YC-1, a Nitric Oxide-Independent Activator of Soluble Guanylate Cyclase, Inhibits the Spontaneous Contractions of Isolated Pregnant Rat Myometrium

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Abstract. The aim of this study was to investigate the effect of YC-1 (3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole) on spontaneous contractions and levels of cyclic GMP (cGMP) of myometrial strips isolated from pregnant rats. It is a nitric oxide-independent soluble guanylate cyclase activator. Myometrial strips were obtained from eight pregnant Wistar albino rats and were mounted in organ baths for the recording of isometric tensions. We evaluated the effect of increasing concentrations of YC-1 on spontaneous myometrial contractions and on contractions of myometrial smooth muscle pretreated with methylene blue (10\textsuperscript{-5} M), tetraethylammonium chloride (TEA) (3 \times 10\textsuperscript{-4} M), and glibenclamide (10\textsuperscript{-6} M). YC-1 (10\textsuperscript{-9} – 3 \times 10\textsuperscript{-5} M) concentration-dependently decreased the amplitude and frequency of spontaneous contractions of myometrial strips. The inhibition of the amplitude and frequency of spontaneous contractions by YC-1 were antagonized with methylene blue (10\textsuperscript{-5} M) and TEA (3 \times 10\textsuperscript{-4} M), but they were not changed by glibenclamide (10\textsuperscript{-6} M); however, the antagonistic effect of methylene blue was significantly more than that of TEA ($P<0.05$). We also evaluated the effect of YC-1 on the levels of cGMP in myometrial strips obtained from pregnant rat uterine horns. YC-1-stimulated myometrial strips showed an excessive elevation in myometrial cGMP that declined slowly during the subsequent washout period. These results show that YC-1 decreases spontaneous contractile activity of myometrial strips isolated from pregnant rat and causes elevation of myometrial cGMP levels in vivo. This effect of YC-1 is significantly reduced by the methylene blue and TEA, suggesting the activation of soluble guanylate cyclase and Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channels as the mechanisms of action.

Keywords: YC-1, soluble guanylate cyclase activator, myometrium (rat)

Introduction

Preterm birth complicates 10% of pregnancies throughout the world and is responsible for 75% to 80% of perinatal mortality in infants without major malformations (1). Prevention of preterm birth continues to be an important subject for the obstetrician. Tocolytic therapy may offer some short-term benefit in the management of it, such as administration of corticosteroids to reduce the severity of fetal respiratory distress syndrome and transferring of the patient to a tertiary care center (2).

Pharmacological inhibition of uterine contractions remains one of the cornerstone treatments for preterm labor. Inhibitors of smooth muscle contraction, like $\beta_2$-agonists, magnesium sulphate, and calcium channel blockers, are the most important agents used to prevent preterm delivery, but their efficacy and safety are questionable (3). New drugs that allow more effective treatment of preterm labor with lower side effects are needed.

Although, the mechanisms to maintain uterine quiescence during pregnancy and initiate uterine contractions during labor are unknown, it is reported that the cyclic guanosine 5'-monophosphate (cGMP) / guany-
late cyclase (GC) system in human myometrium is gestationally modified and potentially plays an important role in mediating quiescence during early pregnancy (4). Nitric oxide (NO) is an activator of soluble guanylate cyclase (sGC) and is clearly an effective agent in relaxing spontaneous contractions of both animal and human myometrium (5–7). It has been claimed that NO promotes human uterine relaxation, as it does in other smooth muscles, by the elevation of cGMP (8–10). Besides NO, only few other sGC-activating substances have been reported. YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole) is an NO-independent sGC activator that provides an about 10-fold increase of enzyme activity (11). Apart from an increase in the formation of cGMP via the stimulation of sGC, the substance also prevents cGMP degradation. Thus, YC-1 may play a role in the relaxation of myometrial smooth muscle and represent a new class of drugs in the treatment of preterm labor.

There is no available data in the literature related to the in vitro relaxant effects of YC-1 on contractile activity of myometrium. The purpose of this study was to investigate the effects of YC-1 on spontaneous contractions of myometrial strips obtained from pregnant rat uterus.

Materials and Methods

Animals

Timed-pregnant (n = 8) Wistar albino rats, 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. Pregnant rats were killed by cervical subluxation at the morning of sperm positivity as day one 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 four full-thickness longitudinal muscle strips (approximately 8 × 2 × 2 mm) from each animal and incubated them in temperature-controlled 10 ml organ baths containing modified Krebs’ solution (125 mM NaCl, 2.4 mM KCl, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 23.9 mM NaHCO₃, and 11.0 mM glucose) aerated with 95% O₂ and 5% CO₂ at 37°C (pH = 7.4).

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 contraction 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.

Four sets of experimental studies were performed with myometrial strips obtained from pregnant rats (n = 8). While conducting the four sets of studies, we used the four myometrial strips isolated from each rat. We evaluated the effect of YC-1 (10⁻⁹–3 × 10⁻⁵ M) on spontaneous contractions of pregnant rat myometrial strips in the first set. In the second, third, and fourth sets, we evaluated the effects of YC-1 (10⁻⁹–3 × 10⁻⁵ M), in the presence of methylene blue (10⁻⁵ M), tetraethylammonium chloride (TEA) (3 × 10⁻⁴ M), and glibenclamide (10⁻⁶ M), respectively. The effects of YC-1 on the spontaneous contractions alone and in the presence of methylene blue (10⁻⁵ M), TEA (3 × 10⁻⁴ M), and glibenclamide (10⁻⁶ M) were measured; and inhibitor effects were compared. Methylene blue blocking GC, TEA blocking Ca²⁺-sensitive K⁺ channels, and glibenclamide blocking ATP-sensitive K⁺ channels were added to the organ baths 15 min before from YC-1 in order to test the effects of GC inhibition and K⁺ channels that may contribute to the myometrial smooth muscle relaxation induced by YC-1.

Determination of myometrial cGMP content

To determine the myometrial cGMP content of rat myometrial strips under experimental conditions, strips were equilibrated for 30 min in Krebs’-Henseleit bicarbonate buffer (KHB) at 37°C and then incubated for a further 60 min with either YC-1 (10⁻⁶–10⁻⁵ M). Segments were collected either immediately by freezing the segments in liquid nitrogen, or allowed to re-equilibrate in KHB without YC-1 (for 5, 20, or 60 min), and then snap-frozen. All segments were pulverized in a liquid nitrogen-cooled stainless steel mortar, and then transferred into 300 μl of 80% ethanol. From this point on, cGMP standards were processed and treated identically to the samples. Samples were homogenized with ethanol and incubated for 30 min at 0–4°C. Then, they were centrifuged at 4°C for 10 min at 12000 × g to
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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 were 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.

Drugs

Chemicals used in the current experiments were YC-1 from A.G. Scientific, Inc. (San Diego, CA, USA) and methylene blue, TEA, and glibenclamide 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.

Data 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 (gram) and frequency (number/min) 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 amplitude (gram) and frequency (number/1000 s) of contractions for 1000-s intervals. Data were presented as means ± S.E.M. and analyzed by repeated measures 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).

Results

YC-1 inhibited spontaneous contractile activity in a concentration-dependent manner in myometrial smooth muscle isolated from pregnant rats (Fig. 1). The inhibition in amplitude and frequency of contractions in myometrial strips reached statistical significance beginning from the concentrations of 10⁻⁹ and 10⁻⁸ M, respectively (n = 8) (P<0.05) (Fig. 2: A and B). In many cases (7 out of 8 strips), spontaneous contractions returned to the initial level after washing out 3 × 10⁻⁵ M YC-1 (Fig. 1). The maximal decrease in the amplitude and frequency of myometrial contractions measured at 3 × 10⁻⁵ M was 80.01 ± 5.14% and 51.25 ± 4.78% of control contractions, respectively.

Pre-incubation of myometrial strips with 10⁻⁵ M methylene blue greatly reduced the inhibitory effect of YC-1 on the amplitude and frequency of spontaneous contractions (n = 8) (P<0.05) (Fig. 2: A and B). The maximal decrease in the presence of methylene blue in the amplitude and frequency of myometrial contractions measured at 3 × 10⁻⁵ M was 49.50 ± 8.38% and 32.50 ± 2.88% of control contractions, respectively.

Fig. 1. Original response to increasing concentrations of YC-1 on spontaneous contractile activity in myometrial strips, alone (A) or pretreated with methylene blue (B), isolated from pregnant rats.
Pre-incubation with $3 \times 10^{-4}$ M TEA significantly reduced the inhibitory effect of YC-1 on the amplitude and frequency of spontaneous myometrial contractions, but $10^{-6}$ M glibenclamide did not (n = 8) ($P < 0.05$) (Fig. 2: A and B). The maximal decrease in the presence of TEA in the amplitude and frequency of myometrial contractions measured at $3 \times 10^{-5}$ M was 56.40 ± 7.99% and 37.00 ± 5.70% of control contractions, respectively. At these concentrations, methylene blue, TEA, glibenclamide, and DMSO (used as solvent vehicle of YC-1) have no effect on amplitude and frequency of spontaneous myometrial contractions (Table 1).

To investigate whether the long lasting effect of YC-1 on contractile responses coincided with elevated cGMP levels, we determined the myometrial cGMP content under identical conditions as above, that is, after YC-1 treatment. Thereby, myometrial strips were preincubated for 60 min with YC-1 $10^{-6}$ and $10^{-5}$ M. Figure 3 shows the myometrial cGMP content of rat uterine horn myometrial strips immediately after the incubation period and after a 5-, 20-, or 60-min washout phase. Myometrial cGMP content is expressed in fmol · mg⁻¹ protein (n = 6).

Table 1. Effect of agents on the amplitude and frequency of spontaneous contractions in myometrium of the rats

<table>
<thead>
<tr>
<th>Agent</th>
<th>n</th>
<th>Amplitude (mg)</th>
<th>Frequency (number/1000 s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16</td>
<td>2624 ± 136</td>
<td>12.0 ± 1.4</td>
</tr>
<tr>
<td>Methylene blue (10⁻⁶ M)</td>
<td>8</td>
<td>2659 ± 124</td>
<td>11.2 ± 1.6</td>
</tr>
<tr>
<td>TEA ($3 \times 10^{-4}$ M)</td>
<td>8</td>
<td>2528 ± 160</td>
<td>10.5 ± 1.8</td>
</tr>
<tr>
<td>Glibenclamide (10⁻⁴ M)</td>
<td>8</td>
<td>2598 ± 144</td>
<td>12.4 ± 1.6</td>
</tr>
<tr>
<td>DMSO</td>
<td>6</td>
<td>2608 ± 108</td>
<td>12.2 ± 1.2</td>
</tr>
</tbody>
</table>
Discussion

Our study was designed to determine the effect of YC-1 on rat myometrial contractile activity and to investigate the role of the GC/cGMP pathway and K\(^+\) channels in these settings. YC-1 concentration-dependently decreased the amplitude and frequency of spontaneous contractions of myometrial strips isolated from pregnant rats. However, the inhibition of the amplitude was more than that of frequency. This may suggest that the inhibitory effect of YC-1 on neuronal mechanisms that regulate the frequency of spontaneous contractions is less significant. Methylene blue (which blocks sGC) and TEA (which blocks Ca\(^{2+}\)-activated K\(^+\) channels) significantly antagonized the effect of YC-1. Furthermore, the antagonistic effect of methylene blue was greater than that of TEA. These results might suggest that the relaxant effect of YC-1 on the rat myometrium could be due to both the stimulation of the sGC and K\(^+\) channels. We also investigated the myometrial cGMP content of rat uterine horns after YC-1-treatment. We confirm that YC-1 is consistent with its ability to directly activate sGC.

The role of the cyclic nucleotides in the maintenance of uterine quiescence during pregnancy remains to be clarified. Evidences from animal and human studies suggest that the adenylate/cAMP signaling pathway is gestationally modified (12–14), but the importance of cGMP in myometrial smooth muscle relaxation during pregnancy remains to be clarified. There are conflicting reports in the literature concerning its role in the regulation of myometrial contractility. Functional studies in the rat and guinea pig reported that the myometrium is relatively insensitive to cGMP (5, 7, 15), especially in comparison to vascular smooth muscle (16). The other study, in contrast, demonstrated that cGMP can modulate myometrial contractile activity (8). In the present study, we suggest that cGMP may play a role for the maintenance of uterine quiescence during pregnancy.

The formation of cGMP is catalyzed by GCs that are subdivided into sGC and the membrane-bound type. They are activated by NO and peptide hormones, respectively (11, 17). NO is clearly an effective agent in relaxing spontaneous contractions in both animal (5, 6, 10, 18) and human myometrium (7, 9, 18, 19). Despite the number of morphological and biochemical analyses evaluating the presence of the enzyme necessary to produce NO in the uterus, only a few investigators have explored the possible mechanism of NO in the uterus (9, 10, 18). These studies offered evidence consistent with current dogma, that the effect of NO on uterine smooth muscle relaxation occurs via elevations in cGMP and concluded that an NO-GC relaxation pathway exists in the uterine smooth muscle. However, the other study (5) reported that NO can indeed alter agonist-evoked uterine contractility, but the effect of NO does not require the activation of cGMP. The novel NO-independent type of sGC activator YC-1 has attracted much attention recently because of its unique action profile. It is a potent and direct activator of sGC (20, 21) and mimics many of the known functions of NO and NO donors, such as inhibition of platelet aggregation (22) and adhesion (23) and inhibition of vascular smooth muscle contraction (21). In this study, we demonstrated that YC-1 concentration-dependently reduced spontaneous contractile activity of myometrial strips isolated from pregnant rats and this reduction was antagonized by inhibition of sGC and K\(^+\) channels.

A number of specific K\(^+\) channel types have been described in the myometrium. More than one type has been detected in individual myometrial cells from a number of species, including rats and humans (24–27). Ca\(^{2+}\)-activated K\(^+\) channels are large voltage-dependent channels and are inhibited by TEA, barium (Ba\(^++\)), and charybdotoxin and iberiotoxin (28). ATP-sensitive K\(^+\) channels have been implicated in myometrial function, but have not been measured directly. Glibenclamide is the inhibitor of ATP-sensitive K\(^+\) channels. It has either a very small effect in nonpregnant myometrium (29) or no effect on uterine contractile activity in nonpregnant or pregnant myometrium (30). In this study, pretreatment of the myometrial strips with TEA produced a significant decrease on YC-1-induced inhibition but not with glibenclamide. These data suggest that the YC-1-induced inhibitory effect in pregnant rat myometrial smooth muscle may be partially mediated through Ca\(^{2+}\)-activated K\(^+\) channels.

In conclusion, YC-1 is the first representative of a novel class of sGC activator compounds that concentration-dependently decreased spontaneous contractile activity of rat myometrium. This effect of YC-1 is suggested to stimulate partially sGC for the production of cGMP that inhibits the contractile activity of myometrial strips. Thus, this study implies that the inhibitory effect of the cGMP system may assist in maintaining pregnancy. However, further investigations and controlled trials to assess the mechanisms of YC-1 on myometrial contractile activity in vitro and to determine the side effects and clinical efficacy especially in preterm labor in vivo are necessary.
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