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RAPID COMMUNICATION

Lack of nitrate tolerance in isosorbide dinitrate- and sodium nitroprusside-induced relaxation of rabbit internal anal sphincter

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Abstract

AIM: To investigate the tolerance development against the relaxant effect of nitric oxide donating drug isosorbide dinitrate (ISDN) and sodium nitroprusside (SNP) in internal anal sphincter (IAS) smooth muscle.

METHODS: Relaxation responses of ISDN, and electrical field stimulation (EFS) were obtained before and after tolerance induction by ISDN incubation.

RESULTS: ISDN (10^{-7}-10^{-4} mol/L) and SNP (10^{-8}-10^{-4} mol/L) caused a concentration-dependent relaxation on the basal tonus of the isolated rabbit IAS strips. After a period of 2 h incubation of the 6 x 10^{-4} mol/L ISDN the relaxation effects of ISDN and SNP did not change compared to control strips. EFS evoked frequency-dependent relaxation in internal anal sphincter smooth muscle and E_{max} obtained from control strips were not changed in ISDN tolerance-inducing condition. In this study nitrate tolerance was not observed in rabbit IAS smooth muscle.

CONCLUSION: This result shows that nitric oxide donating drugs relaxes the internal anal sphincter of the rabbits without the development of tolerance.

Key words: Internal anal sphincter; Nitrate tolerance; Isosorbide dinitrate; Sodium nitroprusside; Nitric oxide

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INTRODUCTION

The internal anal sphincter (IAS) is the specialized continuation of the circular smooth muscle layer of the rectum. It is innervated by autonomic nerve system and plays an important role in anorectal physiology. Prominent nonadrenergic noncholinergic (NANC) innervations of IAS have been demonstrated[1-3]. *In vivo* and *in vitro* studies showed that the NANC neurons caused relaxation of the IAS[4-6]. The tonic resting pressure of IAS is called "anal resting pressure". The IAS pressure has been found to display an overshoot phenomenon in the patients with chronic anal fissure[7,8].

A number of drugs and chemicals were studied to decrease basal and precontracted tonus of IAS *in vitro*[9-14]. It has been showed that some drugs such as glyceryl trinitrate (GTN) and isosorbide dinitrate (ISDN) as nitric oxide (NO) donor might be used in treatment of chronic anal fissure[15,16]. However, prolonged exposure to high levels of nitroglycerine and other organic nitroesters is known to induce tolerance in the cardiovascular system drug both in humans and in experimental animals[17-20]. The same type of tolerance was also shown in rabbit penile tissue against relaxant effect of ISDN[21,22].

It would be reasonable to expect that a similar type of tolerance may develop in internal anal sphincter smooth muscle against the relaxant effect of NO donating drugs. The present study is conducted to test this hypothesis, in rabbit IAS strips smooth muscle using ISDN as a tolerance-inducing agent.

MATERIALS AND METHODS

Isolation of rabbit internal anal sphincter

Twelve white New Zealand rabbit weighing between 2.5-3 kg were used in this study. Rabbits were sacrificed by cervical dislocation after sodium pentobarbital (40 mg/kg per body weight) anesthesia. The entire anal canal was isolated in continuation with a part of the rectum and transferred to oxygenated (95% O_2 and 5% CO_2) Krebs' solution of following composition (mmol/L): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $NaHCO_3$, 25; $MgSO_4$, 1.2; KH2PO4, 1.2, glucose, 11. The anal canal was carefully freed of all extraneous tissues. It was opened along its anterior wall, rinsed quickly, and pinned on a wax block containing oxygenated Krebs' solution, at its *in vivo* length as mucosa facing up. The mucosa was removed by sharp dissection under magnification. Distal 2 mm of the anal canal was used for preparation of 2 mm width and 1 cm long IAS smooth muscle strips. Each end of the IAS strips was tied with fine silk ligatures.

Measurement of isometric tension

IAS strips transferred to 10 mL tissue baths containing oxygenated (95% O_2 and 5% CO_2) Krebs' solution maintained at 37℃. One end of each strip was anchored to the bottom of the tissue bath, and other end was anchored to force transducer (model FT03 Grass Instruments Co., Quincy, MA) under an initial load of 2 g for the measurement of isometric tension on a pen polygraph recorder (model 79E Grass Instruments Co., Quincy, MA). After equilibration, relaxation responds of ISDN, SNP and Papaverine were obtained in different concentrations by adding these agents to the bath in a cumulative manner. Maximum relaxations induced by ISDN and SNP were expressed as the percentage of relaxation induced by papaverine.

Induction of ISDN tolerance

After obtaining pre-incubation concentration-response curves with relaxant agents, IAS muscle strips were incubated by ISDN at a concentration of 6×10^{-4} mol/L for 2 h in different experiments in order to induce the tolerance. After exposure to the tolerance-inducing condition, strips were washed repeatedly with Krebs' solution for 15 min and then exposed cumulative concentrations of SNP, ISDN and papaverine again and post-incubation-response curves were obtained.

Electrical field stimulation (EFS)

In another series of experiments, rabbit IAS muscle strips were electrically stimulated. Stimulation was provided *via* two parallel platinum electrodes. It was conducted at sequential frequencies of 2 Hz, 4 Hz, 8 Hz, 16 Hz as square-wave pulses of 50 V (0.8 ms) delivered by a current amplifier and a stimulator (S 88, Grass). Muscle strips were allowed to return to the base-line of normal basal tonus before each new frequency was delivered during EFS. In all studies, the duration of the electrical stimulation was 10 s. In order to eliminate adrenergic and cholinergic components of nerve stimulation and to study relaxation responses to the non adrenergic, non-cholinergic nerves all experiments were performed in presence of atropine (10^{-5} mol/L) (muscarinic nerve blocker) and guanethidine (10^{-6} mol/L) (adrenergic nerve blocker). The application of the nitric oxide synthase inhibitor L-NAME [N (G)-nitro-L-arginine of methyl ester], a potent inhibitor of NO synthase, inhibited the relaxation responses, which were restored by application of L-arginine, and it has been concluded that the relaxation responses elicited by EFS were nitrergic-mediated activation. After the EFS relaxation was obtained, the same procedure was repeated in the presence of ISDN tolerance-induced conditions.

Drugs

Guanethidine sulphate, atropine sulphate, sodium nitroprusside, L Name, L arginine, and papaverine hydrochloride were dissolved in distilled water. ISDN was dissolved in dimethylsulphoxide and dilutions were made in distilled water. All drugs were purchased from Sigma Chemical Company.

Statistical analysis

All data are expressed as mean ± SE. Tissue relaxant responses were expressed as percentage of papaverine-induced relaxation. In order to evaluate the effect of agonists; maximum responses (E-max) and pD2 values were calculated. The concentration-response data obtained in each individual experiment were plotted as the response/concentration (y) against the response (x). This produced a straight- line relationship in each experiment as predicted from the Scathard equation for drug-receptor interaction: Response/Concentration = $-1/EC_{50} \times$ Response + Maximum response/EC_{50}.

The pD2 value was expressed as the negative logarithm of the EC50 value. Inter-group differences were tested by Wilcoxon test. *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

The IAS muscle strips developed spontaneous basal tonus several minutes after equilibration. ISDN (10^{-7}-10^{-4} mol/L) caused a concentration-dependent relaxation on the basal tonus of the isolated rabbit IAS muscle strips. The maximum relaxation caused by ISDN was 74.4 ± 7.2 percent of the relaxation caused by papaverine (Table 1, Figure 1). pD2 value (negative logarithm of the EC_{50} value) was 5.48 ± 0.05 (Table 1). After a period of 2 h incubation by 6×10^{-4} mol/L ISDN, the relaxation effect induced by ISDN was not change compared to control strips (Figure 2A). pD2 value of ISDN was not changed either by the attempt to induce tolerance with ISDN incubation. When we compared the concentration-response curves of the strips incubating by ISDN and control, there was no statistically significant difference in E_{max} values between groups (Table 1).

SNP (10^{-8}-10^{-4} mol/L) caused a concentration-dependent relaxation on the basal tonus of the isolated

Table 1 Maximum relaxation responses (E_{max}) and pD2 values to agonists in strips of internal anal sphincter smooth muscle from two groups of rabbits (n = 6 each experiments)

		Control	ISDN-induced tolerance
ISDN	E_{max}	74.4 ± 7.2	75.0 ± 6.7
	pD2	5.48 ± 0.05	5.40 ± 0.06
SNP	E_{max}	96.7 ± 3.3	98.8 ± 1.4
	pD2	5.48 ± 0.05	5.57 ± 0.05
EFS	E_{max}	72.5 ± 7.4	78.1 ± 6.3

E_{max}: The maximum relaxation response (percentage of papaverine induced relaxations); pD2: Negative logarithm of the EC_{50} value.

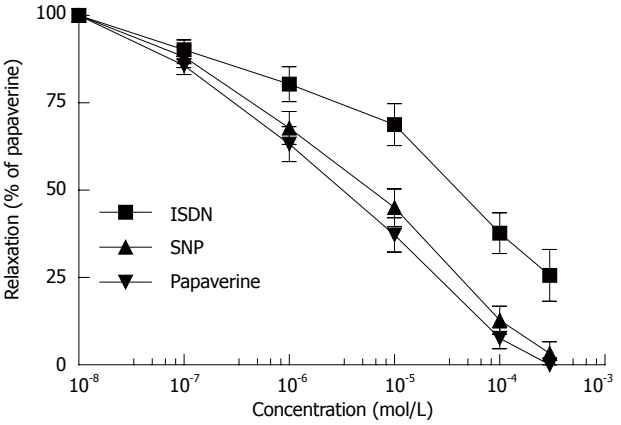

Figure 1 The relaxant effects of ISDN, SNP and papaverine, in isolated rabbit internal anal sphincter smooth muscle strips. Papaverine induced relaxation was accepted as 100%. Maximum relaxations of ISDN and SNP were expressed as percentage of relaxation induced by papaverine. Each curve represents the mean ± SE for 6 experiments.

rabbit IAS muscle strips the maximum relaxation induced by SNP was 96.7 ± 3.3 percent of the relaxation induced by papaverine (Figure 1). The attempt to induce tolerance with ISDN incubation had no significant effect on these responses and there was no change in E_{max} values of drug (Table 1, Figure 2B). pD2 values of SNP in basal tonus and ISDN-induced condition were 5.60 ± 0.04 and 5.57 ± 0.05, respectively.

EFS evoked frequency-dependent relaxation of IAS smooth muscle strips in the presence of 5×10^{-5} mol/L guanethidine and 10^{-6} mol/L atropine. E_{max} (72.5 ± 7.4) obtained from vehicle incubated control strips was not significantly changed in ISDN tolerance-inducing condition (78.1 ± 6.3, Table 1, Figure 2C)

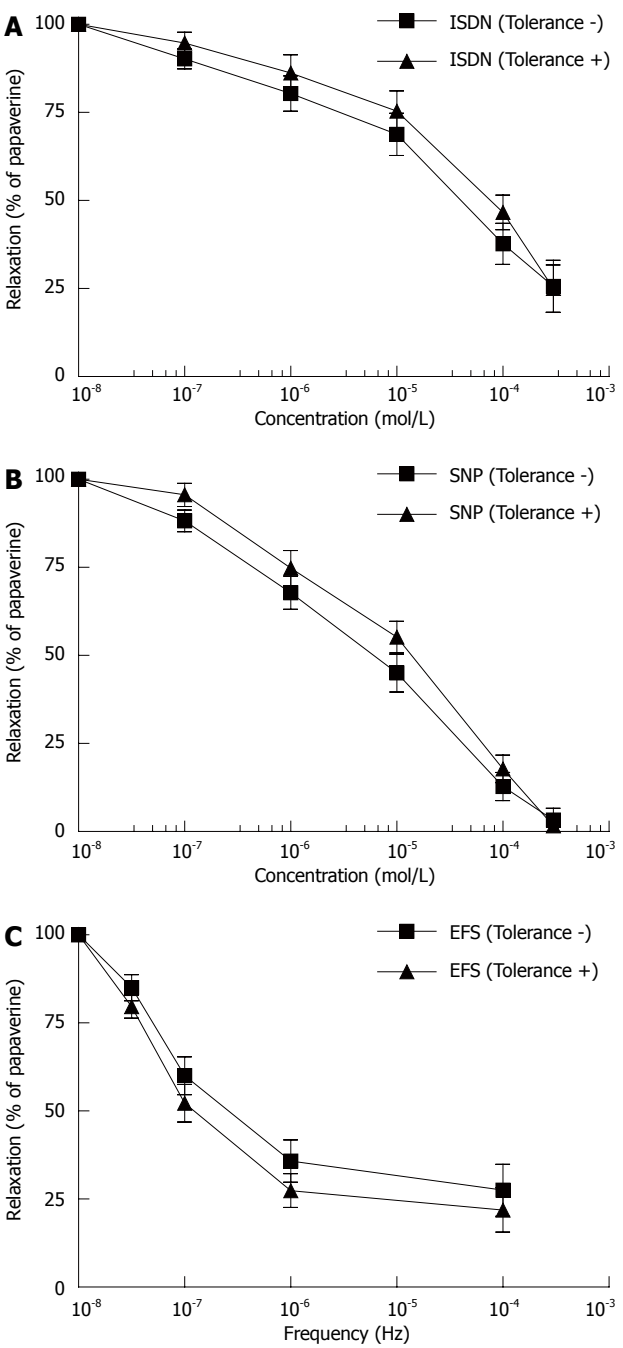

Figure 2 The influence of *in vitro* isosorbide dinitrate (ISDN)-induced tolerance on relaxant effects of ISDN (**A**), relaxant effects of sodium nitroprusside (SNP) (**B**) and relaxant effects of electric field stimulation (**C**) compared with control values in isolated strips of internal anal sphincter smooth muscle. Each curve represents the mean ± SE for 6 experiments.

DISCUSSION

Traditionally, lateral internal sphincterotomy was the gold standard treatment for chronic anal fissures. However this procedure is associated with a risk of incontinence to some degree in 30% of patients[23]. Recent studies have suggested that NO release as an important mediator from enteric inhibitory neurons and causes the relaxation of the IAS muscle[24,25]. In a similar way, NO donating agents lead to IAS relaxation *via* a non-adrenergic non-cholinergic pathway[26]. This effect of NO donating agents such as ISDN and GTN can produce beneficial clinical effects in the healing of anal fissures by relaxing of IAS. Topical application of GTN and ISDN seems to promote the healing process of chronic anal fissures[16,27-30].

In this study, the organic nitrates ISDN and SNP caused concentration-dependent relaxation in rabbit IAS muscle strips. The maximum relaxation effects induced by ISDN and SNP in basal tonus were 74% and approximately 100% respectively as compared to papaverine response.

Organic nitrates have long been used in treating

cardiovascular diseases due to their potent relaxation effects on the vascular smooth muscle. This relaxant effect is generated by *in vivo* metabolic conversion of these compounds to nitric oxide (NO), which then stimulates vascular relaxation by producing of cyclic GMP[31,32].

One of the major drawbacks of NO donating agent therapy in cardiovascular diseases is the rapid development of pharmacological tolerance. It develops shortly after the beginning of treatment and occurs both *in vitro* and *in vivo* conditions[32,33]. Both human and experimental studies showed that prolonged exposure to high levels of nitroglycerin and other organic nitroesters induce tolerance against the cardiovascular effects of the drugs[17-20].

So far, only one study has been reported about NO donating drug tolerance in anorectal smooth muscle[34]. Wang *et al* showed that GTN caused significant and sustained relaxation of anorectal smooth muscle in the anaesthetized rat without evidence of tolerance development[34]. In this study the relaxation response induced by NTG was not diminished in the anorectum in conditions that produced vascular tolerance.

The mechanisms of nitrate tolerance are complex and multi-factorial, including processes such as decreased tissue sulphydryl availability, reduced bioactivation of the parent drug, physiological compensation and tissue oxidative stress[32,33,35]. Some investigators suggested that tolerance may arise from one or more steps involving the biotransformation process of organic nitrates prior to the formation of NO[21,22]. The action of nitrates is dependent on the liberation of from their molecule which subsequently activating guanilate cyclase[36]. The production of cGMP (the alleged mediator of nitrate-induced vascular smooth muscle relaxation) is reduced in tissues, which develop tolerance[37]. cGMP accumulation, was significantly decreased after NTG pre-incubation in the rat aorta but not in the rat anorectal smooth muscle and anal sphincter[34].

Prolonged exposure of rabbit IAS muscle strips to high concentration of ISDN did not change the relaxant effect of the NO donating agents. As far as we know this result is the first *in vitro* evidence showing that rabbit IAS smooth muscle is spared from the tolerance induced by organic nitrates.

In conclusion, the main finding of the current study is that nitrate tolerance is not developing in isolated rabbit internal anal sphincter smooth muscle.

COMMENTS

Background

The internal anal sphincter pressure has been found to display an overshoot phenomenon in the patients with chronic anal fissure. It has been showed that some drugs such as glyceril trinitrate (GTN) and isosorbide dinitrate (ISDN) as nitric oxide (NO) donor might be used in treatment of chronic anal fissure. Prolonged exposure to high levels of nitroglycerin and other organic nitroesters is known to induce tolerance in the cardiovascular system and penile tissue both in humans and in experimental animals.

Research frontiers

In this study, the organic nitrates ISDN and SNP caused concentration-dependent relaxation in rabbit IAS muscle strips. The main finding is that nitrate tolerance is not developing in isolated rabbit internal anal sphincter smooth muscle.

Innovations and breakthroughs

Our study is the first *in vitro* evidence showing that rabbit IAS smooth muscle is spared from the tolerance induced by organic nitrates. Only one *in vivo* study has been reported about NO donating drug tolerance in anorectal smooth muscle before.

Applications

Although animal findings cannot be assumed to apply to human beings and *in vitro* experimentation should not lead straight to clinic conclusions, topical use of NO donating drugs should be continued for the treatment of anal fissure disease without a fear of tolerance development. In the future, this result may be verified in human studies.

Peer review

The paper deals nicely with an interest subject. The main finding of the current study is that nitrate tolerance is not developing in isolated rabbit internal anal sphincter smooth muscle. Topical use of NO donating drugs should be continued for the treatment of anal fissure disease without a fear of tolerance development.

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