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

Florencia Figueroa [a], Roberto Davicino [b], Blas Micalizzi [b], Liliana Oliveros [a], Myriam Forneris [a,*]

[a] Laboratorio de Biología de la Reproducción y, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, San Luis, Argentina
[b] Area Microbiología, Departamento de Bioquímica y Ciencias Biológicas, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, San Luis, Argentina

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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

* Corresponding author at: Laboratorio de Biología de la Reproducción, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Avenida Ejército de los Andes 954, 5700 San Luis, Argentina. Tel.: +54 266 4424689; fax: +54 266 4430224/4431301.
E-mail address: mforn@unsl.edu.ar (M. Forneris).

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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. 3×10^6 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 1×10^6 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).

The following primers were used: AR: (5′-CAGCCCCAGCCCAGCGACAGC-3′ sense, and 5′-CAGGGTGAGGGGCGGGCAGTAGGA-3′ antisense), TNFα: (5′-AAGTTCCCAAATGGCCT CCCTCTCATC-3′ sense and 5′-GAGGCTGACTTTCTCCTGGTATGAAA-3′ antisense), IL-6: (5′-CTTCCAGCCATGTGCCTTCT-3′ sense and 5′-GAGAGCATTGGAAGTTGGGG-3′ antisense), IL-10: (5′-GCCTTCAGTCAAGTGAAGAC-3′ sense and 5′-AAACTCATTCATGGCCTTGTA-3′ antisense), 3β-HSD: (5′-GTCTTCAGACCAGAAACCAAG-3′ sense and 5′-CCTTAAGGCACAAGTATGCAG-3′ antisense), P450 aromatase: (5′-TGCACAGGCTCGAGTATTTCC-3′ sense and 5′-ATTTCCACAATGGGGGCTGTCC-3′ antisense), GAPDH: (5′-GGGCTGCCTTCTCTTGTGAC-3′ sense and 5′-CGCCAGTAGACTCCACGACA-3′ antisense). The predicted sizes of the PCR-amplified products were 423, 485, 506, 346, 489, 266 and 325 base pairs (bp) for AR, TNFα, IL-6, IL-10, 3β-HSD, P450 aromatase and GAPDH, respectively. The PCR products were resolved on 2% agarose gel electrophoresis, containing 0.5 mg/ml ethidium bromide and photographed with a Polaroid camera. Band intensities of RT-PCR products were quantified using NIH Image software. The relative abundance of each band was normalized according to the housekeeping GAPDH gene, calculated as the ratio of the intensity values of each product to that of GAPDH. Thus, results are expressed as mRNA/GAPDH in arbitrary units.

Nitrite assay

Mϕ culture supernatants from control and PCO rats were removed and analyzed for NO by assaying nitrite, a stable product of NO oxidation, using the Griess reagent and absorbance was read at 540 nm (Egami and Taniguchi, 1974). The intra-assay coefficients of variation for the assays were less than 10%. Also, nitrite was measured in the supernatat of Mϕ treated with 1 mM N^G-monomethyl-L-arginine (L-NMMA), a specific inhibitor of nitric oxide formation from L-arginine, in the culture medium for 24 h. The results were expressed as millimole of nitrite per milliliter (mmol/ml).

Determination of TNFα

Mϕ cells were incubated for 24 h and TNFα was quantified on cell-free culture supernatants by an ELISA kit (Chemicon International USA) according to the manufacturer's instructions. The cytokine concentration was determined by extrapolation from the TNFα standard curve and expressed as pg/ml.

Statistical analysis

Results are expressed as mean ± SEM. Significant differences among means were considered at a level of $P<0.05$ and identified by one-way ANOVA and Tukey's test.

Results

Effect of macrophage secretions on the ovarian steroids release

The secretions of Mϕ from PCO as well as control rats, with and without testosterone treatment, had a stimulatory effect on A_2 release from ovaries of control and PCO rats, compared to their respective basal values ($P<0.001$). In all cases, PCO ovaries released more A_2 than control ovaries ($P<0.001$). Interestingly, most of A_2 released from PCO ovaries, was observed with secretions of PCO + T Mϕ, suggesting that testosterone treatment modified the ability of secretion of PCO Mϕ on the A_2 release (Fig. 1). By contrast, testosterone treatment of control Mϕ did not modify their secretion ability on the A_2 release from control and PCO ovaries, compared with untreated control Mϕ. Control ovaries released more A_2 after stimulation with secretions of PCO and PCO + T Mϕ, compared with control Mϕ secretions ($P<0.05$) (Fig. 1).

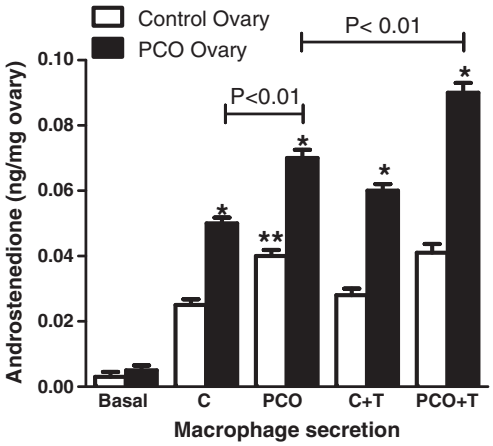

Fig. 1. In vitro androstenedione release from control and polycystic ovaries. Ovaries were stimulated with either basal medium (basal) or culture medium of: control, (C); C + testosterone (T), (C + T); PCO and PCO + T macrophages (all being macrophage secretions). The graph represents the means ± SEM from three experiments with three rats per group. *$P<0.001$ vs respective control ovary, and **$P<0.05$ vs control macrophage secretion on control ovary.

Secretions of control and PCO Mϕ, with and without testosterone treatment, also had a stimulatory effect on E_2 release from PCO and control ovaries, compared to the respective basal values ($P<0.001$). The PCO ovaries had a lower E_2 response after stimulation with secretions from PCO Mϕ ($P<0.05$), and even lower with secretions from PCO + T Mϕ ($P<0.01$), compared to their respective control ovaries. By contrast, testosterone treatment of control Mϕ did not modify their secretion ability on the E_2 release from control and PCO ovaries, compared with untreated control Mϕ. Secretions of PCO and PCO + T Mϕ decreased the E_2 release from control and PCO ovaries, compared with control Mϕ secretions (Fig. 2).

As it was observed with the release of A_2 and E_2, all tested Mϕ secretions from control and PCO ovaries showed higher progesterone release than their respective basal values ($P<0.001$) (Fig. 3). After stimulation with each Mϕ secretion, similar progesterone levels were released among control ovaries as well as PCO ovaries. However, PCO ovaries released more progesterone than control ovaries ($P<0.05$). Thus, the progesterone response could be due to differences between

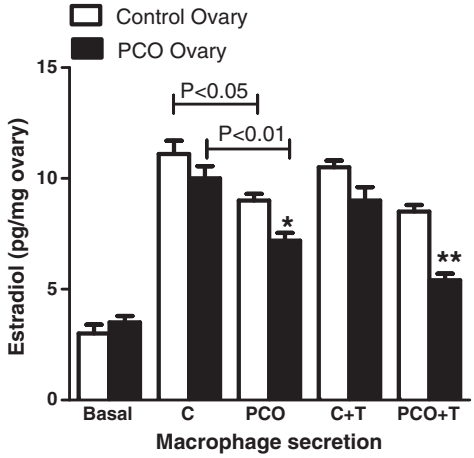

Fig. 2. In vitro estradiol release from control and polycystic ovaries. Ovaries were stimulated with either basal medium (basal) or culture medium of: control, (C); C + testosterone (T), (C + T); PCO and PCO + T macrophages (all being macrophage secretions). The graph represents the means ± SEM from three experiments with three rats per group. *$P<0.05$ and **$P<0.01$ vs respective control ovary.

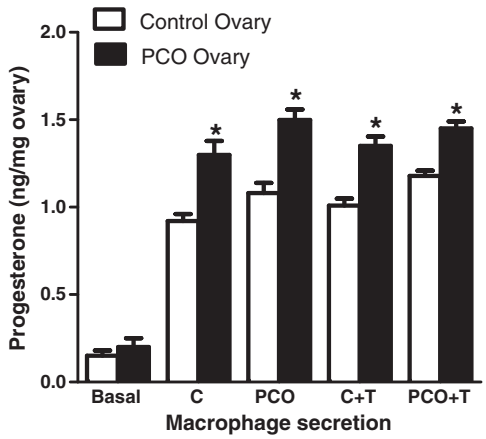

Fig. 3. In vitro progesterone release from control and polycystic ovaries. Ovaries were stimulated with either basal medium (basal) or culture medium of: control, (C); C + testosterone (T), (C + T); PCO and PCO + T macrophages (all being macrophage secretions). The graph represents the means ± SEM from three experiments with three rats per group. *P<0.05 vs respective control ovary.

control and PCO ovaries, but not to differences among Mϕ secretions (Fig. 3).

We have also observed that a 24-h in vitro treatment of PCO Mϕ with 10^{-6} M testosterone plus 10^{-4} M flutamide, used as an androgen AR antagonist, does not modify the steroidogenic ability of their secretions on the progesterone response of PCO and control ovaries, in comparison with their respective untreated Mϕ (1.41 ± 0.03 vs 1.48 ± 0.06, and 1.04 ± 0.07 vs 1.10 ± 0.04, respectively).

Effect of PCO on the TNFα and nitrite release from macrophages

In order to explain the effect observed on the liberation of ovarian hormones, the release of the pro-inflammatory cytokines TNFα and NO, which are related to the synthesis of ovarian steroids, were measured in Mϕ. PCO Mϕ released higher TNFα levels than control Mϕ (P<0.01). Testosterone-treatment did not modify the TNFα release from control Mϕ, but it increased the TNFα release from PCO Mϕ (P<0.05), in relation to untreated Mϕ (Fig. 4).

As shown in Fig. 5, the PCO Mϕ released higher nitrite levels than control Mϕ (P<0.01). Testosterone-treatment did not modify nitrite release from control and PCO Mϕ compared with their respective untreated Mϕ. When control and PCO Mϕ were exposed to 1 mM

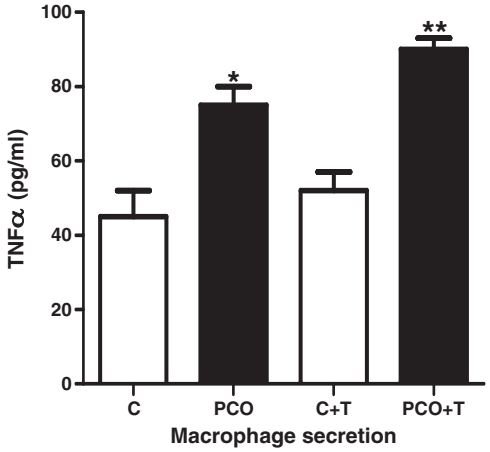

Fig. 4. Levels of TNFα secreted from spleen macrophages of control rats (C), and rats with polycystic ovary (PCO). Control and PCO macrophages were cultured with or without 10^{-6} M testosterone (T), for 24 h. The graph represents the means ± SEM from three experiments with three rats per group. *P<0.01 vs C macrophage, and **P<0.05 vs PCO macrophage.

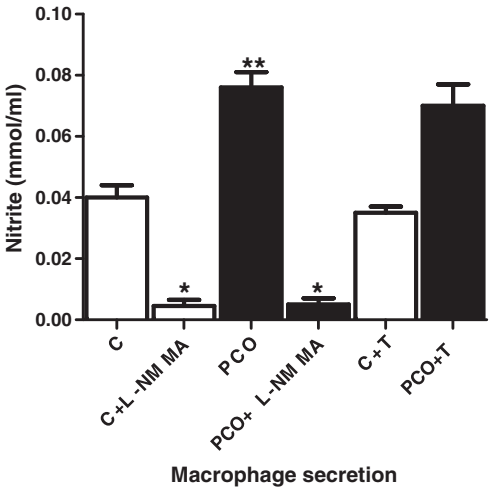

Fig. 5. Levels of nitrites secreted from spleen macrophages of control rats (C), and rats with polycystic ovary (PCO). Control and PCO macrophages were cultured with and without 10^{-6} M testosterone (T), and with or without nitric oxide synthase inhibitor (L-NMMA), for 24 h. The graph represents the means ± SEM from three experiments with three rats per group. *P<0.001 vs macrophage without L-NMMA treatment, and **P<0.01 vs C macrophage.

L-NMMA in the culture medium for 24 h, very low nitrite levels were observed, indicating that nitrite levels released from non-exposed Mϕ can be associated to NO production.

Effect of PCO on mRNA expressions of macrophage interleukins and AR

Other factors that modulate ovarian steroids production are IL-6 and IL-10, which are also known to participate in inflammatory processes. Therefore, their mRNA levels, and the mRNA levels of TNFα, were determined in Mϕ (Fig. 6).

The PCO Mϕ expressed high levels of IL-6 (P<0.01) and TNFα mRNA (P<0.05), without change in the IL-10 mRNA level, compared with control Mϕ. Testosterone treatment increased mRNA expressions of TNFα and IL-6 (P<0.05), but it did not modify IL-10 mRNA levels of PCO Mϕ, compared with the respective untreated Mϕ. The PCO Mϕ expressed low levels of AR mRNA compared with control Mϕ, while control Mϕ as well as PCO Mϕ treated with testosterone, showed a significant decrease of the AR gene expression, in relation to their respective untreated Mϕ (P<0.01). Thus, testosterone environment could modulate the AR expression in both control and PCO Mϕ.

Effect of PCO on mRNA expressions of steroidogenic enzymes

As shown in Fig. 7, the PCO Mϕ and PCO + T Mϕ secretions on control and PCO ovaries, did not modify the ovarian 3β-HSD mRNA expression, compared with BM (basal value). The mRNA expression of P450 aromatase in control ovaries, incubated with secretions from PCO Mϕ and PCO + T Mϕ, was similar to that obtained with incubations with BM. However, the mRNA levels of aromatase of PCO ovaries incubated either with secretions from PCO Mϕ, PCO + T Mϕ or BM, were lower than those observed in their respective control ovaries. The highest decrease was observed with secretions from PCO + T Mϕ.

Discussion

Polycystic ovary syndrome, a complex genetic condition, is a highly prevalent heterogeneous syndrome of clinical and/or biochemical androgen excess, ovulatory dysfunction and polycystic ovaries (Baba et al., 2007; Goodarzi et al., 2011). Immune cells seem to play an important role in the pathology of PCOS, as suggested by the high

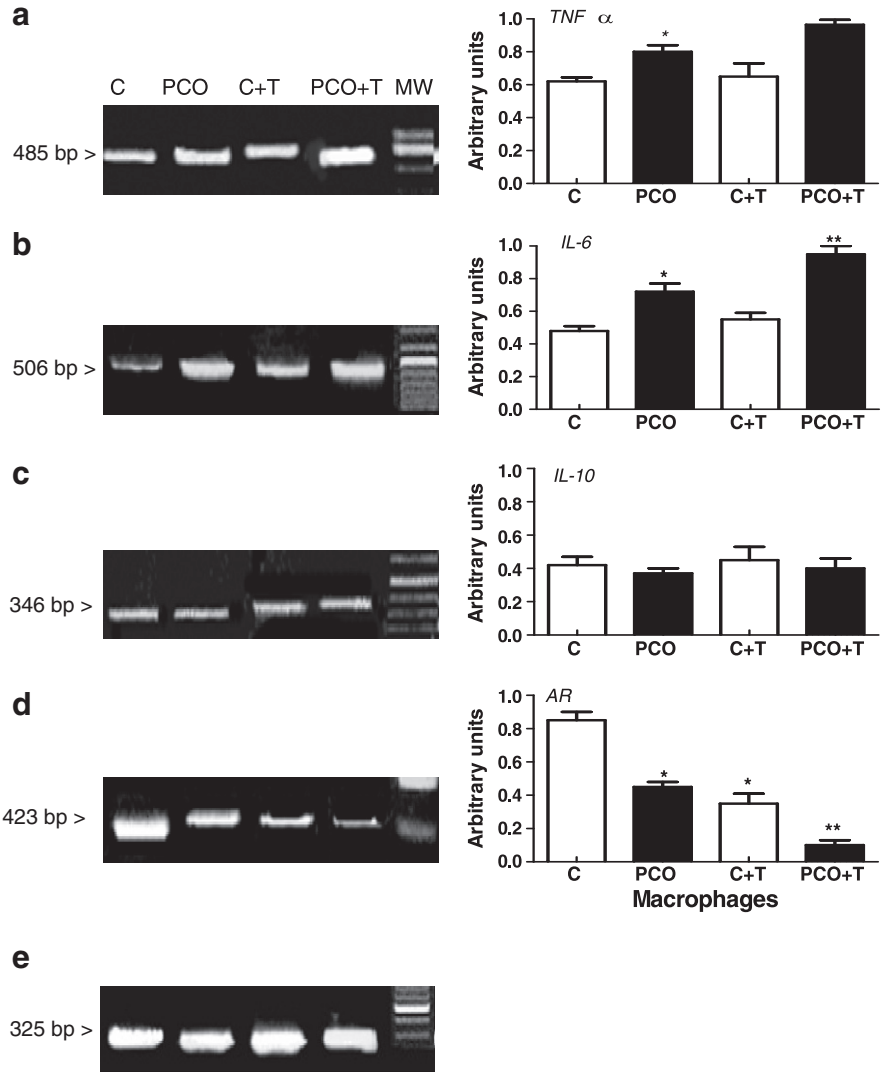

Fig. 6. Expression of spleen macrophage genes from control rats (C) and rats with polycystic ovary (PCO). Representative RT-PCR analysis for (a) TNFα, (b) IL-6, (c) IL-10, (d) androgen receptor (AR) and (e) GAPDH. MW = molecular weight marker. Control and PCO macrophages were cultured with and without 10^{-6} M testosterone (T), for 24 h. On the right is a quantification of the intensity of the fragment bands. Target mRNA was normalized by the level of GAPDH mRNA. Identical results were obtained in four animals per group. TNFα: *P<0.05 vs C and PCO + T; IL-6: *P<0.01 vs C and **P<0.05 vs PCO + T; AR: *P<0.01 vs C and **P<0.01 vs PCO.

concentrations of white blood cells in polycystic ovaries (Bukulmez and Arici, 2000) and the altered leukocyte subset in peripheral blood of women with the disease (Turi et al., 1988; Niccoli et al., 2011). In a previous work, we provided evidence of a functional relation between the ovary and immune spleen cells in PCO rats, by showing that secretions from splenocytes can induce hormone release from the ovary (Forneris et al., 2008). Based on this evidence, we have hypothesized that the characteristic androgen environment of polycystic ovarian condition might modify pro-inflammatory cytokines of Mϕ from spleen, which influence ovarian steroidogenesis.

We observed that PCO Mϕ secretions elicited higher A_2 release and a slightly lower E_2 release from PCO than control ovaries. This was associated with lower mRNA levels of P450 aromatase in PCO than control ovaries. P450 aromatase plays a key role in the conversion of A_2 to E_2; therefore, the increase of A_2 from PCO ovaries could be due, at least in part, to its low conversion to E_2. A low E_2 production has been observed in human PCOS follicles due to insufficient activity and mRNA expression of P450 aromatase (Jakimiuk et al., 1998). Furthermore, when ovaries were incubated with secretions of PCO Mϕ treated with testosterone, the A_2 response of PCO ovaries was even greater, while both E_2 response and aromatase mRNA expression were even lower in comparison with secretions from untreated PCO Mϕ. Results suggest that testosterone environment of Mϕ increases the ability of their secretions to reduce the convertion of A_2 to E_2 in the PCO ovaries.

This steroidogenic effect could be mediated, at least till some extent, by the increase of TNFα released by PCO Mϕ. TNFα and IL-6 are present in the follicular fluid (Deshpande et al., 2000; Tamura et al., 2000) and have the potential to inhibit E_2 production. Preovulatory follicles dissected from ovaries of normal cyclic adult rats have been shown to respond to TNFα with increased A_2 production (Roby and Terranova, 1990). In addition, it has been reported that TNFα activates transcription of an inducible repressor form of 3′,5′-cyclic adenosine 5′-monophosphate-responsive element binding modulator and represses P450 aromatase in ovarian rat granulosa cells (Morales et al., 2006). The increase of mRNA expression of TNFα, as well as the release of TNFα from PCO Mϕ compared with control Mϕ, were even greater after testosterone treatment, suggesting that testosterone environment induces a high production of this cytokine by PCO Mϕ. Elevated levels of TNFα and IL-6 have been

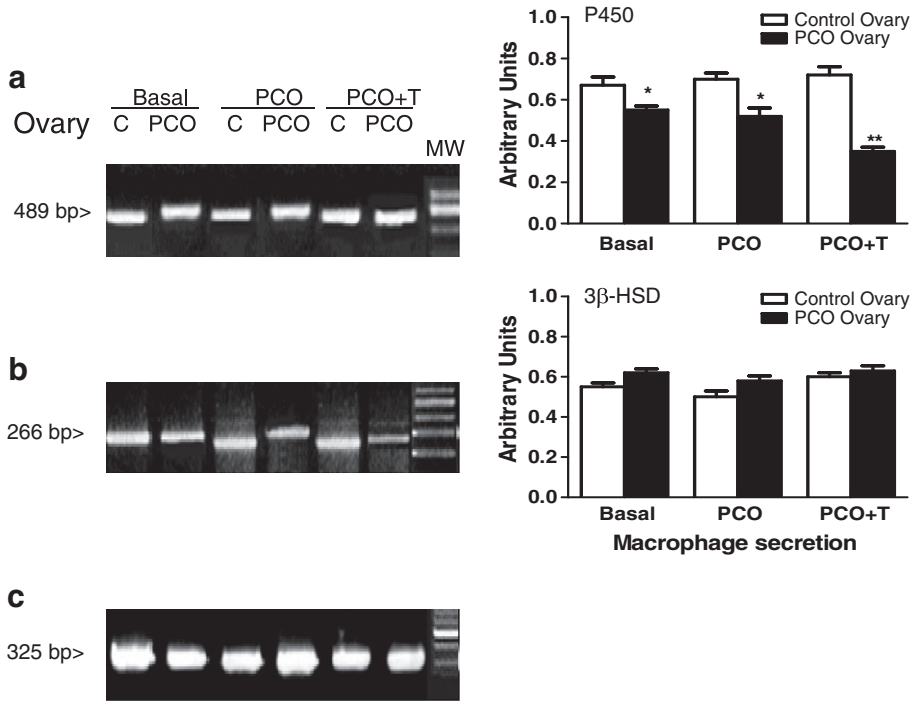

Fig. 7. Expression of genes of ovarian steroidogenic enzymes from control rats (C), and rats with polycystic ovary (PCO). Representative RT-PCR analysis for (a) 3β-HSD, (b) P450 aromatase and (c) GAPDH. MW = molecular weight marker. Ovaries were stimulated with basal medium (basal), or culture medium of: PCO and PCO + testosterone (PCO + T) macrophages (both being macrophage secretions). On the right is a quantification of the intensity of the fragment bands. Target mRNA was normalized by the level of GAPDH mRNA. Identical results were obtained in four animals per group. *P<0.05 and **P<0.01 vs respective control ovary.

observed from peritoneal Mϕ of PCO mice (Deshpande et al., 2000), and in serum of non-obese/non-diabetic PCOS patients treated with gonadotrophins, compared to normal controls (Amato et al., 2003).

Although we have not measured the IL-6 release from Mϕ, we have observed an increase of IL-6 mRNA expression in PCO Mϕ, compared with control Mϕ, which was even greater after treatment with testosterone. This indicates that IL-6 mRNA abundance seems to be modulated, as it was observed with TNFα, by testosterone environment. The increase of IL-6 expression might be mediated by TNFα (Tagashira et al., 2009).

It has been shown that an enhanced IL-6 production may attenuate E_2 production, partially by inhibiting the expression of aromatase mRNA, in follicular cystic ovaries from neonatally E_2-injected mice (Deshpande et al., 2000) and in rat granulosa cells (Tamura et al., 2000). Thus, it is possible that the increased IL-6 expression of PCO Mϕ may contribute to the different steroidogenic ability of Mϕ secretions, in relation to control Mϕ secretions, on the PCO ovary, by decreasing the A_2 convertion to E_2. Furthermore, the NO secreted by PCO Mϕ could be involved in the low E_2 response from PCO ovary, since it has been stated that in vitro synthesis of E_2 is inversely regulated by NO in ovarian rat cells (Dave et al., 1997) and also in human granulosa-luteal cells cultures, being the NO capable of directly inhibiting the activity of aromatase or indirectly decreasing the levels of the mRNA of the enzyme (Snyder et al., 1996).

The Il-10 is an anti-inflammatory cytokine that is crucial for down-regulating pro-inflammatory genes (O'Garra et al., 2008). Synthesis of IL-10 usually occurs as a consequence of acute and chronic inflammatory responses. Recent evidence indicates a condition of low-grade chronic inflammation in women with PCOS, specially associated to an increase of C-reactive protein, that could be considering one of the potential links between hyperandrogenism and the long-term consequences of the syndrome. However, it has been reported that TNFα and IL-6 does not necessarily increase (Repaci et al., 2011; Escobar-Morreale et al., 2011). Despite that in our experimental model we found high levels of TNFα, NO, and high expression of IL-6 in PCO Mϕ, the levels of IL-10 mRNA, with or without testosterone treatment, were not modified compared with untreated Mϕ. In vitro experiments have demonstrated an increased IL-10 production following treatment of CD4+ T lymphocytes, but not of Mϕ, with 5α-dihydrotestosterone, in female mice (Liva and Voskuhl, 2001). In PCO rats, imbalance between Mϕ pro- and anti-inflammatory cytokines could contribute to ovarian hormone alteration.

The significant decrease of the AR mRNA expression in PCO Mϕ, which was very low in the presence of testosterone, suggests a negative regulation of the AR by testosterone. Because it is likely that AR expression at mRNA level indicates AR expression at the protein level, our data suggest that testosterone could be acting directly on PCO Mϕ via the AR to increase TNFα and IL-6 expressions. Thus, it is possible that the high circulating levels of A_2 and testosterone in the PCO rats, which has been previously shown (Forneris et al., 2008), could modulate the Mϕ pro-inflammatory cytokine production through the AR, since both testosterone and A_2 bind to this receptor.

By contrast, the effect of Mϕ secretions on progesterone response from PCO and control ovaries might occur by an AR-independent mechanism since the treatment of PCO and control Mϕ with testosterone, or 10^{-6} M testosterone plus 10^{-4} M flutamide, did not modify the ovarian progesterone release compared with their respective untreated Mϕ.

Furthermore, 3-βHSD mRNA levels were similar after incubation of control and PCO ovaries with all Mϕ secretions. In addition, we have not found changes in serum progesterone levels of PCO rats compared to control (Forneris et al., 2008).

Taken together, our results lead us to assume that testosterone is probably at least one of the factors responsible for the difference in cytokine secretion between Mϕ from control and PCO rats. Testosterone responsiveness of splenic T cells and Mϕ is subjected to a complex regulatory mechanism. Benten et al. (2002) have shown that splenic T cells exhibit responsiveness to 10 nM testosterone by increasing the intracellular $[Ca^{2+}]$. Results also show a possible linkage between TNFα, IL-6 and NO from Mϕ to modulate steroids in the PCO

ovaries. In addition to Mϕ, PCO ovaries also show progressive infiltration of lymphocytes (Bukulmez and Arici, 2000; Niccoli et al., 2011). In this regard, we have previously shown that secretions from splenocytes (mostly lymphocytes) decrease A_2 response from the ovary of PCO rats. Thus, a complex relationship between intraovarian immune cells and the regulation of the ovarian function through a paracrine mechanism may exist in PCO ovaries.

It is still possible that the Mϕ secretions from the spleen might be released into the general circulation to act on hypothalamic–pituitary sites and subsequently produce changes in the ovary.

Conclusions

The present study shows that the steroidogenic ability of Mϕ secretions from PCO rats is modified by the in vitro testosterone environment, stimulating A_2 production by the PCO ovary. Testosterone affects Mϕ pro-inflammatory molecules, such as TNFα, NO, and IL-6, which can contribute to the A_2 increase. Therefore, it is possible to suggest that androgens regulate, in vivo, the macrophage activities of PCO rats, which could provide an additional point of dysfunction that may contribute to the pathogenesis of PCOS.

Conflict of interest statement

The authors of this manuscript declare that there are no conflicts of interest.

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