The effects of metformin on uterine tissue of hyperandrogenized BALB/c mice

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ABSTRACT: The present study investigated the role of the N,N′-dimethylbiguanide metformin (50 mg/kg body weight in 0.05 ml water, given orally with a canulla) in preventing the adverse effects generated by hyperandrogenism on uterine function. Daily injection of dehydroepiandrosterone (DHEA: 6 mg/100 g body weight in 0.1 ml oil) for 20 consecutive days induces polycystic ovaries in BALB/c mice. In this model we found that DHEA produced alterations on uterine histology closely related to the development of pre-cancerous structures concomitantly with increased incidence of uterine apoptosis. The injection of DHEA induced a pro-inflammatory status since uterine prostaglandin (PG) F2 alpha levels and cyclooxygenase 2 were increased although PGE levels were decreased. Furthermore, DHEA promoted a pro-oxidant status since it increased nitric oxide synthase (NOS) activity and decreased superoxide dismutase and catalase activities and the antioxidant metabolite glutathione levels. DHEA also regulated the percentages of CD4+ and CD8+ T lymphocyte that infiltrate uterine tissue. When metformin was administered together with DHEA uterine histology and apoptosis did not differ when compared with controls. Therefore, metformin prevented the pro-inflammatory and pro-oxidative status generated by DHEA and restores the ratios of CD4+ and CD8+ T cells to those observed in controls. We conclude that metformin is able to restore either directly or indirectly uterine function by preventing some inflammatory and oxidative alterations produced by hyperandrogenism.

Key words: PCOS / Metformin / uterus / T lymphocytes / Prostaglandins

Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous disease characterized by hyperandrogenemia, hirsutism, oligo- or amenorrhea and anovulation and is frequently associated with hyperinsulinemia, insulin resistance syndrome, increased cardiovascular risk and diabetes mellitus (Franks, 1995). The detrimental effects of the excess of androgens in the endometrium lead to infertility in women with PCOS (Okon et al., 1998). In addition, androgens can induce hyperplasia and endometrial cancer (Balen, 2001; Pillay et al., 2006). The potential mechanisms underlying these disorders are complex and await complete elucidation. Although during the last decade several clues have emerged from human and animal studies, little is known about the etiology and pathophysiology of PCOS. The battery of animal models used for the study of polycystic ovaries has allowed a focus on different aspects of the pathology. Since dehydroepiandrosterone (DHEA) was found to be one of the most abundant androgens produced by ovaries of women with PCOS, a rodent model was developed by injection of a dose of DHEA equivalent to that of women with PCOS (Roy et al., 1962). Since DHEA is a prohormone, many of its effects, especially endometrial trophic effects, could be mediated by conversion to estrogens. However, subsequent studies established that the DHEA–PCOS murine model exhibits many of the salient features of human PCOS. This model shows hyperandrogenism, insulin resistance, altered steroidogenesis, abnormal maturation of ovarian follicles and anovulation (Lee et al., 1991, 1998; Anderson et al., 1992; Henmi et al., 2001; Luchetti et al., 2004; Sander et al., 2005, 2006; Elia et al., 2006; Solano et al., 2006).

It has been reported that increased levels of androgens induce detrimental effects of the endometrial response resulting in miscarriage (Okon et al., 1998; Tuckerman et al., 2000; Sir-Peterman et al., 2002). In this context, we found that injection of DHEA during early pregnancy in BALB/c mice induces embryo resorption (Sander et al., 2005; Solano et al., 2006) by regulating the expression of uterine progesterone-induced blocking factor, which in turn modulates progestagen (PG) and nitric oxide (NO) systems and cytokine production (Luchetti et al., 2008). In the study of some of the mechanisms...
by which hyperandrogenism alters uterine functions leading to miscarriages, we have previously reported that the injection of DHEA in BALB/c mice induces uterine structures closely related to the development of pre-cancerous structures. These structures are related to a pro-inflammatory and pro-oxidant status of the uterine tissue. These effects are mediated by the PG and NO systems, the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) and the antioxidant metabolite glutathione (GSH) (Elia et al., 2008).

Both infiltrating and resident leukocytes play important roles in the cyclic tissue remodelling events. Reproductive events such as menstruation and implantation are associated with the immune response (Finn, 1986). CD4+ T lymphocytes display a cytolytic activity as they regulate the invasion of extravillous trophoblasts, a crucial process for normal placentation (Finn, 1986). T lymphocytes potentially play a role in the local pathological mechanisms of PCOS (Turi et al., 1988; Luchetti et al., 2004; Sander et al., 2006; Wu et al., 2007). In a previous report we have found that hyperandrogenism diminishes CD8+ and increases CD4+ T lymphocyte infiltration of uterine tissue (Elia et al., 2008).

N,N'-dimethylbiguanide metformin is one of the most common drugs used for the treatment of type 2 diabetes. Metformin decreases hyperglycemia and has beneficial effects on circulating lipids, without affecting insulin secretion (Stumvoll et al., 1995). The glucose-lowering effects of metformin are attributable to both an increase in muscle glucose uptake and a decrease in hepatic glucose production (Hundal et al., 2000). Activation of AMP-activated protein kinase (AMPK) by metformin has been found to be necessary for the decrease in glucose production and the increase in fatty acid oxidation (Fryer et al., 2002; Elia et al., 2006). Unlike the traditionally used glucose-lowering reagents, such as sulfonylureas or insulin, metformin improves cardiovascular functions and reduces cardiovascular risks (Zhou et al., 2001). During the last decades, metformin has been being used for treating women with PCOS (Fedorcsak et al., 2003; Harborne et al., 2003). Metformin decreases androgen levels and improves the frequency of ovulation and menstrual cycles in PCOS patients (Nestler and Jakubowicz, 1996; Ehrmann et al., 1997; Diamanti-Kandarakis et al., 1998; Glueck et al., 2001; Jakubowicz et al., 2001; La Marca et al., 2002). In addition, metformin treatment during early pregnant prevents abortions in women with PCOS (Velázquez et al., 1997; Jakubowicz et al. 2001; Glueck et al. 2004). However, little is known about the mechanisms by which metformin restores uterine functions of women with PCOS (Orio et al., 2005; Palomba et al., 2006).

The aim of the present study was to assess whether metformin is able to improve the pro-inflammatory condition (evaluated by the uterine production of both PGE and PGF2 alpha and the abundance of uterine cyclooxygenase 2 (COX2)) and the pro-oxidative status (evaluated by the SOD, CAT and Nitric oxide synthase (NOS) activities and GSH content) generated in uterine tissue by hyperandrogenism. Considering the important role of resident T cells in uteri, we also investigated whether metformin is able to modulate uterine infiltration of both CD4+ and CD8+ T cells.

**Materials and Methods**

**Animals and experimental protocol**

The hyperandrogenized environment of PCOS was reproduced in mice by injection of DHEA. After examining doses and time of DHEA injection we found that the present model show the salient features of women with PCOS including ovarian cysts, anovulation and insulin resistance (Luchetti et al., 2004). The dose of metformin administered was equivalent to that used in the treatment of women with PCOS. Briefly, female prepuberal (25 days old) mice of the BALB/c strain were injected s.c. daily with DHEA (6 mg/100 g body weight, dissolved in 0.10 ml sesame oil) for 20 consecutive days (DHEA group). The animals of the DHEA + metformin group were injected with DHEA and given metformin orally (50 mg/kg body weight in 0.05 ml water, given orally with a canulla) for 20 days. The controls consisted of two groups: (a) animals injected with oil (0.1 ml) and given water orally (0.05 ml) for 20 consecutive days (control vehicle); (b) the metformin-alone group which consisted of mice treated orally with 50 mg metformin/kg body weight in 0.05 ml water for 20 days. Mice (50 per group) were housed under controlled temperature (22°C) and illumination (14 h light; 10 h darkness; lights on at 05:00 h) and were allowed free access to Purina rat chow and water. All procedures involving animals were conducted in accordance with the Animal Care and Use Committee of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) 1996.

After 20 days of treatment animals were killed by cervical dislocation and freshly dissected uteri from each group (control, metformin, DHEA and DHEA + metformin) were divided as follows: 10 were immediately fixed in 4% (w/v) paraformaldehyde for histological and immunofluorescence studies, 30 were immediately frozen at −70°C until: 10 were used to determine PGF2 alpha by radioimmunoassay, 10 to semi-quantify the abundance of uterine COX2 protein expressions, 10 to evaluate NOS activity. Ten uteri were prepared to quantify uterine CAT and SOD activities and GSH content. All experiments were repeated twice.

**Histological studies**

To study the effect of DHEA and metformin on the uterine tissue, 10 uteri from each group, fixed as described above, were included in paraffin and then were cut (6 mm per section), placed on gelatin-coated slides (Biobond, British Biocell International, Cardiff) and air-dried for 2 h before fixing for 5 min in acetone at 4°C. Then, sections were washed in PBS (pH 7.3) and stained with hematoxylin and eosin (DAKO Corporation, Carpinteria, CA, USA) for histological analysis. Uterine glands were subdivided into a number of morphological types to assess any hyperplastic or neoplastic changes in the endometrium as previously described (Günin et al., 2001; Elia et al., 2008): (i) normal glands: simple tubular glands which can appear in sections as round, oval or elongated with a narrow lumen, and have no branches or daughter glands; (ii) cystic glands: round glands of more than average or large size; (iii) glands with daughter glands: glands with various shapes (round, elongated, tortuous) and sizes, with forming or formed daughter gland or glands inside the epithelium or inside the mother gland lumen, or on the outer surface of the mother gland (budding gland); and (iv) conglomerate of glands: glands with a very complex architecture in which individual glands are closely disposed to each other almost without intervening stroma and have multiple interconnecting lumens; they may develop from glands with daughter glands and are similar to the focus of adenoma or cancer development. Both (iii) and (iv) types of glands are considered to be closely related to the development of malignancy in the endometrium. The epithelium of all glands in randomly selected sections was classified as simple, pseudostratified or stratified (multilayered) epithelia. The number of each type of gland was calculated in randomly selected sections. No less than five sections from each animal were examined. Results are expressed as the proportion (%) of each type of gland.
In situ detection of apoptosis by terminal deoxynucleotidyl transferase mediated dUTP nick-end labelling assay

Nuclear DNA fragmentation was assessed in 4–6 μm thick sections of uteri mounted on silane-coated slides, deparaffinized with xylene and rehydrated, by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labelling method (TUNEL) using the apoptosis detection fluorescein kit according to the specifications of the manufacturer. To avoid artificial DNA fragmentation only nuclease-free solutions were used. Slides were analysed by fluorescence microscopy with a wide band excitation barrier filter suitable for analysing both green (fluorescein-labelled fragmented DNA) and blue (4’,6-diamidino-2-phenylindole; DAPI counterstain identifying cell nucleus) fluorescence. Three independent observers counted apoptotic cells in uteri. The different optical fields (magnification ×400) were selected in a random manner counting at least 1000 cells for each sample. Positive control tissues were uterine samples pretreated with DNase (5 μg/ml type I). To estimate non-specific binding and autofluorescence, negative controls (sections treated without TdT) were included in all the assays. As assessed by morphological criteria, no necrosis was observed.

Prostaglandin radioimmunoassay

The quantification of both PGE and PGF2α were carried out in the incubation media of the uterine tissue as previously reported (Motta et al., 1999). Briefly, the tissue (one uterus per point and ten points per treatment, n = 10) was weighed and incubated in Krebs–Ringer-bicarbonate (KRb) with 11.0 mM glucose as the external substrate, pH = 7.0, for 1 h in a Dubnoff metabolic shaker under an atmosphere of 5% CO2 in 95% O2 at 37°C. At the end of the incubation period, the tissue was removed and the solution acidified to pH 3.0 with 1 M HCl and extracted for PG determination three times with one volume of ethyl acetate. Pooled ethyl acetate extracts were dried under an atmosphere of N2 and stored at −20°C until prostaglandin radioimmunoassay was performed. PGE and PGF2α were quantified by using rabbit anti-serum (Sigma). Sensitivity was 10 pg/tube and cross-reactivity was <0.1% with other prostaglandins. Results are expressed as pg/mg tissue.

Western blotting

Ten uteri from each group (control, metformin, DHEA and DHEA+ metformin) were pooled and lysed for 20 min at 4°C in lysis buffer (20 mM Tris–HCl, pH = 8.0, 137 mM NaCl, 1% Nonidet P-40 and 10% glycerol) supplemented with protease inhibitors (0.5 mM PMSF, 0.025 mM N-CBZ-L-phenylalanine chloromethyl ketone, 0.025 mM N’-p-tosyl-lysine chloromethyl ketone and 0.025 mM L-tosylamide-2-phenyl-ethylchloromethyl ketone). The lysate was centrifuged at 4°C until prostaglandin radioimmunoassay was performed. PGE and PGF2α were quantified by using rabbit anti-serum (Sigma). Sensitivity was 10 pg/tube and cross-reactivity was <0.1% with other prostaglandins. Results are expressed as pg/mg tissue.

Streptavidin–peroxidase complex and diaminobenzidine solution. Consistency of protein loading was evaluated by staining the membranes with Ponceau-S and applying the protein beta actin (43 kDa) (Sigma, USA). The intensities (area × density) of the individual bands on western blots were quantified by densitometry (Model GS-700, Imaging Densitometer, Bio-Rad). The experiment was independently repeated three times. Results are expressed in arbitrary units.

Oxidative stress-related parameters

Ten uteri per treatment were homogenized in homogenization buffer (EDTA (1 mM), KCl (150 mM), β mercaptoethanol (1 mM), Trizma base (20 mM) and sacarose (500 mM), pH = 7.6) and centrifuged at 800 × g for 10 min at 4°C, and supernatant was used for the following stress assays.

SOD assay

SODs are a group of metalloenzymes that detoxify ROS through the conversion of O2•− to hydrogen peroxide and molecular oxygen. Total SOD activity was assayed by a spectrophotometric method based on the inhibition of a superoxide-induced epinephrine oxidation (Sugino et al., 1993). Briefly, supernatants were incubated with 0.05 M carbonate buffer (pH 10.2) containing 0.1 mM EDTA and 30 mM epinephrine in 0.05% v/v acetic acid. The change in SOD activity was measured at 480 nm for 4 min. SOD activity was expressed as the amount of enzyme that inhibited the oxidation of epinephrine by 50%, which is equivalent to 1 U and is expressed in terms of U/mg protein.

CAT activity

The technique used is based on the role of CAT to catalyse the conversion of hydrogen peroxide to molecular oxygen and water (Chance, 1954). Briefly, supernatants (100 μl/point) were incubated with 3 ml of 50 mM phosphate buffer (pH = 7.2) and 100 μl of 3 M H2O2. Since hydrogen peroxide absorbs at 240 nm, its consumption was monitored by a spectrophotometer for 1 min at 10-s intervals. Results are expressed as nmol/mg protein.

GSH content

The GSH assay was carried out as previously described (Motta et al., 2001). The reduced form of GSH comprises the bulk of cellular protein sulphhydryl groups. Thus, measurement of acid-soluble thiol was used for estimation of GSH content in tissue extracts. Briefly, supernatants (50 μl/point) were incubated with 800 μl of 1.5 M Tris buffer (pH 7.4) containing 50 μl of 5 × 10−3 M NADPH and 6 IU of GSH reductase. The reaction involves the enzymatic reduction of the oxidized form (GSSG) to GSH. When Ellman’s reagent (a sulphhydryl reagent 5,5-dithiobis-2-nitrobenzoic acid; Sigma, USA) is added to the incubation medium, the chromophoric product resulting from this reaction develops a molar absorption at 412 nm that is linear for the first 6 min; after which the reaction remains constant. Results were expressed as mM GSH/mg protein.

Uterine NOS activity

NOS activity was measured by monitoring the production of [L-14C] citrulline from [L-14C] arginine as described previously Motta et al. (1999). Briefly, the frozen uterine tissue (one uterus per point and 10 points per treatment, n = 10/group) was homogenized (Tissuemizer Tekmar; Thomas Scientific, Swedesboro, NJ, USA) at 0°C in 3 volumes of 50 mM Hepes, 1 mM Dl-dithiothreitol, 1 mM NADPH and 50 mM L-citrulline, pH = 7.5. Samples were incubated at 37°C for 15 min with 10 μM [14C] arginine (0.3 μCi; C1 = 37 GBq). The samples were centrifuged for 10 min at 1000 × g and then applied to 1 ml of DOWEX AG50W-X8 (Na+ form; Bio-Rad, Hercules, CA, USA) resin.
The radioactivity was measured by liquid scintillation counting. Results were expressed as pmol of [14C]-citrulline produced by g of tissue per min (pmol/g min).

**Immunofluorescence of CD4+ and CD8+ T lymphocytes**

The infiltration of CD4+ T (helper/inducer) and CD8+ T (cytotoxic/suppressor) lymphocytes was evaluated by immunofluorescence of uterine sections using fluorescein isothiocyanate (FITC)-conjugated anti-mouse-CD4+ or with phycoerythrin (PE)-conjugated anti-mouse-CD8+ T cell monoclonal antibodies (Sigma). Briefly, 10 uteri from each group (n = 10), fixed as described above, were include in paraffin and then they were cut (6 μm per section) and placed on gelatin-coated (Biobond, British Biocell International, Cardiff) slides and air-dried for 12 h before they were fixed for 5 min with acetone at 4°C. Sections were washed with 0.01 M PBS (pH 7.3) and treated with 1.25 μl of the respective monoclonal antibody. Incubation was carried out in darkness at room temperature overnight. Isotype controls (IgG1-FITC and IgG2a-PE) were used in paired samples to determine non-specific staining. Cell suspensions obtained from BALB/c lymph nodes were used as positive controls. The CD4+ and CD8+ T cells that infiltrate uterine tissue were determined by fluorescence microscopy and the photographs obtained were analyzed using the WCIF Image J program. The experiment was independently repeated three times. Results are expressed in arbitrary units.

**Flow cytometry for CD4+ and CD8+ T lymphocytes**

To carry out the flow cytometry assay, uterine cells must be dispersed. Briefly, uterine tissue (one uterus from each group, 10 animals for each one) were enzymatically dissociated in culture medium (medium 199, 25 mM NaHCO3, 26 mM Heps, and 50 IU/ml penicillin) with trypsin-free collagenase (740 IU/100 mg tissue) and DNAse (14 IU/100 mg tissue). After 90 min, ovarian cells were washed twice with culture medium, twice with Dulbecco-phosphate-buffered saline (PBS) free of Ca2+ and Mg2+, and twice with culture medium containing EDTA (1 mM). To remove blood cells, suspensions were applied to a Ficoll-hypotique gradient 1.077 (Sigma, USA). centrifuged at 400 × g for 45 min and washed with PBS/0.1% BSA. Cells were counted in a haemocytometer, the viability >80% as assessed by the trypan blue exclusion method, and then processed by direct immunofluorescence. Thus, 100 μl of each cellular suspension at a concentration of 10⁶ cells/ml, was incubated during 30 min at 4°C with: (i) 30 μl of PE mouse IgG2ak isotype control plus 30 μl of FITC mouse IgG2ak isotype control (eBioscience, USA) corresponding the isotype control sample or (ii) 4 μl (=8 μg) PE anti-mouse-CD8+ plus 4 μl (=8 μg) FITC anti-mouse-CD4+ monoclonal antibody (eBioscience, USA) according to the cellular suspension. Antibodies were used at saturating concentration as established after titration by flow cytometry. Then, samples were washed with PBS and PBS–EDTA, fixed with 4% paraformaldehyde and stored at 4°C in darkness and immediately assayed. Fluorescence analysis was evaluated with FACScan® and the Winmdm 2.8 software. Lymphocytes were analysed using different physical characteristics (size and complexity) by gating using forward (FSC: cell size) and side scatter (SSC: cell complexity) parameters. Flow cytometric analysis was performed using standard fluorescence 1 (FL1: FITC anti-mouse-CD4+ T lymphocytes) and fluorescence 2 (FL2: PE anti-mouse-CD8+ T lymphocytes). The analysis was based on measurement of 50 000 nucleated cells/assay within the lymphocyte population, characterized by size and complexity. The percentage of positively labelled CD4+ and CD8+ cells was calculated by subtracting signals from non-specifically labelled cells.

**Statistical analysis**

Statistical analyses were carried out by using the Instant program (GraphPad software, San Diego, CA, USA). Analysis of variance was performed by using the Newman–Keuls test to compare all pairs of columns, and P < 0.05 was considered significant. All results are presented as the mean ± standard error of the mean (SEM).

**Results**

**Uterine histology**

Microscopic examination of uteri from the control and metformin groups (Fig. 1a and b, respectively) revealed normal endometrium, showing normal and cystic glands. Glands were lined with simple cuboidal epithelium. Histological examination of uteri from DHEA-treated mice (Fig. 1c) revealed glands with daughter glands and glands forming conglomerates. Glands were lined with pseudo-stratified or stratified epithelium with atypical cells and nuclei. Histological examination of uteri from DHEA + metformin-treated mice revealed normal endometrium, showing normal and cystic glands like those observed in the control group (Fig. 1d). A semi-quantitative analysis is shown in Fig. 1e and f.

**In situ detection of apoptosis by TUNEL**

The TUNEL method was used to detect the fragmentation of DNA which is one of the first morphological changes of the apoptotic process. Figure 2 illustrates apoptotic uterine cells detected by fluorescence microscope examination. The number of cells showing positive fluorescence nuclear staining in cells from uteri of control and metformin group was limited (Fig. 2a and b, respectively). Conversely, hyperandrogenization with DHEA was associated with a greater number of apoptotic cells as compared with controls (Fig. 2c).

When metformin was administered together with DHEA, the number of cells showing fragmented nuclei was similar to that of controls (Fig. 2d). A semi-quantitative analysis is shown in Fig. 2e.

**Synthesis and secretion of PGE and PGF2 alpha by the uterus**

Treatment with DHEA diminished uterine PGE and increased PGF2 alpha levels in the incubation media when compared with the control group (Fig. 3a and b, respectively). When metformin was administered together with DHEA, the concentration of PGE remained significantly lower than controls, whereas the concentration of PGF2 alpha was similar to that of controls (Fig. 3a and b).

**Uterine cyclooxygenase abundance**

To evaluate how both DHEA and metformin modulate the uterine prostaglandin pathway, we further evaluated its role in uterine COX1 and COX2 abundance. We found that DHEA treatment did not modify COX1 abundance (Fig. 4a) but did significantly decrease the expression of uterine COX2 when compared with the control group (Fig. 4b). When metformin was administered together with DHEA, the abundance of COX2 was similar to that of controls (Fig. 4b).
Uterine oxidative stress
Experiments were performed to determine whether the treatment with metformin was able to prevent uterine oxidative stress induced by hyperandrogenization. The hyperandrogenization with DHEA decreased SOD and CAT activities and uterine GSH content (Fig. 5a, b and c, respectively). Metformin administered with DHEA was not able to prevent the effects of DHEA in the antioxidant defenses (Fig. 5b and c). Metformin given alone decreased SOD activity (Fig. 5a).

NOS activity
DHEA increased uterine NOS activity when compared with controls (Fig. 6). When metformin was administered with DHEA, the uterine NOS activity was similar to that of the control group (Fig. 6).

Lymphocyte infiltration and immunophenotype
Considering that the immune system plays an active role in the uterine function, we were interested in studying the role of DHEA and metformin in modulating the immunophenotype of T lymphocytes that infiltrate the uterine tissue. A representative section of each treatment is shown in Fig. 7 and the analysis by the WCIF Image J software is represented in Fig. 8. We found that the treatment with DHEA diminished the percentages of CD8+ T lymphocytes (Fig. 7c) and increased the percentages of CD4+ T cells (Fig. 7d) when compared with controls (Figs. 7a, b and 8). In contrast, when metformin was administered with DHEA, the percentages of CD8+ (Fig. 7e) and CD4+ T cells (Fig. 7f) were similar to those of controls (Fig. 8). The patterns of CD8+ and CD4+ T cells corresponding to metformin group were similar to controls (data not shown). These data were confirmed by flow cytometry assay. Figure 9
illustrates a representative analysis of the 10 controls, 10 DHEA and 10 DHEA + metformin samples using forward scatter (cell sizes) and side scatter (granularity) parameters. Metformin alone group showed the same pattern that controls (data not shown). The flow cytometry analysis showed that of the lymphocyte population in control samples 64 ± 2% were CD8⁺ and 36 ± 2% were CD4⁺. In the metformin-treated group, 67 ± 2% of lymphocytes were CD8⁺ and 33 ± 3% CD4⁺; and in the DHEA + metformin group the lymphocyte population was made up of 66 ± 2% CD8⁺ and 34 ± 4% CD4⁺ cells. In contrast, in the DHEA-treated group the percentages of CD8⁺ and CD4⁺ cells were 30 ± 1 and 71 ± 2, respectively. Thus in the DHEA-treatment group the percentages of CD4⁺ T lymphocytes were significantly higher than in the control group, as well as in the metformin and DHEA + metformin treatment groups. On the other hand, the percentage of CD8⁺ lymphocytes from DHEA-treated group was significantly lower than the percentages of CD8⁺ lymphocytes from control, metformin and DHEA + metformin group (Fig. 8). Taken together these data show that although DHEA treatment reverses the CD8⁺ :CD4⁺ ratio of lymphocyte infiltration into the uterus, treatment with metformin + DHEA restores the lymphocyte profile to that of control animals.

**Discussion**

The detrimental effects of the excess of androgens in the endometrial function contribute to the infertility of women with PCOS (Okon et al., 1998; Tuckerman et al., 2000; Sir-Peterman et al., 2002). Androgens inhibit human endometrial cell growth and secretory activity. Recent reports demonstrated a negative correlation between both androstenedione and testosterone levels and levels of glycodelin A in uterine flushes in the proliferative phase of the menstrual cycle of women with PCOS (Okon et al., 1998; Tuckerman et al., 2000).
addition, anovulatory PCOS patients have alterations in uterine vascularization (Ajosa et al., 2002). In fact, the pulsatility and the resistance index of uterine artery, two measures of blood impedance inversely related to blood flow, were higher in PCOS patients compared with healthy controls. It has been reported that elevated impedance to blood flow in the uterine arteries is detrimental for endometrial receptivity (Palomba et al., 2006).

In addition to being responsive to the steroid hormones estradiol, progesterone and androgens, the endometrium is also a target for insulin, the receptor for which is cyclically regulated in normo-ovulatory women. In vitro, insulin inhibits the normal process of endometrial stromal differentiation (decidualization). In addition, insulin-like growth factors (IGFs) and their binding proteins (IGFBPs) are regulated by and act on endometrial cellular constituents, and hyperinsulinemia down-regulates hepatic IGFBP-1, resulting in elevated free IGF-I in the circulation. Thus, elevated estrogen (without the opposing effects of progesterone in the absence of ovulation), hyperinsulinemia, elevated free IGF-I and androgens, and obesity all likely contribute to endometrial dysfunction, infertility, increased miscarriage rate, endometrial hyperplasia and endometrial cancer common in women with PCOS (Giudice, 2006). The present study extends our previous investigations about the role of hyperandrogenism on uterine functions. We have also explored the mechanisms by which metformin is able to prevent the adverse effects of excess of androgens. We did not define as ‘to reverse’ the effect of metformin because the drug is given together with DHEA. This has to be in this form because we work with a mouse model; if DHEA is interrupted the ‘PCOS condition’ disappears.

Although the association between hyperandrogenism and endometrial adenocarcinoma has been reported (Balen, 2001) the degree of risk and the possible mechanism involved are still under discussion. In agreement with our previous findings (Elia et al., 2008) we found that hyperandrogenization with DHEA induces a pro-inflammatory and a pro-oxidative status that correlate with the development of pre-cancerous structures. In addition, we reported that the apparition of pre-cancerous structures was related to increased apoptosis of uterine cells via transferase deoxytidyl uridine end labelling staining.

The reproductive events related to the endometrial functions have an inflammatory character (Finn, 1986). Besides sex steroids, PGs regulate uterine functions (Kelly et al., 2002; Boerboom et al., 2004). As we have previously reported (Elia et al., 2008), we found

**Figure 3** Uterine (a) prostaglandin E (PGE) and (b) PGF2 alpha concentrations from mice in control, metformin (M), DHEA and DHEA + M-treated group. Each column represents the mean ± SEM, *P < 0.05, ***P < 0.001 versus controls.

**Figure 4** Protein expression of (a) COX1 and (b) COX2 from uterine tissue from control, metformin (M), DHEA and DHEA + M-treated mice. The corresponding graph shows integrated optical density of the bands. Each column represents the mean ± SEM; ***P < 0.001 versus controls.
that hyperandrogenism increases a pro-inflammatory and vasoconstrictor PG (PGF2 alpha) and decreases an anti-inflammatory and luteotrophic PG (PGE). The enzyme catalyzing the rate-limiting steps in PG synthesis exists in two isoforms: COX1, which is normally constitutively expressed, and COX2, which is generally inducible in response to inflammatory stimuli (Doulla-Bell et al., 1998). Therefore, COX1 and COX2 are independently regulated (Doulla-Bell et al., 1998). The fact that DHEA decreased COX2 abundance without affecting COX1 abundance was the consequence of an established pro-inflammatory process and is in agreement with our previous findings (Elia et al., 2006, 2008). Moreover, in accordance to our previous works (Elia et al., 2006, 2008), we also found that hyperandrogenism inhibits the abundance of uterine COX2 by a feedback mechanism triggered by the accumulation of PGF2 alpha. The relationship between PGs and COX2-apoptosis has been previously described in other systems. It has been reported that PGE and COX2 suppress apoptosis in endometrial carcinomas (Arosh et al., 2004; Zhi et al., 2005; Li et al., 2006; Ohno et al., 2007) and that PGF2 alpha induces apoptosis in corpus luteum (Wang et al., 2003). In summary, we found that hyperandrogenism induces uterine apoptosis concomitantly with the increase in PGF2 alpha and the decrease in uterine PGE production and COX2 abundance.

On the other hand, we found that metformin prevented the hyperandrogenism-induced abnormal endometrial structures and apoptosis, the increase in PGF2 alpha and the decrease in COX2 abundance. Controversial reports have described that metformin can either prevent or enhance apoptosis (Yasuda et al., 2006; Ota et al., 2007; Solano et al., 2008). Our findings are in agreement with those that describe an anti-apoptotic role of metformin in mouse Schwann cells (Ota et al., 2007) and support the hypothesis of a link between PGs and COX2 controlling uterine apoptosis as was described in endometrial carcinomas (Arosh et al., 2004; Zhi et al., 2005; Li et al., 2006; Ohno et al., 2007).

Previous evidences that oxidative stress stimulates the production of PGF2 alpha in both human and mouse endometrial cells (Sugino et al., 2001, 2004; Elia et al., 2008) led us to postulate that hyperandrogenization could induce oxidative stress which in turn might increase uterine PGF2 alpha in a positive feedback. In fact, the relationship between PGs and oxidative stress regulating T lymphocyte proliferation and apoptosis has been previously described (Solano et al., 2008). In agreement with our previous findings (Elia et al., 2008), we also found that hyperandrogenization increased the uterine NOS activity and decreased the anti-oxidative defenses evaluated by SOD and CAT activities and GSH content (Elia et al., 2008). The increase in NOS activity leads to the accumulation of NO, whereas the decrease in SOD activity leads to the accumulation of radical superoxide (O2•-). The reaction between NO and O2•- produces a more aggressive agent, the peroxynitrite radical (ONOO•), which is...
related to the production of abnormal structures (Beckman et al., 1990; Wink et al., 1996; Brune, 2005; Elia et al., 2006, 2008). The fact that metformin by preventing the increase in NOS activity without affecting the antioxidant defenses (including SOD activity which remains lower than controls) was able to prevent the development of pre-cancerous structures would validate our suggestion. In agreement with this, Ota et al. (2007) have reported that metformin prevents apoptosis by the regulation of reactive oxygen species of mouse Schwann cells. It is important to point out that the aminoguanidine-like activity of metformin allows the drug to interact with the heme-group of NOS thus regulating nitrosative stress (Youssef et al., 1999; Elia et al., 2006). Then, the direct effect of metformin on NOS activity prevents the accumulation of NO and in turn the formation of peroxynitrite and the damage of endometrial structures. Unexpectedly, metformin given alone was able to decrease SOD activity. In fact, Tosca et al. (2007) have reported that metformin alone is able to modulate steroidogenesis and oxidative stress in granulosa cells.

Cytotoxic (CD8+) T cells play an important role in the antitumor immunity since they enhance cytotoxicity to cancer cells (Ohno et al., 2006; Zhang et al., 2003). The proportion of CD8+ T cells is used clinically as a measurement of the host-immune status in cancer patients (Ohno et al., 2005). Recent studies both in vitro and in animal models have revealed that COX2 is a critical factor in enabling carcinoma cells to escape host immune defenses (Sharma et al., 2003). Therefore, an immunosuppressive condition has been associated with COX2 since this enzyme modulates CD8+ T cells during the development of tumours (Ohno et al., 2005). It has been reported that endocrine disturbances lead to altered CD4+/CD8+ T ratio both in women with PCOS and in hyperandrogenized mice (Turi et al.,

**Figure 7** Representative sections of uterine T lymphocyte immunophenotype quantified by fluorescent microscopy. (a) CD8+ and (b) CD4+ T cell patterns from mice in the control group; (c) CD8+ and (d) CD4+ T cell patterns from mice in the DHEA group; (e) CD8+ and (f) CD4+ T cell patterns from mice in the DHEA + M-treated group. The CD8+ and CD4+ T cell patterns from mice in the metformin group and were similar to the control group. Magnification: ×100.
Here, we report for the first time that metformin prevents the adverse effects of hyperandrogenism in COX2 abundance and CD4<sup>+</sup> and CD8<sup>+</sup> T percentages that infiltrate uterine tissue. These data are in agreement with previous findings that metformin modulates immune parameters as CD4<sup>+</sup> and CD8<sup>+</sup> T population infiltrating ovarian tissue and retroperitoneal lymph nodes, serum tumour necrosis factor levels and T lymphocyte proliferation (Sander et al., 2006; Solano et al., 2008). We have to point out that the assays have not been addressed whether the total number of T lymphocytes infiltrating the uterus changes with the treatments. Thus, the change in CD4<sup>+</sup>:CD8<sup>+</sup> ratio between groups could be the result of either (a) absolute numbers of CD8<sup>+</sup> cells remaining constant and a massive increase in CD4<sup>+</sup> infiltration as a result of DHEA treatment or (b) the absolute numbers of CD4<sup>+</sup> cells remaining constant and a massive decrease in CD8<sup>+</sup> infiltration as a result of DHEA treatment or (c) changes in both CD4<sup>+</sup> and CD8<sup>+</sup> infiltration. Experiments are designed to clarify these points.

It has been previously reported (Palomba et al., 2006) that metformin acts on uterus from PCOS patients improving several surrogate parameters of endometrial receptivity. Our results demonstrate for the first time that metformin also regulates some related inflammatory, oxidative and immune parameters of uterine functions during the hyperandrogenized status. These actions prevent the formation of pre-cancerous structures and a pro-apoptotic status. For all these reasons our findings represent novel evidences about the clinical implications of metformin-treatment in normalizing not only ovarian functions but also in preventing uterine dysfunction which include the formation of pre-cancerous structures.

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