Nitric oxide regulation of monkey myometrial contractility

[2]Karri A. Kuenzli, Iain L.O. Buxton & [1]Michael E. Bradley

Department of Pharmacology, University of Nevada, Reno, Reno, NV 89557, U.S.A.

1   We evaluated the effect of the nitric oxide (NO) donor CysNO (S-nitroso-L-cysteine) and endogenous NO upon spontaneous contractility in non-pregnant cynomolgus monkeys. We also assessed the role of intracellular guanosine $3',5'$-cyclic monophosphate ([cyclic GMP]$_i$) as a second messenger for NO in monkey uterine smooth muscle.

2   CysNO reduced spontaneous contractility by 84% ($P < 0.05$) at maximal concentrations, and significantly elevated [cyclic GMP]$_i$ ($P < 0.05$). However, increases in [cyclic GMP]$_i$ were not required for CysNO-induced relaxations; CysNO inhibited contractile activity despite the complete inhibition of guanylyl cyclase by methylene blue or LY83,583.

3   Analogues of cyclic GMP had no significant effect upon spontaneous contractile activity. L-arginine produced a 62% reduction in spontaneous activity ($P < 0.05$) while D-arginine had no effect. The competitive nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (L-NOARG) not only blocked L-arginine-induced relaxations, but also significantly increased spontaneous contractile activity when added alone ($P < 0.05$); the inactive D-enantiomer of NOARG had no such effect.

4   While both endogenous NO and the NO donor CysNO relax monkey myometrium, this effect is not causally related to CysNO-induced elevations in [cyclic GMP]$_i$. The failure of cyclic GMP analogues to alter monkey uterine smooth muscle tension also argues against a role for [cyclic GMP]$_i$ in the regulation of uterine contractility. Not only do these findings argue for the existence of a functionally-relevant NOS in the monkey uterus, but increases in contractile activity seen in the presence of NOS inhibitors suggest a role for NO in the moment-to-moment regulation of contractile activity in this organ.

Keywords: Nitric oxide; myometrium; guanosine $3':5'$-cyclic monophosphate; contraction; uterus

Introduction

Recent interest has developed in determining the role of NO in the control of uterine contractility. Evidence exists which suggests that the uterus is an NO-producing organ; most of this evidence stems from morphological and biochemical analyses of uterine samples obtained from the rat (Papka & McNeill, 1992; Conrad et al., 1993; Izumi et al., 1993; Natuzzi et al., 1993; Shew et al., 1993; Yallampalli et al., 1993; 1994), guinea-pig (Weiner et al., 1994), rabbit (Sladek et al., 1993), sheep (Figueroa & Massmann, 1995) and human (Izumi et al., 1993; Buhimschi et al., 1995; Telfer et al., 1995). More recent work suggests that NO may be produced from uterine smooth muscle cells themselves (Nakaya et al., 1996). While the majority of previous studies focused upon the possible role of endogenous nitric oxide in the maintenance of uterine quiescence during pregnancy (Conrad et al., 1993; Natuzzi et al., 1993; Yallampalli et al., 1993; Buhimschi et al., 1995; Izumi & Garfield, 1995; Sladek & Roberts, 1996; Kuenzli et al., 1996), there have been only a few studies on a role for NO in the regulation of function in the non-pregnant uterus. Telfer et al. (1995) have suggested that NO might regulate blood flow in the normally-cycling uterus, and that alterations in this control might lead to non-pregnant uterine dysfunction such as dysmenorrhea.

Despite the number of studies which have established the presence of nitric oxide synthase (NOS) in vivo, only a few studies have explored the possible mechanism of action of NO in the uterus (Izumi et al., 1993; Yallampalli et al., 1993; 1994; Franchi et al., 1994; Buhimschi et al., 1995;

Kuenzli et al., 1996). It has been proposed that a NO-guanylyl cyclase relaxation pathway exists in the uterus, whereby NO affects uterine smooth muscle tone via elevations in intracellular guanosine $3':5'$-cyclic monophosphate [cyclic GMP]$_i$ (Yallampalli et al., 1994; Buhimschi et al., 1995). However, a number of investigators have previously attempted to uncover a role for cyclic GMP in the regulation of uterine smooth muscle contractility, and their findings suggest that cyclic GMP plays a minor role (if any) in the regulation of rodent and human uterine contractility (Diamond, 1983; 1989; Word et al., 1991); we ourselves have found very little evidence for a role for cyclic GMP in the regulation of guinea pig uterine contractility (Kuenzli et al., 1996). The objective of the present study was not only to determine for the first time the effect of NO on monkey uterine contractile activity, but also whether endogenous NO production plays a role in the regulation of contraction in the non-pregnant uterus; we also determined to resolve the apparent paradox implicit in the ability of NO to regulate uterine smooth muscle tone. We have found that while NO inhibition of spontaneous contractions in monkey myometrium was associated with significant elevations in [cyclic GMP]$_i$, these elevations were not causally related to the ability of NO to relax these tissues. Stimulation of NOS activity with excess substrate produced relaxation in myometrial tissues; these effects were blocked by competitive NOS inhibitors. Furthermore, NOS inhibition led to increases in spontaneous activity, suggesting a relaxant contribution by NO in the regulation of contraction in the monkey myometrium. These findings suggest that NO could play a role in the regulation of myometrial contractility in the non-pregnant uterus, perhaps via a mechanism which does not require changes in intracellular cyclic GMP.

[1] Author for correspondence: Department of Pharmacology, MS318, University of Nevada, Reno, NV 89557-0046, USA.
[2] Present address: Department of Physiology and Cell Biology, MS 352, University of Nevada, Reno, NV 89557-0046, USA.

Methods

Source and preparation of uterine samples

Samples of uterine tissue were obtained from mid-menstrual cycle (14 ± 2 days) non-pregnant cynomolgus (*Macaca fascicularis*) monkeys used as control animals in toxicology studies at Sierra Biomedical Inc. (Sparks, Nevada); tissues were taken after animals had been killed with an overdose of sodium pentobarbitone. Samples were transported on ice to the laboratory in an oxygenated, pH 7.40 buffer solution which consisted of the following (in mM): NaCl 120, KCl 5, KH_2PO_4 0.587, Na_2HPO_4 0.598, $MgCl_2$ 2.5, $CaCl_2$ 2.5, α,D-glucose 20 and tris[hydroxymethyl]amino-methane 25.0. Uterine samples for contractile studies were prepared by pinning uterine tissues on a bed of Sylgard (Dow Corning, Midland, MI) so that strips of myometrium (~ 10 mm $\times \sim 2$ mm $\times \sim 2$ mm) could be cut while they were immersed in 37°C in oxygenated buffer. Strips were mounted into water-jacketed organ baths (Radnoti, Monrovia, CA) and attached to isometric force transducers (Kent Scientific, Litchfield, CT) by silk thread. Transducer voltages were amplified and converted to digital signals by an ACJr A/D board mounted within a computer system running the Workbench data acquisition system (Strawberry Tree, Inc., Sunnyvale, CA). Strips were maintained at 37°C, aerated with 100% O_2, and loaded with initial tensions of 0.5 g; tissues were allowed a 1 h equilibration period before the start of experiments. All experiments were completed within 8 h of tissue collection.

Concentration-response relationships

Spontaneously-active myometrial strips were exposed to CysNO at non-cumulative, increasing concentrations for 5 min at each concentration; addition of equivalent concentrations of vehicle (D-cysteine or 'spent' CysNO which had been bubbled with 100% oxygen for 30 min) to paired tissue strips served as controls. Addition of the cyclic GMP analogues 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cyclic GMP) and β-phenyl-1,N^2-ethenoguanosine-3,5′-cyclic monophosphate (PET-cyclic GMP) was accomplished in the same manner, except that they were allowed 10 min of contact with tissue strips. Tissues were allowed a 15 min 'rest' period in buffer solution between treatments, during which time spontaneous contractile activity returned to pretreatment levels.

To inhibit guanylyl cyclase activity, tissue strips were pretreated with methylene blue (10 μM) or LY83,583 (1 μM) for 30 min, after which CysNO at various concentrations was added for 5 min. Spontaneous activity was evaluated both before and following methylene blue pretreatment to determine the effect of methylene blue itself upon spontaneous contractile activity; the concentrations of methylene blue and LY83,583 used were determined empirically as sufficient to inhibit completely guanylyl cyclase, but incapable of altering spontaneous contractility.

Effects of NOS substrates on spontaneous activity

Spontaneously-active tissues were treated with either the biologically-active L-enantiomer of arginine or its inactive D-enantiomer; similarly, the competitive NOS inhibitor N^{ω}-nitro-arginine (NOARG) was also employed as either its L- or D-enantiomer. Inactive (D-) enantiomers also served as controls for effects upon contractility possibly caused by alterations in

bathing solution pH; in addition, arginine solutions were always buffered with 25 mM Tris-HCl and brought to a pH of 7.40 before addition to organ baths. Treatments with arginine were performed for 10 min at 1 mM; other tissues were pretreated with L-NOARG (1 mM) for 30 min, followed by exposure to L-arginine (1 mM) to assess the effects of NOS inhibition upon L-arginine-induced reductions in spontaneous contractility. Finally, experiments were performed in which tissues were treated with either the L- or D-enantiomer of NOARG alone (1 mM, 10 min) to assess the effects of NOS inhibition upon basal spontaneous activity (i.e., in the absence of any NOS stimulation by arginine).

Cyclic GMP quantification

Tissue strips were flash-frozen in liquid nitrogen at various time points (basal tension, at the peak of a spontaneous contraction, or at maximum CysNO-induced relaxation in the presence or absence of methylene blue or LY83,583); maximum relaxation always occurred within 30 s following CysNO addition. Frozen samples were homogenized in 6% trichloroacetic acid in acetone while immersed in a dry ice : methanol slurry. Acetone was removed by lyophilization, samples were resuspended in water and protein was removed by microcentrifugation. Acid was removed by triplicate extraction with diethyl ether and residual ether was evaporated by heating at 70°C for 10 min. Lyophilates were resuspended in 1 ml phosphate-buffered saline and assayed in duplicate for cyclic GMP by enzyme-linked immunoassay (Cayman Chemical Co.).

Preparation of CysNO

CysNO was prepared by the method described by Gibson *et al.* (1992). In essence, L-cysteine-HCl (100 mM) and sodium nitrite (100 mM) were mixed in equal proportions and placed on ice for 30 min. The resulting solution was neutralized with 0.5 M NaOH and kept on ice in an airtight container for the remainder of the day; fresh solutions were prepared daily.

Data analyses

Contractions were quantified by integration of the area under each record for periods of 5 min following addition of CysNO, or 10 min following addition of 8-Br-cyclic GMP, PET-cyclic GMP, L- or D-arginine, or L- or D-NOARG. Relaxations in response to added agents were compared with the amount of spontaneous contractile activity present in each sample during a 10 min period immediately before addition; as a control for time, spontaneous activity was shown to not differ significantly during the course of the experiments. Evaluation of the effect of L-arginine treatment upon L-NOARG-pretreated strips was performed by comparing the level of contractile activity in the presence of the NOS inhibitor with that seen following L-arginine addition; this was necessary because L-NOARG treatment on its own produced significant increases in contractile activity when compared with spontaneous levels. Changes in tissue tensions and cyclic GMP concentrations were evaluated by one-way analysis of variance (ANOVA) by use of Student-Newman-Keuls multiple comparison test. Unless otherwise stated, values for force of contraction are expressed as % of spontaneous activity, $\pm$ one s.e.mean; n = the number of monkeys tested, and statistical significance was assumed when $P < 0.05$. Data analyses and curve fitting were accomplished by use of the Graphpad Prism graphics program (San Diego, CA).

Materials

PET-cyclic GMP was obtained from Ruth Langhorst Biolog International (La Jolla, CA), and cyclic GMP EIA materials were obtained from Cayman Chemical Co. (Ann Arbor, MI). LY83,583 (6-anilinoquinoline-5,8-quinone) was purchased from RBI (Natick, MA). L- and D-arginine and L- and D-NOARG were obtained from Calbiochem (San Diego, CA). All other compounds were reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

CysNO effects on uterine contractile activity

The addition of CysNO to spontaneously-active non-pregnant monkey myometrial strips resulted in the complete inhibition of phasic contractile activity (Figure 1a); the effect of CysNO on spontaneous activity lasted between 5 and 10 min. Phasic contractile activity resumed either upon washout, or spontaneously after 5–10 min (Figure 1a). Figure 1 illustrates not only the inhibitory effect of CysNO on monkey uterine tissue tension, but the typical contractile activity present in monkey uterine tissue strips. Addition of vehicle (D-cysteine alone or 'spent' CysNO) at volumes equivalent to those necessary to deliver CysNO was never observed to affect tissue tension
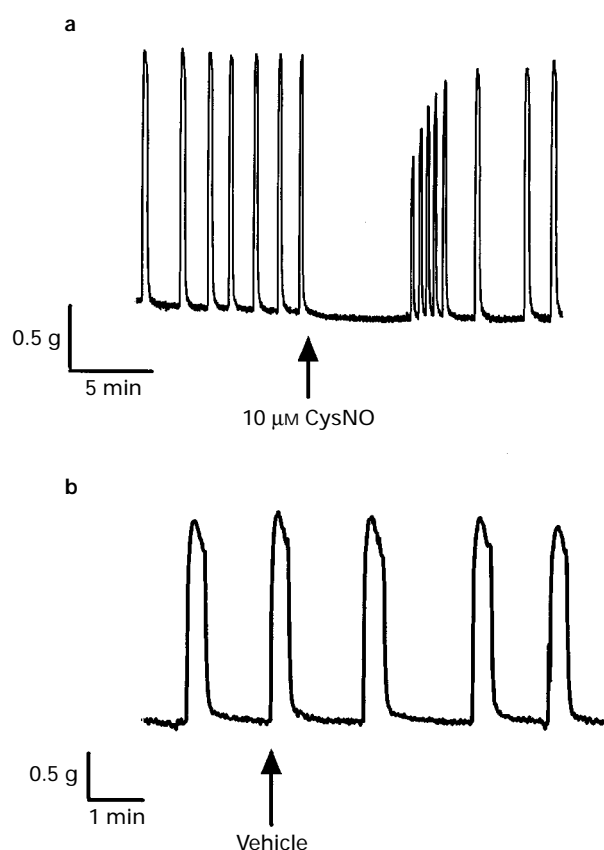
(Figure 1b). Identical inhibitions of spontaneous contractile activity were also observed in tissues treated with gaseous NO (not shown). The concentration-response relationship of CysNO on spontaneous activity was established (Figure 2), and the IC_{50} for CysNO on uterine contractility was determined to be 1.5 μM ($n=4$).

Effects of guanylyl cyclase inhibition

The effects of CysNO upon myometrial tissue tension and [cyclic GMP]$_i$ were simultaneously quantified by flash-freezing tissue samples at precise time points before and following CysNO inhibition of spontaneous contractions. Inhibition of contractile activity by CysNO (100 μM) was found to be associated with a statistically-significant increase in [cyclic GMP]$_i$ (Figure 3; $P<0.05$) when compared to mean values obtained for each of the following conditions: basal (at the trough of a spontaneous contraction), spontaneous (at the peak of a spontaneous contraction), and at maximum (100 μM) CysNO-induced relaxation of spontaneous contractions in the absence and presence of methylene blue (10 μM) or LY83,583 (1 μM). Despite the ability of methylene blue (10 μM) or LY83,583 (1 μM) to block CysNO-stimulated increases in [cyclic GMP]$_i$ (Figure 3), CysNO remained capable of inhibiting spontaneous contractile activity in the monkey uterus in the continued presence of a guanylyl cyclase inhibitor (Figure 4; $P<0.05$). Neither methylene blue nor LY83,583 at the concentrations employed (i.e. concentrations demonstrated to be capable of inhibiting guanylyl cyclase) had any effect upon spontaneous contractile activity, and neither compound was found to interfere with the cyclic GMP assay.

To rule out the possibility that treatment with guanylyl cyclase inhibitors alters the time course of [cyclic GMP]$_i$

Figure 1 CysNO inhibition of spontaneous contractile activity in monkey myometrium. CysNO (10 μM) was added to spontaneously-contracting monkey myometrial tissue strips at the arrow (a) and remained present for the duration of the recording. Resumption of spontaneous activity is also indicated in (a). Addition of vehicle ('spent' CysNO) for CysNO had no discernible effect upon either amplitude or frequency of spontaneous contractions (b). Recordings are representative of results found in 18 tissue strips obtained from 7 monkeys.
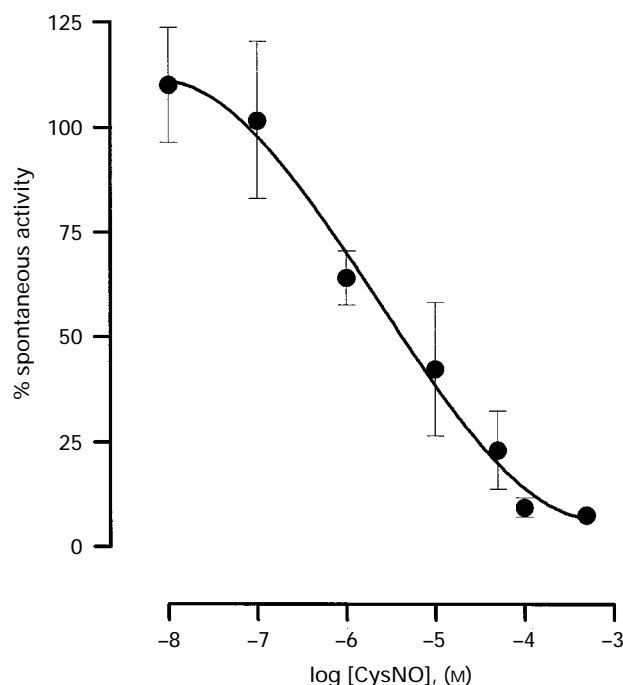

Figure 2 CysNO concentration-response relationship. The concentration-response relationship for the effect of CysNO on spontaneous contractile activity in the non-pregnant monkey uterus was established by adding increasing, non-cumulative concentrations of CysNO to tissue strips. Results are means from at least 4 monkeys, and are expressed as a % of the spontaneous activity, vertical lines show s.e.means. Data were fit by non-linear regression, which was used to calculate an IC_{50} of 1.5 μM.

generation, preliminary experiments established the time courses of cyclic GMP responses in the presence and absence of 10 μM methylene blue; at no point was any significant increase in cyclic GMP concentration detected in response to CysNO in the presence of the inhibitor (not shown).

Effect of cyclic GMP analogues on spontaneous contractions

The effects of two relatively permeant and non-hydrolyzable cyclic GMP analogues on spontaneous contractile activity in monkey myometrium were assessed by establishing non-cumulative concentration-response relationships for 8-Br-cyclic GMP and PET-cyclic GMP on spontaneously-active tissue strips. Neither 8-Br-cyclic GMP nor PET-cyclic GMP at
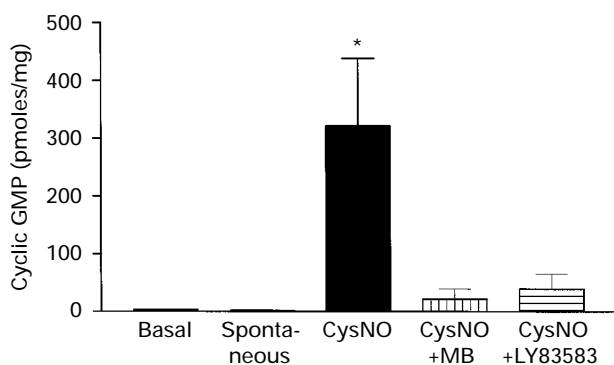
any concentration tested had any significant effect upon spontaneous contractile activity (Figure 5; $P = 0.122$ and 0.159, respectively).

Effect of NOS substrates and inhibitors

Treatment of tissues for 10 min with the NOS substrate L-arginine resulted in an immediate and pronounced 62% reduction in the amount of spontaneous contractile activity present in these tissues when compared to the activity present

Figure 3 CysNO effects on [cyclic GMP]$_i$ in non-pregnant monkey myometrium. Strips of isolated myometrium were flash frozen while tensions were basal, spontaneous and at maximum (100 μM) CysNO-induced relaxations of spontaneous contractions in the absence and presence of methylene blue (MB, 10 μM) or LY83,583 (1 μM). Samples were assayed in duplicate for cyclic GMP content; results are means$\pm$s.e.mean from 3 to 7 animals, and each animal was assayed in quadruplicate for each condition; pmol cyclic GMP were normalized to mg protein present in the homogenate of each sample. Asterisk indicates a statistically-significant difference compared to each individual mean represented by the other 4 columns ($P < 0.05$).
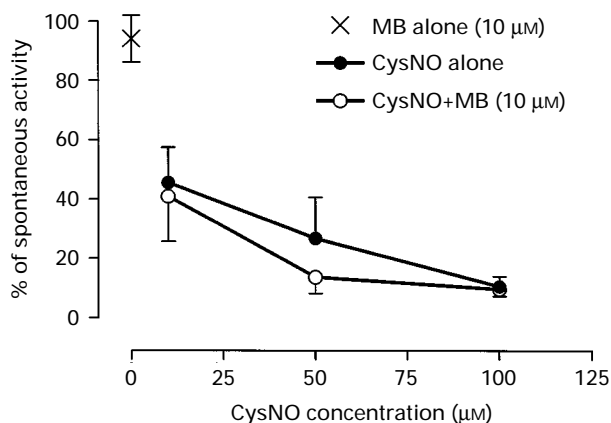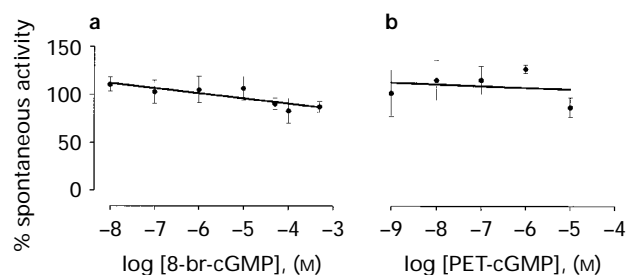

Figure 5 Concentration-response relationships of cyclic GMP analogues on contractile activity in the monkey uterus. Increasing concentrations of 8-Br-cyclic GMP (a) or PET-cyclic GMP (b) were added to spontaneously-active tissues in a non-cumulative manner. At all concentrations tested, neither 8-Br-cyclic GMP nor PET-cyclic GMP had any effect upon spontaneous contractile activity, as assessed by one-way ANOVA ($P = 0.122$ and 0.159, respectively). Results are expressed as a % of spontaneous activity in tissue strips obtained from 6 monkeys; vertical lines show s.e.mean.
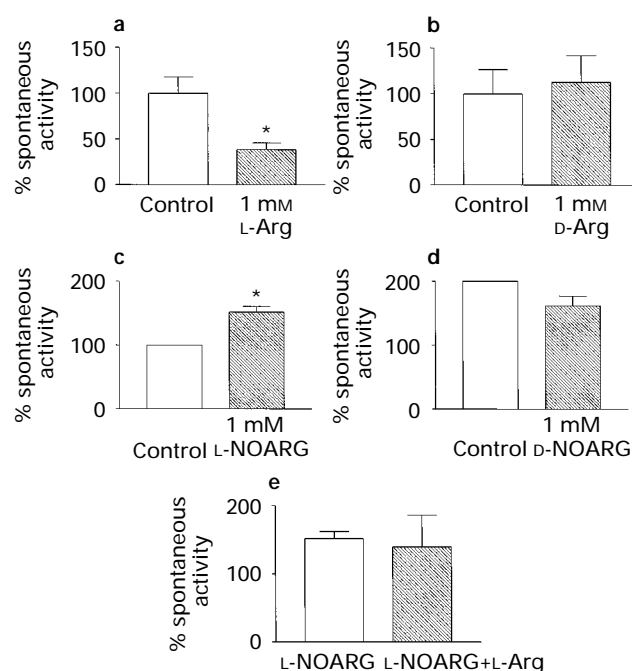

Figure 4 CysNO-inhibition of spontaneous contractions in the presence of methylene blue. Tissue strips were pretreated with methylene blue (MB, 10 μM) for 30 min, and the ability of CysNO (10–100 μM) to relax spontaneous contractions was evaluated by one-way ANOVA and Student-Newman-Keuls multiple comparison analysis. CysNO caused significant ($P < 0.05$) reductions in spontaneous contractile activity at all concentrations tested, in both the presence and the absence of methylene blue when compared to the amount of activity present when methylene blue (MB, 10 μM) was present alone. Results are expressed as a % of the spontaneous activity from 12 tissue strips obtained from 4 different monkeys; vertical lines show s.e.mean.

Figure 6 Effect of L-arginine on spontaneous contractile activity. Excess (1 mM) L-arginine was added to spontaneously-contracting tissue strips obtained from 7 monkeys; integration of the contractile records revealed a significant (62%) reduction in the amount of spontaneous contractility in the presence of L-arginine (a). No reductions were observed when strips were incubated in the presence of 1 mM D-arginine (b). Pretreatment of tissues with 1 mM L-NOARG significantly increased the amount of spontaneous activity present in tissues (c), while D-NOARG had no such effect (d). L-NOARG completely inhibited the ability of L-Arg to inhibit spontaneous activity (e). Results are means$\pm$s.e.mean from studies performed on tissues from 6 monkeys; asterisks represent statistically-significant differences ($P < 0.05$) when compared to control values.

immediately before L-arginine addition ($P<0.05$; Figure 6a). This inhibition persisted in the continued presence of L-arginine for up to 15 min on average, when spontaneous activity resumed. Addition of D-arginine (1 mM) had no effect on spontaneous activity (Figure 6b), and no change in the pH of the bathing solutions was observed following addition of either L- or D-arginine. Addition of 1 mM L-NOARG to tissues resulted in a statistically-significant increase in spontaneous activity when compared to basal levels ($P<0.05$; Figure 6c), while D-NOARG had no such effect (Figure 6d). L-NOARG, in addition to its ability to increase spontaneous contractility, completely blocked the ability of L-arginine to inhibit contractile activity (Figure 6e).

Discussion

Our studies demonstrated that both endogenous NO and the NO donor CysNO are inhibitors of spontaneous contractions in the non-pregnant monkey uterus. Moreover, exogenous NO (in the form of CysNO) exhibited a relatively high order of potency for relaxation of this tissue ($IC_{50} = 1.5$ μM) when compared with other NO donors in other species (Yallampalli *et al.*, 1993; Buhimschi *et al.*, 1995). Support for the notion that NO may be produced endogenously in the monkey uterus stems from our finding that addition of excess L-arginine, the substrate for NOS, to spontaneously-active myometrial tissue strips resulted in significant reductions in spontaneous contractile activity, while D-arginine (which is not a substrate for NOS) had no effect (Figure 6). Nitric oxide, therefore, whether added exogenously (via CysNO or gaseous NO) or produced endogenously, inhibits spontaneous contractions in the monkey myometrium. We have also derived evidence for the existence of a constitutively active NOS in the monkey myometrium, in that addition of L-NOARG to spontaneously-active tissues actually increased the amount of activity – i.e., inhibition of a constitutively active NOS, which otherwise maintains the tissue in a reduced state of spontaneous activity, resulted in a disinhibition of contractile activity.

The objectives of this study were not only to determine the effect of NO on uterine contractility in the monkey, but to evaluate the role of the second messenger cyclic GMP in NO-modulation of uterine tissue tension. Addition of CysNO to spontaneously contracting myometrial tissue strips was found to be associated with significant elevations in [cyclic GMP]$_i$. These results support earlier findings of ours (Kuenzli *et al.*, 1996) and those of others which have demonstrated NO-induced elevations in [cyclic GMP]$_i$ in uterine samples obtained from both human (Buhimschi *et al.*, 1995) and rat (Yallampalli *et al.*, 1994). In the latter studies it was proposed that elevations in [cyclic GMP]$_i$ were responsible for the reductions in tension observed in NO-treated uterine tissues, and that a guanylyl cyclase : cyclic GMP pathway might play an important role in the regulation of uterine smooth muscle contractility. However, we found that while CysNO inhibition of spontaneous contractions in the monkey myometrium was associated with significant increases in [cyclic GMP]$_i$, these increases were not involved in the regulation of myometrial tension, as the prevention of cyclic GMP production by methylene blue treatment had no significant effect upon the ability of CysNO to inhibit spontaneous contractions. We have obtained preliminary findings that relaxations in monkey myometrium elicited by release of endogenous NO (i.e. treatment with L-arginine) also do not require a functional guanylyl cyclase (Kuenzli *et al.*, unpublished data). These results suggest that while guanylyl cyclase does indeed serve as

a target for NO in the monkey uterus, activation of this enzyme is not required for NO-dependent relaxation. Additional support for the lack of involvement of cyclic GMP in the regulation of uterine smooth muscle tone in the monkey stems from the inability of two cyclic GMP analogues, 8-Br-cyclic GMP and PET-cyclic GMP, to affect uterine contractility, particularly as PET-cyclic GMP has been shown to be capable of stimulating the cyclic GMP-dependent protein kinase isoform (1β) known to be present in the uterus (Sandberg *et al.*, 1989; Sekhar *et al.*, 1992). These findings are in agreement with those previously obtained by Word *et al.* (1991) in the human and Diamond (1983, 1989) in the rat uterus, in that intracellular cyclic GMP appears to be incapable of altering tension in the smooth muscle of the uterus.

The cyclic GMP-independent mechanism of action of NO in the monkey myometrium is not clear, although there have been a number of studies demonstrating cyclic GMP-independent effects of NO in a variety of tissues (Garg & Hassid, 1991; Bolotina *et al.*, 1994; Kannan & Johnson, 1995). One possible mechanism involves the activation of a calcium-dependent K^+ channel by NO. We have found, for example, that treatment of human myometrial samples with large-conductance K^+ (BK) channel blockers, such as iberiotoxin, at low concentrations blocks the relaxation effects of the NO donor CysNO (M.E. Bradley *et al.*, unpublished data), suggesting that activation of the BK channel is somehow involved in the NO-mediated relaxation in these tissues. This finding need not necessarily imply a direct effect of NO on the BK channel. For example, Roman and co-workers have found that inhibition of 20-hydroxyeicosatetraenoic acid (20-HETE) production in vascular tissue augments the vasodilator response to NO (Alonso-Galicia *et al.*, 1997); the same group has found that 20-HETE is capable of inhibiting the BK channel (Zou *et al.*, 1996). Given that others have found that NO can inhibit the enzymes responsible for the production of 20-HETE (e.g. cytochrome P450A; Wink *et al.*, 1993), a mechanism by which NO could relax uterine smooth muscle by a cyclic GMP-independent, but P450-dependent, mechanism becomes plausible. Whether this mechanism exists in the myometrium is the subject of current study.

Given the similarities between the present results obtained in monkey tissues and those obtained in human tissues (Buhimschi *et al.*, 1995), it would appear that the monkey could serve as an ideal model in which to study the role and effects of NO in both pregnant and non-pregnant uterus. The significance of our work is our finding of constitutively-active NOS activity in non-pregnant monkey myometrium, and that cyclic GMP is not required for NO-induced relaxation in non-pregnant monkey myometrium. Hence, we suggest not only that disruptions in NO regulation of uterine contractility could lead to contractile disorders such as metralgia in the human, but that development of future tocolytic therapies, designed to take advantage of the potent relaxing effects of NO upon the uterus, should not be based upon the involvement of intracellular cyclic GMP in these effects.

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