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# Macrophage secretions modulate the steroidogenesis of polycystic ovary in rats: Effect of testosterone on macrophage pro-inflammatory cytokines

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ABSTRACT

*Aims:* The macrophage secretions' effect on ovarian steroidogenesis is investigated in a polycystic ovary syndrome rat model (PCO rat). The influence of testosterone environment on the expression of macrophage pro-inflammatory cytokines that participate in ovarian steroidogenesis is studied.
*Main methods:* PCO rats were induced by estradiol valerate. Spleen macrophages were cultured with and without testosterone ($10^{-6}$ M) and their secretions were used to stimulate ovaries from PCO and control rats. Ovarian hormones released and ovary mRNA levels of P450 aromatase and 3β-hydroxysteroid dehydrogenase were measured by radioimmunoassay and RT-PCR, respectively. The tumor necrosis factor alpha (TNFα) and nitric oxide (NO) levels in macrophage culture medium, along with the TNFα, interleukin (IL)-6, IL-10 and androgen receptors (AR) mRNA levels in macrophage cells were determined.
*Key findings:* Macrophages from PCO rats released more TNFα and NO, expressed higher TNFα and IL-6, lower AR, and no change in IL-10 mRNA levels than control macrophages. TNFα, IL-6 and AR changes were greater after macrophage testosterone treatment. Macrophage secretions from PCO rats stimulated androstenedione and decreased estradiol release and ovarian mRNA P450 aromatase expression in PCO rats compared to macrophage secretions from control rats.
These effects were greater when macrophages from PCO rats were treated with testosterone. Ovarian progesterone response was unchanged.
*Significance:* The differential steroidogenic ability of macrophage secretions from PCO rats is associated to the in vitro testosterone environment. Testosterone, probably acting on macrophage AR, induces a greater release of TNFα, modifying ovarian response by increasing androstenedione and slightly decreasing estradiol without affecting progesterone.



## Introduction

It is known that ovarian steroids influence the immune system, or conversely, that the immune system modulates the ovarian function. In particular, macrophages (Mϕ) in the ovary are detected in fluctuating number and phenotype depending on the stages of the estrus and menstrual cycle (Wu et al., 2004, 2007). Mϕ express functional sex hormone receptors and also secrete nitric oxide (NO) along with tumor necrosis factor alpha (TNFα), interleukins (IL)-1, IL-6, IL-10, IL-12 and many other cytokines and growth factors that regulate ovarian function (Adashi, 1990; Miller and Hunt, 1996; Gallinelli et al., 2003). There is evidence that NO decreases ovarian steroidogenesis by inhibiting the steroid acute regulatory protein, 3β-hydroxysteroid dehydrogenase (3-βHSD) and the cytochrome P450 side chain cleavage gene expression (Rekawiecki et al., 2005). TNFα influences the reproductive axis, inducing changes that closely resemble those found in patients with hyperandrogenism. TNFα stimulates proliferation and steroidogenesis in in vitro rat theca cells facilitating the effects of insulin and IGF-I (Spaczynski et al., 1999). Also, TNFα induces apoptosis and anovulation in the ovaries (Greenfeld et al., 2007).

Polycystic ovary syndrome (PCOS) is a common and complex endocrine disorder characterized by anovulation, infertility, hyperandrogenemia, hyperandrogenism and insulin resistance as frequent metabolic traits in women of reproductive age (Goodarzi et al., 2011). Patients with PCOS exhibit chronic low-grade inflammation, which is manifested as elevated levels of classic markers of inflammation such as: C-reactive protein (Escobar-Morreale et al., 2011); TNFα (Gonzalez et al., 1999); IL-6 and IL-18 (Escobar-Morreale et al., 2001). Particularly, a recent metaanalyses of nine studies evaluating the mean difference in serum TNFα concentrations among patients with PCOS and the corresponding controls, revealed no statistically significant differences (Escobar-Morreale et al., 2011).

Ovaries from most women affected by PCOS are characterized by thecal hyperplasia. Theca cells from PCOS patients produced

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testosterone more efficiently than normal theca cells (Nelson et al., 2001). This excessive ovarian androgen production has been related with an increased androgen biosynthesis and cytochrome P450c17 alpha gene expression (Nelson-Degrave et al., 2005).

Several studies suggest that immune regulation may be involved in the etiology of PCOS (Amato et al., 2003; Niccoli et al., 2011). We have shown a functional relationship between the ovarian androgens and immune cells in a rat model of PCOS induced by estradiol valerate (EV). The splenocytes culture from those rats showed a decrease in androgen receptor and IL-12 mRNA expression, and their secretions decreased the ovarian androstenedione ($A_2$) release (Forneris et al., 2008). Moreover, increased levels of pro-inflamatory cytokines as IL-1β, IL-6 and TNFα in follicular fluids have been measured in patients with PCOS (Jasper and Norman, 1995; Amato et al., 2003). However, the role of immune cells on the steroidogenesis of polycystic ovary remains obscure, possibly due to the limited availability of human tissue and animal models for the study of these disorders, and also to the different analysis procedures, different study cell populations and different experimental models used (Jasper and Norman, 1995; Deshpande et al., 2000; Forneris et al., 2008).

Therefore, the aims of this work are to investigate in a rat model of PCOS induced by EV (PCO rats): (1) whether secretions of Mϕ influence the steroidogenic response of the ovary, and (2) if the androgen environment affects the Mϕ pro-inflammatory cytokine expressions and consequently, their effects on the ovarian hormones. For that, secretions of Mϕ from spleen were used to stimulate ovaries from PCO rats.

## Materials and methods

### Chemicals

Estradiol valerate (EV), testosterone (T), fetal bovine serum and RPMI 1640 medium were purchased from Sigma (St. Louis, MO, USA). TRIzol reagent was obtained from Invitrogen/Life Technology. 1,2,6,7-[$^3$H]-Progesterone (107.0 Ci/mmol) and 1,2,6,7-[$^3$H]-androst(4-ene-3,17)dione (115.0 Ci/mmol) were provided by New England Nuclear Products (Boston, MA, USA). Other reagents and chemicals were of analytical grade.

### Animals

Adult Holtzman cycling rats showing at least two regular 4-day cycles were used. They were housed in a controlled environment (22–24 °C, 12 h light–12 h dark). Water and food were available ad libitum. Animals were handled according to the procedures approved in the UFAW Handbook on the Care and Management of Laboratory Animals – vol 1– Terrestrial vertebrates — edn 7, edited by T Poole (1999), and the experimental protocol was approved by the Committee for the Use and Care of Animals of the National University of San Luis. Two groups of rats were used. The first consisted in PCO rats to which the PCOS model was induced at 60 days of age. This was accomplished by the administration of EV as a single intramuscular injection (2 mg/rat diluted in 0.2 ml corn oil) (Brawer et al., 1986) in order to resemble, in some aspects, the human syndrome. This model is characterized by polycystic ovarian morphology, persistent estrus condition and anovulation (Lara et al., 1993) accompanied by increased circulating estradiol levels and hypersecretion of androgens from in vitro incubated ovaries (Brawer et al., 1986; Lara et al., 1993). This is in agreement with what we have previously observed (Forneris et al., 2003). In addition, EV administration to rats is associated with increased ovary sympathetic activity (Lara et al., 1993).

The second group, non-PCO rats (control rats), was injected with vehicle alone. All experiments were performed two months after the injection of EV when cystic follicles were observed by light microscopy. Since PCO rats predominantly showed cornified vaginal smears, control rats were sacrificed by decapitation on estrus day. The spleen and the ovaries were removed to obtain macrophages (for culture and for ovarian incubations, respectively).

### Macrophage culture and treatments

The spleens from PCO and control rats were washed in saline solution and pressed through a sterile nylon screen (200-μm mesh) to obtain the total cell populations. After centrifugation, the cells were resuspended in serum-free RPMI 1640 medium and treated with $NH_4Cl$. Cell number and viability were assessed microscopically using trypan blue exclusion. $3\times10^6$ viable cells/ml of medium were incubated in culture medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), and antibiotics (50 μg/ml streptomycin and 50 units/ml penicillin), defined as basal medium (BM), in culture plates. After incubation for 2 h at 37 °C in 95% air–5% $CO_2$, the non-adherent cells were removed. The adherent Mϕ monolayer showed 90% of purity according to morphologic analysis and nonspecific esterase staining. The Mϕ from PCO rats (PCO Mϕ) as well as the Mϕ from control rats (control Mϕ) were plated at a density of $1\times10^6$ cells/well in a final volume of 1 ml in culture plates, preincubated in BM for 24 h and subsequently cultured in the absence or presence of $10^{-6}$ M testosterone for 24 h. Afterwards, the medium was removed, the Mϕ were washed twice with BM to remove possible residual testosterone and finally, the cells were cultured in BM for an additional 24 h period. The respective culture media were collected and used to stimulate ovaries from PCO rats (PCO ovaries) as well as from control rats (control ovaries) to measure the steroid release. Basal secretions of $A_2$, estradiol ($E_2$) and progesterone were obtained by ovarian incubation only with BM.

### Ovary incubation and steroid assays

After killing the rats, the ovaries were halved and preincubated in 1 ml of BM at 37 °C, in a 95% $O_2$–5% $CO_2$ mixture. After 15 min, the medium was discarded and 1 ml of Mϕ culture medium was added. Incubation was continued for 3 h and then the medium was removed and stored at −20 °C until measuring $A_2$, $E_2$ and progesterone contents. Progesterone and $A_2$ of culture media were determined by radioinmunoassay (Oliveros et al., 2001). The assay sensitivity was less than 5 ng/ml for progesterone, and 0.02 ng/ml for $A_2$. The inter- and intra-assay coefficients of variation for the assays were less than 10.0%. The $E_2$ was measured using an Elecsys estradiol kit. The electrochemiluminescence was detected by analyzer Roche Elecsys 1010 and the analytical sensitivity was 5 pg/ml. The results were expressed as nanograms of progesterone and $A_2$ per milligram of ovarian tissue (ng progesterone/mg tissue and ng $A_2$/mg tissue, respectively), and as picograms of $E_2$ per milligram of ovarian tissue (pg $E_2$/mg tissue).

### RNA isolation and reverse transcription polymerase chain reaction

Total RNA was extracted from Mϕ culture using TRIzol reagent. The RT-PCR, a semi-quantitative analysis of mRNA, was performed using a one-step RT-PCR method (Access RT-PCR system, Promega, Madison, USA). All components for RT and PCR were assembled in 50 μl reactions containing 5× reaction buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 3 mM $MgCl_2$, 10 mM dNTP mixture, 1 μM of each gene specific primers, 2 μg template RNA, 5 units of AMV reverse transcriptase and 5 units of *Tfl* DNA polimerase. The amplification of cDNA was done under the following conditions: denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for AR, 60 °C TNFα, IL-6, IL-10 and GAPDH, and 59 °C for 3β-HSD and P450 aromatase, during 1 min, and extension at 72 °C for 2 min. The reaction was completed with a final extension at 72 °C for 7 min (thermal cycler).