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ORIGINAL ARTICLE

A role for the endocannabinoid system in premature luteal regression and progesterone withdrawal in lipopolysaccharide-induced early pregnancy loss model

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STUDY QUESTION: What is the role of the endocannabinoid system (eCS) in the alterations of the endocrine system in a murine model of lipopolysaccharide (LPS)-induced miscarriage?

SUMMARY ANSWER: In 7-days pregnant wild type, but not cannabinoid receptor type 1 *knockout* (CB1-*KO*) mice, LPS increased COX-2 expression and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production in the uterus leading to lower expression of prolactin receptor in the ovary and a marked regression of corpora lutea (CL), suggesting that the eCS mediates the deleterious effects of LPS on reproductive events.

WHAT IS KNOWN ALREADY: Appropriate systemic progesterone levels are critical for a successful pregnancy outcome. Precocious loss of luteal progesterone (P4) secretion leads to miscarriage in rodents. We have previously shown that LPS administration to pregnant mice induces embryonic resorption accompanied by a dramatic decrease in systemic progesterone levels in a murine model of inflammatory miscarriage, with the eCS mediating these LPS-induced deleterious effects.

STUDY DESIGN SAMPLES/MATERIALS, METHODS: CD1 *wild-type* (WT) and CB1-*KO* mice were randomly allocated to Vehicle (saline; i.p.) or LPS (0.5 µg/g body weight; i.p.) treated groups: (WT-Vehicle; WT-LPS; CB1-*KO*-Vehicle and CB1-*KO*-LPS). A single injection was given on day 7 of pregnancy and tissues (blood, ovary, uterus) were collected 6, 12, 24 and 48 h later. P4 and PGF2α plasma levels were determined by radioimmunoassay. Cyclooxygenase-2 (COX-2) mRNA (RT-PCR) and protein (Western blot) content in uterus was assayed. COX-2 and prolactin receptor (PrlR) mRNA levels in the ovary were assayed by RT-PCR. Tissue morphology of the CL was assessed by haematoxylin–eosin staining.

MAIN RESULTS AND THE ROLE OF CHANCE: Treatment of 7-day pregnant WT mice with LPS induced a P4 withdrawal ($p < 0.05$), increased in uterine COX-2 mRNA and protein expression ($p < 0.05$) as well as an increase in uterine $PGF_{2\alpha}$ production ($p < 0.05$). These changes were absent in LPS-treated 7-day pregnant CB1-*KO* mice. In ovarian tissues, LPS treatment to 7-day pregnant WT mice induced a downregulation of PrlR mRNA expression ($p < 0.05$) together with an increase in COX-2 mRNA expression ($p < 0.05$) and $PGF_{2\alpha}$ content ($p < 0.05$). These effects were absent in the CB1-*KO* mice. Collectively, our results suggest a role for the eCS mediating LPS-induced deleterious effects on reproductive tissues.

LIMITATIONS, REASONS FOR CAUTION: An important caveat of this study is the endocrine differences between mice and humans during pregnancy (e.g. P4 is produced by the CL throughout pregnancy in mice, whereas this is not the case in humans), which limits the extrapolation of the results presented here.

WIDER IMPLICATIONS OF THE FINDINGS: Our findings provide new insights in the role of the endocannabinoid system in the physiopathology of reproduction as well as the role of this endogenous system as a mediator of LPS deleterious effects on reproductive tissues.

LARGE SCALE DATA: None.

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Key words: luteolysis / endocannabinoid system / COX-2 / PGF2α / prolactin receptor / progesterone

Introduction

Following ovulation, the corpus luteum (CL) is formed and luteal phase of the menstrual cycle ensues (Fatemi *et al.*, 2007). The CL is a transient endocrine gland that plays a key role in regulating the estrous cycle and the establishment and maintenance of pregnancy. These functions are carried out mainly by progesterone (P4), which is the main steroid produced by the CL. P4 participates in several events during pregnancy such as endometrial receptivity, improvement of the feto-placental blood flow and oxygen supply, myometrium quiescence and recruitment and differentiation of decidual natural killer (NK) cells (Szekeres-Bartho, 2009). P4 also acts as an immunosteroid by controlling the protective immune milieu during normal pregnancy (Szekeres-Bartho *et al.*, 2008). Endocrine failure, including a drop in P4 levels in the first trimester, is associated with pregnancy loss. Furthermore, P4 administration is critical to support the luteal phase following *in-vitro* fertilization (Nardo and Sallam, 2006), and it is widely used in reproductive medicine and in the management of infertile patients (Ciampaglia and Cognigni, 2015). We have previously shown that P4 had anti-inflammatory effects and protected embryos from lipopolysaccharide (LPS)-induced injury. Moreover, decreased P4 serum levels were associated with embryo loss (Aisemberg *et al.*, 2013; Wolfson *et al.*, 2015), and supplementation with this hormone partially reversed LPS-induced embryo resorption (ER) (Aisemberg *et al.*, 2013).

Infections are known to be one of the principal causes of early pregnancy loss (Giakoumelou *et al.*, 2015). Genitourinary tract infections are very common in women and are one of the most important risk factors for both maternal and fetal health during pregnancy (Cram *et al.*, 2002). Gram negative bacteria, which produce LPS, are one of the most common groups involved. We have previously showed that systemic administration of LPS during early pregnancy in mice results in 100% of ER and fetal loss (Ogando *et al.*, 2003). This process is characterized by an increase of immune cell infiltration in decidua, increased uterine and decidual nitric oxide (NO) and prostaglandin synthesis, decreased P4 plasma levels and an altered serum pattern of endocannabinoid (Ogando *et al.*, 2003; Aisemberg *et al.*, 2013; Wolfson *et al.*, 2015).

Endocannabinoids are unsaturated fatty acid derivatives which act as endogenous ligands for cannabinoid receptors (CB1 and CB2). Anandamide (AEA) is synthesized by N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) and binds both cannabinoid receptors (Mechoulam *et al.*, 1998; Di Marzo *et al.*, 1999). After being produced from membrane precursors, AEA is transported into the cell and subsequently broken down into arachidonic acid (AA) and ethanolamine by an endoplasmic reticular membrane-bound enzyme named fatty-acid amide hydrolase (FAAH) (Devane *et al.*, 1992).

Besides hydrolysis by FAAH, AEA may undergo direct oxygenation by cyclooxygenase-2 (COX-2), resulting in the formation of prostaglandin-ethanolamides, also termed prostamides (Kozak and Marnett, 2002). COX-2 is the inducible form of cyclooxygenase and leads to increased production of prostaglandins and thromboxanes. Moreover, COX-2 is particularly involved in inflammatory events and mediates these processes.

Altogether, receptors, endocannabinoids and enzymes compose the endocannabinoid system (eCS), which is present in many reproductive tissues and is involved in physiological and pathological processes during pregnancy (Sun and Dey, 2012; Battista *et al.*, 2015). For instance, a fine adjustment of uterine endocannabinoid levels is necessary for proper implantation in rodents, with low levels of AEA required at implantation sites and high levels at intersites (Guo *et al.*, 2005). Elevated levels of AEA are associated with inhibition of trophoblast proliferation whereas low levels of this molecule are associated with the opposite effect (Paria and Dey, 2000). Thusly, Habayeb *et al.* (2008) observed that women whose pregnancies ended in spontaneous abortion had higher plasma levels of AEA than those whose pregnancies came to term. In our laboratory, we have previously shown that the eCS has a crucial role in the maintenance of pregnancy, since CB1-*knockout* mice (CB1-*KO*) are resistant to LPS-induced early ER (Wolfson *et al.*, 2015).

The relationship between the eCS and P4 is rather complex and not fully understood (Maccarrone *et al.*, 2000; Taylor *et al.*, 2011; Cecconi *et al.*, 2014; Karasu *et al.*, 2014). We have previously demonstrated that the absence of the CB1 receptor prevented the drop in serum P4 levels after systemic administration of LPS in a murine model of early pregnancy loss (Wolfson *et al.*, 2015). Given that (a) CB1-*KO* mice are resistant to LPS-induced early ER, (b) that this resistance is associated with a lower decrease in P4 serum levels, and (c) that the corpus luteum is the principal producer of P4 during the entire pregnancy in mice; we aimed to study whether the eCS promotes premature luteal regression (via increased prostaglandin F2α production, the major luteolytic factor), which could be involved in LPS-induced withdrawal of serum P4.

Materials and methods

Reagents

LPS from *Escherichia coli* (serotype 05:B55), anti-β-actin antibody and secondary horse radish peroxidase (HRP) conjugated antibody were purchased from Sigma Chemical Co. (St Louis, MI, USA). Radioactive material ([1,2,6,7-^{3}H]-progesterone (115 Ci/mmol, 1 mCi/ml) and [5,6,8,9,11,12,14,15(n)–^{3}H]-prostaglandin $F_{2\alpha}$ (160 Ci/mmol, 200 μCi/ml) were provided by Perkin Elmer (Boston, MA, USA). Western blotting reagents were obtained from Bio-Rad (Hercules, CA, USA) and Sigma Chemical Co. (St Louis, MI, USA). The anti-COX-2 antibody was purchased from Abcam (Cambridge, UK).

The Quick-ZOL reagent for total RNA extraction from tissues was obtained from Kalium Technologies (Bernal, Argentina). Reagents used for mRNA retro-transcription (RT) (ultrapure H_2O, RNAse-free DNAse I, DNAse buffer, random primers, Tris5X Buffer, DTT) were purchased from Invitrogen (Buenos Aires, Argentina), while the RNAse inhibitor and dNTPs were supplied by Genbiotech (Buenos Aires, Argentina). Reverse transcriptase M-MLV, green GoTaq reaction buffer 5X and DNA polymerase used for polymerase chain reaction (PCR) were from Promega (Madison, WI, USA). All other chemicals were analytical grade.

Animals and treatments

Eight to 12-week-old virgin female CD1 (*wildtype,* WT, or CB1-*knockout,* CB1-*KO*) mice were paired with fertile CD1 males of the same genotype: WT × WT and CB1-*KO* × CB1-*KO*. CD1 CB1-*KO* mice were generated as previously described (Ledent *et al*., 1999).

Copulation was verified by the presence of vaginal mucus plug and it was considered as day 0 of pregnancy. Animals received food and water *ad libitum* and were housed under controlled conditions of light (12 h light/12 h dark) and temperature (23–25°C). A single dose of LPS (0.5 μg/g body weight) or vehicle (sterile saline solution) was administered i.p. on day 7 of gestation. This dose of LPS produces a different embryo resorption rate depending on the genotype: 69 ± 22.0% for WT and 3 ± 1.4% for CB1-*KO* (Wolfson *et al*., 2015). Animals were anesthetized under CO_2 and bled by decapitation at 6, 12, 24 and 48 h after each treatment. All efforts were made to minimize suffering (Supplementary Fig. S1).

The experimental procedures reported here were approved by the Animal Care Committee of the Center for Pharmacological and Botanical Studies of the National Research Council (CEFyBO–CONICET) (Resolution Number 1162/2016) and by the Institutional Committee for the Care and Use of Laboratory Animals from the School of Medicine (University of Buenos Aires), and were carried out in accordance with the Guide for Care and Use of Laboratory Animals (NIH).

Radioimmunoassay for progesterone and prostaglandin $F_{2\alpha}$

Blood from control and LPS-treated CD1 WT and CB1-*KO* mice was obtained from euthanized animals at 6 and 24 h after treatment. Blood was allowed to clot, centrifuged at 800*g* for 10 min and the serum fraction was stored at −70°C until used. Progesterone was measured by radioimmunoassay as previously described (Aisemberg *et al*., 2013). Values are expressed as ng/ml of progesterone ($n = 5$ animals per group).

After euthanization, uteri from implantation sites from control and LPS-treated WT and CB1-*KO* mice were isolated and stored at −70°C until used. To measure uterine prostaglandin $F_{2\alpha}$ content, uterine strips were weighed, minced and incubated in Krebs – Ringer – Bicarbonate buffer (KRB: 118 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.22 mM $MgSO_4{\cdot}7H2O$, 25 mM $NaHCO_3$, 11.1 mM glucose) at 37°C for 90 min in a HEPA-filtered air incubator with 5% CO_2 /95% air. The tissue was discarded and the KRB was acidified to pH 3 with 1 N HCl and then 2 mL of ethyl acetate was added. The organic phase was collected and the extraction was repeated two more times and the organic solvent containing PGs was evaporated in a vacuum stove. $PGF_{2\alpha}$ concentration was determined by RIA (Cambell and Ojeda, 1987). $PGF_{2\alpha}$ antiserum was highly specific for $PGF_{2\alpha}$ and showed low cross reactivity with related compounds. Sensitivity was 5–10 pg per tube and Ka = 1.5×10^{10} L/mol. Values are expressed as pg $PGF_{2\alpha}$/mg wet weight ($n = 7$ animals per group).

RT-PCR

Ovaries and uterine tissue from implantation sites were collected from WT and CB1-*KO* mice after euthanization. One milliliter of Quick-ZOL reagent was added to samples which were kept at −70°C until used. Total RNA was isolated according to manufacturer's recommendations. RNA concentration was determined using a micro-volume spectrophotometer (Eppendorf; Hamburg, Germany). The cDNA was generated from 2 μg of RNA pretreated with DNAse. Reverse transcription was performed by incubating the samples with M-MLV enzyme, random primers and triphosphate deoxyribonucleotides in the presence of a recombinant ribonuclease inhibitor for 10 min at 25°C, 50 min at 37°C and 15 min at 70°C. cDNA amplification was performed using specific primers designed with Primer-Blast program (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Primers sequences and PCR conditions are shown in Table I. PCR products were loaded onto 2% agarose gel, stained with ethidium bromide, recorded with a digital camera (Olympus C-5060; Tokyo, Japan) and analyzed using the Image J software package. Data were expressed as the relative amount of each PCR product versus β-actin mRNA ($n = 8$ animals per group).

Western blot analysis

Uteri obtained from both, WT and CB1-*KO* treated (12 h post-LPS treatment) and control (12 h post-vehicle administration) animals were homogenized in lysis buffer (0.02% sodium azide, 0.1% SDS, deoxycholate 0.5%, Nonidet P40 1%, inhibitors cocktail, and PBS), sonicated and centrifuged at 13 000*g* for 10 min.

Supernatants were isolated and total protein was quantified by Bradford method (Bradford, 1976). Next, samples were processed for subsequent electrophoretic separation. Eighty micrograms of protein were loaded in each lane. Samples were separated by electrophoresis on 6–12% gradient

Table I **RT-PCR primers.**

cDNA	Sense pimer	Antisense primer	Product size	Accession number
COX 2	5′-TCCTCCTGGAACATGGACTC–3	5′-CCCCAAAGATAGCATCTGGA–3′	320 bp	NM-011198.4
PrlR	5′-GGATGTGACTTACATTGTTGAACCA–3′	5′-TACCCACAGATATGTTTTTTTGTCTTTT–3′	91 bp	NM-011169.5
β-actin	5′-TGTTACCAACTGGGACGACA-3′	5′-TCTCAGCTGTGGTGGTGAAG-3′	392 bp	NM-007393.5

SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were incubated overnight with anti-COX-2 (1:300) or anti-actin (1:4000) antibodies. Next, blots were washed with T-PBS (PBS and 0.1% (v/v) Tween 20, pH 7.5) followed by 1 h incubation with horse radish peroxidase-conjugated anti-rabbit secondary antibody (1:5000) and developed using the enhanced chemiluminescence western blot system. Images of immunoreactive bands were acquired using the ImageQuant system (GE Healthcare Life Sciences; Buenos Aires, Argentina) and analyzed using the Image J software package. Relative protein levels were normalized to β-actin and results were expressed as relative optical density (COX-2/β-actin) ($n = 8$ animals per group).

Histology

Ovaries from treated and control (12, 24 and 48 h post-LPS or vehicle), WT and CB1-*KO* pregnant females, were obtained and fixed in formaldehyde 3.7% (w/v) in PBS. The ovaries were dehydrated in an increasing gradient of alcohol and embedded in paraffin. Non-consecutive sections of 4 µm per ovary were obtained with a microtome and mounted on silane-coated slides. The sections were stained with haematoxylin–eosin and observed by light microscopy (Nikon Eclipse 200; NY, USA) using 40× magnification to evaluate corpora lutea number in the whole ovary and 100× magnification to observe different morphologies in corpora lutea. Regressing corpora lutea (RCL), in contrast with intact corpora lutea (CL), showed cells with heterogeneous shapes and sizes with a poorly stained perinuclear cytoplasmic area (Taketa *et al.*, 2011). They also presented an increase in the extracellular matrix (Taketa *et al.*, 2011). The numbers of CL and RCL per ovary were calculated by averaging what was observed in three sections. The percentage of RCL was calculated over the total number of corpora lutea counted. Six animals were analyzed for each experimental group.

Statistical analysis

Results were analyzed by one or two-way ANOVA in a completely randomized design. Comparisons were made by Tukey's test. Normality and homoscedasticity were tested by Shapiro–Wilk (modified) and Levene tests, respectively. In the case of data that did not meet the assumptions, normal scores transformation was applied and continued in the same way as with real data. Data were expressed as mean ± SEM. Differences were considered significant when p was less than 0.05. All statistical analyses were performed using the statistical program Infostat (University of Córdoba, Argentina).

Results

Serum progesterone (P4) levels are diminished 6 h post-LPS treatment, and remain low at 24 h, in WT but not in CB1-*KO* mice

LPS treatment significantly decreased P4 levels in WT mice at 6 h and they remained low at 24 h post-treatment (Fig. 1). Conversely, we did not observe a statistically significant drop in P4 serum levels 6 h nor at 24 h post-LPS treatment in CB1-*KO* mice when compared to control mice (Fig. 1). Interestingly, we observed that in control mice, there was a significant decrease on P4 levels between days 7 and 8 of gestation. This drop on P4 serum levels was, however, absent in CB1-*KO* mice.

LPS treatment increases uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) levels in WT but not in CB1-*KO* mice

Uterine $PGF_{2\alpha}$ levels 12 h post-LPS (0.5 µg/g) were significantly increased in samples from WT mice when compared to WT controls (Fig. 2). In contrast, this effect was not observed in LPS-treated CB1-*KO* mice when compared to control CB1-*KO* mice.

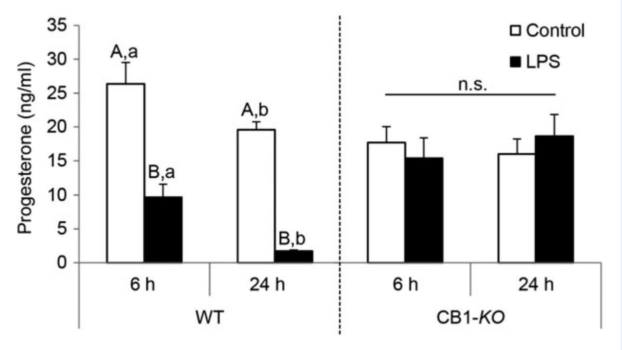

Figure 1 Effect of lipopolysaccharide (LPS) treatment on serum progesterone (P4) levels in wild-type (WT) and CB1-*knockout* (CB1-*KO*) mice. CD1 pregnant mice on day 7 of gestation were injected with LPS (0.5 µg/g) or saline solution (control) and blood was collected 6 and 24 h after treatment. P4 levels were measured by radioimmunoassay. Data are represented as mean ± SEM and analyzed by ANOVA test with $n = 5$ mice per group. Different capital letters denote significant differences between control and LPS treatments. Lower case letters denote significant differences between day 7 and day 8 of pregnancy ($p < 0.05$).

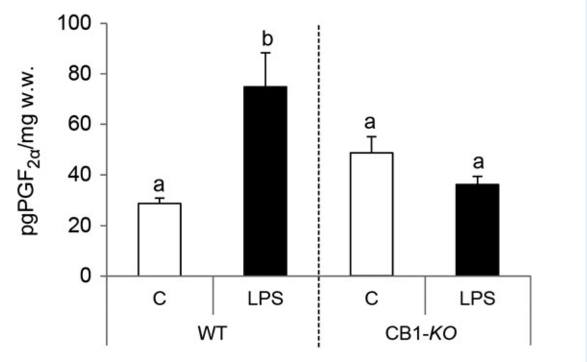

Figure 2 Effect of lipopolysaccharide (LPS) treatment on wild type (WT) and CB1-*KO* mice on uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production. Pregnant mice on day 7 of gestation were euthanized 12 h after LPS (0.5 µg/g) injection and uteri were isolated for $PGF_{2\alpha}$ content determination by radioimmunoassay. C = control. Data are expressed as pg/mg of wet weight (w.w.) and shown as mean ± SEM ANOVA test was used for the statistical analysis with $n = 7$ mice per group. Different letters indicate statistically significant differences ($p < 0.05$).

Cyclooxygenase 2 (COX-2) mRNA and protein levels are increased in uteri from LPS-treated WT mice

We did not observe changes in COX-1 protein levels in uteri of WT nor CB1-*KO* mice after LPS administration (data not shown). However, a significant increase in COX-2 mRNA expression was observed on uteri from WT mice whilst this change was not observed in the uteri of CB1-*KO* mice (Fig. 3A). Nevertheless, uterine COX-2 protein levels were significantly increased in both, LPS-treated WT and CB1-*KO* mice with respect to control WT and CB1-*KO* mice. However, the LPS-induced increase in COX-2 expression was lower in CB1-*KO* mice when compared to WT mice (Fig. 3B).

Cyclooxygenase 2 (COX-2) mRNA levels are increased in ovaries from LPS-treated WT, and CB1-*KO* mice. PGF$_{2\alpha}$ ovarian content is increased in LPS-treated WT but not in *KO* mice

LPS treatment increased COX-2 mRNA levels in the ovaries of both WT and CB1-*KO* mice, when compared to their respective controls. However, this increment was 2.5 times higher in ovaries from WT mice than in CB1-*KO* ones (Fig. 4A). Next, we measured the ovarian content of PGF$_{2\alpha}$ in WT and CB1-*KO* mice 12 h after the treatment with LPS or vehicle. As shown in Fig. 4B, LPS administration induced an increase in the local levels of PGF$_{2\alpha}$ in the ovaries of WT mice when compared to vehicle. Interestingly, this effect was absent in CB1-*KO* mice.

LPS treatment diminishes prolactin receptor (PrlR) mRNA levels in WT, but not in CB1-*KO* mice ovary

PrlR mRNA expression was significantly diminished in the ovaries from LPS-treated WT mice when compared to control WT mice, whereas this effect was not observed in ovaries from LPS-treated CB1-*KO* mice compared to control CB1-*KO* mice (Fig. 5).

LPS treatment produces an increased percentage of regressing corpora lutea in ovaries from WT mice compared to CB1-*KO* mice

No differences were observed in number or morphology of the corpora lutea between ovaries from control and LPS-treated WT and/or CB1-*KO* mice at 12 or 24 h (data not shown). However, 48 h post-LPS treatment, we observed regressing corpora lutea (RCL) amongst the intact corpora lutea (CL) in ovaries from both WT and CB1-*KO* mice (Fig. 6). We proceeded to determine the percentage of RCL and CL respect to the total number of corpora lutea and we observed that the percentage of RCL was higher in the ovaries from LPS-treated WT mice than in the ovaries of LPS-treated CB1-*KO* mice (Table II). We did not observe RCL in the ovaries from control WT or control CB1-*KO* mice.

Discussion

P4 is critical at various stages of pregnancy and low levels of this hormone are associated with an increased risk of miscarriage (Fidel *et al.*, 1998; Ku *et al.*, 2015). We have previously shown that LPS induced a

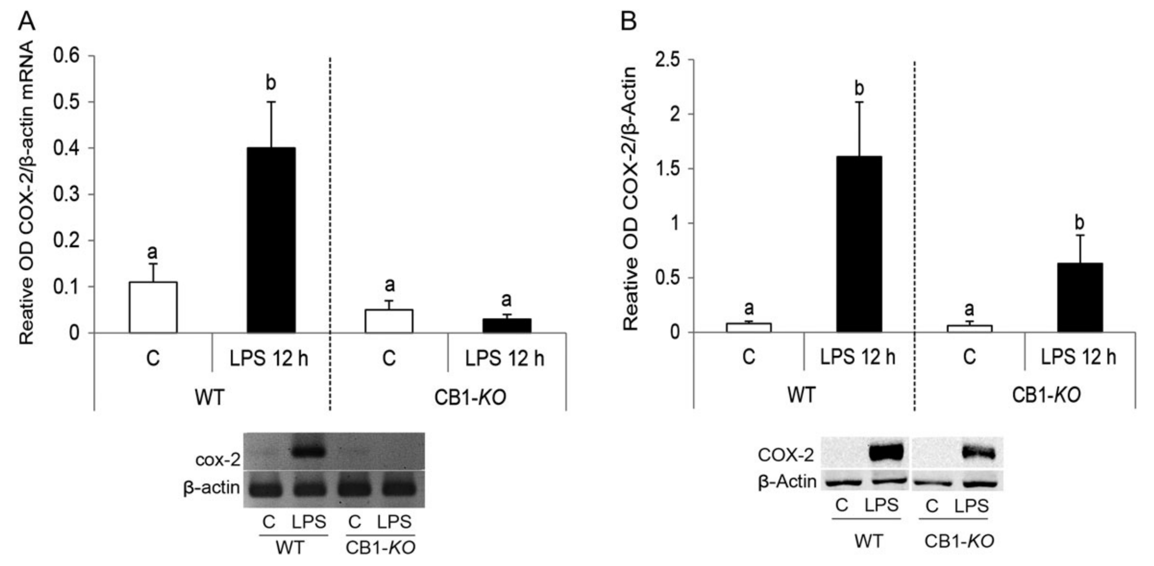

Figure 3 Effect of lipopolysaccharide (LPS) treatment on uterine cyclooxygenase-2 (COX-2) expression. Uteri from wild type (WT) and CB1-*KO* pregnant mice on day 7 of gestation were isolated 12 h after LPS (0.5 µg/g) or saline solution (C = control) injection. (**A**) COX-2 mRNA and (**B**) protein levels were measured by RT-PCR and western blot analysis respectively. All data were normalized to β-actin levels and represented as mean ± SEM. ANOVA test was used for the statistical analysis with $n = 8$ mice per group. Different letters indicate statistically significant differences ($p < 0.05$).

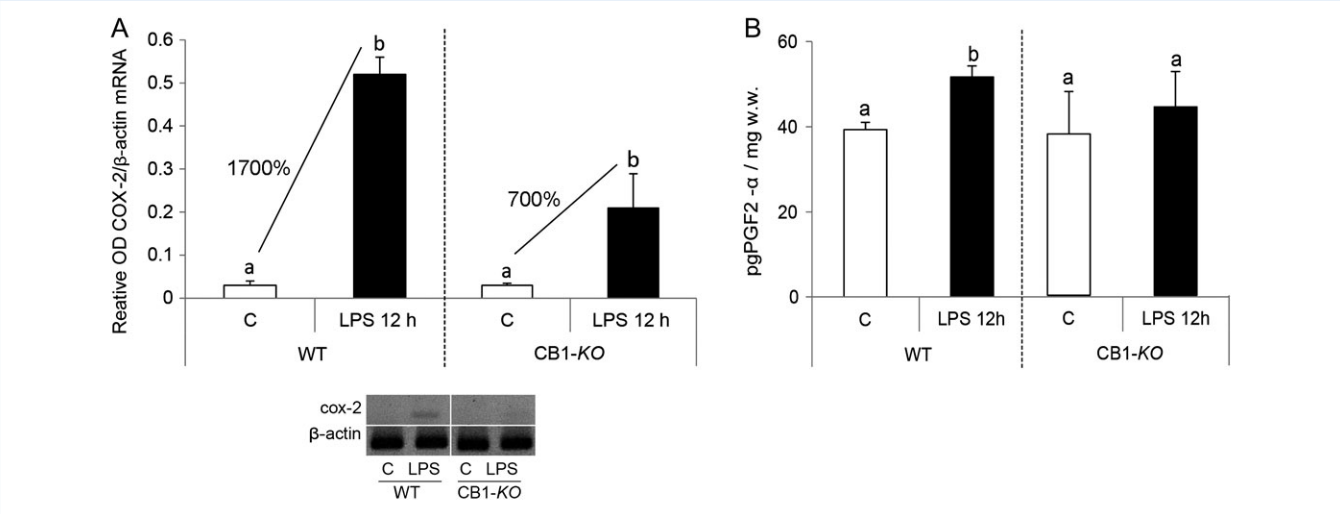

Figure 4 Effect of lipopolysaccharide (LPS) treatment on ovarian cyclooxigenase-2 (COX-2) mRNA levels and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production. Ovaries from wild type (WT) and CB1-*KO* pregnant mice on day 7 of gestation were isolated 12 h after LPS (0.5 µg/g) or saline solution (control) injection. (**A**) COX-2 mRNA levels were assessed by RT-PCR and normalized to the respective β-actin mRNA levels. (**B**) Ovarian $PGF_{2\alpha}$ production was measured by radioimmunoassay and expressed as pg/mg of wet tissue weight (w.w.). All data are expressed as mean ± SEM. ANOVA test was used for the statistical analysis with $n = 8$ mice per group. Different letters indicate statistically significant differences ($p < 0.05$).

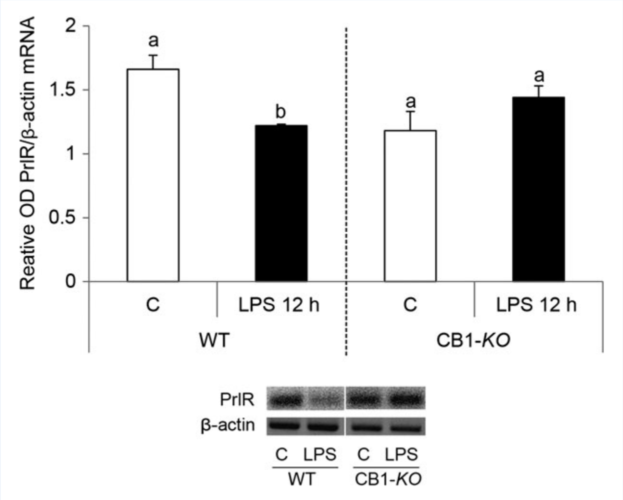

Figure 5 Effect of lipopolysaccharide (LPS) treatment on ovarian prolactin receptor (PrlR) mRNA levels. Ovaries of wild type (WT) and CB1-*KO* pregnant mice on day 7 of gestation were isolated 12 h after LPS (0.5 µg/g) or saline solution (C = control) injection and PrlR mRNA levels were measured by RT-PCR. All data were normalized to β-actin mRNA levels and expressed as mean ± SEM. ANOVA test was used for the statistical analysis with $n = 8$ mice per group. Different letters indicate statistically significant differences ($p < 0.05$).

drop of P4 serum levels in pregnant Balb/c mice (Aisemberg *et al.*, 2013) and CD1 *wild type* mice (Wolfson *et al.*, 2015) at 12 h post-administration. Here, we tested shorter and longer times post-LPS administration to characterize the effect of the endotoxin on P4 levels better. We found that LPS induced a drop in P4 serum levels as early as 6 h post-treatment in CD1 WT mice and that this effect was more pronounced at 24 h post-LPS. Nevertheless, LPS had no effects on P4 plasma levels in CD1 CB1-*KO* mice at 6 or 24 h post-administration. Therefore, the absence of the CB1 receptor resulted in a resistance to LPS actions on P4 levels, suggesting that the eCS mediates some of the endotoxin deleterious effects on reproductive tissues. This is in agreement with reports showing that CB1 and CB2 agonists induced a decrease in P4 secretion from luteal tissues in cows (Weems *et al.*, 1998) and ewes (Tsutahara *et al.*, 2011). More importantly, we have shown that the lower drop of P4 serum levels in LPS-challenged CB1-*KO* mice is associated with the resorption of fewer embryos (Wolfson *et al.*, 2015).

In contrast to humans, prolactin is a major stimulus for P4 production in rodents (Bachelot *et al.*, 2009). Our results show that LPS administration to pregnant WT mice reduced the content of ovarian prolactin receptor (PrlR) mRNA whereas this effect was absent in the ovaries of CB1-*KO* mice. These results are in agreement with Erlebacher et al. (Erlebacher *et al.*, 2004), who have shown that pro-inflammatory cytokines induce a down-regulation of PrlR mRNA in ovarian tissue. Interestingly, $PGF_{2\alpha}$ administration to pregnant rats inhibited PrlR mRNA expression (Stocco *et al.*, 2003) and prolactin signaling (Curlewis *et al.*, 2002), suggesting that elevated levels of this prostaglandin are responsible for the corpus luteum regression and drop in P4 production.

In rodents (except guinea pig) and ruminants, but not in human and nonhuman primates, P4 is secreted by the corpus luteum throughout pregnancy. Therefore, its integrity is of paramount importance for pregnancy outcome. Consequently, a delicate balance between luteotrophic and luteolytic factors must exist within the ovary to prevent premature CL degradation. It is well established that the main signal for luteolysis in ruminants and rodents is the uterine pulsatile release of $PGF_{2\alpha}$ into the uterine vein which is then transported into the ovarian artery via the utero-ovarian-plexus (UOP) (McCracken *et al.*,

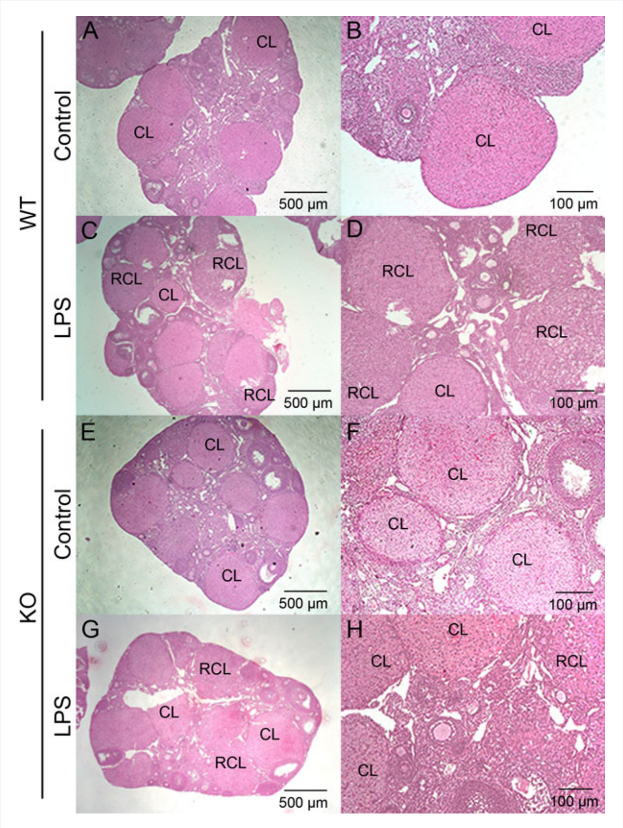

Figure 6 Effect of lipopolysaccharide (LPS) treatment on corpus luteum morphology. Wild type (WT) and CB1-*KO* pregnant mice on day 7 of gestation were injected with LPS (0.5 µg/g) or saline solution (C = control) and ovaries were collected and fixed 48 h post injection to perform haematoxylin–eosin staining. Sections were observed by light microscopy. Panels **A**, **C**, **E** and **G** show a panoramic view of the ovary (40×). Panels **B**, **D**, **F** and **H** show a detail of corpora lutea (100×). CL: Normal corpus luteum; RCL: Regressing corpus luteum.

Table II Percentage of intact corpora lutea (CL) and regressing corpora lutea (RCL) per ovary in lipopolysaccharide (LPS)-treated wild type (WT) and LPS-treated cannabinoid receptor type 1 (CB1)-*KO* mice. Data are shown as mean ± SEM from *n* = 6 animals per group.

	CL	RCL
WT	62 ± 10.7	38 ± 10.7
CB1-*KO*	78 ± 7.3	22 ± 7.3

1972; Bonnin *et al.*, 1999; Lee *et al.*, 2010). Therefore, an abnormal increase in uterine PGF$_{2\alpha}$ production might be involved in a precocious degradation of the corpora lutea and a consequently drop in P4 levels followed by a pregnancy failure. Indeed, our results agree with this hypothesis. We observed that LPS induced an increase in uterine PGF$_{2\alpha}$ production and various signs of regressing corpora lutea at 48 h post-endotoxin administration in 7-days pregnant CD1 WT mice. This effect, however, was lacking in 7-days pregnant CD1 CB1-*KO* mice. When the ovarian production of PGF$_{2\alpha}$ in response to LPS was analyzed, we found an increase in the local levels of this prostaglandin in WT mice, with the endotoxin having no effects on CB1-*KO* mice. Taken together, these results are in agreement with previous observations from our laboratory, where we have shown that the cannabinoid agonist methanandamide, a non-hydrolyzable analog of anandamide, stimulated uterine PGF$_{2\alpha}$ production in pregnant mice (Vercelli *et al.*, 2012). Therefore, we speculate that the endocannabinoid system mediates, at least partially, some of the deleterious effects of LPS-triggered inflammation on reproductive tissues. In this sense, it is well established that CB1 receptors are expressed by the luteal cells (Bagavandoss and Grimshaw, 2010) as well as in uterine tissue in rodents (Das *et al.*, 1995; Vercelli *et al.*, 2009; Bariani *et al.*, 2015). Erlebacher *et al.* (2004) have shown that an inflammatory reaction was associated with ovarian insufficiency and early pregnancy loss. Similarly, LPS has been shown to increase uterine PGF$_{2\alpha}$ production and luteal changes in later stages of pregnancy which was associated with preterm birth (Deb *et al.*, 2004; Bariani *et al.*, 2015).

We have previously shown that LPS increased prostaglandin synthesis at early (Aisemberg *et al.*, 2007) and late pregnancy (Cella *et al.*, 2010; Domínguez Rubio *et al.*, 2014) in Balb/c mice. Here, we show that LPS increases PGF$_{2\alpha}$ synthesis by up-regulating the uterine mRNA content of COX-2 in WT mice but not in CB1-*KO* mice. In addition, we found that LPS induced higher uterine levels of COX-2 protein expression in WT when compared to CB1-*KO* mice. This is in agreement with a previous report from our laboratory where we have found that LPS-induced COX-2 expression in murine uterus was blocked by either CB1 or CB2 antagonists (Vercelli *et al.*, 2012). Altogether, these results points toward the eCS as mediator of LPS-induced increase in COX-2 expression and its deleterious effects during pregnancy. Furthermore, Luchetti *et al.* (2008) have shown that an increased expression of uterine COX-2 was associated with embryo resorption in a model of hyperandrogenization. Nevertheless, it is important to note that a basal level of uterine COX-2 is essential for a proper embryo implantation and spacing (Ye *et al.*, 2005; Pakrasi and Jain, 2007). Similarly, to what happens in the uterus, we found that ovarian COX-2 protein expression was up-regulated by treatment with LPS, although this effect was more pronounced in the ovaries from WT mice as compared to CB1-*KO* mice. Overall, these results suggest that, similar to what happens at uterus level; the eCS mediates LPS-induced tissue damage at ovarian levels as well.

Conclusions

Collectively, our results shed further light into the role of the eCS and the molecular mechanisms associated to LPS deleterious effects in our model of early pregnancy loss. The fact that LPS provokes a higher rate of embryo resorption in pregnant WT as compared to CB1-*KO* mice (Wolfson *et al.*, 2015), could be explained by an ovarian insufficiency caused by the endotoxin with the involvement of the eCS. Namely, when exposed to LPS, CD1 WT mice produce high levels of uterine COX-2 which in turns produces increased amounts of PGF$_{2\alpha}$. This prostaglandin reaches the ovary via the UOP, where it down-regulates the PrlR mRNA levels, activates luteolysis and produces a P4 withdrawal. Taken together, these changes produce a luteal insufficiency which, we propose, is one of the causes responsible for the early embryo loss. Contrariwise, LPS fails to produce these changes in CD1 CB1-*KO* mice, showing these animals a reduced embryo resorption rate when compared to CD1 WT mice.

In summary, the results presented here support our hypothesis that the eCS promotes premature luteal regression, which could be involved in the LPS-induced withdrawal of serum P4.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

Conceived and designed the experiments: J.A.S., M.L.W., A.M.F. Performed the experiments: J.A.S., M.L.W., M.V.B., C.C. Analyzed the data: J.A.S., J.B., M.V.B., M.L.W. Contributed reagents/materials/analysis tools: F.C., F.J., A.M.F. Wrote the paper: J.A.S., F.C., A.M.F.

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Conflict of interest

None to declare.

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