European Journal of Pharmacology ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and inflammation

Progesterone modulates the LPS-induced nitric oxide production by a progesterone-receptor independent mechanism

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ARTICLE INFO

Article history:
Received 7 May 2015
Received in revised form
30 October 2015
Accepted 2 November 2015

Keywords:
Progesterone
Nitric oxide
Glucocorticoid receptors
RU486
Lonaprisan

ABSTRACT

Genital tract infections caused by Gram-negative bacteria induce miscarriage and are one of the most common complications of human pregnancy. LPS administration to 7-day pregnant mice induces embryo resorption after 24 h, with nitric oxide playing a fundamental role in this process. We have previously shown that progesterone exerts protective effects on the embryo by modulating the inflammatory reaction triggered by LPS. Here we sought to investigate whether the *in vivo* administration of progesterone modulated the LPS-induced nitric oxide production from peripheral blood mononuclear cells from pregnant and non-pregnant mice. We found that progesterone downregulated LPS-induced nitric oxide production by a progesterone receptor-independent mechanism. Moreover, our results suggest a possible participation of glucocorticoid receptors in at least some of the anti-inflammatory effects of progesterone.

1. Introduction

Progesterone is a steroid hormone with pleiotropic effects on the female reproductive organs and breasts as well as on extra-reproductive tissues in both male and female, such as brain, bone and the immune system (Kyurkchiev et al., 2010). Progesterone is considered an essential hormone for the initiation and maintenance of pregnancy. Besides its endocrine functions, progesterone is also considered to be an immunosteroid (Szekeres-Bartho et al., 2001). Progesterone-driven modulation of the maternal immune system is required to enable pregnancy to proceed to term since it protects the "semi-allogeneic" conceptus from immunological rejection.

Progesterone exerts its function by binding to specific receptors present in reproductive and non-reproductive tissues, including the immune system. Classically, progesterone binds to the two isoforms of intracellular progesterone receptor (PR-A and PR-B) and actives or suppresses gene transcription, but also triggers rapid non-genomic signaling. PR-A and PR-B expression has been described in the lymphocytes of pregnant (Szekeres-Bartho et al., 1989) and non-pregnant women and men (Hughes et al., 2013); as well as in the peripheral blood mononuclear cells (PBMC) from non-pregnant mice (Wolfson et al., 2013). More recently, new binding sites for progesterone, such as three novel membrane receptors (mPR) (Dosiou et al., 2008; Fernandes et al., 2008) and the progesterone receptor membrane components (PRMC-1 and PRMC-2) (Pru K. and Clark C., 2013), have been characterized. Furthermore, at high physiological concentrations, such as those occurring during pregnancy, progesterone may bind and signal via the glucocorticoid receptors (GR) (Leo et al., 2004; Sugino et al., 1997; Xu et al., 1990). In fact, it has been suggested that some of the immunomodulatory effects of progesterone might be mediated through signaling via GR and not via PR (Jones et al., 2008; Lei et al., 2012). However, more research is still needed in order to fully understand the molecular mechanisms involved in progesterone anti-inflammatory actions.

Genital tract infections by Gram negative bacteria are associated with increased inducible nitric oxide synthase expression and pathological nitric oxide (NO) production (Johnson et al., 2012; Ramsey et al., 2001). NO is a short-lived molecule formed by the action of nitric oxide synthases (NOS), which exist in three isoforms: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) (Kröncke et al., 1997). Typically, local synthesis of NO in reproductive tissues plays an important role regulating

Abbreviations: 21OH-6, 19OP, 21-hydroxy-6,19-epoxyprogesterone; GR, glucocorticoid receptors; i.p., intraperitoneal; iNOS, inducible nitric oxide synthase; LONA, lonaprisan; LPS, lipopolysaccharide; mPR, membrane progesterone receptor; NO, nitric oxide; NOS, nitric oxide synthase; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PR, progesterona receptors; PRMC, progesterone receptor membrane components; s.c., subcutaneous

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http://dx.doi.org/10.1016/j.ejphar.2015.11.005
0014-2999/

endometrial, cervical and myometrial activity (Telfer et al., 1995) as well as in embryo implantation (Battaglia et al., 2003) and embryo development (Chwalisz et al., 1999). However, a dysregulated NO production has been associated with embryo cytotoxicity (Barroso et al., 1999), early embryo loss (Haddad et al., 1995; Ogando et al., 2003) and preterm labor (Cella et al., 2010; Chadha et al., 2007; Törnblom et al., 2005). Therefore, a tight regulation of NO production is required for a successful pregnancy. Indeed, women in their first trimester of pregnancy show lower plasma NO levels when compared to non-pregnant women (Paradisi et al., 2007; Wilson et al., 1997).

Previous works from our lab have highlighted the protective role of progesterone in modulating the innate immune response in an animal model of early pregnancy loss induced by inflammation (Aisemberg et al., 2013; Wolfson et al., 2013). Intraperitoneal administration of lipopolysaccharide (LPS), a component of the cell walls of Gram-negative bacteria, to pregnant mice, induces infiltration of the decidua with granulocytes and large granular lymphocytes (LGL), increased uterine and decidual production of nitric oxide (NO) these changes leads to a 100% of embryonic resorption and fetal expulsion (Ogando et al., 2003). In a previous work from our lab, we have reported that progesterone administration exerted anti-inflammatory effects resulting in the protection of the embryo in our model of LPS-induced miscarriage (Aisemberg et al., 2013). Moreover, we have recently shown that LPS induced a lower embryo resorption rate in CB1 *knock-out* mice compared to wild-type mice (Wolfson et al., 2015). Furthermore, this effect was associated with a feebler decidual NOS activity in the CB1 *knock-out* mice (Wolfson et al., 2015). Similarly, we have previously shown that the *in vivo* administration of progesterone was able to restore the enzymatic activity of the fatty acid amide hydrolase (FAAH), which catabolize endocannabinoids, in a PR-dependent manner, in the PBMC from non-pregnant mice (Wolfson et al., 2013).

The main aim of this work is to analyze the effects of *in vivo* administration of progesterone to the LPS-induced PBMC's production of nitric oxide (NO) and nitric oxide synthase (NOS) activity in our model of LPS-induced embryo loss as well as the participation of the progesterone and/or glucocorticoid receptors.

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). 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). RU486 (mifepristone) was purchased from Biomol (Enzo Life Sciences, Miami, FL, USA). Lonaprisan (LONA, ZK-230211) (Fuhrmann et al., 2000; Afhüppe et al., 2010) was kindly provided by Bayer-Schering (Germany). 21-hydroxy-6,19-epoxyprogesterone (21OH-6,19OP) was a kind gift from Dr. Adalí Pecci (School of Science, University of Buenos Aires, Argentina). 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

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 twelve-week-old virgin female Balb/c mice were paired with eight to twelve-week-old Balb/c males respectively. The day of appearance of a coital plug was taken as day 0 of pregnancy. Next, 7-days pregnant Balb/c mice were divided into four groups: (i) control group received an i.p. and 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 progesterone-treated group received a s.c. injection of progesterone (67 μg/g of body weight in corn oil, as previously used in 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.

Non-pregnant Balb/c mice were divided into eight groups: (i) control group received an i.p. and s.c. injection of vehicle; (ii) progesterone-treated group received a s.c. injection of progesterone (4 μg/g of body weight in corn oil) and 14 h later were administered another dose of progesterone; (iii) LPS-treated group received an i.p. injection of LPS (1 μg/g of body weight in saline solution); (iv) LPS plus progesterone-treated group received a s.c. injection of progesterone and 14 h later were administered another dose of progesterone and the first dose of LPS; (v) LPS plus progesterone plus RU486 group received a i.p. injection of RU486 (10 μg/g of body weight in EtOH:NaCl 0,9%) and progesterone and 14 h later were administered another dose of RU486 and progesterone and the first dose of LPS; (vi) LPS plus progesterone plus LONA group received a i.p. injection of lonaprisan (1 μg/g of body weight in EtOH:NaCl 0.9%) and progesterone and 14 h later were administered another dose of lonaprisan and progesterone and the first dose of LPS; (vii) DEXA group received a i.p. injection of dexametasone (1.5 μg/g of body weight in saline solution) and 14 h later were administered vehicle (saline solution i.p.); (viii) LPS plus DEXA group received a i.p. injection of dexametasone (1.5 μg/g of body weight in saline solution) and 14 h later were administered a dose of LPS.

In all cases, 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.

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

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, 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.

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2.5. Culture of PBMC

PBMC were obtained from control animals as previously described. Cells were cultured in fresh serum-free DMEM and stimulated with LPS (1 μg/ml) in the presence or absence of progesterone (50 ng/ml). In a set of experiments, the GR antagonist 21OH-6,19OP (10 μM) was added 30 min prior the stimulation with LPS and/or progesterone. Cells were incubated for 4 h in 5% CO_2 at 37 °C. Next, cells were centrifuged, washed with ice-cold PBS and processed for total RNA extraction.

2.6. Nitrates and nitrites assay

PBMC from treated mice were cultured in wells that contained 250 μl DMEM supplemented with 10% FBS and antibiotics: 20 iu/ml penicillin G, 20 μg/ml streptomycin and 50 ng/ml amphotericin B. Cells were maintained for 24 h in 5% CO_2 at 37 °C and then culture supernatants were immediately frozen at −70 °C until used for nitrates and nitrites quantification.

Nitric oxide produced by PBMC was measured as nitrate (NO^{3-}) plus nitrite (NO^{2-}) in culture supernatants using the Griess reaction, a colorimetric method for the detection of nitrites, described by Grisham et al. (1996). Briefly, culture supernatants, 2 mM NADPH and 10 U/ml *Aspergillus niger* nitrate reductase were allowed to react in flat-bottomed 96-well culture plates with gentle mixing for 30 min at room temperature. Next, 100 mM pyruvic acid and 1000 U/ml lactate dehydrogenase were added and incubated for 10 min. Later, 10 mg/ml sulphanilic acid was added and incubation continued for 10 min. Finally, 1 mg/ml naphthyl–ethylenediamine was added and incubated for 5 min in the dark. The absorbance of the colored product was measured at 540 nm, using 595 nm readings as reference wavelength to compensate for non-specific absorbance. Media supplemented with FBS were cultured without any cell and used as a blank. The concentration of NO^{3-} plus NO^{2-} was deduced from a standard nitrite curve. Results were expressed as μM $NO^{2-}/10^{-6}$ cells.

2.7. PCR analysis

Total RNA from PBMC from Balb/c mice was isolated using Trizol reagent according to the manufacturer's recommendations (Invitrogen, California, USA). Following extraction, RNA was quantified and further treated with RNAse-free DNAse I to digest contaminating genomic DNA. Subsequently, RNA was quantified and cDNA was synthesized from total RNA (3 μg) using M-MLVRT, random primers and ribonuclease inhibitor. PCR was performed with specific primers designed using the Primer3 Software package and checked for self-complementarity with OligoCalc Software package. iNOS forward primer: 5′-ACCACTCGTACTTGGGATGC-3′, iNOS reverse primer: 5′-CACCTTGGAGTTCACCCAGT-3′; GR forward primer: 5′-TTCTGTTCATGGCGTGAGTACC-3′, GR reverse primer: 5′-CCCTTGGCACCTATTCCAGTT-3′ and β-Actin forward primer: 5′-TGTTACCAACTGGGACGACA-3′, β-actin reverse primer: 5′-TCTCAGCTGTGGTGGTGAAG-3′. PCR cycle parameters were as follows: an initial denaturing step at 94 ºC for 3 min followed by 30 cycles of 94 ºC for 40 s, 60 ºC (GR), 57 ºC (β-actin) or 55 ºC (iNOS) for 40 s and 72 ºC for 40 s followed by 72 ºC for 5 min. A 2% agarose gel was loaded with 18 μl of the PCR reaction and separated DNA bands were visualized on a transilluminator after ethidium bromide staining. Images were taken using a digital camera Olympus C-5060 and analyzed using the Image J software package (open source). Relative amount of target PCR product was calculated as a ratio of each target PCR product versus β-actin PCR product. Next, relative fold change was expressed as the ratio of LPS-treated cases vs control cases.

2.8. Western blot analysis

PBMC were sonicated in RIPA buffer (50 mMTris, pH 7.4; 150 mMNaCl; 1% Nonidet P40; Deoxicolate 0.25%; 10 μg/mlaprotinin; 10 μg/mlleupeptin; 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 sulfate-polyacrylamide gel for iNOS and transferred to a nitrocellulose membrane. Blots were incubated overnight with anti-iNOS (1:500) and 30 minutes with anti-actin (1:4000). Blots were washed with buffer (PBS and 0.1% (v/v) Tween 20, pH 7.5) followed by 1 h incubation with horse radish peroxidase-conjugated anti-rabbit secondary antibody (1:5000) and developed using the enhanced chemiluminescence western blot system. Photographs of the membranes were taken using ImageQuant system (GE Healthcare, Buenos Aires, Argentina) and analyzed using the Image J software package.

2.9. 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).

3. Results

3.1. Effect of LPS on PBMC nitric oxide (NO) production in pregnant mice

We first studied the effect of LPS on NO synthesis in the PBMC from pregnant mice. We observed a significant increase in NO production at 6 h after LPS treatment (Fig. 1). Previously, we have described the protective effect of progesterone on early pregnancy loss by modulating the fatty acid amide hydrolase (FAAH) activity on PBMC from LPS-treated mice (Wolfson et al., 2013). In order to further study the protective actions of progesterone in our model of LPS-induced early pregnancy loss, we analyzed the effects of

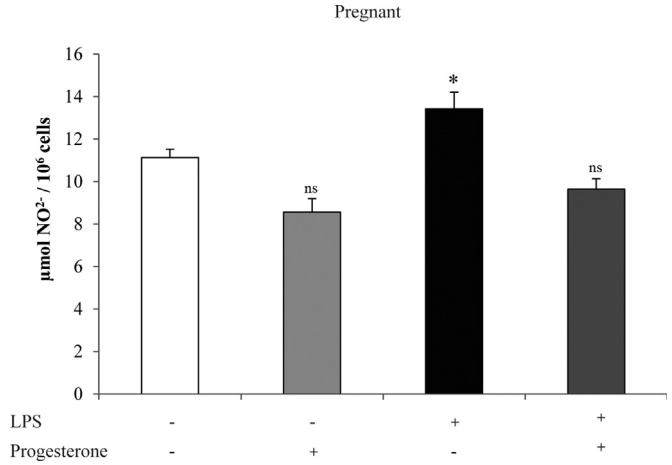

Fig. 1. Six h after injection, LPS (1 μg/g body weight) induces a significant increase in NO production in PBMC from pregnant mice. Co-administration of progesterone (67 μg/g body weight) abrogates this effect. Statistics: $^{*}P < 0.05$ ($n = 4$–8).

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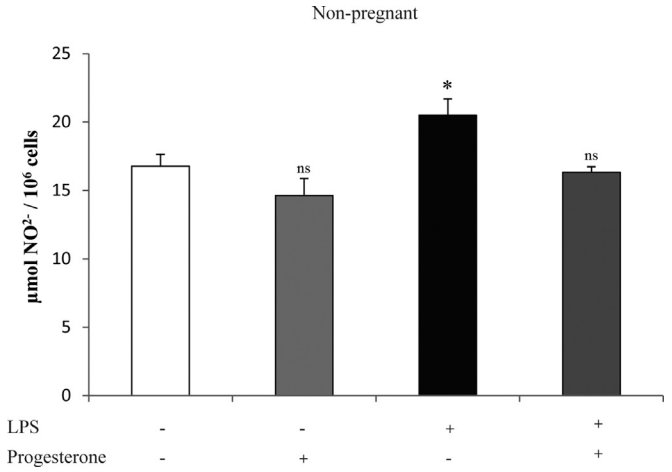

Fig. 2. Six h post-administration of LPS (1 μg/g body weight), PBMC from non-pregnant mice show an increase in NO production. Co-treatment with progesterone (4 μg/g body weight) abrogates this effect. Statistics: $^{*}P < 0.05$ ($n = 4$–6).

this hormone in modulating the levels of nitric oxide in PBMC from 7-day pregnant mice treated in vivo with LPS. In Fig. 1, we show that the administration of progesterone (2 μg/kg) to 7-day pregnant mice downregulated the LPS-induced production of NO in PBMC.

3.2. Effect of LPS on PBMC nitric oxide (NO) synthesis in non-pregnant mice

Next, we studied LPS effect on NO synthesis in PBMC from non-pregnant mice. LPS increased NO production in PBMC from non-pregnant mice (Fig. 2). However, when we compared pregnant with non-pregnant mice, we observed that the basal NO production was lower in pregnant mice when compared to non-pregnant mice (Fig. 3). Therefore, we evaluated whether external administration of progesterone could exert protective effects from a challenge with LPS in non-pregnant mice. As shown in Fig. 2, the *in vivo* administration of progesterone reverted the effects of LPS on NO production of PBMC from non-pregnant mice.

3.3. Role of progesterone on PBMC iNOS protein content in non-pregnant mice challenged with LPS

Next, we decided to analyze whether progesterone modulates the protein expression of iNOS in the PBMC from non-pregnant mice challenged with LPS. In Fig. 4, we show that LPS increased iNOS protein levels and that progesterone treatment abrogated

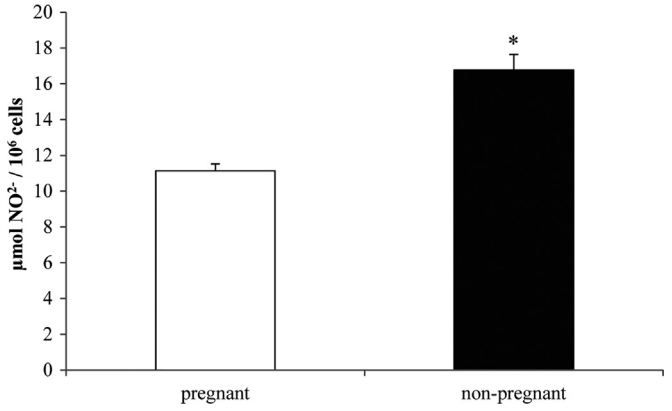

Fig. 3. Basal NO production is lower in pregnant mice when compared to non-pregnant mice. Statistics: $^{*}P < 0.05$ ($n = 6$–8).

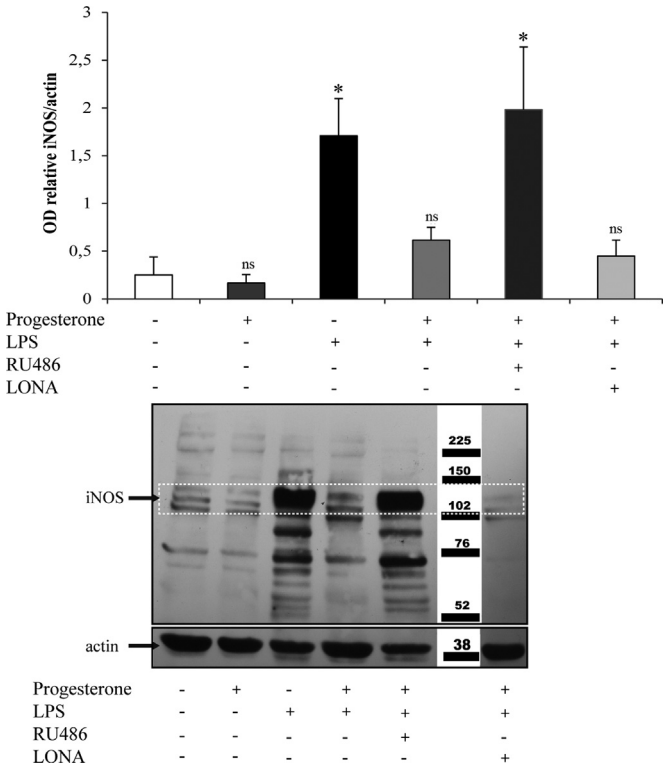

Fig. 4. Western blot analysis of the expression of iNOS in the PBMC from non-pregnant mice. LPS-treated (1 μg/g body weight) mice show an increase in the expression of iNOS and progesterone co-treatment reverts this effect. RU-486 (10 μg/g body weight) completely blocks the effect of progesterone. However, when PBMC from non-pregnant mice are co-treated with LONA (1 μg/g body weight), a more specific PR antagonist, the effect of progesterone remained intact. Statistics: $^{*}P < 0.05$ ($n = 4$).

this effect. We have recently shown the presence of classical progesterone receptors (PR-A and PR-B) in murine PBMC and their role in the protective effects of this hormone by regulating the enzymatic activity of FAAH (Wolfson et al., 2013). In order to evaluate the involvement of progesterone classical receptors, we treated mice with RU486 (also known as mifepristone), a classical PR antagonist. Interestingly, when RU486 was co-administrated, the effect of progesterone on iNOS protein level disappeared, suggesting a PR-mediated mechanism (Fig. 4). However, it is worth noting that RU486 is also an antagonist of GR. Therefore, in order to test the contribution of GR to the actions of progesterone, we decided to use LONA, a highly specific and potent progesterone antagonist (Fig. 4). Interestingly, with this antagonist the effects of progesterone remained undisturbed, suggesting that, at least in the regulation of iNOS protein expression, progesterone exerts its effects via glucocorticoid receptors.

3.4. Progesterone acts via glucocorticoid receptors in the PBMC from non-pregnant mice

The fact that LONA was unable to reverse progesterone effects on LPS-induced iNOS protein expression in PBMC from non-pregnant mice when compared to RU486, suggests that progesterone exerts its effects, at least some of them, via GR. Therefore, we proceeded to evaluate the expression of GR in PMBC by RT-PCR. Additionally, we assessed whether LPS modulates the mRNA expression of GR in these cells. In Fig. 5, we show that murine PBMC express GR and that LPS has no effects in GR expression.

Once established the presence of GR in our model, we next aimed to analyze whether dexamethasone, a glucocorticoid agonist, mimics the effects of progesterone on iNOS protein

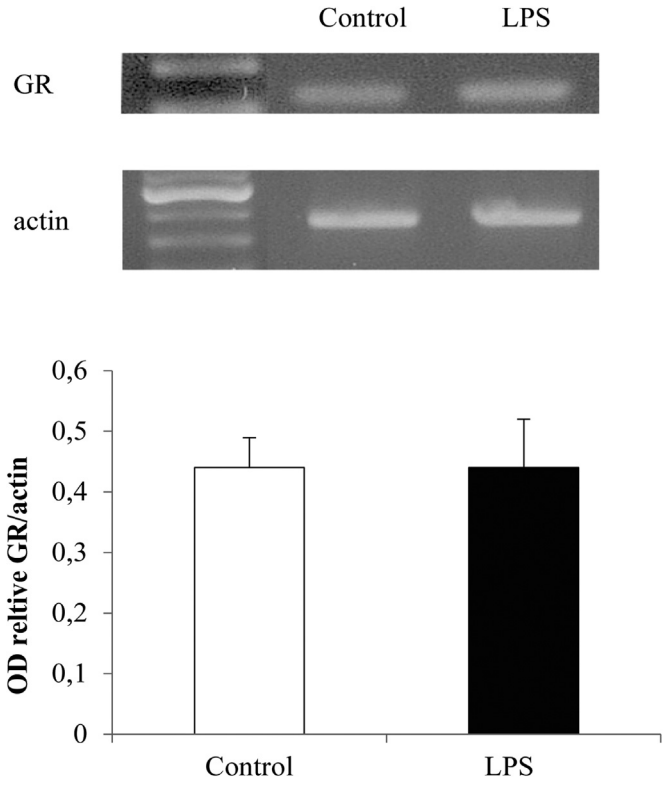

Fig. 5. RT-PCR analyses of the mRNA expression of glucocorticoid receptor in the PBMC from non-pregnant mice showing that LPS (1 μg/g body weight) has no effects in GR expression ($n=4$).

expression in PBMC from non-pregnant mice challenged with LPS. We observed that dexamethasone reverted the effects of LPS in the protein expression of iNOS in PBMC from non-pregnant mice, similarly to progesterone (Fig. 6A). Therefore, we sought to assess the possible participation of GR in the effects of progesterone. PBMC treated *in vitro* with LPS showed an increase in the mRNA expression of iNOS, which was reverted by the co-treatment with progesterone (Fig. 6B). Interestingly, when glucocorticoid receptors were blocked with the specific GR antagonist 21OH-6,19OP, the protective effect of progesterone was lost (Fig. 6B).

4. Discussion

This work aims to analyze the effects of in vivo administration of progesterone on the LPS-induced production of nitric oxide and nitric oxide synthase activity in PBMC in a model of LPS-induced embryo loss. It has been well established that LPS induces a series of inflammatory events that culminate in the production of cytokines, like TNF-α, IL-1β e IL-6, release of prostanoids, NO and other soluble mediators (Morris et al., 2015; Tan and Kagan, 2014). Genital tract infections are one of the main causes of miscarriage (Cram et al., 2002) and the environmental endotoxin has been associated with spontaneous abortions (Clark et al., 2004, 2002). We have previously shown that LPS administration to 7-days Balb/c pregnant mice induced a drop on serum progesterone levels (Aisemberg et al., 2013). A decrease in serum progesterone levels is associated with events leading to pregnancy loss (Babalioğlu et al., 1996; Daya, 1989) and the restoration of the serum levels of this hormone has been shown to abrogate the embryo resorption induced by LPS (Aisemberg et al., 2013).

Nitric oxide (NO) has been proposed to be an important

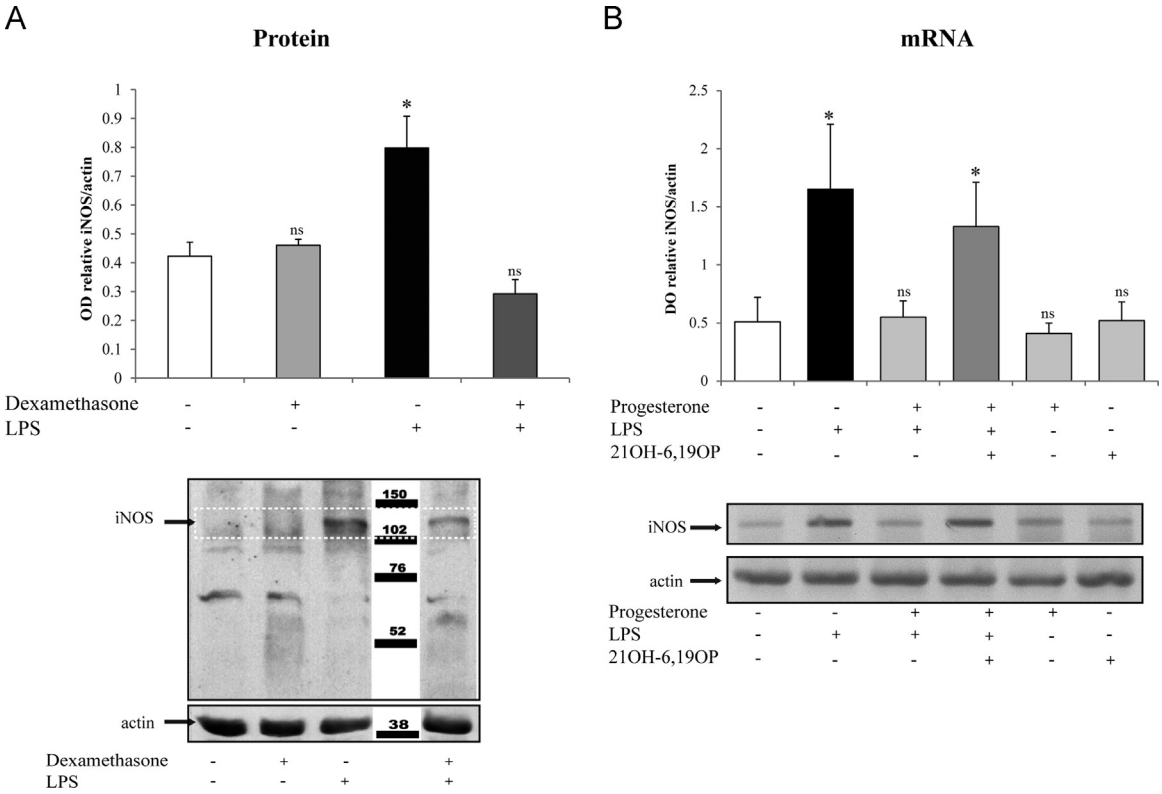

Fig. 6. (A) Western blot analysis of the expression of iNOS in the PBMC from non-pregnant mice. LPS-treated mice show an increase in expression of iNOS and dexamethasone treatment (1.5 μg/g body weight) mimics the protective effect of progesterone by downregulating the LPS-induced iNOS expression. Statistics: $^*P<0.05$ ($n=4$). (B) RT-PCR analyses of the mRNA expression of iNOS in the PBMC from non-pregnant mice showing that *in vitro* treatment with LPS (1 μg/ml) induces iNOS mRNA expression, that progesterone (50 ng/ml) reverts LPS effect and that blocking glucocorticoid receptors with 21OH-6,19-OP (10 μM), a specific GR antagonist, restores LPS-induced iNOS mRNA levels. Statistics: $^*P<0.05$ ($n=6$).

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regulator of the physiology of the reproductive system, exerting important functions during pregnancy and its effects are largely dependent on its levels (Paradisi et al., 2007). Previous results from our lab show an increase in NO levels in deciduas and uterus in our model of LPS-induced embryo resorption, with the highest level at 6 h after the administration of the endotoxin (Ogando et al., 2003). Moreover, the administration of an iNOS inhibitor, such as aminoguanidine, prevented the embryo resorption induced by LPS. It has been shown that the endotoxin produces an increase in NO production and leukocyte infiltration in different tissues (Salter et al., 1991). In the same way, Ogando et al (2003) demonstrated that LPS-treated pregnant mice showed high infiltration of macrophages and granulocytes in the deciduas together with an increased protein expression of iNOS. In agreement with previous reports, we observed here that LPS increase NO release by PBMC from both pregnant and non-pregnant mice. Interestingly, pregnant mice showed a lower basal level of NO production when compared with non-pregnant mice which is in agreement with previous reports showing that pregnant women have lower plasma NO levels than non-pregnant women (Paradisi et al., 2007; Wilson et al., 1997). This observation correlates with a differential hormonal status between pregnant and non-pregnant mice since the former have higher serum progesterone levels than the latter. The role of progesterone in the initiation and maintenance of the pregnancy has been extensively explored (reviewed by Wetendorf and DeMayo, 2014) and it has been suggested that this steroid exerts immunomodulatory effects during pregnancy (Aisemberg et al., 2013; Tan et al., 2015; Wolfson et al., 2013). In this sense, we observed here that the *in vivo* treatment with progesterone prevented the effect of LPS on NO production by PBMC from pregnant and non-pregnant mice. Similarly, Coronel et al. (2014) reported an increase in NO production and a protective effect of progesterone administration in a spinal cord injury model. Similarly, Miller et al. (1996) reported that progesterone reversed the increase in NO production and the iNOS protein level in murine macrophages treated with IFN-γ and LPS. When we analyzed the expression of iNOS in PBMC from non-pregnant mice, we observed that LPS induced an increase in iNOS protein expression, and that the co-treatment with progesterone abrogated this effect, suggesting that progesterone, by downregulating PBMC's iNOS, modulates the production levels of NO in these cells.

To elucidate whether the protective effects of progesterone are mediated by the activation of its own receptors, we used RU486, a PR antagonist. The *in vivo* administration of RU486 blocked the effects of progesterone on the protein expression of iNOS. Many reports have shown that progesterone also activates glucocorticoid receptors (Jones et al., 2008; Lei et al., 2012; Leo et al., 2004), suggesting that, at least partially, some of the effects of progesterone are mediated by the activation of GR instead of PR. Moreover, RU486 is an antagonist of both progesterone and glucocorticoid receptors (Afhüppe et al., 2010). Jones et al. (2008) have reported that both dexamethasone and progesterone, but not norgestrel (a specific PR agonist), significantly reduced the LPS-induced nitric oxide production from murine macrophages, suggesting that this effect was mediated by GR and not via PR. Similarly, Lei et al. (2012) have shown that progesterone, acting via GR, suppressed the IL-1β-induced expression of COX-2 human term myometrial cells. In accordance with previous reports, we observed that the effects of progesterone on iNOS expression remained intact when we used lonaprisan, a more potent and selective antagonist of PR (Afhüppe et al., 2010). Moreover, the *in vivo* administration of dexamethasone mimicked the effects of progesterone on the protein expression of iNOS in PBMC from non-pregnant mice whereas the *in vitro* treatment of PBMC from non-pregnant mice with 21OH-6, 19-OP, a highly specific GR antagonist (Vicent et al., 1997; Álvarez et la., 2008; Presman et al., 2010) reverted the protective effect of progesterone on LPS-induced iNOS mRNA expression. Taken together, these observations suggest that progesterone effects on iNOS expression in PBMC from non-pregnant mice are mediated by GR instead of by PR.

Interestingly, several reports have shown that progesterone might selectively modulate other NOS isoforms. Thus, Amaral et al. (2014) have reported that the progesterone derivative 17-hydroxyorigesterone caproate not only up-regulated placental eNOS but modulated its activity through phosphorylation at serine 1177, which could be associated with an increase in uteroplacental blood flow. In contrast, it has been recently shown that medroxyprogesterone acetate, a progestin, decreased eNOS phosphorylation (Oishi et al., 2011) and reduced the inhibition of platelet aggregation by downregulating eNOS expression in endothelial cells (Zerr-Fouineau et al., 2007) and those effects were mediated by targeting GR (Oishi et al., 2011; Zerr-Fouineau et al., 2007). Conversely, Väisänen-Tommiska et al. (2006) have shown that treatment with RU486 induced iNOS expression in the human cervix without changes in cervical eNOS. Thus, progesterone effects on the different NOS isoforms are seemingly tissue-dependent. However, it is important to keep in mind that these isoforms produce NO differently: eNOS and nNOS synthesize NO in a regulated short pulsative fashion whereas iNOS produces NO in a constant fashion and for a relatively longer period of time (reviewed by Kröncke et al., 1997). These differences account for the physiological and pathophysiological actions of NO in reproductive tissues.

Collectively, our results suggest that progesterone prevents an increase in PBMC's NO production induced by LPS and this effect is mediated via a non-PR mediated mechanism, presumably by targeting glucocorticoid receptors.

Ultimately, further experiments are needed in order to understand the complex role of progesterone in the regulation of the production of nitric oxide.

Acknowledgments

We thank Ramona Morales and Maximiliano Cella for their excellent technical support. We are in debt with Dr. Adalí Pecci from School of Science (University of Buenos Aires, Argentina) for the kind gift of the 21-hydroxy-6,19-epoxyprogesterone (21OH-6,19-OP). This work was supported by Agencia Nacional para la Promoción Científica y Tecnológica (PICT 2010/0813 and PICT 2013/0097), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 2012/0061). Finally, we would like to thank the animal care technicians Vet. Marcela Márquez and Daniel González for their excellent care of the animals used in this study.

References

Afhüppe, W., Beekman, J.M., Otto, C., Korr, D., Hoffmann, J., Fuhrmann, U., Möller, C., 2010. In vitro characterization of ZK 230211–A type III progesterone receptor antagonist with enhanced antiproliferative properties. J. Steroid Biochem. Mol. Biol. 119, 45–55. http://dx.doi.org/10.1016/j.jsbmb.2009.12.011.

Aisemberg, J., Vercelli, C.A., Bariani, M.V., Billi, S.C., Wolfson, M.L., Franchi, A.M., 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. http://dx.doi.org/10.1371/journal.pone.0056161.

Alvarez, L.D., Martí, M.A., Veleiro, A.S., Presman, D.M., Estrin, D.A., Pecci, A., Burton, G., 2008. Exploring the molecular basis of action of the passive antiglucocorticoid 21-hydroxy-6,19-epoxyprogesterone. J. Med. Chem. 51, 1352–1360. http://dx.doi.org/10.1021/jm800007w.

Amaral, L.M., Kiprono, L., Cornelius, D.C., Shoemaker, C., Wallace, K., Moseley, J., Wallukat, G., Martin, J.N., Dechend, R., LaMarca, B., 2014. Progesterone supplementation attenuates hypertension and the autoantibody to the angiotensin II type I receptor in response to elevated interleukin-6 during pregnancy. Am. J. Obs. Gynecol. 211. http://dx.doi.org/10.1016/j.ajog.2014.02.018 158.e1–6.

Babalioğlu, R., Varol, F.G., Ilhan, R., Yalçin, O., Cizmecioğlu, F., 1996. Progesterone profiles in luteal-phase defects associated with recurrent spontaneous abortions. J. Assist. Reprod. Genet. 13, 306–309.

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. http://dx.doi.org/10.1016/S0015-0282(99)00442-2.

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

Cella, M., Farina, M.G., Dominguez Rubio, a P., Di Girolamo, G., Ribeiro, M.L., Franchi, a M., 2010. Dual effect of nitric oxide on uterine prostaglandin synthesis in a murine model of preterm labour. Br. J. Pharmacol. 161, 844–855. http://dx.doi.org/10.1111/j.1476-5381.2010.00911.x.

Chadha, S., Jain, V., Gupta, I., Khullar, M., 2007. Nitric oxide metabolite levels in preterm labor. J. Obs. Gynaecol. Res. 33, 710–717. http://dx.doi.org/10.1111/j.1447-0756.2007.00639.x.

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

Clark, D.A., Chaouat, G., Gorczynski, R.M., 2002. Thinking outside the box: mechanisms of environmental selective pressures on the outcome of the materno-fetal relationship. Am. J. Reprod. Immunol. 47, 275–282. http://dx.doi.org/10.1034/j.1600-0897.2002.01093.x.

Clark, D.A., Manuel, J., Lee, L., Chaouat, G., Gorczynski, R.M., Levy, G.A., 2004. Ecology of danger-dependent cytokine-boosted spontaneous abortion in the CBA x DBA/2 mouse model. I. Synergistic effect of LPS and (TNF-alpha + IFN-gamma) on pregnancy loss. Am. J. Reprod. Immunol. 52, 370–378. http://dx.doi.org/10.1111/j.1600-0897.2004.00237.x.

Coronel, M.F., Labombarda, F., De Nicola, A.F., González, S.L., 2014. Progesterone reduces the expression of spinal cyclooxygenase-2 and inducible nitric oxide synthase and prevents allodynia in a rat model of central neuropathic pain. Eur. J. Pain. 18, 348–359. http://dx.doi.org/10.1002/j.1532-2149.2013.00376.x.

Cram, L.F., Zapata, M.I., Toy, E.C., Baker, B., 2002. Genitourinary infections and their association with preterm labor. Am. Fam. Phys. 65, 241–248.

Daya, S., 1989. Efficacy of progesterone support for pregnancy in women with recurrent miscarriage. A meta-analysis of controlled trials. Br. J. Obs. Gynaecol. 96, 275–280.

Dosiou, C., Hamilton, a E., Pang, Y., Overgaard, M.T., Tulac, S., Dong, J., Thomas, P., Giudice, L.C., 2008. Expression of membrane progesterone receptors on human T lymphocytes and Jurkat cells and activation of G-proteins by progesterone. J. Endocrinol. 196, 67–77. http://dx.doi.org/10.1677/JOE-07-0317.

Fernandes, M.S., Brosens, J.J., Gellersen, B., 2008. Honey, we need to talk about the membrane progestin receptors. Steroids 73, 942–952. http://dx.doi.org/10.1016/j.steroids.2007.12.004.

Fuhrmann, U., Hess-Stumpp, H., Cleve, A., Neef, G., Schwede, W., Hoffmann, J., Fritzemeier, K.H., Chwalisz, K., 2000. Synthesis and biological activity of a novel, highly potent progesterone receptor antagonist. J. Med. Chem. 43, 5010–5016.

Grisham, M.B., Johnson, G.G., Lancaster, J.R., 1996. Quantitation of nitrate and nitrite in extracellular fluids. Methods Enzym. 268, 237–246.

Haddad, E.K., Duclos, A.J., Baines, M.G., 1995. Early embryo loss is associated with local production of nitric oxide by decidual mononuclear cells. J. Exp. Med. 182, 1143–1151.

Hughes, G.C., Clark, E. a, Wong, A.H., 2013. The intracellular progesterone receptor regulates CD4+T cells and T cell-dependent antibody responses. J. Leukoc. Biol. 93, 369–375. http://dx.doi.org/10.1189/jlb.1012491.

Johnson, R.M., Kerr, M.S., Slaven, J.E., 2012. Plac8-dependent and inducible NO synthase-dependent mechanisms clear Chlamydia muridarum infections from the genital tract. J. Immunol. 188, 1896–1904. http://dx.doi.org/10.4049/jimmunol.1102764.

Jones, L. a, Anthony, J.-P., Henriquez, F.L., Lyons, R.E., Nickdel, M.B., Carter, K.C., Alexander, J., Roberts, C.W., 2008. Toll-like receptor-4-mediated macrophage activation is differentially regulated by progesterone via the glucocorticoid and progesterone receptors. Immunology 125, 59–69. http://dx.doi.org/10.1111/j.1365-2567.2008.02820.x.

Kröncke, K.D., Fehsel, K., Kolb-Bachofen, V., 1997. Nitric oxide: cytotoxicity versus cytoprotection – how, why, when, and where? Nitric Oxide 1, 107–120. http://dx.doi.org/10.1006/niox.1997.0118.

Kyurkchiev, D., Ivanova-Todorova, E., Kyurkchiev, S.D., 2010. New target cells of the immunomodulatory effects of progesterone. Reprod. Biomed. Online 21, 304–311. http://dx.doi.org/10.1016/j.rbmo.2010.04.014.

Lei, K., Chen, L., Georgiou, E.X., Sooranna, S.R., Khanjani, S., Brosens, J.J., Bennett, P.R., Johnson, M.R., 2012. Progesterone acts via the nuclear glucocorticoid receptor to suppress il-1??-induced cox-2 expression in human term myometrial cells. Plos One 7. http://dx.doi.org/10.1371/journal.pone.0050167.

Leo, J.C.L., Guo, C., Woon, C.T., Aw, S.E., Lin, V.C.L., 2004. Glucocorticoid and mineralocorticoid cross-talk with progesterone receptor to induce focal adhesion and growth inhibition in breast cancer cells. Endocrinology 145, 1314–1321. http://dx.doi.org/10.1210/en.2003-0732.

Miller, L., Alley, E.W., Murphy, W.J., Russell, S.W., Hunt, J.S., 1996. Progesterone inhibits inducible nitric oxide synthase gene expression and nitric oxide production in murine macrophages. J. Leukoc. Biol. 59, 442–450.

Morris, M.C., Gilliam, E. a, Li, L., 2015. Innate immune programing by endotoxin and its pathological consequences. Front Immunol. 5, 1–8. http://dx.doi.org/10.3389/fimmu.2014.00680.

Ogando, D.G., 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.

Oishi, A., Takahashi, K., Ohmichi, M., Mochizuki, Y., Inaba, N., Kurachi, H., 2011. Role of glucocorticoid receptor in the inhibitory effect of medroxyprogesterone acetate on the estrogen-induced endothelial nitric oxide synthase phosphorylation in human umbilical vein endothelial cells. Fertil. Steril. 95, 1168–1170. http://dx.doi.org/10.1016/j.fertnstert.2010.09.041.

Paradisi, R., Fabbri, R., Battaglia, C., Facchinetti, F., Venturoli, S., 2007. Nitric oxide levels in women with missed and threatened abortion: results of a pilot study. Fertil. Steril. 88, 744–748. http://dx.doi.org/10.1016/j.fertnstert.2006.12.026.

Presman, D.M., Alvarez, L.D., Levi, V., Eduardo, S., Digman, M.a, Martí, M.a, Veleiro, A.S., Burton, G., Pecci, A., 2010. Insights on glucocorticoid receptor activity modulation through the binding of rigid steroids. PLoS One 5, e13279. http://dx.doi.org/10.1371/journal.pone.0013279.

Pru K., J.K., Clark C., N.C., 2013. PGRMC1 and PGRMC2 in uterine physiology and disease. Front. Neurosci. . http://dx.doi.org/10.3389/fnins.2013.00168

Ramsey, K.H., Sigar, I.M., Rana, S.V., Gupta, J., Holland, S.M., Byrne, G.I., 2001. Role for inducible nitric oxide synthase in protection from chronic Chlamydia trachomatis urogenital disease in mice and its regulation by oxygen free radicals. Infect. Immun. 69, 7374–7379. http://dx.doi.org/10.1128/IAI.69.12.7374-7379.2001.

Salter, M., Knowles, R.G., Moncada, S., 1991. Widespread tissue distribution, species distribution and changes in activity of Ca(2+)-dependent and Ca(2+)-independent nitric oxide synthases. FEBS Lett. 291, 145–149.

Sugino, N., Telleria, C.M., Gibori, G., 1997. Progesterone inhibits 20alpha-hydroxysteroid dehydrogenase expression in the rat corpus luteum through the glucocorticoid receptor. Endocrinology 138, 4497–4500.

Szekeres-Bartho, J., Barakonyi, a, Par, G., Polgar, B., Palkovics, T., Szereday, L., 2001. Progesterone as an immunomodulatory molecule. Int. Immunopharmacol. 1, 1037–1048.

Szekeres-Bartho, J., Reznikoff-Etievant, M.F., Varga, P., Pichon, M.F., Varga, Z., Chaouat, G., 1989. Lymphocytic progesterone receptors in normal and pathological human pregnancy. J. Reprod. Immunol. 16, 239–247.

Tan, I.J., Peeva, E., Zandman-Goddard, G., 2015. Hormonal modulation of the immune system—a spotlight on the role of progestogens. Autoimmun. Rev. 14, 536–542. http://dx.doi.org/10.1016/j.autrev.2015.02.004.

Tan, Y., Kagan, J.C., 2014. A cross-disciplinary perspective on the innate immune responses to bacterial lipopolysaccharide. Mol. Cell. 54, 212–223. http://dx.doi.org/10.1016/j.molcel.2014.03.012.

Telfer, J.F., Lyall, F., Norman, J.E., Cameron, I.T., 1995. Identification of nitric oxide synthase in human uterus. Hum. Reprod. 10, 19–23.

Törnblom, S.A., Maul, H., Klimaviciute, A., Garfield, R.E., Byström, B., Malmström, A., Ekman-Ordeberg, G., 2005. mRNA expression and localization of bNOS, eNOS and iNOS in human cervix at preterm and term labour. Reprod. Biol. Endocrinol. 3, 33. http://dx.doi.org/10.1186/1477-7827-3-33.

Väisänen-Tommiska, M., Butzow, R., Ylikorkala, O., Mikkola, T.S., 2006. Mifepristone-induced nitric oxide release and expression of nitric oxide synthases in the human cervix during early pregnancy. Hum. Reprod. 21, 2180–2184. http://dx.doi.org/10.1093/humrep/del141.

Vicent, G.P., Monteserín, M.C., Veleiro, A.S., Burton, G., Lantos, C.P., Galigniana, M.D., 1997. 21-Hydroxy-6,19-oxidoprogesterone: a novel synthetic steroid with specific antiglucocorticoid properties in the rat. Mol. Pharmacol. 52, 749–753.

Wetendorf, M., DeMayo, F.J., 2014. Progesterone receptor signaling in the initiation of pregnancy and preservation of a healthy uterus. Int. J. Dev. Biol. 58, 95–106. http://dx.doi.org/10.1387/ijdb.140069mw.

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

Wolfson, M.L., Aisemberg, J., Salazar, A.I., Domínguez Rubio, A.P., Vercelli, C.A., Franchi, A.M., 2013. Progesterone reverts LPS-reduced FAAH activity in murine peripheral blood mononuclear cells by a receptor-mediated fashion. Mol. Cell. Endocrinol. 381, 97–105. http://dx.doi.org/10.1016/j.mce.2013.07.020.

Wolfson, M.L., Correa, F., Leishman, E., Vercelli, C., Cymeryng, C., Blanco, J., Bradshaw, H.B., Franchi, A.M., 2015. 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. http://dx.doi.org/10.1016/j.mce.2015.04.032.

Xu, X.F., Hoebeke, J., Björntorp, P., 1990. Progestin binds to the glucocorticoid receptor and mediates antiglucocorticoid effect in rat adipose precursor cells. J. Steroid Biochem. 36, 465–471. http://dx.doi.org/10.1016/0022-4731(90)90089-B.

Zerr-Fouineau, M., Chataigneau, M., Blot, C., Schini-Kerth, V.B., 2007. Progestins overcome inhibition of platelet aggregation by endothelial cells by down-regulating endothelial NO synthase via glucocorticoid receptors. FASEB J. 21, 265–273. http://dx.doi.org/10.1096/fj.06-6840com.

Please cite this article as: Wolfson, M.L., et al., Progesterone modulates the LPS-induced nitric oxide production by a progesterone-receptor independent mechanism. Eur J Pharmacol (2015), http://dx.doi.org/10.1016/j.ejphar.2015.11.005