Effects of cGMP and the nitric oxide donors glycercyl trinitrate and sodium nitroprusside on contractions in vitro of isolated myometrial tissue from pregnant women

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The purpose of this study was to determine the relaxant effects in vitro of two nitric oxide donors, glycercyl trinitrate and sodium nitroprusside, which are currently available for use in vivo, on contractions of non-labouring myometrium from pregnant women. Since nitric oxide also mediates relaxation by increasing the concentration of cGMP, sensitivity to 8-bromo-cGMP (a cGMP analogue) was also determined. The effects of the K+-channel opener lemakalim and of the Ca2+-channel blocker nifedipine were studied for comparison. After the addition of glycercyl trinitrate (0.1–100 μmol l−1), sodium nitroprusside (0.1–100 μmol l−1) or 8-bromo-cGMP (0.001–3 mmol l−1), the spontaneous rhythmic contractility of myometrial strips was inhibited in a concentration-dependent manner: the maximum inhibition produced by the highest tested concentration of each drug was 40 ± 7%, 53 ± 8% and 39 ± 8% of the original degree of contraction, respectively. Myometrial contractions were completely abolished by lemakalim and by nifedipine and verapamil at concentrations of ≥ 10−5 mol l−1. The nitric oxide donors, glycercyl trinitrate and sodium nitroprusside, attenuate myometrial contractions and are therefore useful as tocolytic agents. However, at equimolar concentrations in vitro, the ability of glycercyl trinitrate and sodium nitroprusside to attenuate myometrial contractions is less than that of lemakalim, nifedipine and verapamil. Controlled trials are required to determine the side-effects and clinical efficacy of each of these agents in vivo.

Introduction

Nitric oxide (NO), formed from the guanadino nitrogen of L-arginine by the action of NO synthase, was originally described as an endothelium-derived vasodilator (Palmer et al., 1987). NO is widely distributed and its many roles include action as a smooth muscle relaxant, a neurotransmitter and as a bactericidal agent (Moncada and Higgs, 1993). In the reproductive tract, NO is implicated in the physiology of menstruation and, during pregnancy, disorders of the L-arginine–NO pathway may contribute to the pathophysiology of pre-eclampsia and intrauterine growth retardation (Norman and Cameron, 1996). The potential efficacy of NO as a uterine relaxant has been demonstrated in rats, in which NO acts as a smooth muscle relaxant in the myometrium. NO inhibits spontaneous contractions in vitro (Yallampalli et al., 1993a); and a reduction in endogenous NO is implicated in the mechanism of the onset of labour (Yallampalli et al., 1994).

An agent that effectively inhibits myometrial contractions in women is urgently required for the treatment of preterm labour, a condition that contributes to the premature delivery and death of over 1000 infants in the UK annually (Office of Population Censuses and Surveys, 1993). Two previous studies have suggested that NO donors inhibit human myometrial contractility (Buhimschi et al., 1995; Lee and Chang, 1995). However, the NO donors used in these studies, diethylamine/NO, and streptozocin, which releases NO under ultra-violet radiation, are not currently available for use in vivo. In contrast, glycercyl trinitrate (GTN) and sodium nitroprusside (SNP) are widely used clinically and have been used extensively to treat cardiovascular disease. Furthermore, one of these drugs, GTN, has been used in a preliminary uncontrolled study for the treatment of preterm labour, and is currently undergoing controlled trials for this indication (Lees et al., 1994). The purpose of the present study was threefold. (1) We aimed to determine the relaxant effect in vitro of GTN and SNP on the myometrium in pregnant women. (2) In order to
determine the mechanism of action of GTN and SNP, we studied the effect of 8-bromo-cGMP, an analogue of the second messenger cGMP through which NO exerts its actions. To determine the potential confounding effect of endogenous NO production on myometrial contractility, we measured myometrial contractions after adding the NO synthase inhibitor N^\* -nitro-l-arginine methyl ester (l-NNAME). (3) In order to determine the potential efficacy of GTN and SNP in comparison with other potential tocolytic agents, the effects of the K^+ -channel opener lemakalim and of the Ca^{2+} -channel blockers nifedipine and verapamil were also examined. These agents are known to abolish myometrial contractions in vitro when administered in sufficient concentrations (Hollingsworth et al., 1987; Morrison et al., 1993).

**Materials and Methods**

**Subjects and preparation of tissues**

Women undergoing lower segment Caesarean section at term (2 \geq 37 weeks gestation) were recruited into the study. All gave their informed consent and the study was approved by our local ethics committee. After the baby and the placenta had been delivered, a strip of myometrium approximately 4 mm wide was obtained from the upper border of the uterine incision. The myometrium was immediately placed in normal saline at 4°C. Experiments were performed on tissues within 12 h of delivery. Myometrium from labouring and non-labouring women was analysed separately.

**Methods**

Strips of myometrium 15 mm long, 2 mm wide and 2 mm deep were cut and suspended under a resting tension of 2 g in organ baths in Krebs' solution at 37°C containing 118 mmol NaCl l^{-1}, 4.8 mmol KCl l^{-1}, 2.5 mmol CaCl_2 l^{-1}, 1.2 mmol MgSO_4 l^{-1}, 1.2 mmol KH_2PO_4 l^{-1}, 24 mmol NaHCO_3 l^{-1}, and 11.1 mmol glucose l^{-1}, and gassed with 95% O_2 and 5% CO_2. Tension was measured with Grass FTO3 isometric transducers and displayed on a MacLab system. Tissues were equilibrated for 2 h before drugs were added, during which time most tissues developed stable rhythmic activity (see Results). Drugs were then added in cumulative fashion and their effects on the magnitude and frequency of contractions were assessed. Experiments were performed on more than one strip from each woman; the number of strips and the number of patients used for each experiment are described in the figure legends. A control strip was part of each protocol. A full concentration–response curve was produced from each myometrial strip. Tension in the experimental period was taken as the mean of three consecutive contractions once a steady state had been achieved.

**Drugs**

GTN was obtained from Napp Laboratories (Cambridge). 8-Bromoguanosine 3',5' cyclic monophosphate sodium salt (8-bromo-cGMP), nifedipine, SNP and verapamil hydrochloride were obtained from Sigma Chemical Co. (Poole), and lemakalim was a generous gift from S. Trowbridge (SmithKline Beecham Pharmaceuticals, Welwyn Garden City). All drugs were dissolved in saline (0.9% w/v), except for lemakalim, which was prepared as a stock solution of concentration 10 mmol l^{-1} in 70% ethanol, and further dilutions were made in saline.

**Statistical analysis**

Contractions were quantified by measuring muscle tension in grammas. Once a steady state had been reached, a reduction in the magnitude of spontaneous contractions was expressed as a percentage inhibition (mean ± SEM) of the contraction obtained immediately before the first addition of the drug. The frequency of contractions was measured as the number of contractions h^{-1}. Statistical analysis was carried out using one-way analysis of variance followed by Fisher's PLSD test. A value of P < 0.05 was considered significant.

**Results**

**Spontaneous activity of myometrial strips**

When suspended in Krebs' solution, 85% of myometrial strips (58 of 68 strips from 13 patients) taken from pregnant women at term before labour began developed spontaneous rhythmic activity (Fig. 1a). In a series of ten control strips from ten women, rhythmic activity increased steadily with time to reach a magnitude of 5.1 ± 1.1 g and a frequency of 12.7 ± 1.3 contractions h^{-1} within 2 h, and remained constant for at least 3 h thereafter (Fig. 1b, c). In contrast, only 23% of myometrial strips (14 out of 62 strips from 12 patients) from labouring women developed rhythmic activity with a mean magnitude and frequency of contractions of 3.3 ± 0.7 g and 14.7 ± 0.8 contractions h^{-1}, respectively. In each group, the figure for mean amplitude and frequency of contractions is derived from the analysis of spontaneously active strips only. The magnitude and frequency of contractions did not differ significantly between myometrial strips obtained from labouring and non-labouring women. In view of the small proportion of spontaneously contractile myometrial strips obtained from labouring women, it seemed that these strips were not representative of the situation in vivo following the onset of labour. Therefore, further experiments on the effect of each of the test agents were performed on myometrium from non-labouring women only.

**Effects of GTN, SNP, 8-bromo cGMP and l-NNAME**

The spontaneous rhythmic contractility of myometrial strips was inhibited in a concentration-dependent manner following the addition of GTN (0.1–100 μmol l^{-1}), SNP (0.1–100 μmol l^{-1}) and 8-bromo-cGMP (0.001–3 mmol l^{-1}) (Figs 1a and 2a). Maximum inhibitions of contractions produced by the highest concentrations of each drug were not significantly different.
Fig. 1. (a) Individual traces showing the effects of glyceryl trinitrate (GTN; 100 µmol l\(^{-1}\)), nifedipine (NIF; 0.1 µmol l\(^{-1}\)) and lemakalim (LEM; 30 mmol l\(^{-1}\)) on the spontaneous rhythmic contractile activity of strips of myometrium from pregnant women. (b,c) Graphs showing the development of spontaneous rhythmic contractile activity of myometrium from pregnant women after suspension in Krebs' solution at 37°C. The magnitude (b) and frequency (c) increased with time, reaching steady states within 2 h which were maintained for at least a further 3 h. Each point is the mean ± SEM of ten observations from ten patients.

Effects of verapamil and nifedipine

The Ca\(^{2+}\)-channel blocking agents, nifedipine (0.01–10 µmol l\(^{-1}\)) and verapamil (0.01–10 µmol l\(^{-1}\)), each produced powerful, concentration-dependent inhibition of the magnitude of spontaneous rhythmic contractions (Figs 1a and 3a). The highest concentration of each drug completely suppressed myometrial activity. Both drugs caused an initial increase in the frequency of spontaneous contractions at low concentrations (Fig. 3b).

and were 40 ± 7%, 53 ± 8% and 39 ± 8%, respectively. GTN caused a slight but significant fall in the frequency of spontaneous contractions at a concentration of 100 µmol l\(^{-1}\) (Fig. 2b). SNP increased the frequency of spontaneous contractions at concentrations of 30 µmol l\(^{-1}\) and above. 8-Bromo-cGMP had no effect on the frequency of spontaneous contractions (Fig. 2b). The magnitude and frequency of spontaneous contractions was unaffected following treatment with the inhibitor of NO synthase, l-NAME (300 µmol l\(^{-1}\)) (data not shown).
Effects of lemakalim

At concentrations of 10–30 nmol l$^{-1}$, lemakalim had no effect on the magnitude of spontaneous myometrial contractions (Figs 1a and 4a). Higher concentrations (≥100 nmol l$^{-1}$) led to a progressive fall in the magnitude of spontaneous contractions. At concentrations of 0.01–0.3 μmol l$^{-1}$, lemakalim reduced the frequency of contractions in a concentration-dependent manner (Fig. 4b), with the highest concentration suppressing activity completely.

Discussion

In this study, we have determined the effect in vitro of two commonly available NO donors, GTN and SNP, both of which are used clinically to treat cardiovascular disease. Each of these agents could be used in vitro if found to be effective in inhibiting myometrial contractility in vitro. Indeed, a preliminary uncontrolled study has suggested that GTN may be useful in the treatment of preterm labour (Lees et al., 1994). We are not aware of any other studies to determine the effect of GTN or SNP on contractions of myometrium from pregnant women. However, in the myometrium from non-pregnant women.
women, SNP at a concentration of 1 μmol L⁻¹ caused a 43% reduction in force and a 55% reduction in the frequency of spontaneous myometrial contractions (Word et al., 1991). In the present study, GTN (0.1–100 μmol L⁻¹) and SNP (0.1–100 μmol L⁻¹) inhibited the spontaneous rhythmic contractility of myometrial strips from pregnant women in a concentration-dependent manner: the maximum inhibition in the magnitude of contractions produced by the highest concentration of each drug was 40 ± 7% and 53 ± 8% of the control contraction, respectively. The effect on frequency depended on the NO donor used: GTN caused a decrease in the frequency of contractions at concentrations of 100 μmol L⁻¹, whereas SNP caused an increase in the frequency of contractions at concentrations of 30 μmol L⁻¹ and above.

The variety of methods for quantifying myometrial activity and for stimulating contractile activity make it difficult to compare results between different studies. However, previous studies of the action of NO donors in vitro have shown that they inhibit myometrial contractions but fail to abolish contractions completely. The NO-releasing agent dimethylamine/NO (at concentrations up to 0.1 nmol L⁻¹), causes a maximum reduction of 80% in the force (area under the curve) of spontaneous contractions in the myometrium of pregnant women (Buhimschi et al., 1995). Furthermore, the generation of NO from the ultraviolet irradiation of streptozotocin (1 nmol L⁻¹) reduces the amplitude of myometrial contractions by 50% and increases the interval between myometrial contractions by a factor of 1.2 (Lee and Chang, 1995).

When studying the effects of NO donors in vitro, it is important to consider their metabolism and mechanism of action. NO donors are thought to exert their major pharmacological effects via the liberation of NO. GTN must be broken down enzymatically before NO is released; in contrast, SNP and dimethylamine/NO liberate NO spontaneously (Torfgard and Ahlner, 1994; Buhimschi et al., 1995). The enzymes required for the metabolism of organic nitrate esters such as GTN are incompletely defined but are known to be present in a variety of biological tissues such as blood, lung, liver, skeletal muscle and vascular smooth muscle; therefore, we have assumed that these enzymes are present in the myometrial strips in vitro. However, if they are absent or occur at reduced concentrations in our system in vitro, the efficacy of GTN in vivo is likely to be greater than that demonstrated in vitro.

The inhibitory effects of GTN and SNP on the amplitude of myometrial contractions was paralleled by 8-bromo-cGMP in this study. Similar results have been reported previously in the myometrium of pregnant rats (Yallampalli et al., 1993b) but not in that of pregnant women. Our finding that GTN, SNP and 8-bromo-cGMP each produce the same maximum inhibition of the magnitude of contractility supports the assertion that NO donors mediate this effect via an increase in the intracellular concentration of cGMP. GTN and SNP displayed different effects on the frequency of contractions: GTN inhibited contraction frequency while SNP stimulated it. The reasons for this result remain obscure but could be related to different mechanisms of action of SNP and GTN. Since cGMP had no effect on the frequency of uterine contractions, the observed effect of SNP and GTN on this parameter is unlikely to be mediated via cGMP. Several effects of NO as a smooth muscle relaxant that are not dependent on cGMP have been observed, the most important of which is probably the activation of K⁺ channels (Bolotina et al., 1994). Both SNP and GTN have been shown to activate charybdotoxin-sensitive K⁺ channels, and GTN also displays other relaxant effects that are not sensitive to charybdotoxin (Hamaguchi et al., 1992). Although these data help to explain the inhibitory effects of GTN on the frequency of myometrial contractions, the reason for the SNP-induced increase is unknown. A stimulatory effect of SNP on the frequency of contractions has also been described in rat myometrium (Franchi et al., 1994), although this agent has previously been shown to inhibit the frequency of contractions in the myometrium from non-pregnant women (Word et al., 1991). Further
work is required to investigate the mechanisms behind the SNP-induced changes in myometrial contractile frequency.

The concept that the myometrium itself produces NO is suggested by data showing that the myometrium from pregnant women contains cGMP and produces nitrate and nitrite in culture (Buhimschi et al., 1995). The effect of endogenous NO production within the myometrium was determined by applying L-NAME, an inhibitor of NO synthesis, to myometrial strips. In our experiments, L-NAME (0.3 mmol L⁻¹) had no effect on myometrial contractions, implying that there is no endogenous NO production within the myometrium in vitro. These results are in agreement with data reported by Jones and Poston (1997). In contrast, a modest increase in the contractions of myometrium from pregnant women occurred in response to the NO synthase inhibitor Nω-nitro-L-arginine (L-NINA) at concentrations of 0.3 mmol L⁻¹ (Lee and Chang, 1995) and in the contractions of myometrium from non-pregnant women in response to L-NAME (1 mmol L⁻¹) or L-NINA (1 mmol L⁻¹) (Buhimschi et al., 1995). The different results obtained by these studies might be due to differences in sampling technique. It is possible that in cases where an increase in contractility to NO synthase inhibitors can be demonstrated, the myometrial strips being tested are attached to small amounts of NO-generating tissue (for example, endometrium or decidua). Alternatively, myometrial biopsies taken from different areas of the uterus may differ in their NO-generating ability; thus, if myometrium is taken from an area where minimal amounts of NO are generated, then L-NAME would not be expected to have any effect.

The third aim of this study was to determine the potential efficacy of GTN and SNP in attenuating myometrial contractions in comparison with other potential tocolytic agents such as Ca²⁺-channel blockers and K⁺-channel openers. Preliminary clinical studies using Ca²⁺-channel openers to treat preterm labour (Ferguson et al., 1990) have shown that these agents could be beneficial, but further research is required to determine their effects on mother and fetus. The K⁺-channel opener lemakalim has been shown to be effective in inhibiting human myometrial contractions in vitro. Studies with diazoxide, which is now known to exert its actions via K⁺-channel opening, have suggested that it might be useful in treating preterm labour (Caritis et al., 1979; Morrison et al., 1993). In the studies described here, complete inhibition of myometrial contractions was achieved using nifedipine, verapamil or lemakalim. In contrast, no NO donor, including those described here, can inhibit contractions completely in vitro. Clearly, the side-effects and efficacy of each of these agents must be determined in vivo. However, the available data from studies in vitro indicate that nifedipine, verapamil and lemakalim may each be more effective than NO donors in the treatment of preterm labour.

These data suggest that controlled clinical trials of each of the NO donors GTN and SNP, the Ca²⁺-channel blockers nifedipine and verapamil and the K⁺-channel opener lemakalim are warranted in the treatment of preterm labour. The side-effects and efficacy of each of these agents must be studied in vivo. If it is found that these drugs are as effective in attenuating contractions of the myometrium of labouring women in vivo as they are in vitro in myometrium removed before the onset of labour, they could have a major impact in reducing perinatal mortality and morbidity.

This work was supported by grants from Yorkhill National Health Service Trust, Tenovus UK, the Medical Research Council (G9437277) and Wellbeing (the research arm of the Royal College of Obstetricians and Gynaecologists), for which the authors are very grateful.

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