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Lipopolysaccharide-induced murine embryonic resorption involves changes in endocannabinoid profiling and alters progesterone secretion and inflammatory response by a CB1-mediated fashion

Manuel L. Wolfson [a,*], Fernando Correa [a], Emma Leishman [b], Claudia Vercelli [c], Cora Cymeryng [d], Julieta Blanco [a], Heather B. Bradshaw [b], Ana María Franchi [a]

[a] Laboratory of Physiopathology of Pregnancy and Labor, Center for Pharmacological and Botanical Studies, National Research Council, School of Medicine, University of Buenos Aires, Argentina
[b] Department of Psychological and Brain Sciences, Indiana University, Bloomington, IN, USA
[c] Biomedicine Research Institute of Buenos Aires, Partner Institute of the Max Planck Society (MPSP), National Research Council, Ciudad Autónoma de Buenos Aires (CABA), Buenos Aires, Argentina
[d] Laboratory of Molecular Endocrinology, Center for Pharmacological and Botanical Studies, National Research Council, School of Medicine, University of Buenos Aires, Argentina

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ABSTRACT

Genital tract infections are a common complication of human pregnancy that can result in miscarriage. We have previously shown that a lipopolysaccharide (LPS) induces embryonic resorption in a murine model of inflammatory miscarriage. This is accompanied by a dramatic decrease in systemic progesterone levels associated with a robust pro-inflammatory response that results in embryo resorption. Here, we tested the hypothesis that the endogenous cannabinoid system (eCS), through cannabinoid receptor 1 (CB1), plays a role in regulating progesterone levels and, therefore, the pro-inflammatory response. We show that LPS treatment in pregnant mice causes significant changes in the eCS ligands, which are reversed by progesterone treatment. We further show the CB1-*KO* mice maintain higher plasma progesterone levels after LPS treatment, which is associated with a feebler uterine inflammatory response and a significant drop in embryo resorption. These data suggest that manipulation of CB1 receptors and/or ligands is a potential therapeutic avenue to decrease infection-induced miscarriage.

1. Introduction

Genital tract infections caused by Gram-negative bacteria not only induce miscarriage but are also one of the most common complications in human pregnancy (Cram et al., 2002). The systemic presence of the main cell wall component lipopolysaccharide (LPS) in pregnant mice induces infiltration of the decidua with granulocytes and large granular lymphocytes (LGL) (Ogando et al., 2003), increased uterine and decidual production of nitric oxide (NO) and prostaglandins (Aisemberg et al., 2007; Ogando et al., 2003), and decreased plasma levels of progesterone (Aisemberg et al., 2013). These changes were associated with embryonic resorption (EmR) followed by fetal expulsion (Aisemberg et al., 2012, 2013; Ogando et al., 2003). We have previously shown that the endocannabinoid system (eCS) was involved in the effects of LPS on NO and prostaglandin production, and subsequent tissue damage during early embryonic loss (Vercelli et al., 2009a, 2009b, 2012).

Endogenous lipids of the eCS are composed of a growing family of *N*-acylamides and 2-acylglycerol esters, many of which are identified as ligands for specific GPCRs (*e.g.* CB1, CB2, GPR55, GPR18, GPR119) and TRPV receptors that participate in numerous physiological and pathological processes during pregnancy (Cella et al., 2008; Fonseca et al., 2010; Gebeh et al., 2013; Maccarrone et al., 2004; Pertwee et al., 2010; Raboune et al., 2014; Schuel et al., 2002; Sun et al., 2010; Taylor et al., 2011; Vercelli et al., 2009a, 2009b).

Abbreviations: 2-AG, 2-arachidonoylglycerol; 2-LG, 2-linoleoyl-*sn*-glycerol; 2-OG, 2-oleoyl-*sn*-glycerol; AEA, anandamide; AGly, *N*-arachidonoylglycine; CB1, cannabinoid receptor type 1; CB1-*KO*, CB1 receptor *knock-out* mice; CXCL-10, C-X-C motif chemokine 10; DEA, *N*-docosahexaenoylethanolamine; DGly, *N*-docosahexaenoylglycine; eCS, endocannabinoid system; EmR, embryonic resorption; IL, interleukin; i.p, intraperitoneal; LEA, *N*-linoleoylethanolamine; LGly, *N*-linoleoylglycine; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; NAEs, *N*-acylethanolamines; NAGly, *N*-acylglycines; NO, nitric oxyde; NOS, nitric oxyde synthase; OEA, *N*-oleoylethanolamide; OGly, *N*-oleoylglycine; PEA, *N*-palmitoylethanolamine; PCR, polymerase chain reaction; PGly, *N*-plamitoylglycine; RANTES, regulated on activation, normal T cell expressed and secreted; s.c, subcutaneous; SEA, *N*-stearoylethanolamine; SGly, *N*-stearoylglycine; TNFα, tumor necrosis factor alpha; WT, wild-type mice.

* Corresponding author. Centro de Estudios Farmacológicos y Botánicos (CEFyBO), Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Piso 16. C1121ABG, Ciudad Autónoma de Buenos Aires, Argentina. Tel.: +54 11 4508 3680 ext 111; fax: +54 11 4508 3680 ext 102.
E-mail address: manuwolfson@gmail.com (M.L. Wolfson).

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N-arachidonoylethanolamine (anandamide, AEA) was the first endocannabinoid to be isolated and characterized (Devane et al., 1992). *N*-acylethanolamines (NAEs) are the family of AEA endogenous analogs and have a variety of functions at different receptors including but not limited to TRPV1 receptors (Raboune et al., 2014). Similarly, another important endocannabinoid is 2-arachidonoylglycerol (2-AG) and its family of endogenous analogs, 2-acyl-*sn*-glycerols (Sugiura et al., 1999). Cumulative data support the concept that the eCS plays important roles in pregnancy.

Both cannabinoid receptors 1 and 2 (CB1, CB2) are expressed in human endometrium (Taylor et al., 2010a) with CB1 playing an important role in oviductal embryo transport (Wang et al., 2004) and uterine embryo receptivity (Paria et al., 2001). Low levels of AEA are favorable for implantation and trophoblast outgrowth whereas increased AEA concentrations are embryotoxic and lead to arrested embryo development and pregnancy failure (Paria and Dey, 2000). On the other hand, low fatty acid amide hydrolase (FAAH) activity in peripheral lymphocytes has been shown to correlate with miscarriages in humans (Maccarrone et al., 2000). Furthermore, high plasma levels of AEA have been associated with early pregnancy loss in humans (Habayeb et al., 2008). Maccarrone et al. (2002b, 2003) showed that a reduced peripheral FAAH activity and high plasma levels of AEA were correlated with low progesterone levels, even though Taylor et al. (2011) failed to find such correlation. FAAH activity is not limited to AEA; however, and has been shown to metabolize all of the NAEs (Cravatt and Lichtman, 2002). Much less is known about the role of 2-AG in the regulation of pregnancy; however, it has also been shown to be regulated in the uterus as a function of the ovarian cycle (Bradshaw and Allard, 2011). Therefore, the interplay between progesterone and the eCS is complex and not fully understood (Gebeh et al., 2013; Habayeb et al., 2008; Maccarrone et al., 2000; Paria and Dey, 2000; Taylor et al., 2011).

In addition to its role in establishing and maintaining pregnancy, progesterone is considered an immunosteroid (Correale et al., 1998) with a critical function in modulating the immune response during normal gestation (Szekeres-Bartho et al., 1996). It also has been shown that progesterone has anti-inflammatory effects by protecting the embryo from LPS-induced pregnancy loss (Aisemberg et al., 2013). It has been proposed that progesterone upregulates FAAH expression in human lymphocytes through the transcription factor Ikaros (Maccarrone et al., 2003) as well as CB1 mRNA expression in the endometrial stromal cells during the secretory phase (Resuehr et al., 2012). We have recently shown that LPS induced a downregulation of murine peripheral blood mononuclear cells' (PBMC) FAAH activity and that this effect was reversed by progesterone treatment (Wolfson et al., 2013).

Given that (a) CB1 is highly expressed in reproductive tissues (Taylor et al., 2010a; Vercelli et al., 2012), (b) LPS-induced high levels of endocannabinoids are associated with miscarriage (Habayeb et al., 2008; Maccarrone et al., 2000), and (c) progesterone exerts protective effects against pregnancy loss (Aisemberg et al., 2013), our aim for this work was to investigate whether progesterone exerts protective effects from the deleterious actions of LPS in early pregnancy and the role of the eCS.

2. Materials and methods

2.1. Reagents

LPS from *Escherichia coli* 05:B55 and progesterone were purchased from Sigma Chemical Co. (St. Louis, MI, USA). Trizol reagent, RNAse-free DNAse I, Moloney Murine Leukemia virus reverse transcriptase (M-MLVRT) and random primers were purchased from Invitrogen (Life Technologies, Argentina). GoTaq DNA Polymerase was purchased from Promega (Biodynamics, Argentina). All other chemicals were analytical grade.

2.2. Animals and treatments

Eight to 12-week-old virgin female Balb/c or CD1 (*wild-type* or CB1-*knock-out*) mice were paired with 8- to 12-week-old Balb/c or CD1 (*wild-type* or CB1-*knock-out*) males respectively. The day of appearance of a coital plug was taken as day 0 of pregnancy. CD1 CB1-*knock-out* mice were generated as previously described (Ledent et al., 1999). Animals were housed in cages under controlled conditions of light (12 h light, 12 h dark) and temperature (21–25 °C) and received murine chow and water *ad libitum*.

Next, 7-day pregnant Balb/c WT mice were divided into four groups: (1) control group received an i.p. and s.c. injection of vehicle, (2) LPS-treated group received an i.p. injection of LPS (1 μg/g of body weight in saline solution), (3) LPS plus progesterone-treated group received a s.c. injection of progesterone (2 mg/animal in corn oil) and an i.p. injection of LPS, and (4) progesterone-treated group received a s.c. injection of progesterone. Blood from the orbital sinus was extracted under CO_2 anesthesia 12 h after LPS or vehicle administration, followed by animal euthanization by cervical dislocation. The blood was collected in EDTA-coated tubes and centrifuged at 655 *g* for 10 min at 4 °C and the plasma fraction was stored at −70 °C until further use for lipid analysis as will be described later.

In the case of the CD1 mice, 7-day pregnant *wild-type* (WT) or CB1-*knock-out* (CB1-*KO*) mice were caged in two groups each: (1) control WT and control CB1-*KO* received an i.p. injection of vehicle, and (2) LPS-treated WT and LPS-treated CB1-*KO* received an i.p. injection of LPS (1 μg/g or 0.5 μg/g of body weight). Mice were euthanized 6, 12 or 24 h after LPS or vehicle administration. Blood from the orbital sinus was extracted under CO_2 anesthesia, followed by animal euthanization by cervical dislocation. The blood was allowed to clot and was centrifuged at 655 *g* for 10 min and the serum fraction was stored at −70 °C until used for progesterone level determination.

2.3. Ethics statement

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) 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). All blood extractions were performed under CO_2 anesthesia and all efforts were made to minimize suffering.

2.4. Determination of embryonic resorption rate

With the aim of assessing the rate of embryonic resorption, CD1 WT and CB1-*KO* mice were treated on day 7 with LPS (0.5 or 1 μg/g of weight) and euthanized by cervical dislocation 24 h later. The uteri were excised and examined macroscopically to count the number of healthy and reabsorbed embryos. The reabsorbed embryos were identified by their small size, extensive hemorrhage and necrosis. An embryo that fits these criteria was classified as resorbed. Resorption rates were calculated as: [number of resorbed embryos/(total number of embryos)] × 100.

2.5. Radioimmunoassay

Progesterone was measured in serum extracted from LPS treated CD1 WT and CB1-*KO* mice and control mice sacrificed 12 h after treatment. Blood from the orbital sinus was extracted under CO_2 anesthesia. Blood was allowed to clot and was centrifuged at 655 *g* for 10 min and stored at −70 °C until used. Progesterone was

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