

Pharmacological profile of a nitric oxide donor spermine NONOate in the mouse corpus cavernosum

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Background/aim: To investigate the effects of spermine NONOate in the cavernous tissue obtained from mice treated or untreated with sildenafil.

Materials and methods: We studied the effects of spermine NONOate on the tone and nitregeric relaxation responses of isolated mouse corpus cavernosum and compared them with sodium nitroprusside in the absence or presence of L-nitroarginine, hydroxocobalamin, pyrogallol, diethyldithiocarbamate (DETCA), or 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). The neurogenic contractions and relaxations of the tissues were induced by electrical field stimulation. Some mice received a single oral dose of sildenafil and after 1 h the effects of spermine NONOate were evaluated by *in vitro* studies.

Results: Spermine NONOate relaxed mouse corpus cavernosum in a concentration-dependent manner. Spermine-NONOate-induced relaxation was relatively slow to develop and it was reversible and reproducible. These relaxations were significantly suppressed by hydroxocobalamin, diethyldithiocarbamate, or ODQ, but not by L-nitroarginine or pyrogallol. Spermine NONOate potentiated the nitregeric relaxations to electrical field stimulation (EFS), whereas it significantly reduced EFS-induced contractions. Sildenafil treatment can enhance the relaxant responses to spermine NONOate and EFS.

Conclusion: These findings suggest that spermine NONOate has a potent relaxant action in cavernous tissue and this effect can be potentiated by oral sildenafil treatments. Spermine NONOate may be considered an attractive treatment for erectile dysfunction in pathologic disorders with a lack of endogenous NO production.

Key words: EFS, electrical field stimulation, L-NOARG, N-nitro-L-arginine, spermine NONOate, S-NONOate

1. Introduction

It is well known that nitric oxide, which is synthesized by erectile-autonomic nerves and vascular endothelium of the cavernous tissue, has an important modulatory role in penile erection (1). Nitric oxide activates the soluble guanylate cyclase which catalyzes the biosynthesis of cyclic guanosine 3',5'-monophosphate (cGMP). cGMP causes a relaxation in the cavernous tissue thus eliciting an erection (1). It is suggested that some pathologic conditions such as aging and vascular diseases can cause the impaired synthesis, release, or bioavailability of NO in the penile tissue (2). Moreover, diabetic neuropathy can affect nitregeric nerves in the penile tissue (3). This results in erectile dysfunction due to insufficient relaxation of the cavernous smooth muscle and dilation of penile arteries.

Phosphodiesterase type 5 (PDE5) inhibitors such as sildenafil have been very successful in the management of erectile dysfunction (4,5). It improves erectile function by

reducing the breakdown of the active cGMP and causes potentiation of its action (6). However, diabetic patients and patients with cavernosal nerve injury following radical prostatectomy have less success with PDE5 inhibitors than other patient groups because of a lack of endogenous NO production (7,8). It is suggested that a residual NO must be present for a PDE5 inhibitor to be efficient (2). Therefore, we need novel agents that are combinations of a PDE5 inhibitor and NO-releasing compounds. It is well known that some prodrugs that contain nitric oxide or nitric oxide-like species have been used for erectile dysfunction (8–10).

Recently, it was shown that a nitric oxide donor S-NONOate elicited smooth muscle relaxation via stimulating soluble guanylate cyclase (11,12). Since the molecule of S-NONOate can also generate nitric oxide and has a long half-life (around 39–73 min), S-NONOate may be an effective smooth muscle relaxant and vasodilator (13).

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These properties of S-NONOate suggest that it can potentially be used for treatment of erectile dysfunction that has been attributed in part to a lack of endogenous nitric oxide production (2). Mouse corpus cavernosum has been used previously for in vivo and in vitro studies of erectile mechanisms (14–18). Therefore, in the present study, we aimed to demonstrate the pharmacological effects of S-NONOate in the mouse cavernous tissue. We further analyzed the effect of S-NONOate on the tone and nitrenergic relaxation responses of isolated mouse corpus cavernosum and compared the effects with those of a PDE₅ inhibitor, sildenafil, and an NO donor, sodium nitroprusside (SNP).

2. Materials and methods

2.1. Animals

Male Swiss albino mice (n = 50) weighing 25–30 g were used throughout the experiments. The experimental procedure was approved by the animal care committee of the University of Çukurova (TIBDAM) and the experiments were carried out in accordance with the Principles of Laboratory Animal Care (National Institute of Health guideline; publication No. 86–23, revised 1984). All animals were kept under laboratory conditions (12 h dark/12 h light) and allowed access to food and drink ad libitum.

2.2. Organ bath experiments

The mice were killed by cervical dislocation. Penises were removed and placed in a petri dish containing Krebs' solution (composition mM: NaCl 119, KCl 4.6, CaCl₂ 1.5, MgCl₂ 1.2, NaHCO₃ 15, NaHPO₄ 1.2, glucose 11). Corpus cavernosum was prepared according to the previously described method (15). The preparations were mounted under 0.2-g tension in a 15-mL organ bath maintained at 37 °C containing Krebs' solution aerated with 95% O₂ and 5% CO₂. The tissue was allowed to equilibrate for 1 h. During this period, the preparation was washed with fresh Krebs' solution at 15-min intervals. The responses were recorded on a polygraph (Ugo Basile, Gemini 7070) via an isotonic transducer (Ugo Basile, 7006).

After the equilibration period, the tissue was treated with 5 µM of phenylephrine. The active tone reached a stable level within 5 min; at the end of this period, S-NONOate (0.1, 0.3, 1, 3, 10, 30, and 100 µM) or SNP (0.1, 0.3, 1, 3, and 10 µM) was applied cumulatively on the different preparations of corpus cavernosum. Thus, the first series of responses were obtained. After the 30 min washout period, the second series of responses were recorded in a similar manner. In some experiments, after the first series of responses were recorded, we examined the relaxant responses of S-NONOate on the phenylephrine (5 µM)-induced contractions in the presence of N^ω-nitro-L-arginine (L-NOARG; a nitric oxide synthase inhibitor;

100 µM), hydroxocobalamin (a nitric oxide-binding agent; 100 µM), pyrogallol (a superoxide anion generator; 10 µM), DETCA (a superoxidase inhibitor; 8 mM), or ODQ (a selective soluble guanylate cyclase inhibitor; 2 µM). In another experimental group, the relaxant effect of SNP was examined on the contracted tissue by phenylephrine in the presence of 8 mM DETCA or 2 µM ODQ. The incubation period for all antagonists and inhibitors was 30 min.

In some groups, a PDE5 inhibitor sildenafil (30 mg/kg) was given to mice via a stomach tube in a volume of 0.3 mL 1 h before the experiments. Then we examined the S-NONOate-induced relaxations (0.1–100 µM) on the contractions to phenylephrine to test possible interactions between S-NONOate and sildenafil. In these series, at the end of the experiments, the tissue was contracted with phenylephrine once more to monitor the papaverine (100 µM) relaxation.

In experiments in which electrical field stimulation (EFS) was used, atropine (1 µM) and guanethidine (1 µM) were always present in the bathing medium to inhibit cholinergic and adrenergic responses. After the equilibration period, the tissue was treated with 5 µM of phenylephrine. The active tone reached a stable level within 5 min; at the end of this period, EFS (2, 4, 8, 16, and 32 Hz: 30 V, 0.5 ms) was applied to the tissue for 15 s by a Grass S88 stimulator via 2 parallel platinum electrodes embedded in Perspex. We used the same EFS parameters in our previously published papers (11,15). Thus, the first series of responses were obtained. After a 30-min washout period, the second series of responses were recorded in a similar manner. In some experiments, after the first series of responses were recorded, the tissues were incubated with S-NONOate (1 and 5 µM) for 30 min and then the second series of relaxation responses to EFS were examined. In another group, we evaluated the effect of S-NONOate on the neurogenic contractile responses to EFS (2, 4, 8, 16, and 32 Hz: 30 V, 0.5 ms) in the presence of L-NOARG (100 µM). In these experiments, L-NOARG was always present in the bathing medium to inhibit nitric oxide synthase enzyme. After we obtained the first series of EFS-induced contractile responses, we examined the effect of S-NONOate on these contractions to EFS. The incubation period of spermine NONOate was 30 min.

2.3. Drugs and solutions

Stock solutions of phenylephrine, hydroxocobalamin, pyrogallol, papaverine, SNP, spermine NONOate, diethyldithiocarbamate (DETCA), N^G-nitro-L-arginine, guanethidine, and atropine sulfate were dissolved in distilled water. 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was dissolved in dimethyl sulfoxide (final concentration in the bath medium was 0.1%). The S-NONOate stock solution was stored at 20 °C. Except for sildenafil citrate, all drugs were obtained from Sigma.

Sildenafil citrate (Sildegra 50 mg tablet, Fako) was purchased from a drugstore.

2.4. Statistical analysis

Relaxations were expressed as a percentage of the phenylephrine-induced contraction. In the experiments that used sildenafil, relaxations to S-NONOate were expressed as a percentage of the papaverine-induced relaxation. All data were expressed as mean \pm SE. All data were evaluated with the paired and unpaired student-t tests that were used in analysis of variance. P values less than 0.05 are considered significant. Statistical analysis was performed with GraphPad Prism software (San Diego, CA, USA).

3. Results

3.1. Relaxant effects of S-NONOate or SNP in mouse corpus cavernosum

S-NONOate (0.1–100 μ M) or SNP (0.1–10 μ M) relaxed mouse corpus cavernosum in a concentration-dependent manner (Figure 1). S-NONOate-induced relaxation was relatively slow to develop, whereas SNP-induced relaxation developed very rapidly. S-NONOate-induced relaxation was reversible and reproducible (Figure 2).

3.2. Effect of L-NOARG or ODQ on relaxation to S-NONOate or SNP in mouse corpus cavernosum

L-NOARG (100 μ M) did not alter the relaxation to S-NONOate (0.1–100 μ M; Table 1) or SNP (0.1–10 μ M; data not shown), whereas 2 μ M ODQ significantly decreased the relaxations to S-NONOate or SNP at all concentrations (Tables 1 and 2). Thus, both S-NONOate and SNP were apparently endothelium-independent vasorelaxants directly stimulating smooth muscle soluble guanylate cyclase in the cavernosal tissue. The solvent (DMSO; 0.1%) of ODQ had no effect on the responses to S-NONOate (Table 1). DETCA (8 mM) also inhibited the relaxations to SNP (0.1–100 μ M; Table 2). 100 μ M hydroxocobalamin exhibited a similar inhibition on the relaxations to SNP (0.1–100 μ M) (% inhibition: 89.5 ± 7.9 at 1 μ M SNP).

3.3. Effect of hydroxocobalamin, pyrogallol, or DETCA on relaxation to S-NONOate or SNP in mouse corpus cavernosum

Incubation of the tissue with pyrogallol (100 μ M) did not significantly affect relaxations evoked by S-NONOate (0.1–100 μ M; Table 1) or SNP (0.1–10 μ M; data not shown). On the other hand, hydroxocobalamin (100 μ M) or DETCA (8 mM) caused significant inhibition of relaxation to S-NONOate (0.1–100 μ M) or SNP (0.1–10 μ M) (Tables 1 and 2).

3.4. Effect of S-NONOate or SNP on relaxations or contractions induced by electrical field stimulation in mouse corpus cavernosum

EFS (2–32 Hz; 30 V; 0.5 ms) elicited reproducible frequency-dependent relaxation responses that were completely inhibited in the presence of L-NOARG (100 μ M) and ODQ (2 μ M) (% inhibitions were 99 ± 3.1 and 98 ± 2.2 at 4 Hz for L-NOARG and ODQ, respectively). S-NONOate (1–5 μ M) enhanced the magnitude of relaxation responses at all frequencies (2–16 Hz) in the tissue precontracted with phenylephrine concentration dependently (Table 3). SNP also caused an increase in some of the relaxations to EFS, but these enhancements were not statistically significant (not shown). On the other hand, S-NONOate (1 μ M) or SNP (1 μ M) significantly inhibited contraction to EFS (2–16 Hz) in the presence of 100 μ M L-NOARG (Table 4; results obtained with SNP not shown).

3.5. Relaxant effects of S-NONOate or EFS in mouse corpus cavernosum after administration of a single oral dose of sildenafil

Relaxations induced by S-NONOate (0.1–100 μ M) in mouse cavernosum precontracted with phenylephrine (5 μ M) were significantly higher compared to the control group after a single oral dose (30 or 60 mg/kg) of sildenafil (Figure 3). Moreover, relaxations to EFS were significantly higher in the tissues of mice treated with sildenafil (30 or 60 mg/kg) than in those of the control group (Table 5).

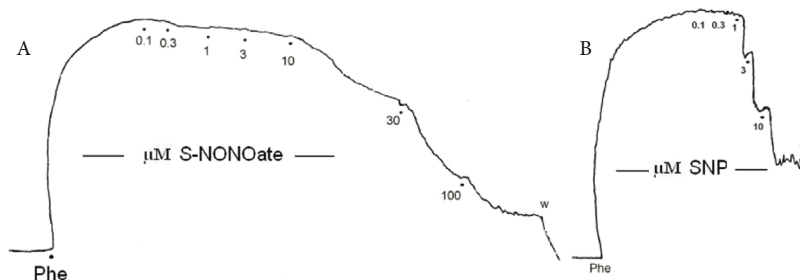


Figure 1. Representative tracings showing the relaxant effects of (A) S-NONOate (0.1–100 μ M) and (B) SNP (0.1–10 μ M) in mouse corpus cavernosum. 'w' represents washout.

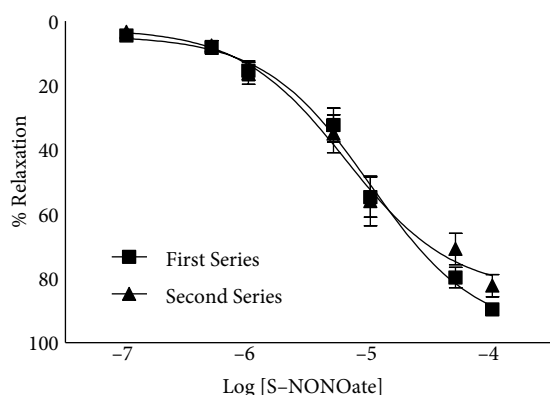


Figure 2. The reversible and reproducible effects of S-NONOate (0.1–100 μ M) in the mouse corpus cavernosum. Results are expressed as mean \pm SE (n = 6–8).

4. Discussion

The results of the present study suggest that S-NONOate induces relaxation of the mouse corpus cavernosum and the relaxant activity of S-NONOate is mediated predominantly by stimulation of soluble guanylate cyclase similar to the effect of S-NONOate in other tissues. Our data show that

this relaxation can be due in part to the generation of nitric oxide from S-NONOate and also by direct activation of soluble guanylate cyclase by S-NONOate via a nitric oxide-dependent mechanism. Another important finding of the present study is that oral sildenafil treatment potentiated the relaxations to S-NONOate.

Spermine NONOate caused a relaxation resembling the effect of SNP and the S-NONOate action developed slowly. In the present study, the ineffectiveness of L-nitroarginine on responses to S-NONOate suggests that an endogenous L-arginine/nitric oxide pathway has minor importance in the mechanism of S-NONOate-induced relaxation. Furthermore, ODQ could inhibit the relaxant responses to both S-NONOate and SNP. This finding suggests that the relaxant activity of S-NONOate is mainly due to stimulation of soluble guanylate cyclase in the cavernous smooth muscle cells. Although there are some studies that suggest that S-NONOate can produce cyclic GMP-independent relaxation in some tissues such as rat pulmonary and femoral arteries (19,20), it appears that most of the effects of S-NONOate are due to direct nitric oxide-dependent interaction of S-NONOate with soluble guanylate cyclase, which enhances cGMP synthesis in this tissue.

Table 1. The effects of DMSO (0.1%), DETCA (8 mM), L-NOARG (100 μ M), ODQ (2 μ M), pyrogallol (10 μ M), and hydroxocobalamin (HC; 100 μ M) on relaxations of S-NONOate of isolated mouse corpus cavernosum contracted by 5 μ M phenylephrine.

S-NONOate	0.1	0.3	1	3	10	30	100 μ M
CONTROL	5.3 \pm 0.4	13.0 \pm 1.2	22.0 \pm 1.5	46.0 \pm 3.2	67.0 \pm 3.1	86.0 \pm 2.9	94.0 \pm 0.5
DMSO	4.9 \pm 0.3	11.2 \pm 0.9	21.3 \pm 1.2	43.8 \pm 2.7	64.3 \pm 1.9	82.9 \pm 3.1	89.5 \pm 2.5
DETCA	0.5 \pm 0.3*	8.2 \pm 1.4*	9.9 \pm 1.7*	36.0 \pm 3.5*	55.0 \pm 5.8*	77.0 \pm 3.7	88.0 \pm 3.5
L-NOARG	4.6 \pm 0.9	8.5 \pm 1.9	18.0 \pm 2.5	42.0 \pm 4.9	63.0 \pm 4.4	80.0 \pm 2.8	89.0 \pm 2.1
ODQ	0.58 \pm 0.6*	3.5 \pm 1.3*	5.3 \pm 1.7*	24.0 \pm 3.1*	36.0 \pm 6.5*	63.0 \pm 4.2*	79.0 \pm 3.0*
PYROGALLOL	5.3 \pm 1.4	11.0 \pm 1.7	17.0 \pm 3.6	38.0 \pm 3.0	58.0 \pm 3.5	76.0 \pm 5.3	85.0 \pm 6.40
HC	1.9 \pm 0.2*	4.9 \pm 0.7*	8.28 \pm 0.7*	18.9 \pm 1.3*	33.3 \pm 1.2*	54.5 \pm 2.8*	68.0 \pm 4.6*

The results are presented as the percentage of the maximal response to phenylephrine-induced contraction at the end of each experiment (mean \pm SE, n = 6). *P < 0.05 shows significant difference from control responses before drug application.

Table 2. The effects of DETCA (8 mM) and ODQ (2×10^{-6} M) on relaxations of SNP of isolated mouse corpus cavernosum induced by phenylephrine.

SNP	0.1	0.3	1	3	10 μ M
CONTROL	7.3 \pm 4.0	25 \pm 7.6	47.8 \pm 6.6	69.8 \pm 4.2	74.4 \pm 4.8
DETCA	1.8 \pm 1.9*	3.6 \pm 3.6*	14.4 \pm 7.0*	29.3 \pm 9.9*	41.9 \pm 12.0*
ODQ	1.43 \pm 1.0*	6.3 \pm 2.3*	12.5 \pm 4.3*	38.3 \pm 15.1*	47.1 \pm 15*

The results are presented as the percentage of the maximal response to phenylephrine-induced contraction at the end of each experiment (mean \pm SE, n = 6). *P < 0.05 shows significant difference from control responses before drug application.

Table 3. The potentiated effects of S-NONOate on the relaxations to EFS (2–32 Hz, 50 V, 0.5 ms) in mouse corpus cavernosum.

EFS	2	4	8	16	32 Hz
Control	18.96 ± 2.40	28.70 ± 2.80	38.34 ± 2.24	45.75 ± 1.77	35.72 ± 2.80
S-NONOate (1µM)	26.55 ± 3.44*	40.35 ± 3.73*	51.75 ± 4.56*	55.83 ± 3.90*	50.33 ± 2.20*
S-NONOate (5µM)	34.90 ± 5.14*	47.72 ± 4.68*	58.70 ± 4.35*	59.38 ± 4.03*	59.33 ± 2.9*

The results expressed as mean ± SE, n = 12. *P < 0.05 shows significant difference from control (n = 6–8).

Table 4. The suppressive effect of S-NONOate (1 µM) on contractions to EFS (2–64 Hz, 50 V, 0.5 ms) in mouse corpus cavernosum.

EFS	2	4	8	16	32	64 Hz
Control	1.60 ± 1.2	5.95 ± 1.32	17.55 ± 3.1	40.55 ± 2.58	68.35 ± 1.39	92.45 ± 1.62
S-NONOate	0.25 ± 0.25*	0.75 ± 0.25*	0.50 ± 0.28*	1.0 ± 0.1*	14.4 ± 4.2 *	78.2 ± 4.1

The results are presented as mean ± SE, n = 8. *P < 0.05 shows significant difference from control (n = 6–8).

Hydroxocobalamin (15,16,21) significantly inhibited the relaxations to S-NONOate and SNP. This finding may suggest that direct NO-dependent sGC activation by S-NONOate plays a critical role in the relaxant activity of the substance. On the other hand, pyrogallol, a superoxide generating agent (22) and putative indirect nitric oxide scavenger (16), did not affect relaxations induced by S-NONOate or SNP. These findings are consistent with our previous studies (16). It was suggested that pyrogallol could not penetrate into cells (23). Therefore, it can be said that S-NONOate generates NO near or at the membrane. Moreover, the finding that DETCA, an irreversible inhibitor of Cu²⁺-Zn²⁺ superoxide dismutase found in cytosol, did inhibit relaxations produced by S-NONOate and SNP supports this suggestion (16,24,25).

In the present study, S-NONOate had a significant effect on the EFS-induced nitrgic relaxations that were completely inhibited by ODQ or L-NOARG (15,26). S-NONOate potentiated the relaxations to EFS. This result may suggest that S-NONOate does not synergize with endogenous nitric oxide and release of the NO from the S-NONOate compound potentiates the nitrgic relaxations via sGC activation in mice corpus cavernosum. On the other hand, some papers suggested that NO donors, NCX-911 or FPPTO (4,7-dimethyl-1,2,5-oxadiazolo[3,4-d]pyridazine 1,5,6-trioxide) did not affect the nitrgic relaxations induced by EFS (14,27).

We also investigated the effect of S-NONOate and SNP on the neurogenic contractions induced by EFS. S-NONOate and SNP inhibited the neurogenic contractions in a dose-dependent manner in the medium containing L-NOARG. S-NONOate and SNP caused a

significant inhibition on the contractions. Administration of L-NOARG results in an EFS-induced contraction beginning immediately after initiation of the stimulus and increased the magnitude and duration of the contraction in the corpus cavernosum tissue (28). It is suggested that lack of contractile response during stimulation is due to release of nitric oxide, which also modulates the magnitude of the contraction (28–30). It was also shown that treatment with ODQ enhanced the nerve-evoked contractions, whereas zaprinast, a cGMP-phosphodiesterase inhibitor, decreased the contractile response to EFS (28). In this tissue, S-NONOate may affect the contractile mechanism of the tissue by generating some nitric oxide-like species thus activating soluble guanylate cyclase.

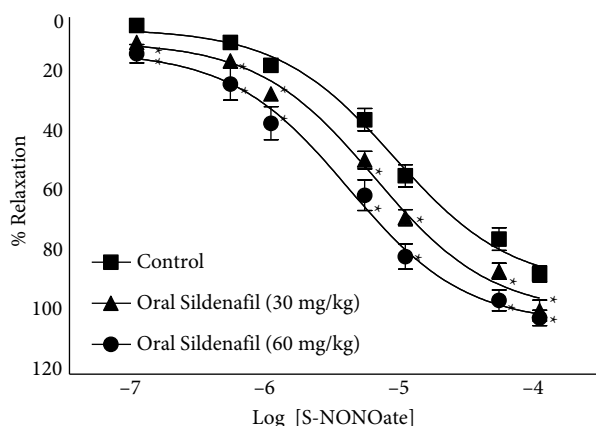
**Figure 3.** The potentiated effects of oral sildenafil treatment (30 or 60 mg/kg) on the relaxations to S-NONOate (0.1–100 µM) in mouse corpus cavernosum. Results are expressed as mean ± SE (n = 6–8). *P < 0.05 shows significant difference from control.

Table 5. The potentiated effects of oral sildenafil treatment on the relaxations to EFS in mouse corpus cavernosum.

EFS	2	4	8	16	32 Hz
Control	20.1 ± 3.4	30.9 ± 4.3	40.4 ± 3.9	43.4 ± 3.8	38.3 ± 2.0
Sildenafil (30 mg/kg)	24.1 ± 2.6	35.9 ± 3.1	43.4 ± 2.7	47.6 ± 3.1	41.1 ± 1.3
Sildenafil (60 mg/kg)	35.1 ± 4.6*	48 ± 5.7*	57.9 ± 5.4*	62.1 ± 6.1*	55.0 ± 3.9*

The results are expressed as mean ± SE, n = 8. *P < 0.05, shows significant difference from control (n = 6–8).

Oral sildenafil treatment significantly enhanced the relaxant responses to EFS and S-NONOate in the mouse corpus cavernosum. Both S-NONOate and sildenafil increased the magnitude of nitrenergic relaxations with similar potencies in the tissues. It was shown that sildenafil enhanced the nitrenergic relaxations in the previous papers (27,31). It is also well known that the action of sildenafil in corpus cavernosum is dependent on the activation of the NO-cGMP system (5). Release of the NO from the S-NONOate or nitrenergic nerves stimulated with EFS causes a sGC activation that results in enhanced cGMP levels. These results suggest that the combination of sildenafil and S-NONOate elicits a synergic effect in the erectile function. On the other hand, we used a commercial sildenafil preparation that contained some additives and preservatives. Therefore, it is possible that these additives and preservatives contributed to the effect.

Our results show that S-NONOate can relax mouse corpus cavernosum and the relaxant activity of this agent is soluble guanylate cyclase-dependent. This effect could be potentiated by sildenafil. The mechanism of the potentiating effect of sildenafil on the relaxant responses of S-NONOate may apparently involve nitric oxide-dependent activation of soluble guanylate cyclase in the tissue. Therefore, S-NONOate may be a novel way of treating male erectile dysfunction in patients with impaired endogenous nitric oxide production.

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