Resistin is a survival factor for porcine ovarian follicular cells

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Abstract

Previously, we demonstrated the expression of resistin in the porcine ovary, the regulation of its expression and its direct effect on ovarian steroidogenesis. The objective of this study was to examine the effect of resistin on cell proliferation and apoptosis in a co-culture model of porcine granulosa and theca cells. First, we analysed the effect of resistin at 1 and 10 ng/ml alone or in combination with FSH- and IGF1 on ovarian cell proliferation with an alamarBlue assay and protein expression of cyclins A and B using western blot. Next, the mRNA and protein expression of selected pro-apoptotic and pro-survival regulators of cell apoptosis, caspase-9, -8 and -3 activity and DNA fragmentation using real time PCR, western blot, fluorescent assay and an ELISA kit, respectively, were analysed after resistin treatment. Furthermore, we determined the effect of resistin on the protein expression of ERK1/2, Stat and Akt kinase. Using specific inhibitors of these kinases, we also checked caspase-3 activity and protein expression. We found that resistin, at both doses, has no effect on cell proliferation. The results showed that resistin decreased pro-apoptotic genes, which was confirmed on protein expression of selected factors. We demonstrate an inhibitory effect of resistin on caspase activity and DNA fragmentation. Finally, resistin stimulated phosphorylation of the ERK1/2, Stat and Akt and kinases inhibitors reversed resistin action on caspase-3 activity and protein expression to control. All of these results showed that resistin has an inhibitory effect on porcine ovarian cell apoptosis by activation of the MAPK/ERK, JAK/Stat and Akt/PI3 kinase signalling pathways.

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Introduction

Resistin is a cysteine-rich polypeptide and signalling molecule that is induced during adipogenesis and is secreted by mature adipocytes (Steppan *et al.* 2001). Levels of resistin increased in diet-induced obesity and genetic models of obesity and insulin resistance (Steppan *et al.* 2001), and therefore it was described as a factor involved in the pathogenesis of insulin resistance, adipogenesis, diabetes and inflammation. Also, resistin promotes proliferation and migration in hepatic stellate cells while inhibiting their apoptosis via an interleukin 6 (IL6) and monocyte chemotactic protein-1 (MCP-1) mechanism (Dong *et al.* 2013). Gao *et al.* (2007) demonstrated that resistin could dramatically decrease apoptosis, thus protecting the heart against ischemia Reperfusion (I/R) injury, using a mouse heart perfusion model. The resistin receptor has not yet been identified, however, in 3T3-L1 preadipocytes resistin modulates adipogenesis and glucose uptake through the tyrosine kinase-like orphan receptor (ROR1) (Sánchez-Solana *et al.* 2012). Further, Daquinag *et al.* (2011) concluded that a product of decorin lacking the glycation site, termed Δ DCN, serves as a functional receptor of resistin

in adipocyte progenitors. In the hypothalamus, resistin can signal through Toll-like receptor-4 (TLR4) (Benomar *et al.* 2013). Similarly, Patel *et al.* (2003) suggested that the resistin-induced inflammatory action of macrophages may occur through peroxisome proliferator-activated receptors type gamma (PPAR γ).

It is a well known fact that resistin, like other adipokines such as leptin (Gregoraszczuk *et al.* 2007, Gregoraszczuk & Rak-Mardyla 2013, Vázquez *et al.* 2015), adiponectin (Chabrolle *et al.* 2007, Palin *et al.* 2012), chemerin (Reverchon *et al.* 2012) or visfatin (Reverchon *et al.* 2013a) can affect reproduction function. The first indication that resistin was involved in the regulation of the female reproductive system may be the discovery that resistin was expressed in rat ovaries throughout the oestrous cycle and was elevated in animals with induced ovarian cysts (Jones *et al.* 2009). Subsequent study demonstrated resistin expression in ovarian cells of bovine (Maillard *et al.* 2011), pigs (Rak-Mardyla *et al.* 2013, 2014), humans (Niles *et al.* 2012, Reverchon *et al.* 2013b) and the vesperilionid bat (Singh *et al.* 2015). We demonstrated that both gonadotropins and steroid hormones significantly stimulated, while insulin growth factor type 1 (IGF1)

and a synthetic agonist of PPAR γ reduced resistin expression and secretion by cultured porcine ovarian follicles, indicating that ovarian resistin expression can be regulated by several factor (Rak-Mardyła & Drwal 2015, Rak *et al.* 2015). Moreover, resistin ovarian expression is a species difference, with both granulosa and thecal cell expression in the cow, but only thecal cell expression in the rat (Maillard *et al.* 2011). These species differences likely account for the differences in steroidogenesis observed. Data in the study of Maillard *et al.* (2011) showed that resistin increased progesterone (P₄) secretion with no effects on estradiol (E₂) secretion in cultured rat granulosa cells, while in cultured cow granulosa cells resistin significantly decreased both steroids' secretion. Our previous study showed that resistin stimulated steroid secretion via 3 beta-hydroxysteroid dehydrogenase (3 β HSD), cytochrome P450 17 alpha-hydroxylase (CYP17A1) and 17 β HSD expression in porcine ovarian follicles (Rak-Mardyła *et al.* 2013, 2014), as in the theca cells of women when the stimulatory action of resistin on androgen production was examined (Munir *et al.* 2005). However, resistin significantly reduced P₄ and E₂ secretion in response to IGF1 in human granulosa cells (Reverchon *et al.* 2013b) and porcine ovarian follicles (Rak *et al.* 2015). In the most recent study, Singh *et al.* (2015) demonstrated also that in vesperilionid bats (*Scotophilus heathi*), resistin alone significantly stimulated P₄ synthesis, but when acting together with the luteinizing hormone (LH), it also stimulated androgen secretion. A previous study demonstrated that PPAR γ activation may represent one mechanism of endocrine action of resistin in porcine ovarian follicles (Rak-Mardyła & Drwal 2015).

Apoptosis is a naturally occurring process in ovarian cells and has been reported to be involved in oogenesis, folliculogenesis, oocyte loss/selection and atresia. A balance of cell proliferation and apoptosis is maintained in healthy individuals and any imbalance of the two processes could lead to pathology. Two general mechanisms operate in apoptosis: one mechanism is triggered by binding of death molecules to cell surface receptors (death receptor-mediated events), while the other is generated by signals arising within the cell (mitochondria-mediated events) (Hussein 2005). The ovarian dynamics are orchestrated by a plethora of molecular mechanisms. The latter are mediated by several pro-apoptotic (Bax, Fas, caspases and p53) and pro-survival molecules (Bcl-2 and TRAIL) (Hussein 2005). Some of these molecules are involved in the process of ovarian follicle atresia such as Bcl-2 family members and caspases, follicle selection such as Bcl-2, Bax, Fas ligand and caspases or luteolysis such as Fas/Fas ligand, caspase-3 and Bax (Hussein 2005). The study of the molecular mechanisms that regulate ovarian cell death is important for understanding normal development and a variety of diseases of the ovary. It is a well-known fact that other adipokines, such as leptin, inhibit

apoptosis in cultured chicken ovarian cells (Sirotkin & Grossmann 2007), immature rats (Almog *et al.* 2001) and porcine prepubertal ovarian cells (Gregoraszczyk *et al.* 2006). However, the effect of resistin on ovarian cell apoptosis is still unknown.

In the present study, we used a co-culture model of porcine ovarian granulosa and theca cells to determine the effect of resistin on cell proliferation and apoptosis measurements by expression of selected apoptosis genes and protein, caspases-9, -8 and -3 activity and DNA fragmentation. We also examined the effect of resistin in combination with follicle stimulating hormone (FSH) and IGF1 on cell proliferation and caspase-3 activity. Gonadotropin and local factors, including IGF1, play an important role in the regulation of ovarian cell survival (Hsu & Hsueh 1997). The ERK1/2, Stat and kinase B (Akt) signalling pathways are widely expressed in ovarian cells and involved in cell survival and apoptosis in the ovary (Westfall *et al.* 2000, Peter & Dhanasekaran 2003, Rak-Mardyła & Gregoraszczyk 2010), so as a molecular mechanism of resistin action on apoptosis, we analysed the activation of the these kinases pathways and their involvement in resistin's action on cell apoptosis.

Material and methods

Reagents and antibodies

M199 medium, foetal bovine serum (FBS, heat inactivated), PBS, and a penicillin/streptomycin solution (penicillin 10 000 units/ml, streptomycin 10 mg/ml) were purchased from GmbH (Bienenweg, Germany). Ac-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), Ac-Ile-Glu-Thr-Asp-7-amido-4-methylcoumarin (Ac-IETD-AMC), Ac-Leu-Glu-His-Asp-7-amido-4-trifluoromethylcoumarin (Ac-LEHD-AFC), HEPES, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), EDTA, glycerol, Tris, Na-deoxycholate, Nonidet NP-40, SDS, protease inhibitors (EDTA-free), DTT, Tween 20, bromophenol blue, 1 bromo-3-chloro-propane, trypsin, staurosporine, PD098059, AG490, FSH from porcine pituitary (cat. #F2293), human recombinant IGF1 (cat. #I3769), rat resistin (cat. #SRP4561) and western blotting luminol reagent (cat. #sc-2048) were obtained from Sigma-Aldrich (St Louis, MO, USA). Rat recombinant resistin was utilised in this experiment because porcine resistin was not readily available at the onset of the experiment. Rat resistin differs from porcine resistin by five amino acids (Adeghate 2004). LY294002 was obtained from Cell Signalling Technology (Danvers, MA, USA). A Bradford protein assay kit was obtained from Bio-Rad Laboratories. PVDF membrane was purchased from Merck Millipore.

Antibodies against cleaved caspase-8 (cat. #9496), caspase-9 (cat. #9502), caspase-3 (cat. #9662), Bax (cat. #2772), Bcl-2 (cat. #2876), cyclin A (cat. #4656), cyclin B (cat. #4138), phospho-p44/42 MAPK (cat. #9101), p44/42 MAPK (cat. #9102), phospho-Stat3 (Tyr705) (cat. #9131), Stat3 (cat. #9132), phospho-Akt (Ser473) (cat. #9271), Akt (cat. #9272) and HRP-conjugated secondary antibody (cat. #7074) were

obtained from Cell Signalling Technology. Anti- β -actin antibody (cat. #A5316) was obtained from Sigma–Aldrich.

Cell culture

Porcine ovaries were collected from mature (7–8 months of age) crossbred gilts (Large White and Polish Landrace) at a local abattoir. Ovaries were collected in a bottle filled with sterilised ice-cold saline with an antibiotic-antimycotic solution and were transported to the laboratory. Approximately 1 h elapsed from slaughter to collection in the laboratory. Medium-size follicles (4–5 mm) were obtained from ovaries of pigs on days 10–12 of the oestrous cycle, as described previously (Gregoraszczyk *et al.* 2000). Since each ovary yielded four to six follicles, the total number of follicles for each preparation varied between 24 and 36. This approach was used to minimise experimental variation throughout the study. Granulosa cells (Gc) and theca interna cells (Tc) from the same follicles were subsequently prepared according to the technique described by Stoklosowa *et al.* (1978) and cultured as a co-culture. The co-culture model of both cell types in ovarian follicles is better than a monoculture of one cell type because all interaction (structural and functional) between granulosa and theca cells are preserved *in vitro*. This *in vitro* model was used in previous studies examining the role of the growth hormone (GH), IGF1 and ghrelin in the porcine ovary (Kolodziejczyk *et al.* 2003, Rak *et al.* 2009, Rak-Mardyla & Gregoraszczyk 2010). Briefly, Gc were scrubbed from the follicular wall with round-tipped ophthalmologic tweezers and rinsed several times with PBS. After isolation, Gc were exposed to DNase I (500 U for 1 min), washed three times in M199, collected and resuspended in M199 supplemented with 10% FBS. The Tc were prepared from the same follicles by placing the theca layers in a drop of saline under the dissecting microscope. Isolated theca interna tissue was then washed with PBS, cleaned, cut with scissors and exposed to 0.25% trypsin in PBS for 10 min at 37 °C. Isolated cells were separated by decantation, and the procedure was repeated three times. Finally, the cells were centrifuged and resuspended in M199/FBS. The viability of the cells was determined before seeding by the Trypan blue exclusion test, and viability was found to be 93% for granulosa cells and 85% for theca cells. For co-culture experiments, granulosa and theca cells were inoculated at concentrations of 6×10^4 and 1.5×10^4 cells/well respectively in 96-well tissue culture plates. Therefore, the ratio of both types of cells was comparable to that observed *in vivo* (Gc:Tc=4:1) (Stoklosowa *et al.* 1982). All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂/95% O₂.

Measurement of cell proliferation and cyclins protein expression

Cell proliferation was measured using the alamarBlue Cell Viability Reagent (Invitrogen) according to the manufacturer's instructions. The co-culture cells were seeded in 96-well culture plates and then incubated in M199 supplemented with 10% FBS. After 24 h, the media were changed to 5% FBS and cells were treated with resistin for 24 h as a control medium or with resistin (1 and 10 ng/ml) alone or in combination with FSH

(100 ng/ml) or IGF1 (30 ng/ml) for 24, 48 and 72 h. Doses of all compounds were chosen based on our previous study (Rak *et al.* 2015). The medium was changed daily, adding new medium and new test compounds. The alamarBlue stock solution was aseptically added to the wells after 24, 48 and 72 h of culture in amounts equal to 10% of the incubation volume. Resazurin reduction was determined after 4 h of incubation by measuring the fluorescence at 560 nm (excitation)/590 nm (emission) using an FLx800 fluorescence microplate reader (BioTek, Winooski, VT, USA). The values, expressed as a relative fluorescence unit, are representative of three independent cultures, with each condition in triplicate.

For the determination of the protein expression of cyclins A and B, ovarian cells were incubated in M199 supplemented with 5% FBS as a control medium or with resistin (1 and 10 ng/ml) for 24 h. The cells were washed with ice-cold PBS and lysed in ice-cold lysis buffer (50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 0.5% sodium deoxycholate, 0.5% NP-40, 0.5% SDS and protease inhibitors) and the lysates were cleared by centrifugation at 15 000 *g* at 4 °C for 30 min. Protein content was determined by a protein assay using bovine serum albumin as a standard and then western blots were performed.

Western blot

Western blotting and quantification were performed as previously described (Rak-Mardyla *et al.* 2013). After 24 h of culture, the cells were washed with ice-cold PBS and lysed in ice-cold lysis buffer and the lysates were cleared by centrifugation at 15 000 *g* at 4 °C for 30 min. Protein content was determined by a protein assay using bovine serum albumin as a standard and then western blots for cleaved caspases-9, -8 and -3 and Bcl2/Bax were performed. In addition, protein expression of ERK1/2, Stat and Akt kinases was evaluated after 5, 15, 30 and 45 min of cell incubation treatment with resistin at 10 ng/ml dose. Our previously study and others demonstrated that during 5–90 min of porcine ovarian cells incubation basal levels of ERK1/2 phosphorylation was at the same levels (Sriraman *et al.* 2008, Rak-Mardyla & Gregoraszczyk 2010). Proteins (30 μ g from each treatment group) were separated by 10% SDS–PAGE and transferred to PVDF membranes (BioRad Mini-Protein 3 apparatus, Bio-Rad Laboratories). The blots were blocked for 2 h with 5% w/v BSA and 0.1% v/v Tween 20 in 0.02 M Tris buffered saline buffer (TBS). Next, the blots were incubated with primary antibodies diluted 1:1000 at 4 °C overnight. Then, the membranes were incubated with a HRP-conjugated secondary antibody diluted 1:1000. Signals were detected by chemiluminescence using Western Blotting Luminol Reagent and were visualised using a ChemiDoc-It Imaging System (UVP, LLC). All bands visualised by chemiluminescence were quantified using ImageJ analysis software (US National Institutes of Health, Bethesda, MD, USA). The blots were stripped and probed for anti- β -actin.

DNA fragmentation

DNA fragmentation was assessed using the Cellular DNA fragmentation ELISA kit (Roche Molecular Biochemicals), following the manufacturer's instructions. This assay is based

on the quantitative detection of bromodeoxyuridine (BrdU)-labelled DNA fragments. Cells were plated in 96-well culture plates in a M199 medium with 10% FBS. After 24 h, the media were changed to 5% FBS and the cells were treated with resitin at 1 and 10 ng/ml and BrdU labelling solution for 24 h. After the removal of the labelling solution, cells were fixed, denatured and incubated for 90 min with the anti-BrdU antibody conjugate, which was subsequently removed by rinsing three times. Finally, cells were incubated in a substrate solution at room temperature and DNA fragmentation was assessed by colorimetric detection. The absorbance was measured at 405 nm using a microplate reader (BioTek Instruments). The values, expressed as a relative absorbance unit, are representative of three independent cultures with each condition in triplicate. In this experiment, staurosporine (0.2 μ M) was used as a positive control of cell apoptosis.

Real time PCR analysis

Ovarian follicular cells were seeded in 96-well culture plates in M199 medium with 10% FBS. After 24 h of cell culturing, the media were changed to 5% FBS and cells were treated with resitin at 1 and 10 ng/ml for 12 h. Total RNA isolation and cDNA synthesis were performed using the *TaqMan* Gene Expression Cell-to-CT Kit (Applied Biosystems) according to the manufacturer's protocol. The lysis solution contained DNase I to remove genomic DNA during cell lysis. Real time PCR analyses were performed using StepOne Real-Time PCR (Applied Biosystems). *TaqMan* Gene Expression Assays were used to quantify mRNA expression of selected apoptosis genes (Table 1). *TaqMan* Gene Expression Assays for caspase-9 were made to order in Applied Biosystems because porcine was not readily available at the onset of the experiment. Matsui *et al.* (2003) described the cDNA sequence of the corresponding domain of caspase-9. GAPDH was used as an internal control.

Quantitative PCR was performed with 100 ng cDNA, 1 μ l *TaqMan* Gene Expression primers, and 10 μ l *TaqMan* PCR master mix (Applied Biosystems) in a final reaction volume of 20 μ l. After a 2-min incubation at 50 °C, thermal cycling conditions were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C to determine the cycle threshold number (*Ct*) for quantitative measurement. The relative mRNA expression levels of apoptosis genes relative to *GAPDH* were determined using the $2^{-\Delta C_t}$ method (Livak & Schmittgen 2001). Briefly, the cycle threshold (*Ct*; defined as the cycle number at which the fluorescence exceeds the threshold level) was determined for each sample. The *Ct* value of the reference gene, *GAPDH*, was

subtracted from the *Ct* value of the gene of interest (ΔC_t) and the relative expression was presented as $2^{-\Delta C_t}$. These $2^{-\Delta C_t}$ values were used to calculate statistical differences. The values, expressed as a relative arbitrary unit, are representative of three independent cultures, with each condition in triplicate.

Caspase activity

The activities of caspases-8, -9 and -3 were measured according to Nicholson *et al.* (1995) using fluorescent substrates (Ac-IETD-AMC, Ac-LEHD-AFC, Ac-DEVD-AMC respectively). Cells were plated in 96-well culture plates in M199 medium with 10% FBS for 24 h. Next, the media were changed to 5% FBS and the cells were treated with resitin at 1 and 10 ng/ml, alone or in combination with FSH (100 ng/ml) or IGF1 (30 ng/ml) for 24 h. To test the activation of signal transduction pathways, cells were pre-treated for 1 h with the JAK/STAT inhibitor AG490 at 50 μ M, the MAPK inhibitor PD098059 at 50 μ M, and the PI3K inhibitor LY294002 at 10 μ M, and resitin at 1 and 10 ng/ml was added at 24 h. The concentrations of the inhibitors were chosen based on our previously published data (Rak-Mardyla & Gregoraszczuk 2010, Ptak *et al.* 2013) and an unpublished study. After cell culture incubation, the culture media were replaced with a caspases assay buffer containing 50 mM HEPES, 100 mM NaCl, 0.1%, CHAPS, 1 mM EDTA, 10% glycerol and 10 mM, DTT (pH 7.4), and the cells were incubated on ice for 10 min. The cell lysate was then incubated at 37 °C with the appropriate caspases substrate at a final concentration of 10 μ M. The amounts of fluorescent products were monitored every 30 min until 5 h using a fluorescence microplate reader (FLx800 BioTek Instruments) at an excitation wavelength of 360 nm and an emissions wavelength of 460 nm for caspase-3 and caspase-8 and an excitation wavelength of 400 nm and emissions wavelength of 505 nm for caspase-9. The culture medium alone was used as a control for nonspecific binding. Lysate protein levels were measured using the Bradford method with bovine serum albumin as a standard. The values, expressed as folds of control, are representative of three independent cultures, with each condition in triplicate.

Statistical analysis

All experimental results are presented as means $\pm$ S.E.M. A Student's *t*-test was used for statistical comparison of the means between two groups. A one-way ANOVA was used for multiple comparisons involving more than two treatment groups.

Table 1 *TaqMan* Gene Expression Assays were used to quantify the mRNA expression for apoptosis genes.

Gene symbol	Gene name	Catalog number	Reference sequence
<i>FADD</i>	Fas (TNFRSF6)-associated via death domain	Ss03379508_s1	NM_001031797.1
<i>FAS</i>	Fas (TNF receptor superfamily, member 6)	Ss03392400_m1	NM_213839.1
<i>TNFSF10 (TRAIL)</i>	Tumor necrosis factor (ligand) superfamily, member 10	Ss03391932_m1	NM_001024696.1
<i>CASP8</i>	Caspase-8, apoptosis-related cysteine peptidase	Ss03379427_u1	NM_001031779.2
<i>CASP3</i>	Caspase-3, apoptosis-related cysteine peptidase	Ss03382792_u1	NM_214131.1
<i>BCL-2</i>	B-cell CLL/lymphoma 2	Ss03375167_s1	AB271960.1
<i>BAX</i>	BCL2-associated X protein	Ss03375842_u1	AJ606301.1
<i>TP53</i>	Tumor protein p53	Ss04248637_m1	NM_213824.3
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	Ss03375629_u1	NM_001206359.1

Tukey's honest significant difference (HSD) multiple range test was performed *post hoc* (GraphPad PRISM v. 4.0; GraphPad Software, Inc., San Diego, CA, USA). Statistical significance is indicated by different letters ($P < 0.05$) or by * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$).

Results

Effect of resistin on ovarian cell proliferation

We observed that resistin has no effect on cell proliferation during 72 h of ovarian cell culturing, whereas in the control group, during 72 h of culturing, we noted a significant increase in ovarian cell proliferation (Fig. 1A, $P < 0.05$). We also determined the effect of resistin combination with FSH and IGF1 on cell proliferation. FSH and IGF1 significantly increased cell proliferation; however, resistin in combination with FSH and IGF1 had no effect (Fig. 1B). Additionally, we also determined that resistin at 1 and 10 ng/ml had no effect on the protein expression of cell proliferation markers cyclins A and B (Fig. 1C).

Effect of resistin on caspase activity and DNA fragmentation

Resistin at both doses significantly decreased caspase-8 (70.5 and 65.5 vs 100% of control respectively), caspase-9 (60.6 and 41 vs 100% of control) and caspase-3 activity (58.2 and 11.3 vs 100% of control) (Fig. 2) ($P < 0.05$). The statistically significant inhibition of all investigated caspases was observed with FSH (100 ng/ml) and IGF1 (30 ng/ml) alone treatment cells ($P < 0.05$), however no change was shown with resistin cultured with FSH and IGF1. The statistically significant inhibition of DNA fragmentation to 0.12 and 0.12 vs 0.19 of control values was observed with resistin treatments of 1 and 10 ng/ml, respectively (Fig. 2D) ($P < 0.05$). Staurosporine as a positive control of cell apoptosis increased significantly DNA fragmentation in porcine ovarian cells ($P < 0.001$).

Effect of resistin on mRNA expression of selected apoptosis genes

Figure 3A showed that resistin at 1 and 10 ng/ml significantly decreased gene expression of *FADD* (3.5 and 3.6 vs 4.8 in control, respectively), *FAS* (3.2 and 3.3 vs 4.2 in control, respectively), caspase-8 (*CASP8*) (4.6 and 4.5 vs 5.7 in control, respectively) and caspase-3 (*CASP3*) (5 and 5.5 vs 7.1 in control, respectively) ($P < 0.05$, $P < 0.01$). We observed that only at 1 ng/ml, resistin was able to significantly decrease *BAX* (1.8 vs 3 in control), and likewise, this occurred at 10 ng/ml for caspase-9 (*CASP9*) (6.7–7.7 in control). We demonstrated that resistin has no effect on *P53* expression.

Figure 3A showed that resistin significantly increased pro-survival *TRAIL* only at 10 ng/ml (7.5 vs

Figure 1 Effect of resistin on ovarian cell proliferation. The cells were treated with (A) resistin at 1 (R1) and 10 (R10) ng/ml for 24, 48 and 72 h alone or (B) in combination with FSH (100 ng/ml) and IGF1 (30 ng/ml), after which cell proliferation was analyzed using the alamarBlue assay as described in Material and methods section. alamarBlue experiments were independently performed and repeated three times ($n = 3$). (C) Effect of resistin on cyclins A and B protein expression. The cells were treated with resistin at 1 and 10 ng/ml for 24 h and western blot for cyclins were performed. The amount of protein (30 µg) in each sample was confirmed by immunoblotting using an anti-β-actin antibody; the proteins levels of cyclin A (55 kDa) and cyclin B (55 kDa) were densitometrically scanned and normalized against the β-actin (42 kDa) signal. Western blot experiments were independently performed and repeated three times ($n = 3$). The intensities of signals were expressed as arbitrary units. The data are plotted as the mean $\pm$ S.E.M. Different letters indicate significant differences among the groups ($P < 0.05$).

6.2 in control) ($P < 0.05$) and had no effect on *BCL-2* expression.

Effect of resistin on protein expression of selected apoptosis peptides

Based on the results obtained using real time PCR, we analysed caspases-8, -9 and -3, as well as Bax, and Bcl-2 protein expression by western blot in resistin treated cells. We observed that resistin at a dose of 10 ng/ml decreased active (cleaved) caspase-8 (Fig. 4A) and caspase-3 (Fig. 4C), while both doses had an inhibitory effect on cleaved caspase-9 (Fig. 4B) protein expression. Figure 4D demonstrates that resistin at 1 and 10 ng/ml increased Bcl-2 protein expression and decreased Bax protein expression ($P < 0.01$ and $P < 0.001$).

Effect of resistin on protein expression of ERK1/2, Stat3 and Akt kinases

Protein expression of signalling pathways of ERK1/2, Stat and Akt kinase phosphorylation from 5 to 45 min was analysed in porcine ovarian follicles cells after resistin treatment at 10 ng/ml. As shown in Fig. 5, resistin significantly increased phosphorylation of ERK1/2 after 15 min of incubation, Stat and Akt kinase after 5–45 min of cell incubation ($P < 0.05$, $P < 0.01$, $P < 0.001$).

Involvement of signalling pathways in resistin antiapoptotic action on ovarian cells

We examined whether the resistin treatment with specific inhibitors of kinases ERK1/2, Stat and Akt affected caspase-3 activity and protein expression. We demonstrated that all investigated inhibitors, PD098059 at 50 μ M, AG490 at 50 μ M and LY294002 at 10 μ M, added with resistin at 1 and 10 ng/ml reversed to control levels the inhibition of caspase-3 activity and protein expression in cells exposed to resistin. These data suggest that the anti-apoptotic effect of resistin is mediated by modulating the MAPK/ERK1/2, JAK/STAT and PI3K signal transduction pathways in porcine ovarian cells (Fig. 6).

Figure 2 Effect of basal resistin at 1 (R1) and 10 (R10) ng/ml and in combination with FSH (100 ng/ml) and IGF1 (30 ng/ml) on caspase activity after 24 h of ovarian cell culture. (A) Caspase-8 activity were analyzed using fluorescent substrate (Ac-IETD-AMC), (B) caspase-9 activity were analyzed using fluorescent substrate (Ac-LEHD-AFC) and (C) caspase-3 activity were analyzed using fluorescent substrate (Ac-DEVD-AMC) (D) effect of resistin on DNA fragmentation measurement by Cellular DNA fragmentation ELISA kit, based on the quantitative detection of bromodeoxyuridine (BrdU)-labeled DNA fragments. Staurosporin (St) was added as a positive control of cell apoptosis. Caspases activity and DNA fragmentation were independently performed and repeated three times ($n = 3$). The data are plotted as the mean $\pm$ S.E.M. Significance between control and resistin treatments is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 3 Effect of resistin at 1 (R1) and 10 (R10) ng/ml on selected apoptosis genes expression after 12 h of porcine ovarian cell culture. (A) Pro-apoptotic gene expression: *FADD*, *FAS*, *BAX*, *CASP8*, *CASP9*, *CASP3* and *P53* and (B) pro-survival genes: *TRAIL* and *BCL-2*. The mRNA expression level of apoptosis genes were determined by real-time PCR and normalized to the expression of GAPDH. The data are plotted as the mean $\pm$ S.E.M., are representative of three to four independent cultures with each condition in quadruplet. Significance between control and resistin treatments is indicated by * $P < 0.05$ and ** $P < 0.01$.

Discussion

In the present study, we demonstrated that in porcine ovarian cells, resistin, at doses of 1 and 10 ng/ml, has no effect on cell proliferation and protein expression of cyclins. The most important results of this study are mechanisms of resistin action on ovarian cell apoptosis. This complex study showed, for the first time, that resistin significantly decreased cell apoptosis by modulating several genes and the protein expression involved in ovarian apoptosis, caspase activity and DNA fragmentation. Moreover, resistin increased the phosphorylation of kinases ERK1/2, Stat and Akt. Finally, we documented that the MAPK/ERK, JAK/Stat and Akt/PI3 kinase signalling pathways mediated the anti-apoptotic effect of resistin in porcine ovarian follicles.

Effect of resistin on cell proliferation

Using the alamarBlue test, which is based on the detection of cellular metabolic activity, we showed that during ovarian cell culturing from 24 to 72 h, resistin

at 1 and 10 ng/ml doses had no effect on cell proliferation. To confirm these results, we checked the effect of resistin on the protein expression of cyclins A and B. Cyclins A and B are well established markers of porcine ovarian cell proliferation, growth and development (Kolesarova *et al.* 2011, Zhen *et al.* 2014). We observed that resistin has no effect on the protein expression of cell proliferation markers, cyclins A and B. Also, we observed that FSH and IGF1 significantly increased cell proliferation, however resistin cultured with FSH and IGF1 had no effect. Our results are in agreement with previous studies that reported that resistin, at a physiological dose (10 ng/ml), had no effect on cell proliferation and cyclin D protein expression in rat granulosa cells (Maillard *et al.* 2011). However, Maillard *et al.* (2011) also demonstrated that resistin at higher doses (100, 333 and 667 ng/ml) significantly increased cell proliferation and in cultures, induced IGF1 resistin decreased the [3 H]thymidine incorporation in cow granulosa cells. In the presented study, we used resistin at doses ranging to physiological (1–10 ng/ml)

Figure 4 Effect of resistin at 1 (R1) and 10 (R10) ng/ml on selected apoptosis peptides expression after 24 h of porcine ovarian cell culture. The amount of protein (30 μ g) in each sample was confirmed by immunoblotting using (A) cleaved caspase-8 (18 kDa), (B) procaspase-9 and cleaved caspase-9 (17 kDa), (C) procaspase-3 and cleaved caspase-3 (17/19 kDa) and (D) Bcl-2 (28 kDa) and Bax (20 kDa) antibody; (E) the proteins levels were densitometrically scanned and normalized against the β -actin signal. The blots were stripped and reprobed with anti- β -actin (42 kDa) antibody. Western blot experiments were independently performed and repeated three times ($n = 3$). Significance between control and resistin treatments is indicated by ** $P < 0.01$ and *** $P < 0.001$.

does, in accordance with the plasma resistin concentration in humans (5–15 ng/ml) (Lu *et al.* 2005, Munir *et al.* 2005, Asimakopoulos *et al.* 2009). Moreover, our results are in agreement with an observation in human preadipocytes where resistin did not influence cell proliferation (Ort *et al.* 2005). However, different results of resistin action on cell proliferation were observed in other models. In human aortic smooth muscle cells, resistin stimulated proliferation through both ERK1/2 and Akt signalling pathways (Calabro *et al.* 2004). Similarly, in human coronary artery endothelial cells (HCAECs), resistin increased cell proliferation and migration, as well as *in vitro* angiogenesis; this effect was also observed in other types of human endothelial cells, such as human umbilical vein endothelial cells (HUVECs) and human lung microvascular endothelial cells (HMVEC-L) (Mu *et al.* 2006). In contrast, in breast cancer cells resistin inhibited proliferation (Pan *et al.* 2007).

Effect of resistin on cell apoptosis

The results of the presented data demonstrated that resistin significantly decreased mRNA expression of several pro-apoptotic molecules, such as *FADD*, *FAS* and *CASP8*, in addition to increasing pro-survival *TRAIL*. We demonstrated that resistin significantly decreased active (cleaved) caspase-8 protein expression and enzyme activity, suggesting resistin action in the extrinsic (receptor) pathways of apoptosis (Fig. 7). However, the present results indicate a dose-dependent effect of resistin on caspase-8 enzyme activity and expression; resistin at 1 ng/ml have no effect only on protein expression of cleaved caspase-8. Lack of correlation between the protein and mRNA expression and enzyme activity may result from transcriptional, post-transcriptional or translation regulation, protein stability or enzyme activity as well as functioning feedbacks, i.e. high protein concentration may suppress mRNA expression or enzyme activity, and a high level of gene expression may diminish the post-transcriptional processes (Kiezun *et al.* 2013, Rak *et al.* 2015). The death receptor-mediated pathway of apoptosis is activated when certain death receptor ligands of the tumour necrosis factor (TNF) family (such as FAS

Figure 5 Effects of resistin on phosphorylation of (A) ERK1/2, (B) Stat and (C) Akt in porcine ovarian cells. Western blot analysis was performed following treatment with resistin (10 ng/ml) in a time-dependent manner (5, 15, 30 and 45 min). Total ERK1/2 (44/42 kDa), Stat-3 (86 kDa) and Akt (60 kDa) were used to normalize the level of phosphorylated ERK1/2 (44/42 kDa), Stat-3 (79 kDa) and Akt (60 kDa), respectively. The blot is representative of three independent experiments ($n=3$). The intensities of signals were expressed as arbitrary units. The data are plotted as the mean $\pm$ S.E.M. Significance between control and resistin treatments is indicated by * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

Figure 6 Involvement of signaling pathways in resistin antiapoptotic action on porcine ovarian cells. Ovarian cells were pretreated for 1 h with the JAK/STAT inhibitor AG490 (50 μ M), the MAPK inhibitor PD098059 (50 μ M) and the PI3K inhibitor LY294002 (10 μ M), and resistin at 1 (R1) and 10 (R10) ng/ml was added at 24 h. (A) Caspase-3 activity was measurement using fluorescent substrates (Ac-DEVD-AMC) and (B) protein expression of procaspase-3 and cleaved caspase-3 (17/19 kDa) by western blot. The blots were stripped and reprobed with anti- β -actin (42 kDa) antibody. Caspase-3 activity and western blot experiments were independently performed and repeated three times ($n=3$). Significance between control and resistin treatments is indicated by ** $P<0.01$ and *** $P<0.001$.

ligand) engage their cognate death receptors on the plasma membrane, leading to caspase-8 activation via FAS-associated death domain proteins (FADD). The role of some death receptor-mediated pathway molecules in ovarian cells has been well described. For example, Porter (2001) demonstrated higher contents of FasL mRNA expression in granulosa and theca cells from atretic compared to healthy follicles. Next, Hakuno *et al.* (1996) showed the ability of Fas for inducing granulosa cell apoptosis. TRAIL and its receptors are expressed in the ovarian follicles (Jääskeläinen *et al.* 2009) and the expression of the TRAIL-decoy receptor-1 is markedly reduced in the porcine granulosa cells of atretic follicles (Wada *et al.* 2002).

In the mitochondria (intrinsic) apoptotic pathway, the death signals cause pro-apoptotic Bax to allow cytochrome *c* to leak out of the mitochondria. The anti-apoptotic proteins Bcl-2 reside in the outer mitochondrial wall and inhibit cytochrome *c* release. The released cytochrome *c* and apoptotic protease-activating factor 1 (Apaf-1) bind to caspase-9, which then activates the caspase cascades, leading to cell death (Hussein 2005). The analysis of the present results regarding the molecules that regulate mitochondrial functions showed that resistin decreased the mRNA and protein expression of Bax. However, it also significantly decreased Bcl-2 protein expression without effect on *BCL-2* mRNA expression, suggesting differences in post-transcriptional regulation

or protein stability. Previous studies have demonstrated that Bcl-2 and Bax are expressed in the granulosa cells of both foetal and adult ovaries, suggesting their possible role in atresia (Nandedkar & Dharma 2001). Bcl-2 is found mainly in developing follicles while Bax is seen mainly in atretic follicles (Van Nassauw *et al.* 1999). Moreover, several previous studies described the important role of Bcl-2 in ovarian apoptosis (Hussein 2005), including decreased numbers of follicles in Bcl-2-deficient mice (Ratts *et al.* 1995). Excessive expression of Bcl-2 leads to decreased follicular apoptosis and atresia (Hsu *et al.* 1996, Morita & Tilly 1999). Bax deficient mice have also been demonstrated to have abnormal follicles with an excessive number of granulosa cells (Perez *et al.* 1999). Additionally, Bax expression is high in atretic follicles compared to healthy ones (Kugu *et al.* 1998). Additionally, in our study we documented that resistin inhibited activity, gene and protein expression of active (cleaved) caspase-9, also acting on the mitochondria apoptotic pathway.

The two pathways converge at the activation of the effector caspases (caspases-3, -7 and -6). Caspases are the main effector molecules in ovarian apoptosis. They are activated in two ways in the granulosa cells: cell surface receptors and members of the Bcl-2 family of proteins (Hussein 2005). In the ovary, caspase-3 is expressed in luteal and theca cells of the healthy corpus luteum, as well as in the granulosa cells of atretic follicles (Hussein 2005). In our study, we observed that resistin in low dose significantly decreased caspases 3 and 8 activity, as well as mRNA expression, while in high doses additionally protein expression. We demonstrated statistically significant inhibition of caspase-3 in cultures with FSH and IGF1 treatment cells, however no change was shown on resistin with FSH and IGF1, suggesting no cooperation between resistin and FSH and IGF1 in ovarian apoptosis.

Several studies demonstrated that p53 is important in ovarian apoptosis (Hussein 2005). Expression of the p53 protein in apoptotic granulosa cells of atretic follicles suggests its possible role in atresia (Kim *et al.* 1999). Inhibition of p53 expression is associated with a marked reduction in the number of apoptotic granulosa cells and atretic follicles (Tilly *et al.* 1995); however, our results demonstrated that resistin has no effect on *P53* mRNA expression.

Using the DNA fragmentation assay, which is based on the detection of BrdU-labelled DNA fragments, we showed that at both doses, resistin significantly decreased DNA fragmentation in ovarian cells. This confirmed resistin's anti-apoptotic effect in porcine ovaries. The obtained results showed a potential new mechanism of resistin action on the ovary, suggested that resistin can control ovarian folliculogenesis, oocyte loss/selection or atresia. Earlier studies have shown that in the ovary of seasonally monoestrous vespertilionid bat, *Scotophilus heathi*, resistin decreased significantly

Figure 7 Schematic diagram illustrating the effect of resistin (red arrow) on porcine ovarian cell apoptosis. Resistin has an inhibitory effect on porcine ovarian cell apoptosis by activation of the MAPK/ERK, JAK/Stat and Akt/PI3 kinase signalling pathways (blue arrow). ↑, stimulatory effect; ↓, inhibitory effect; (–), no effect.

the protein expression of caspase-3, but also had stimulatory effect on active caspase-3 and proliferating cell nuclear antigen (PCNA) protein expression (Singh *et al.* 2014). This difference is probably due to the animal model used. Other adipokines, such as adiponectin and leptin, were earlier shown to inhibit ovarian apoptosis in different species, including pigs (Gregoraszczyk *et al.* 2006, Sirotkin *et al.* 2012), chickens (Sirotkin & Grossmann 2007), humans (Sirotkin *et al.* 2008), rats (Almog *et al.* 2001), mice (Richards *et al.* 2012) and bats (Srivastava & Krishna 2011).

Mechanism of resistin anti-apoptotic action

Results of the presented data demonstrated that resistin at 10 ng/ml significantly increased protein expression of phosphorylated ERK1/2, Stat and Akt kinase in ovarian cells. Activation of these kinases are involved in cell survival and apoptosis in granulosa cells (Westfall *et al.* 2000, Peter & Dhanasekaran 2003, Rak-Mardyla &

Gregoraszczyk 2010). Besides the important mitogenic activity of ERK1/2 in the nucleus, ERK1/2 is localised in the mitochondria and plays a role in cell survival/apoptosis (Poderoso *et al.* 2008). JAK/STAT signalling is essential for numerous developmental and homeostatic processes, including cell apoptosis (Rawlings *et al.* 2004). In addition, phosphorylation of the Akt family of serine/threonine-directed kinases is important in cellular survival and apoptosis. Our results are in good agreement with previous studies. Published studies have indicated that resistin stimulated Akt and p38-MAPK phosphorylation in bovine and rat granulosa cells and ERK1/2 phosphorylation in rats (Maillard *et al.* 2011). Additionally, Reverchon *et al.* (2013b) showed that in human granulosa cells, resistin rapidly activated the Akt and MAPK (ERK1/2 and p38) signalling pathways. In the last published study, Singh *et al.* (2014) documented that resistin significantly increased protein expression of phosphorylated Stat-3 kinase in the ovaries of the vesperilionid bat. In the next series of

experiments, we analysed whether the resistin treatment, with specific inhibitors of kinases ERK1/2, Stat and Akt, affected apoptosis. We demonstrated that all investigated inhibitors (PD098059, AG490 and LY294002) added with resistin reversed to control levels the inhibition of caspase-3 activity and protein expression in cells exposed to resistin. We propose that the molecular mechanism of resistin anti-apoptotic action in ovarian cells is mediated by modulating the MAPK/ERK1/2, JAK/STAT and PI3K signal transduction pathway.

In summary, the results of the presented data provide new evidence for the molecular mechanism of resistin's action on ovarian cell apoptosis. We found that resistin, at physiological doses, has no effect on cell proliferation. However, this complex study showed that resistin significantly decreased cell apoptosis by modulating the expression of several genes and proteins that are involved in death receptor- and mitochondria-mediated ovarian apoptosis, caspase activity and DNA fragmentation. Moreover, in ovarian cells, resistin increased ERK1/2, Stat and Akt phosphorylation, suggesting that its anti-apoptotic effect is mediated by MAPK/ERK, JAK/Stat and Akt/PI3 kinase signalling pathways in porcine ovarian follicles.

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.

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