α-Tocopherol Potentiates the Cervical Resistance Decreasing Effects of COX Inhibitors in Pregnant Rats: The Putative Role of Cyclooxygenase-2 Inhibition

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ABSTRACT

Vitamin E and their analogs as antioxidant and lipid-soluble compounds can have diverse effects on the physiologic processes. By binding to receptors and enzymes, they may modify the action of drugs. It has been proved that α-tocopherol succinate modifies the effects of β_2 agonist terbutaline and cyclooxygenase (COX) inhibitors on rat trachea and myometrium. Our aim was to investigate how α-tocopherol and COX inhibitors may influence cervical resistance in rats. The cervical resistance of nonpregnant and 22 day–pregnant Sprague-Dawley rats was determined in an isolated organ bath in vitro. α-Tocopherol-succinate (10^{-7} M) was used, whereas the COX-nonselective diclofenac (10^{-6} M), the COX-2–selective rofecoxib (10^{-6} M), and the COX-1–selective SC-560 (10^{-6} M) were applied as inhibitors. The COX activities of the cervices were measured by enzyme immunoassay. The modifying effect of single doses of COX inhibitors and tocopherol on the onset of labor was investigated in vivo. The cervical resistance of nonpregnant samples was not changed by either α-tocopherol or COX inhibitors. On pregnant cervices, tocopherol, diclofenac, or rofecoxib pretreatment decreased cervical resistance that was further reduced by COX inhibitors after pretreatment with tocopherol. α-Tocopherol elicited a significant COX-2 enzyme inhibition in cervical samples from pregnant rats. By coadministration of tocopherol and rofecoxib, the parturition was initiated earlier than in the other groups. It is supposed that COXs play a significant role not only in cervical ripening, but also in the contraction of the cervical smooth muscle a few hours before parturition. This latter action may be developed by COX-2–liberated prostaglandins.

Introduction

In the nature, vitamin E presents as a mixture of eight analogs (α-, β-, γ-, and δ-tocopherols and tocotrienols). In recent years, the biologic effects of vitamin E and its analogs have been widely investigated (Zingg, 2007). The analogs have different biologic activities; however, α-tocopherol is one of the most prevalently used analogs. It is commonly known that vitamin E has high peroxyl scavenger ability, in addition, as a lipid-soluble compound it can get across the cell membrane, so it can bind to several intracellular receptors and enzymes. Because of these actions, vitamin E may modify not only the effect of receptors and enzymes (e.g., cAMP, phospholipase A_2, and protein kinase C) but also that of some drugs (Zingg, 2015). In our previous study, we demonstrated that antioxidant α-tocopherol inhibited the tracheal tone and uterine-relaxing effect of β_2-agonist terbutaline on rat trachea (Hódi et al., 2014).

The function of the cervix is alternating during pregnancy. Throughout gestation, the cervix has to be closed and rigid to protect and hold the fetus inside the uterus, while at term and during labor, it must be dilated and soften to help the newborn's passage. This transformation is supported by cervical ripening (Nott et al., 2016). Collagen, elastin, macromolecular components, and smooth muscle, which constitute the cervix, are altered by a complex biologic mechanism in cervical remodeling; however, the full process has not yet been fully clarified. Disorder in this remodeling process can lead to preterm or post-term birth, which increases the risk of complications.

Prostaglandins (PGs), mainly PGE_2 and $PGF_{2\alpha}$, play an essential role in cervical ripening and parturition. Moreover, in clinical practice, the synthetic PGE_1 analog misoprostol and PGE_2 formulation dinoprostone are used for the induction of labor (Bakker et al., 2017). PGs are produced by cyclooxygenase (COX) enzymes. Both COX-1 and COX-2 enzymes are expressed in the epithelial and smooth muscle cells of the cervix, and their levels increase during pregnancy. It seems that the COX-2–related PGs liberation has higher importance in cervical ripening and spontaneous labor (Dong et al., 1996).

Few studies were published about the association of vitamin E and COX enzymes. Vitamin E inhibited COX activity in human aortic endothelial cells (Wu et al., 2005) and reduced

ABBREVIATIONS: COX, cyclooxygenase; COXi, cyclooxygenase inhibitor; CSM, cervical smooth muscle; PG, prostaglandin; PGE_2, prostaglandin E_2; SD, Sprague-Dawley.

PGE$_2$ in rat macrophages (Sakamoto et al., 1991) and mouse macrophages (Beharka et al., 1997; Wu et al., 1998). Kim et al. (2012) reported that analogs of tocopherols have diverse effects on COX enzymes. In human lung epithelial cells, α-tocopherol succinate possessed the most efficient inhibition effect of lipopolysaccharide-stimulated PGE$_2$ and COX activity (Lee et al., 2006). Moreover, we have proved that the pretreatment with tocopherol shifted the COX-1/COX-2 ratio toward COX-2, which led to a more powerful relaxation effect of COX inhibitors (COXis) in the pregnant uterus (Kothencz et al., 2018).

Since PGs have a crucial role in the cervical ripening process and tocopherols may alter the COX activity, we hypothesized that tocopherols may modify the COX activity and the effect of COXis on the cervix. Accordingly, the aim of this study was to investigate how α-tocopherol acid succinate (tocopherol) influences the effects of nonselective and selective COXis on cervical resistance in nonpregnant and 22 day–pregnant rat cervix.

Materials and Methods

All experiments involving animal subjects were carried out with the approval of the National Scientific Ethical Committee on Animal Experimentation (permission number IV/198/2013). The animals were treated in accordance with the European Communities Council Directives (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII).

Housing and Handling of the Animals. Sprague-Dawley (SD) rats were purchased from INNOVO Ltd. (Gödöllő, Hungary) and were maintained at 22 ± 3°C, with 30%–70% relative humidity, on a 12-hour dark/light cycle. The animals were kept on a standard rodent pellet diet (Charles River, Budapest, Hungary) and given tap water ad libitum.

Detection of Estrus Cycle in Nonpregnant Rats. All nonpregnant animals were in the estrous phase. On the day of the experiment, the vaginal impedance of mature female SD rats (180–200 g) was measured by Estrus Cycle Monitor EC40 (Fine Science Tools, Foster City, CA). The rats whose vaginal impedance was 7–10 kΩ were used in isolated organ bath studies and measurement of COX enzyme activity.

Mating of the Animals. The mature female SD rats (180–200 g) were chosen by the estrus cycle. The estrus cycle was detected by the vaginal impedance of rats with Estrus Cycle Monitor EC40. The female rats in estrus and the sexually mature male rats (240–260 g) were placed separately into a special mating cage, which had a time-controlled movable metal door. This door was pulled up before dawn.

Within 4–5 hours after the possible mating, vaginal smears were taken from the female rats. Copulation was successful if sperm was detected in the native vaginal smear or a copulation plug was present. This day was regarded as the first day of pregnancy.

Isolated Organ Bath Studies. The 22 day–pregnant and nonpregnant rats were terminated by CO$_2$ inhalation. After the dissection of the cervices, the two rings of the cervix were separated and mounted with their longitudinal axis vertically by hooks in an organ bath containing 10 ml of de Joung solution (in mM: 137 NaCl, 3 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 12 NaHCO$_3$, 4 NaH$_2$PO$_4$, and 6 glucose, pH 7.4). The temperature of the organ bath was held at 37°C and carbogen (95% O$_2$ + 5% CO$_2$) was bubbled into the chambers. After the setting of the initial tension (1.00 g), the cervical samples were incubated with a buffer change every 15 minutes for about 1 hour. The tissues were equilibrated for another 60 minutes with α-tocopherol succinate (10^{-7} M). Tocopherol (Sigma-Aldrich Hungary Kft., Budapest, Hungary) was applied to tissues after every wash of buffer solution. The control preparations were incubated for 1 hour without

tocopherol. The nonselective COXi diclofenac (Sigma-Aldrich Hungary Kft.) (10^{-6} M), selective COX-1 inhibitor SC-560 (10^{-6} M) and selective COX-2 inhibitor rofecoxib (Sigma-Aldrich Hungary Kft.) (10^{-6} M) were added to the organ bath and the cervices were incubated for 5 minutes. The tension of the cervical rings was measured with a gauge transducer (SEN-03; MDE Ltd., Budapest, Hungary) and recorded with a SPEL Advanced ISOSYS Data Acquisition System (MDE Ltd.). The cervices were stretched in growing steps and allowed to relax for 5 minutes, the tension after 5 minutes was read from the record by the analyzing software. After every 5 minutes, the next initial tension was set, in 1-g steps, between 1 and 12 g. The tension was set up manually via the control screw of a gauge transducer. The developed stress-strain curves had a saw-tooth shape (Fig. 1, A–D). In the evaluation of cervical resistance, the initial tension of the cervix was plotted versus the stretch after 5 minutes. Straight lines were suited by linear regression (Fig. 1E) and the slopes of the lines were applied to express the degree of resistance. A steeper slope reflected a higher resistance (Gáspár et al., 2005).

Measurement of COX Enzymes Activity. The cervical samples (22 day–pregnant and nonpregnant, $n = 6$/group) were incubated in an organ bath as described above. After the incubation period, tissues were rinsed with cold Tris buffer at 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 the experiment, the samples were homogenized in 5 ml of cold buffer (0.1 M Tris-HCl, pH 7.8, containing 1 mM EDTA) per gram of tissue, and centrifuged at 10,000g for 15 minutes at 4°C. In the removed supernatant, the activity of COX enzymes was measured using the COX Activity Assay Kit (Cayman chemicals, Ann Arbor, MI) This kit determines the peroxidase activity of COX enzymes. The peroxidase activity is assayed with the colorimetric method by monitoring the appearance of oxidized N,N,N′,N′-tetramethyl-p-phenylenediamine at 590 nm.

In Vivo Studies. The pregnant rats were divided into the following four groups ($n = 8$/group): 1) control; 2) tocopherol treated; 3) rofecoxib treated; and 4) tocopherol + rofecoxib treated. The animals received a single treatment with 1 ml of water (control), 250 mg/kg tocopherol (John et al., 2001), 5 mg/kg rofecoxib (Halpin et al., 2000), or 250 mg/kg tocopherol + 5 mg/kg rofecoxib on the 21st day of pregnancy at 4:00 PM by oral gavage. After the treatment, the onset of deliveries was observed and the elapsed hours were registered. The presence of blood or the first fetus in the bedding was considered to be the onset of labor.

Statistical Analysis. All data were analyzed using Prism version 5.01 (GraphPad Software, San Diego, CA). The values were statistically evaluated with the unpaired t test or analysis of variance with Tukey's multiple-comparison test.

Results

Isolated Organ Bath Studies. The resistance of control nonpregnant cervices was 0.99 ± 0.01. The pretreatment with tocopherol itself did not alter this value significantly. Furthermore, neither the investigated COXis (diclofenac, SC-560, and rofecoxib) nor tocopherol combined with COXs changed cervical resistance compared with the control (Fig. 2).

In the samples from 22 day–pregnant rats, the control cervical resistance was 0.85 ± 0.01. Tocopherol dramatically decreased resistance to 0.72 ± 0.02. The same reduction in resistance (0.72 ± 0.02) was detected in the case of nonselective COXi diclofenac, and it was further reduced to 0.62 ± 0.0218 by pretreatment with tocopherol. The selective COX-1 inhibitor SC-560 had no effect on cervical resistance. However, in the presence of tocopherol, the resistance value was abated to 0.69 ± 0.02. The selective COX-2 inhibitor rofecoxib also decreased cervical resistance to 0.70 ± 0.01. Rofecoxib with
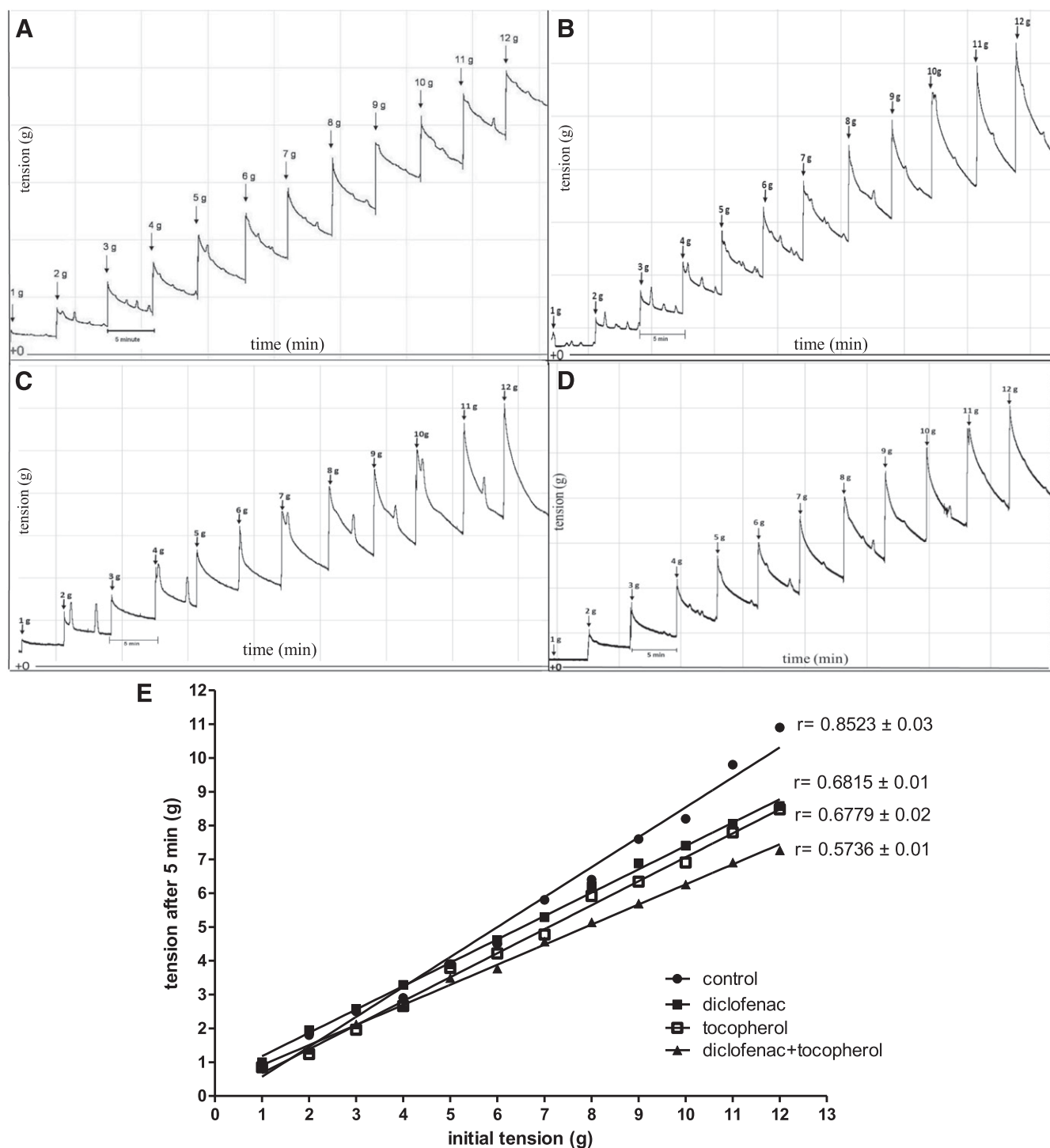
Fig. 1. The recorded saw-tooth shape stress-strain curves of 22 day–pregnant cervices: control (A); in the presence of diclofenac (10^{-6} M) (B); after pretreatment with α-tocopherol (10^{-7} M) and diclofenac (10^{-6} M) (C); and α-tocopherol alone (10^{-7} M) (D). (A–D) The linear regression of curves. (E) The steeper the slope of the line (r), the higher the cervical resistance.

tocopherol induced the strongest reduction in cervical resistance (0.60 ± 0.02) (Fig. 3).

Measurement of COX Enzymes Activity. The activity of total COX enzymes in nonpregnant and pregnant cervices was quite similar (Fig. 4). The ratio of COX-1 to COX-2 activity was equal in nonpregnant samples, whereas in tissues from pregnant rats the ratio shifted to COX-2 predominance. In cervix samples from tocopherol-pretreated nonpregnant rats,

the activity of COX-1 was not altered significantly. However, in cervix samples from pregnant rats the activity of COX-2 was decreased significantly from 19.4 to 12.5 U/ml by pretreatment with tocopherol (Fig. 5).

In Vivo Studies. The delivery occurred 40 hours after the water treatment in control rats. Neither tocopherol nor rofecoxib treatment was able to change the time of delivery compared with control groups. However, in case of
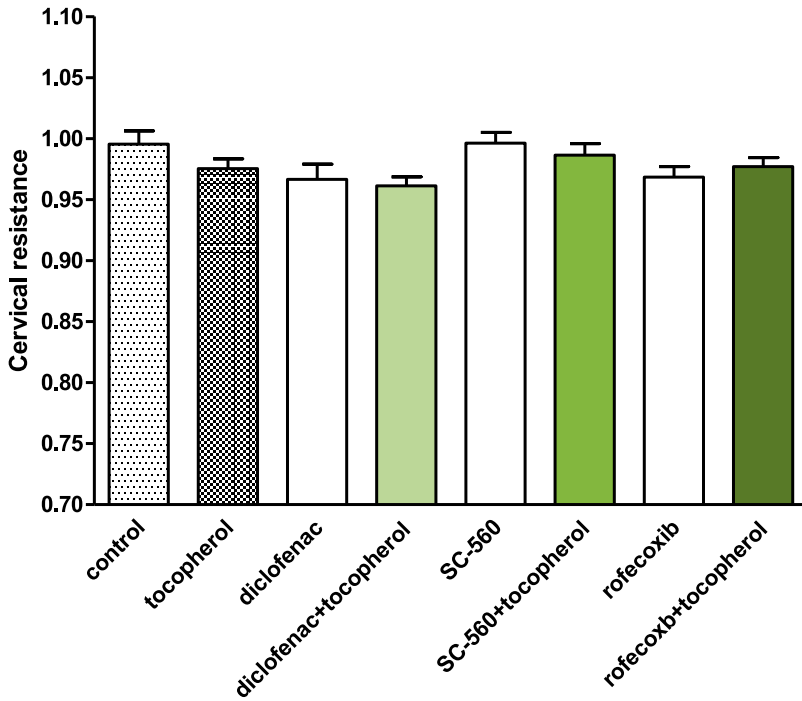
Fig. 2. Effects of α-tocopherol (10^{-7} M), COXis (10^{-6} M), and COXis combined with tocopherol on nonpregnant cervical resistance. Neither tocopherol nor COXis change cervical resistance significantly. Each value denotes the mean $\pm$ S.E.M. ($n = 6$).

the coadministration of tocopherol and rofecoxib, the labor had been initiated 16 hours earlier compared with the control group (Fig. 6).

Discussion

In this study, we have found that pretreatment with α-tocopherol is able to decrease cervical resistance via the inhibition of COX-2 activity and to amplify the resistance-reducing effect of COXis in 22 day–pregnant rats in vitro.

Additionally, the coadministration of α-tocopherol and the selective COX-2 inhibitor rofecoxib before the last day of pregnancy shortens the gestational period.

It is well known that the functions and structures of nonpregnant and pregnant cervices are quite different. The nonpregnant cervix is firm, consists mostly of collagen in about 90% of rats, with a minority of smooth muscle cells (10%). The pregnant cervix, especially near term, is dilated and softened (Vink and Feltovich, 2016); thus, the nonpregnant cervical resistance is much higher than the pregnant
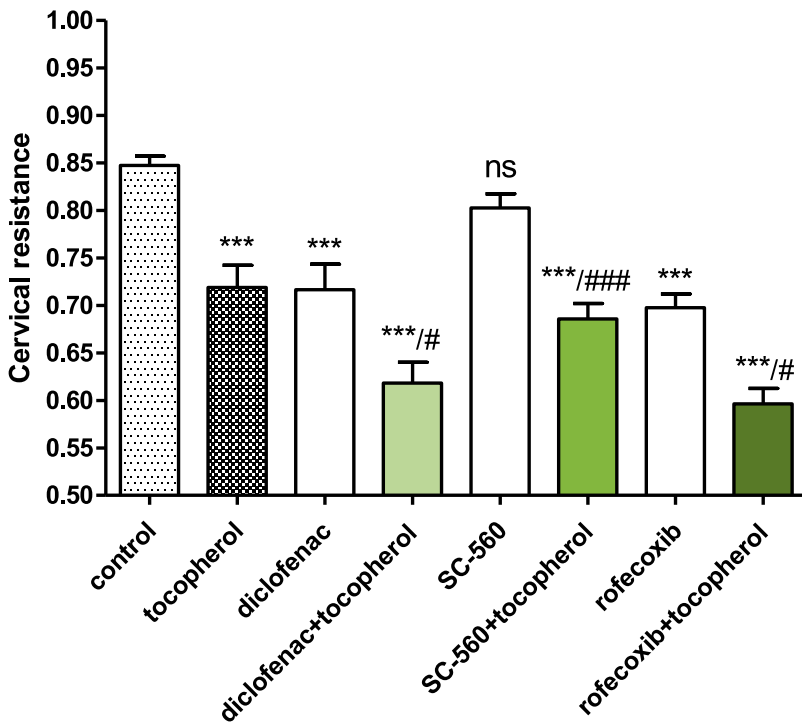

Fig. 3. Effects of α-tocopherol (10^{-7} M), COXis (10^{-6} M), and COXis combined with tocopherol on 22 day–pregnant cervical resistance. α-Tocopherol, diclofenac, and rofecoxib caused the reduction of resistance. Additionally, COXis combined with tocopherol further lowered cervical resistance in all cases. ns, not significant. ***$P < 0.001$ compared with the control column; #$P < 0.05$, ###$P < 0.001$ compared with the previous column. Each value denotes the mean $\pm$ S.E.M. ($n = 6$).
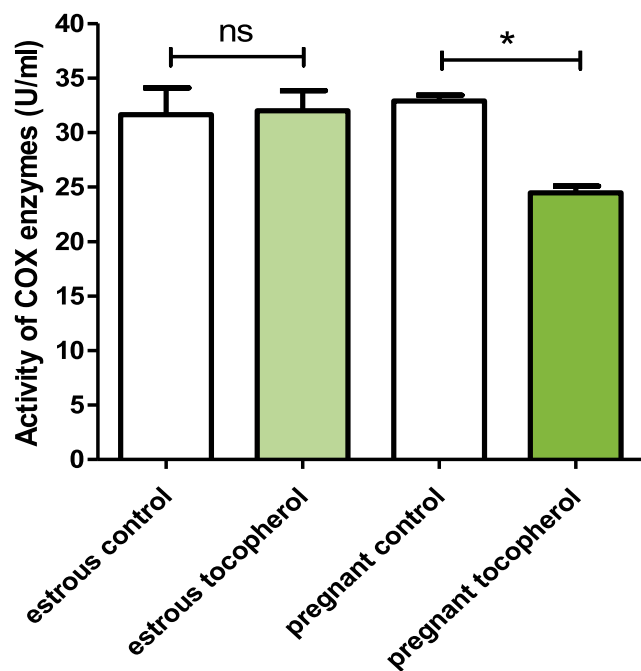
Fig. 4. Changes in the total activity of COX enzymes in nonpregnant and 22 day–pregnant cervices. α-Tocopherol decreased the activity of COX enzymes solely in pregnant samples. ns, not significant ($*P < 0.05$) Each value denotes the mean $\pm$ S.E.M. ($n = 6$).

resistance (Shi et al., 1999). This difference has clearly been confirmed in our study.

During pregnancy, the cervix undergoes a drastic transformation, which is defined as cervical ripening. This is induced by various endogenous substances: PGs, cytokines, and hormones such as progesterone, estrogens, and relaxin. As a result of these compounds, at the term of pregnancy collagen is degraded and the cervix becomes hydrated, which causes the softness (Sennström et al., 2000). Several studies have investigated the exact role of PGs in cervical remodeling. PGE$_2$ increased the activity of collagenase in the human cervix (Osmers et al., 1991) and the inflammatory response, which characterizes cervical ripening and remodeling (Ravanos et al., 2015). PGF$_{2\alpha}$ enhanced the synthesis of hyaluronic acid and glycosaminoglycans (Murota et al., 1977). Moreover, PGs were able to elevate cytokine synthesis (Denison et al., 1999) and PGE$_2$ stimulated cervical softening, whereas the nonselective COXi indomethacin inhibited this effect (Shi et al., 2000). However, those cervices were analyzed on pregnancy day 17 and not on day 22, as was done in our experiment.

In the last decade, COXis have been investigated as potential tocolytic drugs. It was found that nonsteroidal anti-inflammatory drugs, especially indomethacin, can decrease the contractility of uteri and inhibit the ripening of cervix (King et al., 2005; Antonucci et al., 2012; MacIntyre et al., 2012). On the other hand, very limited studies are available about the effect of COXis on uteri or cervices near term. In one study (Klauser et al., 2016), the tocolytic efficacy of magnesium sulfate, nifedipine, and indomethacin was investigated in women in acute preterm labor with advanced cervical dilation. It was found that indomethacin could stop the preterm labor and gain 15.7 ± 20.6 days, but no difference was found in the efficacy of the investigated tocolytic agents. Other authors (Berghella et al., 2009) did not find any significant

difference in benefit between indomethacin-treated and control women who were in acute preterm labor at 14–25 gestational weeks with a 1 cm–dilated cervix. In rats, the COX-2 enzyme is localized in cervical smooth muscle (CSM) throughout gestation in a normal term pregnancy, and its expression was increased during labor on the 22nd day of pregnancy (Marx et al., 2006). It was also demonstrated that the enhanced level of COX-2 enzyme and 15-hydroxyprostaglandin dehydrogenase downregulation are crucial for the PGE$_2$-inducated cervical ripening (Kishore et al., 2017).

Based on these earlier findings, we presumed that COXis probably would enhance cervical resistance through lowering the levels of PGs. Surprisingly, the COX-1–selective inhibitor SC-560 had no action on cervical resistance, whereas the nonselective inhibitor diclofenac and COX-2–selective inhibitor rofecoxib reduced the pregnant cervical resistance.

Cervical remodeling is divided into different phases: softening, ripening, dilatation, and postpartum. The dilatation phase is assigned directly before labor (Read et al., 2007). Isolated organ bath experiments were made on pregnancy day 22. Since the anticipated delivery of SD rats occurs on the 22nd to 23rd day of pregnancy, the cervical samples therefore had probably undergone the ripening process. There is a new approach regarding the significance of CSM in pregnancy. Over the past 60 years, the cervix has been known as a particularly collagenous structure (Danforth, 1947, 1954; Hughesdon, 1952). Researchers have underrated the existence of smooth muscle in the cervix, have believed that CSM stays inactive in pregnancy and during labor, and have interpreted the premature cervical failure to be a disorder of the cervical collagen network (Rechberger et al., 1988; Oxlund et al., 2010; Gedikbasi et al., 2016). The function of CSM has received more attention over the few last years; the article by Ferland et al. (2015) determined that CSM stayed active during pregnancy and labor and, in addition, that it may have contributed efficiently to cervical remodeling in rats. Vink et al. (2016) suggested that CSM plays a possible role in uterine contraction and remodeling. At the end of cervical ripening, collagens are degraded by matrix metalloproteinase, while the content of CSM remains unchanged. Hence, it is possible that CSM may influence all phases of cervical remodeling, and it might have a key function in the dilatation phase as well.

Furthermore, the differences in the selectivity of COXis imply that COXs may affect the dilatation of CSM. COXs catalyze the liberation of PGs in the arachidonic acid cascade. In the cervix, both COX enzymes were determined; however, their levels were different in nonpregnant and pregnant cervices. Dong et al. (1996) demonstrated that the cervical expression of COX-2 was elevated 2-fold at the end of pregnancy and that the activity of COX-2 also rose during labor. Therefore, COX-2 seems to be more important for cervical ripening and spontaneous labor than COX-1. Although the activity of COX-2 did not change significantly in pregnancy, it is apparent that the COX-1/COX-2 ratio of pregnant cervices was shifted toward the predominance of COX-2. This result has a discrepancy with earlier findings that proved the presence of increased COX-2 expression in late-term rats (Dong et al., 1996), but in that experiment only the protein expressions of COXs were measured without detecting the real enzymatic activity.

Vitamin E is necessary for reproduction (Mohd Mutalip et al., 2018). Only a few studies have investigated the relationship
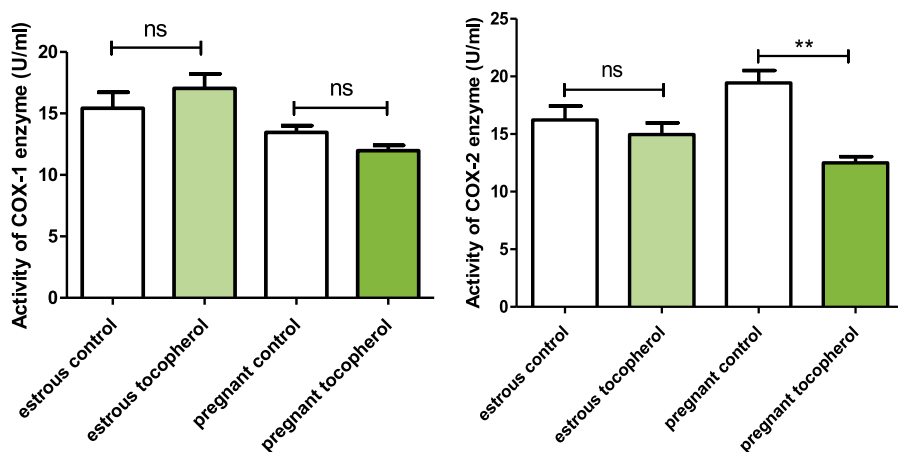
Fig. 5. Alteration of the activity of COX-1 (A) and COX-2 (B) enzymes by α- tocopherol in nonpregnant and 22 day–pregnant cervices. Significant reduction was observed in the activity of COX-2 enzyme in tocopherol-pretreated pregnant cervices. ns, not significant (**$P < 0.01$). Each value denotes the mean ± S.E.M. ($n = 6$).

among vitamin E, its analogs, and COXs (Sakamoto et al., 1991; Beharka et al., 1997; Wu et al., 1998, 2005; Lee et al., 2006; Kim et al., 2012). The drawback of those studies was that experiments were carried out in cell cultures, so their applicability is limited. Although the association of α-tocopherol and COXs had already been demonstrated in the rat uterus by our group (Kothencz et al., 2018), no research had previously been carried out for the cervix. We have found that tocopherol can reduce the pregnant cervical resistance and enhance the resistance inhibition effect of diclofenac and rofecoxib. Its effect was maintained in the presence of COX-1–selective inhibitor SC-560. These results can be explained by COX activity measurements in which α-tocopherol decreased the activity of COX-2 in cervical samples from pregnant rats. These findings suggest that COX-2–mediated PG liberation may have a crucial role in the contraction of CSM during delivery. Moreover, tocopherol may have a synergistic effect with COXis on rat cervical resistance.

The in vivo experiments verified that tocopherol and rofecoxib have a synergistic effect on the reduction of cervical resistance, since together they were able to shorten the gestational period by 16 hours. In our earlier study, we found that the coadministration of these compounds significantly reduced the myometrial contractions in vitro (Kothencz et al., 2018) that would predict a delay in delivery. However, this in vivo result suggests that the joint effect of tocopherol and rofecoxib on the reduction of

cervical resistance is predominant over their myometrium-relaxing effect.

In conclusion, our findings indicate that COX-2 inhibitors can decrease the resistance of late-term and ripened rat cervix. Additionally, α-tocopherol potentiates this action via the inhibition of COX-2 activity. Finally, single oral administration with tocopherol and rofecoxib can shorten the gestational period and accelerate the onset of labor. It seems that COXs play a significant role not only in cervical ripening, but also in the contraction of CSM before parturition. This latter action may be developed by the COX-2–liberated PGs. Interestingly, α-tocopherol has an opposite effect on COX-2 activity pregnant cervices compared with tissue from pregnant myometrium. Further studies are required to reveal the putative clinical significance of this multidirectional activity of α-tocopherol.

Authorship Contributions

Participated in research design: Kothencz and Gáspár.

Performed the experiments: Kothencz, Hajagos-Tóth, Szűcs, and Schaffer.

Wrote or contributed to the writing of the manuscript: Kothencz and Gáspár.

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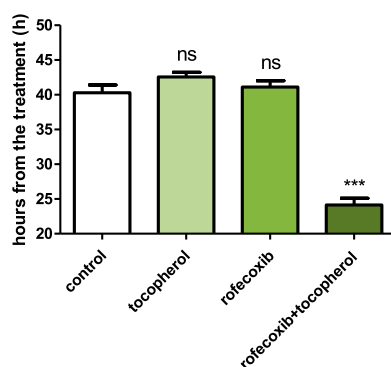
Fig. 6. Changes in the initial time of delivery effected by tocopherol, rofecoxib, and tocopherol + rofecoxib. The delivery occurred earlier after treatment with tocopherol combined with rofecoxib. ns, not significant (***$P < 0.001$). Each value denotes the mean ± S.E.M. ($n = 8$).

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