THE JOURNAL OF
Obstetrics and Gynaecology Research

The official Journal of Asia and Oceania Federation of Obstetrics and Gynaecology
Japan Society of Obstetrics and Gynecology

doi:10.1111/j.1447-0756.2012.01929.x J. Obstet. Gynaecol. Res. Vol. 39, No. 1: 31–39, January 2013

Uterus-relaxing effect of β_2-agonists in combination with phosphodiesterase inhibitors: Studies on pregnant rat *in vivo* and on pregnant human myometrium *in vitro*

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Abstract

Aims: Our aims were to examine the effects of a simultaneous stimulation of β_2-adrenergic receptors and inhibition of uterine phosphodiesterases (PDE), in the pregnant rat uterus *in vivo* and on human uterine tissue *in vitro*. We also set out to measure cAMP levels and detect the expressions of the isoenzymes PDE4B and PDE4D in human uterine tissue samples.

Material and Methods: Preterm birth was induced in Sprague-Dawley rats with bacterial lipopolysaccharide. The uterine effects of terbutaline alone or in combination with rolipram were tested *in vivo*. Human myometrial strips from cesarean sections at full-term pregnancy and at preterm labor were stimulated with oxytocin, and the inhibitory effects of theophylline, rolipram and terbutaline were studied. The myometrial accumulation of cAMP in the presence of rolipram and terbutaline was determined by enzyme immunoassay. The expressions of PDE4B and PDE4D proteins were detected by Western blotting.

Results: The selective PDE4 inhibitor rolipram was more effective than the non-selective PDE inhibitor theophylline in inhibiting the oxytocin-induced contractions in the human uterus. The uterus-relaxing effects of low doses of terbutaline were markedly potentiated by rolipram, both in rats and in human tissues. The changes in uterine cAMP levels correlated with these results. At preterm labor, PDE4B was the predominant form of PDE4 expressed; at full term, PDE4D was expressed more strongly.

Conclusions: A combination of selective PDE4 inhibitors and β_2-agonists should be considered for the treatment of preterm contractions.

Key words: β_2-mimetics, phosphodiesterase, preterm labor, rolipram, terbutaline.

Introduction

Preterm birth, often associated with genital inflammation, is the leading cause of perinatal mortality and morbidity.[1] Although the management of threatened preterm labor by tocolytic therapy can prolong gestation,[2] evidence regarding its efficacy is limited.

A major signaling pathway implicated in maintaining myometrial relaxation is the cAMP/cAMP-dependent protein kinase A (PKA) pathway.[3] While β_2-adrenergic receptor (β_2-AR) agonists stimulate the generation of cAMP in the myometrium,[4] and hence are commonly used to treat preterm labor, local phosphodiesterases (PDE) ensure the termination of signaling by the degradation of cAMP to the inactive 5′-AMP.[5]

PDE4 (formerly known as cAMP-PDE) is the predominant isoenzyme in the majority of inflammatory cells and in the human myometrium.[6] The four genetically distinct PDE4 subtypes, termed PDE4A-D,[7] are

Received: November 15 2011.
Accepted: March 21 2012.
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encoded by alternative mRNA splices, involving at least 35 different PDE4 proteins.[8] In the early 1970s, rolipram, a cAMP-PDE inhibitor, was developed as an antidepressant, although its side-effects (nausea and gastrointestinal disturbances) ruled out its clinical development.[7] Emesis is associated with the inhibition of PDE4D,[9] while PDE4B has been shown to be required for lipopolysaccharide (LPS)-evoked inflammatory responses. LPS, an endotoxin of gram-negative bacteria, causes preterm birth in animals and has been implicated as a factor triggering preterm labor in humans.[10] The prototype selective PDE4 inhibitor rolipram and also β-AR agonists have been reported to modulate the production of inflammatory mediators.[11]

In a previous study, we demonstrated that rolipram potentiated the uterus-relaxing effect of the β_2-AR agonist terbutaline in intact and in LPS-treated rats *in vitro*, the most pronounced relaxation being detected in LPS-treated rats. In the present study, we investigated the tocolytic effects of rolipram and terbutaline in intact and LPS-treated late-pregnant rats *in vivo* and on human uterine specimens *in vitro*. Cyclic AMP levels and the expressions of PDE4B and PDE4D proteins were also determined in human uterine tissues from term and preterm labor.

Methods

Animals

Animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32.§). Experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV./01758-2/2008).

Sexually mature female Sprague-Dawley rats (body mass: 140–160 g, 50–60 days old) were mated in the early morning hours. Copulation was confirmed by the presence of a copulation plug or spermatozoa in the vagina. The day of copulation was considered to be the first day of pregnancy. The animals were housed in temperature- (20–23°C), humidity- (40–60%) and light- (12 h of light, 12 h of dark) regulated rooms, with water and food intake available *ad libitum*.

In vivo treatments

The animals were divided into two groups ($n = 10$ in each): (i) intact pregnant rats on day 22 of pregnancy (full term); and (ii) rats treated with LPS (i.p. 125 μg/day; Sigma-Aldrich, Budapest, Hungary) for 3 consecutive days from day 18 of pregnancy, in the early morning hours, to evoke preterm birth, which occurred in the early afternoon of day 20.[12]

In vivo myographic studies

The *in vivo* myographic studies were done on intact rats (on day 22 of pregnancy; at full term) and on LPS-treated rats (on day 20 of pregnancy; at preterm), in the morning hours between 08.00 and 10.00 hours. Rats were anesthetized with a mixture of ketamine and xylazine (36 and 4 mg/kg, respectively) i.p.; and the jugular vein was cannulated for i.v. drug administration. After laparotomy, the left uterine horn was exposed, and an implantable force/displacement transducer (SEN-04-FSG2; Experimetria, Budapest, Hungary) was sutured onto the myometrial surface, leaving the amniotic membranes untouched. There was no leak of amniotic fluid during the experiments. The animals with the sensors were then placed into a Faraday cage made from iron to filter out environmental electromagnetic noise.

The mechanical displacements elicited by the contractions of the uterus were converted to electrical impulses by the transducer, and amplified by a bridge amplifier (AMP-01-SG, Experimetria, Budapest, Hungary). The amplified electric signal was detected and analyzed by the S.P.E.L. Advanced ISOSYS Data Acquisition System (Experimetria, Budapest, Hungary). The basal tone was registered for 4 min. The area under the curve (AUC) of this 4 min was always used as the control, and the inhibition was calculated as the percentage of this initial period. The AUC of 4-min periods were evaluated; the effects of terbutaline or the terbutaline + rolipram were expressed as percentages of the spontaneous activity.

Doses of terbutaline (Sigma-Aldrich, Budapest, Hungary), and rolipram (Sigma-Aldrich, Budapest, Hungary), were administered i.v. and the contraction signals were recorded. Two 0.5 μg/kg doses of terbutaline were followed by 10 doses of 1 μg/kg at 5-min intervals; 0.25 mg/kg of rolipram was administered similarly to terbutaline alone. In combination, rolipram was given in a single dose of 500 μg/kg. The applied dose was chosen upon the dose-response curve of rolipram, hence the dose eliciting 30% relaxation. This dose (500 μg/kg) is in accordance with some previous *in vivo* studies done on rats.[13,14]

In vitro contractility studies

Thirty biopsy specimens of human myometrial tissue were obtained at cesarean section in the third trimester

of pregnancy in two groups: at full-term pregnancy (37–41 weeks of gestation; $n = 19$) and at preterm birth (32–36 weeks; $n = 11$). At full-term pregnancy cesarean delivery was indicated by a previous cesarean delivery, breech presentation, suspected cephalopelvic disproportion or myopia. Parity varied from 0 to 3, and mean maternal age was 30.8 years (22–41 years). None of the women received a tocolytic agent, and there were no signs of labor.

Preterm delivery occurred in mothers with twin pregnancies, or labor was indicated by an ongoing infection, leukocytosis, toxemia, fetal distress or growth restriction. In the preterm group, parity varied from 0 to 3, mean maternal age was 32.7 years (26–42 years). Three out of 11 patients received tocolytic therapy (magnesium sulfate) to arrest preterm uterine contractions, which proved to be ineffective. All the operations were performed under spinal anesthesia. The Ethical Committee of the University of Szeged approved the clinical protocol (registration number: 114/2009).

Tissue samples ($10 \times 10 \times 20$ mm) from the upper edge of a lower-segment transverse incision were cut after delivering the child, but before oxytocin was given to the mothers. Tissues were stored in Krebs–Henseleit solution (containing in mM: 118 NaCl, 5 KCl, 2 $CaCl_2$, 0.5 $MgSO_4$, 1 K_2SO_4, 25 $NaHCO_3$, 10 glucose; pH 7.4) at 4°C, and were taken into the experiment within 12 h of collection. Each tissue was cut into at least four strips (app. $3 \times 5 \times 10$ mm), mounted vertically in an organ bath (Krebs–Henseleit solution at 37°C; perfused with 95% O_2 + 5% CO_2), and tested in parallel. After mounting, the strips were equilibrated for ~2 h before experiments were undertaken, with a solution change every 15 min. The specimens exhibited spontaneous contractions over the incubation period. The initial tension was set to 3.00 g, which was relaxed to 1.5 g at the end of equilibration. The tension of the myometrial strips was measured with an isometric force transducer (SG-02; Experimetria, Budapest, Hungary) and recorded with an S.P.E.L. Advanced ISOSYS Data Acquisition System.

Rhythmic contractions were elicited by 10^{-6} M oxytocin. After stimulation, theophylline, rolipram, or terbutaline was added in a non-cumulative manner. After each concentration of the tested drug, the strips were washed three times, allowed to recover for 5 min, and then contracted again with oxytocin. Slight tissue fatigue was observed only before the very last dose which did not influence our measurements significantly. The effect of terbutaline was also tested in the presence of 10^{-6} M rolipram. AUC of 4-min periods were evaluated; the effects of rolipram and terbutaline were expressed as percentages of the oxytocin-induced contractions.

Measurement of uterine cAMP accumulation

Human uterine tissue samples from full-term pregnancy and preterm birth were incubated under the same conditions in an organ bath as described above. Basal cAMP accumulations were detected in the presence of rolipram only (10^{-6} M; 10 min). In the presence of rolipram (10^{-6} M; 10 min), the cAMP accumulation was stimulated by terbutaline (10^{-8}; 10^{-6}; 10^{-4} M; 10 min) and finally forskolin was added to the bath (10^{-5} M; 10 min). After stimulation, the samples were immediately frozen in liquid nitrogen in which they were stored until the extraction of cAMP.[15] Uterine cAMP accumulation was measured with a cAMP EIA Kit (Sigma-Aldrich, Budapest, Hungary) and tissue cAMP levels were expressed in pmol/mg tissue.

Western blotting

Proteins from human uterine specimens were isolated with the Macherey-Nagel Nucleospin Kit (Izinta, Budapest, Hungary) according to the manufacturer's instructions. Protein concentrations were determined by BioSpec-nano (Shimadzu, Budapest, Hungary). Sixty micrograms of protein per well was subjected to electrophoresis on 4–12% NuPAGE Bis-Tris Gel in XCell SureLock Mini-Cell Units (Invitrogen, Hungary). Proteins were transferred from gels onto nitrocellulose membranes, using the iBlot Gel Transfer System (Invitrogen, Hungary). The blots were incubated on a shaker with PDE4B, PDE4D and β-actin polyclonal antibodies (Santa Cruz Biotechnology, CA, USA; 1:200) in the blocking buffer, immunoreactivity was detected with the WesternBreeze Chromogenic Western blot immunodetection kit (Invitrogen, Hungary). The optical density (OD) of each immunoreactive band was determined with Kodak 1D Images analysis software. OD values were calculated in arbitrary units after local area background subtraction.

Statistical analyses

In the animal and human contractility studies, AUC were evaluated statistically and maximal inhibitory effects (E_{max}) were calculated with the Prism 4.0 (Graphpad Software Inc. San Diego, CA, USA) computer program. For statistical evaluations, data were analyzed by use of the unpaired *t*-test or one-way ANOVA tests with the Newman–Keuls post-test.

Results

In vivo contractility studies in rats

The effects of the β_2-agonist terbutaline and rolipram were investigated *in vivo*. The contractions were inhibited dose-dependently by both compounds. Maximal inhibitory effect of rolipram in intact rats was 96.7 ± 3.1 standard error of the mean (SEM)% (IC50 = 0.8 ± 0.1 mg/kg; Fig. 1a). The maximal inhibitions achieved with terbutaline + rolipram were not statistically different in intact rats on day 22 of pregnancy (86.1 ± 8.9 SEM%; Fig. 1b) and in LPS-treated rats on day 20 (89.0 ± 9.5 SEM%; Fig. 1c). In case of the combination, rolipram (500 μg/kg i.v.) potentiated the effect of terbutaline, and this effect primarily prevailed at the low doses of terbutaline. In the presence of rolipram and at the lowest dose of terbutaline (0.5 μg/kg), however, the inhibition of the contractions was significantly higher ($P < 0.001$) in the LPS-treated rats (48.1 ± 4.7 SEM%; Fig. 1c) than in the intact rats (33.3 ± 1.4 SEM%; Fig. 1b).

In intact rats at term, the IC50 values for terbutaline (IC50 = 2.94 ± 1.31 μg/kg) were not different from the combination of terbutaline and rolipram (IC50 = 3.53 ± 1.54 μg/kg). Similarly, in the LPS-treated rats on day 20, the IC50 values for terbutaline (IC50 = 2.69 ± 0.98 μg/kg) were not different from the combination of terbutaline and rolipram (IC50 = 5.05 ± 2.71 μg/kg).

In vitro contractility studies in human uterine samples

Uterine activity was characterized by the AUC of the concentration-response curves of human uterine strips. Time control experiments revealed that the oxytocin-stimulated contractions did not decrease significantly through the experiment. Theophylline and rolipram reduced the oxytocin-induced contractions by 24.9 ± 3.6 SEM%, and 61.9 ± 4.5 SEM%, respectively. The contraction-inhibiting effect of rolipram was significantly greater than that of theophylline ($P < 0.001$) (Fig. 2).

The effects of the combination of terbutaline and rolipram were also tested. Whereas terbutaline alone inhibited the oxytocin-stimulated contractions by 71.6 ± 4.5 SEM%, at full-term pregnancy, in the additional presence of 10^{-6} M rolipram, the contractions were inhibited by 78.3 ± 4.7 SEM% (Fig. 3a). At preterm birth, the maximum contraction-inhibiting effect of terbutaline alone was 39.7 ± 4.6 SEM%, but this was increased to 63.5 ± 4.7 SEM% in the presence

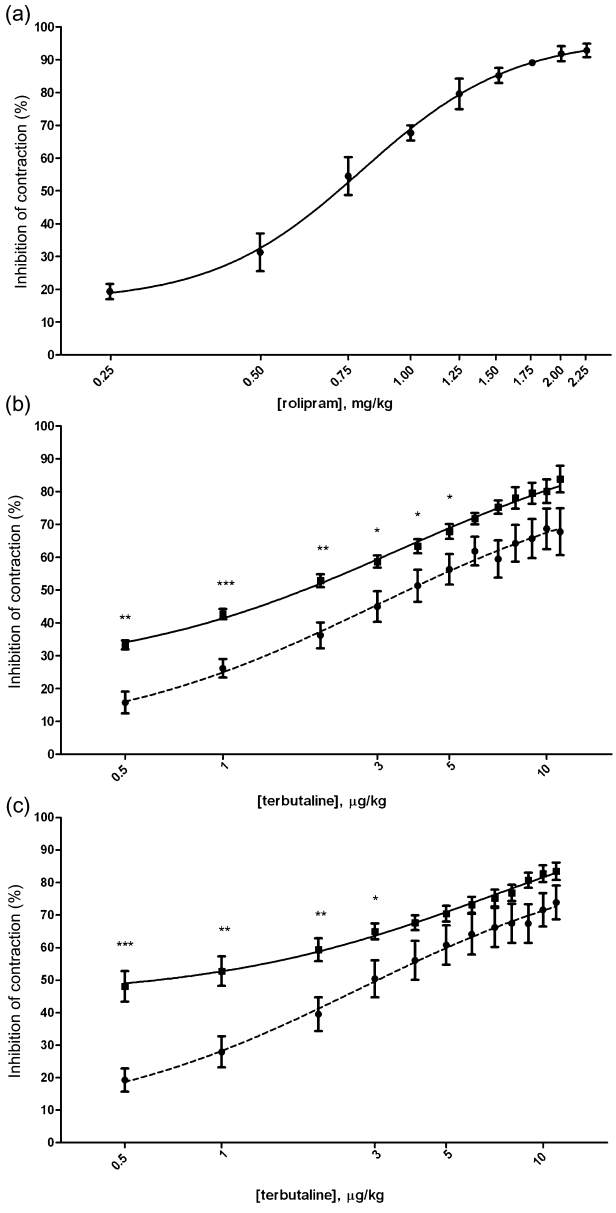

Figure 1 Effect of rolipram on the contraction-inhibiting effect of terbutaline, *in vivo*. Contraction-inhibiting effects are expressed as percentages of the spontaneous uterine activity. (a) On the basis of the dose-response curve of rolipram, we determined the sufficient dose of rolipram to potentiate the contraction-inhibiting effect of terbutaline. In the presence of (■) 500 μg/kg rolipram, the contraction-inhibiting effects of 0.5–11 μg/kg terbutaline were significantly higher in low dose both in (b) intact rats on day 22 and in (c) LPS-treated rats on day 20 of pregnancy than the effects of (●) terbutaline alone. Group comparisons were made by unpaired *t*-test. $n = 10$ in each group; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

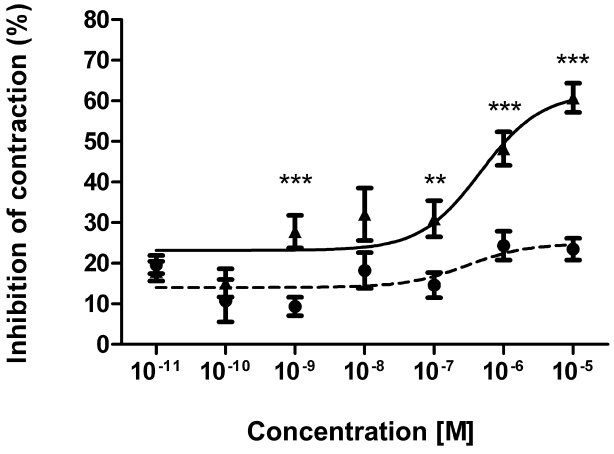

Figure 2 Effects of selective and non-selective phosphodiesterase inhibitors on human uterine contractions at full-term pregnancy, *in vitro*. The contractions were elicited with 10^{-6} M oxytocin and inhibited with (▲) 10^{-11}–10^{-5} M rolipram and (●) theophylline. Group comparisons were made by unpaired *t*-test. $n = 3$ in each group; $^{**}P < 0.01$, $^{***}P < 0.001$.

of rolipram. The effect of 10^{-10} M terbutaline was more than doubled in the presence of 10^{-6} M rolipram ($P < 0.001$) (Fig. 3b).

cAMP levels in human uterine samples

The cAMP levels in human uterine tissue samples from preterm birth and from full-term pregnancy were measured in the presence of 10^{-8}, 10^{-6}, 10^{-4} M terbutaline in combination with 10^{-6} M rolipram. Basal cAMP levels (10^{-6} M rolipram only) were not significantly different between the term pregnant and preterm birth groups. There was a concentration-dependent increase of the terbutaline stimulated cAMP levels in both groups. At 10^{-6} M concentration, terbutaline elicited significant elevation of the cAMP levels ($P < 0.01$) as compared to the basal level, in the preterm birth group. Besides, at 10^{-8} M concentration terbutaline elicited significant elevation of the cAMP levels ($P < 0.01$) as compared to the basal level, in both the full-term and the preterm groups. In the preterm birth samples the cAMP levels were significantly higher ($P < 0.05$) in the presence of 10^{-6} M terbutaline, than in those from full-term pregnancy. There were no significant differences between the groups at 10^{-8} M or 10^{-4} M terbutaline (Fig. 4).

Western blot studies

Western blot analysis revealed the presence of PDE4B and PDE4D in the uterine tissues from term and

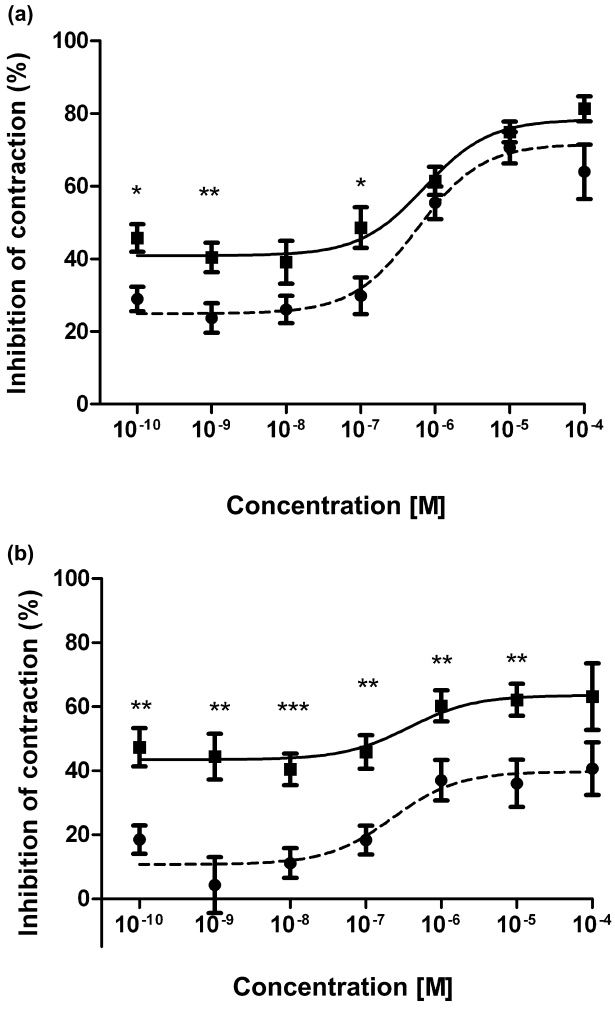

Figure 3 Effects of rolipram on the contraction-inhibiting effects of terbutaline, *in vitro*. The contractions were elicited with 10^{-6} M oxytocin on human uterine samples. The contraction-inhibiting effects of 10^{-10}–10^{-4} M terbutaline were investigated (●) alone or (■) in combination with 10^{-6} M rolipram at (a) full-term pregnancy and at (b) preterm birth. Group comparisons were made by unpaired *t*-test. $n = 3$ in each group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

preterm labor. The expression of PDE4B (Fig. 5a) was significantly higher at preterm labor than at full-term pregnancy. The OD of PDE4D, however, was significantly higher in the uterine tissues from the term pregnancies (Fig. 5b) than at preterm labor.

Discussion

In view of the relatively high rates of adverse maternal and fetal events during tocolysis, and in the hope of

improving the perinatal outcome, there is growing interest in experimental studies in the use of different drug combinations. Numerous experiments have suggested the combinations of tocolytic agents, such as the PDE5 inhibitor sildenafil with the Ca^{2+} channel blocker nifedipine,[16] the oxytocin antagonist atosiban with progesterone,[17] β_2-AR agonists with nifedipine,[18] and β_2-AR agonists with gestagens.[19] We have now tested the tocolytic effects of combinations of the β_2-AR agonist terbutaline and the PDE4 inhibitor rolipram.

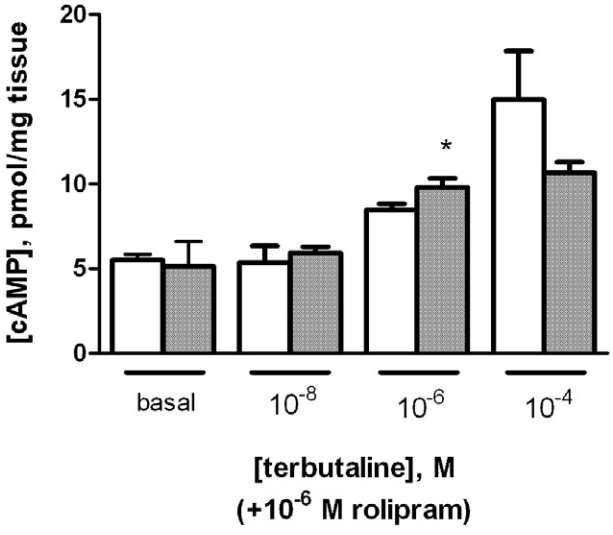

Figure 4 Intracellular cAMP levels stimulated by 10^{-5} M forskolin, in the presence of 10^{-6} M rolipram and 10^{-8}, 10^{-6} or 10^{-4} M terbutaline in human uterine tissue samples from (□) full-term pregnancy and (■) preterm birth. Basal cAMP levels (10^{-6} M rolipram alone) were not significantly different between the term pregnant and preterm birth groups. In the preterm birth group, at 10^{-6} M, terbutaline elevated cAMP levels ($P < 0.01$) relative to the basal level. At 10^{-8} M, terbutaline elevated cAMP levels ($P < 0.01$) as compared to the basal level, in both groups. At 10^{-6} M terbutaline, the cAMP levels were higher (*$P < 0.05$) in the preterm birth samples than in those from full-term pregnancy. These results were analyzed by using one-way ANOVA with the Dunnett post-test. $n = 3$ in each group.

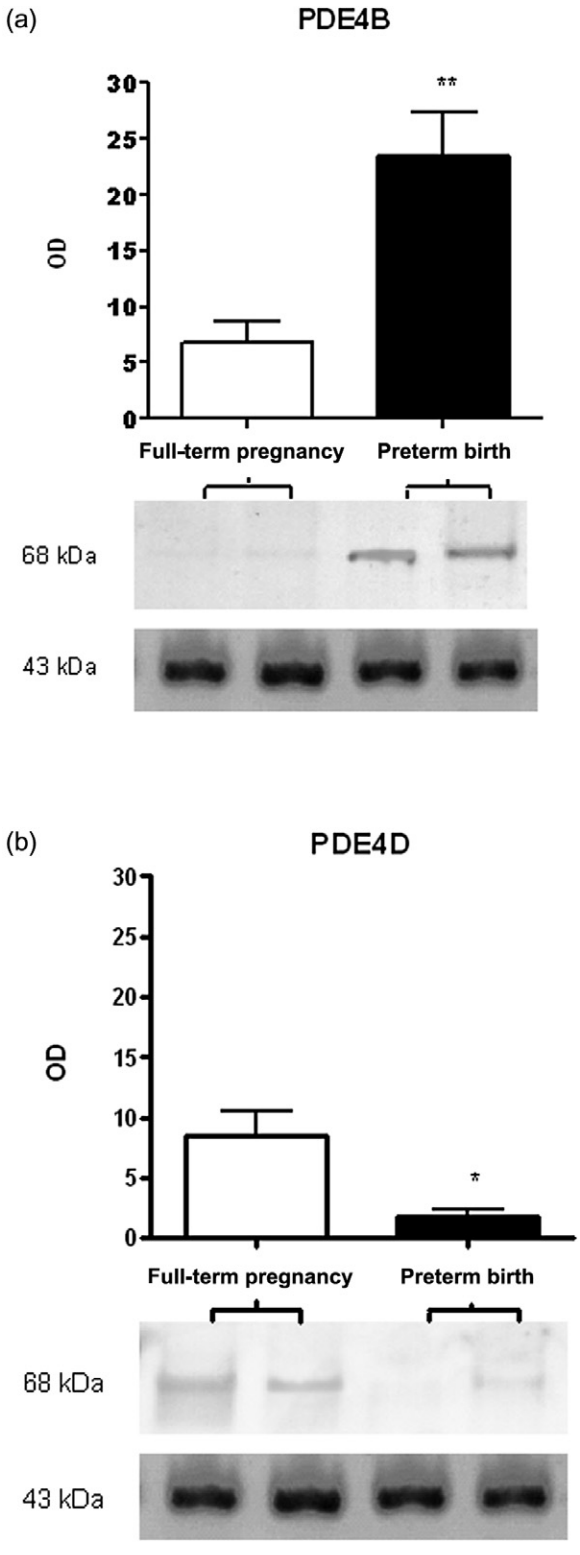

Figure 5 Changes in PDE4B and PDE4D levels in human uterine samples at full-term pregnancy and at preterm birth. The expression of (a) PDE4B was significantly higher at preterm labor than at full-term pregnancy (**$P < 0.01$). The expression of PDE4D was significantly higher in the uterine tissues from (b) term pregnancies than at preterm labor (*$P < 0.05$). Group comparisons were made by unpaired *t*-test. $n = 4$ in each group.

The β_2-AR agonists exert an immediate and profound relaxing effect on the uterine smooth muscle.[20] Recently published systematic reviews (*Lancet*, Cochrane Database) agree that β_2-AR agonists can delay delivery by 48 h, but long-lasting therapy with β_2-AR agonists often fails. The reasons why long-term administration frequently leads to a poorer therapeutic outcome are a drug-induced desensitization of the β_2-AR, and the physiological changes in late pregnancy.[21,22] Despite their unfavorable side-effects (tachycardia and the risk of pulmonary hypertension), the β_2-AR agonists still have an irreplaceable role in tocolytic therapy.[23,24]

On the other hand, the potential beneficial effect of the PDE4 selective inhibitor rolipram as a tocolytic agent has not yet been confirmed. We earlier provided evidence that rolipram potentiates the tocolytic effect of terbutaline both in intact and in LPS-treated pregnant rats, due to the higher cAMP levels.[25] In the present study, we set out to investigate their effects on pregnant rats *in vivo*. Detection of the myometrial activity *in vivo* is accompanied by the benefit of allowing studies of the efficacy of these drugs according to the principles of integrative pharmacology.[26] Experiments on anesthetized rats revealed that the co-administration of rolipram potentiated the uterus-relaxing effect of terbutaline. This effect was more pronounced at lower terbutaline doses. The extent of relaxation achieved with the first dose of terbutaline was 2–2.5 times higher in the intact and in the LPS-treated rats, respectively, when it was co-administered with rolipram. As regards the first three doses of terbutaline, the combination was more effective at the time of LPS-induced preterm labor than at normal term, which is in accordance with our earlier *in vitro* findings.

In human experiments, theophylline exerted a very limited effect, similarly to previous studies on PDE4-transfected cells.[27] The selective PDE4 inhibitor rolipram, however, proved to be much more effective in inhibiting oxytocin-induced uterine contractions. These results correspond with earlier findings on the human uterus in full-term pregnancy.[6,28–31] Bardou *et al*. reported at first that PDE inhibitors potentiate the effects of β_2-AR agonists in the human near-term myometrium *in vitro*.[28] Franova *et al*. investigated the *in vitro* effects of the combination of rolipram and the β_2-AR agonist salbutamol, both given in a single dose of 10^{-4} M, on the human myometrium at term.[32] They found that the oxytocin-induced contractions were inhibited by about 70% by the combination of these two drugs, although the applied doses were very high. Nevertheless, no study has been performed with rolipram and terbutaline on human samples from preterm birth.

Rolipram also potentiated the uterus-relaxing effect of terbutaline in the term and preterm human uterus, its effect being most pronounced at lower terbutaline concentrations and at preterm birth. The findings concerning the tissue cAMP levels were in harmony with the results of the *in vitro* contractility assays, suggesting that the main interaction occurs at an intracellular cAMP level.

Previous studies have demonstrated that the four PDE4 subtypes are expressed in the cytosolic fraction of the human term myometrium,[6] and PDE4-selective inhibitors have been reported to exhibit greater efficacy on the pregnant than on the non-pregnant myometrium.[31] The isoenzymes of PDE4 play a role in inflammatory processes,[32] but there are as yet no data as to whether these proteins are expressed in the preterm human uterus. Our Western blot study demonstrated the upregulation of PDE4B in human samples at preterm birth. This might well be a consequence of ongoing inflammatory processes leading to preterm labor. Intrauterine infection has been recognized as the primary cause of preterm delivery.[33] At preterm birth, the expression of PDE4D was less pronounced than that of PDE4B, which is similar to earlier findings at term.[34] Thus, the inhibition of PDE4D is probably not required for effective tocolysis, which would allow the development of PDE4B-selective inhibitors, with the benefit of no adverse emetic effects.

β_2-AR agonists and PDE4 inhibitors may be of great value for tocolysis, given that they both relieve inflammation and prolong pregnancy.[33] Although the expression of PDE4 has been reported in the cardiac ventricles, PDE4 inhibitors did not produce any positive inotropic effects, which is a typical action of PDE3 inhibitors.[35] Besides the favorable tocolytic effect of the above combination, a lower incidence of cardiac side-effects may also be expected.

Given the low frequency of premature cesarean sections, we believe that every premature sample should be regarded as an almost unique possibility for research. We acknowledge that our sample size is relatively low, and the reasons for such an intervention are diverse. Yet, in spite of the diverse pathology, the expressions of PDE4B were always higher in the preterm tissues, which suggests that this enzyme may be considered a crucial target for drug development.

We conclude that putative therapeutic combinations of β_2-AR agonists and selective PDE4 inhibitors may

enhance the efficacy of human tocolytic therapy. Rolipram alone displays a strong inhibitory effect *in vivo*, although, in human therapy such a high dose may lead to severe side-effects. On the other hand, the co-administration of rolipram in a low dose with terbutaline resulted in a uterus relaxation equivalent to that of rolipram alone. Such drug combinations may also have the benefit of allowing the administration of lower doses of β_2-AR agonists, thereby delaying the desensitization of β_2-AR receptors. We presume that the development of new PDE4B-selective inhibitors may further enhance the efficacy of tocolysis.

Acknowledgment

This work was supported by TÁMOP grant 4.2.1/B-09/1/KONV-2010-0005.

Disclosure

The authors have no relationships with companies that may have a financial interest in the information contained in the manuscript.

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