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Comparative relaxant effects of YC-1 and DETA/NO on spontaneous contractions and the levels of cGMP of isolated pregnant rat myometrium

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Abstract

This study was designed to compare the effects of YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole), a nitric oxide (NO)-independent soluble guanylate cyclase activator, and diethylenetriamine-NONOate (DETA/NO), a NO donor, on spontaneous contractions and the levels of cyclic GMP (cGMP) of myometrial strips isolated from timed-pregnant rats. Myometrial strips were obtained from timed-pregnant Wistar albino rats ($n=10$) and were mounted in organ baths and tested for changes in isometric tension in response to YC-1 and DETA/NO. We also evaluated the effect of YC-1 and DETA/NO on the levels of cGMP in myometrial strips obtained from timed-pregnant rat uterine horns ($n=20$). YC-1 (10^{-9}–3×10^{-5} M) and DETA/NO (10^{-7}–10^{-4} M) concentration-dependently decreased the amplitude and frequency of spontaneous contractions of myometrial strips isolated from term-pregnant rats. The inhibitions of the amplitude and frequency of spontaneous contractions by YC-1 and DETA/NO were antagonized with methylene-blue (10^{-5} M). Antagonistic effect of methylene-blue (10^{-5} M) was more on DETA/NO responses than that of YC-1 ($P<0.05$). In addition, YC-1-stimulated myometrial strips showed more elevation in myometrial cGMP than that of DETA/NO ($P<0.05$). We demonstrated that YC-1 and DETA/NO induce relaxations in the amplitude and frequency of spontaneous contractions of myometrial strips with different potencies. We also found that YC-1 and DETA/NO-induced relaxations are associated with significant increases in cGMP. These results might suggest that the relaxant effects of YC-1 and DETA/NO on the rat myometrium could be due to the stimulation of the soluble guanylate cyclase and cGMP may play a role for the maintenance of uterine quiescence during pregnancy.

Keywords: YC-1; DETA/NO; Soluble guanylate cyclase activator; Nitric oxide donor; Myometrium

1. Introduction

Prematurity is one of the major unresolved problems in obstetrics. Neonatal mortality and morbidity associated with premature labor have stimulated a search for new agents those diminish or eliminate uterine contraction. Although tocolytics based on β-adrenergic receptor and calcium channel antagonists are in use, their efficacy and safety are questionable (Koks et al., 1998). Knowledge of the actions of nitric oxide (NO) as a vascular and gastrointestinal smooth muscle relaxant together with initial clinical evidence has suggested the use of NO in the treatment of preterm labor.

NO is an important mediator of many biologic functions, including the relaxation of smooth muscle of vascular, gastrointestinal, tracheal, and cavernous tissues (Moncada et al., 1991; Li and Rand, 1990; Ozaki et al., 1992; Li and Rand, 1991; Pickard et al., 1991). It is an activator of soluble guanylate cyclase and is effective agent in relaxing spontaneous contractions of both animal and human myometrium (Kuenzli et al., 1996; Kuenzli et al., 1998; Bradley et al., 1998). Several studies provide evidence for the presence of an L-arginine-NO system in the pregnant uterus (Yallampalli et al., 1993; Yallampalli et al., 1994;

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Buhimschi et al., 1995; Sladek and Roberts, 1996). Furthermore, this system is up regulated during pregnancy, and it inhibits uterine contractility until term but not during delivery, suggesting that the NO system may contribute to the maintenance of uterine quiescence during pregnancy and the initiation of labor (Yallampalli et al., 1994). It has been claimed that NO promotes human uterine relaxation, as it does other smooth muscle, by the elevation of cyclic guanosine monophosphate (cGMP) (Yallampalli et al., 1993; Buhimschi et al., 1995; Izumi and Garfield, 1995). However, several other studies have demonstrated that a rise in cGMP fails to cause a relaxation of some smooth muscles, including human myometrium (Kuenzli et al., 1996; Bradley et al., 1998; Buxton et al., 2001).

Besides NO, only few other soluble guanylate cyclase-activating substances have been reported. In 1995, the benzylindazole derivative YC-1 ([3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole]) was described as a novel, apparently NO-independent activator of soluble guanylate cyclase (Wu et al., 1995). It is a direct activator of soluble guanylate cyclase and sensitizes the enzyme for activation by NO and carbon monoxide (Schmidt et al., 2001). YC-1 provides an about 10-fold increase of enzyme activity (Koesling and Friebe, 1999). Apart from an increase in the formation of cGMP via the stimulation of soluble guanylate cyclase, the substance also prevents cGMP degradation. In our previous study, we demonstrated that YC-1 inhibits spontaneous contractions of myometrial strips obtained from timed-pregnant rat uterus and methylene-blue (which blocks soluble guanylate cyclase) or tetraethylammonium (which blocks Ca^{2+}-activated K^+ channels) antagonizes the inhibitor effect of YC-1 (Cetin et al., 2004).

In the present study, we attempt to compare the actions of YC-1 and DETA/NO on spontaneous contractions and levels of cGMP of myometrial strips isolated from timed-pregnant rats.

2. Material and methods

2.1. Animals

Timed-pregnant ($n=10$) Wistar albino rats obtained from Cumhuriyet University Animal House, weighing 180–200 g, were used throughout the study. After obtaining Institutional Review Board approval, all procedures were performed under the guidelines of the Animal Care and Use Committee of Cumhuriyet University. Rats were housed in a 22 °C temperature room with water and food ad libitum. Virgin female rats were placed in separate cages with one male each and left overnight. Pregnancy was dated by accepting the morning of sperm positivity as day one of gestation. The normal length of gestation of rats was 22 days. None of the rats used was in labor. Pregnant rats were killed by cervical subluxation at 21 days of gestation. A midline abdominal incision was made; the uterine horns were rapidly excised, carefully cleaned of surrounding connective tissues, and then opened longitudinally along the mesenteric border. Fetuses were removed and non-uterine tissues were dissected away. We obtained five full-thickness longitudinal muscle strips (approximately $8\times2\times2$ mm) from each animal and incubated them in temperature-controlled 10 ml organ baths containing modified Krebs' solution (NaCl 125 mM, KCl 2.4 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 0.5 mM, $NaHCO_3$ 23.9 mM and glucose 11.0 mM) aerated with 95% O_2 and 5% CO_2 at 37 °C (pH=7.4).

2.2. Measurement of myometrial contractile activity

The myometrial strips were allowed to equilibrate at 1 g tension for 60 min before the addition of the experimental drugs and washed every 15 min. The myometrial tension was recorded isometrically with a Grass FT03 force-displacement transducer and registered on a Grass model 79E polygraph (Grass, Quincy, MA, USA). The recorder was calibrated so that 1 g tension was represented as 1 cm vertical displacement. Paper speed was set at 2.5 mm/min. Myometrial contractions started within 10 min after they were mounted in the organ bath and stabilized in 60 min. Preliminary time-control experiments with no further drug additions showed that strips exhibit stable uterine activity for at least 4 h after preparation in this manner.

Three sets of experimental studies were performed with myometrial strips obtained from pregnant rats ($n=10$). While conducting the three sets of studies, we used the three myometrial strips isolated from each rat. The effect of YC-1 (10^{-9}–3×10^{-5} M) on the spontaneous contractions alone and in the presence of methylene blue (10^{-5} M) was evaluated in the first. In the second set, we evaluated the effect of DETA/NO (10^{-7}–10^{-4} M) alone and in the presence of methylene blue (10^{-5} M) on the spontaneous contractions of rat myometrium. Methylene blue was added to the organ baths 15 min before from YC-1 and DETA/NO in order to test the role of guanylate cyclase which could have a contribution to myometrial smooth muscle relaxation induced by YC-1 and DETA/NO. In the third set, we determined the effect of dimethyl sulfoxide (DMSO) that have used as a solvent of the YC-1 on the spontaneous contractions of rat myometrium.

2.3. Determination of myometrial cGMP content

To determine the myometrial cGMP content of rat myometrial strips under experimental conditions, 80 myometrial strips isolated from term-pregnant rats were used ($n=20$). They were equilibrated for 60 min in Krebs–Henseleit bicarbonate buffer at 37 °C, and then incubated for a further 1, 5, 20, or 60 min with YC-1 (10^{-6} M, $n=4$, and 10^{-5} M, $n=4$), DETA/NO (10^{-6} M, $n=4$, and 10^{-5} M, $n=4$), and DMSO ($n=4$). Strips were collected either immediately by freezing the strips in liquid nitrogen. All strips were pulverized in a liquid nitrogen-cooled stainless steel mortar, and then transferred into 300 μl of 80% ethanol. From this point on, cyclic GMP standards were processed and treated identically to the samples. Samples were homogenized with ethanol and incubated for 30 min at 4 °C. Then, they were centrifuged at 4 °C for 10 min at 12,000 $\times g$ to precipitate cell debris and proteins. To remove ethanol, we poured the supernatants into a clean test tube and dried the supernatant in a vacuum at 50 °C. The 100 μl aliquot of these supernatant fractions was used for cGMP quantitation by radioimmunoassay (IBL, Hamburg, Germany). The amount of cGMP in each myometrial strip was standardized to fmol cGMP mg^{-1} protein.

2.4. Drugs

Chemicals used in the current experiments were YC-1 and DETA/NO from A.G. Scientific, Inc. (San Diego, CA, USA), and methylene blue from Sigma (St. Louis, MO, USA). All chemicals were dissolved in distilled water except for YC-1, which was dissolved in DMSO. All drugs were freshly prepared on the day of the experiments.

2.5. Statistical analyses

At the start of each experiment for experimental procedures, the amplitude and frequency of spontaneous myometrial contractions was considered as a reference response. Changes in the amplitude and frequency of myometrial contractions were expressed as percentage of the initial reference response. The characteristics of the contractions analyzed immediately before and after the addition of drugs included mean length of amplitude (gram) and frequency (number/1000 s) of contractions for 1000 s intervals. The effects of cumulative concentrations of YC-1 and DETA/NO in the presence and absence of methylene blue on oxytocin-stimulated myometrial contractions were measured, and values for $-\log$ (10) EC_{50} and mean maximal inhibition (E_{max}) were compared. Maximal inhibitor effects were calculated for each concentration–response curve. The EC_{50} value of each drug, which represents 50% of the maximal inhibitor effect. EC_{50} values were calculated by linear regression of the probit of response versus $\log_{10}$ molar concentration for each of the YC-1 and DETA/NO. Data were presented as means±S.E.M. and analyzed by repeated measures of analysis of variance (ANOVA) with the Dunnett test. A P value of <0.05 was considered significant. All statistical analyses were performed using Statistica for Windows 6.0. (Statsoft, Inc., Tusla, OK, USA).

3. Results

YC-1 (10^{-9}–3×10^{-5} M) inhibited spontaneous contractile activity in a concentration-dependent manner in myometrial smooth muscle isolated from pregnant rats. The inhibition in amplitude and frequency of contractions in myometrial strips

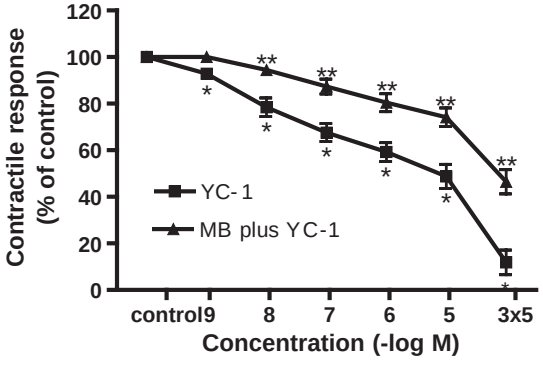

Fig. 1. The effect of YC-1 (alone) and YC-1 in the presence of 10^{-5} M methylene blue (MB) on the amplitude of spontaneous contractions in myometrial strips isolated from timed-pregnant rats. Data were expressed as the means±S.E.M. of ten experiments. *Significantly different from the control for YC-1 ($P<0.05$). **Significantly different from the YC-1 for the MB plus YC-1 at the same doses ($P<0.05$).

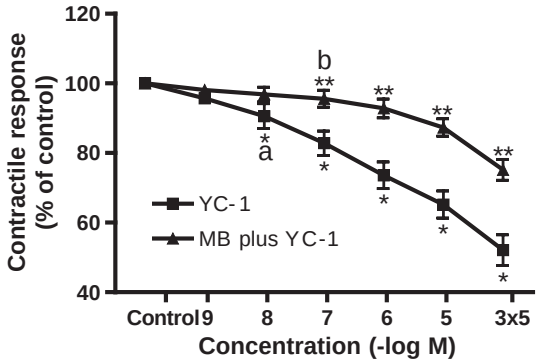

Fig. 2. The effect of YC-1 (alone) and YC-1 in the presence of 10^{-5} M methylene blue (MB) on the frequency of spontaneous contractions in myometrial strips isolated from timed-pregnant rats. Data were expressed as the means±S.E.M. of ten experiments. *Significantly different from the control for YC-1 ($P<0.05$). **Significantly different from the YC-1 for the MB plus YC-1 at the same doses ($P<0.05$).

reached statistical significance beginning from the concentrations of 10^{-9} and 10^{-8} M, respectively ($n=10$) ($P<0.05$) (Figs. 1 and 2). Pre-incubation of myometrial strips with 10^{-5} M methylene blue significantly reduced the inhibitory effect of YC-1 on the amplitude and frequency of spontaneous contractions ($n=10$) ($P<0.05$) (Figs. 1 and 2). Table 1 presents values for E_{max} and $-\log$ (10) EC_{50} of YC-1 in the presence and absence of methylene blue on myometrial contractions of term pregnant rats myometrium. The E_{max} and $-\log$ (10) EC_{50} values for YC-1 in the presence of methylene blue were significantly decreased ($P<0.05$).

DETA/NO (10^{-7}–10^{-4} M) inhibited spontaneous contractile activity in a concentration-dependent manner in myometrial smooth muscle isolated from pregnant rats. The inhibition in amplitude and

Table 1
E_{max} and $-\log$ (10) EC_{50} values for YC-1 and DETA/NO in the presence and absence of methylene blue (MB) on contractions of term pregnant rats myometrium

Drugs	Amplitude	Frequency
YC-1		
E_{max}(%)	88.21±5.32[a]	47.95±4.41[b]
$-\log$ 10 EC_{50}	6.12±0.06[c]	6.08±0.07[b]
MB+YC-1		
E_{max}(%)	53.62±5.30	24.85±3.11
$-\log$ 10 EC_{50}	5.20±0.06	5.14±0.05
DETA/NO		
E_{max}(%)	30.21±4.51[d]	20.15±4.45[e]
$-\log$ 10 EC_{50}	5.96±0.04	5.64±0.05
MB+DETA/NO		
E_{max}(%)	5.61±4.12	3.44±2.20
$-\log$ 10 EC_{50}	5.84±0.03	5.26±0.04

Data are expressed as means±S.E.M. ($n=10$).
[a] Significantly different from all other groups for E_{max} values of YC-1 ($P<0.05$).
[b] Significantly different from all other groups for E_{max} values and $-\log$ 10 EC_{50} of YC-1 ($P<0.05$).
[c] Significantly different from MB+YC-1 for $-\log$ 10 EC_{50} of YC-1 ($P<0.05$).
[d] Significantly different from MB+DETA/NO for E_{max} values of DETA/NO ($P<0.05$).
[e] Significantly different from MB+YC-1 for $-\log$ 10 EC_{50} of YC-1 ($P<0.05$).

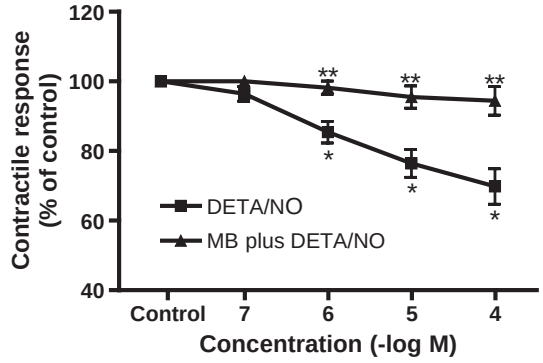

Fig. 3. The effect of DETA/NO (alone) and DETA/NO in the presence of 10^{-5} M methylene blue (MB) on the amplitude of spontaneous contractions in myometrial strips isolated from timed-pregnant rats. Data were expressed as the means ± S.E.M. of ten experiments. *Significantly different from the control for DETA/NO ($P<0.05$). **Significantly different from the DETA/NO for the MB plus DETA/NO at the same doses ($P<0.05$).

frequency of contractions in myometrial strips reached statistical significance beginning from the concentrations of 10^{-6} and 10^{-5} M, respectively ($n=10$) ($P<0.05$) (Figs. 3 and 4).

Pre-incubation of myometrial strips with 10^{-5} M methylene blue almost completely abolished the inhibitory effect of DETA/NO on the amplitude and frequency of spontaneous contractions ($n=10$) ($P<0.05$) (Figs. 3 and 4). Table 1 presents values for E_{max} and $-\log$ (10) EC_{50} of DETA/NO in the presence and absence of methylene blue on myometrial contractions of term pregnant rats myometrium. The E_{max}, but not $-\log$ (10) EC_{50}, values for DETA/NO in the presence of methylene blue was significantly decreased ($P<0.05$).

At these concentrations, methylene blue, and DMSO (used as solvent vehicle of YC-1) have no effect on amplitude and frequency of spontaneous myometrial contractions (data not shown).

To investigate whether the long lasting effect of YC-1 and DETA/NO on contractile responses coincided with elevated cGMP levels, we determined the myometrial cGMP content under identical conditions as above, that is, after YC-1 and DETA/NO treatment. Thereby, myometrial strips were preincubated for 1, 5, 20, and 60 min with YC-1 (10^{-6} and 10^{-5} M), DETA/NO (10^{-6} and 10^{-5} M), and DMSO. Table 2 shows the myometrial cGMP content of rat uterine strips. Both YC-1-and DETA/NO-stimulated myometrial strips showed elevation in myometrial cGMP. The levels of myometrial cGMP pretreated with YC-1 at 10^{-5} M were significantly higher than those of pretreated with YC-1 at 10^{-6}, DETA/NO at 10^{-5} and 10^{-6} M at 5 min. At these concentrations, DMSO has no effect on the levels of cGMP of myometrial strips isolated from pregnant rats (Table 2).

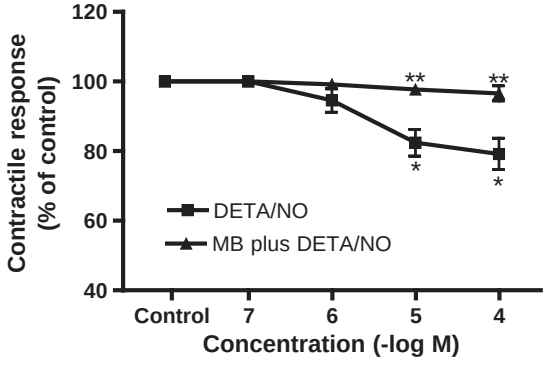

Fig. 4. The effect of DETA/NO (alone) and DETA/NO in the presence of 10^{-5} M methylene blue (MB) on the frequency of spontaneous contractions in myometrial strips isolated from timed-pregnant rats. Data were expressed as the means ± S.E.M. of ten experiments. *Significantly different from the control for DETA/NO ($P<0.05$). **Significantly different from the DETA/NO for the MB plus DETA/NO at the same doses ($P<0.05$).

Table 2
cGMP content of rat myometrial strips immediately after 1, 5, 20 and 60 min incubation period with YC-1 (10^{-6}–10^{-5} M) and DETA/NO (10^{-6}–10^{-5} M)

Drugs	1 min	5 min	20 min	60 min
DMSO	0.15 ± 0.03	0.13 ± 0.03	0.17 ± 0.04	0.16 ± 0.02
YC-1 (10^{-6})	0.41 ± 0.05[a]	0.82 ± 0.07[b]	0.30 ± 0.08[a]	0.20 ± 0.05
YC-1 (10^{-5})	0.52 ± 0.05[a]	0.98 ± 0.10[b,c]	0.34 ± 0.07[a]	0.22 ± 0.06
DETA/NO (10^{-6})	0.38 ± 0.03[a]	0.54 ± 0.05[b]	0.24 ± 0.07[a]	0.18 ± 0.04
DETA/NO (10^{-5})	0.40 ± 0.04[a]	0.76 ± 0.06[b]	0.28 ± 0.06[a]	0.21 ± 0.055

Myometrial cGMP content is expressed in fmol mg^{-1} protein. Data are expressed as means ± S.E.M. ($n=20$).
[a] Significantly different from DMSO values ($P<0.05$).
[b] Significantly different from all other groups ($P<0.05$).
[c] Significantly higher from all other results ($P<0.05$).

4. Discussion

Our study was designed to determine the effects of YC-1 and DETA/NO on timed-pregnant rat myometrial contractile activity and to investigate the role of guanylyl cyclase/cGMP pathway in these settings. YC-1 and DETA/NO decreased the amplitude and frequency of spontaneous contractions of myometrial strips isolated from timed-pregnant rats. Methylene-blue, which blocks soluble guanylate cyclase, was significantly antagonized the inhibitor effect of YC-1, but was almost completely antagonized the inhibitor effect of DETA/NO. We also investigated the myometrial cGMP contents of rat uterine horns after YC-1- and DETA/NO-treatment. We found out that YC-1-and DETA/NO-induced relaxations are associated with significant increases in cGMP. These results might suggest that the relaxant effect of YC-1 and DETA/NO on the pregnant rat myometrium could be due to the stimulation of the soluble guanylate cyclase.

NO is an important modulator of contractile activity in smooth muscle in a variety of organs, exhibiting predominantly relaxing effects (Moncada et al., 1991). It is generated from L-arginine by a group of enzymes called NO synthase. Studies on the human pregnant uterus have demonstrated myometrial NO synthase activity (Telfer et al., 1995; Thomson et al., 1997; Ramsay et al., 1996). Of great clinical interest is the plausible role of NO in the maintenance of uterine quiescence during pregnancy and the efficacy of NO donors to inhibit premature contractions. However, it is still not clear whether NO is involved in the regulation of uterine quiescence during pregnancy (Jones

and Poston, 1997). A preliminary uncontrolled study during preterm labor reported that NO may be an important inhibitor of premature contractions (Lees et al., 1994). Similarly, it has been suggested that an endogenous NO system is present in the rat uterus and is up-regulated during pregnancy, followed by down-regulation during labor (Sladek and Roberts, 1996; Natuzzi et al., 1993). In our study, DETA/NO less inhibited the amplitude and frequency of spontaneous contractions of myometrial strips isolated from timed-pregnant rats compared to YC-1. This effect of DETA/NO may be due to the use of myometrial strips isolated from timed-pregnant rats. In addition, the half time values for nitric oxide release from DETA/NO are 57 h at 24 °C and 20 h at 37 °C (Wolf et al., 1998). This fact may explain why less inhibitor effect on contractile activity was registered following addition of the nitric oxide donor. YC-1 is more potent inhibitor agent in myometrial strips isolated from timed-pregnant rats. Inhibitor effect of YC-1 may be due to both the stimulation of the sGS and Ca^{2+}-activated K^+ channels (Cetin et al., 2004).

While the majority of previous studies focused upon the possible role of endogenous NO in the maintenance of uterine quiescence during pregnancy, only a few studies have explored the possible mechanism of action of NO in the uterus (Bradley et al., 1998; Yallampalli et al., 1994; Buhimschi et al., 1995; Sladek and Roberts, 1996; Izumi and Garfield, 1995). It has been proposed that a NO-soluble guanylate cyclase relaxation pathway exists in the uterus, whereby NO affects uterine smooth muscle tone via elevations in intracellular cGMP (Yallampalli et al., 1994; Buhimschi et al., 1995). However, a number of studies (Kuenzli et al., 1998; Bradley et al., 1998; Buxton et al., 2001) reported that NO can indeed alter agonist-evoked uterine contractility, but the effect of NO does not require the activation of cGMP. Similarly, Weiner et al. (1994) reported that pregnancy dramatically increases myometrial cGMP by a pathway independent of NO and the increase in myometrial cGMP is at least one mechanism by which the myometrial stretch reflex is suppressed during pregnancy. In the present study, increase in cGMP levels of myometrial strips preincubated with DETA/NO was moderate, while the increase in cGMP levels of myometrial strips preincubated with YC-1 was excessive at the same concentrations (10^{-6}–10^{-5} M).

The role of the cyclic nucleotides in the maintenance of uterine quiescence during pregnancy is controversial at present. Evidences from animal and human studies suggest that the adenylate cyclase/cAMP signaling pathway is gestationally modified (Europe-Finner et al., 1994; Lopez et al., 1995; Lindeman et al., 2000), but the importance of cGMP in myometrial smooth muscle relaxation during pregnancy remains to be clarified. Whereas increase in cGMP correlated with relaxation in a time- and concentration-dependent manner in vascular smooth muscle, the role cGMP as a uterine relaxant is not as well established (Lincoln, 1989). For example, several studies reported that there is no correlation between increases in cGMP and relaxation in myometrial smooth muscle (Kuenzli et al., 1996; Bradley et al., 1998; Hennan and Diamond, 1998). In the other studies, in contrast, demonstrated that cGMP can modulate myometrial contractile activity (Yallampalli et al., 1994; Buhimschi et al., 1995; Izumi and Garfield, 1995; Weiner et al., 1994). In the present study, we suggest that cGMP may play a role for the maintenance of uterine quiescence during pregnancy.

There is no available data in the literature related to compare the in vitro relaxant effects of YC-1-and DETA/NO on timed-pregnant rat contractile activity and to investigate the role of soluble guanylate cyclase /cGMP pathway in these settings. In the present study, we also found out that YC-1-and DETA/NO-induced relaxation is associated with significant increases in cGMP. YC-1 is a more potent relaxant than DEA/NO and causes more elevation of cGMP levels in myometrial strips isolated from timed-pregnant rat. These results might suggest that the relaxant effects of YC-1 and DETA/NO on the rat myometrium could be due to the stimulation partially and/or completely of the soluble guanylate cyclase and cGMP may play a role for the maintenance of uterine quiescence during pregnancy.

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