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

J. E. Norman¹, L. M. Ward^{1,2}, W. Martin², A. D. Cameron³,
J. C. McGrath², I. A. Greer¹ and I. T. Cameron¹

¹University of Glasgow, Department of Obstetrics and Gynaecology, Queen Elizabeth Building, Glasgow Royal Infirmary, 10 Alexandra Parade, Glasgow G31 2ER, UK; ²Institute of Biomedical and Life Sciences, West Medical Building, University of Glasgow, Glasgow G12 8QQ, UK; and ³The Queen Mother's Hospital, Yorkhill, Glasgow G3 8SJ, UK

The purpose of this study was to determine the relaxant effects *in vitro* of two nitric oxide donors, glyceryl 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 glyceryl 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, glyceryl trinitrate and sodium nitroprusside, attenuate myometrial contractions and are therefore useful as tocolytic agents. However, at equimolar concentrations *in vitro*, the ability of glyceryl trinitrate and sodium nitroprusside to attenuate myometrial contractions is less than that of lemakalin, 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 streptozotocin, which releases NO under ultra-violet radiation, are not currently available for use *in vivo*. In contrast, glyceryl 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

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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*^ω-nitro-L-arginine methyl ester (L-NAME). (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 Ca²⁺-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 (≥ 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 CaCl₂ l⁻¹, 1.2 mmol MgSO₄ l⁻¹, 1.2 mmol KH₂PO₄ l⁻¹, 24 mmol NaHCO₃ l⁻¹, and 11.1 mmol glucose l⁻¹, and gassed with 95% O₂ and 5% CO₂. 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 grammes. Once a steady state had been reached, a reduction in the magnitude of spontaneous contractions was expressed as a percentage inhibition (mean $\pm$ 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-NAME

The spontaneous rhythmic contractility of myometrial strips was inhibited in a concentration-dependent manner following the addition of GTN (0.1–100 μ mol l⁻¹), SNP (0.1–100 μ mol 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; $100 \mu\text{mol l}^{-1}$), nifedipine (NIF; $0.1 \mu\text{mol l}^{-1}$) 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 $\pm$ SEM of ten observations from ten patients.

and were $40 \pm 7\%$, $53 \pm 8\%$ and $39 \pm 8\%$, respectively. GTN caused a slight but significant fall in the frequency of spontaneous contractions at a concentration of $100 \mu\text{mol l}^{-1}$ (Fig. 2b). SNP increased the frequency of spontaneous contractions at concentrations of $30 \mu\text{mol l}^{-1}$ 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 ($300 \mu\text{mol l}^{-1}$) (data not shown).

Effects of verapamil and nifedipine

The Ca^{2+} -channel blocking agents, nifedipine (0.01 – $10 \mu\text{mol l}^{-1}$) and verapamil (0.01 – $10 \mu\text{mol l}^{-1}$), 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).

Fig. 2. Concentration–response curves showing the effects of sodium nitroprusside (●), glyceryl trinitrate (■), and 8-bromo-cyclic guanosine monophosphate (8-bromo-cGMP; ▼) on (a) the magnitude and (b) the frequency of spontaneous contractions of strips of myometrium from pregnant women. Values are means $\pm$ SEM. Twenty-five strips from nine patients were treated with sodium nitroprusside, 17 strips from five patients were treated with glyceryl trinitrate, and five strips from five patients were treated with 8-bromo-cGMP. * $P < 0.05$ indicates a significant change in the frequency of contractions induced by the drug.

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 μ mol l⁻¹, lemakalim reduced the frequency of contractions in a concentration-dependent manner (Fig. 4b), with the highest concentration suppressing activity completely.

Fig. 3. Concentration–response curves showing the effects of nifedipine (■) and verapamil (●) on (a) the magnitude and (b) the frequency of spontaneous contractions of strips of myometrium from pregnant women. Values are means $\pm$ SEM. Seven strips from five patients were treated with nifedipine and 17 strips from five patients were treated with verapamil. * $P < 0.05$ indicates a significant change in the frequency of contractions induced by the drug.

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 vivo* 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

Fig. 4. Concentration–response curves showing the effects of lemakalim on (a) the magnitude and (b) the frequency of spontaneous contractions of strips of myometrium from pregnant women. Each point is the mean $\pm$ SEM of 16 strips from six patients. * $P < 0.05$ indicates a significant reduction in the frequency of contractions by lemakalim.

women, SNP at a concentration of $1 \mu\text{mol l}^{-1}$ 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 – $100 \mu\text{mol l}^{-1}$) and SNP (0.1 – $100 \mu\text{mol l}^{-1}$) 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 $40 \pm 7\%$ and $53 \pm 8\%$ 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 $100 \mu\text{mol l}^{-1}$, whereas

SNP caused an increase in the frequency of contractions at concentrations of $30 \mu\text{mol l}^{-1}$ 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 mmol 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 mmol 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 *N*^o-nitro-L-arginine (L-NNA) 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-NNA (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 Ca^{2+} -channel blockers and K^{+} -channel openers. Preliminary clinical studies using Ca^{2+} -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 Ca^{2+} -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.

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