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**Antioxidants may reduce the smooth muscle-  
relaxing action of  $\beta_2$ -adrenergic receptor agonists**

Ph.D. Thesis

By

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## List of abbreviations

**ATP:** adenosine triphosphate

**$\beta_2$ -AR:**  $\beta_2$ -adrenergic receptor

**Ca<sup>2+</sup>:** calcium ion

**cAMP:** cyclic adenosine monophosphate

**DAG:** diacyl-glycerol

**E2:** 17-beta-estradiol valerate

**GPCR:** G-protein-coupled receptor

**IP3:** inositol-1,4,5-triphosphate

**NADPH:** nicotinamide adenine dinucleotide phosphate

**NOX:** NADPH oxidases

**OS:** oxidative stress

**OSI:** oxidative stress index

**P4:** progesterone

**PIP<sub>2</sub>:** phosphatidyl-inositol-4,5-bisphosphate

**PKC:** protein kinase C

**PM:** plasma membrane

**PLC:** phospholipase C

**ROS:** reactive oxygen species

**RNS:** reactive nitrogen species

**SMC:** smooth muscle cells

**TOS:** total oxidant status

**TAS:** total antioxidant status

**Tris-HCl:** tris-(hydroxymethyl)-aminomethane hydrochloride

## List of publication

This thesis based on the following publications:

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  $\beta$ -adrenergic signaling: the significance of oxidative stress index. Acta Pharm Hung. 2014 84(3):111-9.

# 1. Introduction

## *1.1. Free radicals and they role in cell signaling*

Free radicals is 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 (Kohen et al. 2002). They are generated from physiological processes to produce energy and metabolites. In most biological systems, the free radicals of interest are often referred to as reactive oxygen species (ROS), as the most biologically significant free radicals are oxygen centered. There is another major group of free radicals which are nitrogen-centered, and many other free radical groups depending the nature of the compound (carbon, lipids and generic radical). Free radicals tend to be transformed into ROS, which are considered to be the most important reactive species (Robles et al. 2013). The reason for this transformation is that the final product of the reaction will be H<sub>2</sub>O, which has no toxic value. So the most commonly produced and well studied ROS are generated during the oxidative phosphorylation or one-electron reduction of O<sub>2</sub> in the mitochondria as natural byproduct. The apparent paradox in the roles of ROS as toxic byproducts of metabolism and as essential biomolecules in the regulation of cellular function may be related to differences in concentrations. They have many important biological functions in our body, some can even act as messengers through a phenomenon called redox signaling. They are ideally suited in many ways to be signalling molecules: they are small, and can diffuse short distances; there are several mechanisms for their production, some of which are rapid and controllable; and there are numerous mechanisms for their rapid removal (Thannickal VJ et al. 2000). Under normal conditions, ROS are safely neutralized by the antioxidant defense systems, including superoxide dismutase, catalase, glutathione peroxidases and glutathion reductase. These systems are complex, located in diferrent 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 is occur, 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. Higher production of ROS in body may change DNA structure, result in modification of proteins and lipids, activation of several stress-

induced transcription factors, and production of proinflammatory and anti-inflammatory cytokines (Birben et al. 2012).

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 characteristic include some vitamins, such as vitamin C and E, that have a direct activity like scavengers and also some indirect activities related to different mechanism that may have an impact on the OS. Tocopherol (vitamin E), a lipid-soluble membrane-localized antioxidant, prevents the propagation of free radical reactions and preserves cell membranes by reacting with lipid peroxyl radicals and undergoing conversion to a nonreactive tocopheroxyl radical. Tocopherol is one of the most frequently consumed antioxidants in both nutrition and dietary supplements. Moreover there are many extensive and varied structural compounds occurring in nature. Terpenes display a wide range of biological and pharmacological activities. Limonene is a major component of several plant essential oils, including orange, lemon, mandarin, lime and grapefruit. Because of its pleasant lemon-like sweet smell it is used widely as an additive in perfumes, soaps, foods, chewing gums and beverages (Lappas et al 2012).

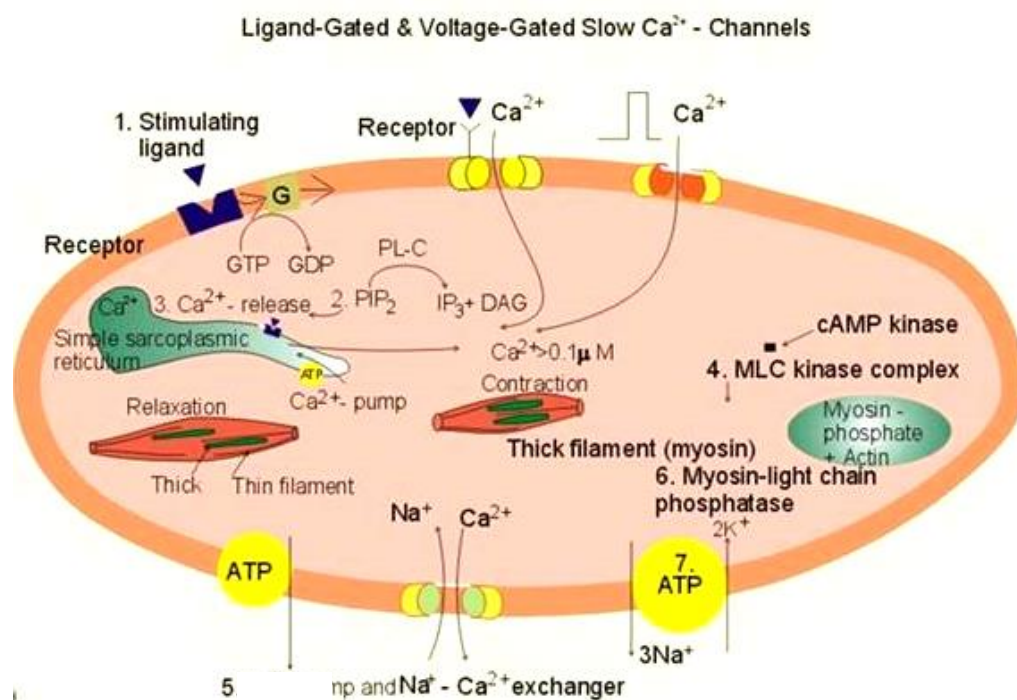
There is mounting evidence over the past decade implicating ROS are important molecules in intracellular signal transduction. For example, the generation of ROS attenuates epidermal growth factor-mediated activation of extracellular signal-regulated kinase, leading to the suggestion that ROS production is an intrinsic signal transduction desensitizer (Maziere et al. 2003). Moreover, the engagement of B cell receptors with immunoglobulin in lymphoma cells promotes ROS-dependent amplification of the cell signal, leading to the conclusion that ROS are rather signal transducers (Singh et al. 2005). They also play key roles in response to a variety of stimuli, including peptide growth factors and cytokines. ROS can also covalently modify proteins and such oxidative modifications, which can greatly alter protein function, have been implicated in certain conditions (Oberley 2003). In addition, hydrogen peroxide and superoxide that can mediate oxidative pathology also mediate changes in  $\text{Ca}^{2+}$ -channel function via alterations of thiol groups (Hool et al. 2007). Agonist binding to certain tyrosine kinase and G protein-coupled receptors regulates 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 (Thannickal and Fanburg 2000). 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 (Mukhin et al. 2000, Yang et al. 2006). Moreover,  $\beta_2$ -AR agonists were shown to stimulate alterations in the redox states of the receptor (Marques et al. 1997). G protein mediated ERK 1/2 phosphorylation was also blunted by ROS depletion, suggesting that ROS are indispensable for  $\beta_2$ -AR-mediated G protein signaling. A similar effect was seen with  $\beta_2$ -AR-mediated  $\beta$ -arrestin signaling. However, the inhibition of ROS attenuates  $\beta_2$ -AR signal transduction, suggesting that a low level of intracellular ROS may influence the whole  $\beta_2$ -AR signal transduction (Moniri et al 2007). Thus, ROS seems to act as second messengers of specific ligands, including different growth factors and adrenergic signaling (Corbi et al, 2013). Previous study show, that acute  $\beta$ -adrenergic stimulation with isoproterenol induces a cAMP- and PKA-dependent increase in mitochondrial ROS production in intact ventricular mouse cardiomyocytes. In addition, this increase in ROS plays a crucial role in the  $\beta$ -adrenergic inotropic effect, because the isoproterenol-induced increases in  $\text{Ca}^{2+}$  transient amplitude is diminished in the presence of the general antioxidant NAC. So isoproterenol is known to increase mitochondrial ROS production in the cardiomyocytes and this action is part of the  $\beta_2$ -AR signal transduction (Andersson et al. 2011).

### ***1.2. Signal transduction pathways in smooth muscle***

Smooth muscle cells are a class of contractile cells with diverse phenotypes that are necessary for the proper function of many organs. The voltage-activated L-type calcium ( $\text{Ca}^{2+}$ ) channels at the plasma membrane (PM) play a central role in the function of SMC by controlling their contraction. Increase in intracellular  $\text{Ca}^{2+}$  is attributed to stimulation of phospholipase C by activation of G protein-coupled plasma membrane receptors. This enzyme stimulates the breakdown of the membrane phospholipid phosphatidyl-inositol-4,5-bisphosphate ( $\text{PIP}_2$ ), resulting the production of the two intracellular second messengers inositol-1,4,5-triphosphate ( $\text{IP}_3$ ) and diacyl-glycerol (DAG).  $\text{IP}_3$  diffuses through the cytosol and binds to specific sites on intracellular calcium stores, resulting in release of  $\text{Ca}^{2+}$  into the cytoplasm. DAG, the other product of  $\text{PIP}_2$  hydrolysis, is able to activate protein kinase C (PKC), may also be able to stimulate  $\text{Ca}^{2+}$  entry (**Fig.1**). SMC also express nonvoltage-dependent  $\text{Ca}^{2+}$  channels that are typically activated by mechanical stimuli or downstream of phospholipase C (PLC) isoforms in response to stimulation by vasoactive and growth factor receptors (Chung et al. 2000). These intracellular signalling pathways stimulate contractile responses by G protein-

coupled receptors. One of the most-studied GPCRs is the  $\beta_2$ -AR, which usually mediates relaxation in different smooth muscles.



**Figure 1.** Contraction and relaxation in smooth muscle cells. (Paulev et Zubieta-Callej 2004)

Relaxation is brought about predominantly by stimulation of  $G_s$ -protein-coupled adenylyl cyclase (AC), which enzyme catalyses the breakdown of ATP to cAMP. Increased levels of cAMP are therefore responsible for the  $\beta_2$ -AR-mediated relaxation of smooth muscle (Xiao *et al.* 2006). The stimulation of  $\beta_2$ -AR has therapeutic importance in relaxation of the bronchial tract (bronchial asthma) and the pregnant uterus (preterm birth) (Cazzola *et al.*, 2013, Gáspár *et al.*, 2005). For both, one of the most frequently used betamimetics is terbutaline, although its use in threatened preterm labor is strongly debated (Sayed *et al.*, 2013, Dodd *et al.*, 2012).

### 1.3. Hormonal effects on $\beta_2$ -ARs

Sex hormones play role in the regulation of G-proteins of  $\beta_2$ -ARs in the myometrial, cervical and tracheal smooth muscles. Previous studies have demonstrated that acute estradiol ( $E_2$ ) administration can induce protein kinase G dependent relaxation in the cardiovascular system mediated by cAMP. (Keung *et al.* 2011). The colocalization of  $\beta_2$ -AR and estrogen



receptors (ER) may play a role in the interaction between these receptors in potentiating relaxation. Furthermore, both estrogens and  $\beta_2$ -AR activate a common mechanism, cAMP/PKA, raising the possibility of using estrogens to enhance  $\beta_2$ -AR agonist effects to induce bronchodilation (Townsend et al. 2012). It is a potentially novel route for enhancing  $\beta_2$ -AR-induced bronchodilation by modulation of estrogen signaling. The relevance of such pathway lies in the possibility of reducing  $\beta_2$ -AR utilization and thus desensitization. Clinically relevant concentrations of estrogen can potentiate  $\beta_2$ -AR-induced reduction in intracellular calcium of airway smooth muscle, as well as relaxation.

Earlier findings suggested that P4 play a role in the regulation of G-proteins in the myometrium (Elwardy et al. 1994). The presence of progesterone is a determining factor for the pregnant uterine-relaxing action of terbutaline. This correlation can be explained by the  $\beta_2$ -AR density increasing effect of progesterone. Importantly, P4 treatment causes an elevation in the number of myometrial  $\beta_2$ -ARs (Gaspar et al. 2005). Thus, higher progesterone level means better G-protein activation and a stronger inhibitory action of betamimetics on myometrial contractions. Theoretically, when the signal mechanism is increased, the linked ROS production should also be increased.

## **2.Aims of the study**

The main focus of our study was to investigate how antioxidant agents alter the  $\beta_2$ -AR signaling and contractility in uterine and tracheal smooth muscles in rats.

1. The first aim was the study to investigate 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 contractility or tones.

3. The sexual hormones influence the activity of  $\beta_2$ -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  $\beta_2$ -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  $\beta_2$ -agonist action. The cAMP and oxidative status of the smooth muscles were investigated by ELISA method.

### **3. Materials and methods**

#### ***3.1. Housing and handling of the animals***

The animals were treated in accordance with the European Communities Council directives (86/609/EEC) and the Hungarian Act for the protection of animals in research (XXVIII.tv.32.§). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (permission number: IV./198/2013). Sprague-Dawley rats were kept at  $22 \pm 3$  °C, with a relative humidity of 30-70%, under a lights-darkness cycle of 12 h : 12 h. The animals were maintained on a standard rodent pellet diet (Charles-River Laboratories, Budapest, Hungary), with tap water available *ad libitum*. The animals were terminated by CO<sub>2</sub> inhalation.

#### ***Mating of the animals***

Mature female Sprague-Dawley rats in estrus were collected. The estrous cycle was detected by measurement of the vaginal impedance with an Estrus Cycle Monitor EC40 (Fine Science Tools, Foster City, CA, USA). The selected female and male rats were mated in a special mating cage. A metal door separated the rooms for the male and the female animals. The separating door was opened before dawn by a small electric engine controlled by a timer. In the morning, within 4-5 h after the possible mating, vaginal smears were taken from the female rats. 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 (n=8) 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. On day 22, the uteri were collected and the contractility and molecular pharmacological studies were carried out as described below.

The estrogen treatment was started with non-ovariectomized female rats (n=8) in the estrous phase. The animals were injected subcutaneously with 5 µg/kg of 17βestradiol-valerate (E2) dissolved in olive oil once a day for a period of 4 days.

### ***3.3. Determination of myometrial, cervical and tracheal $\beta_2$ -AR mRNA by real-time reverse transcription-PCR and Western blot***

*Tissue isolation.* The myometria, cervixes 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.

*Total RNA preparation.* Total cellular RNA was isolated by extraction with guanidinium thiocyanate-acid-phenol-chloroform according to the procedure of Chomczynski and Sacchi (1987). After precipitation with isopropanol, the RNA was washed with 75% ethanol and then resuspended in diethyl pyrocarbonate-treated water. RNA purity was controlled at an optical density of 260/280 nm with BioSpec Nano (Shimadzu, Japan); all samples exhibited an absorbance ratio in the range 1.6-2.0. RNA quality and integrity were assessed by agarose gel electrophoresis.

*Real-time quantitative reverse transcription-PCR (RT-PCR).* Reverse transcription and amplification of the PCR products was performed by using the TaqMan RNA-to-CT<sup>TM</sup> 1-Step Kit (Life Technologies, Hungary) and the ABI StepOne Real-Time cycler. RT-PCR amplifications were performed as follows: 48 °C for 15 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. The generation of specific PCR products was confirmed by melting curve analysis. The following primers were used: assay ID Rn00560650\_s1 for  $\beta_2$ -adrenergic receptor and Rn00667869-m1 for  $\beta$ -actin as endogenous control. All samples were run in triplicates. The fluorescence intensities of the probes were plotted against PCR cycle numbers. The amplification cycle displaying the first significant increase of the fluorescence signal was defined as the threshold cycle ( $C_T$ ).

*Western blot analysis of myometrial, cervical and tracheal  $\beta_2$ -ARs*

50  $\mu$ g 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  $\beta_2$ -adrenergic receptor and  $\beta$ -actin polyclonal antibody (Santa Cruz Biotechnology, California, 1:200) in the blocking buffer. Images were captured with the EDAS290 imaging system (Csertex Ltd., Hungary), 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***

Myometrial rings were dissected from the horns of nontreated (n=8) and P4-treated 22-day-pregnant rats (n=8). Muscle rings 5 mm long were mounted vertically in an organ bath containing 10 ml de Jongh solution (composition in mM: 137 NaCl, 3 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 12 NaHCO<sub>3</sub>, 4 NaH<sub>2</sub>PO<sub>4</sub>, 6 glucose, pH 7.4). The organ bath was maintained at 37 °C and carbogen (95% O<sub>2</sub> + 5% CO<sub>2</sub>) was bubbled through it. The initial tension of the preparation was set to about 1.5 g. After mounting, the rings were equilibrated with a solution change every 15 min for about 1 h before the experiments were undertaken. After the equilibration period, the myometrial rings were incubated for another 60 min with tocopherol (10<sup>-7</sup> M) or limonene (10<sup>-13</sup> M). The tissues were washed and further tocopherol or limonene was administered every 15 min. The control samples were incubated for 1 h without antioxidants. A dose-response curve of 10<sup>-10</sup> - 10<sup>-5</sup> M terbutaline was obtained in a cumulative manner

Cervical tissues of nontreated (n=8) and P4-treated 22-day-pregnant rats (n=8) were removed on pregnancy day 22. Two cervical rings were separated and mounted (with their longitudinal axis vertically) by hooks in an organ bath containing 10 ml de Jongh buffer. The initial tension was set to about 1.00 g. After mounting, the rings were equilibrated for 1 h, and then another 1 h with tocopherol (10<sup>-7</sup> M) before the experiments was undertaken, with a buffer and tocopherol change every 15 min. The control samples were incubated for 1 h without tocopherol. Terbutaline (10<sup>-6</sup> M) was added to the organ bath and the cervixes were incubated for 5 min. The cervixes were stretched in incremental steps and allowed to relax for 5 min. After every 5 min, the next initial tension was set, in 1-g steps between 1 and 10 g. The tension was increased manually via the control screw of a gauge transducer. The resultant stress-strain curves had a sawtooth shape. In the evaluation of the cervical resistance, the initial tension of the cervix was plotted versus the stretch after 5 min. Straight lines were fitted by linear regression and the slopes of the lines were used to express the degree of resistance. A steeper slope reflected a higher resistance (Gaspar et al. 2005).

Trachea tissues were dissected from female rats (160-260 g, n=8) in the estrous phase. The isolated trachea was cut transversally into small rings, which were placed in Krebs solution (composition in mM: 118 NaCl; 4.75 KCl; 2.5 CaCl<sub>2</sub>; 1.19 K<sub>2</sub>HPO<sub>4</sub>; 25 NaHCO<sub>3</sub>; 1.2 MgSO<sub>4</sub>; and 11 glucose). The tracheal tube was cut into rings 4–5 mm wide, which were mounted with their longitudinal axis vertically by hooks. The initial tension was set to about 2.00 g. The tissue was incubated for 60 min. The buffer solution was renewed every 15 min.

After the equilibration period, the tracheal muscle preparation was incubated with tocopherol ( $10^{-7}$  M)/limonene ( $10^{-13}$  M) for another 60 min with renewal of the buffer solution and the administration of further tocopherol or limonene every 15 min. The control samples were incubated for 1 h without tocopherol. A cumulative dose–response curve of  $10^{-9}$  -  $10^{-4}$  M terbutaline was obtained.

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 and total oxidant and antioxidant status***

#### *Measurement of cAMP accumulation*

The tissue samples (myometrium, cervix and trachea, n=6/group) 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 in the presence of the nonspecific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine ( $10^{-3}$  M) and the adenylyl cyclase activator forskolin ( $10^{-5}$  M). Tocopherol/limonene incubation was performed 1 h before terbutaline treatment. Terbutaline was added to the samples for 10 min. The samples were then immediately frozen in liquid nitrogen and stored until the extraction of cAMP (Gáspár et al. 2007). Frozen tissue samples were ground, weighed, homogenized in 10 volumes of ice-cold 5% trichloroacetic acid, and centrifuged at 1000 g for 10 min. The supernatants were extracted with three volumes of water-saturated diethyl ether. After drying, the extracts were stored at  $-70^{\circ}\text{C}$  until the cAMP assay. 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.

#### *Measurements of total oxidant and antioxidant status*

The myometrial, cervical and tracheal tissues of rats (n=6/group) were excised and rinsed with ice-cold saline. Tissue homogenates were prepared from a known amount of tissues, homogenized in 10% (w/v) 20 mM Tris-HCl buffer, pH 7.4 at  $4^{\circ}\text{C}$ , with an IKA Ultra-Turrax T-25 Digital Homogenizer (Janke & Kunkel, Staufen, Germany). The homogenates were centrifuged at 5000 g for 10 min at  $4^{\circ}\text{C}$ . The supernatant was collected as tissue homogenate, and was used for the biochemical estimations (Unal et al. 2012).

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 oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion yields a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically at 530 nm, is proportional to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of  $\mu\text{mol H}_2\text{O}_2$  equiv./L. The assay characteristics were described earlier (Erel 2005).

The total antioxidant status (TAS) was measured with a 3<sup>rd</sup>-generation kit provided by RelAssay Diagnostics (Gaziantep, Turkey). Antioxidants in the sample reduce the dark blue-green ABTS radical to the colorless ABTS form. The change in absorbance at 660 nm is proportional to the total antioxidant level of the sample. The assay is calibrated with a standard stable antioxidant solution, traditionally referred to as Trolox equivalent, which is a vitamin E analog. The assay characteristics were described earlier (Erel 2004).

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  $\text{OSI (arbitrary unit)} = \text{TOS } (\mu\text{mol H}_2\text{O}_2 \text{ equiv./L}) / \text{TAS (mmol Trolox equiv/L)}$  (Erel 2004).

### ***3.6. Statistical analysis***

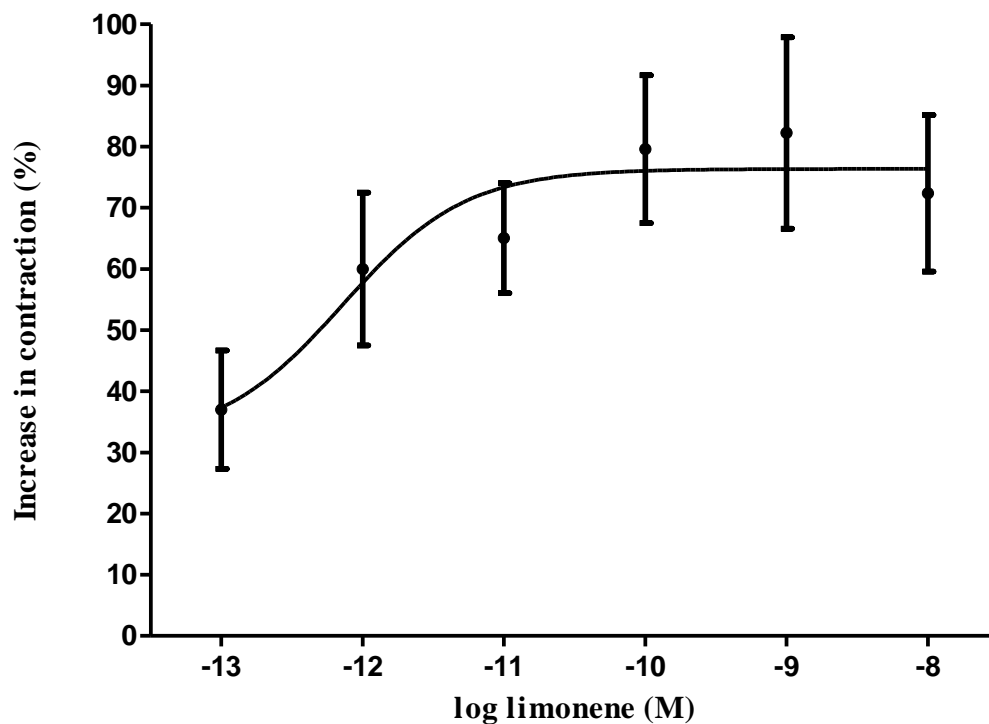
All data were analyzed with the Prism 4.0 (GraphPad Software, USA) computer program, and the values were statistically evaluated with the unpaired t-test or with ANOVA with Dunnett's Multiple Comparison Test.

## 4. Results

### 4.1. Effect of D-limonene and tocoherol on smooth muscle contractility

D-limonene caused myometrial contraction in a dose-dependent manner (**Fig.2.**) The compound has no effect on cervical resistance and on tracheal tone (result is not shown).

Tocopherol at  $10^{-7}$  M did not significantly alter the myometrial contractions, cervical resistance or tracheal tone (result is not shown).

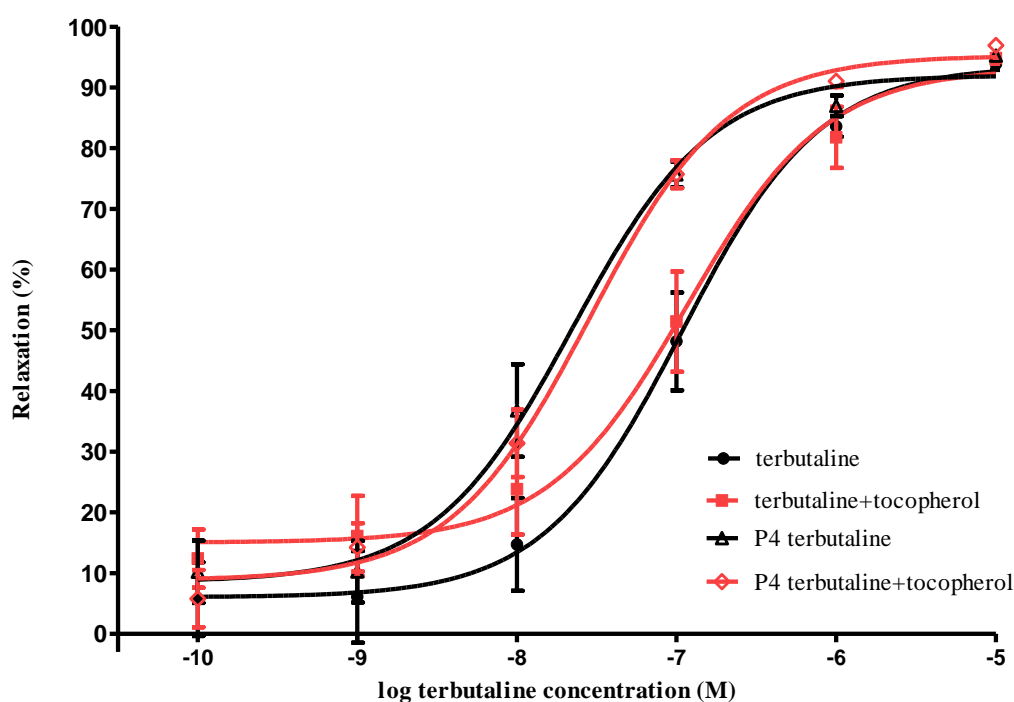


**Figure 2.** The effect of D-limonene ( $10^{-13}$  –  $10^{-8}$ M) on 22 day pregnant rat myometrial contraction, *in vitro*. (n=6)



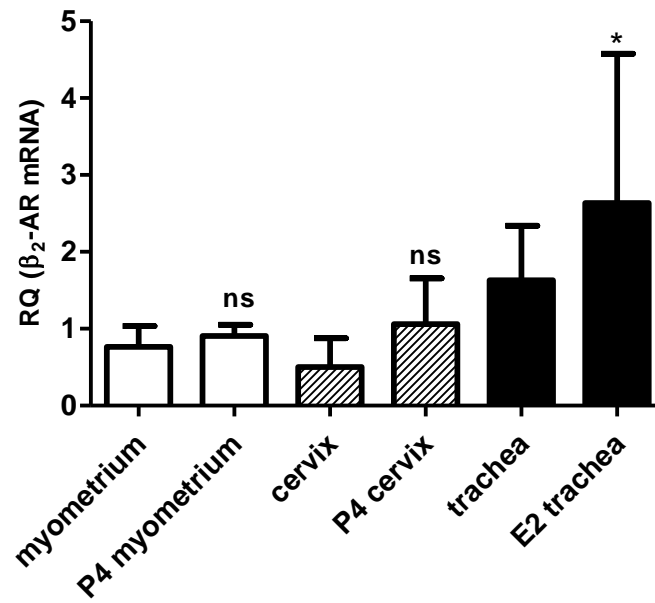
#### 4.2. Effects of tocopherol and limonene on the myometrial action of terbutaline

Terbutaline 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 (**Fig. 3.**). The concentration–response curve of terbutaline ( $EC_{50}$ :  $1.1 \times 10^{-7} \pm 0.6 \times 10^{-8}$  M) was shifted to the left for myometrial samples from P4-pretreated animals ( $EC_{50}$ :  $2.2 \times 10^{-8} \pm 1.4 \times 10^{-8}$  M). The progesterone treatment did not alter the expression of  $\beta_2$ -AR mRNA in pregnant myometria. However, the Western blot study revealed that the progesterone treatment increased the expressions of  $\beta_2$ -ARs in the uterine tissues, respectively (**Fig. 4.**).

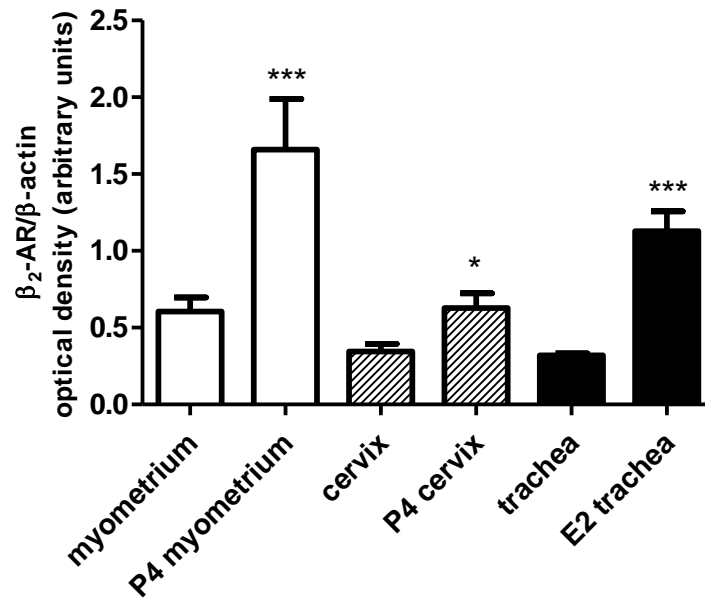


**Figure 3.** Effects of terbutaline on spontaneous contractions of nontreated and P4-pretreated 22-day-pregnant rat myometria, alone and in the presence of tocopherol ( $10^{-7}$  M). The concentration–response curve of terbutaline was shifted to the left by P4-pretreatment (black lines). The presence of tocopherol did not change the effects of terbutaline (red lines)

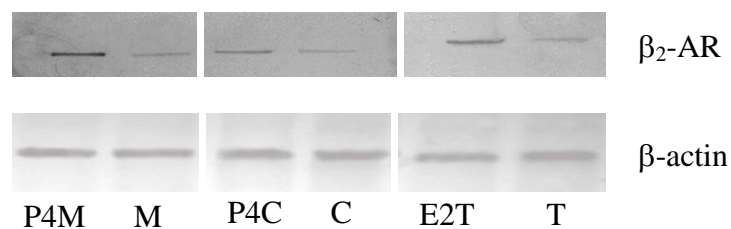
A.



B.



C.



**Figure 4.** β<sub>2</sub> receptor expression in rat myometrium, cervix és trachea. (n=6) \*:  $p < 0,05$ , \*\*\* $p < 0.001$

P4M: P4-treated myometrium, M: myometrium

P4C: P4-treated cervix, C: cervix

E2T: E2-treated trachea, T: trachea

D-limonene ( $10^{-13}$ - $10^{-8}$  M) enhanced the myometrial contractions in concentration dependent manner. 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 (**Table 1.**). The P4 treatment enforced the terbutaline effect on myometrial relaxation, but the D-limonene did not modify the concentration-response curve of terbutaline (**Fig. 5.**).

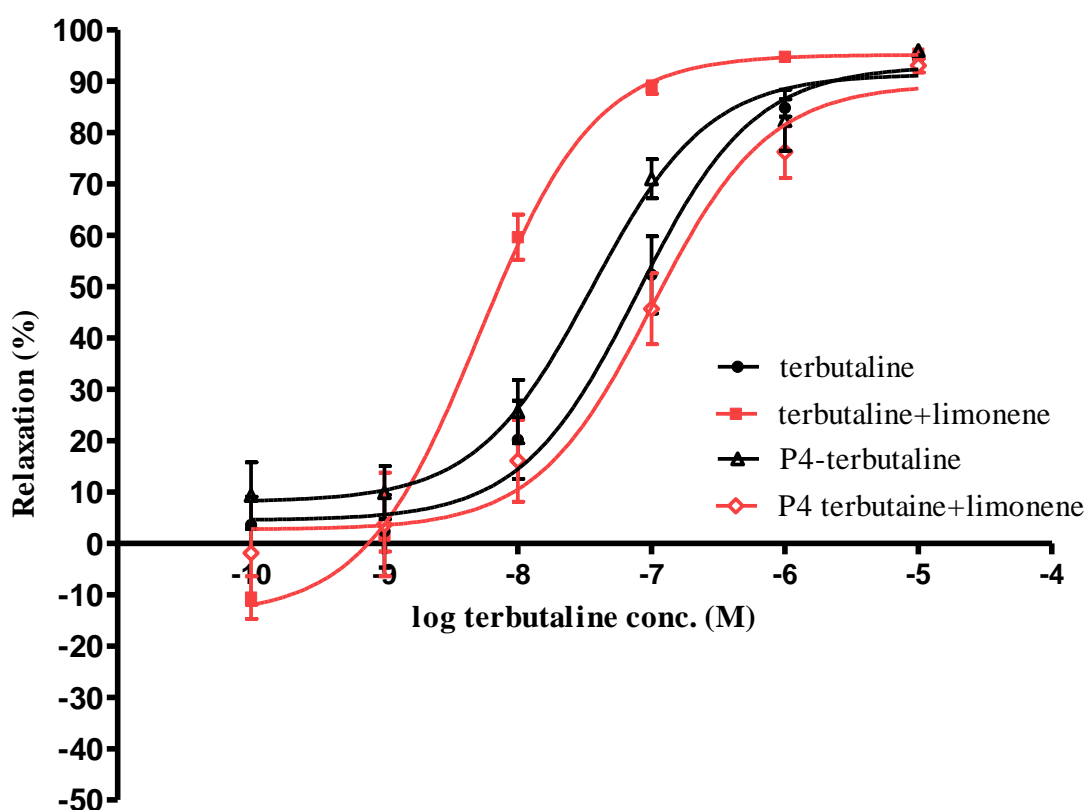
**Table 1.** Parameters of concentration-response curve of terbutaline in non-treated (a) and in P4-treated (b) myometrium in the presence or absence of limonene

a.

Non-treated myometria	EC <sub>50</sub> (M±S.E.M)	E <sub>max</sub> (M±S.E.M.)
terbutaline	$1,4 \times 10^{-7} \pm 3,7 \times 10^{-8}$	$93,7\% \pm 4,8$
limonene+terbutaline	$6,5 \times 10^{-9} \pm 1,48 \times 10^{-9**}$	$95,4\% \pm 1,6^{ns}$

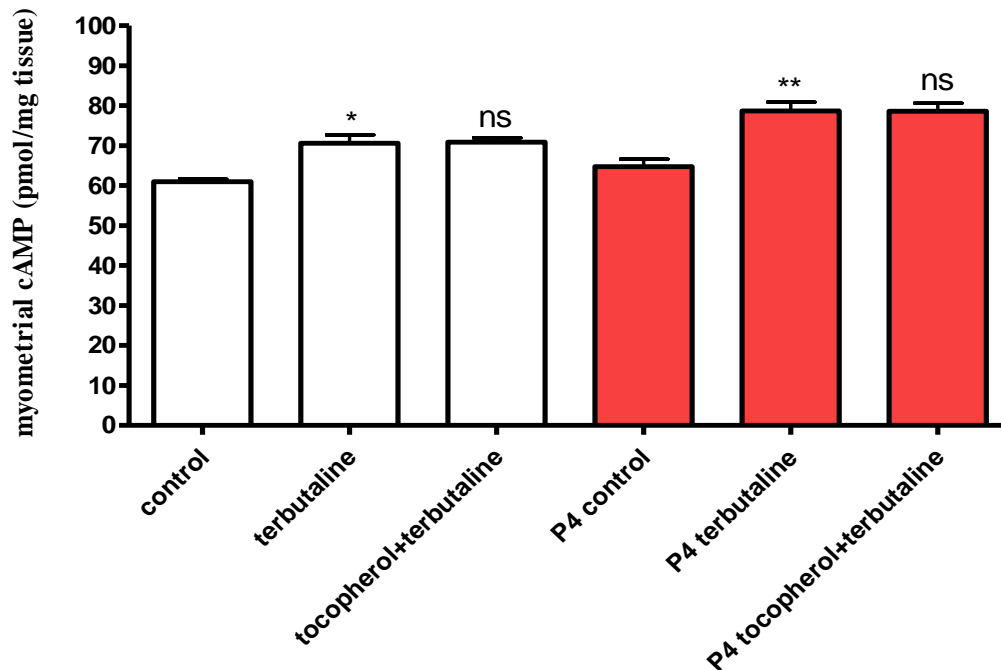
b.

P4-treated myometria	EC <sub>50</sub> (M±S.E.M)	E <sub>max</sub> (M±S.E.M.)
terbutaline	$8,6 \times 10^{-8} \pm 3,35 \times 10^{-8}$	$88,5\% \pm 3,7$
limonene+terbutaline	$4,9 \times 10^{-7} \pm 2,56 \times 10^{-7}^{ns}$	$89,5\% \pm 6,3^{ns}$



**Figure 5.** Effects of terbutaline on spontaneous contractions of nontreated and P4-pretreated 22-day-pregnant rat myometria, alone (black lines) and in the presence of limonene ( $10^{-13}$  M) (red lines).

Terbutaline ( $10^{-6}$  M) increased the cAMP level in the nontreated myometria. The presence of tocopherol ( $10^{-7}$  M) did not alter the myometrial cAMP level induced by terbutaline (**Fig. 6.**). In the P4-treated myometria, the terbutaline-induced increase in cAMP level was higher (12.7% increase,  $p<0.05$ ) as compared with nontreated samples, but tocopherol again remained ineffective (**Fig. 6.**)

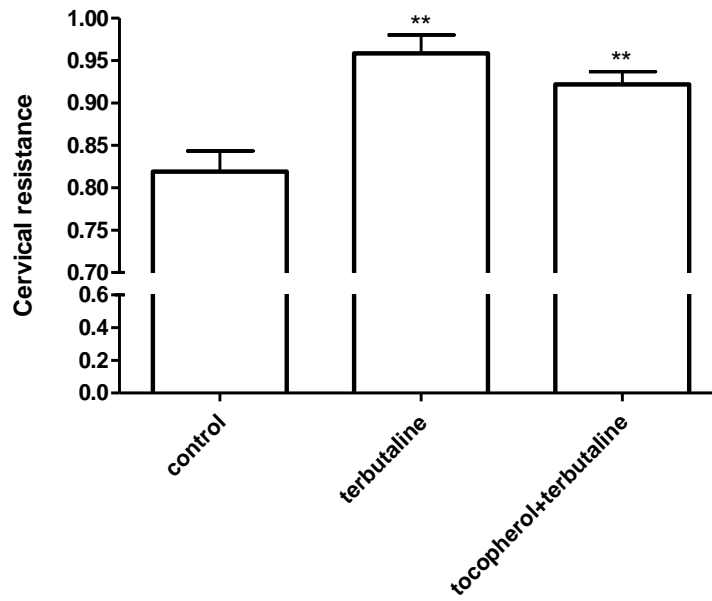


**Figure 6.** The myometrial cAMP level-increasing effect of terbutaline in the absence or presence of tocopherol. Terbutaline treatment ( $10^{-6}$  M) increased the cAMP level in the myometrium; tocopherol ( $10^{-7}$  M) was ineffective on this action. In P4-pretreated animals, terbutaline further increased the myometrial cAMP, but tocopherol did not elicit any effect.  
ns: not significant, \* $p<0.05$ ; \*\* $p<0.01$  as compared with the control pair

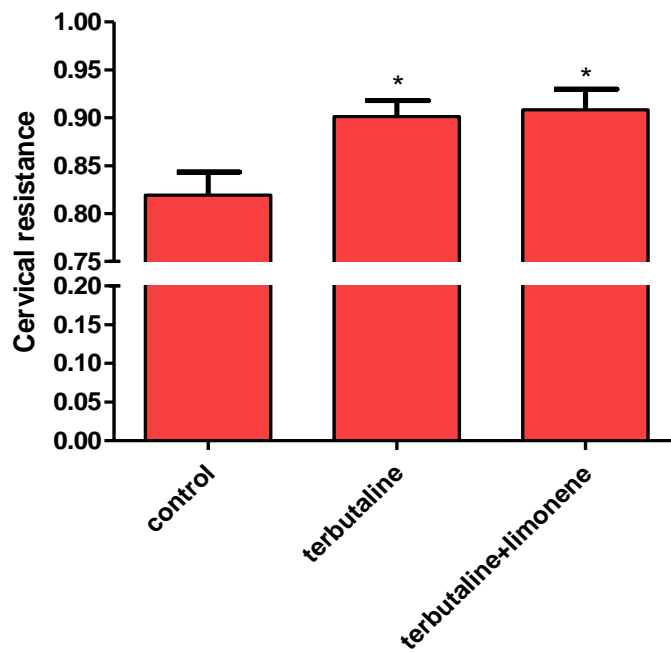
#### ***4.3. Effects of tocopherol and limonene on the cervical action of terbutaline***

Terbutaline ( $10^{-6}$  M) enhanced the cervical resistance of 22-day-pregnant cervical samples ( $0.96 \pm 0.02$ ) as compared with the control value ( $0.82 \pm 0.02$ ). Tocopherol ( $10^{-7}$  M) and D-limonene ( $10^{-13}$  M) did not significantly alter the cervical resistance-increasing effect of terbutaline ( $0.92 \pm 0.01$ ) (**Fig. 7.**).

A.



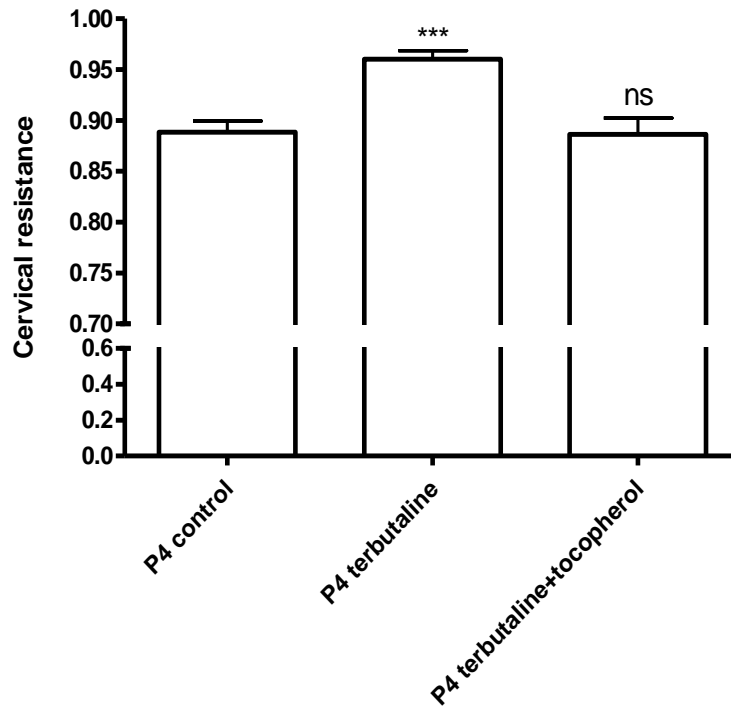
B.



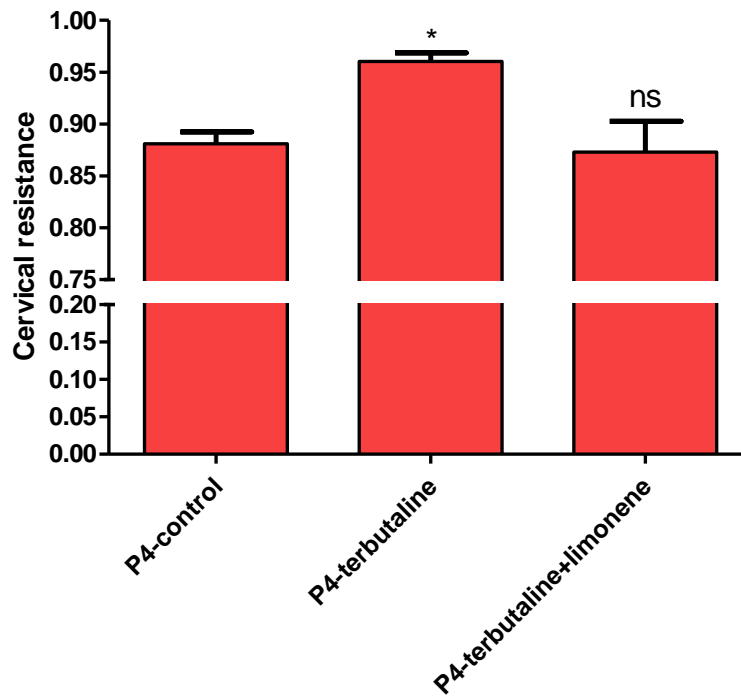
**Figure 7.** Effects of terbutaline on the resistance of nontreated 22-day-pregnant rat cervixes, alone and in the presence of tocopherol (A) and limonene (B). Resistance is expressed as the slope of the regression lines fitted to the stress-strain curves. Terbutaline ( $10^{-6}$  M) increased the cervical resistance, the effect was not altered neither by tocopherol ( $10^{-7}$  M) nor limonene ( $10^{-13}$  M). \* $p < 0.05$ ; \*\* $p < 0.01$  as compared with the control pair

P4 pretreatment increased the cervical resistance ( $0.89 \pm 0.02$ ,  $p < 0.05$ ) and left the resistance-increasing action of terbutaline unchanged ( $0.96 \pm 0.01$ ). However, the presence of tocopherol, and also D-limonene eliminated the effect of terbutaline in P4-pretreated cervical samples (**Fig. 8.**). The progesterone treatment did not alter the expression of  $\beta_2$ -AR mRNA in pregnant cervixes (**Fig. 4.**).

A.

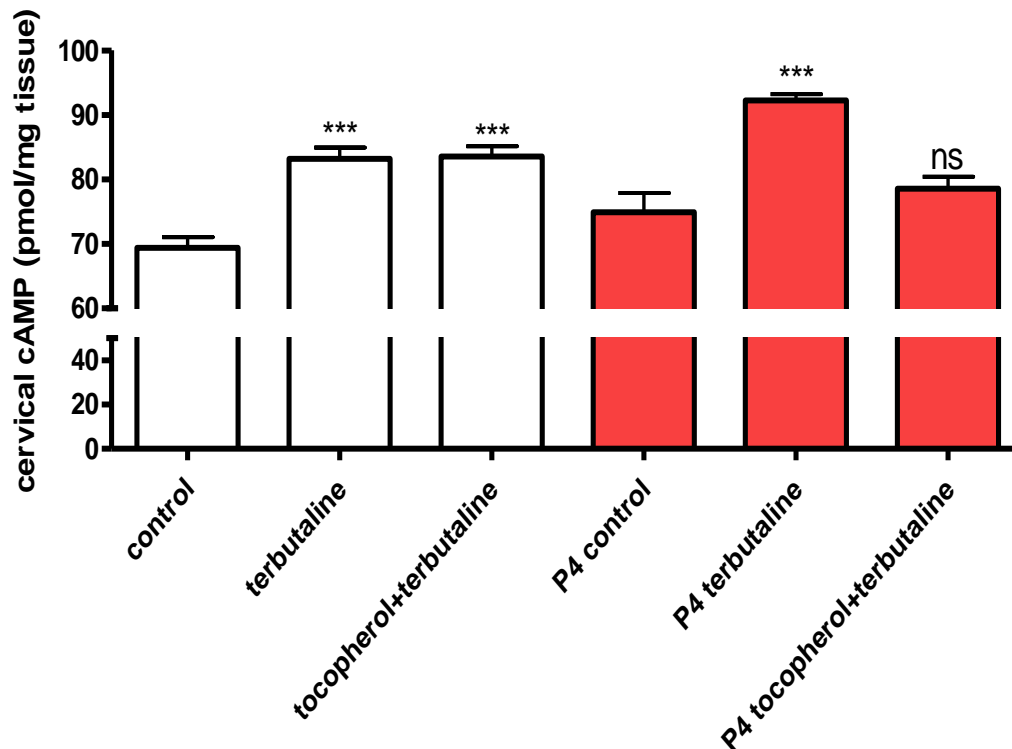


B.



**Figure 8.** 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). Resistance is expressed as the slope of the regression lines fitted to the stress–strain curves. *In vivo* P4-pretreatment did not increase the cervical resistance-increasing effect of terbutaline further, but tocopherol and also limonene blocked the action of terbutaline. ns: not significant; \* $p < 0.05$ ; \*\*\* $p < 0.001$  as compared with the control pair

Terbutaline ( $10^{-6}$  M) elevated the cAMP content in the pregnant cervical tissues. In the presence of tocopherol, there were no further changes in cAMP level. After P4 pretreatment, the cervical cAMP was elevated by terbutaline as compared with nontreated samples, and tocopherol was able to block the cervical resistance-increasing effect of terbutaline. The level of cAMP induced by terbutaline was increased in the P4-treated samples (10.9% increase,  $p < 0.001$ ) (Fig. 9.).



**Figure 9.** The cervical cAMP level-increasing effect of terbutaline in the absence or presence of tocopherol. Cervical samples were collected from 22-day-pregnant rats. Terbutaline treatment ( $10^{-6}$  M) increased the cAMP level in the cervix, but tocopherol ( $10^{-7}$  M) was ineffective. In P4-pretreated animals, terbutaline further increased the cervical cAMP, while this effect was reduced significantly by tocopherol.

ns: not significant; \*\*\* $p < 0.001$  as compared with the control pair.

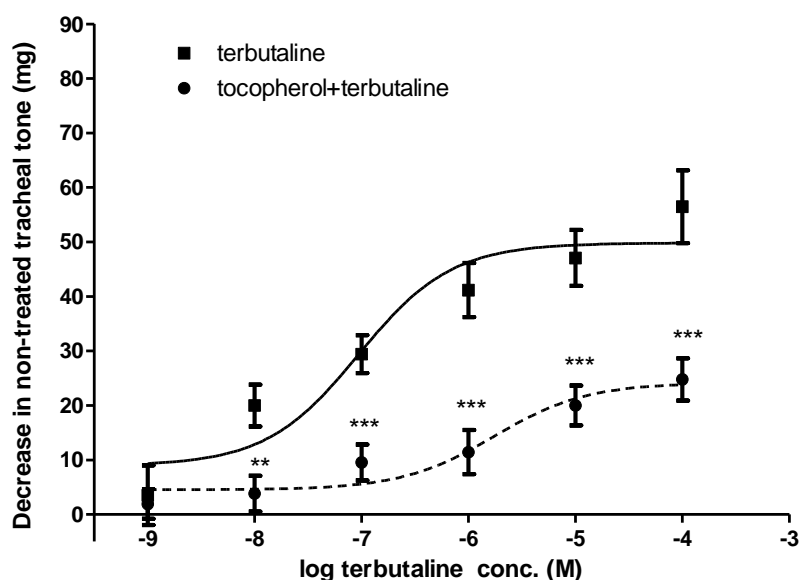
#### 4.4. Effects of tocopherol and limonene on the tracheal action of terbutaline

Terbutaline ( $10^{-9}$  M –  $10^{-4}$  M) decreased the tone of tracheal segments with an average of  $56 \pm 3$  mg. In the presence of tocopherol ( $10^{-7}$  M), the relaxing effect of terbutaline was reduced significantly ( $24 \pm 3$  mg) (Fig. 10.A.). D-limonene ( $10^{-13}$  M) also reduced the relaxing effect of terbutaline (Fig. 10.B.). After *in vivo* E2-pretreatment, the tracheal tone-reducing effect of terbutaline was stronger ( $74 \pm 7$  mg), but the presence of tocopherol led to the same reduction as in nontreated samples ( $24 \pm 4$  mg) (Fig. 11.A.). In the case of d-limonene, the reducing effect was also enhanced (Fig. 11.B). The estrogen treatment enhanced the expression of the

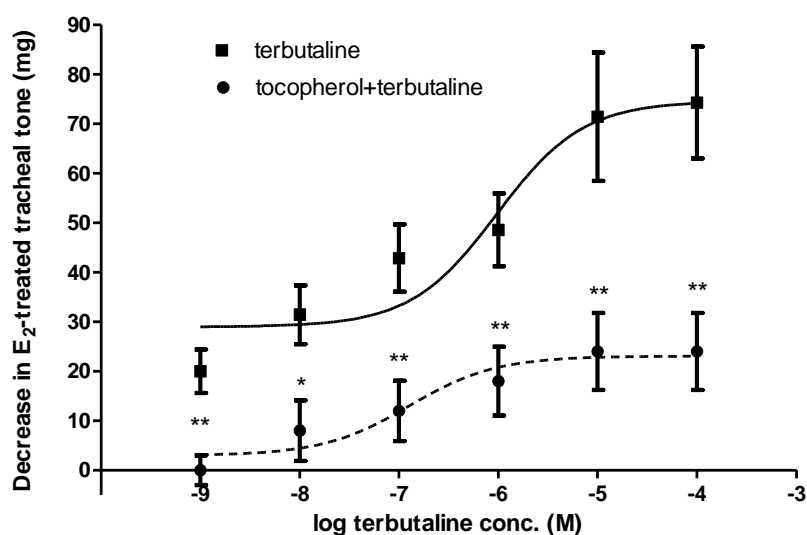


receptor mRNA in the trachea. The Western blot study revealed that the estrogen treatment increased the expressions of  $\beta_2$ -ARs in tracheal tissues, respectively (**Fig. 4.**).

A.

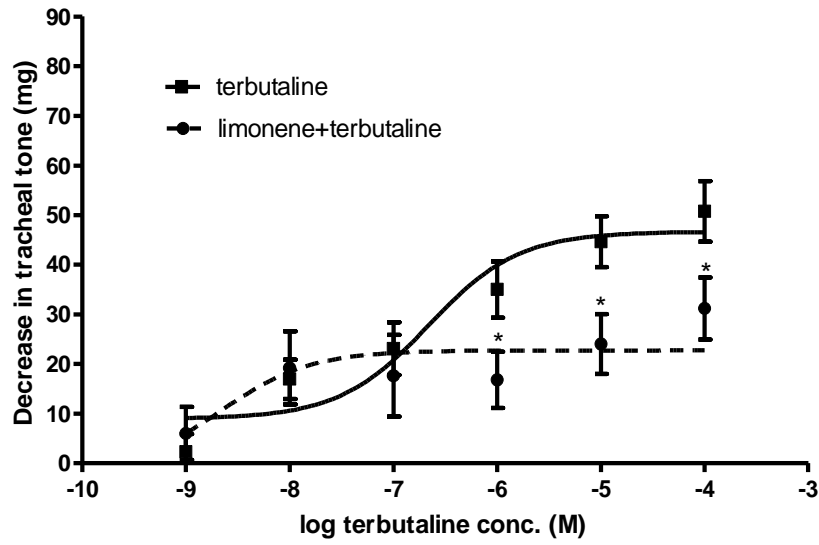


B.

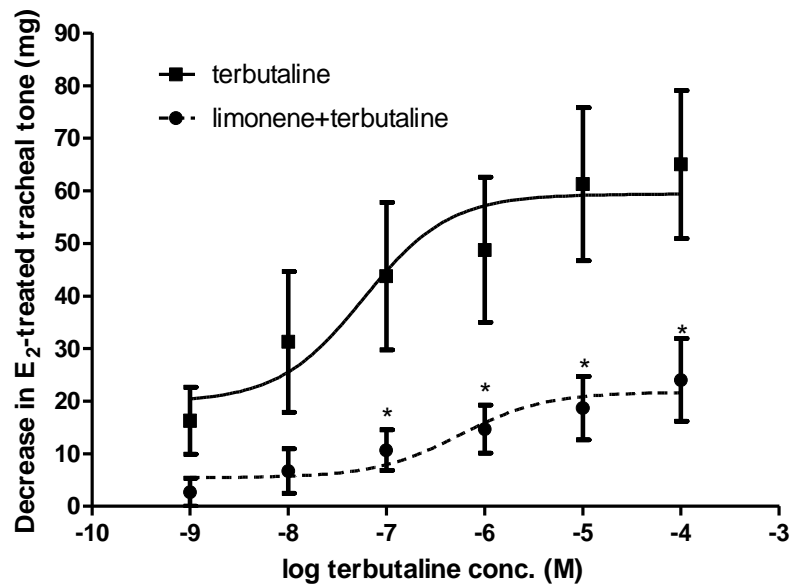


**Figure 10.** Tone-reducing effect of terbutaline on isolated rat tracheal preparations, with or without tocopherol. Tracheal samples were collected from female rats in the estrous cycle. **(A)** In the presence of tocopherol ( $10^{-7}$  M), the effect of terbutaline was significantly decreased. **(B)** After *in vivo* E<sub>2</sub>-treatment, the relaxing effect of terbutaline was even higher. Tocopherol decreased the effect of terbutaline measured without E<sub>2</sub>-pretreatment. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  as compared with terbutaline effect alone

A.

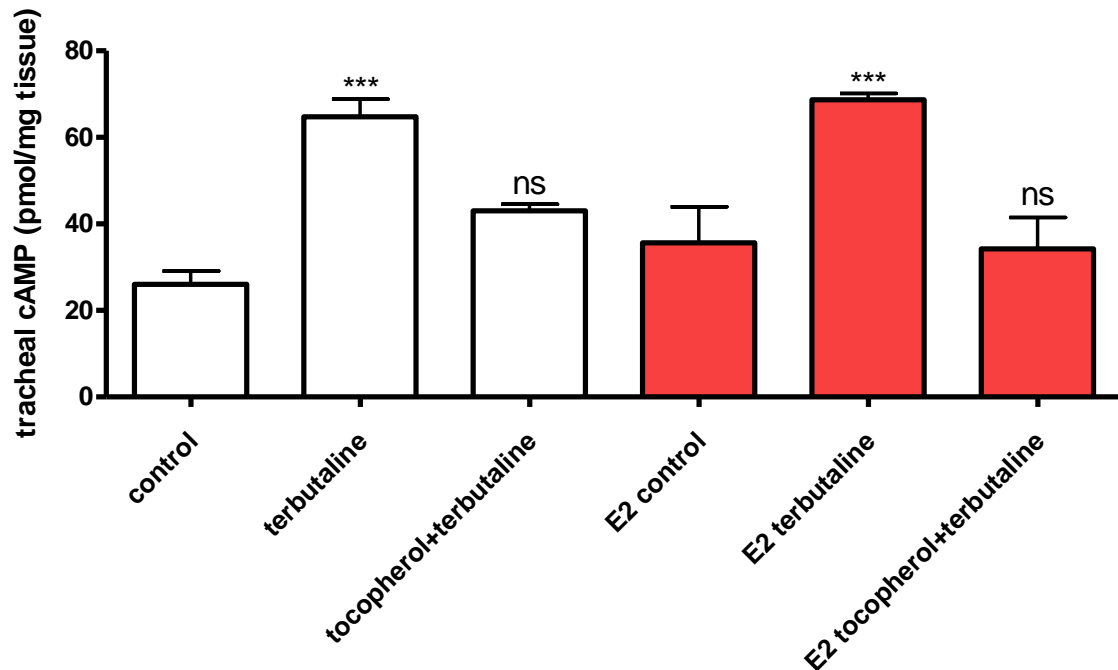


B.



**Figure 11.** Tone-reducing effect of terbutaline on isolated rat tracheal preparations, with or without limonene. Tracheal samples were collected from female rats in the estrous cycle. (A) In the presence of limonene ( $10^{-13}$  M), the effect of terbutaline was significantly decreased. (B) After *in vivo* E<sub>2</sub>-treatment, the relaxing effect of terbutaline was even higher. Limonene decreased the effect of terbutaline measured without E<sub>2</sub>-pretreatment. \* $p < 0.05$ ; as compared with terbutaline effect alone

In the tracheal smooth muscle, the cAMP content was elevated by terbutaline ( $10^{-5}$  M) both in nontreated and in E2-treated samples (**Fig.12.**). The level of cAMP induced by terbutaline was increased in the E2-treated samples (30.0% increase,  $p<0.05$ ). Tocopherol inhibited the action of terbutaline in both cases.

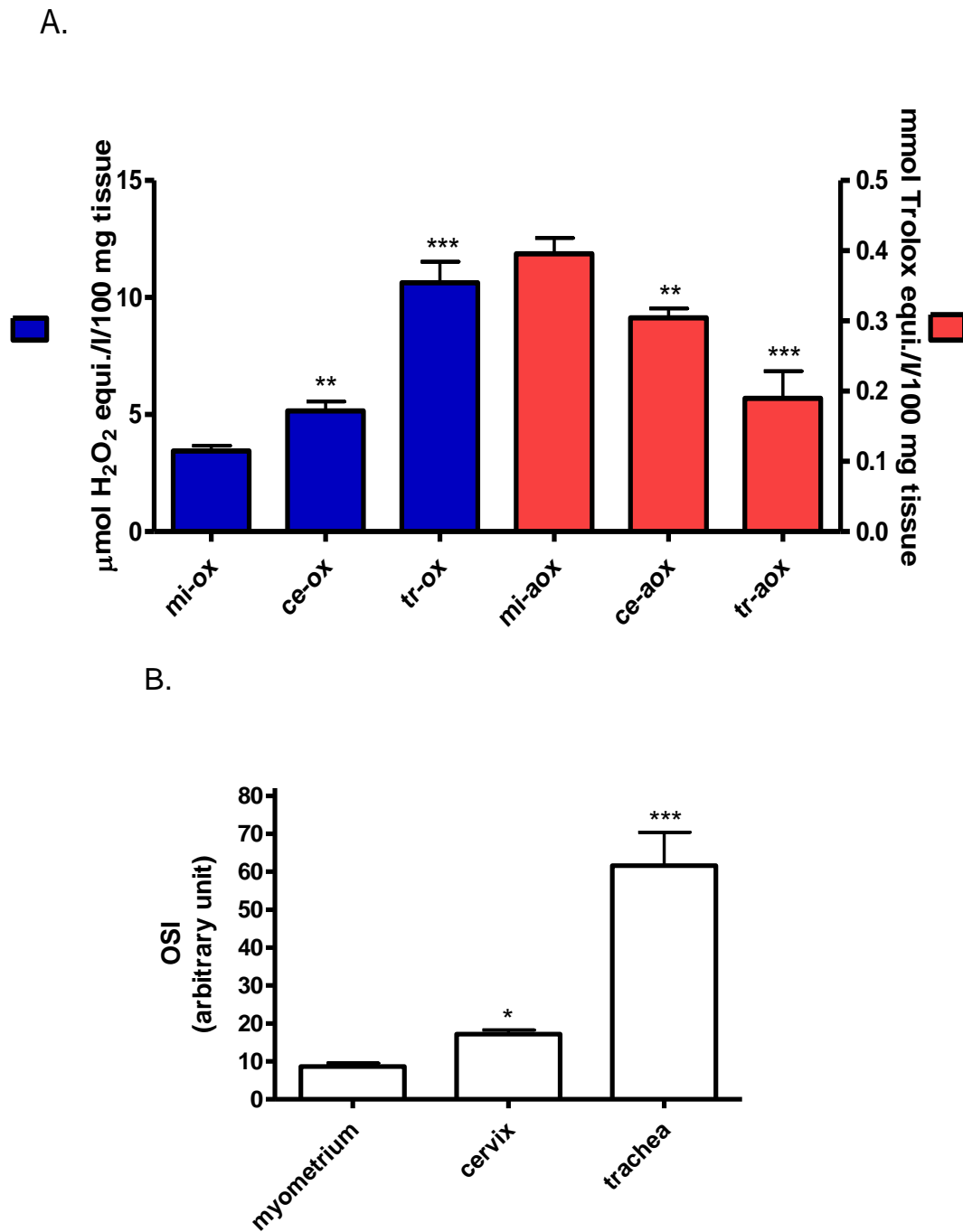


**Figure 12.** The tracheal cAMP level-increasing effect of terbutaline in the absence or presence of tocopherol. Tracheal samples were collected from female rats in the estrous cycle. Terbutaline treatment ( $10^{-5}$  M) increased the cAMP level in the trachea. In the presence of tocopherol ( $10^{-7}$  M), the effect of terbutaline was reduced. In E2-pretreated animals, terbutaline further increased the tracheal cAMP. The inhibitory effect of tocopherol was more pronounced as compared with non-E2-treated samples.

ns: not significant, \*\*\* $p<0.001$  as compared with the control pair.

#### 4.5. 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 nonpregnant 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 nonpregnant myometrium and cervix were lower than those for the pregnant samples (**Fig. 13.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 nonpregnant sample (**Fig. 13.B.**).



**Figure 13.** The oxidative/antioxidant status in the myometrial, cervical and tracheal tissues (empty columns). Nonpregnant samples and trachea were collected from female rats in the estrous cycle. (filled columns). The total oxidative status (TOS) values of trachea and pregnant cervix were significantly higher as compared with pregnant myometrial value. The differences between the pregnant and nonpregnant tissues were nonsignificant. (A) The total antioxidant status (TAS) values were the lowest in the trachea and the highest in the pregnant myometrium. The differences between the pregnant and nonpregnant tissues were significant. 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

ROS are generated during crucial processes of  $O_2$  consumption. Under physiological conditions, there is a balance between the mitochondrial production of ROS and the antioxidant systems. ROS seem to act as second messengers of specific ligands, including different growth factors and adrenergic signaling (Corbi et al, 2013). We therefore hypothesized that an antioxidant effect may interact with the signal mechanism of  $\beta$ -ARs. Since  $\beta_2$ -ARs are of therapeutic importance in the relaxation of uterine and bronchial smooth muscles, we examined whether the antioxidant agents alter the beta-mimetic action in these tissues. The significance of this investigation is enhanced by the fact that patients often consume abundant multivitamin and antioxidant supplements containing tocopherol derivatives in order to improve or maintain their general health condition (Huang et al. 2006). Tocopherol appears to have a wide range of effects on cellular systems, with the ability to inhibit protein kinase C, activate diacylglycerol kinase and protein phosphatase 2A (Azzi et al. 2000), and regulate specific gene expression (Rimbach et al. 2010); in this way, tocopherol can influence a number of biological functions and metabolisms. Additionally, the antioxidant effects of terpenoids are also known (Grassmann et al. 2005), and D-limonene is widely used as flavour and fragrance.

Therefore our aim was to investigate the effect of these agents alone and on beta-mimetic action. D-limonene caused myometrial contraction in a dose-dependant manner, while tocopherol had no significant action. Like numerous cell membrane receptors,  $\beta_2$ -ARs are coupled to their effector units via G-proteins. Traditionally, signal transduction by the  $\beta_2$ -ARs has focused largely on the activation of  $G_s$  proteins and their downstream effectors, including cAMP-generation AC. cAMP and its effectors are considered to be the chief mediators of the cell response to  $\beta_2$ -AR activation (Lefkowitz and Shenoy 2005). However, GPCRs, including  $\beta$ -ARs, have also been found to regulate the production of ROS, which determine the nature and duration of the cell signal (Andersson et al. 2011). As a  $\beta_2$ -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 (Gaspar et al. 2005; Gaspar et al. 2007, Chou et al. 2010). In the presence of the antioxidant agents 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  $\beta_2$ -AR signal mechanism in different smooth muscles.

Sexual hormones are known to play a role in the regulation of G-proteins of  $\beta_2$ -ARs in the myometrial, cervical and tracheal smooth muscles. P4 enhances the signaling of myometrial and cervical  $\beta_2$ -ARs (Elwady et al. 1994), while E2 is able to increase the second messenger processes in airway smooth muscles (Townsend et al. 2012). Theoretically, when the signal mechanism is increased, the linked ROS production should also be increased. Pregnant animals were therefore treated with P4 for 7 days to stimulate the  $\beta_2$ -AR signaling in the myometrium and the cervix (Gaspar et al. 2005), while nonpregnant rats were treated with E2 for 4 days (Song et al. 2002) in order to increase the  $\beta_2$ -AR signaling in the tracheal tissue. Although the P4 treatment was not able to increase the myometrial and cervical mRNA expressions of  $\beta_2$ -AR, the increase in protein expression was significant. The E2 treatment was able to elevate both the expressions of tracheal mRNA and protein. The increased receptor function, and signal mechanism were also proved by the enhanced cAMP levels in hormone-treated samples after terbutaline administration as compared with the hormonally nontreated tissues. After the hormonal induction of the  $\beta_2$ -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  $\beta_2$ -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  $\beta_2$ -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 (Bozkus et al. 2013, Ellidag et al. 2013). 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 (Badziuk et al., 2011, Loukides S et al., 2011). 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/free radicals dependence of the  $\beta_2$ -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/free radicals to provide uninterrupted intracellular signals. Our results suggest that a high OSI in a tissue implies a higher importance of ROS/free radicals in the signal mechanism of  $\beta_2$ -AR.

The OSI values of the nonpregnant myometrium and cervix were higher as compared with the pregnant samples (although the difference was not significant in case of myometria). This result reconfirms the earlier findings of upregulation of antioxidant enzymes during pregnancy. (Kaya S. et al, 2013).

## **6. Conclusion**

We suppose that stimulation of  $\beta_2$ -AR leads to formation of ROS, and that ROS play key role in receptor coupling to G proteins, as well as expression of receptor on the plasma membrane. Thus the generation of ROS seem to be essential for ‘classical’ receptor-mediated signaling events. Antioxidants are able to reduce the coupling of  $\beta_2$ -ARs that may lead to the weakening of the smooth muscle action of  $\beta_2$ -AR agonists. The up-regulation of the receptors means the up-regulation of ROS production as well. However, the extend of the role of ROS in  $\beta_2$ -AR signaling is mainly depends on the oxidative stress index of the given smooth muscle. Our finding suggests that significant decrease in  $\beta_2$ -AR agonist effect by antioxidants might be possible in the antiasthmatic therapy.

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# Appendix