Expression and effect of resistin on bovine and rat granulosa cell steroidogenesis and proliferation

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Abstract

Resistin, initially identified in adipose tissue and macrophages, was implicated in insulin resistance. Recently, its mRNA was found in hypothalamo–pituitary axis and rat testis, leading us to hypothesize that resistin may be expressed in ovary. In this study, we determined in rats and cows 1) the characterization of resistin in ovary by RT-PCR, immunoblotting, and immunohistochemistry and 2) the effects of recombinant resistin (10, 100, 333, and 667 ng/ml) ± IGF1 (76 ng/ml) on steroidogenesis, proliferation, and signaling pathways of granulosa cells (GC) measured by enzyme immunoassay, [3H]thymidine incorporation, and immunoblotting respectively. We observed that resistin mRNA and protein were present in several bovine and rat ovarian cells. Nevertheless, only bovine GC abundantly expressed resistin mRNA and protein. Resistin treatment decreased basal but not IGF1-induced progesterone (P < 0.05; whatever the dose) and estradiol (P < 0.005; for 10 and 333 ng/ml) production by bovine GC. In rats, resistin (10 ng/ml) increased basal and IGF1-induced progesterone secretion (P < 0.0001), without effect on estradiol release. We found no effect of resistin on rat GC proliferation. Conversely, in cows, resistin increased basal proliferation (P < 0.0001; for 100–667 ng/ml) and decreased IGF1-induced proliferation of GC (P < 0.0001; for 10–333 ng/ml) associated with a decrease in cyclin D2 protein level (P < 0.0001). Finally, resistin stimulated AKT and p38-MAPK phosphorylation in both species, ERK1/2-MAPK phosphorylation in rats and had the opposite effect on the AMPK pathway (P < 0.05). In conclusion, our results show that resistin is expressed in rat and bovine ovaries. Furthermore, it can modulate GC functions in basal state or in response to IGF1 in vitro.

Introduction

Resistin is an adipocyte-derived cytokine that plays an important role in the development of insulin resistance and obesity in rodents (Lazar 2007). This finding was supported by in vitro experiments using cultured cells as well as animal experiments (Steppan et al. 2001a, Moon et al. 2003, Banerjee et al. 2004, Kim et al. 2004, Rangwala et al. 2004, Satoh et al. 2004). Resistin is a cysteine-rich protein of around 12 kDa that belongs to a family of polypeptides named resistin-like molecules (RELMs). These molecules contain three domains: an N-terminal signal sequence, a variable middle portion, and a highly constant C-terminal sequence (Holcomb et al. 2000, Steppan et al. 2001b). Resistin is secreted in circulation mainly as a homodimer (Banerjee & Lazar 2001, Rajala et al. 2002). Several types of cell can express resistin. In mice, adipocytes may be the major source of resistin (Steppan et al. 2001a, 2001b), whereas in humans, resistin mainly come from monocytes and macrophages (Nagaev & Smith 2001, Savage et al. 2001, Patel et al. 2003). Beside its effects on glucose metabolism and insulin sensitivity, resistin regulates a plethora of various functions through its action on multiple cell targets in both rodents and humans. Thus, resistin is able to exert proinflammatory processes in adipose tissue (Nagaev et al. 2006) and vascular endothelium (Li et al. 2007), promote vascular smooth muscle cell proliferation (Calabro et al. 2004), and stimulate in vitro angiogenesis (Di Simone et al. 2006, Mu et al. 2006, Robertson et al. 2009). Despite much research on resistin’s action, the receptor(s) mediating its biological effects has not yet been identified, and little is known on the intracellular signaling pathways activated by this protein.

Recently, some data have suggested that resistin could affect male and female fertility. Indeed, expression of resistin (mRNA and protein) has been reported in several reproductive tissues including hypothalamus (Morash et al. 2002, Tovar et al. 2005, Wilkinson et al. 2005), pituitary (Morash et al. 2002, Brown et al. 2005), and testis (Nogueiras et al. 2004). In hypothalamus, resistin has been shown to inhibit feeding (Tovar et al. 2005, Vazquez et al. 2008). In pituitary, resistin expression is...
regulated in a nutritional-, age-, and gender-specific manner (Morash et al. 2004). Resistin mRNA expression increases to a peak level in pituitary of pre-pubertal mice (Morash et al. 2002, 2004). Furthermore, at this stage, pituitary mRNA resistin levels are strongly stimulated by corticosteroids (Brown et al. 2005). Administration of resistin to dispersed rat anterior pituitary cells increases GH release (Rodríguez-Pacheco et al. 2009). In rat testis, resistin protein is detectable throughout postnatal development, and its mRNA is under the control of several hormones and mediators such as gonadotropins, leptin, and nutritional status (Nogueiras et al. 2004). In this organ, resistin is more precisely present in interstitial Leydig cells and, to a lesser extent, in Sertoli cells within seminiferous tubules (Nogueiras et al. 2004). Furthermore, in rodent testis, resistin is able to induce in vitro testosterone secretion in the basal state and in response to hCG (Nogueiras et al. 2004). In cultured human theca cells, 17α-hydroxylase activity is increased by resistin in the presence of forskolin or forskolin ± insulin, suggesting a role of resistin in stimulation of androgen production by theca cells (Munir et al. 2005). Moreover, some studies have shown elevated concentrations of serum resistin in women with polycystic ovary syndrome (Panidis et al. 2004, Carmina et al. 2005, Munir et al. 2005, Yilmaz et al. 2009), which is known to be associated with hyperinsulinemia, hyperandrogenism, and insulin resistance (Gambarini et al. 2002). In mammals, insulin as well as insulin-like growth factor 1 (IGF1) is well known to play a key role on the development of antral follicles, i.e. steroidogenesis and granulosa cell (GC) proliferation (Silva et al. 2009). We and others reported that adipokines, such as adiponectin and leptin, influence GC function (Zachow & Magoffin 1997, Spicer et al. 2000, Chabrolle et al. 2007a, 2007b, 2009, Maillard et al. 2010). Some studies showed that resistin could interfere with some components of IGF1 receptor signaling, such as insulin receptor substrate 1 (IRS1; Barnes & Miner 2009). All these findings led us to hypothesize that resistin could be expressed in the ovary and could modulate IGF1 effects on ovarian cells.

The objectives of this study were to investigate in two different species, bovine and rat, 1) the mRNA and protein expression of resistin in ovary and 2) the effect of recombinant resistin with or without IGF1 on proliferation, steroidogenesis, and different signaling pathways (AKT, AMPK, and pERK1/2 and p38-MAPK) in GCs in vitro.

**Results**

**Characterization of resistin in bovine and rat ovaries**

In bovine ovary, RT-PCR analysis revealed the amplification of one cDNA corresponding to a fragment of resistin (300 bp) in whole ovary, corpus luteum, small and large follicles, GCs, immature and in vitro matured cumulus cells, and oocytes (Fig. 1A). More precisely, we determined by real-time PCR the resistin mRNA levels in fresh and cultured GCs from small follicles. As shown in Fig. 1A, resistin mRNA expression was about fivefold higher in fresh than in cultured GCs. In rat ovary, RT-PCR analysis also resulted in the amplification of one cDNA, which corresponds to a fragment of resistin (321 bp) in whole ovary and corpus luteum, but not in GCs (Fig. 1C). As shown in Fig. 1C, the expression of rat resistin mRNA is very low compared with those of rat cyclophilin A in fresh GCs. Furthermore, it is undetectable in cultured rat GCs.

As shown in Fig. 1B, resistin protein (as the dimer form, about 23 kDa) was expressed in bovine large and small follicles, whole ovary, corpus luteum, cumulus cells, immature and in vitro matured oocytes, and fresh isolated and cultured GCs. In rat species, immunoblotting of protein extracts revealed the presence of resistin (as the dimer form, about 23 kDa) in whole ovary, corpus luteum and a weak expression in cultured GCs (Fig. 1D).

**Effect of recombinant resistin on basal and IGF1-induced steroid production by primary GCs**

We next investigated whether the supplementation of various doses of recombinant human (rh) resistin or recombinant rat (rr) resistin ± exogenous rh IGF1 (76 ng/ml) affected the steroidogenesis of primary bovine and rat GCs respectively. The progesterone and estradiol secretions were measured by EIA protocols in the culture medium after 48 h of treatment.

We found that rh resistin decreased the basal progesterone and estradiol secretions by primary bovine GCs whatever the dose used (10–667 ng/ml; Fig. 4A and C). The decrease in progesterone secretion was significant for the four doses of rh resistin (10, 100, 333, and 667 ng/ml; about −22%, P<0.05; Fig. 4A), whereas only the treatment with 10 and 333 ng/ml of resistin significantly decreased the estradiol production (about −30%, P<0.005; Fig. 4C). As expected, the progesterone and estradiol secretions by cultured bovine GCs were significantly increased by IGF1 treatment compared with the basal release, by 2.3-fold respectively (P<0.0001 and P<0.005; Fig. 4B and D). In the presence of IGF1, no significant effect of rh resistin treatment was observed on bovine GC steroidogenesis (Fig. 4B and D). In the rat, the resistin supplementation at 10 ng/ml significantly increased the progesterone secretion in the presence or not of IGF1 (about 1.9-fold, P<0.0001 for both; Fig. 5B and A). No significant effect of rr resistin was found on both basal and IGF1-induced estradiol secretion by primary rat GCs (Fig. 5B).
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Effect of recombinant resistin on bovine and rat GC proliferation

We examined whether the treatment with various doses of recombinant resistin affected the DNA synthesis of primary GCs. [3H]thymidine incorporation by bovine or rat GCs was measured after 24 h of culture in serum-free medium in the presence or absence of rh (10, 100, 333, and 667 ng/ml) or rr (10 and 100 ng/ml) resistin respectively ± rh IGF1 (76 ng/ml). Resistin did not modify the basal DNA synthesis of rat GCs (data not shown). Conversely, resistin (100, 333, and 667 ng/ml) significantly increased the basal DNA synthesis of bovine GCs by about 1.3-fold (P<0.0001; Fig. 6A). We observed in the two species that IGF1 significantly increased [3H]thymidine incorporation induced by about 5.8- and 1.4-fold in bovine and rat GCs respectively (P<0.0001; Fig. 6B and data not shown for rat GCs). Whereas no effect of resistin was shown on rat GC proliferation induced by IGF1 (data not shown), resistin at 10, 100, and 333 ng/ml doses significantly decreased [3H]thymidine incorporation induced by IGF1 in bovine GCs (Fig. 6B): this decrease in cell proliferation was more marked for the dose of 10 ng/ml resistin (−21.2%, P<0.0001). At this latter dose of resistin, we observed that the protein level of cyclin D2 induced by IGF1 (76 ng/ml, 3 h) was significantly reduced (−45%, P<0.0001) compared with the stimulation with IGF1 alone on bovine GCs (Fig. 6C).

Various signaling pathways modulated by recombinant resistin treatment in bovine and rat GCs

The pattern of AKT, ERK1/2, p38-MAPK, and AMPKα phosphorylation from 1 to 120 min was analyzed in primary bovine and rat GCs, which were overnight serum starved and then supplemented with rh and rr resistin respectively. In both species, a rapid and transient increase in phosphorylated AKT (Figs 7A and 8A) and phosphorylated p38-MAPK (Figs 7C and 8C) was observed after 1 min of recombinant resistin treatment.
(P<0.05 and P<0.1 respectively for cows, and P<0.005 and P<0.05 respectively for rats). Recombinant resistin significantly and transiently increased phosphorylation of ERK1/2-MAPK in rat GCs after 1 min (P<0.005; Fig. 8B), but not in bovine ones (Fig. 7B). Finally, phosphorylated AMPKα was significantly increased in cultured bovine GCs by resistin treatment from 1 to 120 min (P<0.05; Fig. 7D), whereas a significant and transient decrease in AMPKα phosphorylation was found in primary rat GCs after 1 min of supplementation (P<0.05; Fig. 8D).

Discussion

In this study, we reported for the first time that resistin mRNA and protein were present in various structures of bovine and rat ovaries. Besides, we showed that a 48 h treatment with rh resistin decreased progesterone and estradiol secretions by primary bovine GCs. In the rat, rr resistin induced the progesterone production by GCs, without effect on estradiol release. Whereas resistin treatment did not affect the rat GC proliferation, in the cow it increased the basal proliferation and decreased the IGF1-induced proliferation of GCs associated with a decrease in cyclin D2 protein level. Finally, in both species, recombinant resistin stimulated AKT and p38-MAPK phosphorylation and had the opposite effect on the AMPK pathway in GCs. ERK1/2-MAPK phosphorylation was only affected in rat GCs.

Protein and mRNA expression of resistin have been described in several tissues of the reproductive axis (Morash et al. 2002, Nogueiras et al. 2004, Brown et al. 2005, Tovar et al. 2005, Wilkinson et al. 2005). Furthermore, resistin mRNA is expressed in the bovine mammary gland, and its expression in this tissue decreases during lactation (Komatsu et al. 2003). Our results of RT-PCR, immunoblotting, and immunohistochemistry showed the presence of resistin in rat and bovine whole ovary. In the cow, resistin was widely expressed in small and large follicles, corpus luteum, oocyte and cumulus, theca and GCs. In their communication, Jones et al. (2009) also revealed the mRNA expression of resistin in rat whole ovary. We demonstrated in addition that resistin mRNA was present in rat corpus luteum, but very weakly in fresh GCs (and undetectable in cultured GCs), and that resistin protein was localized in rat oocyte, theca cells, corpus luteum and weakly present in GCs. Previously, we found a similar distribution of adiponectin, another adipokine, in rat (Chabrolle et al. 2007b), chicken (Chabrolle et al. 2007a), and human (Chabrolle et al. 2009) ovarian cells, whereas it was equally expressed in all structures (including fresh and cultured GCs) in bovine ovary (Maillard et al. 2010). If we hypothesize that the specificity of primary antibodies used in our study is the same in each species, our results are in favor of a specific expression of adipokines in the bovine ovary. Thus, our present data underline an in situ production of resistin in ovarian cells and a different expression of this adipokine in rat and bovine GCs. This local production of resistin in bovine cultured GCs and not in rat cultured GCs could contribute to ‘species’ differences in responses including steroidogenesis and proliferation.

Subsequently, we explored the effects of recombinant resistin on steroidogenesis and proliferation of primary bovine and rat GCs, in the presence or absence of IGF1. We tested a physiological dose of resistin (10 ng/ml), referring to published resistin concentrations in human (Munir et al. 2005, Asimakopoulous et al. 2009, Hansen et al. 2010) and rodent (Chabrolle et al. 2008, Shankar et al. 2010) plasma (between around 5 and 20 ng/ml) and higher resistin suppletions (100, 333, or 667 ng/ml), since resistin concentration in bovine plasma is still unknown. Moreover, we chose rr resistin for tests on rat GCs and rh resistin for tests on bovine cells, since the protein sequence of Bos taurus resistin is...
closer to the sequence of Homo sapiens resistin (similarity: around 83%) than that of other species.

In this study, in the cow, the basal but not the IGF1-induced progesterone and estradiol productions by primary GCs from small follicles were significantly decreased by resistin without any dose effect (at the four doses and only at the two doses 10 and 333 ng/ml respectively), while resistin at the physiological dose enhanced progesterone but not estradiol release by cultured rat GCs. As suggested previously, these different responses observed between the bovine and rat cultured GCs could be explained by species differences in resistin protein expression. Leptin, another adipokine, has been shown to exert an inhibitory effect on steroidogenesis in rat (Zachow & Magoffin 1997) and bovine (Spicer et al. 2000) cultured GCs. In this study, resistin did not significantly regulate the estradiol secretion of primary rat GCs in the presence of IGF1, and the progesterone release in this model was not influenced by IGF1 treatment. Further investigations are required to elucidate the role of resistin in the presence of IGF1 on GCs of different species. Other ovarian cells have also been described to respond to a resistin stimulation. Munir et al. (2005) had demonstrated in primary human theca cells that recombinant resistin (notably 10 and 100 ng/ml) increased 17α-hydroxylase activity and CYP17 (steroidogenic enzyme) mRNA expression induced by forskolin alone or in combination with insulin. All these data suggest that physiological levels of resistin could affect ovarian functions.

Resistin has been reported to stimulate proliferation of different cellular types (Calabro et al. 2004, Ort et al. 2005, Mu et al. 2006, Park et al. 2008). Conversely, resistin-13 peptide, a fragment of human resistin possessing some biological activity of the entire hormone, inhibited the proliferation of the breast cancer.

Figure 3 Localization of resistin in rat ovary by immunohistochemistry. Resistin was detected in ovary from immature rat (A), in large follicles in ovary from rat treated with PMSG for 48 h (C, D, and E) and in corpus luteum (CL) in ovary from rat treated with PMSG for 48 h and then with hCG for 24 h (F, G, and H). Resistin was localized more precisely in theca cells (TC), CL and oocyte (O), and weakly in cumulus (Cum) and granulosa (GC) cells. Follicular fluid (FF). Negative controls (B) included a section incubated with rabbit IgG (n = 3).

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MDA-MB-231 cells (Pan et al. 2007). Moreover, other adipokines, such as adiponectin and leptin, were able to modulate basal or hormone-induced proliferation of GCs from different species (Spicer et al. 2000, Sirotkin & Grossmann 2007, Sirotkin et al. 2008, Maillard et al. 2010, Sirotkin & Meszarosova 2010). For these reasons, we evaluated the potential effects of several doses of recombinant resistin ± IGF1 (76 ng/ml) on the proliferation of primary bovine and rat GCs for 24 h. As already shown for adiponectin (Chabrolle et al. 2007b) and leptin (Duggal et al. 2002) on rat GC proliferation, no effect of rr resistin (10 and 100 ng/ml) was observed on basal and IGF1-induced mitosis of rat GCs in our study, suggesting that adipokines including resistin do not play a role in rat GC growth. On the other hand, we found in cows that resistin was able to affect GC proliferation: the basal cell proliferation was stimulated with high levels of resistin (100, 333, and 667 ng/ml) but not with the physiological dose (10 ng/ml), whereas the IGF1-induced proliferation was significantly decreased with 10, 100, and 333 ng/ml resistin. This partial inhibitory effect of resistin was greater for the physiological supplementation. To investigate the molecular mechanism involved in this impaired mitotic response of IGF1-stimulated bovine GCs to resistin, we evaluated the expression of the cyclin D2 in GCs treated with resistin (10 ng/ml) for 24 h and then with IGF1 (76 ng/ml) for 3 h. The cyclin D2 is a well-established marker of mammalian cell proliferation and more particularly one of the crucial factors of the G1/S transition of the cell cycle (Sherr 1993, Sicinski et al. 1996, Sherr & Roberts 1999). We observed a significantly decrease in cyclin D2 protein expression induced by IGF1, suggesting that reduction in IGF1-induced proliferation of bovine GCs by resistin could be due to in part an inhibition of cyclin D2.

Until now, few data were available on the intracellular signaling pathways activated by resistin. This hormone has been shown to modulate phosphorylation of MAPK (ERK1/2, p38, and JNK; Di Simone et al. 2009, Chen et al. 2010), AKT (Palanivel et al. 2006), and AMPK (Satoh et al. 2004) in different cell types. These signaling pathways have been described to play a role in steroidogenesis and/or proliferation of GCs in response to various hormones such as FSH, IGF1, and insulin, as well as more recently to some adipokines including leptin and adiponectin (Moore et al. 2001, Kayampilly & Menon 2004, Tosca et al. 2005, Yu et al. 2005, Ryan et al. 2008, Kayampilly & Menon 2009). Since the majority of resistin effects on rat and bovine steroidogenesis and proliferation were observed from the physiological level (10 ng/ml) in our study, we then examined the effect of this dose on phosphorylation of AKT, ERK1/2, p38-MAPK, and AMPKα in bovine and rat GCs for 1–120 min. We chose these times of stimulation because some studies showed that other adipokines including adiponectin or leptin activate rapidly these signaling pathways in GCs (Chabrolle et al. 2007b, Lin et al. 2009). Resistin supplementation led to different and even opposite effects on ERK1/2-MAPK and AMPK pathways. Indeed, phosphorylation of ERK1/2-MAPK...
was transiently induced in rat GCs after 1 min of resistin treatment, whereas no effect was observed in bovine GCs. The activation of ERK1/2-MAPK pathway may explain in part the increased secretion of progesterone measured in medium of rat GCs after 48 h of resistin treatment, since the use of the specific U0126 inhibitor showed in bovine and rat ovarian cells. More precisely, resistin expression is species-dependent in GCs. Furthermore, in these cells, we observed that recombinant resistin can modulate steroidogenesis and proliferation in basal state or in response to IGF1 in vitro. Thus, resistin could be a metabolic signal involved in the reproductive functions. Further investigations in several species are required to understand the different molecular mechanisms involved in resistin effects and its possible interaction with different hormones (such as FSH, insulin, IGF1, and other adipokines) on steroidogenesis and proliferation of cultured ovarian cells. Particularly, it seems important to measure plasma resistin in different species in order to compare the ovarian local and plasma concentration of this adipokine. However, the role of resistin will not be able to be fully understood until its receptor is identified.

Materials and Methods

Chemicals, hormones, and antibodies

Unless otherwise stated in the text, chemicals were obtained from Sigma–Aldrich.

Rh and rr resistins produced in Escherichia coli were purchased from Biovendor Research and Diagnostic Products (Heidelberg, Germany). Rh IGF1 was obtained from Sigma–Aldrich.

Rabbit polyclonal antibodies to rat resistin and human resistin were purchased from Chemicon International (Millipore, Guyancourt, France) and Biovendor Research and Diagnostic Products respectively. Rabbit polyclonal antibodies to cyclin D2 (C-17), p38α (C-20), ERK2 (C-14), and phospho- AKT1/2/3 (Ser473)-R were from Santa Cruz Biotechnology (Euromedex, Souffelweyersheim, France). Rabbit polyclonal antibodies to phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), phospho-p38 MAP kinase (Thr180/Tyr182), phospho-AMPKα (Thr172), AMPKz, and AKT were obtained from Cell Signaling Technology (Ozyme, Saint Quentin Yvelines, France). Mouse MABs to vinculin (clone hVIN-1) and γ-tubulin (clone GTU-88) were purchased from Sigma–Aldrich. HRP-conjugated anti-rabbit and anti-mouse IgG were purchased from Eurobio (Les Ulis, France).

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Figure 5 Effect of rr resistin on progesterone (A) and estradiol (B) secretions by rat granulosa cells. Progesterone (A) and estradiol (B) amounts were determined by EIA protocol in culture medium of granulosa cells after 48 h of culture in enriched McCoy’s 5A medium (without FBS) with different concentration of rh resistin (10, 100, and 667 ng/ml) ± IGF1 (76 ng/ml). The data are expressed as the amount of steroids (pg/ml) secreted per 48 h per basal amount. The concentration of progesterone in the culture medium at the basal state (no resistin or IGF1 treatment) was 20±6 ng/ml, and the concentration of estradiol was 42.4±4.9 pg/ml. The results, expressed as means ± S.E.M., are representative of three to four independent cultures with each condition in quadruplet. Different letters indicate significant difference (P<0.05).
clusin D2/vinculin was determined in bovine granulosa cells cultured for 24 h in enriched McCoy’s 5A medium (without FBS) with various doses of rh resistin (10, 100, 333, and 667 ng/ml) as described in the Materials and Methods section. The data are expressed as mean±S.E.M., and the measurement unit of [3H]thymidine incorporation is counts per min (c.p.m.). The results are representative of three independent cultures with each condition in triplicate.

Figure 6 Effect of rh resistin on cell proliferation (A and B) and cyclin D2 expression (C) in bovine granulosa cells. (A and B) [3H]thymidine incorporation was determined in bovine granulosa cells cultured for 24 h in enriched McCoy’s 5A medium (without FBS) with various doses of rh resistin (10, 100, 333, and 667 ng/ml)±IGF1 (76 ng/ml) as described in the Materials and Methods section. The data are expressed as mean±S.E.M., and the measurement unit of [3H]thymidine incorporation is counts per min (c.p.m.). The results are representative of three independent cultures with each condition in triplicate. Bars with different superscripts are significantly different (P<0.05).

(C) Serum-starved granulosa cells were pre-incubated or not for 24 h with rh resistin (10 ng/ml) and then stimulated or not with IGF1 (76 ng/ml) for 3 h. Protein extracts were separated by electrophoresis on 12% SDS-polyacrylamide gel. After transfer to nitrocellulose membranes, the proteins were probed with antibodies against anti-cyclin D2. The blots on 12% SDS-polyacrylamide gel. After transfer to nitrocellulose (76 ng/ml) for 3 h. Protein extracts were separated by electrophoresis with rh resistin (10 ng/ml) and then stimulated or not with IGF1 (76 ng/ml) for 24 h in enriched McCoy’s 5A medium (without FBS) with various doses of rh resistin (10, 100, 333, and 667 ng/ml)

**Ethics, animals, and tissues samples**

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

Mature (60-day old) and immature (21-day old) female rats of the Wistar strain were purchased from Charles River (L’Arbresle, France). Protein and mRNA characterization of resistin were performed on ovaries, uterus (+ovisuct), and adipose tissue from mature female rats. For immunohistochemistry analyses, ovaries were collected from immature (21-day old) rats that were treated with 25 IU pregnant mares serum gonadotropin (PMSG) alone for 48 h to induce follicular growth (n=6) or from immature rats that received a single i.p. injection of 25 IU hCG after 48 h of PMSG treatment to induce ovulation and luteinization (n=6).

Bovine ovaries, mammary gland, and abdominal adipose tissue were collected at a local slaughterhouse. Tissue samples for mRNA and protein characterization were frozen in liquid nitrogen and stored at −80 °C.

**Isolation and culture of GCs and cumulus–oocyte complexes**

Bovine ovaries from a slaughterhouse were transferred to saline solution until the dissection. Immature female Wistar rats were injected subcutaneously with diethylstilboestrol (DES, 1 mg/day) for 3 days. On the third day of DES treatment, the animals were killed, and the ovaries were removed aseptically.

GCs were isolated by puncturing follicles (follicles <6 mm for cow), allowing the expulsion of cells in modified McCoy’s 5A medium, supplemented with androstenedione (0.1 µmol/l). The composition of the complete medium was previously described (Maillard et al. 2010). After several steps of centrifugation/washes in fresh medium, recovered cells were incubated in modified McCoy’s 5A medium with amphotericin B (2.7 µg/ml; Eurobio) and 10% fetal bovine serum (FBS, PAA Laboratories, Les Mureaux, France) for 24 or 48 h, followed by an overnight serum starvation. Then, cells were cultured in the presence or absence of test reagents for different time according to the analyzed biological function. Cultures were performed in water-saturated atmosphere containing 5% CO2 in air at 37 °C.

Cumulus–oocyte complexes (COC) were aspirated from 3 to 6 mm antral follicles. COC with a compact and complete cumulus were selected and washed three times in HEPES-buffered tissue culture medium 199 (TCM-199) with gentamicin (50 mg/l). Groups of 50 COC were matured in vitro in 500 µl serum-free TCM-199 supplemented with ascorbic acid (75 µg/ml), l-cysteine (90 µg/ml), epidermal growth factor (EGF) (10 ng/ml), and fibroblast growth factor (FGF) from bovine pituitary (mainly FGF2, 2.2 ng/ml), b-glycerol (720 µg/ml), glutamine (0.1 mg/ml), hCG (5 IU/ml), IGF1 (19 ng/ml), insulin (5 µg/ml), mercaptoethanol (0.1 mM), PMSG (10 IU/ml), pyruvate (110 µg/ml), selenium (5 ng/ml), and transferrin (5 µg/ml) (Donnay et al. 2004). In vitro maturation (IVM) was performed for 24 h at 38.8 °C in humidified atmosphere consisting of 5% CO2 and 95% air. Oocytes and cumulus cells (Cum) were mechanically separated.

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Invitrogen (Fischer Scientific, Strasbourg, France). The PCR designed on bovine or rat resistin (Table 1) and purchased from Promega in a 20 μl final volume of reaction mixture for 1 h at 37 °C. Protein extracts were performed with 2 μM of each specific primer designed on bovine or rat resistin (Table 1) and purchased from Invitrogen (Fischer Scientific, Strasbourg, France). The PCR conditions were 94 °C for 6 min, 58 °C for 1 min, 72 °C for 1 min, and 72 °C for 7 min for 35 cycles. After visualization on 1.5% agarose gel stained with ethidium bromide, all PCR products were sequenced by Genome Express (Meylan, France).

For real-time RT-PCR, 1 μg total RNA of fresh or cultured GCs was reverse transcribed in a final volume of 20 μl using RNase H–MMLV reverse transcriptase (SuperScript II, Invitrogen) and oligo(dT) 15 primers (Promega). cDNA was then diluted to 1:20. A 15 μl master mix containing 10 μl iQ SYBR Green supermix (Bio-Rad), 0.25 μl forward primer (10 μM), 0.25 μl reverse primer (10 μM), and 4.5 μl water was then prepared to perform real-time PCR. Specific sets of primer pairs are shown in Table 1. cDNA dilution (5 μl) was added to the PCR master mix to a final volume of 20 μl. The following PCR protocol was used on MyiQ Cycler system (Bio-Rad): initial denaturation (5 min at 95 °C), followed by a three-step

and collected after COC aspiration (immature stage, germinal vesicle stage for oocyte) or after 24 h of IVM (mature stage). They were then frozen in liquid nitrogen and stored at −80 °C before RNA or protein analyses.

**RNA isolation, RT-PCR, and real-time RT-PCR**

As described previously (Maillard et al. 2010), total DNA-free RNA was extracted from whole ovary, dissected healthy follicles (small follicles < 6 mm ≤ large follicles), corpus luteum, oocytes, cumulus and GCs, and adipose tissue (as positive control), and was stored at −80 °C until RT.

RT and PCR were carried out according to previous protocols (Maillard et al. 2010). Briefly, total RNA (1 μg or from 50 oocytes) was reverse transcribed from oligo(dT) 15 Primer (Promega) in a 20 μl final volume of reaction mixture for 1 h at 37 °C. cDNA was performed with 2 μM of each specific primer designed on bovine or rat resistin (Table 1) and purchased from Invitrogen (Fischer Scientific, Strasbourg, France). The PCR

![Figure 7](image-url) Effect of rh resistin on phosphorylation of AKT (A), ERK1/2 (B) and p38 (C) MAPK, and AMPKα (D) in bovine granulosa cells. After overnight serum starvation, granulosa cells from small follicles were incubated in serum-free medium with rh resistin (10 ng/ml) from 1 to 120 min. Protein extracts were separated by electrophoresis on 12% (w:v) SDS-polyacrylamide gel. After transfer to nitrocellulose membranes, the proteins were probed with anti-phospho-AKT1/2/3 (A) or anti-phospho-ERK1/2 (B) or anti-phospho-p38 (C) or anti-phospho-AMPKα (D). The blots were stripped and reprobed with antibodies against AKT, ERK2, p38, or AMPKα respectively. The immunoblots shown are representative of three independent experiments. Bands on the blots were quantified, and the phosphorylated/total protein ratio was calculated. The results are reported as means ± S.E.M. Different letters indicate significant difference (P < 0.05).

![Figure 8](image-url) Effect of rr resistin on phosphorylation of AKT (A), ERK1/2 (B) and p38 (C) MAPK, and AMPKα (D) in rat granulosa cells. After overnight serum starvation, granulosa cells were stimulated with rr resistin (10 ng/ml) in serum-free medium from 1 to 120 min. Protein extracts were separated by electrophoresis on 12% (w:v) SDS-polyacrylamide gel. After transfer to nitrocellulose membranes, the proteins were probed with anti-phospho-AKT1/2/3 (A) or anti-phospho-ERK1/2 (B) or anti-phospho-p38 (C) or anti-phospho-AMPKα (D). The blots were stripped and reprobed with antibodies against AKT, ERK2, p38, or AMPKα respectively. Bands on the blots were quantified, and the phosphorylated/total protein ratio was calculated. Values represent means ± S.E.M. from at least three independent experiments. Bars with different superscripts are significantly different (P < 0.05).
amplification program (30 s at 95 °C, followed by 30 s at 60 °C and 30 s at 72 °C) repeated 40 times. At the end of the PCR, dissociation was performed by slowly heating the samples from 60 to 95 °C and continuous recording of the decrease in SYBR Green fluorescence resulting from the dissociation of double-stranded DNA. The threshold cycle (C\textsubscript{t}) defined as the cycle at which an increase in fluorescence above a defined baseline can be first detected, was determined for each sample. Bovine and rat resistin mRNA levels were estimated on the basis of PCR efficiency and C\textsubscript{t} deviation of an unknown sample versus a control according to the equation proposed by Pfaffl (2001): 
\[ \Delta C_t \text{target} = \frac{C_{target} - C_{reference}}{C_{reference}} \]

Cyclophilin A was chosen as the reference gene. The results were expressed as the bovine or the rat resistin mRNA/cyclophilin A mRNA ratio. Each PCR run included a no template control and replicates of control and unknown samples. Runs were performed in duplicates.

**Table 1 Oligonucleotide primer sequences for RT-PCR amplification.**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Accession number</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine resistin</td>
<td>5'-TGT GCC CCA TAG ATA AAG CC-3'</td>
<td>NM_183362</td>
<td>300</td>
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<tr>
<td>Sense</td>
<td>5'-CAG GCC TGC AGC AGT CTT AG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-CTT TTG CCT TTG CTG CC-3'</td>
<td>NM_144741</td>
<td>321</td>
</tr>
<tr>
<td>Rat resistin</td>
<td>5'-AAG CAA CCC GCA GGG TAC AG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5'-GCA TACA GGT CCT GGC ATC T-3'</td>
<td>NM_178320</td>
<td>217</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-TGT CCA CAG TCA GCA ATG GT-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Protein isolation and immunoblotting**

Protein extraction and separation and immunoblotting were performed as reported previously (Maillard et al. 2010). Analyses were conducted on total protein extracts from whole ovary, dissected healthy follicles (small follicles <6 mm ≤ large follicles), corpus luteum, fresh isolated and cultured GCs, cumulus cells, oocytes and mammary gland, uterus (+-oviduct), and adipose tissue (as positive controls).

Protein lysates were subjected to electrophoresis on 12 or 15% (w:v) SDS-polyacrylamide gel and electrotransferred. The membranes were then incubated overnight at 4°C with appropriate primary antibodies at a 1/10000 final dilution. The blots were washed with Tris-buffered saline containing 0.1% Tween 20 several times and were further incubated for 2 h at room temperature with a HRP-conjugated anti-rabbit or anti-mouse or anti-goat IgG (dilution 1/5000). The signal of specific bands, detected by ECL (Western Lightning Plus-ECL, Perkin Elmer, Life and Analytical Sciences, Courtaboeuf, France), was quantified with the software Scion Image for Windows (Scion Corporation, Frederick, MA, USA).

**Immunohistochemistry**

After fixation and dehydration, rat and cow ovaries were embedded in paraffin and serially sectioned (7 μm thickness). Immunohistochemistry was performed as described previously (Maillard et al. 2010). Sections were incubated overnight at 4 °C with rabbit antibody raised against either human resistin (dilution 1/200) for bovine ovaries or rat resistin (dilution 1/200) for rat ovaries or with rabbit IgG (dilution 1/200), as negative control) in PBS with 5% lamb serum. After several washes in PBS, sections were incubated for 30 min at room temperature with a ready-to-use labeled polymer HRP anti-rabbit antibody (Kit DakoCytomation EnVision Plus System-HRP; Dako, Trappes, France). Immunoreactivity was revealed by incubation in DAB at room temperature (Kit DakoCytomation EnVision Plus System-HRP). The slides were counterstained with hematoxylin and observed using an Axioplan Zeiss transmission microscope.

**Progestosterone and estradiol measurements**

The steroid concentration was determined in serum-free medium from bovine and rat GCs after 48 h of culture in the presence or absence of several doses of resistin (10, 100, 333, or 667 ng/ml) ± IF1 (76 ng/ml). Initially, GCs were grown in 48-well dishes (1.25×10\(^5\) viable cells/250 μl medium/well) in modified McCoy’s 5A medium with androstenedione (0.1 μmol/l), amphotericin B (2.7 μmol/l), and 10% FBS for 24 h. After an overnight serum starvation, GCs were incubated with the different treatments for 48 h. The concentration of progesterone in the culture medium from bovine and rat GCs was measured by an EIA protocol as described previously (Canepa et al. 2008). For a range of progesterone concentrations between 0.4 and 10 ng/ml, the intra-assay coefficients of variation (CV) were in the majority inferior to 10%. The concentration of estradiol was performed by using the Estradiol EIA kit from Cayman Chemical (Intechim, Montluçon, France) according to the manufacturer’s procedure. The intra-assay CV ranged from 22% to about 12% for estradiol concentrations between 16.4 and 256 pg/ml. The results are expressed as the amount of steroids (pg/ml) secreted per 48 h per basal amount. They are representative of three to four independent cultures with each condition in quadruplicate.

**Measurement of GC proliferation and cyclin D2 expression in GCs**

Cell proliferation was assessed by the measurement of \(^{3}H\)thyimidine incorporation after 24 h of culture. GCs (2×10\(^5\) viable cells/400 μl medium/well) were cultured into 24-well dishes in modified McCoy’s 5A medium supplemented with amphotericin B (2.7 μmol/l) and 10% FBS for 48 h. Cells were then serum starved overnight followed by the addition of
0.25 μCi [³H]thymidine (Perkin Elmer; Life and Analytical Sciences) in the presence or absence of resistin (10, 100, 333, or 667 ng/ml) ±IGF1 (76 ng/ml). 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 50% trichloroacetic acid for 15 min, and lysed by 0.5 mol/l NaOH. The radioactivity was determined in Ultima Gold MV scintillation fluid (Perkin Elmer) by counting in a β-photomultiplier. The values, expressed as counts per min (c.p.m.), are representative of three independent cultures with each condition in triplicate.

For the determination of the protein levels of cyclin D2, bovine GCs were starved overnight. Cells were then pre-incubated in new serum-free medium without or with rh resistin (10 ng/ml) for 24 h, and then IGF1 (76 ng/ml) was added or not for 3 h in the serum-free medium. Cells were then lysed, and immunoblotted were performed.

**Statistical analysis**

All experimental results are presented as means ± S.E.M. Statistical analyses were carried out using an one-way (for data on signaling pathways and cyclin D2 expression) or a two-way (for results on steroidogenesis and cell proliferation) factorial ANOVA test followed by Fisher’s PLSD test, when the ANOVA revealed significant effects. In the various graphs, bars with different superscripts were considered statistically significant at P<0.05.

**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**

This study was supported by the GIS-AGENAE Programme, ANR, and APIS-GENE. V Maillard is a recipient of a grant from INRA.

**Acknowledgements**

We thank Thierry Delpuech and Pascal Papillier for the collection of bovine ovaries; Claude Cahier and his staff for the rat care; and Gilles Gomot, Jean-Philippe Dubois, and Albert Arnoud for the collection of bovine tissue samples.

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Received 7 October 2010
First decision 8 November 2010
Revised manuscript received 26 December 2010
Accepted 14 January 2011