Obesity induced by high-fat diet promotes insulin resistance in the ovary

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Abstract

Besides the effects on peripheral energy homeostasis, insulin also has an important role in ovarian function. Obesity has a negative effect on fertility, and may play a role in the development of the polycystic ovary syndrome in susceptible women. Since insulin resistance in the ovary could contribute to the impairment of reproductive function in obese women, we evaluated insulin signaling in the ovary of high-fat diet-induced obese rats. Female Wistar rats were submitted to a high-fat diet for 120 or 180 days, and the insulin signaling pathway in the ovary was evaluated by immunoprecipitation and immunoblotting. At the end of the diet period, we observed insulin resistance, hyperinsulinemia, an increase in progesterone serum levels, an extended estrus cycle, and altered ovarian morphology in obese female rats. Moreover, in female obese rats treated for 120 days with the high-fat diet, the increase in progesterone levels occurred together with enhancement of LH levels. The ovary from high-fat-fed female rats showed a reduction in the insulin receptor substrate/phosphatidylinositol 3-kinase/AKT intracellular pathway, associated with an increase in FOXO3a, IL1B, and TNFA protein expression. These changes in the insulin signaling pathway may have a role in the infertile state associated with obesity.


Introduction

Insulin signaling begins when insulin binds and activates its receptor, resulting in tyrosine phosphorylation of several substrates, including the insulin receptor substrate (IRS) 1–4. IRS proteins, in turn, bind and activate the enzyme phosphatidylinositol 3-kinase (PI3K; Backer et al. 1992, Cheatham & Kahn 1995, Patti et al. 1995). AKT is a key downstream target of PI3K, activated by serine and threonine phosphorylation (Kohn et al. 1996, Bandyopadhyay et al. 1997). The PI3K/AKT pathway has an important role in the metabolic effects of insulin. AKT also phosphorylates and inactivates the members of the forkhead transcription factor subfamily (FOXO) and glycogen synthase kinase 3B (GSK3B). Upon insulin receptor autophosphorylation, there is also recruitment of the SHC protein and GRB2, leading to the activation of the extracellular signal-regulated kinase (ERK) pathway (Skolnik et al. 1993, Giorgetti et al. 1994, Saltiel & Pessin 2002).

The ability of insulin to stimulate steroidogenesis in ovarian cells in vitro (Barbieri et al. 1983) and the presence of insulin receptor in both stromal and follicular compartments of human ovary (Poretsky et al. 1984) have established the ovary as a target organ for insulin action. Indeed, insulin signaling has been shown to have a role in ovarian function, including the regulation of ovarian steroidogenesis, follicular development, and granulosa cell proliferation (Willis et al. 1996, Adashi et al. 1997, Poretsky et al. 1999).

The prevalence of obesity is constantly on the rise and constitutes a major worldwide epidemic. Obesity increases the risk of type 2 diabetes mellitus and cardiovascular disease, and has also a negative effect on fertility. The Nurses’ Health Study reported that the risk of infertility in women increases with increasing body mass index value (Rich-Edwards et al. 1994). Polycystic ovary syndrome is a condition commonly associated with anovulatory infertility, and obesity may play a role in the development of the syndrome in susceptible women (Pasquali & Casimirri 1993, Legro 2000, Metwally et al. 2007).

The major factor underlying the adverse metabolic consequences of obesity is believed to be insulin resistance. The reduction in the sensitivity to the biological actions of insulin affects not only glucose metabolism, but also all aspects of insulin action. However, the obesity effect on insulin signaling in the ovary has not yet been evaluated.

The aim of the present study was to analyze the effect of high-fat diet-induced obesity on the insulin signaling in the ovary. We also verified if insulin signaling impairment is time dependent in relation to the period during which the ovary was submitted to adverse effects of obesity. We demonstrated...
that insulin resistance in the ovary occurred in a similar fashion in relation to the classical target tissues of insulin, providing evidence for intracellular mechanisms that are possibly involved in the impaired reproductive function in the obese female model.

Materials and Methods

The present experimental protocols were approved and performed in accordance with the guidelines of the Committee for Ethics in Animal Research of the Institute of Biomedical Sciences, University of São Paulo, Brazil.

Animals

Female Wistar rats (8–10 weeks of age, weighing 150–180 g at the beginning of the experiments) were obtained from our breeding colony at the Institute. The animals were randomized into two matching weight groups and assigned to receive two types of diet: a standard rat chow (control rats; 3.8 kcal/g – 63.4% carbohydrate, 25.6% proteins, and 11.0% fat), or a high-fat diet (obese rats; 5.4 kcal/g – 25.9% carbohydrate, 14.9% proteins, and 59.0% fat; Rhoster (2010)). To receive two types of diet: a standard rat chow (control rats; 3.8 kcal/g – 63.4% carbohydrate, 25.6% proteins, and 11.0% fat), or a high-fat diet (obese rats; 5.4 kcal/g – 25.9% carbohydrate, 14.9% proteins, and 59.0% fat; Rhoster (2010)).

Characterization of high-fat diet-induced obese female rats

At the end of the diet period, the body weight, retroperitoneal and gonadal fat pad, and HOMA index ((blood glucose (mM)×serum insulin (μU/ml)) /22.5) were determined. After 5 h of food withdraw, female rats were anesthetized with thiopental (5 mg/100 g, i.p.; Cristália, São Paulo, Brazil), and a tail vein blood sample was obtained for glucose monitor (Roche). Blood samples were taken from the abdominal aorta of anesthetized rats for the determination of glucose (mM) and 0.37 (n=7) were determined. After 5 h of food withdraw, female rats were anesthetized with thiopental (5 mg/100 g, i.p.; Cristália, São Paulo, Brazil), and a tail vein blood sample was obtained for determination of glucose concentration using a blood glucose monitor (Roche). Blood samples were taken from the abdominal aorta of anesthetized rats for the determination of serum concentrations of insulin, estradiol, progesterone, FSH, LH, and testosterone using RIAs.

Estrous cycle analysis and ovarian morphology

Estrous cycle was determined once a day by cytological examination of vaginal smears obtained for two consecutive weeks before the end of the period of treatment with the high-fat diet. Ovaries were collected from control and high-fat diet-treated rat females (120 and 180 days), anesthetized with thiopental (5 mg/100 g, i.p.) and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for morphological analysis. The specimens were routinely processed for Histosec (Merck) embedding. Sections of 5 μm were used for hematoxylin and eosin staining and for histological analysis with standard light microscopy.

Table 1 Characteristics of high-fat diet-induced obese female rats. The values were expressed as mean±S.E.M.

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<tr>
<th></th>
<th>120 days</th>
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<tr>
<td></td>
<td>Control</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>238±00±0±0 (n=10)</td>
<td>283±69±7±85 (n=15)</td>
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<tr>
<td>Retroperitoneal fat (g/100 g body weight)</td>
<td>0±85±0±15 (n=4)</td>
<td>3±48±0±64 (n=7)</td>
</tr>
<tr>
<td>Gonadal fat (g/100 g body weight)</td>
<td>1±59±0±16 (n=7)</td>
<td>3±82±0±30 (n=12)</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>5±68±0±32 (n=10)</td>
<td>5±96±0±17 (n=15)</td>
</tr>
<tr>
<td>Serum insulin (μU/ml)</td>
<td>15±23±2±08 (n=9)</td>
<td>32±42±3±22 (n=12)</td>
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<tr>
<td>HOMA-IR</td>
<td>3±90±0±71 (n=9)</td>
<td>8±79±0±93 (n=12)</td>
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*P<0.05, **P<0.01, and ***P<0.001 in comparison with female age-matched control rats.
Sex steroid levels, estrus cycle, and ovarian morphology in females submitted to high-fat diet

In the estrous phase, the serum levels of estradiol, testosterone, and FSH did not change after treatment with high-fat diet for 120 or 180 days (Table 2). On the other hand, progesterone levels were increased in both obese groups, but LH levels were increased only in females fed with high-fat diet for 120 days (Table 2).

Female rats submitted to high-fat diet for 120 and 180 days exhibited an extended estrous cycle due to a prolonged proestrus phase (Table 3). Females fed high-fat diet for 180 days also exhibited a prolonged diestrus phase, and reduced estrus and metestrus phase (Table 3).

Treatment with the high-fat diet for 120 days did not promote significant changes in the ovarian morphology (data not shown). However, morphological differences were found between the ovaries from female rats submitted to high-fat diet for 180 days and their controls. In the control group, the ovaries exhibited morphologically normal antral and pre-antral follicles and corpora lutea. The granulosa layer showed a typical epithelioid arrangement, and were completely embraced by theca cells. Interstitial cells were occasionally found throughout the cortical stroma, among

**Table 2** Serum sex hormone levels in the estrous phase. The values were expressed as mean ± S.E.M.

<table>
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<tr>
<td></td>
<td>Control</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>17-60±2.96 (n=10)</td>
<td>18-65±4.16 (n=8)</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>41-06±2.22 (n=10)</td>
<td>59-32±7.52* (n=10)</td>
</tr>
<tr>
<td>Testosterone (pg/ml)</td>
<td>161-00±26-84 (n=6)</td>
<td>145-40±28-49 (n=10)</td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>14-26±1-79 (n=10)</td>
<td>38-06±4-05* (n=10)</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>3-96±0-44 (n=10)</td>
<td>4-14±0-71 (n=10)</td>
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*P<0.05 and †P<0.001 in comparison with female age-matched control rats.

To reduce nonspecific protein binding to nitrocellulose, the membrane was pre-incubated overnight at 4°C in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose blots were incubated with specific antibodies diluted in blocking buffer (3% nonfat dry milk) overnight at 4°C. The nitrocellulose membranes were treated with Ponceau staining immediately after transfer as a quick visual control of the amount of protein in each lane. To visualize the autoradiogram, commercial enhanced chemiluminescence reagents (GE Healthcare) applied to photographic film were used. The band intensities were quantified by optical densitometry (Scion Image Software, Frederick, MD, USA).

**Antibodies**

Anti-insulin receptor, anti-IRS1, anti-IRS2, anti-phosphotyrosine, anti-pAKT, and anti-GSK3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PI3K, anti-AKT, anti-FOXO3a, anti-ERK, and anti-pERK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-TNFα and anti-IL1β were purchased from Biolegend (San Diego, CA, USA).

**Statistical analysis**

The results were expressed as means ± S.E.M. Statistical analyses were performed using t-test or one-way ANOVA followed by Bartlett’s test for homogeneity of variances and Tukey–Kramer multiple comparisons test when appropriate. The estrous cycle was analyzed by the unpaired t-test with Welch’s correction. The minimum acceptable level of significance was P<0.05.
Insulin signaling pathways on ovaries from females submitted to high-fat diet for 120 days

The protein expression of AKT and FOXO3a in the whole ovary homogenates was modified by 120 days of high-fat diet feeding, whereas the insulin receptor, IRS1, IRS2, ERK1-2, and GSK3 expression were similar to that detected in control rats (Fig. 3A). The AKT and FOXO3a protein levels were enhanced by 69 and 67% respectively above those detected in control rats (Fig. 3B and C respectively).

Acute insulin infusion induced a sixfold increase in the tyrosine phosphorylation degree of the insulin receptor above the baseline, which was similar in the ovaries of control and obese rats (Fig. 4A). There was also a twofold enhancement in the insulin-induced IRS1 association with PI3K above the baseline in ovaries of both groups (Fig. 4B). On the other hand, in the ovary from control rats, there was a sixfold increase in the IRS2 association with PI3K after insulin infusion, whereas in the ovary from obese rats there was only a twofold increase above the baseline (Fig. 4C).

Although the degree of insulin-induced AKT serine phosphorylation was similar in both groups, the phosphorylation on the basal condition was higher in ovaries from obese rats (Fig. 4D). In this regard, insulin promoted a threefold increase in AKT serine phosphorylation in obese female rats, whereas in control female rats that increase was sixfold. Despite the fact that ERK1-2 protein expression was similar in ovaries from both groups (Fig. 3A), the acute insulin infusion-induced ERK1-2 phosphorylation was ~50% lower in obese rats (Fig. 4E).

Insulin signaling pathways in ovaries from females submitted to high-fat diet for 180 days

The maintenance of the high-fat diet for 180 consecutive days had no impact on the protein levels of IRS1, AKT, and GSK3 in the ovary (Fig. 5A). However, there was a reduction in the IRS2 protein level by ~40% (Fig. 5B) and an enhancement of ~30% in the FOXO3a protein level (Fig. 3C) in the ovary from diet-induced obese rats when compared with controls. The acute insulin infusion-induced increase in the IRS1 and IRS2 association with PI3K was detected only in the ovaries from control female rats (Fig. 6A and B). There was a twofold increase above the baseline in the insulin-induced AKT serine phosphorylation in the control female rats, but insulin did not promote any effect in the ovaries from obese rats (Fig. 6C).

Cytokine protein expression in the ovaries from females submitted to high-fat diet

IL1B (Fig. 7A) and TNFα (Fig. 7B) protein expression in the ovary did not change after 120 days of high-fat diet. However, treatment with high-fat diet for 180 days promoted a fourfold increase in IL1B (Fig. 7C) and TNFα (Fig. 7D) protein levels in the ovary when compared with controls.

Discussion

Reproductive health and female fertility are compromised by overweight and obesity (Shaw et al. 1997, Gesink Law et al. 2007). The role of systemic insulin resistance in obesity-impaired reproductive performance is shown by the positive effects of the insulin-sensitizing agents (Nestler et al. 1998, Tang et al. 2006). In the present study, we demonstrated that systemic insulin resistance and hyperinsulinemia occur together with impairment of insulin signaling in the ovary.
Enhancement of progesterone serum levels, and alteration of estrous cycle and of ovarian morphology in high-fat diet-induced obese female rats.

Insulin signaling has been implicated in the regulation of female reproductive function by acting in both central nervous system and ovaries. The hyperinsulinemia can potentiate gonadotropin-stimulated steroidogenesis in granulosa and thecal cells, by increasing the low-density lipoprotein (LDL) receptor, 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, and 17,20 lyase expression (Nestler & Strauss 1991, McGee et al. 1995, Franks et al. 1999, Poretsky et al. 1999, Zhang et al. 2000). In addition, insulin may act on the pituitary to increase LH release (Adashi et al. 1981, Weiss et al. 2003, Dorn et al. 2004, Moret et al. 2009). In agreement with these data, we observed both hyperinsulinemia and enhancement of progesterone serum levels in obese female rats. Moreover, the LH levels were also enhanced in female obese rats receiving the high-fat diet for 120 days, which could explain the progesterone increase. Surprisingly, the LH levels in obese females treated for 180 days with high-fat diet were similar to controls. This suggests that although insulin acts cooperatively with GNRH, chronic hyperstimulation may induce a refractory state ultimately leading to a decreased biosynthetic capacity (Lawson et al. 2008). Such a mechanism would explain the similar LH levels in control and obese females treated for 180 days with high-fat diet, since the hyperinsulinemia persisted at 180 days of high-fat diet treatment and the insulin level was higher than in female rats that received the high-fat diet for 120 days. Moreover, the accumulation of lipid droplets in the stromal and granulosa cells may be a consequence of hyperinsulinemia on the expression of LDL receptor, which would contribute to increase steroidogenesis after 180 days of high-fat diet treatment.

Both low and high progesterone levels have a negative impact on pregnancy outcome (Check et al. 2009). An extended estrous cycle was observed with both periods of high-fat diet treatment, but the 180-day period promoted the alterations that were more pronounced than after 120 days, with a prolonged diestrus phase and short estrus and metestrus phases, which could result in reduced reproductive capacity. Female mice that lack crucial components of the insulin signaling pathway, such as IRS2, are infertile and display ovarian and hypothalamic function deficits, suggesting that the IRS2 pathway mediates the insulin effects upon the reproductive function (Burks et al. 2000). In insulin resistance conditions, such as the polycystic ovary syndrome, the ovary remains sensitive to insulin, whereas other organs exhibit a
Insulin signaling in the ovary from control and high-fat diet (120 days)-induced obese female rats. (A) Representative blots of insulin receptor β-subunit, IRS1, IRS2, AKT, ERK1–2, FOXO3a, and GSK3 protein expression. (B) Densitometric analyses of the AKT protein levels in ovaries from both control and high-fat diet-induced obese rats. (C) Densitometric analyses of the FOXO3a protein levels in ovaries from both control and high-fat diet-induced obese rats. Data are expressed as means ± S.E.M. obtained from six animals. *P<0.05 versus control group.

Figure 3

Insulin signaling in the ovary from control and high-fat diet (120 days)-induced obese female rats. Ovary homogenates were immunoprecipitated with anti-insulin receptor subunit β (IR-β) (A), anti-IRS1 (B), and anti-IRS2 (C) antibodies, followed by immunoblotting with antiphosphotyrosine antibody (A) and anti-p85 kDa subunit of the PI3-kinase (B and C). The membranes containing whole extract of ovary homogenates were incubated with anti-phospho-Ser473-AKT (D) and anti-phospho-Thr185/Tyr187-ERK1–2 (E) antibodies. The phosphorylation and association degree were determined in basal (−) and insulin-stimulated (+) conditions. Data are expressed as means ± S.E.M. obtained from six animals. *P<0.05 versus basal condition of respective group. *P<0.05 versus respective condition of control group.

Figure 4

significant reduction in insulin action (Poretsky 1991, Lima et al. 2006). Since deficits of insulin receptor signaling in the muscle, liver, and adipose tissue are related to obesity-associated insulin resistance, and as it is known that these deficits occur through tissue- and pathway-specific factors, it is interesting to evaluate if the ovary from obese female rats remains sensitive to insulin concomitantly with enhancement of progesterone levels, and alteration of estrous cycle and of ovarian morphology.

In the present study, the ovaries from obese females that received the high-fat diet for 120 days presented AKT phosphorylation similar to controls, despite a reduction in IRS2/PI3K association, probably by a compensatory increase in AKT protein expression. On the other hand, in the ovary from obese females treated for 180 days with the high-fat diet, both IRS1 and IRS2/PI3K association were reduced, concomitantly with a reduction in AKT phosphorylation. These results suggest that the degree of impairment of the insulin signaling pathway exhibits a time-dependent relation to the exposure to obesity-generated deleterious effects. The synergistic action of insulin with LH in the ovary may be due to a positive crosstalk of the intracellular signaling pathway of both hormones. Carvalho et al. (2003) demonstrated that simultaneous infusion of insulin and LH induced higher phosphorylation of AKT in the ovary than each hormone alone. Moreover, PI3K, an upstream protein of AKT in the insulin signaling pathway, is involved in the LH- and insulin-induced upregulation of the LDL receptor expression (Sekar & Veldhuis 2001). Our data could indicate that the IRS/PI3K/AKT pathway may not be involved, at least directly, in the enhancement of steroidogenesis or in the accumulation of lipid droplets observed in ovarian cells of obese female rats.

A balance between IRS/PI3K/AKT pathway-stimulated and MAPK pathway-inhibited steroidogenesis coordinates the ovarian function. Indeed, it was demonstrated that stimulation of ERK1–2 pathway inhibits steroidogenesis in the ovary (Nelson-Degrave et al. 2005). In this regard, the reduced ERK1–2 phosphorylation detected after 120 days of high-fat diet could have a role in the increase in progesterone levels.

Beyond the effect on the ovarian steroidogenesis, insulin is involved in follicular development and granulosa cell proliferation (Willis & Franks 1995, Nestler et al. 1998, Poretsky et al. 1999). In fact, the ovaries from IRS2-null female mice present reduced follicle size, impaired oocyte

E H Akamine and others • Obesity and insulin signaling in the ovary

growth, defective granulosa cell proliferation, as well as reduced insulin-stimulated AKT phosphorylation (Neganova et al. 2007). Several proteins that regulate cell survival and proliferation are downstream effectors of AKT. Members of the FOXO forkhead transcription factor subfamily and GSK3 are phosphorylated and thus inactivated by AKT. Female mice that lack FOXO3a exhibit early depletion of ovarian follicles and secondary infertility due to follicular activation, indicating that this factor functions as a suppressor of the earliest stages of follicular growth (Castrillon et al. 2003). The cascade from granulosa cell-derived AKT/GSK3 pathway also regulates the early ovarian follicular development (Liu et al. 2007). Despite the increase in AKT expression after 120 days of high-fat diet, its phosphorylation degree induced by insulin is similar to the control, which could indicate a lack of effect of the AKT increase upon FOXO3a. On the other hand, the ovary from female rats treated with high-fat diet displayed increased FOXO3a expression and reduced AKT phosphorylation after 180 days of high-fat diet, which could be implicated in obesity-induced impaired reproductive function.

Despite the finding of pre-antral and antral follicles in ovaries of both control and high-fat diet (180-day period) groups, which could indicate that follicular development was not affected, other parameters of the reproductive function were altered in the experimental group. As discussed above, a decrease in the IRS/PI3K/AKT pathway does not seem implicated in the enhancement of steroidogenesis or with accumulation of lipid droplets observed in ovarian cells of obesity and insulin signaling in the ovary. E H AKAMINE and others 71

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Figure 5  Insulin signaling in the ovary from control and high-fat diet (180 days)-induced obese female rats. (A) Representative blots of IRS1, IRS2, AKT, FOXO3a, and GSK3 protein expression. (B) Densitometric analyses of the IRS2 protein levels in ovaries from both control and high-fat diet-induced obese rats. (C) Densitometric analyses of the FOXO3a protein levels in ovaries from both control and high-fat diet-induced obese rats. Data are expressed as means ± S.E.M. obtained from six animals. *P<0.05 versus control group.

Figure 6  Insulin signaling in the ovary from control and high-fat diet (180 days)-induced obese female rats. Ovary homogenates were immunoprecipitated with anti-IRS1 (A) and anti-IRS2 (B) antibodies, followed by immunoblotting with anti-p85 kDa subunit of the PI3-kinase. The membranes containing the whole extract of ovary homogenates were incubated with anti-phospho-Ser473-AKT (C). The phosphorylation and association degree were determined in basal (−) and insulin-stimulated (+) conditions. Data are expressed as means ± S.E.M. obtained from six animals. *P<0.05 versus basal condition of respective group. *P<0.05 versus respective condition of control group.

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obese female rats. On the other hand, progesterone could decrease the IRS/PI3K/AKT pathway. Progesterone is implicated in insulin resistance during pregnancy by decreasing the IRS/PI3K/AKT pathway. Progesterone is able to enhance the expression of inflammatory cytokines such as IL1B and TNFα in different phases of the estrous cycle.

During the ovulation process, expression of inflammatory factors and molecules related to the innate immune response is observed, such as prostaglandins, cytokines, and Toll-like receptors. While regulated synthesis and release of cytokines is essential for follicular development and ovulation (Machelon et al. 2004, Ge´rard et al. 2001, Herath et al. 2002, Bedaiwy et al. 2004, Backer et al. 2004, Burton et al. 2008), blood levels of IL6 are elevated in patients with endometriosis, and could lead to infertility (Odukoya et al. 1997, Bedaiwy et al. 2002, Umezawa et al. 2008). The enhancement of white adipose tissue increases the production of factors related to immune cells, cytokines, and fatty acids, which could contribute to the installation of infertility in the obese condition (Sch¨afler et al. 2007). In the present study, the pro-inflammatory cytokine expression was similar in the ovary from control and obese female rats receiving the high-fat diet for 120 days, but it was increased in the ovary from female rats treated for 180 days. This cytokine enhancement could be involved in the insulin signaling reduction in the ovary after 180 days of a high-fat diet, which could contribute to infertility in obese females.

In summary, our results show that insulin resistance in the ovary occurs in a way which is similar to that observed in the classical target tissues of insulin, and that the insulin signaling alterations is time dependent in relation to the period of exposure to obesity-related deleterious effects. These data suggest that the positive effects of insulin sensitizer agents upon the reproductive function could actually correct insulin signaling directly in the ovary.

Declarations of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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