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Peripheral Blood Mononuclear Cells Infiltration Downregulates Decidual FAAH Activity in an LPS-Induced Embryo Resorption Model

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Maternal infections with gram-negative bacteria are associated with miscarriage and are one of the most common complications during pregnancy. Previous studies from our group have shown that lipopolysaccharide (LPS)-activated infiltrating peripheral blood mononuclear cells (PBMC) into decidual tissue plays an important role in the establishment of a local inflammatory process that results in embryo cytotoxicity and early embryo resorption. Moreover, we have also shown that an increased endocannabinoid tone mediates LPS-induced deleterious effects during early pregnancy loss. Here, we sought to investigate whether the infiltrating PBMC modulates the decidual endocannabinoid tone and the molecular mechanisms involved. PBMC isolated from 7-day pregnant mice subjected to different treatments were co-cultured in a transwell system with decidual tissue from control 7-day pregnant mice. Decidual fatty acid amide hydrolase (FAAH) activity was measured by radioconvertion, total decidual protein nitration by Western blot (WB), and decidual FAAH nitration by immunoprecipitation followed by WB. We found that co-culture of PBMC obtained from LPS-treated mice increased the level of nitration of decidual FAAH, which resulted in a negative modulation of decidual FAAH activity. Interestingly, co-treatment with progesterone or aminoguanidine prevented this effect. We found that LPS-treated PBMC release high amounts of nitric oxide (NO) which causes tyrosine nitration of decidual FAAH, diminishing its enzymatic activity. Inactivation of FAAH, the main degrading enzyme of anandamide and similar endocannabinoids, could lead to an increased decidual endocannabinoid tone with embryotoxic effects.

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Miscarriage caused by systemic or localized gram-negative bacterial infections is one of the most common complications in human pregnancy (Giakoumelou et al., 2016). The presence of systemic lipopolysaccharide (LPS) triggers an inflammatory response in the mother that has the capability of exerting deleterious effect on the conceptus. Thus, high ratios of pro-inflammatory/anti-inflammatory cytokines have been detected in pregnant women with threatened miscarriage (Calleja-Agius et al., 2011). Indeed, high levels of pro-inflammatory cytokines have been shown to activate macrophages, enhancing apoptosis of extravillious trophoblasts (Wu et al., 2012). Furthermore, decidual cells, placental villous stromal cells, and cytotrophoblasts produce and secrete chemokines (Drake et al., 2004), which attract peripheral leukocytes into reproductive tissues. First trimester human decidual cells stimulated with IL-1β or TNF-α have been shown to upregulate the expression of CCL5, CXCL2, CXCL3, CCL2, and CXCL8 (Huang et al., 2006; Lockwood et al., 2006). We have previously shown that LPS administration to 7-day pregnant mice not only induced the decidual expression of the chemokines RANTES, CXCL-10, and MCP-1 in the murine decidua (Wolfson et al., 2015a), but also increased decidual leukocyte infiltration (Ogando et al., 2003; Wolfson et al., 2015a). These pro-inflammatory changes were associated with an increased rate of embryo resorption and fetal expulsion (Ogando et al., 2003; Aisemberg et al., 2007; Wolfson et al., 2015a).

Even though immune cells are abundant in first trimester decidua, representing ~40% of the cells present within the decidua (Amsalem et al., 2008), their role in this tissue is not fully understood. It has been hypothesized that their presence contributes to the appropriate trophoblast invasion at the implantation site (Nakayama et al., 2002). However, during

Abbreviations: AEA, anandamide; AG, aminoguanidine; anti-NT, anti-nitrotyrosine; AA, arachidonic acid; eCS, endocannabinoid system; FAAH, fatty acid amide hydrosale; IP, immunoprecipitation; i.p., intraperitoneal; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; PBMC, peripheral blood mononuclear cells; $ONOO^-$, peroxynitrite; s.c., subcutaneous.

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maternal infection with gram-negative bacteria, the presence of LPS might contribute to the activation and recruitment of peripheral blood mononuclear cells (PBMC) into reproductive tissues (Ogando et al., 2003). Moreover, LPS induced an increased nitric oxide (NO) release by PBMC from both pregnant and non-pregnant mice (Wolfson et al., 2015b).NO is a short-lived molecule that acts as a mediator of multiple cellular functions. In reproductive tissues, local synthesis of NO plays important roles in endometrial, myometrial, and cervical function (Telfer et al., 1995) as well as embryo implantation and development (Chwalisz et al., 1999; Battaglia et al., 2003). Moreover, the levels of NO production and the expression of nitric oxide synthase (NOS) isoforms vary greatly throughout pregnancy (Suzuki et al., 2009). However, excessive production of NO has been associated with peroxidative damage (Franco and Estévez, 2014), embryotoxicity (Barroso et al., 1999), and early pregnancy loss (Haddad et al., 1995; Athanassakis et al., 1999; Ogando et al., 2003). Previous reports from our lab showed that LPS enhanced the production of NO (Wolfson et al., 2015b) and altered components of the endocannabinoid system (eCS) in pregnant mice (Wolfson et al., 2013, 2015b).

The eCS is comprised by ligands of lipid nature, their cognate receptors, and the synthesis and degradation enzymes. High levels of endocannabinoids have been associated with arrested embryo development and pregnancy failure (Paria and Dey, 2000; Habayeb et al., 2008; Wolfson et al., 2015a). Similarly, low levels of FAAH activity in peripheral lymphocytes have been associated with miscarriages in humans (Maccarrone et al., 2000b). Previous studies from our lab have shown that LPS increases the endocannabinoid tone in pregnant mice (Wolfson et al., 2015a) while diminishing the activity of FAAH in murine PBMC (Wolfson et al., 2013). Interestingly, mice lacking CB1 receptor were resistant to the deleterious effects of LPS and showed significantly less leukocyte infiltration in the deciduas (Wolfson et al., 2015a), suggesting a bidirectional relationship between LPS and the eCS. We have shown that the endocannabinoid system (eCS) was a mediator of the effects of LPS on NO production and the ensuing embryotoxicity during early pregnancy loss (Vercelli et al., 2009a,b). FAAH is considered to be the "metabolic gatekeeper" of local levels of anandamide (AEA) and related endocannabinoids in reproductive tissues, maintaining and appropriate "endocannabinoid tone" for a correct development of pregnancy (Wang et al., 2006b). Thus, low levels of FAAH activity are associated with high plasma levels of AEA and increased risk of miscarriage (Maccarrone et al., 2000b). Moreover, deficient FAAH mice show poor outcome of pregnancy (Wang et al., 2006c), suggesting that a proper FAAH expression/activity is important for a successful pregnancy.

Our aim here was to study the role of maternal PBMC-derived NO production on the decidual FAAH activity. We hypothesized that infiltrating PBMCs produce large amounts of NO which negatively alters the decidual FAAH activity by inducing tyrosine nitration of this enzyme. Diminished decidual FAAH activity results in an increased endocannabinoid tone, which are ultimately responsible for the toxic effects of LPS on the embryo.

Materials and Methods

Reagents

LPS from *Escherichia coli* 05:B55, anti-β-actin antibody, aminoguanidin (AG), and progesterone were purchased from Sigma Chemical Co. (St Louis, MI). The anti-FAAH antibody was a gift from Dr. Benjamin Cravatt (Scripps Research Institute, La Jolla, San Diego, CA). Anti-nitrotyrosine (anti-NT) antibody was purchased from Millipore (Billerica, MA). The Western blotting reagents were obtained from Bio-Rad (Tecnolab, Buenos Aires, Argentina). Secondary horse radish peroxidase (HRP) conjugated antibody was purchased from Jackson Immunosearch (Baltimore Pike, Pennsylvania). [^{3}H]-anandamide (specific activity 172.4 Ci/mmol) was provided by Perkin Elmer (Boston, MA). TLC aluminum silica gel plates were purchased from MerkKGaA (Darmstadt, Germany). Protein A/G PLUS-Agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of analytical grade.

Ethics statement

The experimental procedures reported here were approved (Resolution Number 900/2016) by the Animal Care Committee of the Center for Pharmacological and Botanical Studies of the National Research Council (CEFyBO–CONICET) and by the Institutional Committed 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). Blood extractions were performed under CO_2 anesthesia and all efforts were made to minimize animal suffering.

Animals and treatments

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.

Eight to 12-week-old virgin female Balb/c mice were paired with 8–12-week-old Balb/c males. The day of appearance of a coital plug was taken as day 0 of pregnancy. Next, pregnant Balb/c mice were divided into different groups according to each experimental design (Fig. 1).

Determination of decidual FAAH activity in response to in vivo administration of LPS (Fig. 1A): (i) control group received an i.p. injection of vehicle; (ii) LPS-treated group received an i.p. injection of LPS (1 μg/g of body weight in saline solution).

Obtainment of PBMC from LPS and/or progesterone-treated mice (Fig. 1B): (i) control group received an i.p. and/or s.c. injection of vehicle; (ii) LPS-treated group received an i.p. injection of LPS (1 μg/g of body weight in saline solution); (iii) LPS plus

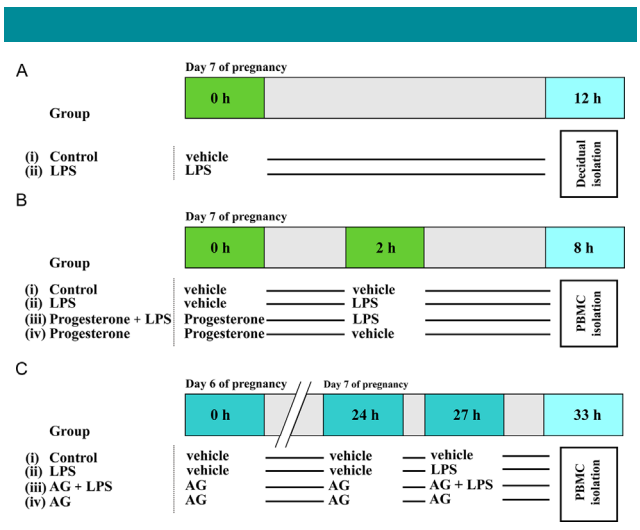

Fig. 1. Sketch of the experimental timeline. (A) Sketch of the in vivo treatment with LPS or vehicle. (B) Sketch showing the treatment schedule of mice on day 7 of pregnancy with LPS and/or progesterone. (C) Sketch showing the treatment of mice on day 6 with AG and on day 7 of pregnancy with LPS and/or LPS. AG, aminoguanidine; LPS, lipopolysaccharide.

progesterone-treated group received a s.c. injection of progesterone (67 μg/g of body weight in corn oil, as previously described [Aisemberg et al., 2013]) and 2 h after an i.p. injection of LPS; (iv) progesterone-treated group received a s.c. injection of progesterone.

Obtainment of PBMC from LPS and/or AG-treated mice (Fig. 1C): (i) control group received an i.p. and/or s.c. injection of vehicle; (ii) LPS-treated group received on day 7 of pregnancy an i.p. injection of LPS (1 μg/g of body weight in saline solution); (iii) LPS plus AG-treated group received on day 6 of pregnancy an i.p. injection of AG (0.2 mg/g of body weight in saline solution, as previously described [Ogando et al., 2003]), a second dose of AG 24 h later (day 7 of pregnancy) followed by a third dose of AG 3 h later. With the third dose of AG, an i.p. injection of LPS (1 μg/g of body weight in saline solution) was administered; (iv) AG-treated group received a i.p. injection of AG on day 6 of pregnancy, a second dose of AG 24 h later (day 7 of pregnancy) followed by a third dose of AG 3 h.

For isolating PBMC from treated pregnant mice, blood from the orbital sinus was extracted under CO_2 anesthesia 6 h after LPS or vehicle administration, followed by animal euthanization by cervical dislocation. The blood was collected in sodium citrate buffer-coated tubes and PBMC isolation was performed.

PBMC isolation

Balb/c female mice were anesthetized in a CO_2 atmosphere and blood was collected by orbital sinus bleeding in a tube containing citrate sodium buffer. Anti-coagulated blood was layered onto Histopaque-1083 (Sigma Chemical Co. St Louis, MI) and peripheral blood mononuclear cells (PBMC) were purified by gradient centrifugation (400 g, 30 min) according to the manufacturer's recommendations. Briefly, PBMC were collected from the opaque interface, transferred to a new tube, and washed twice with PBS.

Co-culture of PBMC and decidua

PBMC were obtained from treated animals as previously described. Cells were cultured in fresh serum-free DMEM for 2 h in 5% CO_2 at 37°C. Adherent monocytes were discarded and suspended cells were used for the co-culture.

On day 7 of pregnancy, control female mice were killed by cervical dislocation. In each implantation site, the uterus and the decidua were separated. Decidual explants were cultured in wells that contained 400 μl complete DMEM (supplemented with 10% FCS and antibiotics: 20 IU/ml penicillin G, 20 μg/ml streptomycin, and 50 ng/ml amphotericin B) for 2 h in 5% CO_2 at 37°C.

In each well, a transwell (membrane 0.4 μm pore) were placed with decidua from control animals remaining in the lower chamber and 2.5×10^{-5} PBMC from treated or control animals in the upper chamber. The transwell system were incubated for 12 h in 5% CO2 at 37°C and then deciduas were immediately frozen at −70°C until used.

Peroxynitrite scavenger assay

Decidual explants were cultured in wells containing 400 μl complete DMEM in the presence or absence of quercetin (a peroxynitrite scavenger) for 2 h in 5% CO_2 at 37°C. Next, 2.5×10^5 PBMC from control or LPS-animals were placed in the upper chamber and FAAH activity in the deciduas was measured 12 h later.

Determination of fatty acid amide hydrolase (FAAH) activity

FAAH (EC 3.5.1.4) activity was assayed as described by Paria et al. (1996). The hydrolyzed [^{3}H]-arachidonic acid (AA) was resolved in the organic layer of a solvent system of ethyl acetate: hexane:acetic acid:distilled water (100:50:20:100 v/v) mixture. The plate was exposed to iodine to identify the zones corresponding to AA. The distribution of radioactivity on the plate was counted in a scintillation counter by scraping off the corresponding spots detected in the plate. The area of each radioactive peak corresponding to AA was calculated and expressed as a percentage of the total radioactivity of the plates. Protein concentration was determined by the method of Bradford. Enzyme activity is reported as nmol [^{3}H]-AA/mg protein/h.

Western blot analysis

Deciduas were sonicated in RIPA buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1% Nonidet P40; deoxycholate 0.25%; 10 μg/ml aprotinin; 10 μg/ml leupeptin; 1 mg/ml benzamidine; 1 mg/ml caproic acid; 10 μg/ml soybean trypsinogen inhibitor; and 1 M EDTA). Sixty micrograms of protein were loaded in each lane. Samples were separated by electrophoresis on 10% sodium dodecyl sulphate–polyacrylamide gel (SDS–PAGE) and transferred to a nitrocellulose membrane. Blots were incubated overnight with anti-NT (1:400), with anti-FAAH (1:400) or with anti-actin (1:4,000). Actin was used as a loading control. Blots were washed with buffer (PBS and 0.1% [v/v] Tween 20, pH 7.5) followed by 1-h incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:5,000) and developed using the enhanced chemiluminescence Western blot system. Photographs of the membranes were taken using ImageQuant system (GE Healthcare, Buenos Aires, Argentina) and analyzed using the ImageJ software package.

FAAH immunoprecipitation

All immunoprecipitation (IP) procedures were performed at 4°C. Deciduas were sonicated in RIPA buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1% Nonidet P40; deoxycholate 0.25%; 10 μg/ml aprotinin; 10 μg/ml leupeptin; 1 mg/ml benzamidine; 1 mg/ml caproic acid; 10 μg/ml soybean trypsinogen inhibitor; and 1 M EDTA). Two-hundred micrograms of total proteins were resuspended in 100 μl of homogenization buffer with 5 μl of anti-FAAH antibody (1:20). Samples were incubated for 3 h with rotation at 4°C. This lysate/antibody mixture was subsequently incubated overnight with protein G-sepharose beads with rotation at 4°C. The beads were pelleted by centrifugation at 15,000*g* for 5 min and washed two times in IP buffer A (Tris 1.2 M; NaCl 0.15 M; 10% detergent solution [Tris 24 mM; 10% Igepal; 0.005% sodium deoxycholate]; 1 mM Na3VO4 [pH 7.4], two times in IP buffer B (Tris 1.2 M; NaCl 0.5 M; 1% detergent solution [Tris 24 mM; 10% Igepal; 0.005% sodium deoxycholate]; 0.5 mM Na3VO4 [pH 7.4]) and two times in IP buffer C (Tris 1.2 M; 0.5% detergent solution [Tris 24 mM; 10% Igepal; 0.005% sodium deoxycholate]; 0.5 mM Na3VO4 [pH 7.4]). The protein–antibody complexes were released from beads by adding the loading buffer and heating at 100°C for 5 min. Next, proteins were subjected to Western blot analysis after separation by SDS–PAGE.

Statistical analyses

Treatments were assigned completely random to experimental units. Data were analyzed by means of one- or two-way ANOVA. Procedures and means were compared by Tukey post hoc tests. Differences between means were considered significant when P-value was 0.05 or less. Different letters indicate significant differences between means. Normality and homoscedasticity were tested by Shapiro–Wilk (modified) and Levene test, respectively. Statistical analysis was performed using the software Infostat (Córdoba, Argentina).

Results

Effect of PBMC 's soluble factor on decidual FAAH activity

We have previously shown that LPS administration to 7-day pregnant mice induced an inflammatory environment in the decidua (Wolfson et al., 2015a). We have also shown that LPS downregulated FAAH activity in PBMCs from both pregnant and non-pregnant mice (Wolfson et al., 2013). Therefore, we sought to study here the effect of in vivo administration of LPS on decidual FAAH activity in pregnant mice. As shown in Figure 2, we observed a significant decrease in FAAH activity at 12 h after LPS treatment.

In order to investigate the role of leukocyte infiltration in our model of early embryonic resorption, we used a transwell system in which two chambers are separated by a permeable membrane that only allows the passage of soluble factors. The decidua from control mice was placed in the lower chamber and the PBMC from mice subjected to different treatments were placed in the upper chamber. Co-cultures were maintained for 12 h (Fig. 3A). We observed that deciduas co-cultured with PBMC from control animal showed no difference in FAAH activity from deciduas cultured in the absence of PBMC (Fig. 3B). However, when deciduas from control mice were co-cultured with PBMC from LPS-treated pregnant mice, a statistically significant decrease in FAAH activity was observed (Fig. 3B). Next, we analyzed the effects of progesterone in modulating the levels of decidual FAAH activity induced by PBMC from LPS-treated mice. In the presence of PBMC from mice co-treated with progesterone and LPS, the levels of decidual FAAH activity from control mice were restored (Fig. 3B). These results suggest that PMBC release a soluble factor which modulates the decidual FAAH activity.

NO released from PBMC modulates decidual FAAH activity

Next, we sought to investigate whether NO could be the soluble factor modulating decidual FAAH activity. We obtained the PBMC from animals treated with LPS and AG, a selective inhibitor of iNOS (Fig. 4A). Using the same transwells system, we observed that in vivo administration of AG reverted the effects of LPS-treated PBMC on the decidual FAAH activity from control mice (Fig. 4B).

Since NO could be transformed in peroxynitrite, we studied whether this potent oxidizing agent was involved in the inhibition of decidual FAAH activity. In order to do so, deciduas from control mice were pre-incubated with quercetin, a scavenger of peroxynitrite, and exposed to PBMC from pregnant mice treated with LPS or vehicle (Fig. 4C). Again, PBMC from LPS-treated mice induced a decrease in decidual FAAH activity; however, when quercetin was present, this effect was lost (Fig. 4D), suggesting a participation of the free radical peroxynitrite in LPS-treated PBMC modulation of decidual FAAH activity.

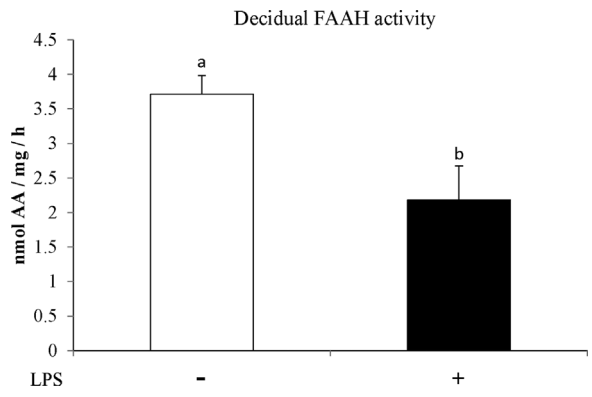

Fig. 2. Effect of in vivo administration of LPS on decidual FAAH activity. LPS treatment induces a significant decrease of FAAH activity in the deciduas of 7-day pregnant mice. ANOVA test; statistics: a≠b, $P < 0.05$ (n = 5).

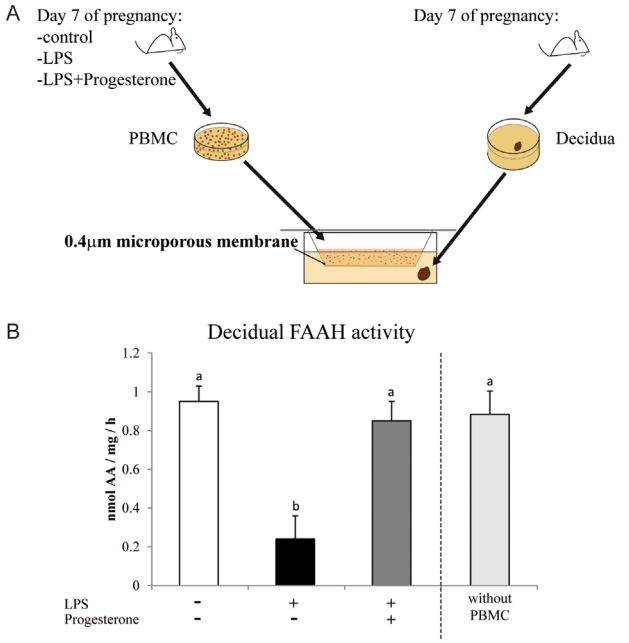

Fig. 3. Effect of PBMC from LPS and/or progesterone-treated mice on decidual FAAH activity from control mice. (A) Sketch of the transwell system: decidual tissue from control 7-day pregnant mice was placed in the lower chamber. PBMC from 7-day pregnant mice subjected to different treatments were placed in the upper chamber. Co-cultures were maintained for 12 h. (B) Co-culture with PBMC from LPS-treated mice negatively modulates the activity of decidual FAAH. When mice are co-administered with progesterone, this effect is prevented. ANOVA test, statistics: a≠b, $P < 0.05$ (n = 6).

NO modulates decidual FAAH enzymatic activity by nitrating FAAH protein

Since peroxynitrite is a highly oxidizing and nitrating agent, we sought to investigate the protein nitration levels in deciduas exposed to PBMC from control or LPS-treated mice in our transwell system. We observed an increase in protein nitration in deciduas co-cultured with PBMC from LPS-treated mice and this effect was reversed when animals were co-administered with AG (Fig. 5).

In order to analyze whether decidual FAAH activity could be modulated by protein nitration in our model, we performed a FAAH immunoprecipitation using anti-FAAH antibody in order to precipitate all nitrated proteins followed by a Western blot detection using anti-nitrotyrosine antibody. We observed an increased level of nitrated FAAH protein in deciduas exposed to PBMC from LPS-treated mice. Interestingly, this effect was reverted when deciduas were exposed to PBMC from LPS and progesterone co-treated mice (Fig. 6).

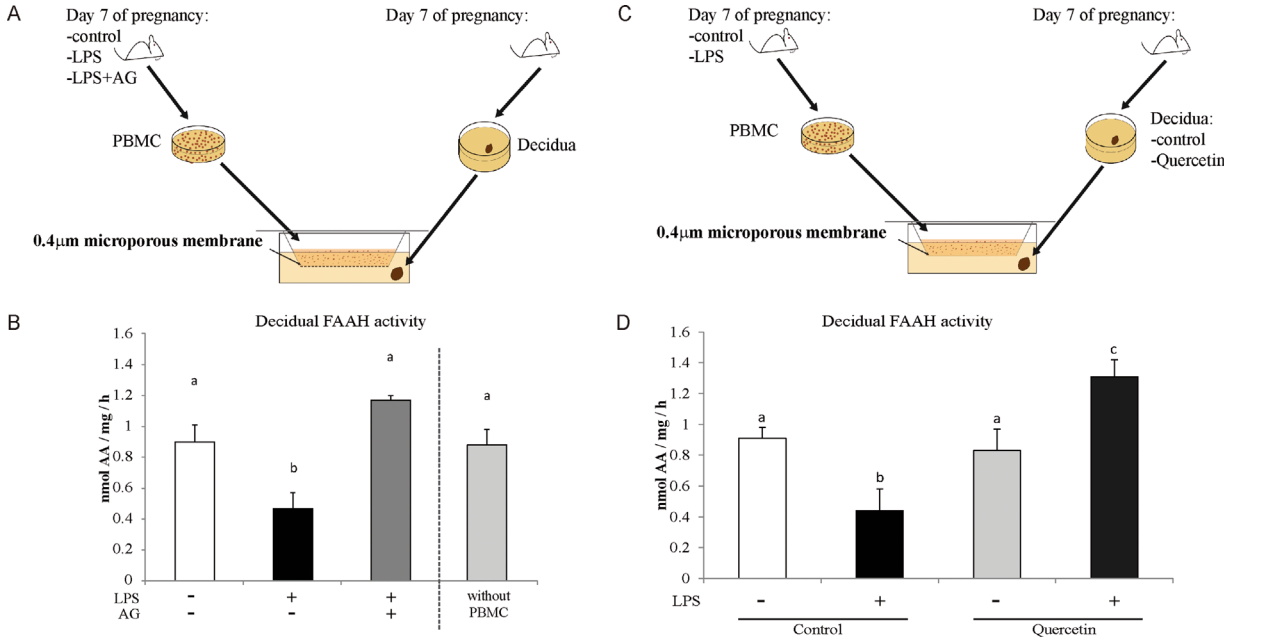

Fig. 4. Effect of aminoguanidine (AG; an iNOS inhibitor) or quercetin (a peroxynitrite scavenger) on decidual FAAH activity. (A) Sketch of the transwell system: decidual tissue from control 7-day pregnant mice was placed in the lower chamber. PBMC from 7-day pregnant mice subjected to different treatments were placed in the upper chamber. Co-cultures were maintained for 12 h. (B) Co-culture with PBMC from LPS-treated mice negatively modulates the activity of decidual FAAH. When mice are co-treated with AG, this effect is prevented. ANOVA test, statistics: a≠b, $P < 0.05$ (n = 6). (C) Sketch of the transwell system: decidual tissue from control 7-day pregnant mice was placed in the lower chamber. Decidua tissue cultures were pretreated with quercetin before exposing them to PBMC from LPS-treated mice placed in the upper chamber. Co-cultures were maintained for 12 h. (D) Co-culture with PBMC from LPS-treated mice negatively modulates the activity of the decidual FAAH. When deciduas are pre-treated with quercetin, this effect is lost. ANOVA test, statistics: a≠b, $P < 0.05$ (n = 5).

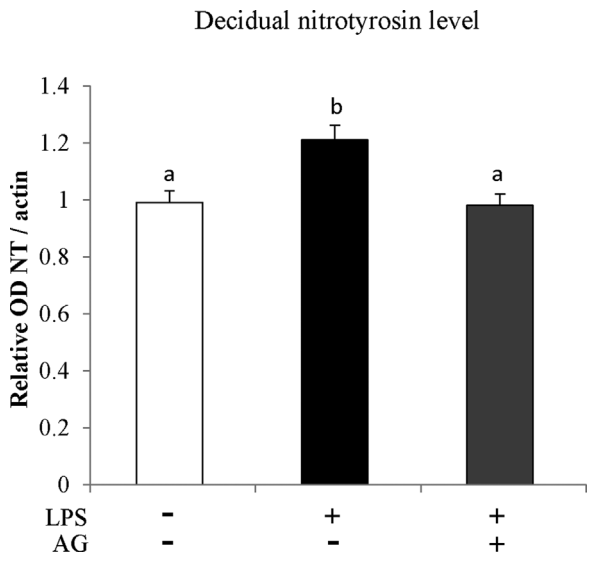

Fig. 5. PBMC-derived NO induces an increase in total decidual protein nitration, which is prevented with aminoguanidine (AG). Co-culture of PBMC obtained from LPS-treated 7-day pregnant mice increases the protein nitration levels in deciduas from control 7-day pregnant mice. This effect is reversed when animals are co-treated with AG. Densitometric analysis of immunoblot assays. ANOVA test, statistics: a≠b, $P < 0.05$ (n = 5).

Discussion

The molecular mechanisms by which decidua infiltrating PBMC play a role in LPS-induced embryo loss are still poorly understood. It has been proposed that decidual cells, placental villous stromal cells, and cytotrophoblasts have the capacity to produce and secrete chemokines (Drake et al., 2004). These chemokines are well-known leukocyte chemoattractants and have an important role in promoting PBMC invasion of the decidua. These invading activated leukocytes exert, in turn, deleterious effects on decidual cells by releasing excessive pro-inflammatory mediators (Wu et al., 2012; Wolfson et al., 2015a). Here, we sought to explore the role of infiltrating PBMC on the regulation of decidual FAAH activity. The endocannabinoid system plays an important role in the establishment and maintenance of pregnancy and a dysregulation of this system could lead to retarded embryo development, fetal loss and pregnancy failure (Paria and Dey, 2000; Guo et al., 2005; Wang et al., 2006a). In this sense, cumulative evidence suggests that FAAH is a metabolic gatekeeper of the in situ AEA levels maintaining an in vivo endocannabinoid tone in reproductive tissues.

We have previously shown that in vitro treatment with LPS induced an increase in FAAH activity in decidua explants (Vercelli et al., 2009a). However, when we administered LPS in vivo, we observed a decrease in decidual FAAH activity. These seemingly disparate observations could be explained due to the differences in an in vivo and an in vitro approach. Indeed, in vivo administration of LPS to 7-day pregnant mice is accompanied with an increased leukocyte infiltration of the decidua (Ogando et al., 2003; Wolfson et al., 2015a) which is not present in the in vitro experiments. Moreover, in our in vivo approach, the

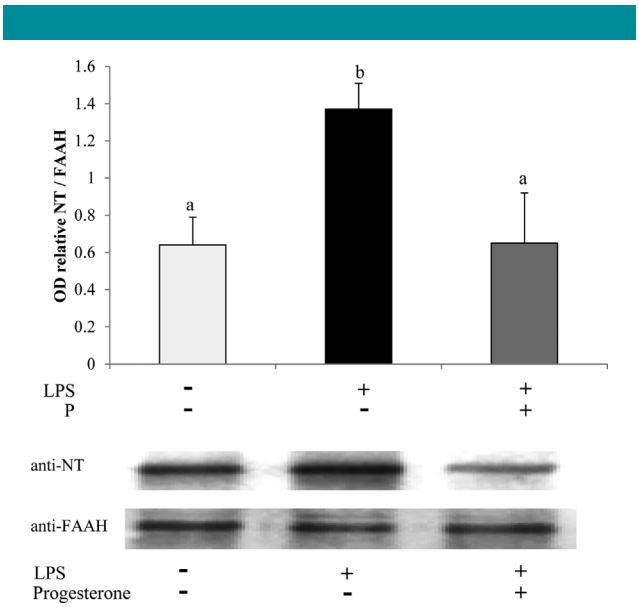

Fig. 6. PBMC-derived NO induces decidual FAAH nitration. (A) Co-culture of PBMC obtained from LPS-treated 7 day-pregnant mice increases the level of nitration of decidual FAAH. This effect is reversed when animals are co-treated with progesterone. (B) Densitometric analysis of immunoblot assays. ANOVA test, statistics: a≠b, $P < 0.05$ (n = 4).

PBMC from LPS-treated mice showed increased NO production (Wolfson et al., 2015a) and the leukocyte-infiltrated decidua from these mice expressed higher mRNA levels of cytokines and chemokines (Wolfson et al., 2015b). Overall, the results presented here might resemble more faithfully a pathophysiological process than our previous in vitro approach.

The first trimester decidua is abundant in immune cells, which comprise ~40% of the total cells within the decidua (Amsalem et al., 2008; Warning et al., 2011). It has been shown that infiltrating PBMCs derived from the mother during an early stage of pregnancy promote trophoblast invasion at the implantation site (Nakayama et al., 2002; Blois et al., 2011; Croy et al., 2012). Under certain conditions, such us maternal infection with gram-negative bacteria, these infiltrating PBMC could be involved in the termination of pregnancy (Amsalem et al., 2008). Using a transwell system, we found that in the co-cultures of PBMC from LPS-treated mice with deciduas from untreated mice, there was a reduction in decidual FAAH activity and that this effect was reversed when PBMC were obtained from LPS-treated mice co-treated with progesterone. Similarly, we had previously shown that in vitro cultured PBMC from in vivo LPS-treated pregnant mice showed and increased NO production and that in vivo co-administration with progesterone reversed this effect (Wolfson et al., 2015b). These results are in agreement with previous observations showing that progesterone and other progestins exert immunomulatory effects during pregnancy (Raghupathy et al., 2005; Aisemberg et al., 2013; Wolfson et al., 2013; Tan et al., 2015).

NO has a central role as a mediator of a plethora of physiological processes. However, it becomes cytotoxic when produced in excess. In the presence of superoxide anion, NO generates peroxynitrite ($ONOO^-$) which is highly oxidant and contributes to peroxidative damage. Excessive production of NO, which in turn generates high amounts of $ONOO^-$, has been associated with early pregnancy loss (Haddad et al., 1995; Athanassakis et al., 1999; Ogando et al., 2003). In this sense, it has been reported that NO levels in the blood from women with recurrent pregnancy loss was higher than in control patients (Makino et al., 2004) and that increased serum NO levels were present in pregnant women with a history of recurrent spontaneous abortion when compared to control pregnant women (Wilson et al., 1997). Similarly, Raffaelli et al. (2010) have recently reported that women with spontaneous miscarriages or a history of recurrent spontaneous miscarriages showed increased platelet NO and $ONOO^-$ levels when compared to controls. Previous results from our lab showed an increase of iNOS expression and NO production in our model of LPS-induced early pregnancy loss (Ogando et al., 2003; Aisemberg et al., 2007; Wolfson et al., 2015b). Moreover, we have also shown that there is a complex interaction between the NO system and the endocannabinoid system, with cannabinoids inducing NO production in placenta (Cella et al., 2008), uterus (Vercelli et al., 2009b), and decidua (Vercelli et al., 2009a) and with NO modulating the uptake of endocannabinoids (Maccarrone et al., 2000a). Consistent with previous reports, we show here that PBMC-released NO in turn modulates decidual FAAH activity and that this effect is blocked in the presence of AG (an iNOS inhibitor) or of quercetin (a peroxynitrite scavenger).

An increase in protein nitration levels is a hallmark of oxidative injury caused by leukocyte infiltration to inflamed tissues (Matata and Galiñanes, 2002). Moreover, it has been proposed that tyrosine nitration is a marker of tissue damage and cell death (Franco and Estévez, 2014). Here, we observed increased total nitrotyrosine levels in deciduas from untreated mice when exposed to LPS-activated PBMC. This effect was abrogated when LPS-treated mice were co-treated with AG, suggesting that, indeed, NO production mediates the oxidative damage induced by activated PBMC on the decidua from untreated mice. One of the mechanisms responsible of tissue damage that has been proposed is the ability of NO to alter protein activity by covalent modifications, such as nitration/nitrosylation (Balafanova et al., 2002; Vadseth et al., 2004). Indeed, it has been shown that nitration/nitrosylation of proteins, such as prostacyclin I2 synthase (Salvemini et al., 2006), superoxide dismutase (Ferrer-Sueta et al., 2002; Radi et al., 2002), and tyrosine hydroxylase (Blanchard-Fillion et al., 2001), reduced their enzymatic activity. We propose that NO produced by activated PBMC induces tyrosine nitration of decidual FAAH, therefore reducing its activity. The reduced catabolic activity of FAAH leads to higher local levels of AEA and other endocannabinoids which, in turn, leads to early embryo loss and pregnancy failure. Furthermore, a reduced FAAH activity could favor the AEA oxidative metabolism by COX-2 with the production of prostamides, which have been recently shown to be involved in pregnancy failure (Almada et al., 2015). Overall, our results are in agreement with the hypothesis of FAAH functioning as a metabolic gatekeeper for the on-site levels of AEA in order to maintain an appropriate endocannabinoid tone for a proper development of pregnancy. In this sense, Wang et al. (2006c) have shown that FAAH-deficient mice present impaired normal embryo development and implantation, leading to pregnancy failure. Moreover, it has been reported that low FAAH activity and high AEA plasma levels were associated with failure to achieve pregnancy in women subjected to in vitro fertilization (Maccarrone et al., 2002). Furthermore, Maccarrone et al. (2000b) found a correlation between decreased FAAH activity in peripheral lymphocytes and higher risk of miscarriage.

Taking together, our results point towards the participation of infiltrating leukocyte-derived NO on the modulation of the decidual endocannabinoid tone as an important mediator of the embryotoxic effects of LPS.

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Authors' Contributions

MLW performed the experiments, the statistical analyses, and prepared the figures. JA and AMF designed the experiments. FC and AMF wrote the manuscript text. All authors reviewed the manuscript.

Literature Cited

Aisemberg J, Vercelli CA, Bariani MV, Billi SC, Wolfson ML, Franchi AM. 2013. Progesterone is essential for protecting against LPS-induced pregnancy loss. LIF as a potential mediator of the anti-inflammatory effect of progesterone. PLoS ONE 8:e56161.

Aisemberg J, Vercelli C, Billi S, Ribeiro ML, Ogando D, Meiss R, McCann SM, Rettori V, Franchi AM. 2007. Nitric oxide mediates prostaglandins' deleterious effect on lipopolysaccharide-triggered murine fetal resorption. Proc Natl Acad Sci USA 104:7534–7539.

Almada M, Piscitelli F, Fonseca BM, Di Marzo V, Correia-da-Silva G, Teixeira N. 2015. Anandamide and decidual remodelling: COX-2 oxidative metabolism as a key regulator. Biochim Biophys Acta 1851:1473–1481.

Amsalem H, Gaiger A, Mizrahi S, Yagel S, Rachmilewitz J. 2008. Characterization of a lymphocyte subset displaying a unique regulatory activity in human decidua. Int Immunol 20:1147–1154.

Athanassakis I, Aifantis I, Ranella a, Giouremou K, Vassiliadis S. 1999. Inhibition of nitric oxide production rescues LPS-induced fetal abortion in mice. Nitric Oxide 3:216–224.

Balafanova Z, Bolli R, Zhang J, Zheng Y, Pass JM, Bhatnagar A, Tang XL, Wang O, Cardwell E, Ping P. 2002. Nitric oxide (NO) induces nitration of protein kinase Cepsilon (PKCepsilon), facilitating PKCepsilon translocation via enhanced PKCepsilon -RACK2 interactions: A novel mechanism of no-triggered activation of PKCepsilon. J Biol Chem 277:15021–15027.

Barroso G, Barrionuevo M, Rao P, Graham L, Danforth D, Huey S, Abuhamad A, Oehninger S. 1999. Vascular endothelial growth factor, nitric oxide, and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients. Fertil Steril 72:1024–1026.

Battaglia C, Ciotti P, Notarangelo L, Fratto R, Facchinetti F, Aloysio de D. 2003. Embryonic production of nitric oxide and its role in implantation: A pilot study. J Assist Reprod Genet 20:449–454.

Blanchard-Fillion B, Souza JM, Friel T, Jiang GC, Vrana K, Sharov V, Barrón L, Schöneich C, Quijano C, Alvarez B, Radi R, Przedborski S, Fernando GS, Horwitz J, Ischiropoulos H. 2001. Nitration and inactivation of tyrosine hydroxylase by peroxynitrite. J Biol Chem 276:46017–46023.

Blois SM, Klapp BF, Barrientos G. 2011. Decidualization and angiogenesis in early pregnancy: Unravelling the functions of DC and NK cells. J Reprod Immunol 88:86–92.

Calleja-Agius J, Muttukrishna S, Pizzey AR, Jauniaux E. 2011. Pro- and antiinflammatory cytokines in threatened miscarriages. Am J Obstet Gynecol 205:83.e8–83.e16.

Cella M, Leguizamón GF, Sordelli MS, Cervini M, Guadagnoli T, Ribeiro ML, Franchi AM, Farina MG. 2008. Dual effect of anandamide on rat placenta nitric oxide synthesis. Placenta 29:699–707.

Chwalisz K, Winterhager E, Thienel T, Garfield RE. 1999. Synergistic role of nitric oxide and progesterone during the establishment of pregnancy in the rat. Hum Reprod 14:542–552.

Croy BA, Chen Z, Hofmann AP, Lord EM, Sedlacek AL, Gerber SA. 2012. Imaging of vascular development in early mouse decidua and its association with leukocytes and trophoblasts. Biol Reprod 87:125.

Drake PM, Red-Horse K, Fisher SJ. 2004. Reciprocal chemokine receptor and ligand expression in the human placenta: Implications for cytotrophoblast differentiation. Dev Dyn 229:877–885.

Ferrer-Sueta G, Quijano C, Alvarez B, Radi R. 2002. Reactions of manganese porphyrins and manganese-superoxide dismutase with peroxynitrite. Methods Enzymol 349:23–37.

Franco MC, Estévez AG. 2014. Tyrosine nitration as mediator of cell death. Cell Mol Life Sci 71:3939–3950.

Giakoumelou S, Wheelhouse N, Cuschieri K, Entrican G, Howie SEM, Horne AW. 2016. The role of infection in miscarriage. Hum Reprod Update 22:116–133.

Guo Y, Wang H, Okamoto Y, Ueda N, Kingsley PJ, Marnett LJ, Schmid HH, Das SK, Dey SK. 2005. N-acylphosphatidylethanolamine-hydrolyzing phospholipase D is an important determinant of uterine anandamide levels during implantation. J Biol Chem 280:23429–23432.

Habayeb OMH, Taylor AH, Finney M, Evans MD, Konje JC. 2008. Plasma anandamide concentration and pregnancy outcome in women with threatened miscarriage. JAMA 299:1135–1136.

Haddad EK, Duclos AJ, Baines MG. 1995. Early embryo loss is associated with local production of nitric oxide by decidual mononuclear cells. J Exp Med 182:1143–1151.

Huang SJ, Schatz F, Masch R, Rahman M, Buchwalder L, Niven-Fairchild T, Tang C, Abrahams VM, Krikun G, Lockwood CJ. 2006. Regulation of chemokine production in response to pro-inflammatory cytokines in first trimester decidual cells. J Reprod Immunol 72:60–73.

Lockwood CJ, Arcuri F, Toti P, Felice C, Krikun G, Guller S, Buchwalder LF, Schatz F. 2006. Tumor necrosis factor-alpha and interleukin-1beta regulate interleukin-8 expression in third trimester decidual cells: Implications for the genesis of chorioamnionitis. Am J Pathol 169:1294–1302.

Maccarrone M, Bari M, Lorenzon T, Bisogno T, Di Marzo V, Finazzi-Agrò A. 2000a. Anandamide uptake by human endothelial cells and its regulation by nitric oxide. J Biol Chem 275:13484–13492.

Maccarrone M, Bisogno T, Valensise H, Lazzarin N, Fezza F, Manna C, Di Marzo V, Finazzi-Agrò A. 2002. Low fatty acid amide hydrolase and high anandamide levels are associated with failure to achieve an ongoing pregnancy after IVF and embryo transfer. Mol Hum Reprod 8:188–195.

Maccarrone M, Valensise H, Bari M, Lazzarin N, Romanini C, Finazzi-Agrò A. 2000b. Relation between decreased anandamide hydrolase concentrations in human lymphocytes and miscarriage. Lancet 355:1326–1329.

Makino A, Nakanishi T, Sugiura-Ogasawara M, Ozaki Y, Suzumori N, Suzumori K. 2004. No association of C677T methylenetetrahydrofolate reductase and an endothelial nitric oxide synthase polymorphism with recurrent pregnancy loss. Am J Reprod Immunol 52:60–66.

Matata BM, Galiñanes M. 2002. Peroxynitrite is an essential component of cytokines production mechanism in human monocytes through modulation of nuclear factor-kappa B DNA binding activity. J Biol Chem 277:2330–2335.

Nakayama T, Fujiwara H, Maeda M, Inoue T, Yoshioka S, Mori T, Fujii S. 2002. Human peripheral blood mononuclear cells (PBMC) in early pregnancy promote embryo invasion in vitro: HCG enhances the effects of PBMC. Hum Reprod 17:207–212.

Ogando DG, Paz D, Cella M, Franchi a M. 2003. The fundamental role of increased production of nitric oxide in lipopolysaccharide-induced embryonic resorption in mice. Reproduction 125:95–110.

Paria BC, Deutsch DD, Dey SK. 1996. The uterus is a potential site for anandamide synthesis and hydrolysis: Differential profiles of anandamide synthase and hydrolase activities in the mouse uterus during the periimplantation period. Mol Reprod Dev 45:183–192.

Paria BC, Dey SK. 2000. Ligand-receptor signaling with endocannabinoids in preimplantation embryo development and implantation. Chem Phys Lipids 108:211–220.

Radi R, Cassina A, Hodara R. 2002. Nitric oxide and peroxynitrite interactions with mitochondria. Biol Chem 383:401–409.

Raffaelli F, Nanetti L, Vignini A, Mazzanti L, Giannubilo SR, Curzi CM, Turi A, Vitali P, Tranquilli AL. 2010. Nitric oxide platelet production in spontaneous miscarriage in the first trimester. Fertil Steril 93:1976–1982.

Raghupathy R, Al Mutawa E, Makhseed M, Azizieh F, Szekeres-Bartho J. 2005. Modulation of cytokine production by dydrogesterone in lymphocytes from women with recurrent miscarriage. BJOG 112:1096–1101.

Salvemini D, Doyle TM, Cuzzocrea S. 2006. Superoxide, peroxynitrite and oxidative/nitrative stress in inflammation. Biochem Soc Trans 34:965–970.

Suzuki T, Mori C, Yoshikawa H, Miyazaki Y, Kansaku N, Tanaka K, Morita H, Takizawa T. 2009. Changes in nitric oxide production levels and expression of nitric oxide synthase isoforms in the rat uterus during pregnancy. Biosci Biotechnol Biochem 73:2163–2166.

Tan IJ, Peeva E, Zandman-Goddard G. 2015. Hormonal modulation of the immune system—A spotlight on the role of progestogens. Autoimmun Rev 14:536–542.

Telfer JF, Lyall F, Norman JE, Cameron IT. 1995. Identification of nitric oxide synthase in human uterus. Hum Reprod 10:19–23.

Vadseth C, Souza JM, Thomson L, Seagraves A, Nagaswami C, Scheiner T, Torbet J, Vilaire G, Bennett JS, Murciano JC, Muzykantov V, Penn MS, Hazen SL, Weisel JW, Ischiropoulos H. 2004. Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species. J Biol Chem 279:8820–8826.

Vercelli CA, Aisemberg J, Billi S, Cervini M, Ribeiro ML, Farina M, Franchi AM. 2009a. Anandamide regulates lipopolysaccharide-induced nitric oxide synthesis and tissue damage in the murine uterus. Reprod Biomed Online 18:824–831.

Vercelli CA, Aisemberg J, Billi S, Wolfson ML, Franchi AM. 2009b. Endocannabinoid system and nitric oxide are involved in the deleterious effects of lipopolysaccharide on murine decidua. Placenta 30:579–584.

Wang H, Dey SK, Maccarrone M. 2006a. Jekyll and hyde: Two faces of cannabinoid signaling in male and female fertility. Endocr Rev 27:427–448.

Wang H, Xie H, Dey SK. 2006b. Endocannabinoid signaling directs periimplantation events. AAPS J 8:E425–E432.

Wang H, Xie H, Guo Y, Zhang H, Takahashi T, Kingsley PJ, Marnett LJ, Das SK, Cravatt BF, Dey SK. 2006c. Fatty acid amide hydrolase deficiency limits early pregnancy events. J Clin Invest 116:2122–2131.

Warning JC, McCracken SA, Morris JM. 2011. A balancing act: Mechanisms by which the fetus avoids rejection by the maternal immune system. Reproduction 141:715–724.

Wilson R, McInnes I, Leung B, McKillop JH, Walker JJ. 1997. Altered interleukin 12 and nitric oxide levels in recurrent miscarriage. Eur J Obstet Gynecol Reprod Biol 75:211–214.

Wolfson ML, Aisemberg J, Salazar AI, Domínguez Rubio AP, Vercelli CA, Franchi AM. 2013. Progesterone reverts LPS-reduced FAAH activity in murine peripheral blood mononuclear cells by a receptor-mediated fashion. Mol Cell Endocrinol 381:97–105.

Wolfson ML, Correa F, Leishman E, Vercelli C, Cymeryng C, Blanco J, Bradshaw HB, Franchi AM. 2015a. Lipopolysaccharide-induced murine embryonic resorption involves changes in endocannabinoid profiling and alters progesterone secretion and inflammatory response by a CB1-mediated fashion. Mol Cell Endocrinol 411:214–222.

Wolfson ML, Schander JA, Bariani MV, Correa F, Franchi AM. 2015b. Progesterone modulates the LPS-induced nitric oxide production by a progesterone-receptor independent mechanism. Eur J Pharmacol 769:110–116.

Wu ZM, Yang H, Li M, Yeh CC, Schatz F, Lockwood CJ, Di W, Huang SJ. 2012. Pro-inflammatory cytokine-stimulated first trimester decidual cells enhance macrophage-induced apoptosis of extravillous trophoblasts. Placenta 33:188–194.