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The regulatory action of acetylcholine and its receptors on B4 and C4 leukotriene formation in the porcine endometrium after experimental inflammogenic *Escherichia coli* infection

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Abstract

Introduction: Endometritis is a very common pathology in animals which changes endometrial leukotriene (LT) formation and muscarinic 2 and 3 receptor subtypes (M2R/M3R) and α-7 nicotinic acetylcholine (ACh) receptor (α-7 nAChR) expression patterns. With the relationship between ACh, its receptors and LT production remaining unclear, the role of M2R, M3R and α-7 nAChR in action of ACh on the 5-lipoxygenase (5-LO), LTA4 hydrolase (LTAH) and LTC4 synthase (LTCS) protein abundances in the inflamed porcine endometrium and on the tissue secretion of LTB4 and LTC4 were studied. **Material and Methods:** On day three of the oestrous cycle in gilts aged 7–8 months, 50 mL of either saline solution (control group, n = 5) or an *E. coli* suspension at 10^9 colony-forming units/mL (*E. coli* group, n = 5), was injected into each uterine horn. Endometrial explants obtained eight days later, were incubated with ACh alone, antagonists of M2R, M3R and α-7 nAChR alone, or with ACh together with particular antagonists for 16 h. Enzyme abundances in endometrial tissue were estimated by Western blotting, and LT concentrations in medium by ELISA. **Results:** Severe acute endometritis developed in the *E. coli* group. In the endometrial explants from both groups, ACh elevated 5-LO, LTAH and LTCS protein abundances and LTB4 and LTC4 release. In the *E. coli* group, ACh-induced 5-LO and LTCS abundances and LTB4 release were increased versus the control group. In both groups, the M3R antagonist with ACh reduced all ACh-stimulated enzyme abundances and LT release in comparison to the abundances and release mediated by ACh alone. This effect on LTCS protein abundance and LTB4 release was also produced by the M2R antagonist with ACh in the *E. coli* group. Compared to the effect of ACh alone, exposure of the *E. coli* group endometrium to the α-7 nAChR antagonist with ACh led to a rise in LTAH and LTCS protein abundances and LTB4 and LTC4 secretion. **Conclusion:** In the inflamed pig endometrium, ACh increased 5-LO, LTAH and LTCS protein abundances and LTB4 and LTC4 release by M3R, and LTCS protein abundance and LTB4 release also by M2R. By interaction with α-7 nAChR, ACh reduced LTAH and LTCS protein abundances and the release of these LTs. Thus, in an indirect manner, ACh can affect LT-controlled processes.

Keywords: endometritis, acetylcholine, B4 and C4 leukotriene production, pig.

Introduction

In pigs, the uterine parasympathetic nerve fibres derive from the paracervical ganglion and contain other neurotransmitters in addition to acetylcholine (ACh). They supply the blood vessels, glands and myocytes of the myometrium (25, 42). Acetylcholine is also an autocrine/paracrine-secreted hormone from non-neuronal origins, including inflammatory cells. This neurotransmitter acts by muscarinic receptors (mAChRs of the subtypes M1R–M5R) and nicotinic receptors (nAChRs) which are expressed in neuronal and non-neuronal tissues (9). Three such receptors – M2R, M3R and α-7 nAChR – were identified in the healthy porcine uterus (12), and MR-mediated uterine contractility was reported in mice (20) and pigs (11). Subtype 3 muscarinic receptors are mainly involved in the ACh-induced contraction activity of the canine uterine artery (33). During inflammation

via M2R and M3R, ACh potentiates neutrophil cells migration and regulates pro-inflammatory mediator secretion from airway cells (35). Alpha-7 nAChR expression takes place in normal placentae and those in patients with severe preeclampsia (24), as well as in endometriotic lesions, limiting their formation (46). Acetylcholine modulates inflammatory reactions by α-7 nAChR, present in the endothelial (32) and immune (37) cells. These factors play roles in the development of colon (43) and lung inflammation (47).

The synthesis of leukotrienes (LTs) from arachidonic acid is initiated by the enzyme 5-lipoxygenase (5-LO), which requires the 5-LO-activating protein for its catalytic activity. Leukotriene A4, the precursor of LT species formed under the influence of 5-LO from arachidonic acid, is further hydrolysed by LTA4 hydrolase (LTAH) to generate leukotriene B4 (LTB4) or conjugated with reduced glutathione by the integral membrane protein leukotriene C4 synthase (LTCS) to yield leukotriene C4 (LTC4). Generation of this LT is the initial step in the generation of cysteinyl LTs (cys-LTs), others of which are leukotriene D4 (LTD4) and leukotriene E4 (LTE4) (41). Cysteinyl LTs are ligands for cysLT1 and cysLT2 receptors, and LTB4 is the agonist for BLT1 and BLT2, the receptors of LTB4 (34). The actions of LTs in the physiological state relate to the control of reproductive processes (18). As inflammatory mediators, LTs are implicated in intestinal (43) and respiratory (27) diseases.

Uterine inflammation (endometritis and metritis) very often occurs in domestic animals and women, developing mainly after parturition. The severe form of inflammation leads in cows and pigs to disturbances in reproductive processes and lower farm production profits (29, 30, 40). This pathology is mainly caused by bacteria and develops as a result of disturbances in the endometrial immune defence mechanisms and/or in the contractility of the myometrium (31, 44). In the inflamed uterus, the synthesis and release of LTB4 and LTC4 – metabolites derived from the 5-LO pathway – are known to be markedly increased (1, 2, 14). Inflammatory mediators influence 5-LO, LTAH and LTCS expression and LTB4 and LTC4 secretion from the inflamed pig endometrium (6) and pig endometrial epithelial (13) and endothelial (7) cells. When considered for its cholinergic regulation of function, the inflamed pig uterus presented changes in M2R, M3R and α-7 nAChR expression (12), and ACh *via* M2R and M3R impaired the contractility of this organ (11). The significance of ACh and its receptors in the synthesis and secretion of LTs by the inflamed uterus has not been entirely recognised yet. As reported earlier, ACh raises LTB4 release from the sputum cells during inflammation by M2R and M3R (36). On the basis of these findings we hypothesised that ACh along with M2R, M3R and α-7 nAChR play roles in LTB4 and LTC4 production and release by the inflamed uterus. Insight into these relationships will achieve better understanding of the neuro-immune mechanisms underlying uterine inflammation, which will significantly improve the prevention and treatment of uterine inflammation in animals and women. Elucidating the actions of ACh alone or combined with antagonists of M2R, M3R and α-7 nAChR on the 5-LO, LTAH and LTCS protein abundances in the inflamed porcine endometrium and in the release of LTB4 and LTC4 from this tissue were the aim of the study.

Material and Methods

Animals. All study procedures were approved by the Local Ethics Committee for Experiments on Animals (University of Warmia and Mazury in Olsztyn, Poland, Consent No. 65/2015). The guidelines in EU Directive 2010/63/EU for animal studies were also followed. The experiment was carried out on sexually mature Large White × Landrace gilts aged 7–8 months and weighing 107.3 ± 1.8 kg /mean ± standard error of the mean (SEM). Behavioural oestrus was detected by a tester boar. There was no vaginal discharge from the experimental pigs and their second oestrous cycles were regular. For acclimatisation, the gilts were transported from a commercial farm (Agro-Wronie Sp. z o.o., Wronie, Wąbrzeźno, Poland) to the animal house at the University of Warmia and Mazury (Olsztyn, Poland) three days prior to the beginning of the study. Each gilt was housed in an individual pen measuring approximately 5 m^2 and kept at a temperature of 18 ± 2°C, in 14.5 ± 1.5 h of natural daylight and 9.5 ± 1.5 h of darkness. The gilts were given a commercial diet and had access to water.

Experiment design. At the end of the acclimatisation period (day 3 of the second oestrous cycle and day 0 of the experiment), the gilts were randomly assigned to two groups: the control group (n = 5), in which the gilts received saline injections into their uterine horns and the *E. coli* group (n = 5), in which the gilts received *E. coli* injections into their uterine horns. The study procedures were reported earlier (11). Briefly, the animals were premedicated with atropine (Atropinum sulfuricum WZF; Warszawskie Zakłady Farmaceutyczne Polfa S.A., Warsaw, Poland), azaperone (Stresnil; Janssen Pharmaceutica, Beerse, Belgium) and ketamine hydrochloride (Ketamina; Biowet, Puławy, Poland). General anaesthesia was induced by ketamine hydrochloride. After median laparotomies had been performed, one injection of 10 mL was given into each of five equidistant sites of each uterine horn in the *E. coli* group and in the control group. The experimental group received a suspension of the *E. coli* serotype O25:K23/a/:H1 at 10^9 colony forming units/mL concentration, which was provided by the Department of Microbiology at the National Veterinary Research Institute (Puławy, Poland). The quantity of injected bacteria was the same as was used in a previous experiment which revealed that injections into pig uterine horns of the same strain of *E. coli* caused severe (mostly) or moderate acute endometritis after eight days (15). The animals from both groups were

untreated in the period from surgery until euthanasia, which was performed by an overdose of sodium pentobarbital on day 8 of the study (the expected day 11 of the oestrous cycle). The collected uteri were transported on ice to the laboratory within 20 min.

Preparation and treatment of endometrial explants. The uterine horns were washed twice using sterile phosphate-buffered saline. Next, a fragment of the wall was harvested from the middle part of each uterine horn. The wall was divided into endometrial and myometrial layers with the help of a scalpel blade. The endometrium fragments were cut into slices weighing 60–70 mg and then rinsed in Medium 199 with 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) modification and Earle's salts, L-glutamine and 2 mM HEPES and without sodium bicarbonate (catalogue No. M2520; Sigma-Aldrich, St. Louis, MO, USA). Single endometrial explants were put into glass vials with 2 mL of Medium 199 and 0.1% bovine serum albumin (BSA; catalogue No. A2058, Sigma-Aldrich), gentamicin at 500 µL/500 mL (catalogue No. G1272) and neomycin at 100 µL/500 mL (catalogue No. N1142 (both from Sigma-Aldrich). Endometrium slices underwent preincubation for 1.5 h and incubation for 16 h in a shaking water bath at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Incubation was with fresh medium (as controls) or with the addition of 10^{-5} M of ACh alone, 10^{-4} M of AChR antagonists alone for M2R, M3R and α-7 nAChR, or with ACh together with particular antagonists all in the same amounts as when used alone. The ACh was catalogue No. A6625 (Sigma-Aldrich); the M2R antagonist was AF-DX 116/11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one, catalogue No. 1105; the M3R antagonist was 4-DAMP/1,1-dimethyl-4-diphenylacetoxypiperidinium iodide, catalogue No. 0482; and the α-7 nAChR antagonist was 03B1-bungarotoxin, catalogue No. 2133 (all from Tocris Bioscience, Bristol, UK). Initial dilutions of the antagonists were made in accordance with the manufacturer's instructions, in dimethyl sulfoxide (catalogue No. W387509; Sigma-Aldrich) for the antagonists for M2R and M3R, and in 0.2 mm-filtered distilled water for the antagonist for α-7 nAChR, and the solutions were then stored at −20°C. The final antagonist solutions and ACh solution were prepared using the same medium as for preincubation and incubation of explants. Each separate study (one per gilt, n = 5 in each group) was performed in triplicate. Prostaglandin F2α (PGF2α) secretion after exposure to 10^{-4} M of a nitric oxide donor (NONOate, catalogue No. 82150; Cayman Chemical, Ann Arbor, MI, USA) was measured to evaluate the reactivity of the endometrial explants. The doses of ACh, antagonists and NONOate and the time of incubation were selected based on the findings from preliminary studies or according to methodology in the authors' previous experiments. After the end of incubation, the endometrial explants were blotted with a paper filter, weighed and stored at −80°C until estimation of 5-LO, LTAH and LTCS protein abundances was carried out. The medium was placed into tubes with a solution of 5% ethylenediaminetetraacetic acid (EDTA, catalogue No. 118798103; Chempur, Piekary Śląskie, Poland), 1% acetylsalicylic acid (catalogue No. 107140422; POCH, Gliwice, Poland) at pH 7.4 and stored at −20°C until determination of LTB4 and LTC4 concentrations was made.

Western blot analysis. For endometrial explant homogenisation, a buffer with the following composition was used: 50 mM Tris–HCl at pH 7.4, 10 mM EDTA, 150 mM NaCl, 1% Triton X100 with 1 mM pepstatin A, 5 mg/mL of leupeptin, 5 mg/mL of aprotinin and 1 mM phenylmethylsulphonyl fluoride. All reagents were purchased from Sigma-Aldrich. The obtained homogenates were centrifuged for 10 min at 2,500 × *g* and 4°C. The supernatants were centrifuged for 1 h at 17,500 × *g* and 4°C, and pellets were frozen at −80°C for enzyme estimation. The total protein concentration was determined (4). Equal 20 µg amounts of protein isolate were dissolved in sodium dodecyl sulphate (SDS, catalogue No. L3771; Sigma-Aldrich) and a gel-loading buffer, heated for 4 min at 95°C and separated by 12% SDS-polyacrylamide gel electrophoresis. The separated proteins were then electroblotted onto 0.45 µm pore size Immobilon-P PVDF membranes (catalogue No. IOVH00010; Sigma-Aldrich) in a transfer buffer. In order to block nonspecific binding sites, incubation was carried out with 5% fat-free dry milk (Spółdzielnia Mleczarska, Gostyń, Poland) in Tris -buffered saline with Tween 20 (respective catalogue Nos T1503 and P1379; Sigma-Aldrich) for 1.5 h at 21°C. Next, the membranes were incubated for 18 h at 4°C with polyclonal rabbit antibodies for 5-LO at 1:200 dilution (catalogue No. 160402; Cayman Chemical Co.), polyclonal rabbit antibody for LTAH at 1:200 dilution (catalogue No. 1600250; Cayman Chemical Co.) and polyclonal rabbit antibody for LTCS at 1:1000 dilution (catalogue No. DF14129; Affinity Biosciences, Changzhou, China). Subsequently, the membranes were incubated for 1.5 h at 21°C with alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies for all enzymes at 1:10,000 dilution (catalogue No. 111-055-003; Jackson ImmunoResearch, West Grove, PA, USA). Protein immune complexes were visualised with a standard alkaline phosphatase method (nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP), catalogue No. 72091; Sigma-Aldrich). Analysis was made three times. To demonstrate the specificity of the primary antibodies, specific binding peptides to 5-LO (catalogue No. 360402; Cayman Chemical Co.) and to LTCS (catalogue no. DF14129-BP; Affinity Biosciences) were used as negative controls (Supplementary Fig. 1). The specificity of the LTAH antibody was previously reported for the porcine uterus (14). Polyclonal rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, at 1:5,000 dilution, catalogue No. G9545; Sigma-Aldrich) was used as an internal control for protein loading. Images were acquired and

quantified using a ChemiDoc Imaging System (Image Lab Touch 5.2; Bio-Rad Laboratories, Hercules, CA, USA).

ELISA procedure. Concentrations of LTB4 and LTC4 in the incubation medium were estimated with ELISA kits (catalogue Nos 502390 and 501070, respectively; Cayman Chemical Co.) according to the manufacturer's instructions. The standard curve for LTB4 ranged from 1.96 to 1,000 pg/mL, and the assay's effective concentration for 50% inhibition (IC_{50}) was 2.5 pg/mL. The intra- and interassay coefficients of variation were 4.5% and 7.2%, respectively. The standard curve for LTC4 ranged from 0.98 to 500 pg/mL, and the assay's IC_{50} was 1.85 pg/mL. The intra- and interassay coefficients of variation were 4.7% and 6.2%, respectively.

Statistical analysis. The only results obtained from the incubation of endometrium which were taken into account were those for which the PGF2α release under the influence of NONOate was statistically significant. A two-way analysis of variance (group and treatment) followed by the Bonferroni test (InStat; Graph Pad, San Diego, CA, USA) was applied to compare the mean ± SEM values. Differences with P-values < 0.05 were considered significant.

Results

The effects of ACh alone, antagonists of M2R, M3R and α-7 nAChR alone and ACh and antagonist combinations on the 5-LO protein abundance in the endometrium. In the control group endometria, the 5-LO enzyme abundance under the influence of ACh was higher than it was under the influence of M2R, M3R or α-7 nAChR antagonists (P-value < 0.01 for M2R and P-value < 0.05 for M3R and α-7 nAChR) (Table 1). This enzyme's abundance in the *E. coli*-challenged endometria in response to ACh was increased *versus* the antagonists' influences (P-value < 0.001). A rise in the enzyme abundance was also caused by the exposure of the *E. coli* group tissue to M2R, M3R and α-7 nAChR compared to the control group (P-value <0.001 for M2R and P-value < 0.01 for M3R and α-7 nAChR).

In the control and *E. coli* groups, ACh used alone increased the 5-LO abundances *versus* the control values obtained from endometria of the control or *E. coli* groups that had not undergone any *in vitro* treatment (P-value < 0.01 for the control group and P-value 0.001 for the *E. coli* group) (Fig. 1A). In the *E. coli* group, the control value of the protein abundance and the value in response to ACh were higher than the value in the control group (P-value < 0.001). In both groups, when the M2R antagonist was added together with ACh, it did not significantly change the 5-LO abundances in relation to these abundances produced by the ACh action alone (Fig. 1A). In the *E. coli* group, the enzyme abundance was increased by the M2R antagonist with ACh *versus* the control group abundance (P-value < 0.001). In the control and the *E. coli* groups, when the M3R antagonist was added together with ACh, it diminished the 5-LO enzyme abundances compared to the 5-LO abundance produced by ACh alone (P-value < 0.05 for the control group and P-value < 0.001 for the *E. coli* group) (Fig. 1A). In both groups, the pairing of the α-7 nAChR antagonist with ACh did not significantly alter 5-LO enzyme abundances from their levels when ACh influenced them alone (Fig. 1A). The enzyme abundance was increased by the α-7 nAChR antagonist in concert with ACh in the *E. coli* group compared to the control group (P-value < 0.001).

The effects of ACh alone, antagonists of M2R, M3R and α-7 nAChR alone and ACh and antagonist combinations on the LTAH protein abundance in the endometrium. The LTAH abundances in the control and *E. coli* groups were reduced in response to the M2R, M3R and α-7 nAChR antagonists *versus* the ACh action (P-value < 0.001) (Table 1). In the *E. coli* group, the enzyme abundance after using the M2R antagonist was higher than in the control group (P-value < 0.01). Acetylcholine without the antagonists increased the LTAH abundances in the control and *E. coli* groups compared to the control values (P-value < 0.001) (Fig. 1B). In both groups, the enzyme abundances in response to the M2R antagonist with ACh did not differ significantly from the abundance produced by the ACh action singly (Fig. 1B). In both groups, the M3R antagonist with ACh decreased the LTAH protein abundances compared to the effect of ACh alone (P-value < 0.001) (Fig. 1B). In the control group, the LTAH protein abundance in response to the α-7 nAChR antagonist with ACh did not differ significantly from that resulting from the action of ACh in isolation (Fig. 1B). In the *E. coli* group, the α-7 nAChR antagonist with ACh increased the LTAH enzyme abundance from the level it reached through ACh action (P-value < 0.05). The enzyme abundance in this group was also greater after the α-7 nAChR antagonist and ACh had acted than it was in the control group (P-value < 0.001).

The effects of ACh alone, antagonists of M2R, M3R and α-7 nAChR alone and ACh and antagonist combinations on the LTCS protein abundance in the endometrium. The LTCS abundances in both groups were reduced by M2R, M3R and α-7 nAChR antagonists compared to the ACh action (P-value < 0.001 for M2R and M3R and P-value < 0.01 for α-7 nAChR) (Table 1). In the *E. coli* group, this enzyme's abundances after exposure to M2R, M3R and α-7 nAChR antagonists were higher than in the control group (P-value < 0.001 for M2R and M3R and P-value < 0.01 for α-7 nAChR). In the control and *E. coli* groups, ACh elevated the LTCS abundances over the control values (P-value < 0.01 for the control group and P-value < 0.001 for the *E. coli* group) (Fig. 1C). In the *E. coli* group, the control value of LTCS enzyme abundance and the value after using ACh were increased *versus* the control group value (P-value < 0.001). In the control group, the LTCS abundance after using the M2R antagonist with ACh did not differ significantly from the abundance when only ACh acted (Fig. 1C). The enzyme abundance in the *E. coli* group was lower under the influence of the M2R antagonist in combination with ACh than under the influence of ACh alone (P-value < 0.001).

In the control and *E. coli* groups, the M3R antagonist with ACh reduced the LTCS enzyme abundances compared to those caused by ACh without the antagonist (P-value < 0.05 for the control group and P-value < 0.001 for the *E. coli* group) (Fig. 1C). In the *E. coli* group, higher LTCS abundance resulted from treatment with this combination than the abundance in the control group (P-value < 0.001). In the control group, the LTCS abundance produced by the action of the α-7 nAChR antagonist used with ACh did not differ significantly from what was observed by the action of ACh without the antagonist (Fig. 1C). In the *E. coli* group, the enzyme abundance was increased by the α-7 nAChR antagonist with ACh from what was caused by the ACh action (P-value < 0.01). After exposure to the α-7 nAChR antagonist with ACh, the LTCS abundance was higher in the *E. coli* group compared to the control group (P-value < 0.001).

The effects of ACh, antagonists of M2R, M3R and α-7 nAChR alone and ACh and antagonist combinations on the secretion of LTB4 from the endometrium. In the control group, the endometrial LTB4 secretion under the influence of the M2R, M3R and α-7 nAChR antagonists was reduced *versus* the ACh action (P-value < 0.05 for M2R and M3R and P-value < 0.01 for α-7 nAChR) (Table 2).

Table 1. Western blot results for the effects of acetylcholine (ACh) alone or antagonists of muscarinic 2 and 3 receptor subtypes (M2R/M3R) and α-7 nicotinic ACh receptor (α-7 nAChR) alone on the 5-lipoxygenase (5-LO), leukotriene (LT)A4 hydrolase (LTAH) and LTC4 synthase (LTCS) protein abundances in unchallenged and *E. coli*-challenged endometria of gilts

Treatment	Group	Protein expression (arbitrary units)		
		5 LO	LTAH	LTCS
Control value	Control	0.12 ± 0.01^{a}	1.82 ± 0.24^{a}	0.73 ± 0.04^{a}
	E. coli	$0.76 \pm 0.12^{a, ***}$	1.71 ± 0.07^{a}	$2.31 \pm 0.36^{a, ***}$
ACh	Control	0.65 ± 0.07^{b}	3.06 ± 0.11^{b}	2.15 ± 0.09^{b}
	E. coli	$1.63 \pm 0.21^{b, ***}$	2.81 ± 0.13^{b}	$5.16 \pm 0.42^{b, ***}$
M2R antagonist	Control	0.09 ± 0.01^{a}	1.91 ± 0.16^{a}	0.62 ± 0.06^{a}
	E. coli	$0.83 \pm 0.09^{a, ***}$	$1.23 \pm 0.09^{a, **}$	$3.29 \pm 0.23^{a, ***}$
M3R antagonist	Control	0.13 ± 0.05^{a}	1.87 ± 0.08^{a}	0.54 ± 0.07^{a}
	E. coli	$0.69 \pm 0.14^{a, **}$	1.65 ± 0.11^{a}	$2.86 \pm 0.31^{a, ***}$
α-7 nAChR antagonist	Control	0.11 ± 0.08^{a}	1.41 ± 0.05^{a}	0.63 ± 0.05^{a}
	E. coli	$0.68 \pm 0.15^{a, **}$	1.48 ± 0.07^{a}	$1.99 \pm 0.38^{a, **}$

Data are shown as the mean ± standard error of the mean. $^{a, b}$ – different superscript letters for any pair of values within a group for the same enzyme indicate significant difference (P-value < 0.05–0.001) between the control value, action of acetylcholine (ACh) and actions of particular antagonists; **, *** – significant difference between groups for the same enzyme and type of treatment (P-value < 0.01, P-value < 0.001)

Table 2. Effects of acetylcholine (ACh) alone or antagonists of muscarinic 2 and 3 receptor subtypes (M2R/M3R) and α-7 nicotinic ACh receptor (α-7 nAChR) alone on leukotriene (LT)B4 and LTC4 secretion from the endometrium of gilts determined by ELISA

Treatment	Group	Concentrations (ng/g tissue)	
		LTB4	LTC4
Control value	Control	2.41 ± 0.14^{a}	0.85 ± 0.11^{a}
	E. coli	$3.37 \pm 0.18^{a, **}$	$1.29 \pm 0.08^{a, *}$
ACh	Control	3.41 ± 017^{b}	1.45 ± 0.1^{b}
	E. coli	$4.25 \pm 0.26^{b, *}$	1.82 ± 0.09^{b}
M2R antagonist	Control	2.42 ± 0.23^{a}	0.98 ± 0.09^{a}
	E. coli	$3.26 \pm 0.22^{a, *}$	1.33 ± 0.11^{a}
M3R antagonist	Control	2.49 ± 0.15^{a}	0.81 ± 0.12^{a}
	E. coli	3.09 ± 0.27^{a}	$1.27 \pm 0.15^{a, *}$
α-7 nAChR antagonist	Control	2.31 ± 0.17^{a}	0.92 ± 0.07^{a}
	E. coli	2.99 ± 0.26^{a}	1.16 ± 0.14^{a}

Data are shown as the mean ± standard error of the mean. $^{a, b}$ – different superscript letters for any pair of values within a group for the same leukotriene (LT) indicate significant difference (P-value < 0.05–0.001) between the control value, action of acetylcholine (ACh) and actions of particular antagonists; *, ** – significant difference between groups for the same LT and type of treatment (P-value < 0.05, P-value < 0.01)

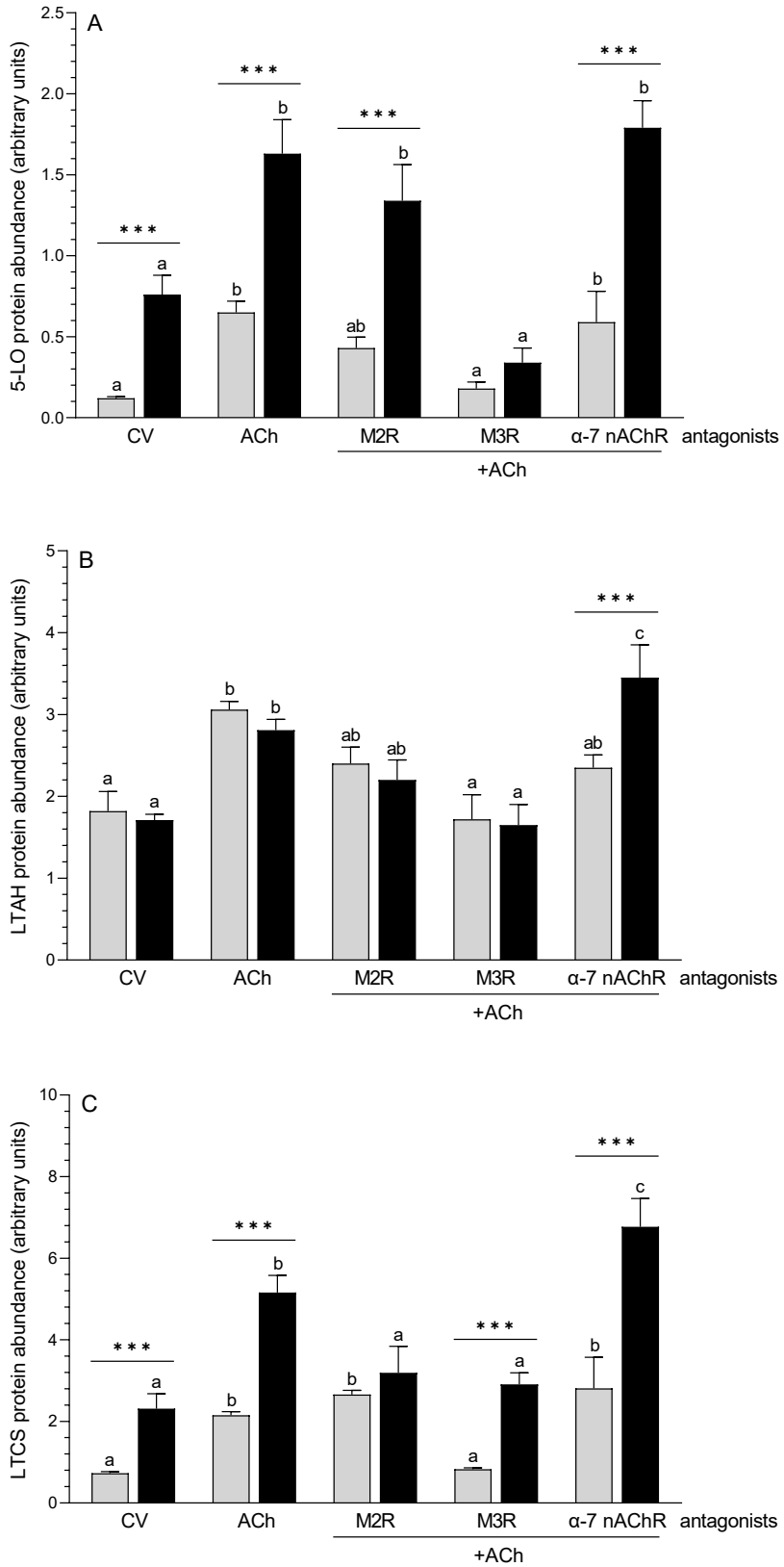

Fig. 1. Western blot results for the effects of acetylcholine (ACh) alone or with antagonists of muscarinic 2 and 3 receptor subtypes (M2R/M3R) and α-7 nicotinic ACh receptor (α-7 nAChR) on 5-lipoxygenase (5-LO; A), leukotriene (LT) A4 hydrolase (LTAH; B) and LTC4 synthase (LTCS; C) protein abundances in the endometrium of gilts from the control (grey bars, n = 5) and *E. coli* (black bars, n = 5) groups. Blots with representative bands for each enzyme in particular groups are depicted in Supplementary Figure 2. a, b, c – different letters for any pair of values within a group indicate significant difference (P-value < 0.05–0.001) between the control values (CV), action of ACh and actions of particular antagonists with ACh; *** – statistical difference between groups for the same type of treatment (P < 0.001)

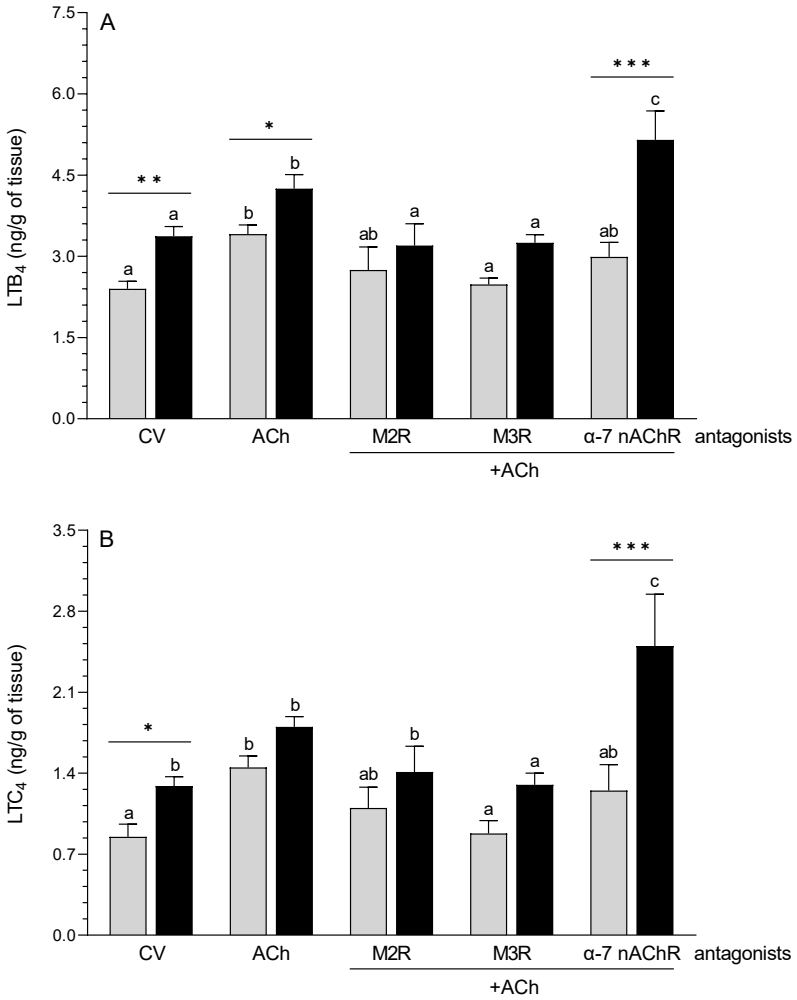

Fig. 2. ELISA results for the effects of acetylcholine (ACh) alone or with antagonists of muscarinic 2 and 3 receptor subtypes (M2R/M3R) and α-7 nicotinic ACh receptor (α-7 nAChR) on the leukotriene (LT)B4 (A) and LTC4 (B) secretion from the endometrium of gilts from the CON (grey bars, n = 5) and *E. coli* (black bars, n = 5) groups. a, b, c – different letters for any pair of values within a group indicate significant difference (P-value < 0.05–0.001) between the control values (CV), action of ACh and actions of particular antagonists with ACh; * P < 0.05, ** P < 0.01, *** P < 0.001 – statistical difference between groups for the same type treatment

The LTB4 release in the *E. coli* group was not as high after exposure to the antagonists for M2R, M3R and α-7 nAChR as it was after exposure to ACh alone (P-value < 0.05 for M2R and P-value < 0.01 for M3R and α-7 nAChR). The M2R antagonist induced more LTB4 release in the *E. coli* group than in the control group (P-value < 0.05).

The LTB4 release mediated by ACh in both groups was higher than the control values (P-value < 0.05) (Fig. 2A). In the *E. coli* group, the control value and the value in response to ACh exceeded that of the control group (P-value < 0.01 for the control value and P-value < 0.05 for ACh). The M2R antagonist in concert with ACh did not significantly change the LTB4 release in the control group (Fig. 2A), while in the *E. coli* group, it did so to diminish LTB4 release from that caused by ACh alone (P-value < 0.01). In both groups, the LTB4 secretion was reduced by the M3R antagonist with ACh *versus* the ACh action uncombined with the antagonist (P-value < 0.05) (Fig. 2A). In the control group, the LTB4 production mediated by the α-7 nAChR antagonist with ACh did not differ significantly from the production mediated by ACh alone (Fig. 2A). Leukotriene B4 was more heavily secreted from the *E. coli* group endometrium in response to the α-7 nAChR antagonist with ACh than in response only to ACh. In this group, the LTB4 release triggered by the α-7 nAChR antagonist with ACh was higher than the release in the control group (P-value < 0.001).

The effects of ACh alone, antagonists of M2R, M3R and α-7 nAChR alone and ACh and antagonist combinations on the on the secretion of LTC4 from the endometrium. In the control group, the LTC4 secretion in response to M2R, M3R and α-7 nAChR antagonists was lower *versus* the ACh action (P-value < 0.05 for M2R and P-value < 0.01 for M3R and α-7 nAChR) (Table 2). The LT release in the *E. coli* group by M2R, M3R and α-7 nAChR antagonists was reduced compared to the ACh effect (P-value < 0.05 for M2R and M3R and P-value < 0.01 for α-7 nAChR). After the M3R

antagonist was used, the *E. coli* group endometrium secreted more LTC4 than that of the control group (P-value < 0.05). The LTC4 release by ACh in the control and *E. coli* groups was higher than the control values (P-value < 0.01 for the control group and P-value < 0.05 for the *E. coli* group) (Fig. 2B). In the *E. coli* group, the control value was increased *versus* the CON group (P-value < 0.05). In neither group did the LTC4 release by the M2R antagonist with ACh change significantly in relation to the influence of ACh alone (Fig. 2B). The LTC4 secretion in the control and *E. coli* groups was downregulated by the M3R antagonist with ACh compared to the ACh effect in isolation (P-value < 0.01 for the control group and P-value < 0.05 for the *E. coli* groups) (Fig. 2B). In the control group, the α-7 nAChR antagonist with ACh did not significantly change the LTC4 release from the release triggered by ACh (Fig. 2B). In the *E. coli* group, the LTC4 activity mediated by the α-7 nAChR antagonist with ACh was higher than that mediated by ACh (P-value < 0.001). The α-7 nAChR–ACh pair elevated the leukotriene's activity in the *E. coli* group over this activity in the control group (P-value < 0.001).

Discussion

The present experiment is the first to reveal the parasympathetic control of synthesis and release of metabolites derived from the 5-LO pathway during uterine inflammation. More precisely, the 5-LO, LTAH and LTCS protein abundances and LTB4 and LTC4 secretion from the inflamed porcine endometrium were determined after exposure to ACh and/or M2R, M3R and α-7 nAChR antagonists. Macroscopically, injections of *E. coli* suspension into the uterine horns led to the appearance of inflammatory exudate in the horns and redness and swelling of the endometrium. In this uterine layer, a severe acute inflammatory state was diagnosed based on the presence of oedema, hyperaemia, damage to the luminal and glandular epithelium and a statistically significantly higher number of neutrophils in comparison to the healthy uterus (26).

To our knowledge, this is also the first report on the role of ACh in the synthesis and secretion of the LTs by the healthy uterus. We found that ACh increased the 5-LO, LTAH and LTCS protein abundances and LTB4 and LTC4 secretion in the control group endometrial explants. Previously it was found that ACh exerted a stimulatory action on LTB4 release from human bronchial epithelial (16-HBE) cells (35), a myelomonocytic cell line, bronchial epithelial cell line and alveolar macrophages (5, 38). Thus, our showing of the importance of ACh in the endometrial synthesis and release of LTs expands knowledge of the neuronal control of arachidonic acid metabolite formation in a healthy uterus.

Similarly to how it did in the control group, ACh increased the 5-LO, LTAH and LTCS protein abundances and the release of LTB4 and LTC4 from the inflamed endometrial tissue of the *E. coli* group. However, the 5-LO and LTCS protein abundances and release of both LTs were elevated in the *E. coli* group. There is no data in the available literature on the role of ACh in LT synthesis and secretion in the reproductive organs. With regard to the respiratory system, it has been reported that this neurotransmitter raises LTB4 release by the sputum cells of chronic obstructive pulmonary disease (COPD) patients (36), and that cigarette smoke extract increased LTB4 secretion from the 16-HBE cells (35). This LT showed a trend towards higher levels in bronchoalveolar lavage fluid after cigarette smoke experimentally induced inflammation in mice (45). Moreover, ACh increased the levels of LTs (C4, D4 and E4) in acutely inflamed circular oesophageal muscle (19).

There had hitherto been no data on the involvement of receptors in the effect of ACh on the synthesis and secretion of LTs from the uterus under physiological and pathological conditions. In the endometrial explants from the control and *E. coli* groups, we indicated the significance of M3R in ACh-stimulated LTB4 and LTC4 secretion and 5-LO, LTAH and LTCS protein abundances. We also indicated M2R as mediating the ACh stimulatory action on LTB4 release and LTCS protein abundance in the *E. coli* group. It was revealed in previous research that M1R, M2R and M3R are involved in the ACh-induced LTB4 release from the sputum cells of COPD patients (35, 36). Acetylcholine also increased LTB4 release from bovine alveolar macrophages by M3R (38). The participation of M3R in the stimulatory action of ACh on the 5-LO, LTAH and LTCS protein abundances and LTB4 and LTC4 release by the inflamed endometrial explants, as demonstrated in the present study, was associated with increased immunoreaction for this receptor in the glandular epithelial and stromal cells of the endometrium (12). In regard to α-7 nAChR, we found that under physiological conditions this receptor had no part in the mediation by ACh of the formation and release of LTs by the porcine endometrial explants. Under pathological conditions, in turn, the role of α-7 nAChR was detected in the inhibiting action of ACh on the LTB4 and LTC4 release by the inflamed endometrial explants. This coincided with the reduction by ACh of the LTAH and LTCS protein abundances through α-7 nAChR. It is known that dimethylphenylpiperazinium, a nonselective nAChR agonist, reduced induction by platelet-activating factor of LTC4 production in human blood eosinophils (3), and that nicotine inhibited this LT release by mast cells *via* α-7-nAChRs, as it also did *via* α-9- and α-10-nAChRs (28).

With regard to the importance of cholinergic stimuli in the formation of arachidonic acid metabolites, it was indicated that inflammation potentiated the effect of carbachol – an mAChR agonist – on prostaglandin (PG)E2 production in murine fibroblasts (8). Carbachol also induced PG-endoperoxidase synthase (PTGS)-1 and PTGS-2 mRNA expression in the intact bovine tracheal smooth muscle (17). Activation of α-7 nAChR

inhibited tumour-necrosis factor α (TNF-α) release in lung inflammation (10) and TNF-α and interleukin (IL)-1β expression in immune cells (46). In uterine inflammation, the present data showed the participation of ACh in LT synthesis and secretion and have expanded knowledge of the mechanisms regulating LT formation in porcine endometrial tissue. Besides influencing the pro-inflammatory (lipopolysaccharide, TNF-α and IL-1β) and anti-inflammatory (IL-4 and IL-10) mediators (6), ACh also influences the 5-LO, LTAH and LTCS protein abundances and LTB4 and LTC4 secretion by the inflamed pig endometrium. Our data clearly suggested that ACh may indirectly affect endometrial processes controlled by LTs, and specifically that it modulates inflammatory reaction and secretory activity. It is possible that acting *via* M3R (and M2R), ACh induces LT formation and release during endometritis, and this results in pro-inflammatory ACh activity. As a possible example of this mechanism in other tissue, ACh increased LTB4 release from bovine bronchial epithelial cells (23) and alveolar macrophages (38) to levels that were chemotactic for inflammatory cells. Leukotriene B4 is involved in activation and prolongation of inflammatory cell survival (39); additionally, besides its action in the development of inflammation, it is also involved in the maintenance and regeneration of damaged tissues (16). Both LTB4 and LTC4 increased PGE2, PGF2α and IL-6 secretion by *E. coli*-challenged bovine uterine explants (21) and the contents of these PGs in the bovine endometrium under physiological conditions (22). We suppose that the contrary effect of ACh in its action *via* α-7 nAChR in decreasing LT synthesis and release may be important to its anti-inflammatory activity, and may limit the endometrial inflammatory process. Knowledge of the receptor mechanism of LT synthesis and secretion during uterine inflammation can be used to develop drugs (antagonists and/or agonists of particular ACh receptors) that will allow the inflammatory process and endometrial function to be controlled.

Conclusion

The current data provide evidence that ACh has a stimulatory action on 5-LO, LTAH and LTCS protein abundances and the secretion of LTB4 and LTC4 by the porcine inflamed endometrium. These results suggest that by altering LT synthesis and secretion in an inflamed endometrium, ACh may indirectly affect the processes controlled by LTs. In the inflamed pig endometrium, ACh increases the 5-LO, LTAH and LTCS protein abundances and LTB4 and LTC4 release, mainly acting *via* M3R, which indicates the role of this kind of receptor in the pro-inflammatory activity of ACh. On the other hand, by reducing the LTAH and LTCS protein abundances and LTB4 and LTC4 release through α-7 nAChR, ACh's effects indicate the presence of a cholinergic anti-inflammatory pathway in the inflammatory endometrium. We suggest that the therapeutic effectiveness of the receptors studied here to restore normal uterine function should be further examined.

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