Dehydroepiandrosterone and metformin modulate progesterone-induced blocking factor (PIBF), cyclooxygenase 2 (COX2) and cytokines in early pregnant mice

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1. Introduction

The establishment and maintenance of pregnancy are associated with a Th2-dominant peripheral cytokine profile, while miscarriage is characterized by a Th1-dominant peripheral cytokine production [1,2]. The Th2/Th1 balance is controlled by sex hormones, in particular, progesterone, which promotes the development of a Th2 response. The biological effects of progesterone are mediated by a complex network of effectors, including a protein called progesterone-induced blocking factor (PIBF). PIBF, synthesized by lymphocytes of healthy pregnant women in the presence of progesterone, inhibits arachidonic acid liberation and acts on the cytokine balance to control NK activity [3–5]. Moreover, neutralization of endogenous PIBF results in altered cytokine production and pregnancy termination in mice [6] and low PIBF concentrations in the uteri of pregnant women suggest a risk for spontaneous pregnancy termination [7]. Both in vitro [8] and in vivo studies [9] show that PIBF induces a Th2-biased cytokine production.

Dehydroepiandrosterone (DHEA) is an androgen precursor mainly secreted by the adrenal cortex in humans [10]. Women in the first and second trimesters of pregnancy show increased levels of DHEA compared to non-pregnant women [11]. Treatment with DHEA (60 mg/kg, s.c. 24 and 48 h post-implantation) induces embryo resorption of early pregnant BALB/c mice while simultaneous treatment with metformin (240 mg/kg, oral 24 and 48 h post-implantation) prevents it. During pregnancy progesterone-induced blocking factor (PIBF) modulates prostaglandins (PGs) and cytokine production. These findings prompted us to investigate the effect of DHEA and metformin on both PIBF and cyclooxygenase 2 (COX2) expressions at the implantation sites, as well as cytokine production. PIBF and COX2 expression were detected by immunohistochemistry from DHEA and DHEA+ metformin treated 8 days-pregnant mice and serum cytokine levels of these animals were determined by ELISA. DHEA treatment both abolished PIBF expression and increased COX2 expression. Embryo resorption correlates with the lack of PIBF expression, diminished IL-6 levels and increased IL-2 concentration while metformin was able to reverse the effect of DHEA on both PIBF and COX2 expression and IL-6 levels. We concluded that hyperandrogenization induces embryo resorption in early pregnancy diminishing PIBF in implantation sites, having a pro-inflammatory effect. Metformin is able to prevent such effects.

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in recurrent miscarriages [22–25]. Given that DHEA is one of the main androgens produced by ovaries of women with PCOS, a rodent model of PCOS was developed in DHEA treated mice [26,27]. The DHEA–PCOS murine model exhibits many of the salient features of human PCOS [18,19,28–31]. Previously, we demonstrated that the post-implantation hyperandrogenization with DHEA of pregnant BALB/c mice induces endocrine and immune disturbances resulting in 88% of mice with total embryo resorption [18]. Embryo resorption was accompanied by diminished serum progesterone levels [19].

Metformin, a biguanide derivative (N,N′-dimethylbiguanide) is one of the most commonly used drugs for the treatment of type 2 diabetes. Metformin decreases hyperglycemia and has beneficial effects on circulating lipids, without affecting insulin secretion [31]. The glucose-lowering effects of metformin are attributable to both an increase in muscle glucose uptake and a decrease in hepatic glucose production [32]. Activation of AMP-activated protein kinase (AMPK) by metformin was found to be required for the decrease in glucose production and the increase in fatty acid oxidation [33,34]. Unlike traditionally used glucose-lowering reagents, such as sulfonylureas or insulin, metformin improves cardiovascular functions and reduces cardiovascular risks [35]. Recently metformin is being used for treating women with PCOS [36,37]. Metformin decreases androgen levels and improves the frequency of ovulation and menstrual cycles in PCOS patients [38–45] and the treatment with metformin of early pregnant women with PCOS prevents abortions [44,46,47], however, the mechanisms of the protective effect remains unknown. In previous studies we have reported that metformin prevents embryo resorption in hyperandrogenized early pregnant BALB/c mice, by restoring progesterone and estradiol production, preventing the development of insulin resistance, and ovarian oxidative stress [19].

The aim of the present study was to assess the events resulting from the diminution of serum progesterone levels caused by DHEA that lead to embryo resorption. We also studied whether metformin was able to prevent it.

2. Materials and methods

2.1. Animals and experimental protocol

Early pregnant BALB/c mice were post-implantation hyperandrogenized as previously described [19]. Briefly, virgin female BALB/c mice (8- to 12-week-old) were paired with BALB/c males (8- to 12-week-old). The day of appearance of a coital plug was taken as day 0 of pregnancy. Implantation occurs in the morning of the 5th day, therefore, on days 6 and 7 animals were treated orally (by canulla) with 240 mg/kg of metformin in 0.1 ml of water, or injected s.c. with 60 mg/kg of DHEA in 0.1 ml of sesame oil. A third group of animals were treated under the same conditions with DHEA and metformin. Animals that were given 0.1 ml of water and injected s.c. with 0.1 ml of sesame oil on days 6 and 7 of pregnancy served as controls. This procedure induces total embryo resorption in 88% of mice from the DHEA group, accompanied with diminished serum progesterone levels [19]. Both effects were prevented in the DHEA+ metformin group. On day 8 of pregnancy, animals were anesthetized with ether and euthanized by cervical dislocation. Blood was collected and serum was stored at −70 °C to quantify cytokine levels (interleukin-6: IL-6, IL-4, IL-2 and interferon gamma: IFN gamma). Uterine tissues from 16 animals/each group were immediately collected and divided as follows: after embryos were removed, 8 uteri of each group were frozen at −70 °C to determine COX2 expression by western blotting. The remaining eight uteri of each group were fixed in 4% (w/v) paraformaldehyde to carry out both histological analysis of embryo resorption and to determine the expression of PIBF and COX2 on implantation sites. All the experiments were performed three times.

In order to test any long-term adverse effects of the treatments two additional control groups (10 animals each) including DHEA+ metformin treated and control mice were allowed to proceed to term. We found that these animals exhibited normal labor and number and morphology of pups. The mice were housed under controlled temperature (22 °C) and illumination (14 light, 10-h darkness; lights on at 05:00 h) and were allowed to free access to Purina rat chow and water. All procedures involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the studies were approved by the “Comité Independiente de Etica en Investigación” de la Facultad de Medicina de la Universidad de Buenos Aires.

2.2. Histological studies and embryo resorption

Paraformaldehyde 4% (w/v)-fixed uterine tissues were embedded in paraffin, 6 μm-section, cut and placed on gelatin-coated glass slides (Biobond; British Biocell International, Cardiff). Only sections that passed through the center of the implantation sites were selected. Then, slides were air-dried for 2 h before being fixed for 5 min with acetone at 4 °C, washed in PBS (pH 7.3) and stained with haematoxylin and eosin (DAKO Corporation, Carpinteria, CA, USA). Ten different sections from each uterus were analyzed.

2.3. Immunohistochemical localization of PIBF and COX2 expression

Uterine sections were stained with the immunoperoxidase staining kit CSA/HRP (Dako). Briefly, tissue sections were deparaffinized, rehydrated in PBS and blocked in TMB. Endogenous peroxidase activity was blocked with 0.1% (v/v) hydrogen peroxide for 15 min. Non-specific binding sites were blocked by 1% (v/v) goat serum (Bio-Rad). Sections were incubated overnight at 4 °C with the primary rabbit polyclonal anti-human COX2 antibody (Cayman, USA) diluted 1:50 in blocking buffer, or with the primary polyclonal anti-PIBF antibody diluted 1:100 in blocking buffer at room temperature for 60 min. Polyclonal anti-recombinant human PIBF IgG was generated by immunizing rabbits with the 48-kDa N-terminal recombinant PIBF [48]. Control sections were made without primary antibody. After washing with PBS, slides were incubated at room temperature for 30 min with 1:100 diluted biotin-conjugated secondary polyclonal anti-rabbit IgG-HRPO developed in goat (Sigma Chemical Co., St. Louis, MO, USA). Finally slides were revealed by streptavidin–peroxidase complex and diaminobenzidine solution; a counterstaining was made with haematoxylin and mounted with gelatin–glycerol.

2.4. Expression of COX2 on implantation sites—Western blotting

Uteri were homogenized with a Teflon homogenizer at 4 °C in lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl and 1% Triton X-100, v/v) supplemented with protease inhibitors (0.5 mM PMSF, 0.025 mM N-CBZ-L-phenylalanine chloromethyl ketone and 0.025 mM L-1-tosylamide-2-phenyl-ethylchloromethyl ketone). The lysates from three uteri/treatment were centrifuged at 1500 × g for 10 min and supernatants were pooled. Protein concentrations were measured by Bradford assay (Bio-Rad). After boiling for 5 min, 90 μg of protein from each sample was applied to an 8% SDS-polyacrylamide gel and electrophoresis was performed at 100 V for 4 h. The separated proteins were transferred onto...
2.5. Determination of cytokines (IL-6, IL-4, IL-2 and IFN gamma)

The mouse Th2/Th1 ELISA Ready-Set Go (eBioscience, USA) was used to measure IL-6, IL-4, IL-2 and IFN gamma concentrations in serum samples from 7 mice/group. Experiments were carried out following the manufacturer’s instructions. Briefly, pre-coated goat anti-rabbit antibody plates were incubated overnight with 100 μl of sample or standard at 4 °C. Then, 100 μl/well of assay diluent was added and incubated at room temperature for 2 h. After aspirating and washing five times with assay diluent, biotin-conjugated anti-mouse cytokine antibody was added for 1 h at room temperature. After washing twice, the plates were incubated with 100 μl/well of 1:250 diluted avidin-HRP for 30 min. The reaction was finished by addition of 100 μl/well of stop reaction and optical density was measured at 450 nm. The sensitivity of the test is 0.78 pg/ml for each cytokine. Data below detection limit were excluded. Results were expressed as pg cytokine/ml serum.

2.6. Statistical analysis

Statistical analyses were carried out using the Instat program (GraphPAD software, San Diego, CA, USA). One-way ANOVA test (Tukey post-test Multiple Comparison that compares all the pairs of columns) was used. A P value <0.05 was considered significant. Results are presented as mean values ± S.E.M.
3. Results

3.1. Histology of implantation sites

Eighty-eight percent of mice from the DHEA-treated group showed total embryo resorption. None of the control animals showed embryo resorption. The effects of DHEA and metformin treatments on the structure of implantation sites were studied using the hematoxylin–eosin staining. The uteri form 8 days-pregnant mice from the control group showed a well organized deciduas corresponding to endometrial cells transformed in decidual cells (d) and divided in two regions: mesometrial (md) and...
antimesometrial (am) deciduas. The blastocyst (formed by the trophoblast (tr) and the embryo (e)) invades the deciduas following the antimesometrial–mesometrial direction. Fig. 1E is a magnification of the control trophoblast. The mesometrial decidua (md) shows abundant lacunae (lac). These structure and organization are also found in both the metformin and DHEA+ metformin groups (Fig. 1A, B and D corresponding to control, metformin and DHEA+ metformin groups, respectively).

In contrast, in the uteri from the DHEA group mice, the decidual structure is disorganized (d) and the embryo is resorbed (re) (Fig. 1C). The trophoblast disappeared and only few trophoblastic cells remain in the central region of the decidua (Fig. 1F). The decidua shows more and larger lacunae (lac), present not only in the mesometrial decidua (md), as it was seen in the control group, but also occupying the antimesometrial (am) region.

3.2. Localization of PIBF on implantation sites

The effects of DHEA and metformin treatments on the localization of PIBF were determined by immunohistochemistry. The uteri from 8 days-pregnant mice of the control, metformin and DHEA+ metformin groups showed nuclei stained with hematoxylin and cytoplasms reacting to PIBF both in the trophoblast (tr) and decidua (d) stained with streptavidin peroxidase (Fig. 2A, B and D, respectively). In uteri from mice of the DHEA group there are nuclei stained with hematoxylin but there are no immunoperoxidase staining the cytoplasms, indicating no expression of PIBF (Fig. 2C). Magnifications of the decidual cells from mice in the control, metformin, DHEA and DHEA+ metformin groups (Fig. 2E–H, respectively) show that the immunoperoxidase staining for PIBF was only observed in cytoplasms of control, metformin and DHEA+ metformin decidual cells.

3.3. Localization of COX2 on implantation sites

To address the question whether hyperandrogenization with DHEA alters the localization of the enzyme responsible for prostaglandin production, the immunolocalization of COX2 was evaluated on implantation sites. We found that the COX2 immunoreactivity in the control and DHEA+ metformin groups are located both in the trophoblast (tr) and in the inner side of lacunae (lac) (Fig. 3A and C for control and DHEA+ metformin treatment, respectively). The uteri obtained from metformin-treated mice showed an appearance similar to that of controls (data not shown). In the DHEA-treated mice, COX2 was located not only in the trophoblast (tr) and in the inner side of lacunae (lac), as in the control and DHEA+ metformin group, but also the immunoperoxidase staining was present in the decidua (d) (Fig. 3B). Fig. 3D shows the negative control (slide without primary antibody).

3.4. Western blotting of COX2 on implantation sites

Western blot revealed that DHEA treatment increased COX2 protein expression on implantation sites while metformin treatment prevented the effect of DHEA (Fig. 4: ‘a’ vs. ‘c’ and ‘a’ vs. ‘d’, respectively).

3.5. Effect of DHEA and metformin on the peripheral cytokine pattern

In order to investigate whether DHEA and metformin regulate the Th1/Th2 type cytokine balance, we evaluated the production of IL-6 and IL-4 as Th2 type cytokines and IL-2 and IFN gamma as type 2 cytokines in mice treated with DHEA and/or metformin. Serum IL-6 concentrations were significantly increased in metformin treated and significantly decreased in DHEA treated animals (Fig. 5A; ‘a’ vs.

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**Fig. 3.** Immunolocalization of cyclooxygenase 2 (COX2) on implantation sites. A representative implantation site from: (A) controls, (B) DHEA, (C) DHEA+ metformin and (D) negative control to both PIBF and COX2. Reactivity to COX2 was revealed by streptavidin–peroxidase complex and diaminobenzidine solution; a counterstaining was made with haematoxylin. (A)–(D): 100×. e: Embryo, lac: lacunae, tr: trophoblast, md: mesometrial decidua, am: antimesometrial decidua.
Fig. 4. Western blotting for expression of cyclooxygenase 2 (COX2) on implantation sites. Lane 1: implantation sites from mice in the control group, lane 2: implantation sites from mice in the dehydroepiandrosterone (DHEA) group and lane 4: implantation sites from mice in the DHEA+metformin group. The graph shows integrated optical density of the bands. ‘a’ vs. ‘c’ \( P < 0.001; \) ‘a’ vs. ‘d’ \( P < 0.05.\)

‘b’ and ‘a’ vs ‘c’, respectively) and metformin treatment avoided the effect of DHEA (Fig. 5A; ‘a’ vs. ‘d’). None of the treatments affected IL-4 concentrations (Fig. 5B). DHEA treatment resulted in significantly increased IL-2 levels (Fig. 5C; ‘a’ vs. ‘c’) and simultaneous metformin treatment failed to correct this effect (Fig. 5C; ‘a’ vs. ‘d’). IFN gamma was not detectable in the sera of either group (data not shown).

4. Discussion

The present study extends our previous investigations on the effects of DHEA and metformin on the outcome of murine pregnancy. In agreement with our previous reports [18,19] we found that DHEA injected during the post-implantation period induced embryo resorption, and for the first time we present an evidence for an effect of DHEA on PIBF expression. In addition we found that both trophoblast and decidua showed reactivity to PIBF representing the first evidence that other cell types different from lymphocytes producing PIBF. Previously we had reported diminished progesterone synthesis in DHEA-treated pregnant mice [18,19], and the present study revealed the consequent lack of PIBF expression in these animals. It was previously reported that neutralization of PIBF results in pregnancy loss [7,9]. In agreement we found a direct correlation between the rate of resorption and the lack of uterine PIBF expression.

Although metformin is being widely used for the treatment of pregnant women with PCOS [44,46,47] its mechanism of action remains unknown. Data presented here show that metformin restored PIBF expression and also avoid the reduction of IL-6 levels (a Th2 type cytokine) induced by DHEA. Although metformin failed to correct the IL-2 production (a Th1 type 1 cytokine) increased by DHEA, pregnancy rates were improved with metformin treatment, which might suggest that high levels of IL-2 are less critical than low levels of IL-6 for the maintenance of early pregnancy in mice. This concept is supported by the finding that IL-6 acts as a trigger for the synthesis of asymmetric antibodies, an important protective mechanism during pregnancy [3,49]. Moreover, Blois et al. [49] reported that the lack of IL-6 production by trophoblast and decidual cells is fundamentally involved in the pathology of abortion. Unexpectedly the treatment with metformin alone increased serum IL-6 levels. We have previously found that metformin alone is able to increase antioxidant defenses of T lymphocyte higher than controls (unpublished data), then, we might assume that metformin is able to improve “basal conditions”. In agreement with this suggestion, Palomba et al. [50] found that metformin is able to induce a high reproductive potential with lower-than-expected rates of spontaneous miscarriages. We found that IFN gamma was not detectable in sera from control early pregnant mice and it correlates with those reported during early pregnant women [51]. In addition, none of treatments was able to induce IFN gamma production.

PIBF inhibits the release of arachidonic acid [5] that is converted in prostaglandins by the enzyme COX2. Here, for the first time we describe a direct effect of both DHEA and metformin on the localization as well as the expression of COX2 in the implantation sites. In DHEA-treated animals we observed an increased uterine COX2 expression, together with the lack expression of PIBF. This effect was avoided by metformin administration. Considering that the enzyme prostaglandin E2 9 ketoreductase, which converts PGE (a luteoprotective mediator responsible for relaxation of uterine muscle), to PGF2 alpha (luteolytic and vasoconstrictor of uterine muscle) is down regulated during pregnancy, studies are being carried out to assess the prostaglandin pathway. These...
studies will focus on the activity of this enzyme as well as on the PGE: PGF2 α ratio on hyperandrogenized and metformin-treated-pregnant murine model.

In summary, the possible mechanism that results in embryo resorption in DHEA-treated mice includes: (i) the lack of PIBF production due to reduced availability of progesterone, (ii) diminished IL-6 production due to the lack of PIBF and (iii) increased expression of COX2.

The understanding of the mechanisms used by metformin during the hyperandrogenized early pregnancy could contribute additional benefits in the treatment of PCOS especially in the combination with other drugs.

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References


