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Progesterone reverts LPS-reduced FAAH activity in murine peripheral blood mononuclear cells by a receptor-mediated fashion

Manuel L. Wolfson [a,*], Julieta Aisemberg [a], Ana I. Salazar [a], Ana P. Domínguez Rubio [a], Claudia A. Vercelli [b], Ana M. 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] Biomedicine Research Institute of Buenos Aires, National Research Council, Partner Institute of the Max Planck Society (MPSP), Ciudad Autónoma de Buenos Aires (CABA), Buenos Aires, Argentina

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ABSTRACT

Increased anandamide concentrations are associated with pregnancy failure. Anandamide levels are regulated by the fatty acid amide hydrolase (FAAH). The aim of the study was to investigate the role of progesterone (P) on FAAH modulation in murine peripheral blood mononuclear cells (PBMC) under septic conditions. We observed that *in vivo* administration of LPS to non-pregnant (NP) mice decreased FAAH activity of PBMC while in pregnant mice no changes in FAAH activity were observed. NP animals administered with P had a similar response to LPS as the pregnant animals. Also, NP mice injected with P antagonist and P showed that the effect of P on LPS-reduced FAAH activity was impaired. Furthermore, LPS produced a decrease in the ratio of PR-B/PR-A in NP animals.

Our results showed that, in our model the endotoxin decreased PBMC's FAAH activity and this condition was reverted by P in a receptor-mediated fashion.

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1. Introduction

Endocannabinoids are amides, esters, and ethers of long-chain polyunsaturated fatty acids found in several human tissues (Fowler et al., 2001; Hanus et al., 2001). N-arachidonoylethanolamine, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the main endocannabinoids described to date (Howlett and Mukhopadhyay, 2000; Sugiura and Waku, 2000). They bind to both brain cannabinoid receptors (CB1 and CB2), which are widely distributed in mammalian tissues (Sun and Dey, 2012) and their effects are terminated by their rapid uptake and subsequent intracellular degradation. Strong genetic and pharmacological evidence has demonstrated that fatty acid amide hydrolase (FAAH) inactivates AEA (Cravatt et al., 2001; Kathuria et al., 2003; Maccarroneet al., 2010). On the other hand, it has been reported that 2-AG is inactivated by monoacylglycerol lipase (MAGL) (Dinh et al., 2002; Bisogno et al., 1997, 2001; Beltramo and Piomelli, 2000). However, when MAGL protein is fully knocked down by RNA interference (RNAi), 50% of the 2-AG hydrolyzing activity remains in cell homogenates, indicating that additional enzymes may hydrolyze this lipid (Dinh et al., 2004). Prime candidates are FAAH and the cyclooxygenases (Kozak et al., 2000; Goparaju et al., 1998).

Anandamide and 2-AG are two of the best-studied members of the endocannabinoid family and they have been described as the major endocannabinoids present in the uterus suggesting that they might play a role in reproduction (Wang et al., 2007). The endogenous tone of AEA is the checkpoint for the regulation of its action. Evidence has been provided that low levels of AEA are favorable for implantation and trophoblast outgrowth while increased AEA concentrations are associated with retarded embryo development, fetal loss and pregnancy failure (Paria and Dey, 2000). Cravatt and Lichtman (2003) suggested that both *in vivo* AEA "tone" and biological activity are regulated by FAAH. A similar pattern of 2-AG was noted in the uterus during early pregnancy (Wang et al., 2007). Moreover, it has been reported that high plasma AEA levels are associated with early pregnancy loss in humans (Habayeb et al., 2008). In fact, in women undergoing *in vitro* fertilization and

Abbreviations: AEA, anandamide; 2-AG, 2-arachidonoylglycerol; CB, cannabinoid receptor EmRe: embryonic resorption FAAH: fatty acid amide hydrolase; LPS, lipopolysaccharide; LONA, Lonaprisan; NAPE-PLD, N-arachidonoyl phosphatidylethanolamine; MAGL, monoacylglycerol lipase; PBMC, peripheral blood mononuclear cells; P, progesterone; PR, progesterone receptor.

* Corresponding author. Address: Paraguay 2155, 16th floor, (C1121ABG), Ciudad Autónoma de Buenos Aires (CABA), Buenos Aires, Argentina. Tel.: +54 11 4508 3680x111; fax: +54 11 4508 3680x106.
E-mail address: manuwolfson@gmail.com (M.L. Wolfson).

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embryo transfer, high plasma AEA levels and low lymphocyte FAAH levels at 6 weeks after embryo transfer were associated with failure to achieve an ongoing pregnancy (Maccarrone et al., 2002). It has been suggested that FAAH present in peripheral T cells has a crucial role in controlling pregnancy (Maccarrone and Finazzi-Agrò, 2004; Bambang et al., 2012). Also, it was demonstrated recently that peripheral FAAH activity is significantly reduced in ectopic pregnancies (Gebeh et al., 2013).

Progesterone (P), a hormone essential not only for the establishment but also for the maintenance of pregnancy, is also known to modulate immune function (Correale et al., 1998) and to elicit an immunological response critical for normal gestation (Szekeres-Bartho et al., 1996). The need for progesterone is shown by the fact that blocking progesterone binding sites causes abortion or preterm labor in humans and various animal species (Elger et al., 1987; Winer et al., 2009). It has been reported that P upregulates FAAH activity in human lymphocytes through up-regulation of gene expression at transcriptional and translational level (Maccarrone et al., 2001a).

On the other hand, genital tract infections caused by Gram-negative bacteria induce miscarriage and are one of the most common complications of human pregnancy (Cram et al., 2002). Our previous results show that exposure of pregnant female mice (through i.p. injections) to lipopolysaccharide (LPS), a component of the cell walls of Gram-negative bacteria, leads to embryonic resorption (EmRe) followed by fetal expulsion (Ogando et al., 2003). Histological analysis of implantation sites from pregnant female mice challenged *in vivo* with LPS showed that the part of the decidua closer to the uterus was highly infiltrated by granulocytes and LGL cells (large granular lymphocytes) (Ogando et al., 2003). Moreover, LPS-induced EmRe is associated with an increased uterine and decidual production of nitric oxide (NO) and prostaglandins (PGs), two molecules that play key roles in this process (Ogando et al., 2003; Aisemberg et al., 2007). Furthermore, our previous work suggests that LPS could augment AEA levels in uterine explants by inhibiting its degradation Indeed, we demonstrated that AEA regulate LPS-induced NO production and tissue damage in the uterus of pregnant mice (Vercelli et al., 2009).

In addition, Maccarrone et al. (2001b) showed that LPS downregulates FAAH expression and increases AEA levels in human peripheral lymphocytes while Liu et al. showed that LPS induces AEA but not 2-AG in murine macrophages (Liu et al., 2003).

Taking into consideration that: (1) in our model of LPS-induced EmRe the decidua was highly infiltrated by granulocytes and LGL cells, (2) that LPS and P regulate endocannabinoid metabolism in human T lymphocytes and, (3) that high levels of endocannabinoids are associated with early pregnancy loss, our aim was therefore to investigate the role of P on FAAH modulation in murine peripheral blood mononuclear cells (PBMC) in our model of LPS-induced EmRe and to determine which P receptors if any were involved in this effect.

2. Materials and methods

2.1. Reagents

LPS from Escherichia coli 05:B55, anti-β-actin antibody and progesterone, were purchased from Sigma Chemical Co. (St Louis, MI, USA), [^{3}H]-anandamide (specific activity 172.4 Ci mmol^{-1}) was provided by Perkin Elmer (Boston, MA, USA). Thin Layer Chromatography (TLC) aluminum Silica Gel plates were purchased from Merk KGaA (Darmstadt, Germany). The western blotting reagents were obtained from Bio-Rad (Tecnolab, Argentina). Secondary horse radish peroxidase (HRP) conjugated antibody was purchased from Jackson Immunosearch (Baltimor Pike, USA). The anti-FAAH antibody was a gift from Dr. Benjamin Cravatt (Scripps, La Joya, San Diego USA). The anti-PR (C-19) rabbit polyclonal antibody was provided by Santa Cruz biotechnology (Tecnolab, Argentina). Anandamide and RU-486 were purchased from Biomol (Enzo Life Sciences, Miami, FL, USA).As RU486 is also an antagonist of glucocorticoid receptors, we decided to use the more specific and potent progesterone antagonist, Lonaprisan (ZK-230211) (Fuhrmann et al., 2000; Afhüppe et al., 2009), which was kindly provided by Bayer-Schering (Germany). 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

8- to 12-week-old virgin female BALB/c mice were paired with 8- to 12-week-old BALB/c males and the day of appearance of a coital plug was taken as day 0 of pregnancy. Animals were housed in cages under controlled conditions of light (14 h light, 10 h dark) and temperature (23–25 °C) and received murine chow and water ad libitum.

Non-pregnant mice were divided into six groups (Supplementary Fig. 1A): (i) *control*: females received an i.p. injection of vehicle and 14 h later were administered another i.p. injection of vehicle; (ii) *RU486/Lonaprisan/progesterone*: females received an i.p. injection of RU486 (10 μg g^{-1} of body weight) or Lonaprizan (LONA. 1 μg g^{-1} of body weight in 1:2 EtOH:NaCl 0.9%) or progesterone (P, subcutaneous (s.c.), 4 μg g^{-1} of body weight in oil) and 14 h later were administered another dose of RU486 or LONA or P; (iii) *LPS*: females received an i.p. injection of vehicle and 14 h later were administered an i.p. injection of LPS (1 μg g^{-1} of body weight); (iv) *LPS plus P*: females received a s.c. injection of P and 14 h later were administered an i.p. injection of LPS plus another s.c. injection of P; (v) *LPS plus P plus RU486*: females received a s.c. injection of P plus an i.p. injection of RU486. Fourteen hours later females were administered an i.p. injection of LPS plus another s.c. injection of P and another i.p. injection of RU486; (vi) *LPS plus P plus LONA*: females received a s.c. injection of P plus an i.p. injection of LONA. Fourteen hours later females were administered an i.p. injection of LPS plus another s.c. injection of P and another i.p. injection of LONA. Animals were euthanized 6 h after LPS administration.

Pregnant mice were divided into four groups (Supplementary Fig. 1B). On day 7 of pregnancy: (i) *control:* females received an i.p. injection of vehicle and 2 h later were administered another i.p. injection of vehicle; (ii) *LPS:* females received an intraperitoneal (i.p.) injection of vehicle and 2 h later were administered an i.p. injection of LPS (1 μg g^{-1} of body weight); (iii) *RU486*: females received an i.p. injection of RU486 (10 μg g^{-1} of body weight) and 2 h later were administered an i.p. injection of vehicle; (iv) *LPS plus RU486*: females received an i.p. injection of RU486 and 2 h later were administered an i.p. injection of LPS. Animals were euthanized 6 h after LPS or vehicle administration.

Note: administration of RU486 to pregnant animals produced embryonic resorption and fetal expulsion within a time frame of 12 h. Due to this, the dosing schedule was reduced from a 14 h to a 2 h time frame.

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

2.4. PBMC isolation

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, USA) 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.

2.5. T cell isolation

CD3 + T cells were obtained by negative selection using the Pan T Cell Isolation Kit II mouse (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacture's protocols. Briefly, PBMCs were incubated with biotin-conjugated monoclonal antibodies against CD11B, CD11c, CD19, CD45, CD45R (B220), CD49b (DX5), CD105, Anti-MHC class II, and Ter-119 as primary labeling. In a second step, cells were mixed with Anti-biotin monoclonal antibodies conjugated to MicroBeads. The magnetically labeled non-target cells are depleted by retaining them on a MACS column in the magnetic field of MACS separator, while the unlabeled T cells passed through the column.

2.6. 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]-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^{-1}) 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 (1976). Enzyme activity is reported as nmol [^{3}H]-AA (mg protein)$^{-1}$ h^{-1}. The optimal reaction conditions were previously determined (data not shown).

2.7. PCR analysis

Total RNA from PBMC was isolated using Trizol reagent according to the manufacturer's recommendations. Following extraction, RNA was quantified and further treated with RNAse-free DNAse I to digest contaminating genomic DNA. cDNA was synthesized from total RNA as described by Vercelli et al. (2009). Oligonucleotide primers for *PR-A+PR+B* and *PR-B* were synthesized using Primer 3 Input free Software (v 0.4.0) (Rozen and Skaletsky, 2000). *PR-A+PR-B* sense and antisense primers used were 5′-GGTGGGCCTTCCTAACGAG-3′ and 5′-GACCACATCAGGCTCAATGCT-3′, respectively, yielding a product of approximately 121 bp. PCR cycle parameters were as follows: an initial denaturizing step at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min followed by 72 °C for 5 min. *PR-B* sense and antisense primers used were 5′-GGTCCCCCTTGCTTGCA-3′ and 5′-CAGGACCGAGGAAAAAGCAG-3′, respectively, yielding a product of approximately 121 bp. PCR cycle parameters were as follows: an initial denaturizing step at 94 °C for 5 min followed by 40 cycles of 94 °C for 40 s, 61 °C for 40 s and 72 °C for 45 s followed by 72 °C for 5 min. Oligonucleotide primers for *FAAH* and *β-actin* were synthesized as described by Vercelli et al. (2009) and PCR cycle parameters were described in the same work. Seventeen microliters of the PCR reaction were loaded onto a 1.5% agarose gel, stained with ethidium bromide, recorded with a digital camera Olympus C-5060 under UV light and analyzed using the Image J software package (open source). Data were expressed as the relative amount of each PCR product versus β-actin mRNA.

2.8. Western blot analysis

PBMC were sonicated in RIPA buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1% Nonidet P40; Deoxicolate 0.25%; 10 μg ml^{-1} aprotinin; 10 μg ml^{-1} leupeptin; 1 mg ml^{-1} benzamidine; 1 mg ml^{-1} caproic acid; 10 μg ml^{-1} 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 sulfate–polyacrylamide gel for PR and FAAH and transferred to a nitrocellulose membrane. Blots were incubated overnight with anti-FAAH (1:500) or anti-PR (1:200) and 30 min with anti-actin (1:4000). Blots were washed with buffer (PBS and 0.1% (v v^{-1}) 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. Photographs of the membranes were taken using ImageQuant system (GE Healthcare, Buenos Aires, Argentina) and analyzed using the Image J software package.

2.9. Flow cytometry assay

PBMCs were stained with a fluorochrome-conjugated monoclonal antibody against CD3 (145-2C11, BD Biosciences. Franklin Lakes, NJ, USA) and subjected to flow cytometry analysis. Negative control samples were incubated with an isotype-matched Ab (PerCP-cy5.5 Hamster IgG1, k (A19-3), BD Biosciences. Franklin Lakes, NJ, USA). PBMC were acquired on a FACS Calibur cytometer (Becton Dickinson, CA, USA.) Data were analyzed by using Cyflogic software (Version 1.2.1 for non-commercial use).

2.10. Intracellular staining for detection of endogenous PR

Cell-surface staining was performed using anti-mouse monoclonal anti-FITC-CD3 (BD Biosciences, Franklin Lakes, NJ, USA) for 30 min at room temperature (RT) followed by PBS washing. Intracellular staining for detection of endogenous PR was as follows: PBMC were fixed in 1 ml of PBS with 1% paraformaldehyde. Cells were permeabilized for 20 min at RT with PBS + Triton X-100 0.3 + 5% normal goat serum. Cells were incubated with mouse monoclonal antibody anti-PR (hPRa7, Thermo Scientific, Kalamazoo, MI, USA) for 1 h at RT and washed with permeabilization solution. PR mAb binding was detected using Cy3 goat anti-mouse-IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and washed as described above. PBMC were mounted and examined by fluorescence microscopy (Nikon, Japan).

2.11. Statistical analyses

Treatments were assigned completely random to experimental units. Data were analyzed by means of ANOVA procedures and means were compared by Tukey's test. Differences between means were considered significant when p value was 0.05 or less. Different letters indicate significant differences between means. Normality and Homogeneity of variances were tested by Shapiro–Wilks (modified) and Levene test, respectively. In each figure, dissimilar letters denote significantly different values. Statistical analysis was performed using the Graph Pad Prism (San Diego, CA, USA).

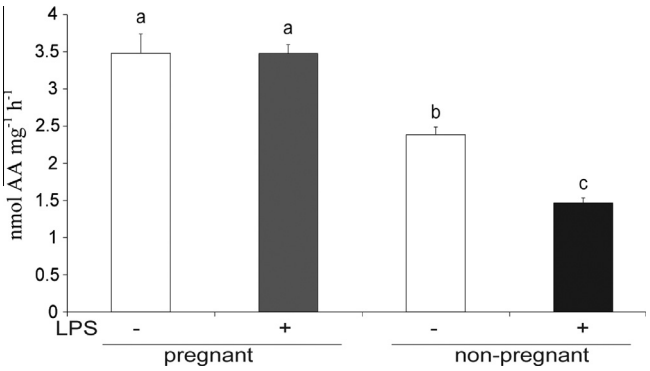

Fig. 1. Effect of LPS on PBMC FAAH activity in pregnant and non-pregnant mice. Pregnant (day 7 of pregnancy) and non-pregnant mice received an i.p. injection of vehicle or LPS and were sacrificed 6 h after LPS administration to assess PBMC's FAAH activity. ANOVA test. Different letters indicate statistically significant differences ($p < 0.05$). $n = 5$ animals per group.

3. Results

3.1. PBMC FAAH activity in pregnant and non-pregnant mice

We first studied whether PBMC from pregnant and non-pregnant mice showed differences in the activity of this enzyme. An important finding in this study was the statistically significant increase (almost 32%) in PBMC FAAH activity in the pregnant group compared to the non-pregnant group (Fig. 1).

3.2. Effect of LPS on PBMC FAAH activity in pregnant and non-pregnant mice

Next we evaluated the effect of LPS on PBMC FAAH activity from pregnant and non-pregnant mice. Fig. 1 shows that *in vivo* administration of LPS to non-pregnant mice significantly decreased FAAH activity of PBMC. Nevertheless, PBMC from pregnant mice challenged *in vivo* with the endotoxin showed no changes in FAAH activity when compared to the control group (Fig. 1).

3.3. Role of P on PBMC FAAH activity in pregnant mice challenged in vivo with LPS

To evaluate whether the differences in FAAH activity between the LPS-challenged group and the control group were attributable to P, pregnant mice challenged with LPS were administered with RU486 (an antagonist of P receptors (PR)) and PBMC FAAH activity was assessed. Our results showed that PBMC FAAH activity remained unchanged when pregnant mice were challenged with LPS or RU486 alone (Fig. 2A). However, when pregnant animals were co-administered both drugs, PBMC FAAH activity was significantly reduced. On the other hand, we observed that PBMC FAAH activity was diminished 12 h post-administration of LPS (Fig. 2B).

To verify if P was modulating FAAH activity, non-pregnant mice were injected with P plus LPS plus RU486. Consistent with the results showed above, we observed that administration of LPS alone to non-pregnant mice significantly reduced PBMC FAAH activity (Fig. 3). When animals were administered with P plus LPS, the effect of the endotoxin on PBMC FAAH activity was prevented (Fig. 3). Moreover, when mice were injected with P plus LPS plus RU486 the effect of P on LPS-reduced FAAH activity was significantly more decreased (Fig. 3). In agreement with our previous results, Fig. 3 shows that when mice were injected with P plus LPS plus LONA the effect of P on LPS-reduced FAAH activity was also highly significantly impaired. These results suggest that P could be modulating FAAH activity in pregnant mice challenged with LPS, most likely through P receptors rather than glucocorticoid receptors.

3.4. Role of P on PBMC FAAH protein and mRNA content in non-pregnant mice challenged with LPS

We next evaluated whether P could modulate FAAH protein and mRNA levels in PBMC from non-pregnant mice challenged with LPS. Our results show that administration of LPS to non-pregnant mice significantly reduced PBMC FAAH protein and mRNA content (Fig. 4). Moreover, when non-pregnant animals were injected with P plus LPS, the effect of the endotoxin on PBMC FAAH protein and mRNA levels was prevented. In addition, when non-pregnant mice were administered with P plus LPS plus RU486 or LONA, the effect of P on LPS-reduced FAAH protein and mRNA content was significantly impaired. Similarly to the PBMC FAAH activity results shown above, these results suggest that P could be modulating FAAH protein and mRNA levels in non-pregnant mice challenged with LPS, probably through P receptors rather than glucocorticoid receptors.

3.5. Effect of LPS on PBMC progesterone receptors (PR) in non-pregnant mice

We first sought to determine the presence of progesterone receptors, PR-A and PR-B, by RT-PCR and western blot in PBMC from non-pregnant mice. Both PR-A and PR-B are products of a single gene. PR-A is a truncated form of PR-B, lacking the N-terminal

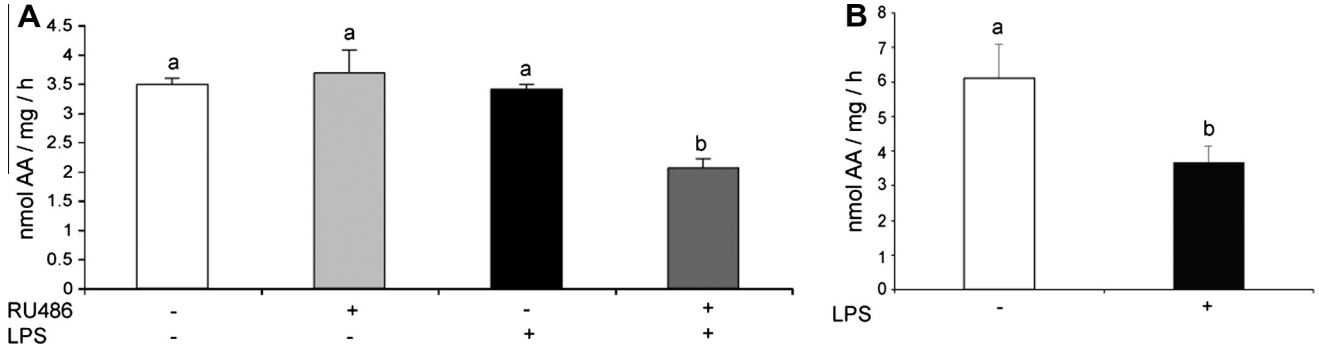

Fig. 2. Role of P on PBMC FAAH activity in pregnant mice challenged *in vivo* with LPS. A- Pregnant mice received an injection of vehicle, RU486, LPS or LPS plus RU486 on day 7 of pregnancy and were sacrificed 6 h after LPS administration to assess PBMC's FAAH activity. ANOVA test. Different letters indicate statistically significant differences ($p < 0.05$). $n = 7$ animals per group. B- Pregnant mice received an injection of vehicle or LPS on day 7 of pregnancy and were sacrificed 12 h after LPS administration to assess PBMC's FAAH activity. Student *T* test. Different letters indicate statistically significant differences ($p < 0.05$). $n = 5$ animals per group.

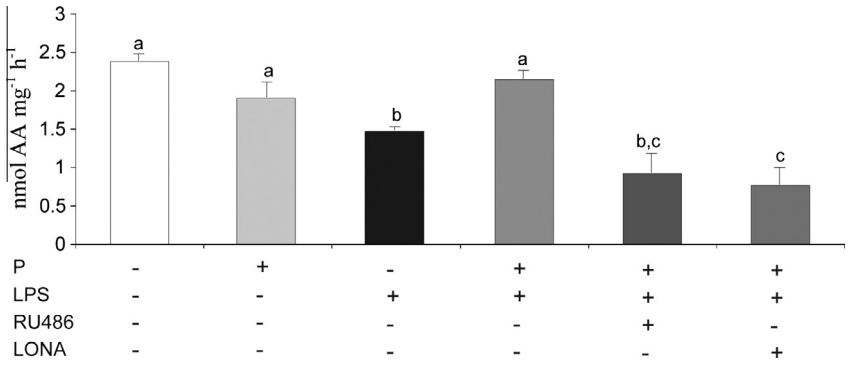

Fig. 3. P modulated FAAH activity through P receptors. Non-pregnant mice were injected with the different drugs and were sacrificed to assess PBMC's FAAH activity. ANOVA test. Different letters indicate statistically significant differences ($p < 0.05$). $n = 4$ animals per group.

164 amino acids. (Conneely et al., 1989; Kastner et al., 1990). For this reason, two sets of primers were designed: the first set was designed to amplify cDNA from a region common to both PR subtypes; the other set was designed to amplify cDNA from a region common to PR-B only. Reverse transcription-PCR amplification of mRNA showed a single band of the expected size for both cDNA products (PR-A plus PR-B and PR-B alone) (Fig. 5) in PBMC from non-pregnant control animals.

We next assessed the presence of PR-A and PR-B by western blot in PBMC from non-pregnant mice. Fig. 6 shows that both receptors are present in these cells. Also, we showed that PR-B protein levels are down-regulated in PBMC from animals challenged with LPS (Fig. 6B) although PR-A protein levels remained unchanged (Fig. 6A). As mentioned before, our results suggest that P could modulate FAAH activity, protein and mRNA levels in PBMC from non-pregnant mice challenged with LPS most likely through P receptors. In this sense, we next determined whether P could be involved in the regulation of the expression of its own receptors. Fig. 6B shows that when animals were co-injected with P plus LPS, the effect of the endotoxin on PBMC PR-B protein levels was abrogated. In addition, when mice were co-administered P plus LPS plus RU486, the effect of P on LPS-reduced PR-B protein content was significantly impaired. These results suggest that administration of the endotoxin to non-pregnant mice reduced PR-B

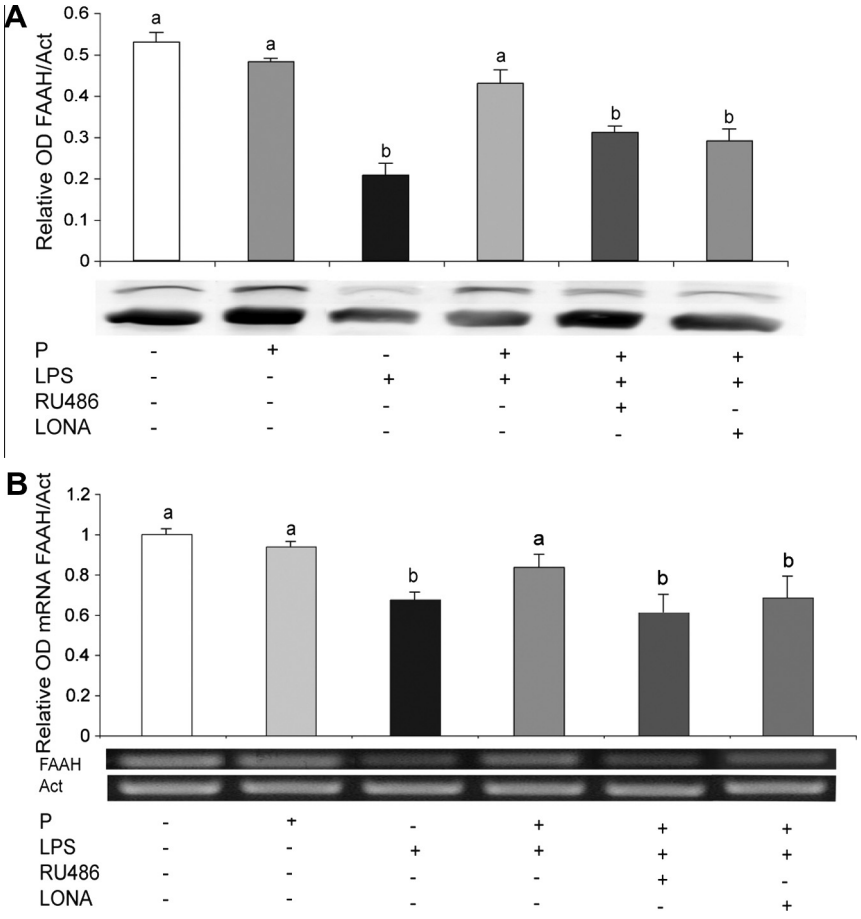

Fig. 4. Role of P on PBMC FAAH protein and mRNA content in non-pregnant mice challenged with LPS. Non-pregnant mice were injected with the different drugs and were sacrificed to assess PBMC's FAAH expression. Densitometric analysis of (A) protein and (B) mRNA expression of FAAH. ANOVA test. Different letters indicate statistically significant differences ($p < 0.05$). $n = 5$ animals per group. One representative blot/gel is shown.

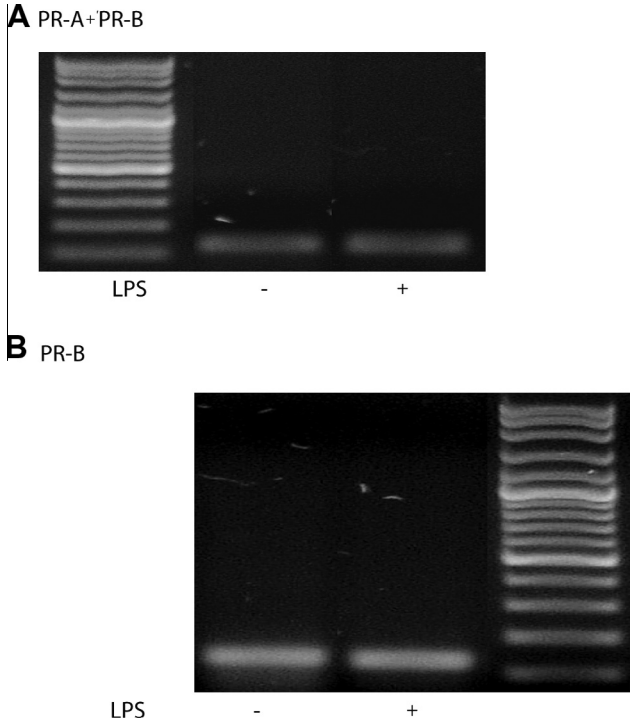

Fig. 5. Presence of PR mRNA in non-pregnant mice PBMCs. Non-pregnant mice received an i.p. injection of vehicle or LPS and were sacrificed 6 h after LPS administration to assess PR mRNA on PBMCs. Top panel, PR-A + PR-B. Low panel, PR-B alone. $n = 4$ animals per group. One representative gel is shown.

protein levels and this effect could be mediated through its own receptors.

3.6. T lymphocytes: possible candidates among PBMCs?

We evaluated the proportion of $CD3^+$ cells in Histopaque-derived cell preparation. FACS analysis of PBMC from non-pregnant mice indicated that almost 75% of the cell population was $CD3^+$ (data not shown). Next we isolated T-cells from the whole PBMC population by means of the MACS cell separation system (Fig. 7A) and the presence of PR-A and PR-B receptors was determined by RT-PCR and immunocytochemistry (ICC). Reverse transcription-PCR amplification of mRNA showed a single band of the expected size for both cDNA products (PR-A plus PR-B and PR-B alone, Fig. 7B). Moreover, ICC analysis showed that cells that were $CD3^+$ were also PR^+ (Fig. 7C).

4. Discussion

Previous work showed that FAAH serves as a metabolic gatekeeper for the regulation of on-site anandamide levels in order to maintain an *in vivo* endocannabinoid tone conducive to normal development of preimplantation embryos and their on-time homing into uterus. We first investigated the role of P on murine PBMC FAAH modulation and what first caught our attention was the fact that there was a statistically significant increase (almost 32%) in PBMC FAAH activity in the pregnant group compared to the non-pregnant group. Murine P serum levels rise almost ten times during pregnancy (Virgo and Bellward, 1974) and taking into account that P upregulates FAAH activity, this was the first clue that led us to suspect that P could be responsible for the difference in FAAH activity between groups. We observed that *in vivo* administration of LPS to non-pregnant mice significantly decreased PMBC's FAAH activity. Moreover, administration of the endotoxin to pregnant mice showed different effects after 6 h and 12 h post-injection. While LPS was unable to affect the enzyme activity of PBMCs from pregnant mice after 6-h LPS treatment, the endotoxin produced a significant reduction in PBMC's FAAH activity after 12 h. In agreement with these results, Aisemberg et al. (2013) showed that mice treated with LPS (1 μg/g) on day 7 of pregnancy display only a 10% reduction in progesterone serum levels 6 h post-injection whereas animals treated with LPS for 12 h showed a 60% decrease. These results not only suggest that LPS modulates FAAH activity (in accordance to previously reported results (Maccarrone et al., 2001b; Liu et al., 2003)), but also that P acts as a "protective" agent in pregnant mice preventing a fall in FAAH activity. In keeping with these data, pregnant mice challenged with LPS were previously administered with the P receptor antagonist RU486. We observed that LPS had the same effect on FAAH activity in pregnant animals injected with RU486 than in non-pregnant mice.

Our hypothesis was corroborated with the observation that P abrogated LPS-reduced FAAH activity in non-pregnant mice. In this sense, non-pregnant animals administered with P had a similar response to LPS as the pregnant mice. With the aim of investigating whether this effect of P was mediated by the hormone receptors, we injected non-pregnant mice with RU-486 before the administration of P and then the animals were challenged with the endotoxin. In these animals, the effect of P on LPS-reduced FAAH activity was significantly impaired. Indeed, when we tried a more specific and potent P antagonist (LONA), the effect of P on LPS-reduced FAAH activity was also significantly impaired suggesting that P modulates FAAH activity most likely through P receptors rather than glucocorticoid receptors. In the same way, our results show that P modulated FAAH protein and mRNA levels in non-pregnant mice challenged with LPS probably through the same type of receptors. As we mentioned before, in addition to its role in the establishment and maintenance of pregnancy (Conneely et al., 2002), several studies have demonstrated that P has immune suppressive properties (Butts et al., 2007, 2008). In our case, PBMC's FAAH activity remained constant in pregnant mice challenged *in vivo* with LPS probably due to the presence of high levels of serum P, which are characteristic during pregnancy. This elevated P levels could be preventing a decrease in FAAH activity thus, keeping plasma endocannabinoid levels low to avoid its negative effects on pregnancy.

The majority of cellular actions of P are mediated through intracellular P receptors (PRs), PR-A and PR-B, which are well-studied gene regulators (Conneely et al., 2002). De León-Nava et al. (2009) described the presence of PRs on murine splenocytes. We showed the presence of these receptors in murine PBMCs. We also demonstrated that LPS reduced PR-B protein levels and this effect could be modulated through P receptors. It is known that P regulates the levels of its own receptors (Katzenellenbogen, 1980), and these levels as well as the stoichiometric ratio of PR-A to PR-B in reproductive tissues vary as a consequence of developmental (Shyamala et al., 1990) and hormonal status (Duffy et al., 1997). PR-B has been shown to function as a strong activator of transcription of several PR-dependent promoters and in a variety of cell types in which PR-A is inactive. In addition, when the PR-A and PR-B proteins are co-expressed in cultured cells, the PR-A can repress the activity of PR-B as well as the activity of other nuclear receptors (Giangrande and McDonnell, 1999). Our results are in line with previous reports that show that the predominant isoform of PR in fetal membranes of women undergoing elective cesarean delivery at term is PR-B, while patients with a vaginal delivery show a PR-A isoform predominance. Also, the ratio of PR-A/PR-B in fetal membranes is significantly higher in women in labor than in those who were not in labor (Oh et al., 2005). Tan et al. (2012) also reported that when human myometrial cells are PR-B dominant, P promotes myometrial quiescence through PR-B-mediated

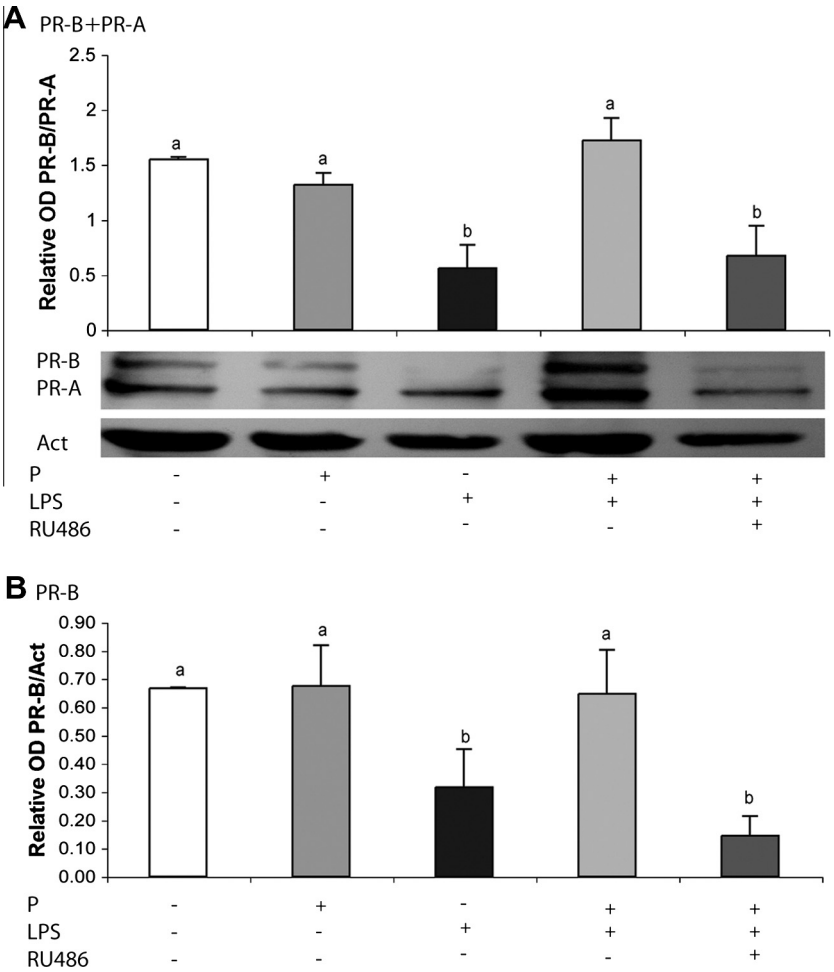

Fig. 6. LPS reduced PR-B protein levels through P receptors. Non-pregnant mice were injected with the different drugs and were sacrificed to assess PR protein levels on PBMCs. (A) Top panel, PR-B/PR-A. (B) Low panel, PR-B alone. ANOVA test. Different letters indicate statistically significant differences ($p < 0.05$). $n = 3$ animals per group. One representative blot is shown.

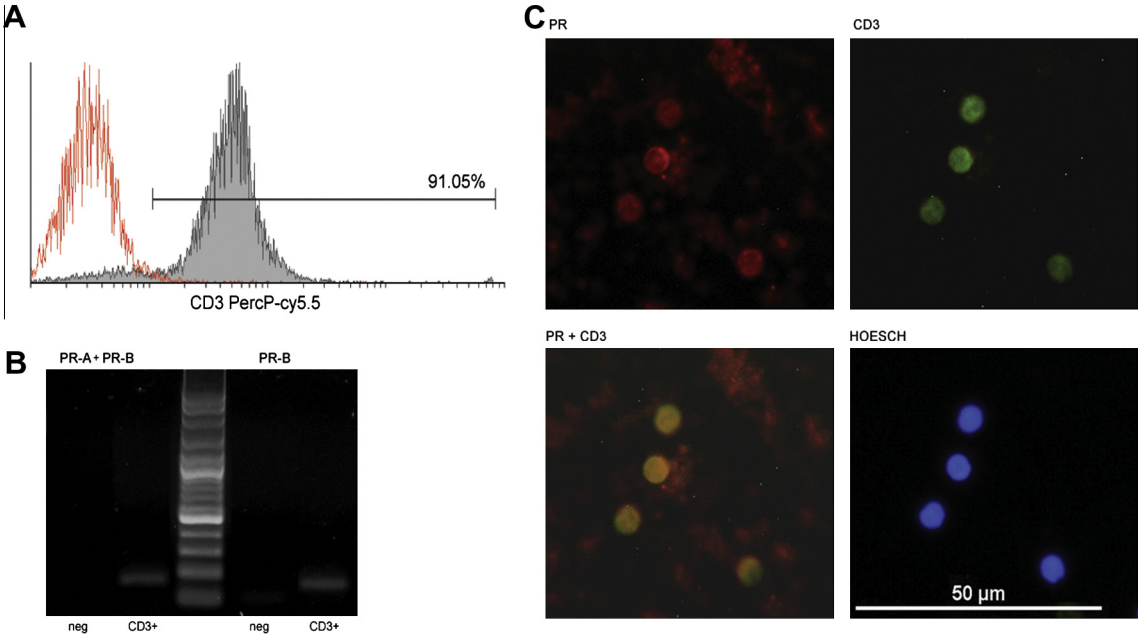

Fig. 7. CD3 + cells also express PR. T-cells were isolated from the whole PBMC population by means of the MACS cell separation system and the presence of PR-A and PR-B receptors was determined by RT-PCR and immunocytochemistry (ICC). (A) Left top panel, FACS analysis of CD3 + cells; (B) left low panel, mRNA expression of PRs (one representative gel is shown); (C) right panel, ICC analysis for CD3 + PR + cells. $n = 4$ animals per group.

anti-inflammatory actions. At parturition, the rise in PR-A expression promotes labor by inhibiting the anti-inflammatory actions of PR-B and stimulating pro-inflammatory gene expression in response to P (Tan et al., 2012). This would promote a fall in FAAH activity that could increase plasma endocannabinoid levels and probably augment pro-inflammatory gene expression which would contribute to embryonic resorption.

Maccarrone et al. (2000) showed that FAAH activity was lower in the lymphocytes of women who had miscarriages than in those of women who did not. According to this, the role of FAAH seems to be critical as there is an association between decreased FAAH activity and expression in maternal peripheral lymphocytes and early pregnancy failure in humans, demonstrating that an impairment of endocannabinoids degradation might be connected with reduced fertility (Gebeh et al., 2013). On the other hand, taking into consideration that high plasma AEA levels are also associated with early pregnancy loss in humans (Habayeb et al., 2008; Bambang et al., 2012) and high plasma AEA levels at 6 weeks after embryo transfer are associated with failure to achieve an ongoing pregnancy in women undergoing *in vitro* fertilization and embryo transfer (Maccarrone et al., 2002), it would be interesting to measure endocannabinoid concentrations in plasma after LPS challenge in our model to confirm the association mentioned above.

Peripheral lymphocytes have a critical role in embryo implantation and successful pregnancy in humans (Piccinni et al., 1998). As previously reported, the early stages of spontaneous abortion in humans (<8 weeks) are characterized by decreased activity and expression of FAAH in maternal T cells and by increased levels of AEA in blood (Maccarrone et al., 2002; Butts et al., 2007). On the other hand, P upregulates the levels of FAAH, but not those of NAPE-PLD, the AEA transporter (AMT) or CB receptors, in human lymphocytes, thus reducing the AEA content of these cells (Maccarrone et al., 2000, 2001a). Based on this evidence, and taking into account that there are no published results that describe the presence of nuclear PR in murine peripheral lymphocytes, we isolated T-cells from the whole PBMC population by means of the MACS cell separation system and assessed the presence of PR-A and PR-B receptors. Our results showed that CD3+ cells expressed PR-A + PR-B mRNA and PR-B mRNA. Also, we showed that cells that were CD3+ were also PR+. These results suggest that, among PBMCs, T-lymphocytes have the machinery to respond to P. Although we have performed all our experiments on PBMCs and hypothesized that T-lymphocytes could be the main cell population that contributes to the modulatory effect of P on FAAH activity in our model, we cannot rule out that other cell populations are present in the Histopaque-derived cell preparation (almost 25%). Moreover, among mononuclear cells, monocytes are included and it has been reported that mouse RAW264.7 macrophages express FAAH. Nonetheless, LPS treatment of these cells upregulates FAAH expression and activity (Liu et al., 2003). On the other hand, despite the fact that LPS-activated lymphocytes could contribute to fetal expulsion, and FAAH might be critical in this process, we cannot rule out the participation of other cells and pro-inflammatory molecules such as cytokines, prostaglandins and lipid mediators that participate in LPS-induced EmRe (Ogando et al., 2003; Aisemberg et al., 2007).

In conclusion, our work shows that in our model the endotoxin decreased PBMC's FAAH activity and this condition was reverted by P.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2013.07.020.

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