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Alpha-tocopherol succinate increases cyclooxygenase-2 activity: Tissue-specific action in pregnant rat uterus in vitro

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ABSTRACT

Aims: Lipid soluble vitamin E plays a role in several physiological mechanisms, however, the mechanism of this action is controversial. We investigated how tocopherol (α-tocopherol acid succinate) influences the effects of cyclooxygenase inhibitors (COXi) in the smooth muscles.

Main methods: The contractility of the samples from 22-day-pregnant myometrium and non-pregnant myometrium and trachea was determined in an isolated organ bath in vitro. The activity of cyclooxygenase enzymes (COX) was also measured in the tissues.

Key findings: Diclofenac (10^{-9}–10^{-5} M) and rofecoxib (10^{-10}–10^{-5} M) decreased the contractions in non-pregnant and 22-day-pregnant uteri. Tocopherol (10^{-7} M) increased the relaxant effect only in pregnant uteri. Both diclofenac (10^{-9}–10^{-5} M) and rofecoxib (10^{-10}–10^{-5} M) reduced the tracheal tones, while they were slightly intensified by pretreatment with tocopherol (10^{-7} M). Tocopherol enhanced the contractions of pregnant uteri. Tocopherol (10^{-7} M) itself can induce the cyclooxygenase activity and shift the COX-1 and COX-2 ratio to COX-2. The lowest COX activity was found in non-pregnant uteri, while the highest one was in the trachea.

Significance: The COX enzymes, especially COX-2, play an important role in the contraction of pregnant uteri in rat. Tocopherol has a tissue specific COX-2 activity increasing effect in pregnant rat uterus but has no such action in non-pregnant uteri or tracheal tissue. Hereby, tocopherol may intensify selectively the uterine relaxing effect of COX-2 inhibitors in preterm contractions. However, tocopherol can enhance the contractile response of pregnant uterus that may increase the risk of premature contractions.

1. Introduction

Vitamin E is a lipid-soluble compound with high peroxyl radical scavenger ability. Vitamin E includes eight natural analogs, such as α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols, however, their activity is quite various [1]. α-tocopherol is the most frequently used analog. Over the past thirty years, many studies have been published on the biological effects and functions of vitamin E, e.g. it is needed for reproduction [2] and may help to prevent Alzheimer's disease, non-alcoholic fatty liver diseases (NAFLD and NASH) of non-diabetic patients, atherosclerosis and certain types of cancer [3–7]. The mechanism of action of vitamin E has not yet been fully clarified. It can get across the cell membrane and can bind to many intracellular receptors and enzymes and may lead to modifying their functions [8]. For example, tocopherols can increase the activity of estrogen receptor β (ERβ) [9], inhibit activities of protein kinase C alpha [10], phospholipase A2 [11] and protein tyrosine kinases [12]. Moreover, vitamin E can also influence the action of other drugs by its antioxidant effect that shows tissue specificity. The tracheal tone-reducing effect of β_2-agonist terbutaline was decreased in female estrous rat by pretreatment with α-tocopherol, while in pregnant uterus the pretreatment was inefficient. This difference was explained by the various oxidative states of smooth muscles [13].

The interaction between tocopherols and cyclooxygenase enzymes (COX) was investigated in a few studies. Wu et al. [14] reported vitamin E inhibited the activity of cyclooxygenase enzymes in human aortic endothelial cells. Moreover, vitamin E can affect the different steps of the arachidonic acid cascade, but this effect may be diverse in tissues. According to literature, prostaglandin E2 (PGE_2) production was reduced in mouse [15,16] and rat [17] macrophages by vitamin E. Analogs of tocopherols have different effects on COX. Alpha-tocopherol succinate inhibited more efficiently the LPS-stimulated PGE_2 production in macrophages and the COX activity in human lung epithelial cells than the other analogs [18,19].

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit COX-1 or COX-2 enzymes, hereby decrease the liberation of prostaglandins (PG).

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The low level of PGE_2 induces the relaxation of the uterine smooth muscle, although the clinical use of NSAIDs for the prevention of preterm birth is limited because of the side effects [20]. Non-COX selective NSAIDs, especially acetyl-salicylic acid, are responsible for aspirin-induced asthma (AIA), while COX-2 selective compounds seem to be helpful not to exacerbate asthmatic symptoms [21].

We hypothesize that tocopherols modifies the COX activity and the effect of NSAIDs that may have significance in smooth muscle contractions. Accordingly, the aim of this study is to investigate how α-tocopherol acid succinate (tocopherol) influences the effects of non-selective and selective COX inhibitors (COXi) in the non-pregnant and pregnant uterine, and tracheal smooth muscle contractions in rats in vitro.

2. Materials and methods

All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (permission number: IV/198/2013). The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII).

2.1. Housing and handling of the animals

Sprague-Dawley rats were procured from INNOVO Ltd. (Gödöllő, Hungary) and were kept at a controlled temperature (22 ± 3 °C), with 30–70% relative humidity, on a 12 h dark-light cycle. The animals were fed with standard rodent pellet diet (Charles-River Laboratories, Budapest, Hungary) and given tap water ad libitum.

2.2. Mating of the animals

The mature female Sprague-Dawley rats (180–200 g) were selected by the estrus cycle. The estrus cycle was measured by the vaginal impedance of rats with an Estrus Cycle Monitor EC40 (Fine Science Tools. Foster City, CA, USA). The female in estrus and sexually mature male (240–260 g) rats were placed into a special mating cage with two rooms. There was a time-controlled movable metal door between the rooms. The separating door was pulled up before dawn.

In the morning, vaginal smears were taken from the female rats. Successful copulation was indicated by the presence of sperm in the native vaginal smear or a copulation plug. This day meant the first day of pregnancy.

2.3. Isolated organ bath studies

2.3.1. Preparation of uteri

The animals were terminated by CO_2 inhalation. Two horns of uteri were excised, 5-mm-long muscle rings were sliced and mounted vertically in an organ bath containing 10 ml of de Joung 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 heated at 37 °C and carbogen (95% O_2 + 5% CO_2) was bubbled into the chambers. After the setting of initial tension (1.5 g), myometrial rings were incubated with a buffer change every 15 min for about 1 h. Tocopherol was dissolved in mixture of ethanol 96%: Macrogol 400 (1:14) and diluted further in Macrogol 400. The control tissues have been treated with the solvent without tocopherol and results with tocopherol have been corrected with these values. The samples were equilibrated for another 60 min with tocopherol (10^{-7} M). Tocopherol was added to tissues after every wash of buffer solution. The control preparations were incubated for 1 h without tocopherol. Cumulative dose-response curves of non-selective COXi diclofenac (10^{-9}–10^{-5} M) and COX-2 selective inhibitor rofecoxib (10^{-10}–10^{-5} M) were obtained.

2.3.2. Preparation of trachea

Trachea tissues were dissected from non-pregnant estrous rats (160–260 g, n = 8), then the esophagus and blood vessels were removed. The tracheal tube was cut transversally into 4–5- mm-wide rings, which were placed in Krebs buffer (composition in mM: 118 NaCl; 4.75 KCl; 2.5 $CaCl_2$; 1.19 K_2HPO_4; 25 $NaHCO_3$; 1.2 $MgSO_4$ and 11 glucose). The tracheal rings were mounted with their longitudinal axis vertically by hooks. The initial strain was set to about 2.00 g. The samples were equilibrated for 1 h, while the buffer solution was changed in every 15 min. After that, the tissues were incubated for another 60 min with tocopherol (10^{-7} M) with further buffer change in every 15 min. Except for the control preparations, tocopherol was applied after every wash of buffer solution. Cumulative dose-response curves of non-selective COXi diclofenac (10^{-9}–10^{-5} M) and COX-2 selective inhibitor rofecoxib (10^{-10}–10^{-5} M) were obtained.

2.3.3. Measurement of COX enzymes activity

The smooth muscle samples (22-day-pregnant and non-pregnant myometrium, trachea, n = 6/group) were incubated in an organ bath as described above. After the incubated period, tissues were perfused with cold Tris Buffer pH 7.4 to remove any red blood cells and clots, frozen in liquid nitrogen and stored at − 80 °C until assay. On the day of measurement, samples were homogenized in 5 ml of cold buffer (0.1 M Tris-HCl, pH 7.8, containing 1 mM EDTA) per gram tissue, centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant was stored on ice. The activity of COX enzymes was determined by COX Activity Assay Kit (Cayman chemicals, Ann Arbor, MI). This Kit measures the peroxidase activity of COX. The peroxidase activity is assayed with the colorimetric method by monitoring the appearance of oxidized *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm.

2.4. Statistical analysis

All data were analyzed by the Prism 5.01 (GraphPad Software, USA) computer program. The values were statistically evaluated with the unpaired *t*-test or ANOVA with Tukey Multiple Comparison Test.

3. Results

3.1. Isolated organ bath studies

Pretreatment with tocopherol significantly enhanced the contractions of the 22-day-pregnant myometrium (Fig. 1 striped columns) but did not alter it in non-pregnant uteri (Fig. 1 empty columns).

The non-selective COX inhibitor diclofenac (10^{-9}–10^{-5} M) and the selective COX-2 inhibitor rofecoxib (10^{-10}–10^{-5} M) inhibited the contractions of non-pregnant uteri in a concentration-dependent manner. In the presence of tocopherol, the relaxant effects of diclofenac and rofecoxib did not change (Fig. 2).

In 22-day-pregnant smooth muscle uteri the relaxant effect of selective COX-2 inhibitor rofecoxib was higher than that of diclofenac. After pretreatment with tocopherol, the relaxant effect was increased significantly in each concentration in the case of both compounds (Fig. 3).

Both diclofenac (10^{-9}–10^{-5} M) and rofecoxib (10^{-10}–10^{-5} M) also reduced the tone of tracheal tissues. The average tone decrease by diclofenac and rofecoxib was 46.8 ± 5.0 mg and 32.6 ± 10.4 mg, respectively. Tocopherol influenced the effect of diclofenac and rofecoxib only in lower concentrations (Fig. 4).

When one dose of selective COX-2 inhibitor rofecoxib (10^{-7} M) was applied before KCl-evoked control contractions on 22-day pregnant uteri, the activity of diclofenac stayed low, while in the presence of tocopherol it augmented slightly (Fig. 5**A**).

Besides, if we added one dose of selective COX-1 inhibitor SC-560 before eliciting control contraction, the relaxing effect of rofecoxib was enhanced and the presence of tocopherol kept on increasing this effect

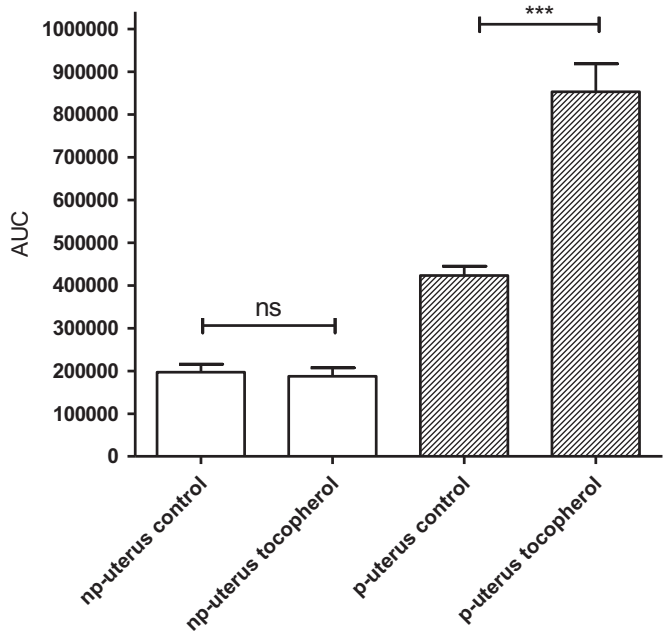

Fig. 1. The area under the curve of KCl-evoked uterus contractions after the incubation with tocopherol. The presence of tocopherol did not alter the contraction of non-pregnant myometrium (empty columns), but increased it significantly in 22-day-pregnant uteri (striped columns). The statistical analyses were carried out with the two-tailed unpaired *t*-test. (ns: not-significant; *** $p < 0.001$). Each value denotes the mean ± S.E.M, n = 6.

(Fig. 5**B**).

3.2. Measurement of COX enzymes activity

The highest level of total COX activity was found in trachea tissues, while the lowest was in non-pregnant uteri. After pretreatment with tocopherol, neither the COX activity of the trachea nor the COX activity of non-pregnant uteri changed. In 22-day-pregnant myometrium the total cyclooxygenase enzymes activity was significantly increased by pretreatment with tocopherol. The activity of COX-1 enzyme was not altered in the tissues in the presence of tocopherol. However, the activity of COX-2 enzyme was enhanced significantly in tocopherol pretreated 22-day-pregnant uteri (Fig. 6).

4. Discussion

In this study, we have demonstrated that α-tocopherol succinate modifies the relaxant effect of NSAID's via the increase of the activity of COX-2 in 22-day-pregnant rat uteri. These findings may open up a new therapeutic use for tocopherols. On the other hand, the use of tocopherol during pregnancy may intensify the premature contractions.

The levels of PGE_2, $PGF_{2\alpha}$, COX enzymes and other proteins and enzymes which play a role in reproduction, pregnancy and parturition are rather various in non-pregnant and pregnant uteri [22–24]. Moreover, in the last weeks of human pregnancy, different biological mechanisms are developed to prepare uteri for labor. Gap junctions and ion channels protein are activated in phase 1 of labor, while myometrial contractions are enhanced by oxytocin and prostaglandins in phase 2 [25].

Cyclooxygenase enzymes (COX-1, COX-2) catalyze the transformation of arachidonic acid to prostaglandins. It is generally accepted that PGs play an important role in parturition by inducing myometrial contraction and relaxation via EP1, EP3 and EP2, EP4 receptors [26]. On the other hand, there is no general agreement in literature as to which isoenzyme of cyclooxygenase leads to the liberation of prostaglandins at the time of parturition.

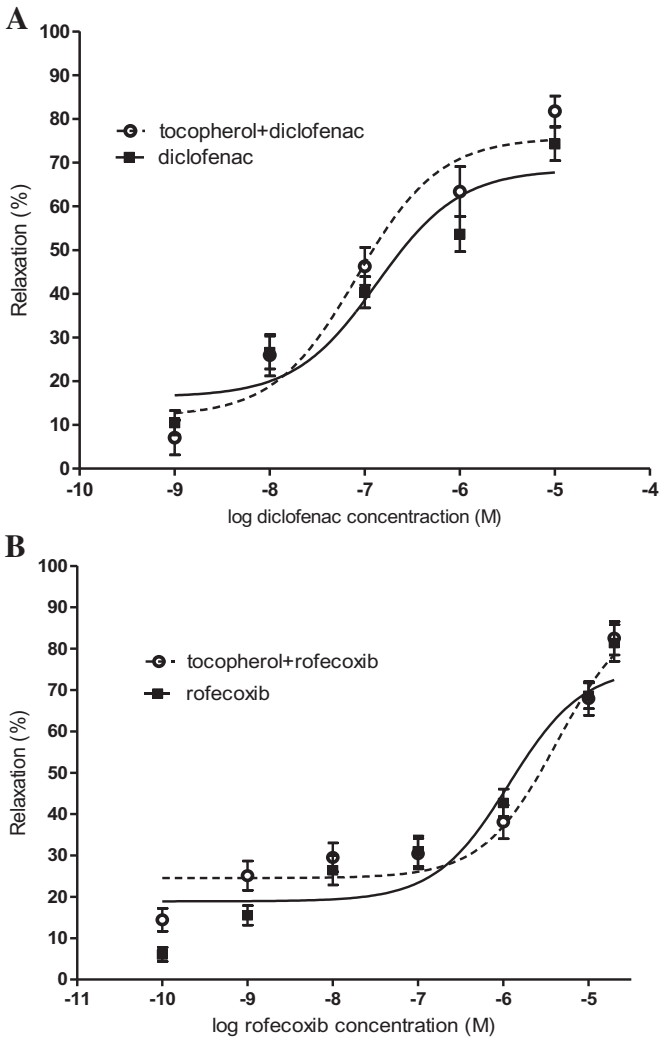

Fig. 2. Effect of non-selective COXi diclofenac (**A**) and selective COX-2 inhibitor rofecoxib (**B**) on KCl-evoked control contraction of non-pregnant rat uteri alone and in the presence of tocopherol (10^{-7} M). Tocopherol changed the effect of neither diclofenac nor rofecoxib. The statistical analyses were carried out with the two-tailed unpaired *t*-test. Each value denotes the mean ± S.E.M, n = 6.

St-Louis et al. [27] described that the expression of COX-1 enzyme was elevated at time of parturition, while the presence of COX-2 was less in pregnant rat endometrium. In the case of non-pregnant rat endometrium, the amounts of both COX-1 and COX-2 enzymes were equal in the estrous phase. In other studies, the expression of COX-2 was enhanced in rodent myometrium during parturition, hence COX-2 derived PGs were determinant in the final pathway of parturition [28]. Furthermore, the increased expression of COX-2 led to a high level of PGE_2, since the expression of COX-1 remained low and was not altered with gestational age in human uteri [29]. In our experiment, in non-pregnant uteri the activity of the COX-1 and COX-2 ratio was about equal, while in pregnant uteri the activity of COX-2 was moderately lower than that of COX-1 enzyme. In addition, the total COX activity of pregnant uteri was higher than in non-pregnant samples. The maximum effect of selective COX-2 inhibitor rofecoxib was more powerful than that of non-selective diclofenac both in pregnant and non-pregnant uteri. However, diclofenac as non-selective COX inhibitor is 20-fold more potent for COX-2 than COX-1 [30,31]. These results suggest that both COX-1 and COX-2 enzymes play a role in uterine contractions, nevertheless, the relaxation mostly depends on COX-2 inhibition. The relaxant effect of diclofenac and rofecoxib was stronger in non-pregnant than in pregnant uteri, which can be explained by the activities of COX-1 and COX-2 in these

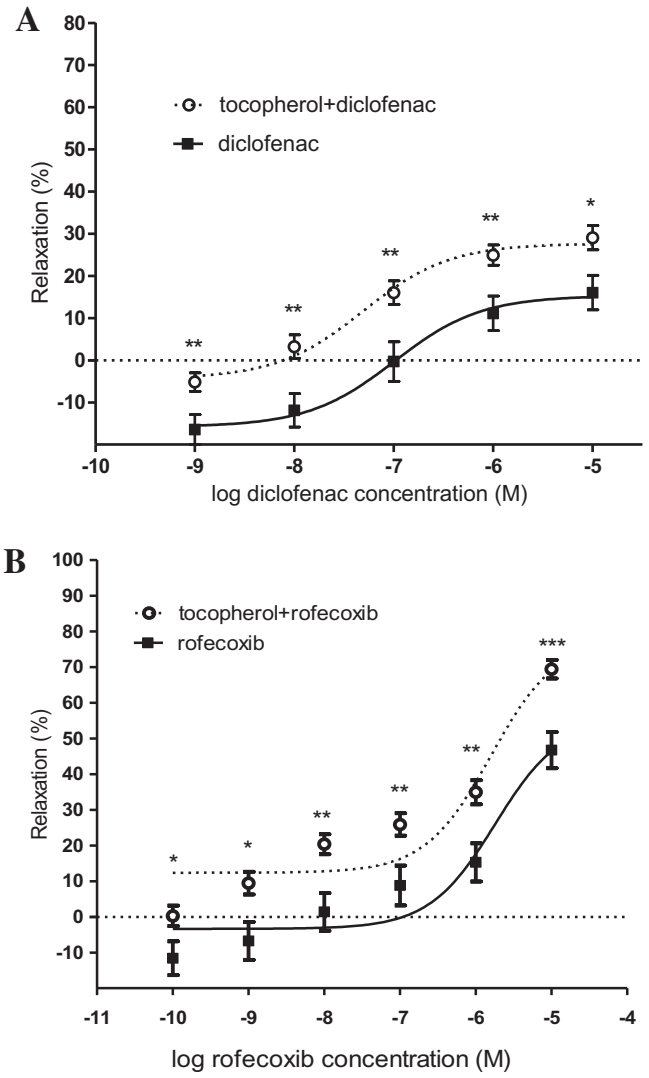

Fig. 3. Effect of non-selective COXi diclofenac (**A**) and selective COX-2 inhibitor rofecoxib (**B**) on KCl-evoked control contraction of 22-day-pregnant rat uteri with or without tocopherol (10^{-7} M). After pretreatment with tocopherol, the relaxation effect of diclofenac and rofecoxib was significantly enhanced in all concentrations. The statistical analyses were carried out with the two-tailed unpaired *t*-test. Each value denotes the mean ± S.E.M, n = 6. (*p < 0.05; **p < 0.01; ***p < 0.001).

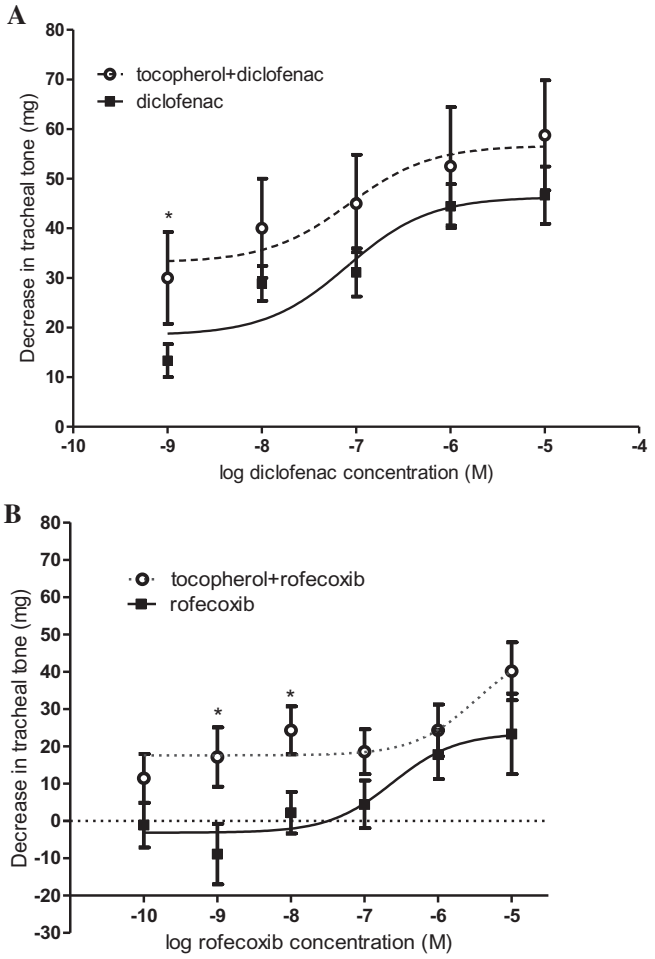

Fig. 4. Tone-reducing effect of non-selective COXi diclofenac (**A**) and selective COX-2 inhibitor rofecoxib (**B**) on estrous rat trachea tissues. In the presence of tocopherol the effect of both compounds was significantly increased only in low concentrations. The statistical analyses were carried out with the two-tailed unpaired *t*-test. Each value denotes the mean ± S.E.M, n = 6. (*p < 0.05).

tissues. In non-pregnant uteri, the COX1/COX2 ratio is 1.09, which means that the two isoenzymes have similar activity. However, in pregnant uteri the COX-1 activity was 1.22, showing the greater activity of COX-1. This is further evidence that the amount of COX-2 determines the relaxing efficacy of COXi: less COX-2 means less efficacy, while more COX-2 means higher efficacy in the relaxing effect of COXi.

Several studies have shown the relationship between tocopherol and cyclooxygenase enzymes. Abate et al. [32] demonstrated that vitamin E reduced LPS-mediated COX-2 induction alone and in combination with aspirin in macrophages. Moreover, O'Leary et al. [33] reported that tocopherols decreased the activity of COX-2 in Caco2 cells. The limitation of these reports is that the investigations were done in cultured cells. Additionally, only few papers have focused on the connection of tocopherol and cyclooxygenase enzymes in uteri. According to our results, pretreatment with tocopherol unambiguously raised the area under the curve of KCl-evoked contractions in pregnant uteri. This implies that tocopherol may enhance the contractibility of pregnant myometrium. Interestingly, the influence of tocopherol was not detectable in the case of non-selective COX inhibitor diclofenac either in non-pregnant or in 22-day pregnant uteri. These results suggest that, in contrast to earlier findings, pretreatment with tocopherol may increase the contractility of uteri by enhancing the activity of COX-2 enzyme, and hereby the relaxant effect of COX inhibitors may be more pronounced, especially in the case of selective COX-2 inhibitors. To confirm this hypothesis, the alteration of the activity of COX enzymes was measured in the tissues before and after the incubation with tocopherol. Tocopherol itself induced the COX activity and shifted the COX-1 and COX-2 ratio to COX-2 in pregnant uteri. When COX-1 enzymes were blocked by selective inhibitor SC-560, the relaxant effect of rofecoxib increased alone and further increased in the presence of tocopherol. However, when COX-2 was inhibited by the selective blocker rofecoxib, the dose-response curve of diclofenac was shifted slightly left, and after pretreatment with tocopherol the significant difference between the curves practically ceased. These findings provide further evidence that COX-2 is predominant in pregnant uterine contraction and the tocopherol-induced modification of the COX-1 and COX-2 ratio led to the increased relaxing efficacy of COX inhibitors.

In trachea tissues the levels of COX-1 and COX-2 activity were equal (the COX-1/COX-2 ratio was 0.97), which correlates with the previous findings in literature [34,35]. Diclofenac decreased the tracheal tone more than rofecoxib, suggesting that COX-1 derived prostaglandins may play a larger role in airway smooth muscle contraction in rat. Interestingly, pretreatment with tocopherol increased the relaxing effects of rofecoxib and diclofenac, but only in low concentrations of the drugs. We have no clear explanation for this phenomenon, but it might be

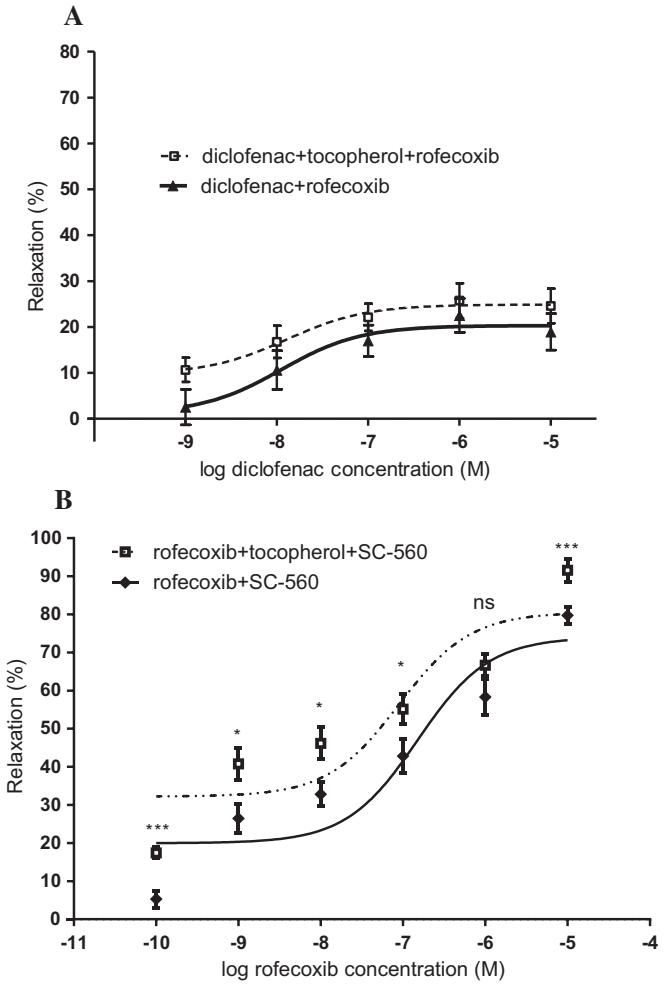

Fig. 5. The relaxant effect of non-selective COXi diclofenac after pretreatment with selective COX-2 inhibitor rofecoxib (10^{-7} M) (**A**) and the concentration-response curve of selective COX-2 inhibitor rofecoxib after pretreatment with selective COX-1 inhibitor SC-560 (**B**) on the KCl-evoked contractions of 22-day-pregnant rat uteri alone and in the presence of tocopherol. If COX-2 enzymes were inhibited, the action of diclofenac did not change. In case of COX-1 inhibition the relaxing effect of rofecoxib were increased with and without tocopherol.
The change in contraction was calculated via the area under the curve and expressed in % ± S.E.M. The statistical analyses were carried out with the two-tailed unpaired *t*-test. (ns: not significant; *p < 0.05; **p < 0.01; ***p < 0.001).

related to the weak antioxidant capacity of rat trachea as compared with pregnant uterus [13].

5. Conclusions

Pretreatment with tocopherol can strengthen COX-2 activity in pregnant uteri, leading to the stronger relaxant effect of COX-2 inhibitor. Our results suggest that COX enzymes, especially COX-2, play an important role in the contraction of pregnant uteri in rat. Tocopherol has a tissue specific COX-2 activity increasing effect in pregnant rat uterus but has no such action in non-pregnant uteri or tracheal tissue. Hereby tocopherol may intensify selectively the uterine relaxing effect of COX-2 inhibitors in preterm contractions. However, tocopherol alone can enhance the contractile response of pregnant uterus that may increase the risk of premature contractions. The use of vitamin preparations containing Vitamin E derivatives may modify the contractility of the pregnant uterus and its pharmacological response to contractile agents and COX-inhibitors. Our results raise the necessity of studies for further preclinical and clinical clarification of the correlation between tocopherol level and pregnant uterine contraction.

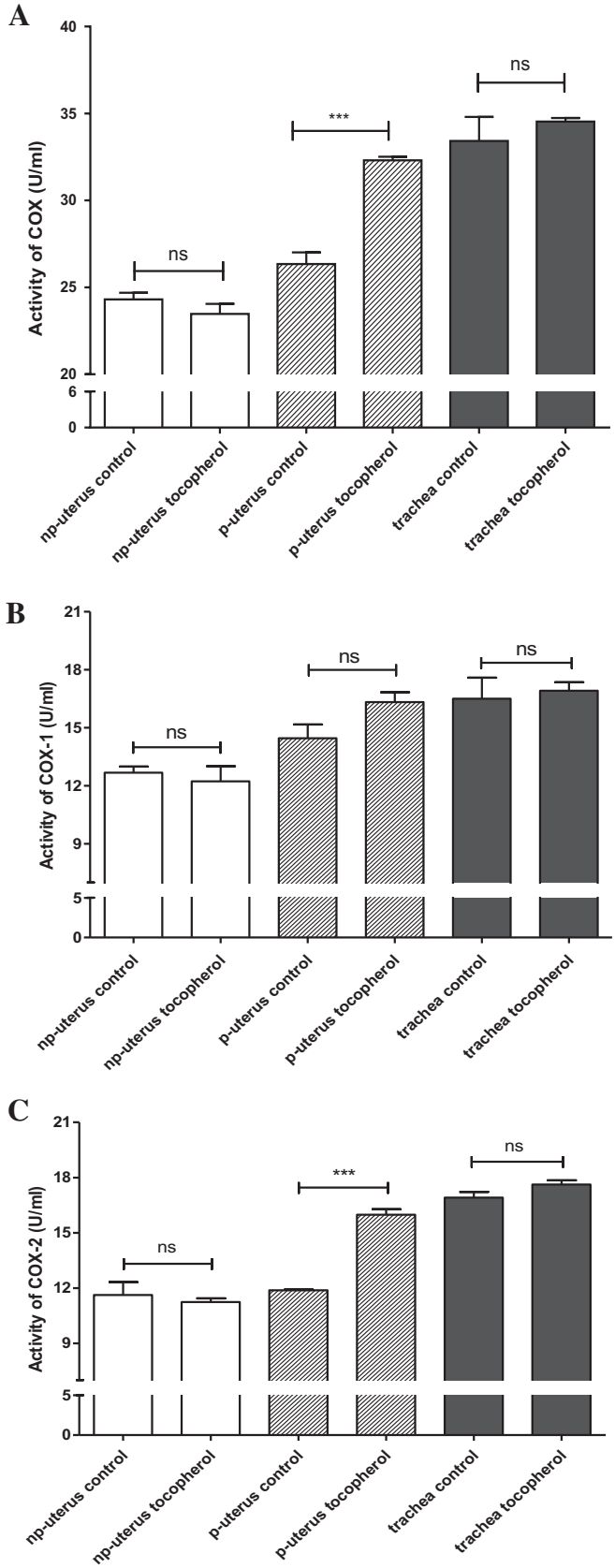

Fig. 6. The effect of tocopherol on the activity of COX-enzymes in non-pregnant uterus (np) (empty columns), 22-day-pregnant uterus (p) (striped columns) and estrous trachea (filled columns). The pretreatment with tocopherol increased the activity of total COX- enzymes only in pregnant uterus (**A**). In the case of COX-1 activity, no significant alteration was determined (**B**). The activity of COX-2 was enhanced by the presence of tocopherol in pregnant animals (**C**). The statistical analyses were carried out with ANOVA Tukey test. (ns: not significant; ***p < 0.001) Each value denotes the mean ± S.E.M., n = 6.

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