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Tocopherol inhibits the relaxing effect of terbutaline in the respiratory and reproductive tracts of the rat: The role of the oxidative stress index

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ABSTRACT

Aims: Reactive oxygen species play a role in the signal transduction of beta-adrenergic receptors. We investigated whether an antioxidant (tocopherol) can reduce the effect of terbutaline in beta-2-adrenergic receptor (β_2-AR)-regulated smooth muscles.
Main methods: Contractility of the tissues from nonpregnant (trachea) and 22-day-pregnant (myometrium and cervix) rats was investigated in an isolated organ bath. The tracheal and uterine β_2-AR expressions were increased by 17-beta-estradiol valerate (E2) and progesterone (P4), respectively. The accumulation of cyclic-AMP (cAMP), and the total oxidant (TOS) and total antioxidant status (TAS) were also measured. The oxidative stress index (OSI) was defined as the ratio of TOS and TAS.
Key findings: Terbutaline (10^{-10}–10^{-5} M) decreased the contractions in the nontreated and the P4-pretreated myometria, but tocopherol (10^{-7} M) did not alter these actions. Terbutaline (10^{-6} M) increased the cervical resistance both in the nontreated and in the P4-treated samples, while tocopherol reduced this action only in the P4-treated cervices. Terbutaline (10^{-9}–10^{-4} M) reduced the tracheal tones both in the nontreated and in the E2-treated tissues, while tocopherol reduced these effects. The changes in the intracellular cAMP levels of the tissues were in harmony with the isolated organ results. The OSI was highest in the trachea and lowest in the pregnant myometrium.
Significance: A higher OSI is linked to a higher tocopherol sensitivity of beta-mimetic-induced relaxation. Our results suggest that the antiasthmatic effect of beta-mimetics may worsen, while their tocolytic effect may remain unchanged during parallel tocopherol administration.

Introduction

The process of smooth muscle cell contraction is principally regulated by the receptor and mechanical (stretch) activation of the contractile proteins myosin and actin, which is an adenosine triphosphate (ATP)-dependent process. In organs, the ATP is produced via oxidative phosphorylation in the mitochondria. During this process, molecular oxygen (O_2) is reduced to H_2O_2 by cytochrome C oxidase. In 95% of the reactions, this mechanism proceeds flawlessly, but in 5% of the reactions the O_2 is only partially reduced, leading to the production of reactive oxygen species (ROS) (Slavić et al., 2006).

During recent years, substantial evidence has accumulated concerning the roles of ROS in the transduction of the different intracellular signals. The ROS are involved primarily in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system. ROS and free radicals in general are essential for cell signaling and other vital physiological functions. For instance, a low level of endogenous ROS is required for regulation of the vital sperm function (Aprioku, 2013). ROS interacts directly with critical signaling molecules to initiate signaling in a broad variety of cellular processes, such as proliferation and survival, ROS homeostasis and antioxidant gene regulation, mitochondrial oxidative stress, apoptosis, aging, iron homeostasis and the DNA damage response (Ray et al., 2012). 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. In the case of G-protein-coupled receptors (GPCRs), the stimulation of the respective receptors has also been linked to the formation of ROS (Thannickal and Fanburg, 2000). Thus, ROS are also important in the functions of adrenergic receptors. The adrenergic system plays a main role in stress signaling, which is often associated with the increased production of ROS. The β-adrenergic agonist isoproterenol is known to increase mitochondrial ROS production in the cardiomyocytes (Andersson et al., 2011).

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The β_2-adrenergic receptor (β_2-AR) is a prototypical GPCR that mediates an intracellular cAMP increase by activating G_s (Rasmussen et al., 2011). Via G_s-protein-coupled adenylyl cyclase (AC), increased levels of cAMP are therefore responsible for the β_2-AR-mediated relaxation of smooth muscle. The stimulation of β2-AR (betamimetics) is of therapeutic importance in relaxation of the bronchial tract (bronchial asthma) and the pregnant uterus (preterm birth) (Cazzola et al., 2013; Gáspár et al., 2005b). 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). However, the inhibition of ROS attenuates β_2-AR signal transduction (Moniri and Daaka, 2007), suggesting that a low level of intracellular ROS may influence the whole β_2-AR signal transduction.

ROS inhibitors have emerging roles in the therapy and prevention of diseases, but may clearly have an impact on ROS-dependent physiological processes. 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 (Clarke et al., 2008). Tocopherol is one of the most frequently consumed antioxidants in both nutrition and dietary supplements.

The main focus of our study was to investigate how tocopherol (α-tocopherol acid succinate) alters the effects of terbutaline in the myometrial, cervical and bronchial smooth muscles in rats *in vitro*.

Materials and methods

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 ± 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 killed by CO_2 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 sexually mature male Sprague–Dawley 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.

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.

Determination of myometrial, cervical and tracheal β_2-AR mRNA by real-time reverse transcription-PCR

Tissue isolation

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.

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 of 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 were performed by using the TaqMan RNA-to-C_T™ 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 s 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 β_2-adrenergic receptor and Rn00667869-m1 for ß-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 β_2-ARs

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 immundetection 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 (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.

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 $CaCl_2$, 1 $MgCl_2$, 12 $NaHCO_3$, 4 NaH_2PO_4, 6 glucose, pH 7.4). The organ bath was maintained at 37 °C and carbogen (95% O_2 + 5% CO_2) 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^{-7} M). The tissues were washed and further tocopherol was administered every 15 min. The control samples were incubated for 1 h without tocopherol. A cumulative dose–response curve of 10^{-10}–10^{-5} M terbutaline was obtained.

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