The mechanisms involved in the action of metformin in regulating ovarian function in hyperandrogenized mice

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The aim of this study was to investigate the mechanisms by which N,N'-dimethylbiguanide metformin (50 mg/100 g body weight (BW) in 0.05 ml of water, given orally with a cannula) prevents the ovarian disorders provoked by the hyperandrogenization with dehydroepiandrosterone (DHEA) in prepuberal BALB/c mice. The injection of DHEA (6 mg/100 g BW in 0.1 ml of oil) for 20 consecutive days re-creates a mouse model that resembles some aspects of the human polycystic ovary syndrome (PCOS). The treatment with DHEA increased ovarian oxidative stress because it enhanced lipid peroxidation (LPO) and diminished both catalase (CAT) activity and glutathione (GSH) content. Therefore, the treatment with DHEA diminished both ovarian nitric oxide synthase (NOS) activity and prostaglandin E (PGE) production. When metformin was administered together with DHEA, the ovarian GSH content, NOS activity and PGE production did not differ when compared with controls. However, metformin was not able to prevent the effect of DHEA on ovarian LPO or CAT activity. Finally, DHEA increased the ovarian protein expressions of inducible NOS (iNOS), inducible cyclooxygenase (COX2) and the phosphorylated AMP-dependent kinase α (AMPK-α) (Thr172). Metformin administered together with DHEA was able to prevent the increase of ovarian iNOS and COX2 expressions and to enhance the activation of phosphorylated AMPK-α expression.

Key words: AMP-dependent kinase α/cyclooxygenase/dehydroepiandrosterone/polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous disease characterized by hyperandrogenaemia, hirsutism, oligo- or amenorrhea and anovulation. PCOS is frequently associated with hyperinsulinaemia, insulin resistance syndrome, increased cardiovascular risk and diabetes mellitus (Franks, 1995; Asuncion et al., 2000; Abbott et al., 2002). Although it has been difficult to evaluate the aetiology and development of PCOS in patients, the similarities in key steps of mammalian reproduction make animal models attractive for studying the pathogenesis of this pathology. After Malesh and Greenblatt (1962) found that dehydroepiandrosterone (DHEA) levels were increased in women with PCOS, Roy et al. (1962) developed an animal model using DHEA to induce PCOS in rodents. The DHEA-PCOS murine model induces increased levels of serum testosterone, androstenedione and 5α-dihydrotestosterone similar to the levels observed in women with PCOS. Subsequent studies established that the DHEA-PCOS murine model exhibits some of the salient features of human PCOS, such as hyperandrogenism, abnormal maturation of ovarian follicles and anovulation, and for these reasons it is well accepted in the literature (Lee et al., 1991, 1998; Anderson et al., 1992; Henmi et al., 2001; Luchetti et al., 2004; Sander et al., 2006).

Reactive oxygen species (ROS), toxic oxygen-derived products, are generated in all aerobic cells and include production of superoxide radical (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (OH⁻) (Halliwell and Gutteridge, 1984). Damage to biological systems caused by the excess of ROS is referred to as oxidative stress. One consequence of the increased generation of ROS within ovarian cells is associated with the loss of ovarian function (Motta et al., 1999, 2001a,b) and in the case of PCOS could impair ovarian steroidogenesis. In addition, it has been well established that the enhanced oxidant status of women with PCOS increases the risk of cardiovascular diseases (Franks, 1995; Sabuncu et al., 2001; Yilmaz et al., 2005). Nitric oxide (NO) has emerged as a messenger that controls many physiological processes that are essential for maintaining homeostasis (Ignarro, 1990). It has also been reported that NO modulates ovarian physiology (Zackrisson et al., 1996; Motta et al., 2001a; Estevez et al., 2004). Like ROS, the production of reactive nitrogen oxide species (RNOs) damages specific cellular targets; however, RNOs are more aggressive oxidant species than ROS (Wink et al., 1996). Protection against oxidative stress in cells is provided by enzymes [catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase, GPX], metabolites, e.g. glutathione (GSH) or antioxidant vitamins (Aten et al., 1992), and it has been reported that the oxidant–antioxidant balance is endocrine regulated (Sugino et al., 1999; Motta et al., 2001a).

Multiple concomitant therapies have been applied in PCOS. Biguanides, which were primarily used as antidiabetic drugs, were later associated with beneficial effects in the treatment of PCOS.
Metformin is useful in the reduction of both insulin resistance and circulating androgens, as well as in restoring ovulation (Harbome et al., 2003; Yilmaz et al., 2005; Diamanti-Kandarakis et al., 2006; Tang et al., 2006). However, metformin is being clinically used without a complete understanding of the mechanism involved. Investigations towards the mechanisms involved in the action of metformin will be useful to adjust the treatments using a combination of drugs.

In previous studies, we have reported that hyperandrogenization of prepuberal BALB/c mice with DHEA induced ovarian cysts and altered endocrine parameters such as serum 17β-estradiol (E), progesterone (P) and tumour necrosis factor α (TNFα) levels and ovarian prostaglandin E (PGE) concentration. In addition, the treatment with DHEA modified ovarian CD4+ and CD8+ T-lymphocyte expression and induced an imbalance in the ovarian oxidant–antioxidant equilibrium, evidenced by the increase of lipid peroxidation (LPO) index and the diminution of GSH content when compared with controls (Luchetti et al., 2004). We also investigated some aspects of the role of metformin in the treatment of the endocrine and immune disorders provoked by DHEA. In this regard, we found that metformin administered together with DHEA prevented endocrine (serum insulin levels, E and P concentration and ovarian PGE production) and immune alterations (ovarian T-lymphocyte expression and TNFα levels) (Sander et al., 2006).

The aim of the present work was to extend our previous studies by means of investigating the possible role of metformin in preventing the ovarian oxidative stress induced after hyperandrogenization with DHEA. Considering the role of NO as a generator of oxidant species, the interaction between metformin and NO described in other systems (Zou et al., 2004) and the role of NO in the ovarian function, we also evaluated the ovarian nitric oxide synthase (NOS) activity and the protein expression of inducible NOS (iNOS). In view of the fact that dyslipidemia is frequently associated with PCOS (Franks, 1995; Asbellion et al., 2000; Abbott et al., 2002) and the fact that PGE is altered in women with PCOS (Navarra et al., 1996), and also taking into consideration our previous findings (Luchetti et al., 2004), we also evaluated the efficacy of metformin in modulating ovarian PGE synthesis. To study the mechanism involved, we quantified the protein expression of the inducible cyclooxygenase (COX2), the rate-limiting enzyme of prostaglandin synthesis.

It has been reported that metformin activates the AMP-dependent kinase α (AMPK-α) pathway to decrease glucose production, increase fatty acid oxidation and promote the uptake of glucose by cells (Zhou et al., 2001; Fryer et al., 2002; Zou et al., 2004). Furthermore, metformin activates the AMPK pathway during states of stress where ATP is depleted (Hardie, 2003; Zang et al., 2004). In addition, it has been recently demonstrated that metformin activates AMPK via the RNS pathway (Zou et al., 2004) and a direct cross-talk mechanism among AMPK, NO and metformin has been recently described in bovine aortic endothelial cells (Davis et al., 2006). For these reasons, we also studied whether the ovarian AMPK pathway is involved in metformin action.

Materials and methods

Animals and experimental protocol

The hyperandrogenized environment of PCOS was reproduced in mice by injection of DHEA (Luchetti et al., 2004). Briefly, female prepuberal (25-day-old) mice of the BALB/c strain were daily injected with DHEA [6 mg/100 g body weight (BW)] dissolved in 0.10 ml of 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/100 g BW in 0.05 ml of water given orally with a canula) daily for 20 days. The dose of metformin administered was equivalent to that used in the treatment of women with PCOS. The control group consisted of animals injected with oil (0.1 ml) and given water orally (0.05 ml) daily for 20 consecutive days. The metformin group consisted of mice treated orally with 50 mg metformin/100 g BW in 0.05 ml of water for 20 days. Thirty mice per group were housed under controlled temperature (22°C) and illumination (14 h light : 10 h dark; lights on at 05:00 hours) 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 anaesthetized with ether and killed by decapitation. Ovaries were collected and divided as follows: 15 were used to determine LPO, 15 for CAT, 15 for GSH, 10 for NOS activity and 5 for PGE production. To determine oxidant–antioxidant parameters, ovarian tissues were collected and immediately processed. The ovaries destined for the PGE assay were immediately frozen at −70°C until assayed.

To analyse the intracellular pathway, the protein expressions of COX2, iNOS and phospho AMPK-α were determined by western blotting. Five mice per group were treated for 20 consecutive days as described before. The collected tissue was homogenized in the western blotting buffer and stored at −20°C until assayed. All experiments were repeated three times.

Oxidative stress-related parameters

Lipid peroxidation

The amount of malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids may be taken as an index of peroxidation reaction. The method was performed as previously described (Motta et al., 2001b). It quantifies MDA as the product of LPO that reacts with trichloroacetic acid–thiobarbituric acid–HCl [15% (w/v); 0.375% (w/v) and 0.25 M, respectively], yielding a red compound that absorbs at 535 nm. Homogenates of ovarian pooled tissue (three ovaries per point and five points per treatment, n = 5) were treated with trichloroacetic acid–thiobarbituric acid–HCl and heated for 15 min in a boiling water bath. After cooling, the precipitate was removed by centrifugation at 1000 g for 10 min. The absorbance of samples was determined at 535 nm. Content of thiobarbituric acid reactants was expressed as μmol MDA formed/g ovarian tissue.

Catalase activity

The technique used is based on the role of the enzyme CAT to catalyse the conversion of hydrogen peroxide into water and oxygen (Chance, 1954). Three ovaries per point and five points per treatment (n = 5) were homogenized in 50 mM phosphate buffer, pH = 7.2. Homogenates (100 μl per point) were incubated with 3 ml of 50 mM phosphate buffer. Given that hydrogen peroxide absorbs at 240 μm, the consumption of hydrogen peroxide was monitored by spectrophotometer for 1 min with intervals of 10 s. Results were expressed as μmol CAT activity/mg protein.

Glutathione content

The GSH assay was carried out as previously described (Motta et al., 2001a). The reduced form of GSH comprises the bulk of cellular protein sulphydryl groups. Thus, measurement of acid-soluble thiol is used for estimation of GSH content in tissue extracts. Briefly, 300 μl of homogenates obtained from pooled tissues (three ovaries per point and five points per treatment, n = 5) in 0.5% (v/v) trichloroacetic acid was incubated with buffer—1.75 M Tris (pH = 7.4) containing NADPH and GSH reductase. The reaction involves the enzymatic reduction of the oxidized form (GSSG) to GSH. When Ellman’s reagent (5,5-dithiobis-2 nitrobenzoic acid; Sigma and Co, St Louis, MO, USA) is added to the incubation medium, the chromophoric product resulting from this reaction develops a molar absorption at 412 nm that is linear to the first beyond 6 min; after this, the reaction remains constant. Results were expressed as μmol GSH/mg protein.

Nitric oxide synthase activity

NOS activity in ovarian homogenate was determined by monitoring the formation of L-[14C] citrulline from L-[14C] arginine as described by Motta et al. (2001a). Briefly, the frozen ovarian tissue (two ovaries per point and five points per treatment, n = 5) was homogenized (Tissuemizer Tekmar; Thomas Scientific, Swedesboro, NJ, USA) at 0°C in 3 volumes of 50 mM Heps, 1 mM DL-dithiothreitol, 1 mM NADPH and 50 mM L- ALA, pH = 7.5. Samples were incubated at 37°C for 15 min with 10 μM [14C] arginine (0.3 μCi;
1 Ci = 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/g/min.

Prostaglandin radioimmunoassay

The measurement of PGE was carried out as previously reported (Luchetti et al., 2004). Briefly, the tissue (one ovary per point and five points per treatment, n = 5) was weighed and incubated in Krebs-Ringer-bicarbonate (KRB) with 11.0 mM glucose as 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 prostaglandin determination three times with 1 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 was quantified by using a rabbit antiserum from Sigma Chemical Co. Sensitivity was 10 pg/tube and cross-reactivity was 100% with PGE and <0.1% with other prostaglandins. Results were expressed as pg PGEx/g protein.

Western blotting

Pooled samples of ten ovaries from each group (control, DHEA, DHEA + metformin) were 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 L-N-tosyl-lysine chloromethyl ketone, 0.025 mM L-tosylamide-2-phenyl-ethylchloromethyl ketone). The lysate was centrifuged at 4°C for 10 min at 10 000 g, and the pellet was discarded. Protein concentrations in the supernatant were measured by Bradford method (Bradford, 1976).

Levels of ovarian proteins

Ovarian protein content was determined by Bradford method (Bradford, 1976).

Statistical analysis

Statistical analyses were carried out by using the Instat 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 ± SEM.

Results

Effect of metformin on hyperandrogenization-induced ovarian oxidative stress

The experiments were performed to determine whether the treatment with metformin was able to regulate ovarian oxidative stress induced by hyperandrogenization. The hyperandrogenization with DHEA of prepuberal BALB/c mice increased LPO (Figure 1a) and diminished both CAT activity (Figure 1b) and ovarian GSH content (Figure 1c).

Metformin administered together with DHEA was not able to modify the effect of DHEA either on LPO (Figure 1a) or on CAT activity (Figure 1b). However, control and DHEA + metformin groups did not show significant differences in ovarian GSH content (Figure 1c), thus suggesting that metformin prevented the effect of DHEA on diminishing GSH levels.

<table>
<thead>
<tr>
<th>Control</th>
<th>Metformin</th>
<th>DHEA</th>
<th>DHEA+Metf</th>
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<tr>
<td>a) Lipid peroxidation (umol MDA/g tissue)</td>
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<tr>
<td>Control</td>
<td>Metformin</td>
<td>DHEA</td>
<td>DHEA+Metf</td>
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<tr>
<td>b) Catalase (pmol/mg protein)</td>
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<tr>
<td>Control</td>
<td>Metformin</td>
<td>DHEA</td>
<td>DHEA+Metf</td>
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<tr>
<td>c) Total glutathione (umol/g protein)</td>
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Figure 1. Role of dehydroepiandrosterone (DHEA) in (a) lipid peroxidation index, (b) catalase (CAT) activity and (c) total glutathione (GSH) in ovarian tissue from control, metformin, DHEA, DHEA + metformin-treated mice. Each column represents the mean ± SEM, *P < 0.001.
Metformin action on ovarian NOS activity

The hyperandrogenization with DHEA diminished ovarian NOS activity (Figure 2). When metformin was given together with DHEA, the ovarian NOS activity was similar to that of the control group (Figure 2).

Effect of metformin on ovarian prostaglandin E production

The treatment with DHEA diminished ovarian PGE levels, whereas when metformin was given together with DHEA, PGE levels were similar to those observed in controls (Figure 3).

Role of metformin in modulating ovarian iNOS expression

The western blotting for iNOS expression showed that hyperandrogenization with DHEA induced an increased protein expression of iNOS (Figure 4). This effect was partially reversed when metformin was administered together with DHEA (Figure 4).

Role of metformin in the regulation of ovarian COX2 expression

To evaluate how metformin modulates ovarian prostaglandin pathway, we further evaluated the role of biguanide in ovarian COX2 expression. As can be seen in Figure 5, the hyperandrogenization with DHEA induced the increased expression of COX2 protein. When metformin was given together with DHEA, the expression of COX2 protein was similar to that in controls (Figure 5).

Activation of ovarian AMPK by metformin

To evaluate whether, as in other systems, metformin acts on ovarian tissue via the chronic activation of AMPK pathway, we studied the rate of phospho-AMPK-α (Thr172) activation. We found that DHEA increased the activation of AMPK when compared with controls (Figure 6). When metformin was administered together with DHEA, the activation of AMPK was significantly higher than that observed in the DHEA group (Figure 6: b versus c).

Discussion

Metformin is an antidiabetic drug that increases glucose utilization in insulin-sensitive tissues. As PCOS and diabetes share some altered
A DHEA+Met

PCOS (Yilmaz et al., 2001) ever, metformin fails to decrease serum LPO in lean patients with hepatic antioxidant levels in rats (Ewis and Abdel-Rahman, 1995) and from high fructose-fed rats (Faure et al., 2001). Infertility caused by PCOS (Vandermolen et al., 1999; Pavlovic et al., 2005) and is not able to scavengE O2 and H2O2. In this point, we have to consider first that we have previously found increased LPO in rat (Motta et al., 2001a). Second, we have to remember that reactive nitrogen oxide species (RNOs)—such as peroxynitrite radical (ONOO−, formed by the combination of NO and O2)—result in a more aggressive pro-oxidant species than NO itself (Rosenberg et al., 1999; Motta et al., 2001a). On the other hand, this presence of O2, which in turn could produce ONOO−, might indirectly support our above suggestion that O2 could be one of the most abundant ROS species produced by hyperandrogenized ovaries. In agreement with previous reports, the diminution of ROS activity after hyperandrogenization could be the result of down-regulation by a direct effect of NO on the enzyme (Rengasamy and Johns, 1993; Motta et al., 2001a). We also found that metformin administered together with DHEA showed an ovarian ROS activity similar to that observed in the control group.

Although a cross-talk mechanism between metformin and the NO/NOS system has been described during restoration of microvascular reactivity in diabetic rats (Sartoretto et al., 2005), in bovine aortic cells (Zou et al., 2004) and hepatic cells (McCarty, 2004a), our data provide novel evidence that metformin regulates the ovarian NO/NOS system during the hyperandrogenized condition.

To study the intracellular mechanism involved in metformin action, we determined the expression of ovarian iNOS by western blotting assay. We found that hyperandrogenization with DHEA enhanced the expression of ovarian iNOS protein, suggesting that, as it has been recently reported in mesenteric arteries (Briones et al., 2005), the enhancement of iNOS expression produced after DHEA treatment could be a compensatory mechanism following a decrease in NOS activity.

Different from ROS, there is agreement with respect to the modulation of GSH by metformin. It has been reported that metformin regulates GSH levels during in vitro maturation of oocyte (Lee et al, 2005), in the liver of diabetic rats (Yanardag et al., 2005), in pancreatic islets from type 2 diabetic patients (Guigas et al., 2004; Marchetti et al., 2004), in erythrocytes from type 2 diabetic patients (Pavlovic et al., 2000) and during carbon tetrachloride hepatotoxicity in mice (Poon et al., 2003). However, our data represent the first evidence that metformin regulates ovarian GSH content in hyperandrogenized oocytes.

The present study also shows that hyperandrogenization diminished ovarian PGE and increased COX2 expression, showing a pattern similar to that observed in the case of NO activity—inNOS expression. These data could suggest, as has been previously reported, that the biosynthesis and release of NO and PGE share a number of pathways (Salvemini et al., 1993; Mollace et al., 2005; Schrage et al., 2005). In fact, it has been demonstrated that NO modulates COX activity by combining with the haem-group of COX (Salvemini et al., 1993). In the present study, we demonstrate for the first time that metformin reversed both ovarian PGE production and COX2 expression. It is important to point out that it has been reported that the aminoguanidine-like activity of metformin allows the drug to interact with the haem-group of both NOS and COX (Yousuf et al., 1999).

In addition, it has been proposed that metformin (acting via the chronic activation of AMPK pathway) suppresses excess androgen production in PCOS (McCarty, 2004b). Phosphorylation of Thr172 within the activation loop of the catalytic domain of the α subunit is necessary for AMPK activity (Crute et al., 1998; Stein et al., 2000). It has been established that women with PCOS present an enhanced oxidant status in blood cells, which may contribute to increase the risk of cardiovascular disease (Franks, 1995; Sabuncu et al., 2001; Yilmaz et al., 2005). In addition, it has been documented that both ROS and metformin activate phosphorylation in Thr172 of AMPK (Zhou et al., 2001; Musi et al., 2002; Zou et al., 2002; Bonnefont-Rousselot et al., 2003). In the present study, we demonstrate for the first time that hyperandrogenization generates increased oxidative stress on ovarian tissue via activation of the phosphorylation of AMPK. We found that
the co-treatment with metformin produced an enhanced activation of AMPK and that this chronic activation was necessary to increase the ovarian GSH content. These findings are in agreement with those reported previously where even during a condition of increased stress, a chronic AMPK activation achieved by metformin has to be present to restore the oxidant status (Fryer et al., 2002; McCarty, 2004a,b).

In summary, we have demonstrated for the first time that metformin is able to regulate ovarian GSH content, NO synthase activity and PGE production, but it is not effective in modulating either LPO or CAT activity of hyperandrogenized ovaries. It appears that these actions involve the regulation of iNOS and COX2 expressions and the phosphorylation of AMPK-α. The understanding of the mechanisms used by metformin during the treatment of PCOS could contribute to additional benefits especially to those related to the combination with other drugs.

Acknowledgements

These studies were supported by PIP CONICET, reference 6051 and Fundación Alberto Roemmers.

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Submitted on March 27, 2006; resubmitted on May 19, 2006; accepted on May 23, 2006

Metformin in hyperandrogenized ovaries