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Modification of the myometrial relaxing effect of nifedipine *in vitro* and *in vivo*

Ph.D. Thesis

By

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Introduction

Tocolysis is one of the greatest challenges in obstetrical practice. Despite research directed to the development of drugs to inhibit myometrial contractions, there has been no reduction in the incidence of PTB for more than 30 years. A number of agents are used clinically as tocolytics, but the efficacy of the current modes of pharmacological treatment has been questioned.

In recent years, Ca^{2+} channel antagonists have been used increasingly as tocolytic agents. These agents act to inhibit Ca^{2+} influx across the cell membrane, thereby decreasing the tone in the smooth muscle vasculature. The dihydropyridines are the most potent inhibitors of uterine tension development among the Ca^{2+} entry blockers and are therefore of considerable interest for both therapeutic and experimental purposes. Nifedipine and its analogs have recently been considered as tools for tocolytic therapy. With a view to decreasing the potentially maternal and foetal adverse events and improving the perinatal outcome, there is growing interest in experimental studies of the possible use of different tocolytic combinations.

Aims

The main focus of our study was to enhance the tocolytic effect of nifedipine. Since no extensive experiments have been carried out to investigate the *in vivo* and *in vitro* uterus-relaxing effect of a combination of nifedipine and β_2-mimetics or progesterone in the rat and also on human myometrium, the following aims were set:

1. The first aim of the study was to investigate the effects of nifedipine on the potassium chloride (KCl)-evoked rat uterine contractions on different days (15, 18, 20, and 22) of pregnancy *in vitro*.

2. Our further aim was to alter the effect of nifedipine on rat myometrium by applying a combination with terbutaline, progesterone or BK_{Ca}-channel inhibitors, which can have effects on the $Ca_v1.2$ channel.

3. The efficacy of the $Ca_v1.2$ channel blocker – β_2-agonist combination has not been investigated on isolated human myometrium. In our study we investigate how terbutaline and nifedipine modify the contractions of the isolated human myometrium.

4. And finally, our last aim was to investigate the effects of nifedipine – salmeterol and nifedipine – progesterone combination in hormone-induced PTB model *in vivo*, whether these drug interactions apply under *in vivo* circumstances.

Materials and methods

Mature female (180-200 g) and male (240-260 g) Sprague-Dawley rats were mated in a special mating cage. Vaginal smears were taken from the female rats and a sperm search was performed under a microscope at a magnification of 1200 x. When the smear provided positive, the female rats were separated as first-day pregnant animals.

In vitro studies

Uterus preparation, investigation the effect of nifedipine on rats' myometrium

Uteri were removed from rats on day 15, 18, 20 or 22 of pregnancy. Muscle rings 5 mm long were sliced from the uterine horns and mounted vertically in an organ bath containing 10 ml de Jongh solution, which was maintained at 37 °C and carbogen was bubbled through it. Contractions were elicited with 25 mM or 100 mM KCl, and noncumulative dose–response curves were constructed in each experiment in the presence of nifedipine (10^{-11}–10^{-6} M). Concentration–response curves were fitted, and areas under curves (AUCs) were evaluated and analyzed statistically with the Prism 4.0 computer program. For statistical evaluations, data were analyzed by the ANOVA Neuman-Keuls test. .

Investigation the effect of BK_{Ca}-channel blockers on nifedipine action

Uteri were removed from rats on day 22 of pregnancy and mounted vertically in the organ bath as described above. Contractions were elicited with 10^{-6} M oxytocin and cumulative nifedipine (10^{-11} – 10^{-6} M) dose-response curves were constructed in each experiment in the presence of paxilline (5 μM) or TEA (10^{-3} M). For statistical evaluations, data were analyzed by the ANOVA Neuman-Keuls test.

Nifedipine combination with terbutaline

Uteri were removed from rats on day 22 of pregnancy and mounted vertically in the organ bath as described above. Contractions were elicited with 25 mM KCl, and non-cumulative dose–response curves were constructed in each experiment in the presence of nifedipine (10^{-11}–10^{-6} M) and terbutaline (10^{-7} M) or terbutaline (10^{-10}–10^{-4} M) and nifedipine (10^{-7} M). The effects of the nifedipine – terbutaline combination were also investigated in the absence of Ca^{2+} ion *in vitro*. De Jongh solution containing 0.5 mM Ca^{2+} ion was used to induce a low Ca^{2+} environment. After the equilibration period, the normal De Joung solution was changed to the low Ca^{2+}-containing solution. The E_{max} and EC_{50} values of the curves obtained with the combinations were calculated. For statistical evaluations, data were analyzed through the unpaired t test.

In vivo studies

Induction of preterm labour

The animals were treated with mifepristone (3 mg per 0.1 ml) and PGE_2 (0.5 mg/animal) on day 19 of pregnancy. Mifepristone was suspended in olive oil and given as a subcutaneous injection at 9:00 A.M. At 4:00 P.M, PGE_2 was applied intravaginally. The delivery time of the first foetus was noted as the duration in hours from the time of mifepristone administration.

Treatments of the animals

Alzet osmotic pumps were loaded with nifedipine, salmeterol xinofoate solution or vehicle. In combination studies two different osmotic pumps were inserted subcutaneously into the back skin of rats on days 16 or 18 of pregnancy under isoflurane anesthesia. The progesterone treatment of the pregnant animals was started on day 15 of pregnancy and was injected subcutaneously every day up to delivery (days 19 or 20).

Group A was the control group, while group B was treated with vehicle, group C with nifedipine, group D with the nifedipine–salmeterol combination and group E with the nifedipine–P4 combination. There were 8 rats in each group. Statistical analyses were carried out with the analysis of Dunnett's Multiple Comparison Test.

Human myometrial studies

Biopsy specimens of human myometrial tissue were obtained at caesarean section in the third trimester of pregnancy. Uterine smooth muscle tissue samples were collected at 37 to 40 weeks of gestation.

Longitudinal myometrial strips were mounted vertically in an organ bath containing Krebs-Henseleit solution. The organ bath was maintained at 37 °C and carbogen was bubbled through it. After eliciting contractions with 10^{-6} M oxytocin, noncumulative dose–response curves were constructed in each experiment with nifedipine (10^{-11}–10^{-5} M) and terbutaline (10^{-7} M) or terbutaline (10^{-11}–10^{-5} M) and nifedipine (10^{-7} M). Concentrations-response curves were fitted, and areas under curves (AUCs) were evaluated. For statistical evaluations, data were analyzed by use of the unpaired t test.

Results

Animal studies

Organ bath studies

The 25 mM and 100 mM KCl-stimulated uterine contractions were inhibited concentration-dependently by nifedipine in the range of 10^{-11}–10^{-6} M (Fig. 1).

The presence of selective or non-selective BK_{Ca}-channel inhibitors (paxilline or TEA) had no effect on the maximal relaxing effect of nifedipine and did not modify the EC_{50} values of nifedipine. Although, paxilline shifted the nifedipine curve to the left, but it was not significant.
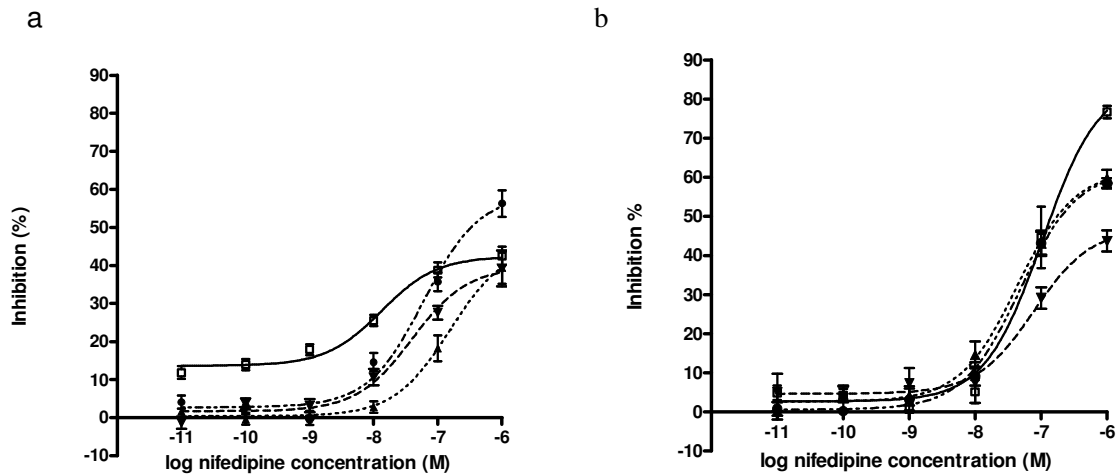
Fig. 1: Contraction inhibiting effect of nifedipine on isolated pregnant rat uteri evoked by 100 mM (**a**) and 25 mM (**b**) potassium chloride *in vitro* on different days of pregnancy. (● day 15, ▲ day 18, ▼ day 20, € day 22).

The concentration–response curves for nifedipine in the presence of 10^{-7} M terbutaline were shifted to the left and a decrease in the maximal inhibitory effect was observed. In the presence of 0.5 mM Ca^{2+} (Ca^{2+}-poor buffer), terbutaline did not alter the effect of nifedipine.

The concentration–response curves for terbutaline in the presence of 10^{-7} M nifedipine were also shifted to the left, but this shift was greater than that of the nifedipine curve by terbutaline. Nifedipine also significantly increased the E_{max} of terbutaline. In the Ca^{2+}-poor buffer, the presence of nifedipine increased the EC_{50} of terbutaline, but did not alter its E_{max}.

In vivo studies

In group A (control), preterm labour occurred within 24 hours after mifepristone treatment, at about 9:00 a.m. on pregnancy day 20. The vehicle (group B) did not alter the time of delivery relative to that in group A.

Nifedipine (group C) treatment started on pregnancy day 16 was effective in delaying the hormone-induced preterm delivery by 6.6 hours. In group D (nifedipine–salmeterol combination), the treatment was extremely effective; preterm birth was delayed by ~ 24 hours as compared with group A.

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When started on day 18 of pregnancy (Fig. 2a.), nifedipine treatment (group C) was not effective. In contrast in group D (combination therapy) the treatment was effective; labour was delayed by ~ 25 hours. The difference in efficacy between groups C and D was most expressed for the treatment started on day 18.

With the nifedipine – P4 combination (Fig. 2b.), the P4 pre-treatment (group E) abolished the effect of nifedipine (group C).
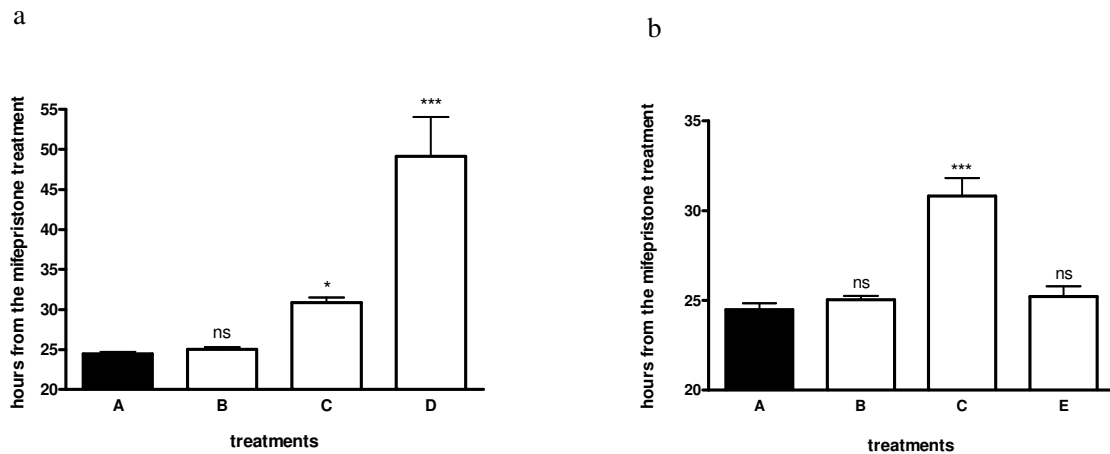
Fig. 6: The effects of treatment with nifedipine (3.89 mg/day) or the nifedipine – salmeterol combination (a) and nifedipine – progesterone (b) on hormone-induced preterm delivery in the rat (n = 8 for each group). Treatment was started on gestation day 16.

A: control group; B: vehicle-treated (PEG400 : ethanol : physiological salt solution = 6:6:4 + methanol : water =1:1 in osmotic minipump); C: nifedipine-treated; D: nifedipine – salmeterol combination-treated; E: nifedipine – progesterone treated animals.

The *bar graphs* show means ± SD. The effects were compared with the results on group A.

ns: not significant; *: p<0.05; ***: p<0.001.

Human myometrial studies

The 10^{-6} M oxytocin-stimulated human uterine contractions were inhibited concentration-dependently by nifedipine and terbutaline in the range 10^{-5}–10^{-11} M (Fig. 3. a, b). The addition of 10^{-7} M terbutaline to nifedipine did not alter the E_{max} of nifedipine, but decreased EC_{50} from 1.8 x 10^{-8} M to 1.5 x 10^{-6} M (p<0.05) (Fig. 3a). The addition of 10^{-7} M nifedipine to terbutaline increased the E_{max} of terbutaline (Fig. 3b), from 60.8% to 76.8 % (p<0.01) but EC_{50} was not changed.

a   b
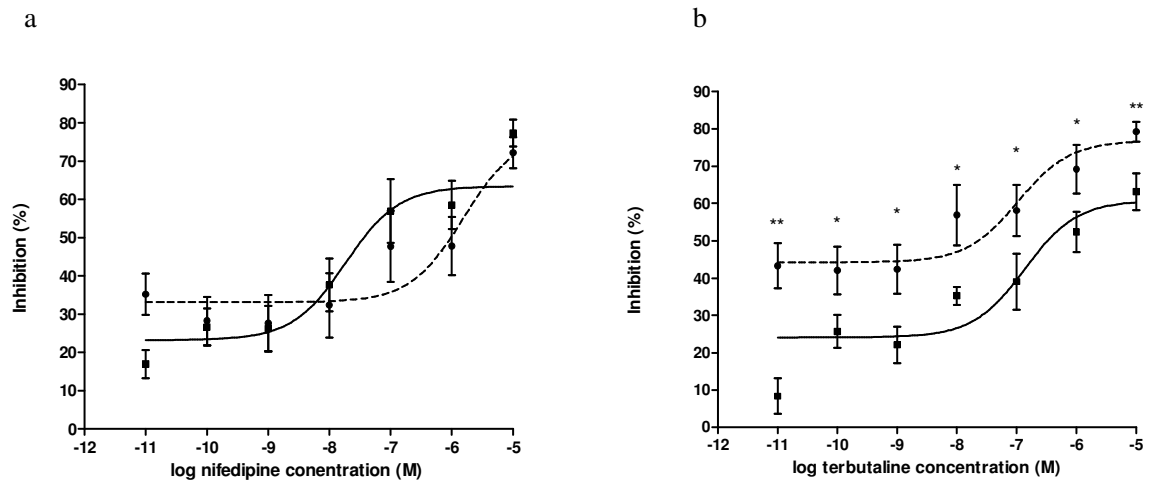
Fig. 3: The effects of treatment with the nifedipine – terbutaline combination on oxytocin-evoked human myometrial contractions *in vitro*.

a: ■ nifedipine (10^{-11}-10^{-5} M); ● terbutaline (10^{-7} M) + nifedipine (10^{-11}-10^{-5} M)

b: ■ terbutaline (10^{-11}-10^{-5} M); ● nifedipine (10^{-7} M) + terbutaline (10^{-11}-10^{-5} M

Discussion

The most important factor controlling force in the myometrium is the concentration of intracellular Ca^{2+}, and membrane potential is the major factor governing Ca^{2+} entry into the cell. In the presence of 25 mM KCl the uterine contractions were rhythmic and the relaxing effect of nifedipine was the highest on the last day of pregnancy. These results led us to conclude that stimulation with 25 mM KCl is much more appropriate for investigations of the action of the Ca^{2+} channel blocker nifedipine. As the relaxing effect of nifedipine was highest on the last day of pregnancy, further experiments were carried out on day 22.

The activity of the $Ca_v1.2$ channel is regulated by several factors. The studies with the selective BK_{Ca} channel blocker paxilline and non-selective blocker TEA were carried out by oxytocin-induced contractions, because in these cases the stimulation with KCl was not useful. The results revealed that BK_{Ca} channels and any other K^+ channels are not involved in the relaxing effect of nifedipine in pregnant rat myometrium.

Progesterone is regarded as a preventive drug against PTB, especially in late-preterm birth. Unfortunately, the *in vivo* progsterone pre-treatment decreased the maximal inhibitory effect of nifedipine and increased its EC_{50}.

The other investigated factor which regulates the $Ca_v1.2$ channel was the β_2-adrenergic system. We found synergism in the uterus-relaxing effect of nifedipine and the β_2-AR agonist terbutaline, although the extent of potentiation depended on the sequence of administration of the two compounds. When terbutaline was added first in a single dose, synergism was found in EC_{50} (the nifedipine curve was shifted to the left), but the maximal inhibitory effect of nifedipine was lower. When nifedipine was administered first, the relaxing effect of terbutaline was obviously stronger.

The entry of the Ca^{2+} into the cells through the voltage-gated Ca^{2+} channel is one of the crucial factors in the generation of smooth muscle contraction. Terbutaline possibly activates the $Ca_v1.2$ channels and decreases the maximal relaxing effect of nifedipine. The resultant effect of the increase of cAMP level and activation of $Ca_v1.2$ channels causes a weaker smooth muscle relaxation. In the opposite case, when nifedipine is administered first, the $Ca_v1.2$ channels are blocked; hence, there is only a low possibility that terbutaline can activate them.

To check on the above-mentioned hypothesis, the synergism between the two compounds was investigated in Ca^{2+}-poor buffer. A Ca^{2+}-poor environment theoretically decreases the terbutaline-induced Ca^{2+} influx and may alter the extent of the synergism. The Ca^{2+}-poor environment shifted the nifedipine dose-response curve to the left, and the maximal inhibitory effect of nifedipine was so high that its effect could not be enhanced by terbutaline. In contrast, the Ca^{2+}-poor environment shifted the terbutaline dose-response curve to the left, but nifedipine was able to enhance the shift. However, it could not increase the maximal uterus-relaxing effect of terbutaline, possibly because of the very strong blocking effect of the β-mimetic. These results indicate that, in a Ca^{2+}-poor environment, terbutaline is not able to worsen the maximal effect of nifedipine, which suggests the role of the Ca^{2+} inflow in the weakening effect of terbutaline.

Synergism between nifedipine and the β_2-agonist terbutaline was also investigated on human myometrium tissue, which revealed that both nifedipine and terbutaline inhibit the oxytocin-induced myometrial contractions dose-dependently. When terbutaline was added first, it decreased the EC_{50} of nifedipine, though it did not alter the E_{max} of nifedipine. In the opposite case, nifedipine administered first increased the relaxing effect of terbutaline, though there was no difference in EC_{50}. The results suggest a parallelism between the human situation and that in the rat myometrium.

In view of these results, the effects of nifedipine – salmeterol and nifedipine – progesterone combination were investigated also in hormone-induced PTB model *in vivo*, whether these drug interactions apply under *in vivo* circumstances. We found that treatment with nifedipine alone started on pregnancy day 16 was more effective in delaying delivery than salmeterol treatment alone. Similarly, the nifedipine – salmeterol combination also had a great effect.

The short-term effects of nifedipine and its combination with salmeterol were also tested in our experiments. Nifedipine treatment started on day 18 did not delay labour, but the nifedipine – salmeterol combination was as effective as the combination started on the pregnancy day 16. The ability of nifedipine to delay labour was tripled by its combination with the β_2-agonist. In our *in vivo* study two osmotic pumps were implanted subcutaneously in the rat and the administration of the two agents was simultaneous and continuous. The results suggested that the parallel administration of the two compounds may lead to a similar benefit as that of nifedipine-potentiated terbutaline treatment.

Progesterone pre-treatment also abolished the ability of nifedipine to delay labour in hormone-induced preterm delivery in rats *in vivo*. These results correlate with the hypothesis that progesterone decreases the activity of the L-type Ca^{2+} channels.

In the light of our results, we can conclude that progesterone pre-treatment abolished the ability of nifedipine to delay labour in hormone-induced preterm delivery in rat. These results correlate with the hypothesis that progesterone decreases the activity of the L-type Ca^{2+} channels.

There is growing interest in nifedipine as a potentially effective and well-tolerated form of tocolysis. Nifedipine has been demonstrated to have fewer side-effects and leads to a better neonatal outcome than β_2-mimetics. We presume that the effect of nifedipine in tocolytic therapy might be intensified through combination with β_2-adrenerg agonists and enhanced by low concentrations of β_2-mimetics. However, our results indicate that the administration of β_2-adrenergic agonists can not precede that of nifedipine. The significance of these experimental findings remains to be validated in clinical trials, including human side effects.

Appendix

Publications related to the Ph.D. thesis:

Hajagos-Tóth J, Falkay G, Gáspár R. Modification of the effect of nifedipine in the pregnant rat myometrium: The influence of progesterone and terbutaline. Life Sci 2009; 85:568–572. **IF: 2.56**

Hajagos-Tóth J, Kormányos Zs, Falkay G, Pál A, Gáspár R. Potentiation of the uterus-relaxing effects of beta-adrenergic agonists with nifedipine: studies on rats and the human myometrium. Acta Obstetricia et Gynecologica 2010; 89:1284-1289 **IF: 1.618**

Hajagos-Tóth J, Kormányos Zs, Falkay G, Pál A, Gáspár R. Nifedipin myometrium relaxáló hatásának vizsgálata terbutaline és K^+-csatorna gátlók jelenlétében in vitro. Acta Pharm Hung 2010;3: 109-114

Other publications:

Klukovits A, Tekes K, Gündüz Cinar O, Benyhe S, Borsodi A, Deák BH, **Hajagos-Tóth J**, Verli J, Falkay G, Gáspár R. Nociceptin inhibits uterine contractions in term-pregnant rats by signaling through multiple pathways. Biol Reprod. 2010 83(1):36-41 **IF: 3,300**

Abstracts:

Hajagos-Tóth J, Gáspár R, Falkay G. Investigation of uterine-relaxing effect of nifedipine on late-pregnant rat myometrium *in vitro*. The First Conference of PhD Students in Medicine and Pharmacy, July 9-11, 2008, Targu Mures, Romania (presentation)

Hajagos-Tóth J, Falkay G, Gáspár R. Nifedipin uterusz kontrakciót gátló hatásának vizsgálata *in vitro*. A Magyar Tudomány Ünnepe; Szeged, 2008. November 28. (presentation)

Hajagos-Tóth J, Falkay G, Gáspár R. Investigation of uterus-relaxing effect of nifedipine on late pregnant myometrium *in vitro*. 2nd Meeting of the Egon & Ann *Diczfalusy* Foundation; Szeged, 2008.09.30-10.01. (poster)

Hajagos-Tóth J, Falkay Gy, Gáspár R. Nifedipin uterusz kontrakciót gátló hatásának módosítása ptogeszteronnal és terbutalinnal *in vitro*. XIV. Congressus Pharmaceuticus Hungaricus; Budapest, 2009. november 13-15. (poster)

Hajagos-Tóth J, Kormányos Zs, Falkay Gy, Gáspár R. A nifedipin tocolyticus hatásának módosítása béta-mimetikumokkal. A Magyar Élettani Társaság (MÉT) LXXIV. Vándorgyűlése és a Magyar Kísérletes és Klinikai Farmakológiai Társaság (MFT) II. Közös tudományos konferenciája; Szeged, 2010. június 16-18. (presentation)

Hajagos-Tóth J, Kormányos Zs, Falkay G, Gáspár R. Potentiation of uterus-relaxing effects of nifedipine with β-adrenergic agonists: Studies on rats and human myometrium. 16th World Congress on Basic and Clinical Pharmacology , 17-23 July, 2010, Copenhagen, Denmark (poster)