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University of Szeged, Faculty of Pharmacy**

PhD thesis

**The effects of antioxidants on the smooth muscle-relaxing action of
terbutaline**

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1. Introduction

Free radicals are defined as any chemical species that contains unpaired electron in its outer orbit. Because of these unpaired electrons, they are highly reactive and readily take part in chemical reactions with all cell components (lipids, proteins and nucleic acids) in the body. They are generated from physiological processes to produce energy and metabolites. Under normal conditions, ROS are safely neutralized by the antioxidant defense systems, including superoxide dismutase, catalase, glutathione peroxidases and glutathione reductase. These systems are complex, located in different cellular compartments and are often redundant or complementary in various conditions. Oxidative stress occurs when the balance between antioxidants and ROS are disrupted because of either depletion of antioxidants or accumulation of ROS. When it occurs, cells attempt to counteract the oxidant effects and restore the redox balance by activation or silencing of genes encoding defensive enzymes, transcription factors, and structural proteins. ROS inhibitors have emerging roles in the therapy and prevention of diseases, but may clearly have an impact on ROS-dependent physiological processes. Typical compounds with these characteristics include some vitamins, such as vitamin C and E, that have a direct activity like scavengers and also some indirect activities related to different mechanisms that may have an impact on the oxidative stress.

There is mounting evidence over the past decade implicating ROS as important molecules in intracellular signal transduction. G protein-coupled receptors regulate production of ROS in turn, determine the nature and duration of the cell signal. In the case of GPCRs, the stimulation of the respective receptors linked to the formation of ROS. Stimulation of several GPCRs such as muscarinic acetylcholine, angiotensin II-1, dopamine, as well as the serotonin receptors either increase or decrease ROS generation with significant downstream signaling consequences, suggesting that GPCR-mediated ROS signaling may have an important role in homeostatic balance which may be altered in pathophysiological states. Moreover, β_2 -AR agonists isoproterenol were shown to increase mitochondrial ROS production in the cardiomyocytes. It stimulates the Ca^{2+} influx eliciting stronger contraction. The inhibition of ROS attenuates β_2 -AR signal transduction, suggesting that a low level of intracellular ROS may influence the whole β_2 -AR signal transduction. Thus, ROS seems to act as second messengers of specific ligands. Since antioxidants are very popular agents nowadays, we would like to examine the smooth muscle-relaxing action of β_2 -adrenergic receptor agonists terbutaline in the presence of limonene or tocopherol.

2. Aims of the study

The main focus of our study was to investigate how antioxidant agents alter the β_2 -AR signaling and contractility in uterine and tracheal smooth muscles in rats.

1. The first aim was to study the separate effects of tocopherol or limonene on pregnant rat myometrium and cervix, and on trachea from non-pregnant rat. Myometrial contractions, cervical and tracheal tones were measured in organ bath.

2. Since ROS have a role in cell-signaling of cardiac muscle, our further aim was to investigate how tocopherol or limonene may alter the effect of terbutaline on myometrial, cervical and tracheal smooth muscle activity.

3. The sexual hormones influence the activity of β_2 -ARs. While progesterone enhances the receptor function in the uterus, the estrogen increases the receptor activity in the trachea. Therefore our next aim was to reveal the consequences of sexual hormone pre-treatment on the β_2 -AR-mediated smooth muscle response in the presence of antioxidants.

4. Our last aim was to clarify the reasons of the possible differences in response among the smooth muscle tissues toward the modifying action of tocopherol or limonene during β_2 -agonist action. The cAMP and oxidative status of the smooth muscles were investigated by ELISA method.

3. Materials and methods

3.1. Mating of the animals

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. Copulation was determined by the presence of a copulation plug or the presence of sperms in a native vaginal smear. The day of conception was regarded as the first day of pregnancy.

3.2. In vivo sexual hormone treatments of the rats

The progesterone (P4) treatment of the pregnant animals was started on day 15 of pregnancy. P4 was dissolved in olive oil and injected subcutaneously every day up to day 21 at 0.5 mg/0.1 ml. The estrogen treatment was started with non-ovariectomized female rats in the estrous phase. The animals were injected subcutaneously with 5 μ g/kg of 17 β estradiol-valerate (E2) dissolved in olive oil once a day for a period of 4 days.

3.3. PCR and Western blot studies

PCR

The myometria, cervices from pregnant rats (day 22) and the trachea from female rats in estrous cycle were rapidly removed and placed in RNAlater Solution (Sigma-Aldrich, Hungary). The tissues were frozen in liquid nitrogen and then stored at -70 °C until the extraction of total RNA.

Western blot

50 µg of protein per well was subjected to electrophoresis on 4-12% NuPAGE Bis-Tris Gel in XCell SureLock Mini-Cell Units (Life Technologies, Hungary). Proteins were transferred from gels to nitrocellulose membranes, using the iBlot Gel Transfer System (Life Technologies, Hungary). The antibody binding was detected with the WesternBreeze Chromogenic Western blot immunodetection kit (Life Technologies, Hungary). The blots were incubated on a shaker with β_2 -adrenergic receptor and β -actin polyclonal antibody (Santa Cruz Biotechnology, California, 1:200) in the blocking buffer. Images were captured with the EDAS290 imaging system, and the optical density of each immunoreactive band was determined with Kodak 1D Images analysis software. Optical densities were calculated as arbitrary units after local area background subtraction.

3.4. In vitro contractility studies

Cervical and myometrial rings were dissected from the rats of non-treated and P4-treated 22-day-pregnant rats. Muscle rings were mounted in an organ bath containing 10 ml de Jongh solution. Trachea tissues were cut transversally into small rings, which were placed in Krebs solution.

The myometrial, cervical and tracheal activities were measured with a gauge transducer (SG-02, Experimetria Ltd, Budapest, Hungary) and recorded with a SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd, Budapest, Hungary).

3.5. Measurement of cAMP accumulation

The tissue samples (myometrium, cervix and trachea) were incubated in an organ bath as described above. The effects of terbutaline (10^{-6} M) and/or tocopherol (10^{-7} M) on cAMP accumulation were detected. Tissue cAMP accumulation was measured with a commercial competitive cAMP enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, Michigan, USA), and expressed as pmol/mg tissue.

3.6. Measurement of total oxidant and antioxidant status

The total oxidant status (TOS) was measured with a kit provided by RelAssay Diagnostics (Gaziantep, Turkey). Oxidants present in the sample oxidize the ferrous ion-*o*-dianisidine complex to ferric ion. The color intensity is proportional to the total amount of oxidant molecules present in the sample. The results were expressed in terms of $\mu\text{mol H}_2\text{O}_2$ equiv./L. The total antioxidant status (TAS) was measured by RelAssay Diagnostics (Gaziantep, Turkey). Antioxidants in the sample reduce the dark blue-green ABTS radical to the colorless ABTS form. The results were expressed as Trolox equivalent (mmol Trolox equiv/L).

The oxidative stress index (OSI) was defined as the ratio of TOS and TAS. A standardized value does not exist for OSI levels, which were used only for comparisons. OSI was calculated via the formula $\text{OSI (arbitrary unit)} = \text{TOS } (\mu\text{mol H}_2\text{O}_2 \text{ equiv./L}) / \text{TAS (mmol Trolox equiv/L)}$.

3.7. Statistical analysis

All data were analyzed with the Prism 4.0 (GraphPad Software, USA) computer program, and the values were statistically evaluated with the unpaired t-test or with ANOVA with Dunnett's Multiple Comparison Test.

4. Results

4.1. β_2 -adrenergic receptor expression in the smooth muscle cells

The progesterone treatment did not alter the expression of β_2 -AR mRNA in pregnant myometria and cervixes. However, the Western blot study revealed that the progesterone treatment increased the expressions of β_2 -ARs in the uterine tissues, respectively. The estrogen treatment enhanced the expression of the receptor mRNA, and increased the expressions of β_2 -ARs in tracheal tissues, respectively (**Fig. 1.**).

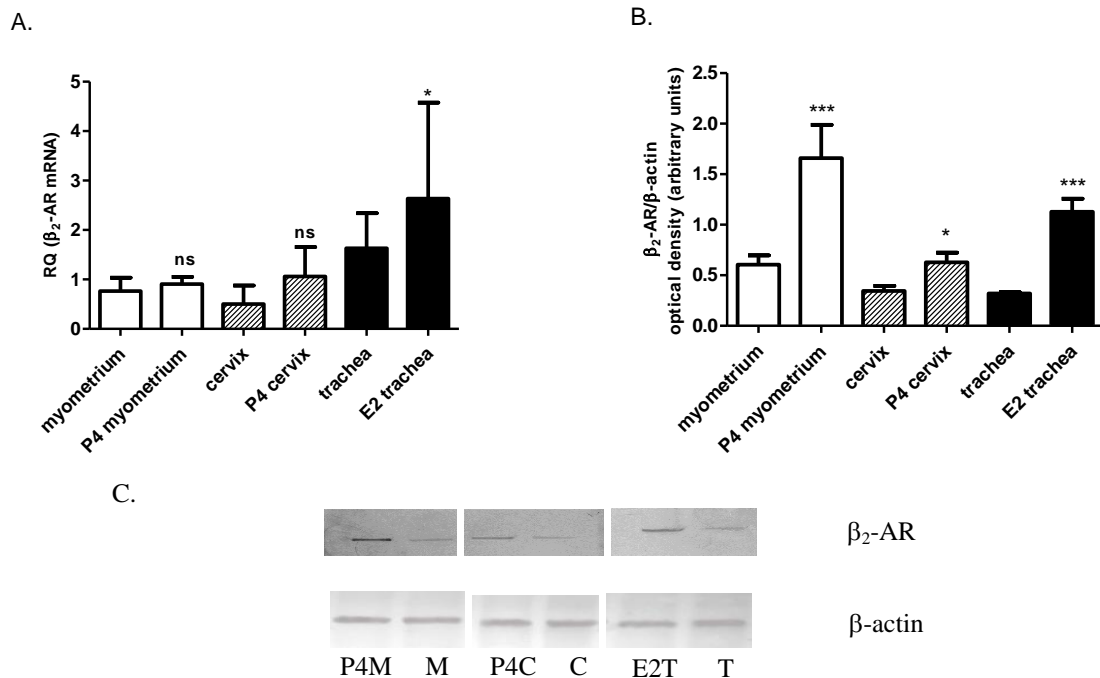


Figure 1. : β₂-receptor expression in rat myometrium, cervix és trachea. *: $p < 0,05$, *** $p < 0,001$

ns: non-significant

P4M: P4-treated myometrium, M: myometrium

P4C: P4-treated cervix, C: cervix

E2T: E2-treated trachea, T: trachea

4.2. Organ bath studies

Myometrium. Terbutaline (10^{-10} – 10^{-5} M) elicited a concentration-dependent inhibitory effect on the spontaneous contractions of the 22-day-pregnant myometria. The presence of tocopherol (10^{-7} M) did not change the effect of terbutaline in either non-treated or P4-treated tissues. The concentration–response curve of terbutaline was shifted to the left for myometrial samples from P4-pretreated animals (**Fig.2A.**).

In the presence of d-limonene (10^{-13} M) the concentration–response curve of terbutaline was shifted to the left; however the maximal effect of terbutaline remained unchanged. The P4 treatment enforced the terbutaline effect on myometrial relaxation, but the D-limonene did not modify the concentration-response curve of terbutaline (**Fig.2B.**).

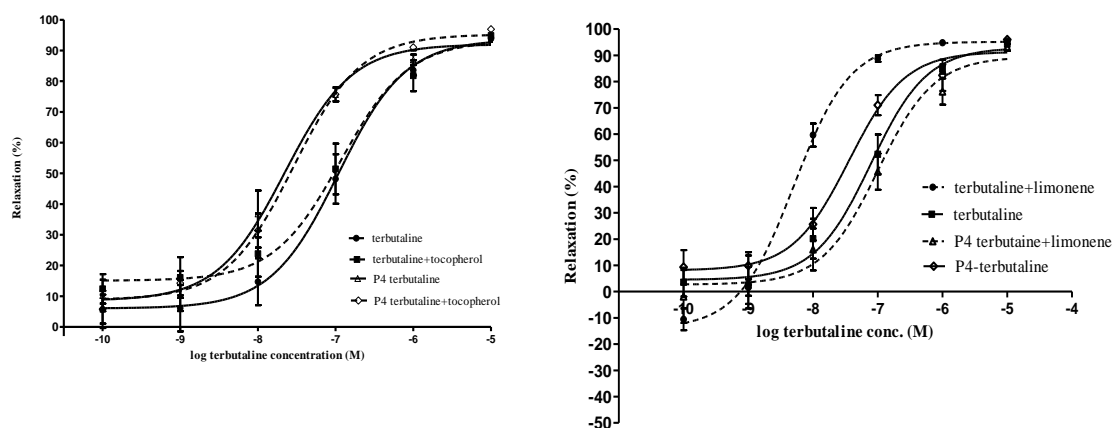


Figure 2. Effects of terbutaline on spontaneous contractions of non-treated and P4-pretreated 22-day-pregnant rat myometria, alone (curves with continuous line) and in the presence of limonene (10^{-13} M) or tocopherol (10^{-7} M) (curves with dashed lines).

Cervix. Terbutaline (10^{-6} M) enhanced the cervical resistance of 22-day-pregnant cervical samples as compared with the control value. Tocopherol (10^{-7} M) and D-limonene (10^{-13} M) did not significantly alter the cervical resistance-increasing effect of terbutaline (**Fig. 3.A.**). P4 pretreatment increased the cervical resistance and left the resistance-increasing action of terbutaline unchanged. However, the presence of tocopherol, and also D-limonene eliminated the effect of terbutaline in P4-pretreated cervical samples (**Fig. 3.B.**).

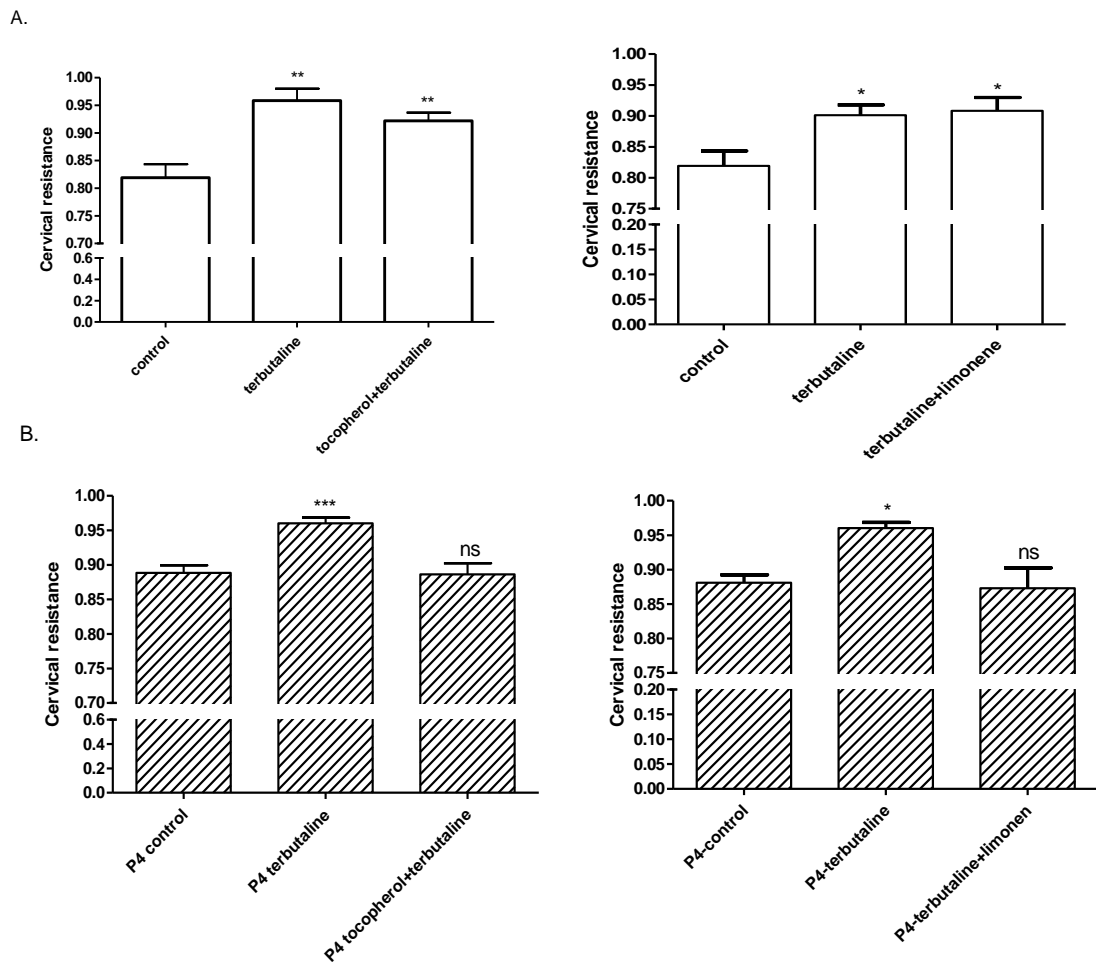


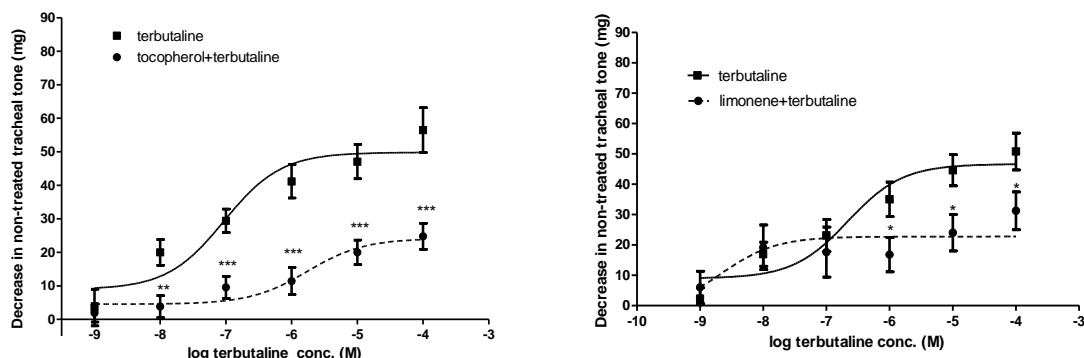
Figure 3. Effects of terbutaline on the resistance of P4-pretreated 22-day-pregnant rat cervixes, alone and in the presence of tocopherol (A) and limonene (B).

ns: non- significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ as compared with the control.

Trachea. Terbutaline (10^{-9} M – 10^{-4} M) decreased the tone of tracheal segments with an average of 56 ± 3 mg. In the presence of tocopherol (10^{-7} M), the relaxing effect of

terbutaline was reduced significantly. D-limonene (10^{-13} M) also reduced the relaxing effect of terbutaline (**Fig. 4A.**). After *in vivo* E2-pretreatment, the tracheal tone-reducing effect of terbutaline was stronger. The reducing effect was enhanced in the presence of tocopherol and d-limonene (**Fig 4.B.**).

A.



B.

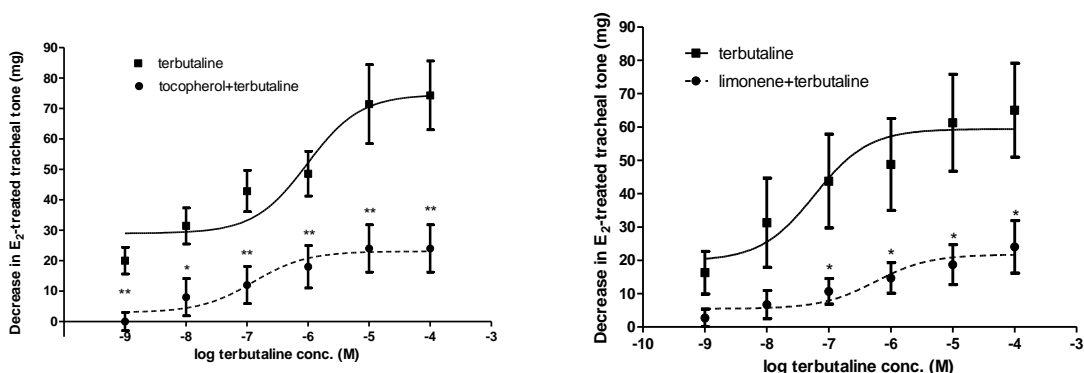


Figure 4. Tone-reducing effect of terbutaline on isolated rat tracheal preparations, with or without tocopherol or limonene. (A) After *in vivo* E2-treatment, the relaxing effect of terbutaline was even higher (B). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ as compared with terbutaline effect alone

4.3. Oxidative and antioxidative status of myometrial, cervical and tracheal smooth muscles

TOS was lowest in the pregnant myometrium, and highest in the trachea. The pregnant cervical TOS value was higher than the pregnant myometrial value. The TOS values of the non-pregnant myometrium and cervix were similar to those for the pregnant samples. TAS was highest in the pregnant myometrium, and lowest in the trachea. The TAS values of the non-pregnant myometrium and cervix were lower than those for the pregnant samples (**Fig. 5.A.**). OSI (TOS/TAS) was lowest in the pregnant myometrium, and highest in the trachea. The OSI value of the pregnant cervical cervix was lower than those for the non-pregnant sample (**Fig.5.B.**).

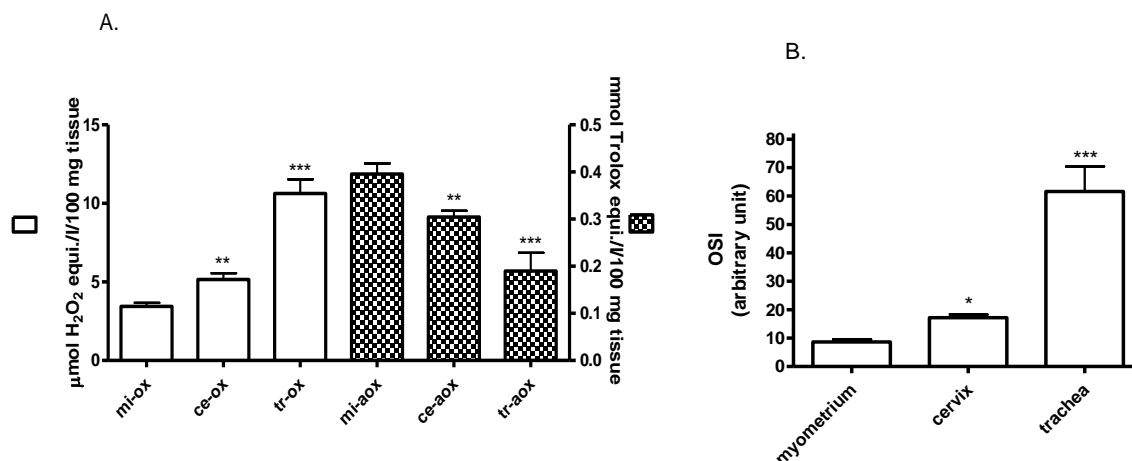


Figure 5. The oxidative (empty columns) /antioxidant status (stripped columns) in the myometrial, cervical and tracheal tissues. (A) The oxidative stress index (OSI) was calculated as the ratio of TOS and TAS. The OSI was highest in the trachea and lowest in the pregnant myometrium. (B)

mi: myometrium, ce: cervix, tr: trachea

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ as compared with the pregnant myometrial values

5. Discussion

Previous studies suggested that, ROS are important molecules in inter- and intracellular signal transduction and in redox homeostasis. Therefore, we hypothesized that an antioxidant effect may interact with the signal mechanism of β -ARs.

Since β_2 -ARs are of therapeutic importance in the relaxation of uterine and bronchial smooth muscles, we examined whether the antioxidant agents (limonene, tocopherol) alter the beta-mimetic action in these tissues. β_2 -AR agonist, terbutaline was added to last-day-pregnant myometrial and cervical rat tissue, and to tracheal samples from estrous phase female rats in an organ bath. The responses of the tissues were in harmony with the literature data: the myometrium was relaxed, the cervical resistance was increased, and the tracheal tone was reduced.

D-limonene caused myometrial contraction in a dose-dependent manner, while tocopherol had no significant action. Interestingly, in the presence of the antioxidant agents reduced the effect of terbutaline was reduced only in the case of the trachea, while the uterine (myometrium and cervix) responses were unchanged. These results indicate that differences may occur in the ROS dependence of the β_2 -AR signal mechanism in different smooth muscles.

Sexual hormones are known to play a role in the regulation of G-proteins of β_2 -ARs in the myometrial, cervical and tracheal smooth muscles. P4 enhances the signaling of

myometrial and cervical β_2 -ARs, while E2 is able to increase the second messenger processes in airway smooth muscle. Theoretically, when the signal mechanism is increased, the linked ROS production should also be increased. After the hormonal induction of the β_2 -AR signal in the myometrium, tocopherol and limonene still remained ineffective on the action of terbutaline. However, the antioxidants significantly reduced the cervical response to terbutaline, and a further decline was found in the trachea. These changes were supported by the altered intracellular cAMP productions of the tissues. These findings suggest that ROS are necessary for the β_2 -AR-linked cAMP production in the trachea and in the hormonally induced cervix, while their contribution is not so important in the pregnant myometrium.

The differences observed between these tissues raised the possibility of the tissue-specific regulation of ROS production after β_2 -AR stimulation.

The ROS and productions of a given tissue can be characterized by the determination of TOS and TAS, the ratio of which gives the OSI, an accepted parameter of the oxidative status. Although our method was not able to distinguish between the ROS and the free radicals, earlier studies showed the predominance of ROS in uterine and tracheal processes. In our studies the highest OSI was found in the trachea and the lowest in the pregnant myometrium. The terbutaline-induced relaxation was inhibited most strongly by tocopherol and limonene in the trachea, where the OSI was highest. On the other hand, the antioxidants were ineffective in the pregnant myometrium, which has the lowest oxidative stress level.

This means that the ROS dependence of the β_2 -AR signal varies with the localization and function of the smooth muscles. It is not yet known which steps of the second messenger mechanisms require the presence of ROS to provide uninterrupted intracellular signals.

Our results suggest that a high OSI in a tissue implies a higher importance of ROS in the signal mechanism of β_2 -AR. So, in the case of tocopherol and limonene the anti-asthmatic effect of beta-mimetics may worsen during parallel antioxidant administration.

Publications related to the Ph.D. thesis:

1. **Hódi A**, Földesi I, Ducza E, Hajagos-Tóth J, Seres AB, Klukovits A, Gáspár R. Tocopherol inhibits the relaxing effect of terbutaline in the respiratory and reproductive tracts of the rat: The role of the oxidative stress index. Life Sci. 105 (1-2):48-55 (2014).
(2.702 impact factor)
2. Judit Hajagos-Tóth, **Ágnes Hódi**, Adrienn B. Seres, Róbert Gáspár: Effects of d- and l-limonene on the pregnant rat myometrium in vitro. Croat Med J. 2015 Oct;56(5):431-8.
(1,373 impact factor)
3. **Hódi A.**, Földesi I., Hajagos-Tóth J., Ducza E., Gáspár R.: The effect of R (+) limonene on β -adrenergic signaling: the significance of oxidative stress index. Acta Pharm Hung. 2014 84(3):111-9.

Poster presentations:

1. Hódi Ágnes, Hajagos-Tóth Judit, Seres Adrien, Klukovits Anna, Gáspár Róbert: Tocopherol interacts with beta-mimetic effect in reproductive and respiratory tracts in the rat
Society for Endocrinology BES, Harrogate, United Kingdom, 2013
2. Hódi Ágnes, Földesi Imre, Hajagos-Tóth Judit, Seres Adrien, Klukovits Anna, Gáspár Róbert: Terbutalin szimuláns relaxáló hatása tocopherol jelenlétében: az oxidatív státusz szerepe
Congressus Pharmaceuticus Hungaricus XV., Budapest, Magyarország, 2014

