Proceedings of the Interdisciplinary STEM Teaching and Learning Conference

Volume 1 Article 8

5-2017

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DOI: 10.20429/stem.2017.010108
Available at: https://digitalcommons.georgiasouthern.edu/stem_proceedings/vol1/iss1/8

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Abstract

It has been widely established that, in addition to its role in reproduction, progesterone (P4) also has potent anti-inflammatory effects. While the precise mechanisms have never been clearly elucidated in RAW 246.7 cells, it seems logical to assume that this response is – at least in part - a consequence of activation of and signaling through the progesterone receptor (P4-R). However, it has recently been shown that in a rat model, this anti-inflammatory effect is – in fact - independent of the progesterone receptor. In this project, the aim was to characterize this response by assaying nitric oxide production from lipopolysaccharide-challenged RAW 264.7 cells and ascertain the involvement of the P4-R. To determine the contribution of the receptor, RAW cells were incubated in the presence and absence of RU-486 – a potent P4-R antagonist. Our results indicate that the anti-inflammatory response of progesterone was in fact through the activation of the P4-R as cells incubated in RU-486 show an approximate 60% reversal of the inhibitory effect of P4 as compared to cells incubated in the absence of the antagonist. However, because we did not observe a complete reversal, suggests that perhaps other receptors come into play which will be addressed in future studies.

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Abstract: It has been widely established that, in addition to its role in reproduction, progesterone (P4) also has potent anti-inflammatory effects. While the precise mechanisms have never been clearly elucidated in RAW 246.7 cells, it seems logical to assume that this response is – at least in part - a consequence of activation of and signaling through the progesterone receptor (P4-R). However, it has recently been shown that in a rat model, this anti-inflammatory effect is – in fact - independent of the progesterone receptor. In this project, the aim was to characterize this response by assaying nitric oxide production from lipopolysaccharide-challenged RAW 264.7 cells and ascertain the involvement of the P4-R. To determine the contribution of the receptor, RAW cells were incubated in the presence and absence of RU-486 – a potent P4-R antagonist. Our results indicate that the anti-inflammatory response of progesterone was in fact through the activation of the P4-R as cells incubated in RU-486 show an approximate 60% reversal of the inhibitory effect of P4 as compared to cells incubated in the absence of the antagonist. However, because we did not observe a complete reversal, suggests that perhaps other receptors come into play which will be addressed in future studies.

Acknowledgements: The authors would like to thank Dr. David Hurley and Natalie Norton – College of Veterinary Medicine, University of Georgia for their experience, assistance and expertise in the establishment of protocols and analysis of data; The School of Science and Technology, Georgia Gwinnett College for provision of facilities and equipment; and the University System of Georgia STEM Initiative for partial funding of this project.

Introduction

Progesterone (P4) has long been known as a steroid hormone associated with reproduction – more specifically in female reproductive physiology as it is commonly referred to as the 'hormone of pregnancy' (King TL, 2010). Its

presence is only observed in appreciable amounts during the luteal phase as its source is the corpus luteum, and its primary role during this time period is in endometrial remodeling as well as endometrial angiogenesis in preparation for the arrival of an early embryo (Patel B, 2014). While this effect is critical for the maintenance of pregnancy, just as important is the inhibitory role that P4 has – along with estrogen and inhibin – on the pituitary gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), respectively (Lesoon LA, 1992). Secondary to these effects, P4 also plays a role as a mediator in many inhibitory pathways such as: 1) modulation of maternal immune response and suppression of key inflammatory mediators given that the luteal progesterone at the decidua level appears to play a major role in the maternal defense strategy, 2) reduction of uterine contractility as adequate progesterone concentrations in the myometrium plays an inhibitory role on prostaglandin and oxytocin's stimulatory activity, and 3) improvement of utero-placental circulation and luteal phase support given that it has been shown that progesterone may promote the invasion of extravillous trophoblasts to the decidua by inhibiting apoptosis of extravillous trophoblasts (Di Renzo GC, 2016). Additionally, P4 is not without its effects in the male in that it serves as precursor to testosterone, has a similar inhibitory effect on the gonadotropins– albeit a weak effect – and is a precursor to capacitation (Oettel M, 2004).

Given the steroid hormone chemistry of P4, it has widely been known to signal through an intracellular receptor which is nuclear in origin – NR3C3, that serves as a transcription factor driving the expression of many genes associated with reproduction (Werner R, 2014). More recently however, a membrane-bound receptor has also been identified which is a G protein-coupled receptor, and has been isolated in many isoforms, namely mPR , mPR , mPR , mPR , and mPR (Wolfson ML, 2016). The advent of signal transduction mechanisms through these receptors have been shown to be primarily anti-inflammatory in nature. However, in the study by Wolfson et al., it was reported that the inhibition of at least one inflammatory mediator (nitric oxide – NO), was through a progesterone independent mechanism. These authors went on to suggest the possible involvement of the glucocorticoid receptors in this immunomodulatory role. Regardless of the receptor involved or cell signaling pathways initiated, the anti-inflammatory properties of progesterone are an exciting avenue for scientific discovery and warrants exploration.

The Immune response is an immunological response that originates via

DOI: 10.20429/stem.2017.010108

activation by antigens, including the response to pathogenic microorganisms, allergens, as well as autoimmunity to self-antigens, and graft ejections. It can be further subdivided into the adaptive immune response involving T cells, B lymphocytes, and the innate immune response involving the monocyte/macrophage system, neutrophils, eosinophils, basophils as well as other tissues of the lymphatic system. The monocyte/macrophage system is of particular interest based on the many inflammatory mediators activated macrophages produce, namely nitric oxide (NO) and tumor necrosis factor-alpha (TNF) because of their integral role in the initiation of the inflammatory response. Nitric oxide is a gas under standard conditions, is one of several oxides of nitrogen, and is classified as a free radical. In mammals it is a potent vasodilator with short half-life, and an important cell signaling molecule that is involved in many physiological and pathological processes (Hou, 1999). It has been reported that localized NO synthesis in reproductive tissue plays an important role in regulating certain reproductive functions such as endometrial, cervical and myometrial activity (Telfer, 1995), embryo implantation (Battaglia, 2003) as well as embryo development (Chwalisz, 1999). While it is apparent from these studies that NO plays key roles in early pregnancy and even parturition, its concentration must be tightly regulated as it has also been reported that aberrant NO levels have been associated with embryo cytotoxicity (Barroso, 1999), early embryo loss (Haddad, 1995), as well as preterm labor (Cella, 2010). Given that progesterone has been shown to have an anti-inflammatory effect, it seems logical to assume that it also plays a major role in the regulation of appropriate NO concentration during pregnancy. Indeed, the protective role of progesterone has been shown by modulating the innate immune response in an animal model of early pregnancy loss induced by inflammation (Aisemberg, 2013; Wolfson, 2013). In fact it was demonstrated that the 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) and these changes leads to a 100% of embryonic resorption and fetal expulsion (Ogando, 2003).

On the other hands, TNFα, is a cell signaling protein (cytokine) involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. It is produced primarily by activated macrophages, although it is also produced by many other cell types such as CD4+ lymphocytes,

natural killer cells, neutrophils, mast cells, eosinophils, as well as neurons (Carswell EA, 1975). The primary role of TNF is in the regulation of immune cells. Being an endogenous pyrogen, TNF is able to induce fever, apoptotic cell death, cachexia, inflammation and to inhibit tumorigenesis and viral replication and respond to sepsis via IL1 & IL6 producing cells. Dysregulation of TNF production has been implicated in a variety of human diseases including Alzheimer's disease (Swardfager W, 2010), cancer (Locksley RM, 2001), and major depression (Dowlati Y, 2010) to name a few. From a reproductive standpoint, TNF has been identified in the ovary, oviduct, uterus, and placenta (Terranova PF, 1995), and it is expressed in embryonic tissues (Kohchi C, 1994) practically at all stages of development. TNFα levels have also been shown to be significantly elevated in the amniotic fluid of women with uterine infections, and its increased production correlates with the incidence of preterm labor (Romero R, 1989). These observations have implicated TNFα as a cytokine involved in triggering immunological pregnancy loss (Clark DA, 1999; R, 2001), i.e. death of embryos owing to failure of defense mechanisms preventing rejection of the semiallogeneic fetoplacental unit.

Materials and Methods

Cell Culture

RAW 264.7 cells were maintained in MEM culture medium (Fisher Scientific) containing 10% Fetal Calf Serum (FCS, Atlanta Biologicals), 1000 IU Penicillin/Streptomycin (Sigma Chemicals), 2 mM glutamine (Sigma) at 37oC, 5% CO2. Prior to stimulation assay, RAW cells were cultured to 80% confluence and harvested by gentle agitation utilizing a 10 mm cell scraper (Fisher). Scraped cells were then pooled in 1X PBS, and washed twice with centrifugation, 1,800 rpm, 5 min. In between the 2nd and 3rd wash cycle, cell viability assays were performed to determine living vs. dead cells. With each cell harvest, an aliquot of RAW cells were returned to culture flasks to maintain the cell line; after 15 passages, cells were discarded as it has been demonstrated that there is a reduction in receptor expression beyond this time.

Cell Stimulation

Cells were prepared for stimulation assays by combining live cells at a density of 75 x 103 live cells/ml in Minimal Essential Media (MEM, Fisher)

DOI: 10.20429/stem.2017.010108

supplemented with 10% FCS in polystyrene tubes. To appropriate tubes, lipopolysaccharide (Sigma) was added to final concentrations of 200, 20 or 2 ng LPS/ml to each tube. One milliliter of each cell suspension was then seeded in triplicate to 24-well Falcon tissue culture plates (Fisher) and incubated at 37oC, 5% CO2. Each well contained a final concentration of 75 x 103 cells in MEM/ FCS, and the appropriate dilutions of LPS in a final volume of 1 ml. Control wells contained MEM/FCS alone. Cells were maintained for 48 hrs., afterwhich growth media was harvested and maintained at -20oC until such time as NO assays were performed.

To determine the effects of the progesterone receptor on NO and TNF production, RAW cells were handled and stimulated with LPS as above with the exception that in appropriate wells, cells were incubated simultaneously in 10 M RU-486 – a selective P4 receptor antagonist. Upon the completion of the 48 hr. incubation period, cell supernatant was handled and NO assays performed as previously described.

Nitric Oxide Assay.

This protocol was adopted with modifications from that of Griess (Griess, 1879). Briefly, 100 l aliquots of samples from the cell stimulation assays above were loaded in triplicate into appropriate wells of a 96-well Corning Costar ELISA plate (Fisher), followed by 10 l of nitrate reductase solution to each well. Plates were then incubated at 37oC, 5% CO2 for one hour. After incubation, 80 ml Griess reagent was added to each well and nitrites (NO2-) read at 540 nm on a plate reader (BioTek). An 8-point sodium nitrate standard was also established to determine the nitrate concentration of the unknowns.

Tumor Necrosis Factor ELISA (Mouse TNF ELISA Ready-Set-Go, Affymetrix, eBioscience, San Diego, CA).

Initially wells from 96-well Corning Costar (Nunc) plates were coated with capture antibody (anti-mouse TNF ,) in coating buffer and incubating overnight at 4oC. Plates were then washed 3 times with 250 l/well wash buffer consisting of 1x phosphate buffered saline (PBS) containing 0.05% Tween-20. Wells were then subsequently blocked with 200 l/well 1x ELISA ELISPOT buffer at room temperature for 1 hour and all wells washed as previously described. TNF standards consisting of eight serial dilutions of mouse TNF were then prepared and 100 l/well of standards were added to corresponding wells. Additionally, 100 l of media from stimulated cells were added to appropriate

wells with two additional wells containing ELISA/ELISPOT buffer which served as plate blanks. Plate was sealed and incubated for 2 hrs. at RT, afterwhich wells were washed as previously described. The detection antibody (Anti-mouse TNF -Biotin) in buffer was added at 100 l/well. Plate was sealed and incubated at RT, for an additional 1 hr. and wells washed as previously described. 100 l/well of Avidin-Horseradish Peroxidase (HRP) in buffer was then added to corresponding wells, and plate was sealed and allowed to incubate for 1 hr at RT, and wells were washed as previously described. TMB solution was then added at 100 l/well and incubated at RT, 15 min. afterwhich 50 l of stop solution (1M H2PO4) as added and plates were read at 450 nm on a plate reader (BioTek)

Results

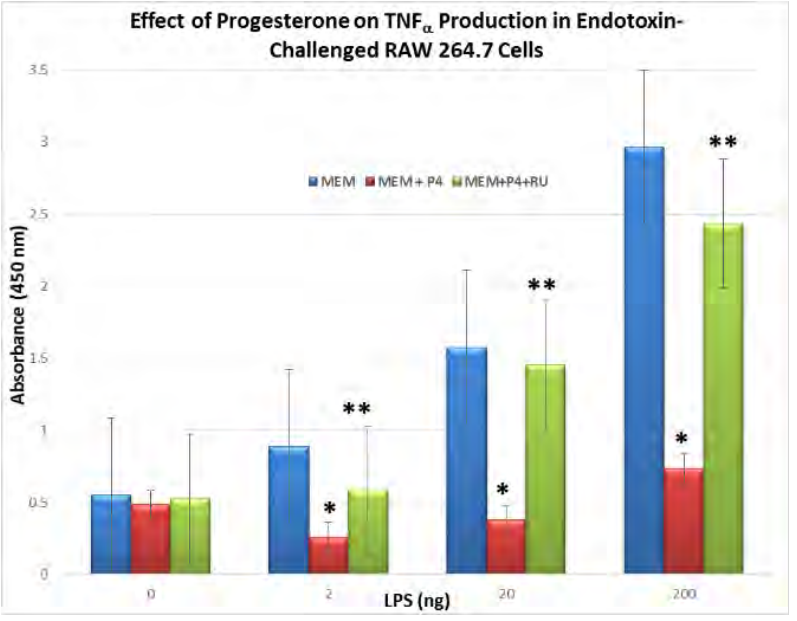
Nitric Oxide Production as a Result of Endotoxin Challenge

Initially, after endotoxin challenge, there was a significant increase ($p < 0.05$) in NO production at all concentrations as compared to controls (Fig. 1, Table 1). Specifically, there was an approximate 40% increase in NO release from RAW cells with each 10-fold increase in endotoxin concentration (2 ng/ml, 20 ng/ml, 200 ng/well). Upon addition of a saturating concentration of progesterone (10 M), there was a significant decrease ($p < 0.05$) in NO production by 12, 46 and 62% at 2, 20 and 200 ng/ml, respectively. To determine if this effect was due to activation of the progesterone receptor specifically, the P4-R antagonist RU-486 was co-incubated in wells containing stimulated cells along with progesterone. In these wells, there was a rebound in NO production – which is to say a reversal of the inhibitory effect – in cells incubated with 10 M RU-486 by 7, 27 and 58%; while there was a significant difference ($p < 0.05$) in the cells stimulated with 20 and 200 ng/well LPS, cells stimulated with 2 ng/ml endotoxin did not surpass this level of statistical scrutiny.

Figure 1. Effect of Progesterone (P4) on Nitric Oxide (NO) Production. RAW cells were treated with a 10-fold increase in LPS to stimulate a significant ($p < 0.05$) concentration-dependent production of NO (blue bars). Treatment of cells in conjunction with 10 M P4 elicited a significant inhibition of NO production (red bars). Treatment of cells with 10 M RU-486 – a potent antagonist of P4

DOI: 10.20429/stem.2017.010108

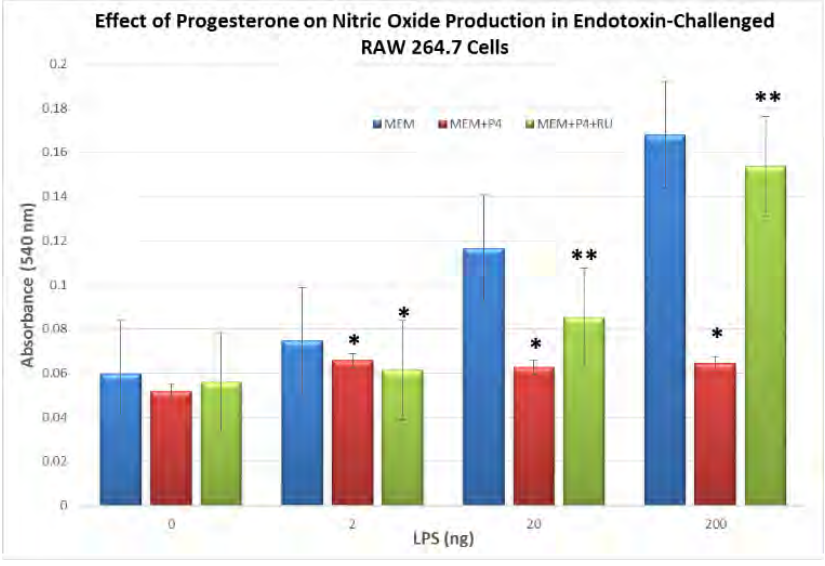
exhibited a partial reversal of this inhibitory effect (green bars). * denotes a significant difference to LPS stimulated cells at that concentration, and ** denotes a significant difference from LPS stimulated cells and LPS + P4 stimulated cells.



Tumor Necrosis Factor-Alpha Production as a Result of Endotoxin Challenge

Stimulation of RAW 264.7 cells with 2, 20 and 200 ng LPS resulted in a significant ($p < 0.05$) concentration-dependent increase in TNF production as compared to controls (Fig. 2, Table 1). Specifically an approximate 35% increase in TNF upon stimulation with 2 ng/ml LPS as compared to controls, followed by a 70% increase in TNF production with two successive 2-fold increases of LPS concentrations. Upon co-incubation with 10 M P4, there was a significant ($p < 0.05$) decrease in TNF production by approximately 70% at all concentrations of LPS. After inclusion of 10 M RU-486 along with the saturating concentration of P4, a similar reversal of the inhibitory effect of P4 alone was seen as was observed in the NO assays. While no significant difference was seen at the low concentration of LPS (2 ng/ml), there was significance at the two higher concentrations (20, 200 ng/ml).

Figure 2. Effect of Progesterone (P4) on Tumor Necrosis Factor-Alpha Production. RAW cells were treated with a 10-fold increase in LPS to stimulate a significant ($p < 0.05$) concentration-dependent production of TNF (blue bars). Treatment of cells in conjunction with 10 M P4 elicited a significant inhibition of NO production (red bars). Treatment of cells with 10 M RU-486 – a potent antagonist of P4 exhibited a partial reversal of this inhibitory effect (green bars). * denotes a significant difference to LPS stimulated cells at that concentration, and ** denotes a significant difference from LPS stimulated cells and LPS + P4 stimulated cells.



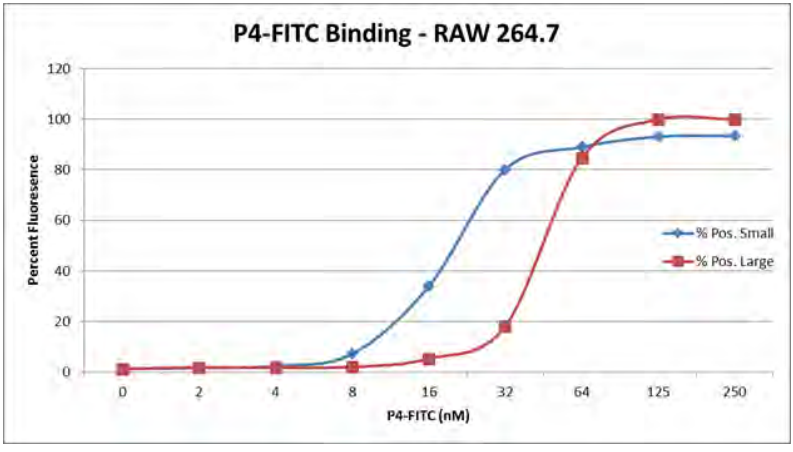
Discussion

These data clearly indicate that stimulation of RAW 264.7 cells with varying concentrations of lipopolysaccharide (LPS) initiate an inflammatory response insofar as the production of nitric oxide (NO) and tumor necrosis factor-alpha (TNF) are concerned. These results are to be expected however as RAW cells are an immortalized murine macrophage leukemic cell line established from an ascites of a tumor induced in a male mouse (*Mus musculus*) by intraperitoneal

DOI: 10.20429/stem.2017.010108

injection of Abselon Leukaemia Virus (A-MuLV) (Raschke WC, 1978). RAW cells were chosen as the experimental model in the present study as they propagate quickly, cell culture needs are minimal - but more importantly - upon stimulation with an inflammatory insult results in a myriad of immunologic responses. In the present study, we were able to show that RAW 264.7 cells express the progesterone receptor, and that progesterone binds with high affinity and saturation at 125 nm (Fig. 3).

Figure 3. P4-FITC Binding Assay. RAW 264.7 cells were treated with 2-fold serial dilutions of FITC-P4 in Ros media and incubated, 1 hr. 100 l of cell-P4-FITC solution was then added to 300 l FACS buffer and incubated an additional hr. Cell suspension was then washed 3x followed by analysis on an Accuri C6 Plus flow cytometer (Becton-Dickinson).



Further, stimulation of RAW cells with LPS initiates an inflammatory response by the production of NO and TNF in a concentration dependent manner. Additionally, we were able to show that upon co-incubation with progesterone this response was ameliorated as shown by the reduction in both inflammatory mediators. That being said, because we have demonstrated this response, it should not be assumed that this response is due to the effects of progesterone acting solely through its receptor. In fact, in a rather elegant study by Wolfson and colleagues (Wolfson ML, 2016), it was shown that in a rat model, stimulation with endotoxin resulted in a significant increase in NO production, and that this

effect was inhibited by treatment with progesterone as seen in the current study. However, it was also reported in this study that this response was independent of progesterone receptor activation as co-treatment with a progesterone receptor antagonist had no apparent effect on this inhibitory effect (Wolfson ML, 2016). These authors went further to suggest that perhaps this response was an effect of glucocorticoid receptors activation as incubation of cells with a glucocorticoid receptor antagonist restored similar NO levels as was seen in cells stimulated with LPS alone. In the present study, we show that in fact the progesterone receptor activation is involved by a partial reversal of progesterone's inhibitory activity of both NO and TNF (Fig's. 1, 2; Table 1).

Table 1. Production of nitric oxide and tumor necrosis factor from LPS-challenged RAW 264.7 cells, and subsequent P4-dependent inhibition. Nitric oxide assays revealed a concentration-dependent increase in NO production followed by a 12, 46 and 62% inhibition following P4 incubation. Tumor necrosis factor assays revealed a similar concentration-dependent increase in cytokine production followed by a 71, 76 and 75% inhibition of TNF following P4 incubation. With the exception of the low dose of LPS in the NO assays, RU-486 revealed a partial reversal of this inhibitory effect.

NO Production in LPS-Challenged RAW 264.7 Cells				
LPS (ng)	NO (pg)	NO Gain	P4 Inhibition	Antagonism RU-486
2	17.3	20.09%	12.05%	NA
20	28.25	48.78%	46.21%	26.56%
200	47.54	64.48%	61.71%	58.13%
TNFα Production in LPS-Challenged RAW 264.7 Cells				
LPS (ng)	TNFα (pg)	TNFα Gain	P4 Inhibition	Antagonism RU-486
2	51.11	37.52%	70.71%	55.56%
20	178.89	64.86%	76.09%	74.12%
200	642.11	81.32%	75.26%	69.84%

pecifically, regarding the production of these two inflammatory mediators, a 58.13% and 69.84% increase in NO and TNF was observed, respectively from cells stimulated with 200 ng LPS (high dose). With this in mind, the question arises as to why the contrasting effects in this study as compared to that reported by Wolfson et al. To this point, the answer may lie in the fact that the study by Wolfson was conducted through the in vivo infusion of LPS to pregnant and

DOI: 10.20429/stem.2017.010108

non-pregnant mice with or without progesterone and two differing antagonist – RU-486 and Lonaprisan – a P4 antagonist with higher affinity for the P4-R than RU-486. Peripheral blood monocytes (PBMC) were then collected, cultured for 24 hrs afterwhich cell supernatants assayed for nitrates and nitrites as described. Additionally, PBMC from these mice were also assayed for inducible nitric oxide synthase (iNOS) mRNA as an indicator of NO production. In the present study, our group directly stimulated RAW 264.7 cells (a macrophage cell line) with a range of LPS concentrations as well as fixed concentrations of both progesterone and RU-486. Given this, it is difficult to compare the two studies due to the fact that in the report by Wolfson et al., LPS (1 g/g), progesterone (67 g/g – pregnant mice and 4 g/g – non-pregnant mice), RU-486 (10 g/g) and Lonaprisan (1 g/g) were administered via intraperitoneal injection while in the present study, RAW cells were stimulated with LPS (2, 20 and 200 ng/ml), and further treated with progesterone (10 M) and RU-486 (10 M) at fixed concentrations. Irrespective of these discrepancies, it seems clear that 1) progesterone plays a centralized role in the maintenance of pregnancy and regulation of NO and TNF concentrations in reproductive tissues and, based on these results, that 2) it is an important inhibitory mediator in the inflammatory response insofar as NO and TNF production are concerned, and 3) the action of which are at least in part, through activation of the progesterone receptor.

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