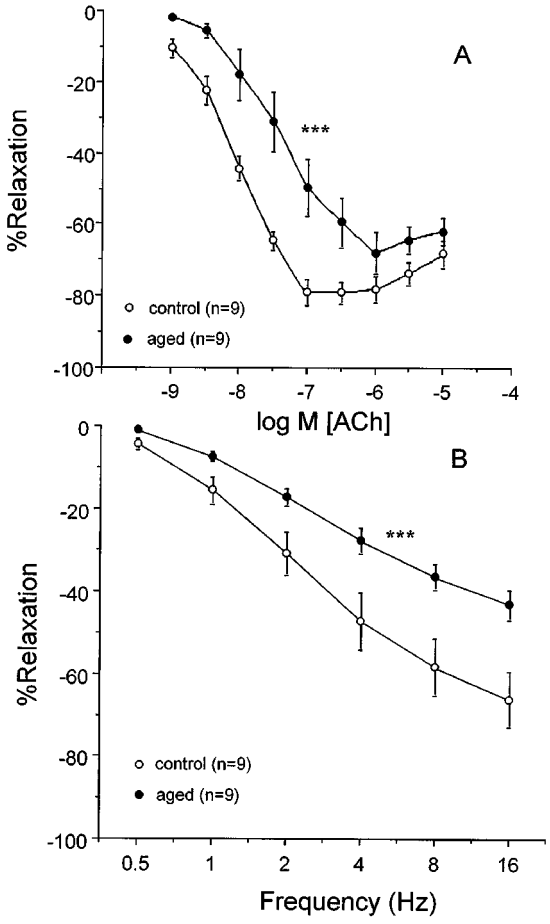

Figure 7 Effects of ageing on endothelium-dependent relaxation induced by acetylcholine (ACh; 1 nM to 10 μM) (A) and neurogenic relaxation induced by transmural electrical stimulation (TES; 0.5 to 12 Hz) (B) in rabbit corpus cavernosum strips contracted with phenylephrine. Control group consisted of young adult rabbits (5-months-old), while aged rabbits were above 20-months-old. Data are expressed as mean $\pm$ s.e.mean of the percentage of total relaxation induced by 0.1 mM papaverine. *n* indicates the number of strips used, each strip from a different animal. *** Indicates $P<0.005$ vs control.

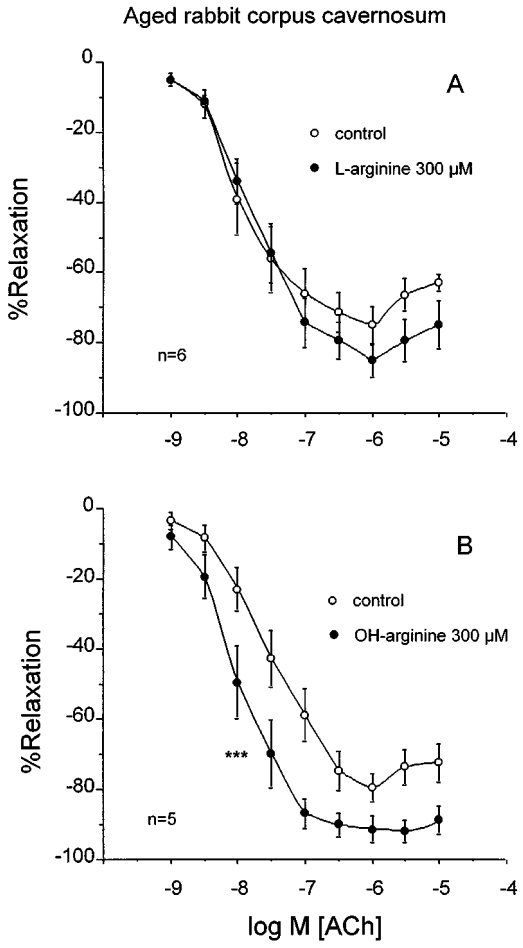

Figure 8 Effects of L-arginine (300 μM) (A) and N^G-hydroxy-L-arginine (OH-arginine; 300 μM) (B) on endothelium-dependent relaxation induced by acetylcholine (ACh; 1 nM to 10 μM) in aged rabbit corpus cavernosum strips contracted with phenylephrine. Data are expressed as mean $\pm$ s.e.mean of the percentage of total relaxation induced by 0.1 mM papaverine. *n* indicates the number of strips used, each strip from a different animal. ***Indicates $P<0.005$ vs control.

month-old) were significantly reduced when compared with the responses to ACh obtained in tissues from adult young rabbits (5-month-old) (Figure 7A). Ageing also produced a significant impairment of neurogenic relaxation of rabbit corpus cavernosum induced by transmural electrical stimulation (TES; 0.5 to 16 Hz) of nitrergic nerves (Figure 7B).

Effects of OH-arginine on ACh-induced and neurogenic relaxations of aged rabbit corpus cavernosum

Acute administration of L-arginine (300 μM) did not significantly alter the endotehlium-dependent relaxation induced by ACh in corpus cavernosum strips from aged rabbits (Figure 8A). In contrast, the treatment with OH-arginine (300 μM) produced a significant potentiation of ACh-induced responses (Figure 8B).

Neurogenic relaxations of corpus cavernosum strips from aged rabbits produced by TES application were not significantly modified by the treatment with L-arginine (300 μM) (Figure 9A), while the incubation with OH-arginine (300 μM) significantly enhanced the neurogenic relaxation of aged rabbit cavernosal strips (Figure 9B).

Discussion

Since the NO/cGMP pathway is crucial for penile erection, many efforts have been done in order to enhance this pathway as a therapeutic approach for the treatment of erectile dysfunction. Pharmacological interventions along the pathway have been tried, starting from the substrate of NOS, L-arginine (Zorgniotti & Lizza, 1994), followed by the intracavernosal application of NO donors (Truss *et al.*, 1994; Martinez-Piñeiro *et al.*, 1998) and at the end of the pathway, the recently developed inhibitors of the cGMP specific phosphodiesterase type 5 (PDE5), for oral therapy (Rajfer *et al.*, 1992; Goldstein *et al.*, 1998; Porst *et al.*, 2001; Padma-Nathan *et al.*, 2001). Here we show that OH-arginine activates and potentiates the NO/cGMP pathway in rabbit corpus cavernosum.

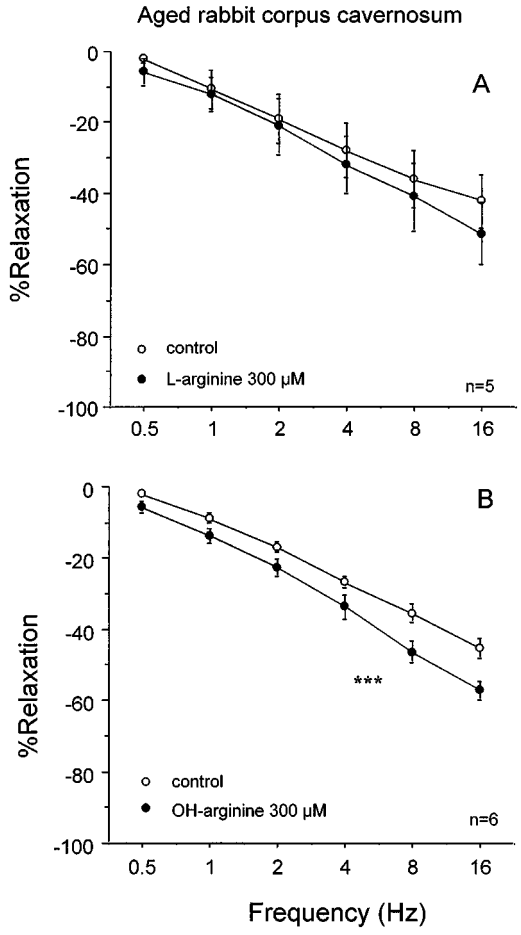

Figure 9 Effects of L-arginine (300 μM) (A) and N^G-hydroxy-L-arginine (OH-arginine; 300 μM) (B) on neurogenic relaxation induced by transmural electrical stimulation (TES; 0.5 to 12 Hz) in aged rabbit corpus cavernosum strips contracted with phenylephrine and treated with guanethidine (5 μM) and atropine (0.1 μM). Data are expressed as mean $\pm$ s.e.mean of the percentage of total relaxation induced by 0.1 mM papaverine. *n* indicates the number of strips used, each strip from a different animal. ***Indicates $P<0.005$ vs control.

OH-arginine is an intermediate product of the enzymatic production of NO from L-arginine and is itself a substrate for the NOS enzyme (Stuehr *et al*., 1991; Klatt *et al*., 1993). This compound has the ability to relax rabbit corpus cavernosum, a property that is not shared by L-arginine. The relaxation of rabbit corpus cavernosum induced by OH-arginine involves guanylyl cyclase activation since the blockade of this enzyme with ODQ abolished its relaxant effect. It has been suggested that the activity of NOS is not required for the formation of NO from OH-arginine (Schott *et al*., 1994). Nevertheless, in our preparations, the generation of NO from OH-arginine is derived from NOS activity since the treatment with a NOS inhibitor prevented OH-arginine-induced relaxations of rabbit corpus cavernosum. Wallace *et al*. (1991) also observed that the relaxation induced by OH-arginine in bovine intrapulmonary artery was reversed by NOS inhibition. The role of P450 cytochrome oxygenases, with structural similarity to NOS, as putative enzymes responsible for NO production from OH-arginine, can be discarded because of the lack of effect of miconazole, an inhibitor of this group of enzymes, on relaxant responses to OH-arginine.

There is evidence that hypoxia inhibits NO production (Kantrow *et al*., 1997; Murata *et al*., 2001). NO synthases are oxygenases that require molecular oxygen to catalyze the reaction from L-arginine to NO. Thus, it seems reasonable that low oxygen availability could reduce the NO generation by NO synthases (Whorton *et al*., 1997). In fact, the regulatory effect of oxygen tension on NO production in rabbit corpus cavernosum tissue has been reported, showing increased NOS activity as the oxygen tension rises, while, at very low oxygen concentrations, the activity of this enzyme is almost abolished (Kim *et al*., 1993). This observation is highly relevant in the understanding of the phsyiology of erection, since the increase of oxygen tension in the penis after the blood inflow produced by arterial dilation, allows to trigger NO synthesis in corpus cavernosum tissue promoting trabecular relaxation and penile erection (Kim *et al*., 1993).

OH-arginine is able to relax rabbit trabecular smooth muscle even at very low oxygen tension ($pO_2 \sim 20$ mmHg). The basis for this effect could likely be the fact that NO synthase requires half the amount of oxygen when it uses OH-arginine as substrate than when the substrate is L-arginine. Furthermore, although hypoxia markedly reduces the increase of cGMP in rabbit corpus cavernosum after exposure to the endogenous release of NO by ACh, OH-arginine significantly potentiates the cGMP accumulation under these conditions. This observation is important with respect to penile erection, since treatment with OH-arginine could reduce the threshold of oxygen tension required to trigger the NO generation after blood inflow to the penis and thus facilitate penile erection.

Ageing is associated with a marked increase in the prevalence of erectile dysfunction (McKinlay, 2000; Martin-Morales *et al*., 2001). It is well recognized that impaired NO-mediated relaxations and defective NO production are present in vasculature from aged humans (Gerhard *et al*., 1996; Toprakci *et al*., 2000; Taddei *et al*., 2001) and experimental models of ageing (Cernadas *et al*., 1998; Matz *et al*., 2000). In fact, aged rats show reduced penile NOS activity and erectile dysfunction (Garban *et al*., 1995), which is reversed by long-term oral administration of L-arginine (Moody *et al*., 1997) or gene transfer of the endothelial NOS enzyme to the penis (Champion *et al*., 1999). The present study shows that the endothelium-dependent relaxation and the neurogenic relaxation induced by the electrical stimulation of nitrergic nerves are both impaired in corpus cavernosum from aged rabbits. These results confirm previous studies carried out in this model (Haas *et al*., 1998; Utkan *et al*., 2002). Interestingly, OH-arginine significantly improves both neurogenic and endothelium-dependent relaxation of corpus cavernosum from aged rabbits, while L-arginine failed to significantly modify these responses. Thus, OH-arginine may be useful in the recovery of penile erections in aged men by enhancing NO-mediated responses. Furthermore, the effects of OH-arginine on aged corpus cavernosum allow us to speculate that this compound could be effective to potentiate NO-mediated relaxation in pathological conditions associated with defective NO production such as diabetes and hypertension (Sáenz de Tejada *et al*., 1989; Panza *et al*., 1990). Other diseases in which OH-arginine administration could be beneficial are those where a metabolic imbalance occurs (Tsai *et al*., 1998), since the requirement of NADPH for NO production from OH-

arginine is also lower. Finally, it is important to note that patients presenting combined vascular risk factors, which are associated with reduced NO production, have decreased plasmatic concentrations of OH-arginine (Garlichs *et al*., 2000).

In conclusion, OH-arginine promotes NO-mediated relaxation of rabbit corpus cavernosum and potentiates the NO-mediated responses induced by stimulation of endogenous NO generation in this tissue. The effects of this molecule are still present under conditions of reduced NO production as low oxygen tension and the ageing of the tissue. Thus, we suggest that OH-arginine cold be a therapeutic tool for the treatment of erectile dysfunction, mainly when defective NO production exists.

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