Rationale for the Combination of PGE₁ and S-Nitroso-glutathione to Induce Relaxation of Human Penile Smooth Muscle¹

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ABSTRACT

Many men with erectile dysfunction have been successfully treated with intracavernosal injection of prostaglandin E₁ (PGE₁) but this treatment is ineffective in 30 to 40% of patients. The goals of this study were to characterize PGE₁-induced relaxation of isolated human penile smooth muscle (penile arteries and trabecular strips), correlating this in vitro response with the clinical response to this drug, and to evaluate the effects of the combination of PGE₁ with S-nitrosoglutathione (SNO-Glu) on relaxation of isolated human penile smooth muscle. Large variability in the EC₅₀ and maximal relaxation induced by PGE₁ was observed between tissues of different patients. Patients with poor clinical response to intracavernosal alprostadil (PGE₁) had significantly larger EC₅₀ values and smaller maximal relaxation compared with patients with partial or complete clinical response to this drug. SNO-Glu consistently produced complete or near complete relaxation of human corpus cavernosum strips and penile arteries, even when the tissue responded poorly to PGE₁. In trabecular strips, the combination of PGE₁ and SNO-Glu in a 1:100 ratio demonstrated a synergistic relaxation effect. The combination of PGE₁ and SNO-Glu simultaneously increased the levels of both cAMP and cGMP in human corpus cavernosum tissue. Our results suggest that the clinical effectiveness of intracavernosal administration of PGE₁ is related to the variability of the relaxation responses of human trabecular tissue and penile arteries to this drug. The synergistic interaction of PGE₁ and SNO-Glu makes this combination an effective method to cause penile smooth muscle relaxation, a necessary step to initiate and maintain penile erection.

Intracavernosal prostaglandin E₁ (PGE₁) has been extensively used as a therapeutic agent for the treatment of erectile dysfunction (Buvat et al., 1996, 1998; Linet and Ogring, 1996). Although some success has been obtained, a considerable number of patients (about 30–40%) have not responded to treatment with PGE₁ (Porst, 1996). The reason for the lack of response to PGE₁ in refractory patients is not known, but could depend on many factors (number of receptors, transduction mechanisms, metabolism, tissue structure, etc.). Indeed, it has not been demonstrated that the efficacy in the clinical response to PGE₁ correlates with the relaxation of human penile smooth muscle (arterial and trabecular) to this drug.

PGE₁ promotes relaxation of penile arterial smooth muscle via prostacyclin (IP) receptors and of trabecular smooth muscle through interaction with prostaglandin E (EP) receptors, with some preliminary data supporting a key role for the EP₂ receptor subtype (Andersson and Wagner, 1995; Sáenz de Tejada et al., 1998). IP and EP receptors, coupled to G-proteins, increase intracellular cAMP levels through adenylyl cyclase stimulation (Andersson and Wagner, 1995). Penile smooth muscle relaxation; however, is not only mediated by the cAMP pathway but also by the nitric oxide (NO)-cGMP pathway, which 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). Activation of the cGMP pathway is brought about by the endogenous generation of NO from lacunar and vascular endothelia and nitrergic nerves. The existence of NO donors allows for pharmacological activation of the cGMP pathway and facilitates penile erection (Truss et al., 1994; Martínez-Piñeiro et al., 1998). S-Nitrosothiols are NO donor molecules, some of which are present in human plasma and tissues in physiological conditions and one of these compounds, S-nitrosoglutathione (SNO-Glu) has been reported to induce vascular (MacAllister et al., 1995) and penile tra-

ABBREVIATIONS: PGE₁, prostaglandin E₁; NO, nitric oxide; SNO-Glu, S-nitrosoglutathione; SIN-1, linsidomine chlorohydrate; PSS, physiological salt solution.

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buccal (Gupta et al., 1995) smooth muscle relaxation. In addition, promising, although preliminary clinical data have shown that intracavernosal coadministration of PGE1 and the NO donor linsidomine chloride hydrate (SIN-1) produces better erectile responses than those obtained with either compound individually (Tordjman, 1993). The mechanism behind such possible clinical benefit has not been determined.

The aim of the present study was to analyze the relaxant responses to PGE1 in human corpus cavernosum and penile resistance arteries from impotent patients and to determine whether the clinical response to PGE1 would depend, at least in part, on the degree of penile smooth muscle relaxation induced by this prostaglandin. We also sought to evaluate possible synergistic effects of combining PGE1 and SNO-Glu to cause relaxation of penile smooth muscle.

**Experimental Procedures**

**Human Corpus Cavernosum Tissues.** Human corpus cavernosum specimens were obtained from impotent men at the time of penile prosthesis insertion. All experimental protocols were approved by the local ethics committee. Tissues were maintained at 4–6°C in M-400 solution (composition per 100 ml: 4.19 g of mannitol, 0.205 g of KH2PO4, 0.97 g of KH2PO4·3H2O, 0.112 g of KCl, 0.084 g of NaHCO3, pH 7.4) until used, which ranged between 2 and 16 h after extraction (Simonsen et al., 1997).

**Vascular Reactivity of Resistance Penile Arteries.** Penile small arteries, helicine arteries (lumen diameter 150–400 µm), which are the terminal branches of deep penile arteries, were dissected carefully removing the adhering trabecular tissue, and arterial ring segments (2 mm in length) were subsequently mounted on two 40-µm wires on microvascular Halpern-Mulvany myographs (J.P. Trading, Aarhus, Denmark) for isometric tension recordings. The vessels were allowed to equilibrate for 30 min in physiological salt solution (PSS) of the following composition: 119 mM NaCl, 4.6 mM KCl, 1.5 mM CaCl2, 1.2 mM MgCl2, 24.9 mM NaHCO3, 11 mM glucose, 1.2 mM KH2PO4, and 0.027 mM EDTA at 37°C continuously bubbled with 95% O2 and 5% CO2 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 mm Hg (L100) were assessed in situ under a transmural pressure of 100 mm Hg (L100), were assessed after extraction (Simonsen et al., 1997).

**Organ Chamber Studies.** Strips of corpus cavernosum tissue (3 × 3 × 7 mm) were immersed in 8-ml organ chambers containing PSS, maintained at 37°C, and aerated with 95% O2, 5% CO2, pH 7.4. Each tissue strip was incrementally stretched to optimal isometric tension, as determined by the maximal contractile response to 1 µM phenylephrine. Then each tissue was exposed to the phosphodiesterase inhibitors zaprinast (30 µM) and 3-isobutyl-1-methylxanthine (100 µM) and allowed to incubate for 15 min, after which time tissues were treated with drug or vehicle. Tissues were allowed to incubate for another 30 min and then immediately frozen in liquid nitrogen and stored at −80°C until extraction for the cyclic nucleotide assay. Tissues were extracted by homogenization in 6% trichloroacetic acid followed by ether (H2O-saturated) extraction and lyophilization. Cyclic nucleotides were determined by enzyme-linked immunosorbent assay using a kit from Cayman Chemical Co. (Ann Arbor, MI).

**Measurement of Cyclic Nucleotides in Human Corpus Cavernosum Tissue.** Corpus cavernosum strips were immersed in 8-ml organ chambers containing PSS, maintained at 37°C, and aerated with 95% O2, 5% CO2, pH 7.4. Each tissue strip was incrementally stretched to optimal isometric tension, as determined by the maximal contractile response to 1 µM phenylephrine. Then each tissue was treated with the phosphodiesterase inhibitors zaprinast (30 µM) and 3-isobutyl-1-methylxanthine (100 µM) and allowed to incubate for 15 min, after which time tissues were treated with drug or vehicle. Tissues were allowed to incubate for another 30 min and then immediately frozen in liquid nitrogen and stored at −80°C until extraction for the cyclic nucleotide assay. Tissues were extracted by homogenization in 6% trichloroacetic acid followed by ether (H2O-saturated) extraction and lyophilization. Cyclic nucleotides were determined by enzyme-linked immunosorbent assay using a kit from Cayman Chemical Co. (Ann Arbor, MI).

**Protein Determinations.** Protein was determined using the Bio-Rad Protein Assay kit microtiter plate assay procedure (Bio-Rad, Hercules, CA) with BSA as the standard.

**Drugs and Materials.** Phenylephrine, norepinephrine (arterenol), and zaprinast were obtained from Sigma Chemical Co. (St. Louis, MO). 3-Isobutyl-1-methylxanthine was obtained from Research Biochemicals International (Natick, MA). PGE1, α-cycloheximide was kindly provided by Schwarz-Pharma (Monheim, Germany). SNO-Glu was kindly provided by Nitromed Inc. (Bedford, MA). Drugs were dissolved in distilled water at the time of the experiment.

**Statistical Evaluation.** A total of 57 patients was included in the study of individual responses to PGE1. We obtained penile resistance arteries from 22 of them and trabecular tissue from 39 (both arteries and cavernosal strips were collected from four patients). Individual EC50 (concentration required to obtain the half-maximum response induced by the compound) and maximum relaxations to PGE1 were calculated.

Patients were divided into two groups according to the clinical erectile response (poor and partial or complete) to PGE1, as assessed by the degree of penile tumescence after intracavernosal injection of alprostadil (PGE1) determined by visual and physical examination of the penis by the attending physician. The comparison of EC50, maximum relaxation, and clinical response to alprostadil between both groups was done by Student’s t test analysis, which was evaluated using StatView software (Abacus Concepts, Inc., Cary, NC) for Apple computers.

Evaluation of the effects of PGE1 and SNO-Glu, alone or in combination, on penile smooth muscle relaxation and cyclic nucleotide accumulation was performed in tissues from another group of 10 patients (five for relaxation studies and five for cyclic nucleotide determinations). Complete concentration-response curves were obtained and compared by a two-factor ANOVA statistical analysis using StatView software for Apple computers. Statistical analysis of tissue cyclic nucleotide levels was performed by ANOVA followed by a Student-Newman-Keuls post hoc test using GraphPad (San Diego, CA) InStat software.

**Determination of synergy for the combination of PGE1 and SNO-Glu was performed according to the “isobole method” Berenbaum, 1989). This method applies the equation Δd/ΔD = 1 to define the additive effects between two agents, A and B, where Δd and ΔD are the concentrations of agents A and B in the combination that is needed to obtain a specified level of the effect (relaxation), and ΔD and ΔA are the single concentrations of the drugs required to achieve the same level of effect. Synergy is assumed when solving this equation gives a value less than unity. Applying this equation, we determined, in each patient, the theoretical concentrations of the PGE1 and SNO-Glu combination (always in a 1:100 M ratio) needed to obtain several specified levels of relaxation of trabecular smooth muscle (10, 20, 40, 60, and 70%) when additive effects between the two agents are assumed.

The following equation is the application of the isobole method described by Berenbaum (Δd/ΔD + Δd/ΔD = 1) to our model. We used
this equation to obtain the theoretical concentration of PGE1 in the combination ([SNO-Glu] is 100-fold higher) needed to obtain a specified level of relaxation when only additive effects are present.

\[
\frac{[\text{PGE1}]}{[\text{PGE1 alone}]} + 100 \cdot \frac{[\text{PGE1}]}{[\text{SNO-Glu}]} = 1
\]

Using these data we constructed a theoretical curve that was compared with the curve experimentally obtained with the combination of PGE1 and SNO-Glu. Comparison of these curves was performed by a two-factor ANOVA using StatView software.

**Results**

**Evaluation of Individual Responses to PGE1 in Human Penile Smooth Muscle.** Relaxations induced by PGE1 were individually studied in strips of corpus cavernosum from 39 impotent patients and in penile resistance arteries from 22 patients (Fig. 1). Notable variability was observed with respect to the relaxant responses produced by PGE1 in trabecular smooth muscle from different patients, resulting in a range of several orders of magnitude in individual EC50 values (mean 0.226 μM, standard deviation 0.418, range from 0.01 to 10 μM) and variability in maximal relaxation (mean 83.5%, standard deviation 12.0, range from <60 to 100%). Similar results were obtained when PGE1-induced relaxations were evaluated in individual penile resistance arteries, showing variability in EC50 values (mean 0.896 μM, standard deviation 1.097, range from 0.01 to 10 μM), and more noteworthy in maximal relaxation (mean 73.3%, standard deviation 23.2, range from 50 to 100%).

Analysis of the data showed a statistically significant correlation between the clinical effectiveness of PGE1 and the magnitude of the relaxations obtained in vitro with PGE1. Lower EC50 values for PGE1 and larger maximal relaxations were observed when corpus cavernosum strips or penile arteries originated from patients with better clinical response to intracavernosal injection of PGE1 (Fig. 2).

**Relaxant Responses Induced by the Combination of SNO-Glu and PGE1 on Human Penile Smooth Muscle.** The nitrosothiol SNO-Glu produced consistent relaxations of human corpus cavernosum strips and penile resistance arteries in a concentration-dependent manner. Administration of SNO-Glu always elicited a near-to-full relaxation of both penile smooth muscle preparations even when tissue from the same patient responded poorly to PGE1. Representative examples of this observation are shown in Fig. 3. The coadministration of PGE1 and SNO-Glu in a 1:100 proportion (PGE1:SNO-Glu) caused concentration-dependent relaxation of both trabecular and arterial smooth muscle.

Figure 4 shows relaxation to PGE1 alone compared with relaxation caused by the combination of PGE1 and SNO-Glu plotted as only PGE1 concentrations. There was a significant shift to the left of the PGE1 concentration-response curve with the coadministration of SNO-Glu. (In trabecular tissue, EC50 for PGE1 alone was 0.083 ± 0.005 μM versus 0.006 ± 0.001 μM for PGE1 + SNO-Glu, P < .01; in penile arteries, EC50 for PGE1 alone was 0.402 ± 0.160 μM versus 0.043 ± 0.023 μM for PGE1 + SNO-Glu, P < .01. Maximum responses for PGE1 in trabecular tissue were 71.0 ± 5.1% versus 95.2 ± 2.1% with the PGE1 + SNO-Glu combination, P < .01; and 52.9 ± 13.9% versus 91.4 ± 2.6% for PGE1 versus PGE1 + SNO-Glu, respectively, P < .01 in penile arteries.)

The relaxation response of the PGE1 and SNO-Glu combination was also compared with the relaxation-response curve induced by SNO-Glu alone, but this time plotted as only the concentration of SNO-Glu in the combination, as shown in Fig. 5. There was a significant shift to the left of the SNO-Glu concentration-response curve with the coadministration of PGE1 in trabecular tissue, but not in penile arteries. The EC50 for SNO-Glu alone was 2.56 ± 1.3 μM versus 0.58 ± 0.16 μM for SNO-Glu + PGE1 (P < .05) in trabecular tissue. Maximum responses were 94.4 ± 4.2% for SNO-Glu versus 95.2 ± 2.1% for SNO-Glu + PGE1 and were not significantly different from each other.

The possible existence of synergism in the relaxation of penile smooth muscle between PGE1 and SNO-Glu was analyzed from the responses obtained with the drug combination in human penile trabecular and arterial smooth muscle. The actual experimental curve with the concentrations of the PGE1 and SNO-Glu combination needed to obtain several
specified levels of relaxation (10, 20, 40, 60, and 70%) was compared with the calculated theoretical curve assuming additive effects of both drugs (under Experimental Procedures). Significantly lower concentrations of the PGE1 and SNO-Glu combination were required than those that would be expected if only additive effects occurred between both compounds. This suggests a synergistic interaction between PGE1 and SNO-Glu in the relaxation of trabecular smooth muscle (Fig. 6, top). However, PGE1 and SNO-Glu did not exert synergistic effects in causing relaxation of penile arterial smooth muscle (Fig. 6, bottom).

**Effect of the Combination of PGE1 and SNO-Glu on Cyclic Nucleotide Accumulation in Corpus Cavernosum Tissue.** Tissue levels of cGMP were not significantly altered by incubation with PGE1 (1 μM), but a remarkable increase was observed when treating the tissues with SNO-Glu (100 μM). Incubation of trabecular tissues with the combination of PGE1 and SNO-Glu induced a significant increase in cGMP content compared with control (Fig. 7, top).

CAMP content was increased by incubation of tissues with PGE1 (1 μM). Incubation of the strips with SNO-Glu (100 μM) did not modify tissue CAMP levels and the combination of SNO-Glu + PGE1 produced an increase in CAMP content that was similar to that obtained with PGE1 alone (Fig. 7, bottom).

**Discussion**

In clinical trials, response rates in the range of 40 to 70% to intracavernosal injection of PGE1 have been reported in patients suffering from erectile dysfunction (Buvat et al., 1996, 1998; Linet and Ogring, 1996; Porst, 1996). This observation, supported by extensive clinical experience, presents intracavernosal injection of PGE1 as an efficacious treatment for impotence. However, a considerable number of patients do not respond to such treatment for unknown reasons. Furthermore, the dose of PGE1 required to achieve satisfactory erections is markedly variable, ranging from 0.5 to 20 μg (Linet and Ogring, 1996), with some patients needing doses as high as 40 μg to obtain responses (Porst, 1996).

Analysis of in vitro responses to PGE1 in penile arterial and trabecular tissues from impotent patients shows a great degree of variability in the relaxation of human penile smooth muscle to this prostanoid. The variability of PGE1-induced relaxations was observed in human penile resistance arteries as well as corpus cavernosum strips and it affected both the sensitivity to PGE1, obtaining EC50 values separated by several orders of magnitude, and the maximum relaxation, which varied, especially in penile arteries, from less than 50% to complete relaxation (100%). It is interesting to note that the in vitro response to PGE1 in human penile tissues correlated with the clinical response to PGE1 in the patients from whom the tissue was obtained. Our results show that poor responses to intracavernosal alprostadil are associated with diminished maximum response and increased EC50 of the relaxations to PGE1 in vitro. These data suggest that a lack of clinical response to PGE1 may be, in many cases, due to the inability of this prostanoid to relax penile arterial and/or trabecular smooth muscle sufficiently.
The mechanisms underlying reduced penile smooth muscle relaxation to PGE1 are currently unknown.

The rationale for the use of a combination of PGE1 with other drugs, for the treatment of erectile dysfunction, is to increase the number of responding patients and to diminish the dose of PGE1 required to achieve adequate responses because this prostanoid may cause penile pain. To date, the most widely used combination therapy includes PGE1, papaverine, and phentolamine, which has been shown to improve response rates (Padma-Nathan, 1990; McMahon, 1991; von Heyden et al., 1993).

In the present study, we have investigated the possibility of combining the administration of PGE1, which activates the cAMP pathway, with the activation of the NO/cGMP pathway, which plays a prominent role in the physiological mechanism of penile erection (Kim et al., 1991; Burnett et al., 1992; Rajfer et al., 1992; Trigo-Rocha et al., 1993). Pharmacological stimulation of cGMP production in penile smooth muscle can be achieved with NO donors, which are compounds that release NO but are more stable, allowing for their use as therapeutic drugs. These NO donors have been reported to relax penile smooth muscle (Sáenz de Tejada et al., 1989; Bush et al., 1992) and have been evaluated for the treatment of erectile dysfunction as a potential alternative treatment to PGE1 (Truss et al., 1994; Martínez-Piñeiro et al., 1998).

Our results show that SNO-Glu, a nitrosothiol present in plasma, is an effective relaxant of human penile resistance arteries and human trabecular smooth muscle. When the tissues, primarily arteries but also trabecular smooth muscle, relaxed poorly to PGE1, SNO-Glu alone was able to induce a strong response (near full relaxation) in those tissues. The combination with SNO-Glu improved the relaxations to PGE1 in human penile arteries and in trabecular smooth muscle, causing a shift to the left of the PGE1 concentration-response curve and a larger maximum relaxation compared with PGE1 alone. Although in penile arteries the combination of PGE1 and SNO-Glu did not improve the relaxation response to SNO-Glu alone, this combination did relax human trabecular smooth muscle more efficiently than SNO-Glu or PGE1 alone. Furthermore, a synergistic interaction between the two compounds was observed when given in combination to relax trabecular smooth muscle, as shown in the analysis of synergism using the isobole method (Berenbaum, 1989). The constructed theoretical curve for the existence of additive effects between PGE1 and SNO-Glu predicted significantly higher concentrations of both drugs than those experimentally determined to produce the same level of relaxation, confirming the existence of synergism. Based on these results, we propose that the combination of PGE1 and SNO-Glu has significant advantages for the relaxation of penile trabecular smooth muscle compared with the separate administration of these drugs.

Improved relaxant properties of the combination of PGE1 and SNO-Glu are probably due to the ability of this treatment to trigger the activity of the two essential pathways responsible of penile smooth muscle relaxation (i.e., cAMP and cGMP pathways). The activation of both pathways was demonstrated in our experiments by the accumulation of both cyclic nucleotides in human cavernosal tissue after...
treatment with the combination. Potentiation of the NO/cGMP pathway with oral sildenafil together with local PGE1 is used on occasions in the clinical management of patients who are partial responders to PGE1. The existence of synergism between NO and cAMP is not well recognized in human penile smooth muscle. Furthermore, other investigators have tested the effect of combining two other molecules, vasoactive intestinal polypeptide and SIN-1, stimulants of cAMP and cGMP, respectively, in human corpus cavernosum and cavernous artery, reporting a lack of synergistic interaction (Hempelmann et al., 1995). The difference between the results of Hempelmann et al. (1995) and those of our study may be due to the specificity of stimulating different receptor pathways. The combination of PGE1 with SIN-1 has been shown to be more effective to produce erections than PGE1 or SIN-1 individually, when evaluated in a clinical trial involving 50 impotent patients (Tordjman, 1993). Our study provides new information that may explain the mechanism underlying this clinical finding. The synergistic interaction of PGE1 and SNO-Glu was specific to trabecular smooth muscle because it was not observed in penile arteries, despite the fact that these two tissues are anatomically related, and functionally key to the erectile process.
In summary, the present study characterizes the responses to PGE₁ in human trabecular smooth muscle and penile resistance arteries, which both show large variability in response to PGE₁. We found a correlation of the in vitro response with the clinical erectile response that had not been previously reported. These results may explain why some patients respond and others do not to intracavernosal PGE1. This study also demonstrates that SNO-Glu consistently relaxes penile smooth muscle whether it relaxes well or not to PGE1. This suggests that the clinical response to PGE₁ may be limited in some patients by the specific lack of response of penile smooth muscle to this prostanoid, while maintaining the ability to relax in response to agents that activate alternative relaxant pathways. We also found that a combination of PGE₁ and SNO-Glu has a synergistic interaction to relax penile trabecular smooth muscle. Such a combination may have significant therapeutic advantages in the treatment of male erectile dysfunction.

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