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Role of the endocannabinoid system in the mechanisms involved in the LPS-induced preterm labor

María Victoria Bariani, Ana Paula Domínguez Rubio, Maximiliano Cella, Juliana Burdet[1], Ana María Franchi and Julieta Aisemberg

Laboratory of Physiopathology of Pregnancy and Labor, Center for Pharmacological and Botanical Studies, National Research Council, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina and [1]Laboratory of Physiopathogeny, Department of Physiology, School of Medicine, University of Buenos Aires, Argentina

Correspondence should be addressed to M V Bariani; Email: vickiba_86@hotmail.com

Abstract

Prematurity is the leading cause of perinatal morbidity and mortality worldwide. There is a strong causal relationship between infection and preterm births. Intrauterine infection elicits an immune response involving the release of inflammatory mediators like cytokines and prostaglandins (PG) that trigger uterine contractions and parturition events. Anandamide (AEA) is an endogenous ligand for the cannabinoid receptors CB1 and CB2. Similarly to PG, endocannabinoids are implicated in different aspects of reproduction, such as maintenance of pregnancy and parturition. Little is known about the involvement of endocannabinoids on the onset of labor in an infectious milieu. Here, using a mouse model of preterm labor induced by lipopolysaccharide (LPS), we explored changes on the expression of components of endocannabinoid system (ECS). We have also determined whether AEA and CB antagonists alter PG production that induces labor. We observed an increase in uterine *N*-acylphosphatidylethanolamine-specific phospholipase D expression (NAPE-PLD, the enzyme that synthesizes AEA) upon LPS treatment. Activity of catabolic enzyme fatty acid amide hydrolase (FAAH) did not change significantly. In addition, we also found that LPS modulated uterine cannabinoid receptors expression by downregulating *Cb2* mRNA levels and upregulating CB1 protein expression. Furthermore, LPS and AEA induced PGF2a augmentation, and this was reversed by antagonizing CB1 receptor. Collectively, our results suggest that ECS may be involved in the mechanism by which infection causes preterm birth.

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Introduction

Prematurity is the leading cause of mortality and morbidity in neonates and children. It is well known that ~30% of all premature deliveries occur in association with an underlying infectious process (Challis *et al*. 2001, Park *et al*. 2009). We have previously developed a murine model of lipopolysaccharide (LPS)-induced preterm labor to mimic the pathological process implicated in prematurity induced by infections. Premature labor was induced by i.p. administration of LPS on day 15 of pregnancy. Exposure to bacterial endotoxin produces 100% of preterm birth with low incidence of maternal mortality (Cella *et al*. 2010, Domínguez Rubio *et al*. 2014).

Endocannabinoids are endogenous unsaturated bioactive fatty acid amides, ethers and esters that bind to and activate cannabinoid receptors CB1 and CB2. Anandamide (AEA) is synthesized on demand from membrane lipid precursors by the action of *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) (Piomelli *et al*. 2000). The biological activity of AEA is terminated by its cellular uptake followed by its intracellular degradation by a fatty acid amide hydrolase (FAAH). The endocannabinoid system (ECS) has been implicated in different reproductive events in women (Paria *et al*. 1995, Habayeb *et al*. 2004, Taylor *et al*. 2007). Uterine tissue contains high concentrations of AEA (Schmid *et al*. 1997), and during labor blood AEA levels increase dramatically, suggesting a role for AEA in normal labor (Habayeb *et al*. 2004). Numerous studies have shown that LPS modulates the ECS in different ways depending on the cell type (Varga *et al*. 1998, Maccarrone *et al*. 2001).

Prostaglandins (PG) production plays a central role in preterm labor induced by intrauterine infection (Gross *et al*. 2000, Adams *et al*. 2008, Timmons *et al*. 2014). Accordingly, previous results from our laboratory have shown that LPS treatment increases uterine PG and

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cyclooxygenase levels (Cella *et al*. 2010, Domínguez Rubio *et al*. 2014).

On the basis of the evidence presented above, the aim of the present study was to investigate the effect of LPS on the ECS in a murine model of LPS-induced preterm labor as well as the relationship between uterine PG levels and ECS.

Materials and methods

Reagents

LPS from *Escherichia coli*, serotype 055:B5, HRP conjugated secondary antibody, anti-b-Actin antibody, FITC–conjugated secondary antibody and PGF2a antiserum were provided by Sigma–Aldrich Co. [5,6,8,9,11,12,14,15(n)–^{3}H]-PGF2a (160 Ci/mmol, 200 µCi/ml) and [5,6,8,9,11,12,14,15-^{3}H]-AEA (172.4 Ci/mmol, 100 µCi/ml) were provided by Perkin-Elmer Life and Analytical Sciences, Inc. (Walthman, MA, USA). TLC aluminum silica gel plates were purchased from Merck KGaA (Darmstadt, Germany). AM251 (CB1 antagonist) was provided by Tocris Cookson, Inc. (Ellisville, MO, USA) and AM630 (CB2 antagonist) by Cayman Chemical Co. (Ann Arbor, MI, USA). CB2 and NAPE-PLD primary antibody were purchased from Abcam, Inc. (Cambridge, MA, USA) and CB1 primary antibody from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). FAAH primary antibody was a kind gift from Benjamin Cravatt (Department of Chemical Physiology, The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA 92037, USA). Western blotting reagents and nitrocellulose membranes (Trans-Blot, 0.45 µm) were from Bio-Rad Inc. and molecular weight marker was purchased from GE Healthcare Bio-Science Corp. (Piscataway, NJ, USA). TRI Reagent, dNTPs and ribonuclease inhibitor were from Genbiotech (Buenos Aires, Argentina). Ultrapure water, DTT, RNAse free DNAse 1, Moloney Murine Leukemia virus reverse transcriptase (M-MLVRT) and random primers were purchased from Invitrogen. GoTaq DNA polymerase was provided by Promega. All other chemicals were of analytical grade.

Animals

Virgin *BALB/c* female mice (8–12 weeks old; 25–30 g of weight) were paired with fertile males of the same strain. Day of vaginal plug detection was considered as day 0 of pregnancy. Under our animal facility conditions, normal term labor occurs at day 19 of pregnancy. Animals received a regular supply of food and water and allowed to feed and drink *ad libitum* and were housed under controlled conditions of light (12 h light:12 h darkness cycle) and temperature (23–25 °C).

Mice were euthanized by CO_2 inhalation. The experimental procedures reported here were approved by the Animal Care Committee of the Center for Pharmacological and Botanical Studies, National Research Council, and by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) from the School of Medicine, University of Buenos Aires, and were carried out in accordance.

Murine model of preterm labor

BALB/c mice on day 15 of pregnancy were injected i.p. with two doses of vehicle (sterile saline solution) or LPS (Escherichia coli, 055:B5), the first one (0.26 mg/kg,10 µg in 0.1 ml of vehicle) at 1000 h and the second at 1300 h (0.52 mg/kg, 20 µg in 0.1 ml of vehicle) (Cella *et al*. 2010). Preterm labor occured in 100% of the animals during the night of day 15 and the morning of day 16, without affecting maternal health. The beginning of preterm labor was defined by the delivery of the first pup.

RT-PCR

Animals were euthanized on day 15 of pregnancy 5 h after the second vehicle or LPS injection. The uterus was immediately removed and cleaned. Decidua was separated and discarded. TRI Reagent was added to samples and tissues were homogenized and frozen at −80 °C until used. Total RNA from uterine strips was isolated according to manufacturer's recommendations (Molecular Research Center, Inc., Cincinnati, OH, USA). Following extraction, RNA was quantified and cDNA was synthesized from total RNA (3 µg) using M-MLVRT, random primers and ribonuclease inhibitor. Specific primers were designed using the Primer3 Software (Rozen & Skaletsky 2000). Primers sequences and PCR conditions are shown in Table 1. Products were loaded onto 2% agarose gel and stained with ethidium bromide. Bands were visualized on a transilluminator under UV light. Photographs were taken with a digital camera (Olympus C-5060) and analyzed using the freely available Image J Software Package. Relative mRNA level was normalized to *b-Actin* and results were expressed as relative optical density (*Faah/b-Actin, Nape-pld/b-Actin, Cb1/b-Actin, Cb2/b-Actin*).

Western blot analysis

A group of animals was euthanized on day 13, 15, 18 or 19 of pregnancy whereas another was euthanized on day 15 of pregnancy 7 h after second vehicle or LPS injection. The uteri were immediately removed and homogenized (Ultra Turrax, T25 basic, IKA Labortechnik, Staufen, Germany) in lysis buffer (10 mM Hepes, 5 mM $MgCl_2$, 142.5 mM KCl, 0.1% SDS, 1% Nonidet-40, 5 mM EDTA, 0.5% sodium deoxycholate in PBS) with a freshly added protease inhibitor cocktail (10 µg/ml leupeptin, 2 µg/ml aprotinin, 100 µg/ml soybean-trypsin inhibitor, 1 mmol/l EDTA, 1 mg/ml benzamidine, 10 µg/ml DTT and 1 mg/ml caproid acid). Tissues were sonicated (Ultrasonic Cell Disrupter, Microson, Heat systems, Inc. New York, NY, USA) for 30 s, centrifuged at 1500 ***g*** for 10 min and protein concentration determined by Bradford assay (Bradford 1976). Eighty micrograms of protein were loaded in each lane. Positive controls were mouse liver for FAAH, mouse brain for NAPE-PLD and CB1 and mouse spleen for CB2. Samples were separated by electrophoresis in 7.5%–12% SDS–PAGE gel and transferred to a 0.45 µm nitrocellulose membrane. Membranes were blocked using 5% w/v dried non-fat milk and then incubated with the primary antibodies. Dilution of antibodies and incubation conditions are shown in

Table 1 Primer sequences and PCR conditions used for RT-PCR in uteri from BALB/c mice.

mRNA	Primers	Conditions
β-Actin	Forward 5′-TGT TAC CAA CTG GGA CGA CA-3′ Reverse 5′-TCT CAG CTG TGG TGG TGA AG-3′	94 °C 5 min (94 °C 40 s, 57 °C 30 s, 72 °C 1 min)×30 72 °C 5 min
Faah	Forward 5′-GAG ATG TAT CGC CAG TCC GT-3′ Reverse 5′-ACA GGC AGG CCT ATA CCC TT-3′	94 °C 5 min (94 °C 40 s, 54 °C 40 s, 72 °C 40 s)×34 72 °C 5 min
Nape-pld	Forward 5′-ATG AGA ACA GCC AGT CTC CA-3′ Reverse 5′-CCA TTT CCA CCA TCA GCG TC-3′	94 °C 5 min (94 °C 40 s, 57 °C 1 min, 72 °C 1 min)×35 72 °C 5 min
Cb1	Forward 5′-ACC TGA TGT TCT GGA TCG GA-3′ Reverse 5′-TGT TAT CTA GAG GCT GCG CA-3′	94 °C 5 min (94 °C 15 s, 55 °C 30 s, 72 °C 30 s)×35 72 °C 5 min
Cb2	Forward 5′-TCT GTG TTA CCC GCC TAC CT-3′ Reverse 5′-GTG GGG AAA GCT CAG AGC AG-3′	95 °C 5 min (95 °C 1 min, 60 °C 1 min, 75 °C 1 min)×40 75 °C 5 min

Faah, Fatty acid amide hydrolase; Nape-pld, *N*-acyl phosphatidylethanolamine specific phospholipase D; *Cb1*,Cannabinoid receptor type 1; *Cb2*,Cannabinoid receptor type 2.

Table 2. Membranes were washed with PBS-T (10 mM Tris, 100 mM NaCl and 0.1% Tween 20, pH 7.5) followed by 1 h incubation with HRP-conjugated anti-rabbit secondary antibody (1:3000) and developed using the ECL system. Images for immunoreactive bands were acquired using the Image-Quant blot documentation instrument (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and analyzed using the Image J Software Package (developed at the National Institutes of Health, free download available at http://rsbweb.nih.gov/ij/). Relative protein level was normalized to β-ACTIN and results were expressed as relative optical density (FAAH/β-ACTIN, NAPE-PLD/β-ACTIN, CB1/β-ACTIN, CB2/β-ACTIN).

Determination of FAAH activity

FAAH activity was assayed as described by Paria *et al.* (1996) with minor modifications. Uteri from mice on day 15 of pregnancy treated with vehicle or LPS (3, 5 or 7 h after second injection) were homogenized (Ultra Turrax, T25 basic, IKA Labortechnik) in Tris-HCl 20 mM (pH = 7.6) buffer containing EDTA 1 mM and protein concentration determined by the Bradford assay (Bradford 1976). One hundred mg of protein were incubated at 37 °C for 15 min in 200 µl Tris/HCl 50 mM (pH = 8.5) buffer whit 100 µM [^{3}H]-AEA and 20 nmols AEA. The reaction was stopped adding a mix of chloroform:methanol (1:1 v/v). The aqueous phase was extracted twice with chloroform. Samples were dried and resuspended in chloroform and were plated on silica TLC plates Gel 60. A solvent mixture (ethyl acetate:hexane:acetic acid:distilled water, 100:50:20:100 v/v) was flowed through the plate. Lipids were visualized with iodine vapor and were identified by co-migration with AEA and arachidonic acid (AA) standards. Radioactivity was counted in a b-scintillation counter. The area of each radioactive peak corresponding to AA was calculated and expressed as a percentage of the total radioactivity of the plates. Enzyme activity is reported as nmol AA/h/mg protein.

Hematoxilin-eosin (H&E) histological staining and immunofluorescence

Uteri and ovaries from mice on day 15 of pregnancy treated with vehicle or LPS (7 h after second injection) and ovaries from mice on day 18 of pregnancy were fixed in 4% formaldehyde. Fixed tissues were dehydrated in an increasing gradient of alcohol and embedded in paraffin. Sections of 4 µm were obtained and mounted on silanized slides. Ovaries were stained with H&E to evaluate its morphology and the proportion of large and small cells of the total luteal cells population. Also, uterine tissue section slides were stained with H&E for quality control. Uterine tissues slides were de-waxed and rehydrated. Endogenous peroxidase enzyme activity were eliminated by the addition of 0.3% hydrogen peroxide (v/v) in methanol for 15 min. Tissues slides were incubated with 5% fetal bovine serum-PBS in a moist chamber at room temperature for 1 h 30 min. Tissues slides were incubated with primary antibody against CB1 (1:50) or CB2 (1:50) at 4 °C for 48 h. After PBS washes, tissues slides were incubated whit anti-FITC secondary antibody (1:200) at room temperature for 1 h 30 min. Mounting medium Glycerol:PBS (1:1 v/v) was placed on the slide and were observed on a Nikon Eclipse E200 (Melville, NY, USA) binocular microscope.

Progesterone electrochemiluminescence immunoassay

Animals were anesthetized in a CO_2 atmosphere on day 15 of pregnancy 3, 5 or 7 h after second vehicle or LPS injection and blood was collected by decapitation. Blood was allowed to clot and was centrifuged at 0.8 g for 10 min to separated serum. Progesterone serum levels were determined by electrochemiluminescence immunoassay (ECLIA). Progesterone serum levels are reported as ng of progesterone/ml serum.

Table 2 Dilution of antibodies and incubation conditions used for western blot analysis in uteri from BALB/c mice.

Antibody	Dilution	Incubation
FAAH	1/250	36 h a 4 °C
NAPE-PLD	1/1500	36 h a 4 °C
CB2	1/300	36 h a 4 °C
CB1	1/200 in 5% BSA	18 h a 4 °C
β-Actin	1/4000	15 min at room temperature

FAAH, *Fatty acid amide hydrolase;*NAPE-PLD, *N*-acyl phosphatidylethanolamine specific phospholipase D; CB1, Cannabinoid receptor type 1; CB2, Cannabinoid receptor type 2.

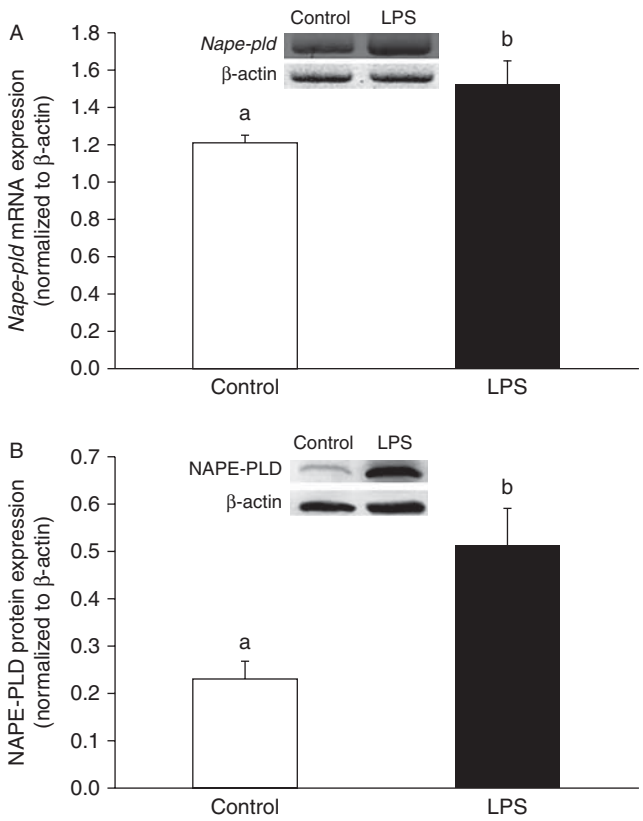

Figure 1 Effect of LPS on NAPE-PLD expression. *BALB*/c female mice in day 15 of pregnancy were injected with LPS or saline solution (control) and uterine strips were collected. *Nape-pld* mRNA (A) or protein (B) levels were measured at 5 or 7 h after the second LPS injection, respectively. Data are mean ± S.E.M. ($n=6$). Bars with different superscript letters denote significant differences. $P<0.05$ by Tukey's test, a ≠ b. Representative images of *Nape-pld* mRNA and protein levels are shown in panel A and B respectively.

PGF2a RIA

Animals were euthanized 7 h after the second vehicle or LPS injection. Uterine strips were incubated in Krebs – Ringer – Bicarbonate buffer (KRB: NaCl 118 mM, KCl 4.7 mM, KH_2PO_4 1.18 mM, $MgSO_4 \cdot 7H_2O$ 1.22 mM, $NaHCO_3$ 25 mM, glucose 11.1 mM) at 37 °C for 1 h in a 95% O_2/5% CO_2 atmosphere. In some cases, incubation was performed in the presence of cannabinoid receptors antagonist AM630 and AM251 (CB1 and CB2 antagonist respectively) at different concentrations (10^{-6} M; 10^{-7} M; 10^{-8} M and 10^{-9} M) or in the presence of AEA at different concentrations (10^{-6} M; 10^{-7} M and 10^{-9} M). For the following experiments, the lower concentration in which we observed changes was used. Protein concentration was determinate by Bradford assay (Bradford 1976). Medium was acidified to pH 3 with 1 M HCl. Two milliliters of ethyl acetate were added to KRB and organic phase was extracted three times. Pooled ethyl acetate extracts were dried. PGF2a concentration was determined by RIA (Campbell & Ojeda 1987). PGF2a antiserum was highly specific for PGF2a and showed low cross-reactivity with related compounds. Sensitivity was 5–10 pg per tube and $Ka = 1.5 \times 10^{10}$ l/mol. Values were expressed as pg of PGF2a/mg of protein.

Statistical analysis

Results were analyzed by one-way ANOVA (LPS or AEA) or by two-way ANOVA (LPS and CB receptor antagonists or AEA and CB receptor antagonists) in a completely randomized design. Comparisons were made with the Tukey's test. Results were considered significant at $P<0.05$. The assumptions of normality and homogeneity of variance were analytically assessed by the Shapiro–Wilks test and the Levene test respectively. All statistical analyses were performed using the statistical program Infostat (FCA, University of Córdoba, Argentina).

Results

Effect of LPS on AEA metabolism

We began analyzing the effect of LPS treatment on AEA biosynthesis in uterine tissue. We observed an increase in *Nape-pld* mRNA (Fig. 1A) and protein expression (Fig. 1B) in uteri from LPS-treated mice when compared to control mice. Next, we evaluated FAAH activity in uteri tissue from LPS-treated and control females. Figure 2 shows that uterine FAAH activity did not change significantly after LPS treatment.

Expression and localization of cannabinoid receptors in murine uterus during pregnancy. Effects of LPS administration

We observed that both CB1 and CB2 receptors were expressed in uterus (Fig. 3A and B respectively) and there was no change in their protein expression from day 13 to day 19 of pregnancy.

Even though we did not find changes in *Cb1* mRNA levels, we found an increase in the protein expression

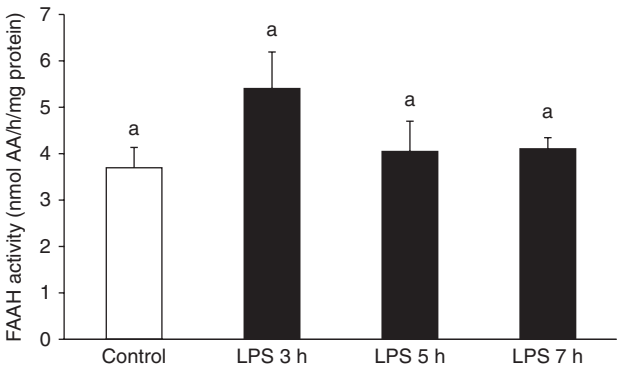

Figure 2 Effect of LPS on FAAH activity. *BALB*/c female mice in day 15 of pregnancy were injected with LPS or saline solution (control) and uterine strips were collected at 3, 5 or 7 h after the second LPS injection, and FAAH activity was measured. Data are mean ± S.E.M. ($n=8$). $P<0.05$ by Tukey's test.

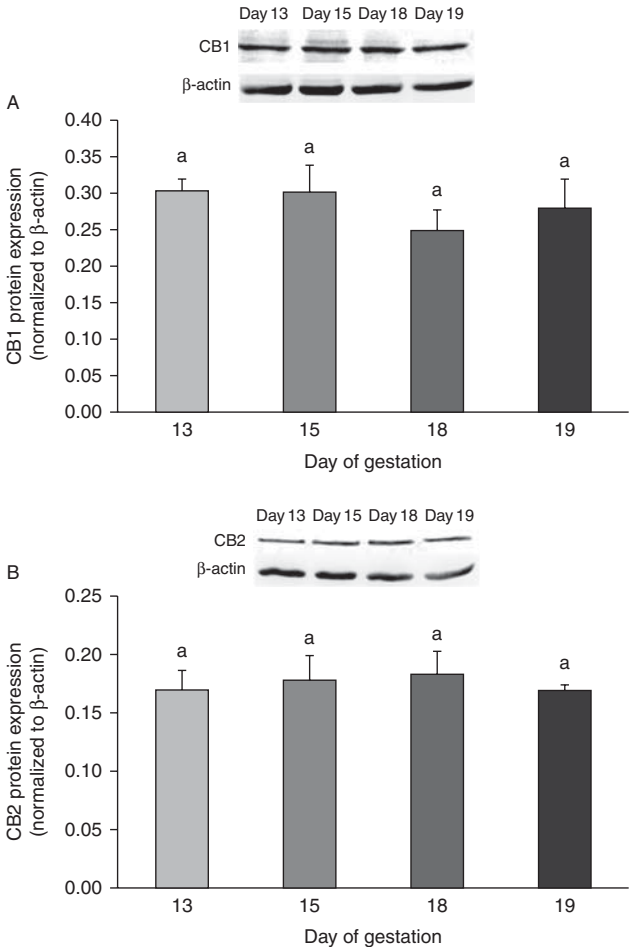

Figure 3 Presence of cannabinoids receptors in mice uterus during late pregnancy. Uterine strips from *BALB*/c female mice were collected on day 13, 15, 18 or 19 of pregnancy, and CB1 (A) and CB2 (B) protein levels were measured. Data are mean ± S.E.M. ($n=5$). $P<0.05$ by Tukey's test. Representative images of CB1 and CB2 protein levels are shown in panel A and B respectively.

of this receptor after LPS treatment (Fig. 4A and B). Interestingly, we observed a decrease in *Cb2* mRNA expression in uteri of pregnant mice after the last LPS injection (Fig. 4C), albeit no changes at the protein levels were observed (Fig. 4D).

Positive immunoreactivity tissue sections for CB1 and CB2 were detected in uteri from LPS-treated and control females. Specifically, staining was restricted to the apical membrane of endometrial epithelial cells, with no staining on the endometrial stromal cells, myometrium and serous. We did not find changes in the uterine localization of cannabinoid receptors between treatments (Fig. 5A and B).

Effect of LPS on maternal serum progesterone levels

To evaluate whether LPS treatment affects maternal progesterone serum levels, we measured this hormone on day 18 of pregnancy (at term) and day 15 of

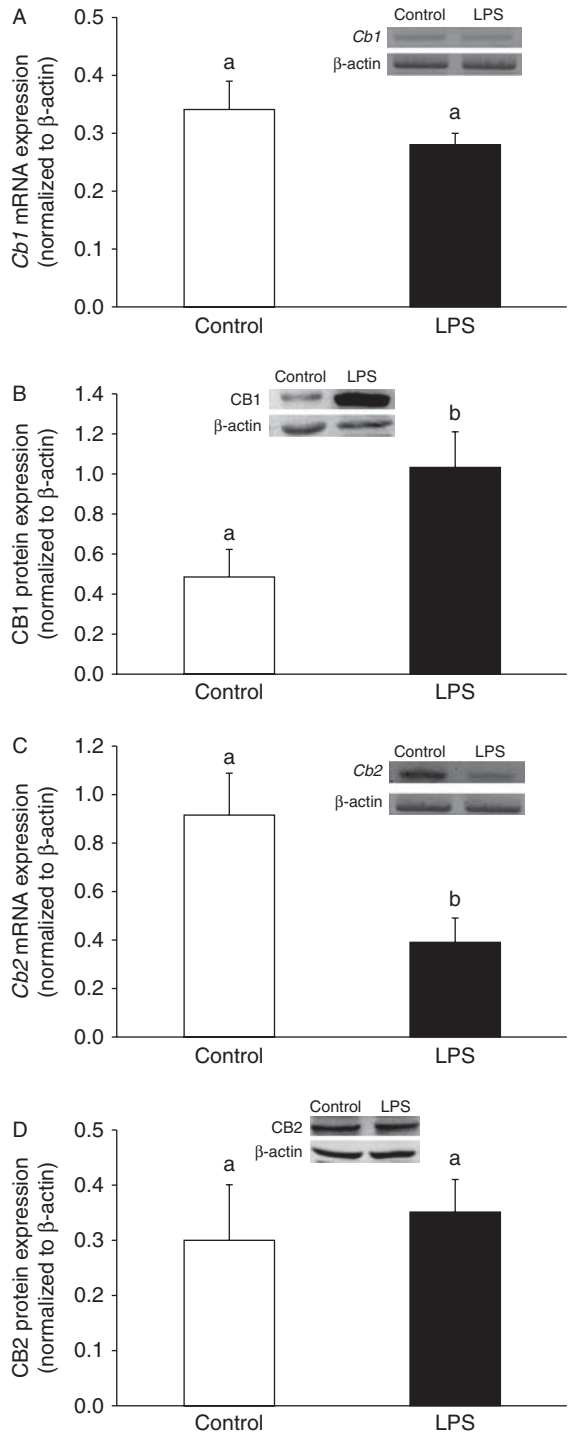

Figure 4 Effect of LPS on cannabinoids receptors expression. *BALB*/c female mice in day 15 of pregnancy were injected with LPS or saline solution (control) and uterine strips were collected at 5 h for mRNA or 7 h for protein determination after the second injection. (A) *Cb1* mRNA levels ($n=8$). (B) CB1 protein levels ($n=6$). (C) *Cb2* mRNA levels ($n=5$). (D) CB2 protein levels ($n=6$). Data are mean ± S.E.M. Bars with different superscript letters denote significant differences. $P<0.05$ by Tukey's test, a≠b. Representative images of *Cb1* mRNA, CB1 protein, *Cb2* mRNA and CB2 protein levels are shown in panel A, B, C and D respectively.

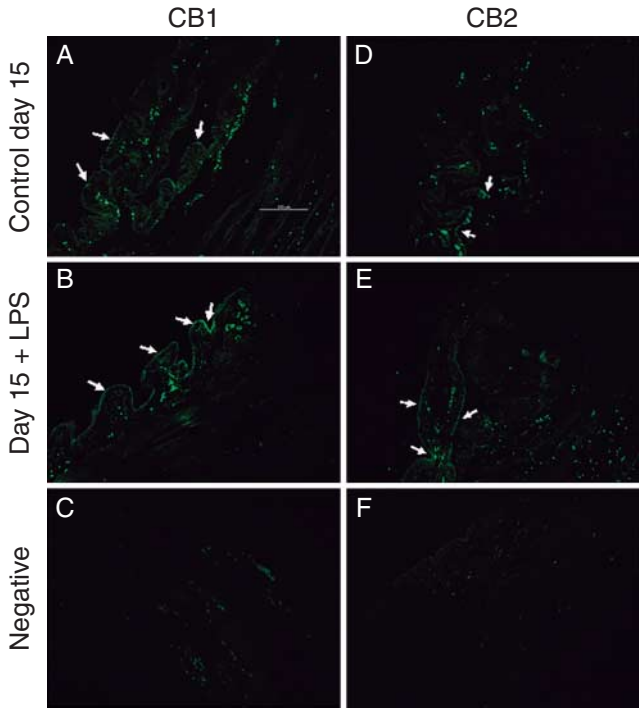

Figure 5 Effect of LPS on CB1 and CB2 uterine localization. Representative immunofluorescence for CB1 (A, B and C) and CB2 (D, E and F) in uterine strips from *BALB*/c female mice in day 15 of pregnancy injected with saline solution (control) (A and D) or LPS (B and E). These were collected at 7 h after the second injection. Arrows indicates endometrial epithelial cell positive for CB1 or CB2. In negative control the primary antibody is not added (C and F). Sections were visualized using fluorescence optical microscope (200× magnification).

pregnancy in LPS-treated and control mice. As expected, prior to delivery maternal progesterone serum levels decreased at day 18 of pregnancy. LPS treatment did not have any effect on progesterone serum levels on day 15 of pregnancy (Fig. 6).

Effect of LPS on ovary morphology and luteal cell types

Ovarian tissue sections were stained with H&E and histopathological analysis was performed. Figure 7 shows that ovaries from mice treated with LPS presented intact tissular and cellular structure (Fig. 7C and F). We observed that ovaries from mice on day 18 of pregnancy (Fig. 7A and D) displayed a smaller population of large cells compared with ovaries from mice on day 15 of pregnancy (Fig. 7B and E). However, we did not find any differences in luteal cell types between LPS-treated and control females on day 15 of pregnancy.

Relationship between uterine PGF2a levels and ECS

As expected, we observed that PGF2a levels increased in uteri from LPS-treated mice (Fig. 8). The presence of CB2 receptor antagonist did not block PGF2a augmentation, whereas CB1 antagonist (10^{-8} M) abrogated LPS induced PGF2a increment (Fig. 8). Next, we decided to analyze whether AEA modulates PGF2a levels in this organ. Therefore, we measured PGF2a in uteri from day 15 pregnant mice incubated with different concentrations of AEA. Figure 9A showed an increase in uterine PGF2a levels at all analyzed concentrations. To evaluate which cannabinoid receptor was involved, we used the lower concentration of AEA (10^{-9} M) that caused an effect on PGF2a levels. We co-incubated uteri from day 15 pregnant mice with AEA and AM251 or AM630 (CB1 and CB2 receptor antagonist respectively). PGF2a augmentation was reversed in the presence of CB1 receptor antagonist (10^{-8} M) (Fig. 9B), whereas incubation with AM630 had no effects (data not shown).

Discussion

Here we evaluated whether LPS affects the ECS in a model of endotoxin-induced preterm labor. Our results suggest that the ECS is involved in the mechanisms triggered by LPS that culminates with preterm labor.

Several studies have examined the expression and role of cannabinoid receptors in the reproductive system (Dennedy *et al.* 2004, El-Talatini *et al.* 2009, Fonseca *et al.* 2009). We are the first group to demonstrate the presence of uterine cannabinoid receptors in the late pregnancy in mice. We show their localization and expression in our LPS-induced preterm labor model.

The immunoreactivity of both receptors was restricted to the apical membrane of endometrial epithelial cells and no changes in CB1 or CB2 localization among treatments were observed. Schuel *et al.* (2002) reported the presence of AEA and others *N*-acylethanolamines in human reproductive fluids, including follicular fluid. Therefore, the localization of CB1 and CB2 on the apical membrane of endometrial epithelial cells suggests the

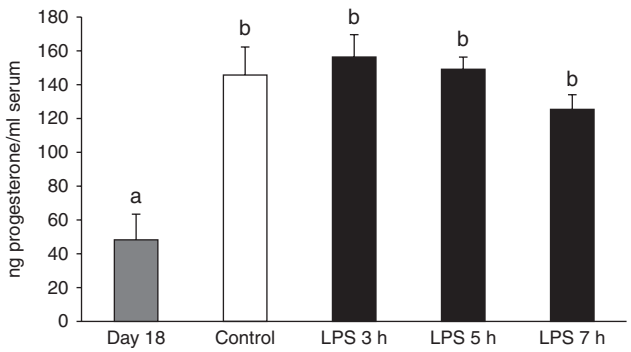

Figure 6 Effect of LPS on maternal serum progestrone levels. *BALB*/c female mice in day 15 of pregnancy were injected with LPS or saline solution (control) and blood were collected at 3, 5 or 7 h after the second injection to measure serum P levels by ECLIA. Sera from mice on day 18 of pregnancy were used as controls of term delivery. Data are mean±S.E.M. (n=8). Bars with different superscript letters denote significant differences. $P<0.05$ by Tukey's test, a≠b.

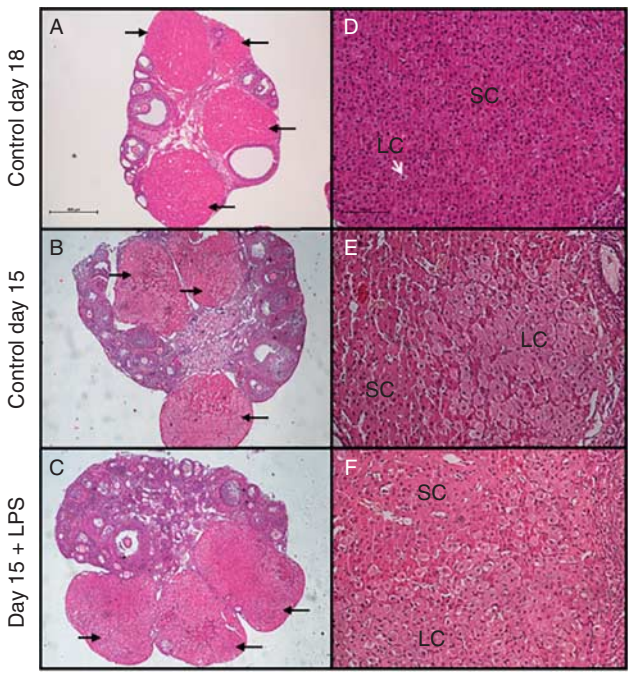

Figure 7 Effect of LPS on ovary morphology and luteal cell types. *BALB*/c female mice in day 15 of pregnancy were injected with LPS or saline solution (control) and ovaries were collected at 7 h after the second injection to perform H&E staining. Ovaries from mice on day 18 of pregnancy were used as controls of term delivery. Sections were visualized using optical microscope. Arrows indicates corpora lutea. SC = small cells. LC = large cells. A, B and C were evaluated at 40× magnification and D, E and F were evaluated at 200× magnification.

availability of these receptors to the AEA present in the uterine fluids. Interestingly, no staining was observed in endometrial stromal cells, myometrium and serous.

Several studies suggest the involvement of CB1 receptor in the regulation of labor. Wang *et al.* (2008) observed that genetic or pharmacological inactivation of CB1, but not CB2, induced preterm labor in C57BL/6J mice, thus suggesting that endocannabinoid signaling via CB1 is critical for a normal gestational length. In contrast, when we evaluated the cannabinoid receptors expression in our model of LPS-induced preterm labor, we observed an increased in CB1 receptor protein levels in uteri of pregnant mice after LPS injection. Therefore, the mechanism triggering preterm labor in this model seems to involve the activation of ECS through an increase on CB1 expression.

Recently, Sun *et al.* (2014) reported the participation of CB2 in regulating inflammation-driven preterm birth. The authors showed that CB2-, but not CB1-deficient mice are resistant to LPS-driven preterm birth. Here, we showed a downregulation of *Cb2* receptor at mRNA levels in uteri of pregnant mice upon LPS injection, albeit no changes in CB2 receptor expression at protein. It is well known that CB2 receptors are expressed in immune, hematopoietic and in almost all peripheral blood immune cells (Pacher & Mechoulam 2011), and its expression can be influenced by various inflammatory agents, e.g., LPS (Carlisle *et al.* 2002, Klein *et al.* 2003). Sun *et al.* (2014) observed that CB2-*KO* mice had higher serum levels of IL-10 and lower levels of pro-inflammatory cytokines in response to LPS challenge compared with WT controls.

The levels of AEA are regulated by a balance between the expression or activity of synthesizing (NAPE-PLD) and degrading (FAAH) enzymes. Furthermore, there is evidence that NAPE-PLD and/or FAAH expression and/or activity change in a spatiotemporal manner, determining AEA levels in different reproductive tissues such as oviduct, uterus and placenta (Guo *et al.* 2005, Maccarrone 2009, Fonseca *et al.* 2010). We are the first group to show uterine NAPE-PLD changes in the last part of pregnancy in mice. Here we demonstrated that, as we have previously shown for early pregnancy stages (Vercelli *et al.* 2009), LPS increases *Nape-pld* mRNA and protein expression in the uterus in the late stages of pregnancy. This finding correlates with Liu *et al.* (2003), who have demonstrated that LPS increases NAPE-PLD activity and AEA levels in RAW264.7 mouse macrophages. In addition, Shynlova *et al.* (2013) have observed that the numbers of neutrophils and macrophages were increased in myometrium in a mice model of preterm labor induced by endotoxin. Therefore, the changes we observed may be due not only to the changes produced in the murine uterus, but also to the modifications in the immune cells infiltrated in this tissue.

Several studies suggest an important role for FAAH in the maintenance and successfulness of a normal pregnancy (Wang *et al.* 2006). A lower enzymatic

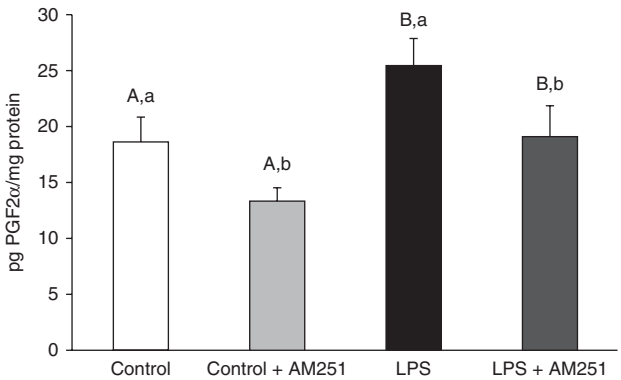

Figure 8 Relationship between uterine prostaglandin F2a levels and ECS. *BALB*/c female mice in day 15 of pregnancy were injected with LPS or saline solution (control) and uterine strips were collected at 7 h after the second injection to measure PGF2a by RIA. In some cases, tissue was incubated in the presence of AM251 (CB1 antagonist, 10^{-8} M), either in control (control + AM251) or in LPS-treated uteri (LPS + AM251). Data are mean ± S.E.M. ($n = 10$). Bars with different superscript capital letters denote significant differences between *in vivo* treatment (LPS and saline solution) and bars with different superscript lowercase letters denote significant differences between *in vitro* treatment (with or without AM251). $P < 0.05$ by Tukey's test, A ≠ B and a ≠ b.

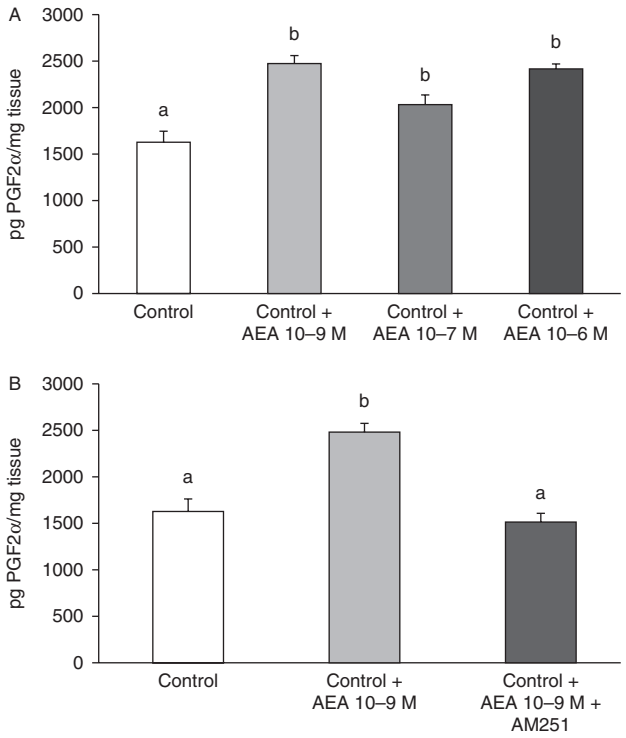

Figure 9 Effect of anandamide on uterine prostaglandin F2a levels. *BALB*/c female mice in day 15 of pregnancy were injected with saline solution (control) and uterine strips were collected at 7 h after the second injection to measure PGF2a by RIA. Tissue was incubated in the presence of AEA at different concentrations (A) or in presence of AEA (10^{-9} M) (control + AEA) or AEA plus AM251 (CB1 antagonist, 10^{-8} M) (control + AEA + AM251) (B). Data are mean ± S.E.M. ($n = 10$) (A) ($n = 10$) (B). Bars with different superscript letters denote significant differences. $P < 0.05$ by Tukey's test, a ≠ b.

activity of FAAH has been described in peripheral lymphocytes from women who subsequently spontaneously miscarried (Maccarrone *et al*. 2000). Accordingly, placental tissues from spontaneously miscarrying women are characterized by very low FAAH levels (Trabucco *et al*. 2009). Conversely, there is a lack of data regarding the participation of FAAH on the pathophysiology of preterm labor. Several studies have shown that LPS affects FAAH activity and/or expression. Indeed, incubation of uterine explants from mice in day 7 of pregnancy with LPS increases FAAH activity (Vercelli *et al*. 2009), whereas in human peripheral lymphocytes, LPS downregulates FAAH expression and increases AEA levels (Maccarrone *et al*. 2001). We were unable to detect any changes in FAAH activity in our model of LPS-induced preterm labor. The discordance between our results and previous reports may suggest that both *in vitro* and *in vivo* regulation of FAAH are context-dependent and tissue-specific. Wolfson *et al*. (2013) observed that *in vivo* administration of LPS to non-pregnant mice decreased FAAH activity in peripheral blood mononuclear cells (PBMC), whereas in pregnant mice the endotoxin was unable to produce changes in PBMC's FAAH activity. The fact that we found increased expression of NAPE-LPD with no changes in FAAH activity points towards a possible rise of uterine AEA levels in this model. In this sense, Habayeb *et al*. (2004) have shown an increase in AEA plasma levels in women during labor, suggesting a participation of AEA during parturition.

The corpus luteum (CL) is a transient endocrine gland that produces progesterone to maintain pregnancy in mice. Luteolysis includes loss of function and the involution of the morphology of CL. We observed that CL from mice at term (day 18) consisted mainly of small luteal cells with a lesser population of large cells, the main producers of P, when compared to ovaries from mice on day 15 of pregnancy. Although it is well known that endotoxin induces luteolysis (Mishra & Dhali 2007), we did not find effects of LPS on tissue and cellular structures or in the luteal cell types. The regression of CL, directly associated with the onset of parturition in mice, occurs first as functional regression, associated with a marked decrease in progesterone synthesis.

We did not detect a decrease in maternal progesterone serum levels in mice treated with LPS. There are potential mechanisms involved in the reduction of PR function in association with the onset of labor, as it occurs in humans that could be involved in our model of LPS-induced preterm labor (Mendelson 2009).

The ECS is also known to interact with gonadal steroid hormones. Lazzarin *et al*. (2004) have shown that changes in progesterone levels and FAAH expression are well correlated during the menstrual cycle. The CB2-*KO* mice are resistant to LPS-driven preterm birth and the endotoxin was unable to decrease maternal progesterone serum levels compared with WT mice (Sun *et al*. 2014), suggesting that ovaries deficient in CB2 are protected from adverse effects of inflammatory insults. In addition, in a model of LPS-induced embryonic resorption, the changes in FAAH activity correlated with changes in progesterone serum levels (Wolfson *et al*. 2013). In our model, we found no changes in maternal progesterone serum levels or in FAAH activity.

PG are crucial mediators in triggering preterm birth. Previous reports, although conflictingly, have determined the relationship between the ECS and PG (Chang *et al*. 2001, Mitchell *et al*. 2008, Vercelli *et al*. 2010). Indeed, it seems that this relationship varies greatly depending on the tissue as well as the studied species. In our model, LPS increased uterine PG and cyclooxygenase levels (Cella *et al*. 2010, Domínguez Rubio *et al*. 2014). Here, we show that the *in vitro* blockade of CB1 receptor decreased the uterine PGF2a levels in control and LPS-treated mice. Similarly to LPS, we observed that *in vitro* incubation with AEA produced an increase in uterine PGF2a levels. The results presented here suggest that LPS may not only increase CB1 expression but also AEA levels, which in turn, and

by targeting CB1 receptors, increases PGF2a levels, as part of the mechanism that triggers LPS-induced preterm labor. It is important to note that AEA is metabolized by FAAH to ethanolamine and AA, a precursor of PG. However, PGF2a augmentation was blocked by the CB1 receptor antagonist AM251, suggesting that this effect occurs by AEA binding to CB1 and not by the metabolism of AEA into PG precursors. In keeping with this, Mitchell *et al*. (2008) showed that endocannabinoids and the synthetic cannabinoid CP55, 940 stimulated the fetal membrane production of PGE2 in a CB1 receptor-dependent manner. Taken together, our previous results as well as the ones presented here indicate that the ECS is able to modulate PG production.

Although there is a continuous effort to find different and inventive ways to address this problem, there are limited options to prevent preterm birth to date. Collectively, our data suggest the possible involvement of the ECS in LPS-induced preterm labor and that targeting this system could provide new therapeutic strategies for preventing preterm labor.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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