Effects of NCX 4050, a new NO donor, in rabbit and human corpus cavernosum

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Summary

The effects of NCX 4050, a drug belonging to a new class of NO donors, was investigated in isolated preparations of human and rabbit corpus cavernosum (CC) and in human foetal corpora cavernosa (hfCC) smooth muscle cells. In strips of rabbit CC, NCX 4050 (0.001–100 μM) induced a concentration-dependent relaxation which was influenced neither by Nω-nitro- L-arginine methyl-ester (L-NAME; 100 μM) nor by endothelium deprivation. The NCX 4050-induced relaxation was significantly reduced by the guanylate cyclase inhibitor 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 1 μM) and enhanced by a specific phosphodiesterase 5 inhibitor, sildenafil (300 nM). Moreover, NCX 4050 (0.01–1 μM), induced a concentration-dependent potentiation of the relaxant response induced by electrical field stimulation (EFS) in rabbit preparations pre-treated with guanethidine and indomethacin. The relaxant effect of NCX 4050 was similar to that obtained by increasing concentrations (0.001–100 μM) of sodium nitroprusside (SNP) in either rabbit or human preparations. To further investigate the activity of NCX 4050 on human corpora cavernosa, we exposed cultured hfCC smooth muscle cells to increasing concentrations of NCX 4050 and SNP. We found that both compounds dose-dependently reduced cell proliferation. The antiproliferative effect of all the concentrations tested of NCX 4050 was completely blocked by ODQ (1 μM). These results suggest that in rabbit and human corpora cavernosa NCX 4050 acts by activating guanylate cyclase activity, induces smooth muscle relaxation and quiescence. Our results provide a rationale for a possible future use of NCX 4050 in the pharmacotherapy of erectile dysfunction linked to an impaired release of NO from the endothelium.

Keywords: corpus cavernosum, NCX 4050, NO donors, NO-NSAIDs, relaxation

Introduction

Penile erection is a physiological event controlled by two opposite functional influences (Andersson & Wagner, 1995; Maggi et al., 2000). The first influence causes smooth muscle relaxation and increase in cavernosal blood flow, thus leading to penile erection. The activity of this system is essentially mediated by the nitrergic network (Ignarro et al., 1990). The second influence, which operates for the large majority of time, induces 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. A complex network of neurohormonal factors ultimately influences the relaxation of penile arteries and sinusoidal smooth muscle cells. The relaxant factors which are involved

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in this effect include acetylcholine from cholinergic nerves and several nonadrenergic noncholinergic transmitters released by nerve terminals and vascular endothelium, such as nitric oxide (NO; Saenz de Tejada et al., 1988; Knispel et al., 1991; Bush et al., 1992). NO is produced as the enzymatic by-product of molecular oxygen (O$_2$) and L-arginine under the control of nitric oxide synthase (NOS). So far, three distinct isoforms of NOS have been identified and termed neuronal (nNOS), endothelial (eNOS) and inducible NOS (iNOS). All three isoforms are present in corpora cavernosa, although with a different cellular localization (Burnett et al., 1993). Non-adrenergic non-cholinergic (NANC) neurones express nNOS, while endothelial and smooth muscle cells express the other two isoforms (Moce-ana et al., 1996). Several synthetic NO donors have been tested in clinical trials or on experimental animals, on the premise that the release of exogenous NO would allow corporal smooth muscle cell relaxation. Although sodium nitroprusside (SNP) substantially relaxes pre-contracted preparations in vitro, contrasting results have been obtained in vivo by directly injecting NO donors into corpora cavernosa in erectile dysfunction (Wegner & Knispel, 1993; Truss et al., 1994; Wang et al., 1994). Recently, an entirely new class of NO donors has been synthesized, which consists in nitro-derivates of non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin (NO-NSAIDs; Del Soldato et al., 1999). A common chemical feature of these drugs is a link of an effective moiety with nitrate through a spacer. NO-NSAIDs are chemically stable agents and NO release is therefore achieved enzymatically following exposure to biological tissues (Keeble & Moore, 2002). NCX 4050 belongs to these novel drugs characterized by the ability to release low concentrations of NO, in a controlled manner. Some NO-NSAIDs have been shown to relax blood vessels in vitro by an NO-dependent mechanism, but they have been found unable to influence mean arterial blood pressure or heart rate in vivo (Keeble et al., 2001). The aim of the present study was to test whether NCX 4050 is able to relax corpus cavernosum (CC) and to investigate the mechanisms underlying the drug effect by using rabbit and human isolated preparations of erectile tissue and cultured human foetal corpora cavernosa (hfCC) smooth muscle cells.

**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 × 0.2 × 0.7 cm). Specimens of human corpora cavernosa (HCC) were obtained from four patients who were undergoing surgical correction of Peyronie’s disease (three patients) or congenital curvature of the penis (one patient). After surgery, biopsies of CC, obtained in the operating room, were immediately placed in cold Krebs solution and transported in the laboratory for in vitro experiments. All the surgical specimens were obtained after informed consent from the patients. 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$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, NaHCO$_3$ 25, 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. The presence of functional endothelium was assessed by testing the vasodilator effect of acetylcholine (ACH), the preparations in which ACh (3 μM) reduced the tone by <40% were not used for the study. To remove endothelium, strips of CC were rubbed between the thumb and index finger for ~20 sec (Saenz de Tejada et al., 1988). The lack of a relaxation response to ACh in pre-constricted preparations indicated that the procedure was successful. Experiments were carried out in corporal smooth muscle pre-contracted with 10 μM phenylephrine (Phe). This concentration of Phe was selected as it was able to produce a stable increase in tension of about 1000 mg. This degree of contractile response was taken as 100% and the relaxant effect induced by different drug concentrations was referred to this value. Drug cumulative concentrations were added to the bath, at 7-min intervals, in order to obtain a concentration–relaxant effect curve. A 15–30 min pretreatment with selected antagonists and/or inhibitors was performed before repeating the concentration-response curve for the drug. Electrical field stimulation (EFS) was performed using two platinum plates, parallel to the preparations, connected to a pulse generator. EFS was conducted at 10 V, 0.5 msec, at a frequency of 5 Hz, for 30 sec. Trains of field pulses were given at 15-min intervals in preparations pre-contracted by Phe and pre-treated with guanethidine 3 μM and indomethacin 3 μM. The drug influence on the recorded relaxant response was tested by administering NCX 4050, at increasing concentrations, 7 min before each train. The response detected in the absence of the drug was taken as 100% and those observed in the presence of the drug were referred to this value. More details about EFS technique are available elsewhere (Maggi et al., 2000).

**Cell cultures**

Human foetal corpora cavernosa (hfCC) cells were obtained from two 8–12-week-old foetuses after spontaneous or therapeutic abortion, as previously described (Granchi et al., 2002). 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 phosphate-buffered saline (PBS).
and cultured in a mixture 1:1 (v/v) of Dulbecco’s modified Eagle’s medium and F-12 Ham (DMEM/F-12 1:1 mix) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM glucose, 100 U/mL penicillin and 100 µg/mL streptomycin and enriched with a mixture of insulin/transferrin/selenium (ITS), in a fully humidified atmosphere of 95% air and 5% CO₂. Cells began to emerge within 24–48 h and were used within the fifth passage. Indirect immunofluorescence technique was used to characterize hfCC cells. Briefly, after growing the cell on sterile slides, cultures were washed twice with PBS pH 7.4, dried overnight and fixed for 15 min at room temperature. The primary antibody (alpha smooth muscle actin, clone NCL-SMA; Novo Castra Laboratories Ltd, Peterborough, UK) was appropriately diluted (1:250) in PBS containing 2% bovine serum albumin (BSA) and added to the slides. Slides were incubated overnight at 4°C and thereafter washed three times (5 min each) in PBS, incubated at room temperature for 45 min with PBS-2% BSA containing the fluorescent second antibody at the appropriate dilution (goat antimouse, 1:100) and examined with microscope equipped with epifluorescence (Nikon Microphot-FX Microscope; Nikon, Kogaku, Tokyo, Japan).

**Cell proliferation assay**

For growth measurement, 2 × 10⁴ cells were seeded onto 12-well plates in growth medium. After 24 h the growth medium was removed, the cells were washed twice in PBS and incubated in phenol red- and serum-free medium containing 0.1% BSA. After 24 h, increasing concentrations (0.1 nM–10 µM) of NCX 4050 or SNP were added. A fixed concentration (1 µM) of 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was also added to the cells stimulated with different concentrations of NCX 4050 or SNP. Cells in phenol red- and serum-free medium containing 0.1% BSA were used as basal controls. After 24 h, cells were trypsinized and each experimental point was derived from counting in the haemocytometer, and then averaging, at least five different fields for each well. In the same experiment, each experimental point was repeated in duplicate or triplicate.

**Statistical analysis**

Results are expressed as mean ± SEM of n experiments. Differences between groups were tested for significance by Student’s test for paired or unpaired data, and p < 0.05 was taken as significant. Cell growth results are expressed as percentage (±SE) of the growth of their relative controls.

The computer program ALLFIT was used to obtain inhibitory half concentration (IC50; De Lean et al., 1978).

**Chemicals and solutions**

Chemicals used included Phe HCl, Ach and Nω-nitro-L-arginine-methyl-ester (L-NAME), indomethacin, guanethidine, SNP, DMEM, collagenase type IV, PBS, antibiotics, glutamine, BSA and atropine from Sigma (St Louis, MO, USA); FBS (Unipath, Bedford, UK); ODQ was obtained from Tocris (Bristol, UK); plasticware for cell culture from Falcon (Oxnard, CA, USA); sildenafil was a gift from Dr Stief (Hannover, Germany). NCX 4050 was synthesized at Research Institute NicOx (Milano, Italy). Stock solution of sildenafil and indomethacin were made in ethanol; stock solution of ODQ and NCX 4050 were made in dimethylsulphoxide (DMSO); the other substances were dissolved daily in double distilled water and further dilutions to the final concentrations were made in Krebs’ solution. Control experiments showed that the concentrations of DMSO used modified neither the vasoconstrictor response to Phe nor the relaxation induced by the different agents.

**Results**

**Relaxant responses to NCX 4050 in rabbit and human preparations**

Rabbit preparations exposed to Phe 10 µM developed a stable increase in vascular tone amounting to 1050 ± 99 mg (n = 15). The addition of increasing concentrations of NCX 4050 to pre-contracted preparations induced a relaxant effect which was evident at concentrations higher than 0.1 µM; this effect was concentration-dependent in the range of concentrations between 0.1 and 100 µM (IC₅₀ = 21 ± 1.7 µM; Fig. 1). The maximum relaxing effect observed with 100 µM NCX 4050 consisted of a reduction in the tone by 77 ± 5.7%. The vasorelaxant response to NCX 4050 developed slowly and reached the maximum value after

![Figure 1](image-url)
SNP also relaxed rabbit preparations, with IC\textsubscript{50} = 12 ± 1.05 µM (Fig. 1). The maximum relaxing effect observed with 100 µM SNP, consisting of a reduction in the tone by 83.3 ± 5.8%, was not statistically different from that detected with NCX 4050.

Human CC preparations treated with 10 µM Phe developed a stable increase in basal tone (990 ± 75.5 mg) of a degree similar to that observed in rabbit preparations. In these preparations, a relaxant response was again detected with NCX 4050, which was concentration-dependent in the range of concentrations between 0.1 and 100 µM (IC\textsubscript{50} = 19 ± 2.3 µM; Fig. 2a). Additionally, in this case, the maximal effect obtained with NCX 4050 was of an extent similar to that induced by concentrations of SNP ranging from 0.001 to 100 µM (IC\textsubscript{50} = 2.6 ± 3.2 µM; Fig. 2a). It is noteworthy that a comparison between the data obtained in rabbit preparations and those detected in human preparations revealed that NCX 4050 was able to induce a relaxant response of the same degree in the two kinds of preparations (Fig. 2b).

**Influence of NO synthase inhibition and of endothelium deprivation on the relaxant response to NCX 4050**

In order to check whether endogenous NO was involved in the relaxant response to NCX 4050, further experiments were carried out in preparations pre-treated with the NOS inhibitor L-NAME. The results showed that, in the presence of 100 µM L-NAME, the degree of the relaxant response to NCX 4050 (0.001–100 µM) was unchanged, because the observed relaxant effect was of a degree similar to that detected in the absence of the inhibitor (Fig. 3). Results similar to those described above were obtained in endothelium-deprived preparations, in which NCX 4050 concentration-dependently relaxed strips of rabbit CC precontracted by Phe at a degree similar to that observed in preparations with intact endothelium (not shown in the figure).

![Figure 2](image1.png)

**Figure 2.** (a) Concentration-relaxant response curves, induced by increasing concentrations of NCX 4050 (open circles) and sodium nitroprusside (SNP-closed circles) in strips of human corpus cavernosum precontracted by phenylephrine. (b) Comparison between the relaxant effects induced by NCX 4050 in rabbit (open circles) and in human (closed circles) preparations pre-contracted by phenylephrine. Each point represents mean ± SEM of at least four experiments.

![Figure 3](image2.png)

**Figure 3.** Concentration-relaxant response curves induced by increasing concentrations of NCX 4050 alone (closed circles) and in the presence of 100 µM L-NAME (open circles) in strips of rabbit corpus cavernosum precontracted by phenylephrine. L-NAME was added to preparations 30 min before obtaining the curve for NCX 4050. Each point represents mean ± SEM of at least four experiments.
Influence of ODQ and sildenafil on the relaxant response to NCX 4050 in rabbit preparations

The hypothesis that the relaxation induced by NCX 4050 was attributable to soluble guanylate cyclase activation because of NO production by the drug, was tested by experiments carried out in preparations pre-treated with either the potent and selective guanylate cyclase inhibitor ODQ (Garthwaite et al., 1995) or the specific phosphodiesterase 5 inhibitor, sildenafil (Boolell et al., 1996). It was observed that 1 μM ODQ antagonized the relaxant response induced by NCX 4050: in fact, the relaxant responses to the higher drug concentrations (10 and 100 μM) were significantly reduced in the presence of the guanylate cyclase inhibitor (IC$_{50}$ = 2587 ± 312 μM; Fig. 4). On the contrary, the relaxant responses to 1–100 μM NCX 4050 were significantly enhanced by 300 nM sildenafil (IC$_{50}$ = 2.3 ± 0.26 μM; Fig. 4).

Influence of NCX 4050 on the response of preparations to field stimulation

In preparations pre-contracted by Phe and pre-treated with concentrations (3 μM) of guanethidine and indomethacin unable to affect the tone of the preparations, trains of field pulses given at 15-min intervals produced relaxant responses whose degree remained stable for more than 90 min. The relaxant response to EFS was unaffected by pre-treatment with atropine 1 μM, but was completely inhibited in the presence of 100 μM L-NAME (not shown). The exposure of the Phe-pre-contracted preparations to increasing concentrations of NCX 4050 for 7 min before each train induced a concentration-dependent potentiation of the relaxant response (Fig. 5a,b). In fact, the degree of the relaxant response to EFS amounted to 111.3 ± 2.2, 125.7 ± 1.0 and 140.2 ± 2.0% in the presence of concentrations of NCX 4050 of 0.01, 0.1 and 1 μM, respectively.

Effect of NCX 4050 on hfCC

Figure 6 shows a typical immunostaining with an antibody against α-smooth muscle actin in hfCC cells. According to their smooth muscle origin, the large majority of hfCC cells (>95%) are positive for α-smooth muscle actin and shows the characteristic morphological features of contractile cells. In these cells we tested whether the NO donor NCX 4050 was able to affect proliferation. Both NCX 4050 and SNP dose-dependently reduced hfCC cell growth, although with different IC$_{50}$ (Fig. 7). Indeed, while NCX 4050 was active in the subnanomolar range (IC$_{50}$ = 55 pm), higher concentrations of SNP were necessary to reduce hfCC-cell proliferation (IC$_{50}$ = 9.9 nm). When sigmoidal curves were analysed using a mathematical modelling (program ALLFIT), differences in IC$_{50}$ was statistically significant (p < 0.01). The antiproliferative effect of NCX 4050, at all the concentration tested, was completely blocked by ODQ (1 μM; Fig. 8). This suggests that the antiproliferative activity of NCX4050 is mediated by cGMP and therefore it is the result of its capacity to release NO.

Discussion

In the present study, we provide evidence that NCX 4050, a drug belonging to a new class of NO-donor drugs called NO-NSAIDs, is provided with relaxing properties on human and rabbit CC with a potency similar to that of SNP. The interest for this drug was not only because of the circumstance that NO-NSAIDs have been never tested before on the erectile tissue, but also the result of the interesting properties displayed by this new class of drugs. In fact, NCX 4050 belongs to a group of drugs characterized by the ability to release NO through a slow metabolic process probably involving cholinesterase activity (Keeble & Moore, 2002). As this process is dissimilar from the sudden spontaneous process involved in SNP activity, these drugs are less prone to interfere with blood pressure regulation (Del Soldato et al., 1999), as shown by the lack of influence on cardiovascular parameters in normotensive animals in vivo (Wallace et al., 1994; Wainwright et al., 2002). In agreement with the known profile of NO-NSAID activity, in the present study the relaxant effect of NCX 4050 was influenced neither by the lack of functional endothelial cells nor by the inhibition of nitrergic transmission. In fact, the drug fully maintained its activity in endothelium-deprived preparations and after treatment with the NOS inhibitor L-NAME, at a concentration (100 μM) able to completely prevent the relaxant response induced by electrical stimulation of nitrergic terminals of the tissue. In order to

![Figure 4](image-url)
demonstrate that the relaxation induced by NCX 4050 was indeed attributable to NO production by the drug, experiments were carried out in preparations pre-treated with drugs able to interfere with the NO transduction pathway. The two drugs selected for this purpose were the guanylate cyclase inhibitor, ODQ (Garthwaite et al., 1995) and sildenafil, a phosphodiesterase (PDE) inhibitor characterized by an affinity for PDE5 much higher than for the other PDE isoenzymes (Boolell et al., 1996). ODQ, at a concentration (1 µM) provided with a selective inhibitory effect on soluble guanylate cyclase (Garthwaite et al., 1995), effectively and significantly inhibited the response to NCX 4050. Conversely, the NCX 4050-induced relaxation was significantly enhanced by 300 nM sildenafil, a drug able to inhibit the main subtype of PDEs present in cavernosal tissue (Ballard et al., 1998), thus increasing cellular cGMP levels. As the above mentioned observations showed that the relaxant response to NCX 4050 was attributable to an NO-donor

Figure 5. (a) Typical experiment showing the effect of increasing concentrations of NCX 4050 on the relaxant response induced by electrical field stimulation (EFS; 5 Hz, 10 V, 30 sec) in rabbit corpus cavernosum preparations precontracted by phenylephrine (Phe). (b) Effect of increasing concentrations of NCX 4050 on the response induced by EFS in rabbit corpus cavernosum preparations pre-contracted by phenylephrine (Phe). Histograms show mean ± SEM of at least four experiments. *p < 0.05 vs. NCX 4050. c = control.

Figure 6. Immunofluorescence of hCCT, as obtained using an antibody against α-smooth muscle actin. Almost all the cells are positive. Note the typical morphological feature of smooth muscle cells.
effect, we finally checked whether or not this drug was able to interfere with the response of CC to relaxant neurogenic influences induced by EFS. These kinds of experiments were carried out in preparations pre-treated with guanethidine and indomethacin in order to inhibit the constrictor influences because of sympathetic terminal activation and to exclude a role for prostanoids in the relaxant response, respectively (Maggi et al., 2000). The observed concentration-dependent enhancement of the neurogenic relaxation induced by NCX 4050 clearly indicated that the supply of exogenous NO can be additive to the endogenous nitrergic system in producing a relaxant response in the corporal muscle.

Experiments conducted in proliferating smooth muscle cells derived by foetal human CC (Granchi et al., 2000) further substantiated the NO-donor properties of NCX 4050. In fact, the antiproliferative activity of NCX 4050 was completely counteracted by blocking guanilate cyclase activity with ODQ. This indicates that the activation of cGMP is absolutely required for growth inhibition, which is compatible with the NO-donor property of the compound. In addition, in growth studies NCX 4050 was even more potent than the well-known NO-donor SNP.

In conclusion, the present study demonstrates that NCX 4050 is provided with a potent relaxant activity in isolated CC preparations and with an antiproliferative effect in cultured smooth muscle cells from human foetal penis. All these effects are compatible to its NO-donor properties. Our study suggests that the CC may represent a new possible target in the spectrum of activity of NO-NSAIDs. The possible employment of NCX 4050 in the treatment of erectile dysfunction obviously needs further studies. However, the NO-releasing properties of the compound are potentially attractive in erectile dysfunctions characterized by functional endothelial damage, such as in diabetes and hyperlipidaemia, where the endogenous production of NO might be compromised.

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