Metformin decreases IGF1-induced cell proliferation and protein synthesis through AMP-activated protein kinase in cultured bovine granulosa cells

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Abstract

Although its mechanism of action is still unclear, metformin is an anti-diabetic drug effective to restore cyclicity and spontaneous ovulation in women with polycystic ovary syndrome. It may also reduce the risk of cancer. We have recently shown that metformin treatment decreases steroidogenesis through AMP-activated kinase (AMPK) in granulosa cells of various species. Here, we investigated the effects and the molecular mechanisms of metformin in IGF1-induced proliferation and protein synthesis in cultured bovine granulosa cells. Treatment with metformin (10 mM) for 24 h reduced cell proliferation and the levels of cyclin D2 and E, and increased the associations cyclin D2/p21 and cyclin D2/p27 without affecting cell viability in response to IGF1 (10⁻⁸ M). It also decreased IGF1-induced protein synthesis and phosphorylation of P70S6 kinase and ribosomal S6 protein. Interestingly, metformin treatment for 1 h decreased MAPK3/1 (ERK1/2) and P90RSK phosphorylation without affecting AKT phosphorylation in response to IGF1. Adenovirus-mediated expression of dominant-negative AMPK totally abolished the effects of metformin on cell proliferation and phosphorylation of P70S6 kinase and ribosomal S6 protein. Interestingly, metformin treatment for 1 h decreased MAPK3/1 (ERK1/2) and P90RSK phosphorylation without affecting AKT phosphorylation in response to IGF1. Taken together, our results strongly suggest that metformin reduces cell growth, protein synthesis, MAPK3/1, and P90RSK phosphorylation in response to IGF1 through an AMPK-dependent mechanism in cultured bovine granulosa cells.


Introduction

Metformin, a biguanide, is the most widely used drug for the treatment of type 2 diabetes but also for the treatment of the polycystic ovary syndrome (PCOS). This syndrome affects 5–10% of women of childbearing age (Franks 1995). It is characterized by the presence of two or more of the following features: chronic oligo-ovulation or anovulation, androgen excess, and polycystic ovaries (Franks 1995). In women with PCOS, a long-term metformin treatment increases ovulation rates, improves menstrual cyclicity, and reduces serum androgen levels (Velazquez et al. 1997, Vandermolen et al. 2001). These beneficial effects have been attributed to a reduction in hyperinsulinemia partly due to an increase in peripheral glucose utilization and a decrease in endogenous glucose production by the liver (Bailey 1992, Scheen 1997). Some evidence indicates that metformin has also significant effects on tumorigenesis and cancer cell growth (Evans et al. 2005, Bowker et al. 2006, Ben Sahra et al. 2008). Although the molecular mechanisms of metformin have been studied in tissues such as liver, muscle, and fat, in relation to glucose homeostasis and insulin action, relatively little is known about the mechanism of action of this compound on the ovarian tissue. Several studies have shown in vitro that metformin decreases steroids production in response to FSH but also to insulin-like growth factor 1 (IGF1) in granulosa cells of different species including rodent (Tosca et al. 2006), human (Mansfield et al. 2003), and bovine (Tosca et al. 2007a). Several studies have shown that there is an increased granulosa cell proliferation in some follicles in women with PCOS (Stubbs et al. 2007, Das et al. 2008). Thus, it is important to determine the effects and the molecular mechanisms of metformin, frequently used for the treatment of PCOS, on proliferation and protein synthesis in granulosa cells. In the present work, we chose to investigate these actions of metformin on cultured bovine granulosa cells, where we have already studied the effects of this drug on steroidogenesis.

Here, we studied the effect of metformin on the proliferation of granulosa cells in response to IGF1. IGF1 is a potent mitogenic factor, and it is critical for ovarian follicle development. In transgenic mice lacking IGF1,
follicle development is arrested at the small antral stage, and mature Graafian follicles are not produced (Baker et al. 1996, Zhou et al. 1997). IGF1 activates two major signal transduction pathways: the phosphoinositide 3'-OH kinase (PI3K) pathway and the MAP kinase pathway (MAPK pathway, also known as the ERK pathway; Dupont et al. 2003). Some of the downstream effectors of PI3K are the serine/threonine protein kinases AKT/PKB and P70S6 kinase. P70S6 kinase (P70S6k) is a key enzyme involved in the control of protein synthesis (Dufrner & Thomas 1999). Activation of the MAPK pathway by IGF1 requires activation of Ras and is mediated by a cascade of successive protein phosphorylation reactions involving Raf, MAPK kinase (MEK-1 and -2), and finally MAPK3 and -1 (ERK1 and -2; Pearson et al. 2001). After activation, MAPK3/1 phosphorylates several downstream elements, including Elk-1 or P90RSK. In granulosa cells, activation of the PI3K or the MAPK pathway by IGF1 has been reported to stimulate proliferation, differentiation, and/or cell survival (Hu et al. 2004, Tosca et al. 2005, Goto et al. 2009). Metformin treatment decreases cell proliferation of various cells including breast cancer cells and endometriotic stromal cells (Takemura et al. 2007, Alimova et al. 2009). However, the mechanisms of action by which metformin mediates cell cycle arrest are not completely understood.

Several studies have shown both in vivo and in vitro that metformin can act through activation of AMPK activated protein kinase (AMPK; Zhou et al. 2001, Musi et al. 2002). AMPK is a heterotrimeric enzyme, consisting of one catalytic subunit, α, and two regulatory subunits, β and γ (Cheung et al. 2000). AMPK activity results partly from phosphorylation of the Thr172 residue of the α-subunit by two known upstream kinases: LKB1 and calcium–calmodulin-dependent kinase kinase (CaMKK; Hardie 2004, Birnbaum 2005). AMPK is activated by a change in AMP:ATP ratio in response to several stimuli, including exercise (Hayashi et al. 1998), hypoxia (Mu et al. 2001), hormones (Minokoshi et al. 2002, Yamauchi et al. 2002), and pharmacological drugs such as metformin (Zhou et al. 2001) or 5-aminoimidazole-4-carboxamide-riboside-5-phosphate (Coroton et al. 1995). AMPK is a multisubstrate enzyme well characterized in many tissues, including the liver, muscle, lung, heart, kidney, brain (Stapleton et al. 1996), and ovary (Downs et al. 2002, Bilodeau-Goseeels et al. 2007, Tosca et al. 2007a, 2007b). We recently characterized AMPK in bovine ovary and demonstrated that metformin-induced AMPK activation decreased MAPK3/1 phosphorylation and steroid secretion by granulosa cells (Tosca et al. 2007a).

The aims of this study were to investigate the effects of metformin on cultured bovine primary granulosa cell proliferation and protein synthesis in response to IGF1 and the underlying molecular mechanisms. More precisely, we tested whether the decrease observed in IGF1-induced cell proliferation and protein synthesis in response to metformin was AMPK dependent by using an adenovirus-mediated expression of dominant-negative AMPK in bovine granulosa cells.

Results

Effects of the metformin treatment on basal and IGF1-stimulated cell proliferation in cultured bovine granulosa cells

We investigated whether the metformin treatment affected the number of granulosa cells in response to IGF1 by inducing mitosis or altering cell viability. [3H]-thymidine incorporation into granulosa cells treated with 10 mM metformin ± IGF1 (10⁻⁸ M) was determined after 24 h of culture. As expected, IGF1 treatment significantly increased [3H]-thymidine incorporation (P<0.001; Fig. 1A). However, metformin treatment decreased IGF1-stimulated [3H]-thymidine incorporation (Fig. 1A) by about 20% (P<0.001) without affecting the level of [3H]-thymidine incorporation in basal state (in absence of IGF1). These effects of metformin on IGF1-induced cell proliferation were confirmed by evaluating cyclin D2 and cyclin E protein levels in response to IGF1 by western blotting (Fig. 1B and C). Indeed, metformin treatment reduced IGF1-induced cyclin D2 protein levels by 30% (P<0.05, Fig. 1B) and IGF1-stimulated cyclin E protein levels by 35% (P<0.05, Fig. 1C). We have also shown that metformin treatment increased significantly the association levels between the cyclin-dependent kinase inhibitors (CDKIs) p21 and p27 with CDK2 in the presence of IGF1 (P<0.05, Fig. 1D and E). These data were observed without any variation of the protein amount of p21 and p27 whatever the treatment used (data not shown). Trypan blue staining demonstrated that metformin treatment had no effect on cell viability in the presence or absence of IGF1 (data not shown).

Effects of the metformin treatment on basal and IGF1-stimulated protein synthesis in cultured bovine granulosa cells

Cell growth is very often accompanied with an increase in protein synthesis. We next determined the effect of metformin treatment on IGF1-induced protein synthesis. [¹⁴C]-phenylalanine incorporation into granulosa cells treated with 10 mM metformin ± IGF1 (10⁻⁸ M) was determined after 4 h of culture. As expected, IGF1 treatment significantly increased [¹⁴C]-phenylalanine incorporation (P<0.001, Fig. 2A). However, metformin treatment halved IGF1-stimulated [¹⁴C]-phenylalanine incorporation (P<0.001, Fig. 2A). To elucidate the molecular mechanism associated with this latter result, we determined the effect of metformin on the IGF1-induced phosphorylation of P70S6 kinase and
ribosomal S6 protein (two key components involved in protein synthesis). Bovine granulosa cells were serum-deprived for 16 h, and then pre-incubated in the presence or in the absence of metformin (10 mM) for various times and then stimulated for 5 min with IGF1 (10^{-8} M). As expected, IGF1 treatment significantly increased phosphorylation of P70S6K on Thr389 and on Ser424 Thr421 residues, and phosphorylation of S6 (Fig. 2B). However, this effect was totally abolished when cells were pre-incubated with metformin (10 mM) for at least 1 h (Fig. 2B). Thus, metformin treatment decreased IGF1-induced protein synthesis, and this was associated with a strong reduction in P70S6K and S6 phosphorylation.

Effects of the overexpression of dominant-negative AMPKα1 on IGF1-induced cell proliferation and P70S6K phosphorylation in response to metformin treatment in cultured bovine granulosa cells

We have demonstrated that metformin treatment activates AMPK in bovine granulosa cells (Tosca et al. 2006, 2007a). We next investigated whether the metformin-induced decrease in cell proliferation and protein synthesis was mediated by AMPK. We used an adenoviral vector (Ad.αDN) to overexpress a dominant-negative AMPKα1 in bovine granulosa cells and treated these cells with IGF1 as indicated in the Materials and Methods. Granulosa cells were infected with Ad.αDN and Ad.GFP (Ad.GFP as a control) adenovirus (100 pfu) and were analyzed by western blotting. Samples contained equal levels of protein, as confirmed by reprobing each membrane with an anti-vinculin antibody. In each panel, immunoreactivity was quantified by scanning densitometry, and the ratio cyclin/vinculin was represented. These results are representative of three independent experiments. Different letters indicate significant differences at \( P<0.05 \).
inhibitory effects of metformin (Fig. 3B and C). Thus, metformin reduces proliferation and P70S6 kinase phosphorylation through the activation of AMPK in bovine granulosa cells.

**Effects of the metformin treatment on IGF1-stimulated MAPK3/1 and p90RSK: potential involvement of AMPK**

We also determined the effects of metformin on IGF1-stimulated MAPK3/1 and P90RSK and tested a potential implication of AMPK by using Ad.αDN. Bovine granulosa cells were serum-deprived for 16 h, and then pre-incubated in the presence or in the absence of metformin (10 mM) for various times and then stimulated for 5 min with IGF1 (10^{-8} M). Lysates (50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-phospho-P70S6K Thr389 and anti-phospho-P70S6K Ser424/Thr421 antibodies. Membranes were then reprobed with P70S6K antibodies to evaluate the ratio phospho-P70S6K Thr389 or Ser424/Thr421/P70S6K in each lane. Membranes were also reprobed with anti-phospho-S6 Ser235/236 antibodies. In this case, the membranes were reprobed with vinculin antibodies to confirm that samples contained equal levels of protein in each lane. We used vinculin antibodies because we did not find good antibodies against the S6 protein that cross-react with bovine species. Representative blots from three independent experiments are shown. Blots were quantified, and the phosphorylated protein/total protein or pS6/vinculin ratio is shown. The results are represented as means ± S.E.M. Different letters indicate significant differences at *P* < 0.05.

Figure 2 Effect of metformin treatment on IGF1-induced protein synthesis in bovine granulosa cells. (A) Effects of metformin on IGF1-induced phenylalanine incorporation. Bovine granulosa cells were cultured for 24 h in serum-free medium and then stimulated or not with IGF1 (10^{-8} M) in the absence or presence of metformin (10 mM) for 4 h. Protein synthesis was determined as described in Materials and Methods. Different letters indicate significant differences at *P* < 0.05. (B) Bovine granulosa cells were serum-deprived for 16 h, and then pre-incubated in the presence or in the absence of metformin (10 mM) for various times (5, 30, 60, and 120 min) and then stimulated for 5 min with IGF1 (10^{-8} M). Lysates (50 μg) were then resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-phospho-P70S6K Thr389 and anti-phospho-P70S6K Ser424/Thr421 antibodies. Membranes were then reprobed with P70S6K antibodies to evaluate the ratio phospho-P70S6K Thr389 or Ser424/Thr421/P70S6K in each lane. Membranes were also reprobed with anti-phospho-S6 Ser235/236 antibodies. In this case, the membranes were reprobed with vinculin antibodies to confirm that samples contained equal levels of protein in each lane. We used vinculin antibodies because we did not find good antibodies against the S6 protein that cross-react with bovine species. Representative blots from three independent experiments are shown. Blots were quantified, and the phosphorylated protein/total protein or pS6/vinculin ratio is shown. The results are represented as means ± S.E.M. Different letters indicate significant differences at *P* < 0.05.

Discussion

We have recently shown that metformin decreases steroidogenesis through AMPK in the presence or absence of FSH (10^{-8} M) and IGF1 (10^{-8} M) in cultured bovine granulosa cells (Tosca et al. 2007a). However, the effects and the mechanisms through which metformin affects cell growth and protein synthesis have never been defined in these granulosa cells. In the present paper, we have shown that metformin inhibits proliferation and protein synthesis in response to IGF1 through an AMPK-dependent mechanism in bovine granulosa cells. We did not observe effects of metformin on IGF1-induced AKT phosphorylation (data not shown).
decrease in the protein synthesis in response to IGF1 is associated with a reduction in P70S6K phosphorylation. Metformin through AMPK activation has also inhibited IGF1-induced phosphorylation of MAPK3/1 and P90RSK, two kinases involved in cell growth and protein synthesis (Fig. 5).

In our study, we used bovine granulosa cells cultured for 48 h in the presence of serum (10%). These culture conditions are luteinizing conditions for this cell type. Thus, our cultured cells are more representative cells from the corpus luteum than from the growing follicles. The fact that metformin treatment reduces cell proliferation induced by IGF1 in cultured bovine granulosa cells is in good agreement with some results observed both in vivo and in vitro in other cell types. Indeed, metformin has inhibitory effects on tumor cell growth in vitro and in vivo (Evans et al. 2005, Bowker et al. 2006, Buzzi et al. 2007, Phoenix et al. 2009). In our study, we have shown that metformin inhibits IGF1-induced cyclins D2 and E protein expression, suggesting that it inhibits cell cycle progression from G0/G1 into S phase in bovine granulosa cells. Cyclin D2 is an important regulator of the cell cycle in granulosa cells. Indeed, cyclin D2-deficient females are sterile, ovarian granulosa cells being unable to proliferate (Sicinski et al. 1996).

Downregulation of cyclin D1 in response to metformin has also been demonstrated in LnCaP prostate cancer cells and in human breast cancer cell lines (Ben Sahra et al. 2008, Zhuang & Miskimins 2008). In mammalian cells, the rate of transit through G1 phase is regulated by coordinated action of CDKs in association with specific regulatory cyclin proteins. The primary known regulators of G1 progression are the D-type cyclins (D1, D2, and D3), cyclin E, and their catalytic partners CDKs-2, -4, and -6 (Dulic et al. 1992, Sherr 1993, 1994). The activity of these CDKs is regulated by changes in cyclin

Figure 3 Metformin-reduced proliferation and P70S6K phosphorylation in the presence of IGF1 in bovine granulosa cells infected with adenovirus expressing AMPKα1 mutant constructs. (A) The expression and phosphorylation levels of AMPKα in bovine granulosa cells infected with adenovirus constructs expressing mutant AMPKα1 (Ad.aDN) or the GFP protein (Ad.GFP). Granulosa cells were infected with 100 Pfu/cell of the viruses and were stimulated 16 h later in the presence or not of metformin 10 mM±IGF1 10^{-8} M for 2 h. Cells were then prepared, and the lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, probed with anti-phospho-AMPKα Thr172, and then reprobed with anti-AMPKα1/2 antibodies. Blots were quantified, and the phosphorylated protein/total protein ratio is shown. The immunoblots shown are representative of three independent experiments. (B and C) Thymidine incorporation (B) and P70S6K Thr389 phosphorylation (C) in granulosa cells expressing a1-DN or GFP in the presence or absence of metformin 10 mM±IGF1 10^{-8} M. (B) Granulosa cells were infected or not with 100 Pl/μl of Ad.aDN or GFP virus for 16 h, and stimulated or not with metformin 10 mM±IGF1 10^{-8} M for 24 h. Thymidine incorporation was then determined as described in the Materials and Methods. The data shown represent means±S.E.M. from three independent experiments. Data are expressed as the percentage of the results obtained from unstimulated cells. Different letters indicate significant differences at P<0.05. (C) Granulosa cells were infected or not with 100 Pl/μl of Ad.aDN or GFP virus for 16 h, and then pre-incubated in the presence or in the absence of metformin (10 mM) for 60 min and then stimulated for 5 min with IGF1 (10^{-8} M). Lysates (50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-phospho-P70S6K Thr389 and then reprobed with anti-P70S6K total antibodies. Representative blots from three independent experiments are shown. Blots were quantified, and the phosphorylated protein/total protein ratio is shown. The results are represented as means±S.E.M. Different letters indicate significant differences at P<0.05.
levels, interaction with CDKIs, and by regulatory phosphorylation (Sherr & Roberts 1999). Two distinct classes of cellular CDKIs are known, the Cip/Kip and Ink4 families. The Cip/Kip family includes p21 and p27, which inhibit the formation of cyclin D-CDK4/6 and cyclin E-CDK2 complexes and consequently the progression of the cell cycle. p27 and p21 play crucial roles in mammalian ovarian development and more precisely in the proliferation of granulosa cells (Jirawatnotai et al. 2003, Rajareddy et al. 2007). In the present paper, we have observed that metformin increased the association CDK2/p21 or CDK2/p27 in the presence of IGF1 without altering the protein level of p21 and p27. Active CDK–cyclin complexes are essential for the activation of genes required for progression through the S phase (Weinberg 1995). Thus, metformin could inhibit IGF1-induced cell proliferation by blocking cyclins D2 and E expression and inhibiting the activity of the cyclin/CDK complexes. In bovine granulosa cells, we have shown that the metformin-induced decrease in proliferation in response to IGF1 was AMPK dependent by using a dominant-negative AMPK adenovirus. Zakikhani et al. (2006) have also shown that siRNAs targeting AMPK prevented growth inhibition of MCF7 cells in response to metformin treatment. Moreover, the compound C, a specific inhibitor of AMPK, blocked metformin-induced downregulation of cyclin D1 in MCF7 cells. Thus, metformin-induced AMPK activation could inhibit the expression of cyclins D2 and E expression and consequently cell proliferation in response to IGF1 in bovine granulosa cells. AKT and MAPK3/1 are the main signaling pathways that control the growth of the granulosa cells in response to IGF1 (Hu et al. 2004, Goto et al. 2009). In our study, metformin did not affect IGF1-induced AKT phosphorylation (data not shown). In contrast, we have observed that metformin decreased IGF1-induced MAPK3/1 and P90RSK phosphorylation, and this was totally abolished when cells were infected with a dominant-negative AMPK adenovirus. P90RSK is one of the downstream elements phosphorylated by MAPK3/1. We have previously shown that metformin-induced AMPK activation reduced steroidogenesis in bovine granulosa, and this was associated with an inhibition of the MAPK3/1 phosphorylation (Tosca et al. 2007a). Thus, in these cells, metformin through AMPK activation and inhibition of MAPK3/1 could...
by an increased number of small antral follicles (Stubbs processes, including growth, proliferation, and proliferation in granulosa cells in women with PCOS. Metformin treatment could reduce these abnormalities of dependent mechanism in bovine granulosa cells. Thus, protein synthesis in response to IGF1 through an AMPK-pathway. We have shown that metformin inhibits proliferation and proliferation of granulosa cell are unknown. In our study, effects and the molecular mechanism of metformin on the (Mansfield et al. 2008). The ovarian phenotype in PCOS is characterized of the polycystic ovaries. Metformin treatment (10 mM) activates AMPK and consequently inhibits MAPK3/1, P70S6K, and P90RSK phosphorylation induced by IGF1. The inhibition of these different pathways leads to a reduction in proliferation and protein synthesis in bovine granulosa cells. The arrow from metformin to AMPK represents a positive effect, and effects downstream of AMPK are negative effects on IGF-mediated pathways.

decrease steroid production and cell proliferation in response to IGF1.

Some studies indicate that there are significant differences in the rate of cell death and proliferation in granulosa cell populations in PCOS patients (Das et al. 2008). The ovarian phenotype in PCOS is characterized by an increased number of small antral follicles (Stubbs et al. 2007). The follicles frequently contain an increased number of steroidogenic cells in the theca interna, although they are not present in all follicles. There is also an increased granulosa cell proliferation. Therefore, it is clear that there are abnormalities of proliferation and differentiation in both the theca and granulosa compartments of the polycystic ovary. Metformin treatment is now widely prescribed in women with PCOS. Several studies have demonstrated that metformin is able to exert direct effects on the ovary, inhibiting progesterone and estradiol production by human granulosa cells (Mansfield et al. 2003, Rice et al. 2009). However, the effects and the molecular mechanism of metformin on the proliferation of granulosa cell are unknown. In our study, we have shown that metformin inhibits proliferation and protein synthesis in response to IGF1 through an AMPK-dependent mechanism in bovine granulosa cells. Thus, metformin treatment could reduce these abnormalities of proliferation in granulosa cells in women with PCOS.

Protein synthesis is required for many cellular processes, including growth, proliferation, and differentiation. In the present study, we have shown that metformin decreased protein synthesis in response to IGF1, and this was associated with an inhibition of P70S6K. Moreover, this inhibition was AMPK dependent. In response to growth factors, the serine/threonine P70S6K phosphorylates several proteins, including eukaryotic initiation factor 4B (Erf4B) and ribosomal protein S6. It also induces dephosphorylation of the eukaryotic elongation factor 2 (EEF2), therefore affecting both the initiation and elongation stages of mRNA translation (Dufner & Thomas 1999, Proud 2006). P70S6K possesses different sites of phosphorylation. One of them, T389 in the linker domain, is considered as critical for kinase function (Dennis et al. 1998). Phosphorylation of T421 and S424 located in the autoinhibitory domain of P70S6K is also supposed to be required for opening the kinase into a fully active conformation, which allows the sequential phosphorylation of other phosphorylation sites, such as T389. Phosphorylation of all these sites of P70S6K was inhibited by metformin in bovine granulosa cells in the presence of IGF1. These results are in good agreement with results observed in breast cancer cells. Indeed, metformin through AMPK activation inhibited protein synthesis in MCF-7 cells, and this was associated with an inhibition of the P70S6 kinase phosphorylation (Dowling et al. 2007). Metformin has also been reported to cause an inhibition of protein synthesis in cardiac myocytes stimulated with phenylephrine (Chan et al. 2004). Some studies demonstrated the importance of MAPK3/1 signaling in the stimulation of P70S6K (Iijima et al. 2002) and protein synthesis by growth factors (Toku-dome et al. 2004). Thus, metformin could inhibit protein synthesis in bovine granulosa cells through inhibition of MAPK3/1 and P90RSK (Fig. 5).

In conclusion, we showed that activation of AMPK by metformin in bovine granulosa cells leads to a reduction in IGF1-induced proliferation and protein synthesis probably due to an inhibition in the cyclins D2 and E protein expression and phosphorylation levels of P70S6K, MAPK3/1, and P90RSK. Our findings have significant implications for the understanding of the mechanism of action of metformin on ovarian cells. However, further investigations are needed in human ovarian cells to determine whether metformin through AMPK activation is important in the treatment of PCOS and/or cancer.

Materials and Methods

Hormones and reagents

Recombinant human IGF1 and metformin were from Sigma Chemical Co. McCoy 5A modified culture medium, penicillin, streptomycin were purchased from Invitrogen. Thymidine methyl-H and phenylalanine C were purchased from Perkin Elmer Life and Technological Sciences (Boston, MA, USA).
Antibodies

Rabbit polyclonal antibodies to phospho-AMPKζ (Thr172), phospho-MAPK3/1 (Thr202/Tyr204), AKT, phospho-P70S6 kinase (Thr389), phospho-P70S6 kinase (Ser424/Thr421), phospho-S6 (Ser235/236), phospho-P90RSK (Thr359/Ser363), and P90RSK were purchased from New England Biolabs Inc. (Beverly, MA, USA). Rabbit polyclonal antibodies to MAPK1 (C14), cyclin D2 (C17), cyclin E (C19), p21 p27, CDK2, and phospho-AKT (Ser 473) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies to AMPKζα/β were obtained from Upstate Biotechnology Inc. (Lake, Placid, NY, USA). Mouse monoclonal antibody to vinculin was obtained from Sigma. All antibodies were used at 1/1000 dilution in western blotting.

Animals, isolation, and culture of bovine granulosa cells

All procedures were approved by the Agricultural Authority and the Scientific Research Authority, and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Adult cow ovaries were collected at the slaughterhouse, removed aseptically, and transferred to culture medium. Granulosa cells from small follicles (<6 mm) were harvested by puncturing the follicles, allowing expulsion of the cells. Cells were recovered by centrifugation, washed with fresh medium, and counted in a hemocytometer. The culture medium used was McCoy’s 5A supplemented with 20 mmol/l Hepes, penicillin (100 U/ml), streptomycin (100 mg/l), l-glutamine (3 mmol/l), 0.1% BSA, 0.1 μmol/l androstenedione, 5 mg/l transferrin, 20 μg/l selenium, and 10% FBS. The cells were initially cultured for 48 h with no other treatment and then incubated in fresh culture medium with or without test reagents for the appropriate time. Even if the cells collected were granulosa cells, after 48 h of culture, the cells have probably become luteinized granulosa cells. All cultures were performed under a water-saturated atmosphere of 95% air/5% CO₂ at 37 °C.

Thymidine incorporation into granulosa cells

Granulosa cells (2 × 10⁵ viable cells/500 μl) were cultured in 24-well dishes in McCoy’s 5A medium and 10% FBS during 48 h, and were then serum-starved for 24 h followed by the addition of 1 μCi/μl of [³H]-thymidine in the presence or absence of metformin (10 mM) and/or IGF1 (10⁻⁸ M). Cultures were maintained at 37 °C under 5% CO₂ in air. After 24 h of culture, excess of thymidine was removed by washing twice with PBS, fixed with cold trichloroacetic acid (TCA) 50% for 15 min, and lysed by 0.5 mol/l NaOH. The radioactivity was determined by scintillation fluid (Packard Bioscience, Perkin Elmer, Courtaboeuf, France) counting in a β-photomultiplier.

Phenylalanine C14 incorporation into granulosa cells

In order to measure protein synthesis in granulosa cells, l-[U-¹⁴C]phenylalanine (0.25 μCi/ml) was added to the medium during the stimulation period. At the end of incubation (4 h), cells were washed once in ice-cold PBS, and then in ice-cold 10% TCA. TCA-insoluble material was washed thrice with 10% TCA and solubilized in 0.5 M NaOH at 37 °C for determination of protein (BCA, Pierce, Fisher Scientific, Strasbourg, France) and radioactivity incorporated into granulosa cell protein. Protein synthesis was expressed as nmol phenylalanine incorporated per mg protein/h (Dardevet et al. 1996) and then as the percentage of the result obtained from unstimulated cells.

Adenoviruses and infection of bovine granulosa cells

Dominant-negative AMPK adenovirus was constructed from AMPKζα carrying the Asp-157 to Ala (D157A) mutation as previously described (Woods et al. 2000). Recombinant adenovirus was propagated in HEK293 cells, purified by cesium chloride density centrifugation, and stored as previously described (Woods et al. 2000). Bovine granulosa cells were pre-infected with 100 pfu/cell adenovirus in serum-starved McCoy’s 5A during 16 h, and then cultured for 24 h with 100 pfu/cell adenovirus and [³H]-thymidine in the presence or absence of IGF1 (10⁻⁴ M) and/or metformin (10 mM) for cell proliferation measurement or for different time as indicated in the legends of figures. Preliminary studies revealed that within 16 h of infection (100 pfu/cell) with a green fluorescent protein (GFP)-expressing virus, 70% of granulosa cells expressed GFP.

Immunoprecipitation and immunoblot

Total proteins from granulosa cells were extracted on ice in lysis buffer A (10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Igepal) containing various protease inhibitors (2 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin) and phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate (Sigma)). Lysates were centrifuged at 13 000 g for 20 min at 4 °C, and the protein concentration in the supernatants was determined using a colorimetric assay (kit BC Assay, Uptima Interchim, Montluçon, France).

After normalization for protein concentration (500 μg), CDK2 proteins were immunoprecipitated from the supernatants using 5 μg of the appropriate antibody at 4 °C overnight. The immunocomplexes were precipitated with 40 μl protein A-agarose for 1 h at 4 °C. After two sequential washes using buffer A with a 1/2 dilution, the resulting pellets were boiled for 4 min in reducing Laemmli buffer containing 80 mM dithiothreitol. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes.

Cell extracts (50 μg) or immunoprecipitated proteins were subjected to electrophoresis on 12% (w/v) SDS-polyacrylamide gel under reducing conditions. The proteins were then electrotransferred onto nitrocellulose membranes (Schleicher and Schuell, Ecquevilly, France) for 2 h 30 min at 80 V. Membranes were incubated for 1 h at room temperature with Tris-buffered saline (TBS, 2 mM Tris–HCl, pH 8.0, 15 mM NaCl, pH 7.6), containing 5% nonfat dry milk powder (NFDMP) and 0.1% Tween-20 to saturate nonspecific sites. Then, membranes were incubated overnight at 4 °C with
appropriate primary antibodies (final dilution 1:1000), in TBS containing 0.1% Tween-20 and 5% NFDM. After washing in TBS–0.1% Tween-20, the membranes were incubated for 2 h at room temperature with a HRP-conjugated anti-rabbit or anti-mouse IgG (final dilution 1:10 000; Diagnostic Pasteur, Marnes-la-Coquette, France) in TBS–0.1% Tween-20. After washing in TBS–0.1% Tween-20, the signal was detected by ECL (ECL, Amersham Pharmacia Biotech). The films were analyzed and signals quantified with the software Scion Image (4.0.3.2 version; Scion Corporation, Frederick, MD, USA).

Statistical analysis

All experimental data are presented as means ± S.E.M. One-way ANOVA was used to test differences. If ANOVA revealed significant effects, the means were compared by Newman’s test, with P<0.05 considered significant. In the various graphs, different letters indicate significant differences.

Declaration of interest

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

Funding

Lucie Tosca is a PhD student supported by the Région Centre. This work was supported by the GIS-AGENAE Programme, ANR and APIS-GENE.

Acknowledgements

We thank Pascal Ferré and Fabienne Foulle for the AMPKz dominant-negative adenovirus.

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Received 14 August 2009
First decision 22 September 2009
Revised manuscript received 8 November 2009
Accepted 11 November 2009