Visfatin is expressed in human granulosa cells: regulation by metformin through AMPK/SIRT1 pathways and its role in steroidogenesis

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ABSTRACT: Visfatin is a cytokine hormone and an enzyme involved in metabolic (obesity, type II diabetes) and immune disorders. Some data suggest a role of visfatin in ovarian function. Here, we identified visfatin in human follicles and investigated the molecular mechanisms involved in the regulation of its expression in response to insulin sensitizers, metformin (MetF) and rosiglitazone, in primary human granulosa cells (hGCs) and in a human ovarian granulosa-like tumour cell line (KGN). We also studied the effects of human recombinant visfatin (RhVisf) on steroid production and on the activation of various signalling pathways. By RT–PCR, immunoblotting and immunohistochemistry, we showed that visfatin is expressed not only in hGCs and KGN cells, but also in human cumulus cells and oocytes. In hGCs and KGN cells, MetF increased visfatin mRNA in a dose-dependent manner (0.1, 1 and 10 mM), and rosiglitazone increased visfatin mRNA expression (only at 10 μM) after treatments for 24 h, whereas both reduced it after 48 h of incubation. This regulation was confirmed at the protein level for the MetF treatment only. Using the compound C and Aicar, inhibitor and activator of AMP-activated protein kinase (AMPK), respectively, and Sirtinol, an inhibitor of sirtuin-1 (SIRT1), we observed that these MetF effects on visfatin expression were mediated through the AMPK/SIRT1 signalling pathways. RhVisf (10 ng/ml) significantly increased insulin-like growth factor-1 (IGF-1) (10 nM)- but not FSH (10 nM)-induced secretion of progesterone and estradiol as determined by radioimmunoassay and IGF-1-induced thymidine incorporation in hGCs and KGN cells. Finally, rhVisf rapidly activates the mitogen-activated protein kinase pathway via ERK1/2, P38 and Akt phosphorylation under basal conditions in primary hGC cells. In conclusion, visfatin is present in ovarian human follicles, and in hGCs and KGN cells, visfatin increases IGF-1-induced steroidogenesis and cell proliferation and MetF regulates visfatin expression through the AMPK/SIRT1 signalling pathway.

Key words: adipokine / ovary / insulin-like growth factors / signalling pathways

Introduction

Visfatin, also known as nicotinamide phosphoribosyltranferase (NAMPT) and pre-B cell colony-enhancing factor (PBEF), is a cytokine hormone and an enzyme involved in metabolic (obesity, type II diabetes) and immune disorders (Rongvaux et al., 2002; Revollo et al., 2004; Luk et al., 2008). It is the most recently identified adipokine that regulates numerous processes, including glucose homeostasis, lipid metabolism, inflammation and angiogenesis (Stofkova, 2010). Visfatin is also the rate-limiting enzyme in the NAD biosynthesis pathway from nicotinamide. It exists in two molecular forms, and both the extracellular (cytokine like) and intracellular (enzymatic) forms seem to be responsible for its biological effects. Some evidence has shown that visfatin-mediated NAD biosynthesis regulates the activity of sirtuin 1 (SIRT1), a NAD-dependent deacetylase involved in the pathogenesis of age-associated complications including type 2 diabetes (Revollo et al., 2004). Visfatin expression is regulated by anti-diabetic thiazolidinediones such as rosiglitazone in rat-isolated adipocytes (Kloting and Kloting, 2005), and visfatin secretion is improved by insulin and glucose in cultured human subcutaneous adipocytes (Haider et al., 2006). Furthermore, Xie et al. have suggested that visfatin can activate insulin receptor and downstream signalling pathways (Xie et al., 2007).
Some data suggest that visfatin, like other adipokines (Tersigni et al., 2011), could regulate reproductive functions. In the male, visfatin is expressed in chicken testis and particularly in Sertoli and Leydig cells and in germinal cells (Ocon-Grove et al., 2010). Furthermore, in this species, sexual maturation is associated with changes in testicular visfatin expression. In the female, a recent study showed that administration of visfatin during ovulation induction improves developmental competency of oocytes and fertility potential in old female mice (Choi et al., 2012), suggesting a role of this adipokine in ovarian function. Visfatin is expressed by human fetal membranes during pregnancy and is present throughout gestation in the amniotic epithelium and mesenchymal cells (Ognjanovic and Bryant-Greenwood, 2002). It may play an important role in placentation for normal pregnancy (Fashhauer et al., 2008; Mazaki-Tovi et al., 2010; Zulfikaroğlu et al., 2010). In humans, Shen et al. reported a positive correlation between follicular fluid visfatin concentrations of women undergoing controlled ovarian stimulation and the number of oocytes retrieved (Shen et al., 2010).

It has recently been shown that visfatin is expressed in human granulosa cells (hGCs), and its expression in vitro is increased by hCG and prostaglandin E2 treatments (Shen et al., 2010). However, the role of visfatin in hGCs steroidogenesis is unknown. A previous study showed that women with polycystic ovary syndrome exhibit higher plasma visfatin levels than control subjects of similar body mass index, suggesting that plasma visfatin concentrations might affect ovarian functions (Chan et al., 2007; Kowalska et al., 2007). Furthermore, Ozkaya et al. found that metformin (MetF) treatment significantly reduced circulating plasma visfatin concentrations in PCOS patients (Ozkaya et al., 2010). In addition to its reproductive consequences (infertility, anovulation, polycystic ovaries and hyperandrogenism), PCOS is a metabolic disorder associated with insulin resistance and hyperinsulinemia (Dunaif, 1997). Peripheral effects of MetF, dependent or not on its insulin-sensitizing action, have been observed in several studies (Palomba et al., 2006; Diamanti-Kandarakis et al., 2010). Several reports have suggested a specific effect of MetF on ovaries, showing that PCOS patients ovulating under treatment have an improved ovarian artery blood flow and better dominant follicle and corpus luteum vascularization (Palomba et al., 2006). MetF is also able to exert direct effects on the ovary, by inhibiting, for example, in vitro steroid production by GCs in various species, including man (Mansfield et al., 2003; Tosca et al., 2006). Furthermore, in vivo MetF treatment reduces the stimulated activity of several steroidogenic enzymes without a reduction in circulating insulin in women with PCOS (Vrbikova et al., 2001). However, although MetF has been used in clinical practice for several years, its mechanism of action remains unclear. One hypothesis is that MetF treatment could affect visfatin expression in ovarian cells. The objectives of this study was to explore the expression of visfatin in the human follicle and to study its regulation in hGCs (luteal GCs from women undergoing in vitro fertilization and KGN cells) in response to two insulin sensitizers, MetF and rosiglitazone. We also investigated the effects of human recombinant visfatin on steroid production and on the activation of various signalling pathways in these cells.

Materials and Methods

Patients

Twenty infertile women (35 ± 4 years old) were recruited at the Service de médecine et Biologie de la Reproduction, CHRU de Tours, in 2011 for mechanical, unexplained or male factor infertility without any known endocrinopathy (polycystic ovarian syndrome, hyperprolactinemia and hyperandrogenism). Patients gave their written informed consent and did not receive any monetary compensation for participating in the study. Only one cycle was studied from each patient.

KGN cell culture

The human ovarian granulosa-like tumour cell line, KGN, was cultured in Dulbecco’s minimal essential medium/F12 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin obtained from Sigma) in a 5% CO2 atmosphere at 37°C. KGN cells were obtained from Drs Masatoshi Nomura and Hajime Nawata, Kyushu University, Japan (Nishi et al., 2001). They are undifferentiated and maintain the physiological characteristics of ovarian cells, including the expression of functional FSH receptor and the expression of aromatase.

Hormones and reagents

Purified ovine FSH-20 (oFSH; lot no.AFP-702BD, 4453 IU/mg. FSH activity = 175 times the activity of oFSH-S1) was a gift from NIDDK, National Hormone Pituitary Program, Bethesda, MD, USA. Recombinant human insulin-like growth factor-1 (IGF-1) used for culture treatment was from Sigma (St Louis, MO, USA). Recombinant human visfatin was from R&D (Lille, France). Human ovary sections embedded in paraffin were obtained from Euromedex (Souffleveysheim, France). The visfatin inhibitor, FK866, the AMP-activated protein kinase (AMPK) inhibitor, compound C, the SIRT1 inhibitor, Sirtinol and the SIRT1 activity assay were obtained from Sigma (St Louis, MO, USA).

Antibodies

Affinity-purified rabbit anti-human PBEF (BL2122) polyclonal antibody and blocking peptides for the polyclonal antibody PBEF (BP2122) were purchased from Bethyl Laboratories Inc. (Montgomery, USA). Rabbit polyclonal antibodies to phospho-ERK1/2 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-Akt (Ser 473) and phospho-AMPK alpha Thr172 were obtained from New England Biolabs Inc. (Beverly, MA, USA). Rabbit polyclonal antibodies to AMPKalpha were purchased from Upstate Biotechnology Inc. (Lake, Placid, NY, USA). Mouse monoclonal antibodies to Vinculin (VLC) were obtained from Sigma (St Louis, MO, USA). Rabbit polyclonal antibodies to ERK2 (C14) and p38 (C20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All antibodies were diluted 1/1000 in western blotting.

Isolation and culture of hGCs

The hGCs were collected from pre-ovulatory follicles during oocyte retrieval for IVF. The ovarian stimulation protocol and IVF and ICSI procedures used have already been reported (Guerif et al., 2004; Reverchon et al., 2012). After isolation of cumulus oocyte complexes (used for IVF), follicular fluids were pooled and then centrifuged (400g, 10 min). To remove most of the red blood cells, the pellet was centrifuged (400g, 20 min) on a two-layer discontinuous Percoll gradient (40, 60% in Ham’s medium, Gibco-BRL; Life Technologies, Cergy Pontoise, France).
France). The 40% fraction was collected and treated with haemolytic medium (NH₄Cl 10 mmol/l in Tris–HCl pH 7.5; Sigma, Isles d’Abeau, France) to remove the remaining red blood cells as many as possible. Following centrifugation, the pellet was washed with fresh medium (Hams F12); cells were counted in a haemocytometer and cell viability was determined using Trypan Blue dye exclusion. Cell were cultured in McCoy’s 5A medium supplemented with 20 mmol/l Hepes, penicillin (100 U/ml), streptomycin (100 mg/l), L-glutamine (3 mmol/l, 0.1% BSA, 0.1 mmol/l androstenedione, 5 mg/l transferrin and 20 μg/l selenium) and 5% fetal bovine serum (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. All cultures were kept under a water-saturated atmosphere of 95% air/5% CO₂ at 37°C. We made four cultures (one per week). Each culture was made by pooling cells obtained from different follicles from one patient. In each culture, each treatment (visfatin in the presence or absence of IGF-1) was applied in quadruplicate or duplicate as indicated in the figure legends.

RNA extraction and RT–PCR
Total RNA from hGCs, KGN cells and human visceral or subcutaneous adipose tissue (Vis AT, Sc AT) was extracted with Trizol reagent according to the manufacturer’s procedure (Invitrogen). RT–PCR was used to detect visfatin expression in hGCs, KGN cells and in Vis and Sc AT. Reverse transcription of total RNA (1 μg) was denatured and retrotranscribed with the reverse transcriptase Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase (15 U) in a 20 μl reaction mixture containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 200 μM of each deoxynucleotide triphosphate (Amersham, Piscataway, NJ, USA), 50 pmol of oligo(dT)15 and 5 μl of ribonuclease inhibitor. All was incubated at 37°C for 1 h. cDNAs were amplified in 50 μl reaction mixture containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 200 mM of each deoxynucleotide triphosphate, 10 pmol of each primer and 1 U of Taq polymerase and 2 μl of the RT mix. PCR was performed with specific primer pairs for visfatin (forward: 5′-AAGGAGACTGTGGCATAGA-3′ and reverse: 5′-ACACAGATAACGGCCTAG-3′) and reverse (forward: 5′-ACCCAGCTGAGTGGC-3′ and reverse 5′-GTCC CAGTTCTCAAGAC-3′). First, the samples were denatured at 94°C for 5 min, then 35 PCR cycles were processed (95°C, 1 min; 58°C, 1 min; 72°C, 1 min), with a final extension step at 72°C for 10 min. PCR products were migrated on 1% agarose gel stained with ethidium bromide and sequenced by the Genome Express company (Lyon, France). MMLV reverse transcriptase and RNase inhibitor (Promega, Promega, Madison, WI, USA) were used. The 40% fraction was collected and treated with haemolytic medium (NH₄Cl 10 mmol/l in Tris–HCl pH 7.5; Sigma, Isles d’Abeau, France) to remove the remaining red blood cells as many as possible. Following centrifugation, the pellet was washed with fresh medium (Hams F12); cells were counted in a haemocytometer and cell viability was determined using Trypan Blue dye exclusion. Cell were cultured in McCoy’s 5A medium supplemented with 20 mmol/l Hepes, penicillin (100 U/ml), streptomycin (100 mg/l), L-glutamine (3 mmol/l, 0.1% BSA, 0.1 mmol/l androstenedione, 5 mg/l transferrin and 20 μg/l selenium) and 5% fetal bovine serum (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. All cultures were kept under a water-saturated atmosphere of 95% air/5% CO₂ at 37°C. We made four cultures (one per week). Each culture was made by pooling cells obtained from different follicles from one patient. In each culture, each treatment (visfatin in the presence or absence of IGF-1) was applied in quadruplicate or duplicate as indicated in the figure legends.

Protein extraction and western blot
Freshly collected hGCs purified on Percoll gradient, KGN cells, in vitro-cultured hGCs and adipose tissues (Vis AT, Sc AT) were homogenized as previously described (Chabrolle et al., 2009; Pierre et al., 2009). Lysates were incubated on ice for 30 min and then centrifuged at 12,000g for 20 min at 4°C. The protein concentration in the resulting supernatants was then determined using the BCA protein assay. After denaturation, the samples were subjected to electrophoresis on 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Schleicher and Schuell, Ecouen, France). The membrane was blocked for 30 min in TBS-Tween-milk 5% and incubated for 1 h with appropriate primary antibodies at a 1/1000 final dilution. Finally, the blots were incubated for 1 h 30 min at room temperature with an HRP-conjugated anti-rabbit or anti-mouse IgG (dilution 1/5000). Proteins were detected by enhanced chemiluminescence (Western Lightning Plus-ECL, Perkin Elmer) using a GBox SynGene (Ozyme) with the GeneSnap software (release 7.09.17). Signals detected were quantified with the GeneTools software (release 4.01.02). The results were expressed as the intensity signal in arbitrary units after normalization, allowed by the presence of ERK2, p38, Akt, AMPK total (for ERK1/2, p38, Akt and AMPK phosphorylation, respectively) and VLC (for visfatin) as an internal standard. The effect of recombinant human visfatin was analysed on four different primary human granulosa cells cultures. Each primary culture was derived from a different patient.

SIRT1 activity assay
A SIRT1 activity assay was performed using the SIRT1 Fluorimetric Drug Discovery Kit (Biomol International). Briefly, total protein (25 μg) and acetylated substrate (Fluor-de-Lys-Sirt1 substrate, 100 μM) were incubated at 37°C in the presence of NAD (100 μM) for 30 min. After incubation, de-acetylated substrates were detected on a TECAN M200pro fluorometric plate reader at ‘Physiologie de la Reproduction et des Comportements’ (Nouzilly, France).

Immunohistochemistry
Human ovary sections were deparaffinized, hydrated and microwaved for 5 min in antigen unmasking solution (Vector Laboratories, Inc., AbCys, Paris, France) and then allowed to cool to room temperature. After washing in a phosphate-buffered saline (PBS) bath for 5 min, sections were immersed in peroxidase-blocking reagent for 10 min at room temperature to quench endogenous peroxidase activity (DAKO Cytomation, Dako, Ely, UK). For rabbit primary antibody, sections were washed twice for 5 min each time in a PBS bath and were incubated for 30 min at room temperature with a ‘ready to use’ labelled Polymer-HRP anti-rabbit antibody (DakoCytomation EnVision Plus HRP system, Dako, Ely, UK). The sections were then washed twice in PBS, and the staining was revealed by incubation at room temperature with 3,3′-diaminobenzidine tetrahydrochloride (Liquid DAB + Substrate Chromogen System, DakoCytomation). Negative controls involved replacing primary antibodies with rabbit IgG.

Progesterone and estradiol radioimmunoassay
The concentration of progesterone and estradiol in the serum-free culture medium of KGN and hGCs cells was measured after 48 h of incubation with or without IGF-1, FSH or FK866 by a radioimmunoassay protocol as previously described (Chabrolle et al., 2009; Pierre et al., 2009). The limit of detection of progesterone was 12 pg/tube (60 pg/well), and the intra- and inter-assay coefficients of variation were less than 10 and 11%, respectively. The limit of detection of estradiol was 1.5 pg/tube (7.5 pg/well), and the intra- and inter-assay coefficients of variation were less than 7 and 9%, respectively. Results were expressed as the concentration of steroids/cell protein concentration/well. Results are mean ± SEM of data obtained from KGN cells (at four different passages) and primary hGCs (four cultures, one per week). Each primary hGCs culture was performed using cells obtained from different follicles from one patient. In each culture, each treatment (in the presence or absence of IGF-1, FSH or FK866) was applied in quadruplicate.

Thymidine incorporation into GCs
hGCs (2 × 10⁵ viable cells/500 μl) were cultured in McCoy’s 5A medium and 10% FBS for 48 h and were then serum starved for 24 h, followed by
the addition of 1 μCi/μl of [3H] thymidine (Amersham Life Science, Arlington Heights, IL, USA) in the presence or absence of visfatin (10 ng/ml) and/or IGF-1 (10 nM). After 24 h of culture, excess thymidine was removed by washing the cells twice with PBS, and then they were fixed with cold trichloroacetic acid 50% for 15 min and lysed by NaOH 0.5 N. The radioactivity was determined by scintillation fluid (Packard Bioscience) counting in a beta-photomultiplier.

**Real-time quantitative PCR**

Targeted cDNAs were quantified by real-time PCR using SYBR Green Supermix (Bio-Rad, Marnes la Coquette, France) and 250 nM of specific primers (visfatin (forward: 5′-AAGAGACTGCTGGCATAGGA-3′ and reverse: 5′-ACCACAGTAGATCAGGACTGA-3′), β-actin (forward: 5′-ACGGACTCCACAGTTTATCATC-3′ and reverse: 5′-GTCCCAGTCTCCAATATCACC3′), ribosomal protein L19 (RPL 19) (forward: 5′-AATCGCCAATG CCACTC-3′ and reverse: 5′-CCCTTTCGCTTACCTATACC-3′) and cyclophilin A (forward: 5′-GCATACAGGTCCTGGCATCT-3′ and reverse: 5′-TGTCGACGTCAGGATTC-3′) in total volume of 20 μl in a MyIq Cycle device (Bio-Rad). Samples were tested in duplicate on the same plate, and PCR amplification with water, instead of cDNA, was done systematically as a negative control. After incubation for 2 min at 50°C and a denaturation step of 10 min at 95°C, samples were subjected to 40 cycles (30 s at 95°C, 30 s 60°C and 30 s at 72°C), followed by the acquisition of the melting curve. Primers’ efficiency (E) was performed from serial dilutions of a pool of obtained cDNA and ranged from 1.8 to 2. Three reference genes were used: cyclophilin A, RPL19 and β-actin. For each gene, expression was calculated according to primer efficiency and Cq: expression \( = E^{-\Delta C_q} \). Then, relative

![Figure 1](https://academic.oup.com/molehr/article-abstract/19/5/313/1227184)  

**Figure 1** Expression of visfatin in hGCs and ovarian follicle. (A) RT–PCR from total RNA of hCGs, ovarian granulosa cell lines (KGN), human visceral (Vis AT) and subcutaneous (Sc AT) adipose tissues was performed with primers designed to amplify one fragment of visfatin (228 pb) and one fragment of actin (188 pb) as a housekeeping gene. Human visceral (Vis AT) and subcutaneous (Sc AT) adipose tissues were used as a positive control for visfatin expression [mRNA (A) and protein (B, see below)]. The expected sizes of the different amplified products by RT–PCR are indicated on the right. RT-corresponds to an RT–PCR carried out without reverse transcriptase. (B) Protein extracts (50 μg) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and incubated with specific antibodies against visfatin. Equal protein loading was verified by a reprobing membrane with an anti-VLC antibody. hCGs from four different patients were used. The data presented are from one representative experiment with only one sample of hCGs from two different patients. Recombinant human visfatin was used as positive control. (C) Visfatin localization in human ovarian follicle by immunohistochemistry. DAB-immunoperoxidase staining was performed on paraffin-embedded human ovary using antibodies against visfatin (2, 3) or no primary antibodies but rabbit IgG (1). Immuno-specific staining is brown. The sections were counterstained with haematoxylin. Visfatin is detected in GCs, CC, theca cells (T) and oocytes (OO) of ovarian follicles. A, antrum. Bars = 100 μm or 20 μm. Immunohistochemistry was performed on two different human ovary slides from each of four patients.
expression of visfatin/reference gene was analysed. Only one reference gene is represented in the figures.

**Statistical analysis**

All experimental results are expressed as the mean ± SEM. Statistical analyses were carried out using a t-test or one-way analysis of variance (ANOVA) (for comparison of various means), and if ANOVA revealed significant effects, it was supplemented with Fisher’s test. A P<0.05 was considered significant.

**Results**

**Visfatin expression in hGCs**

We determined the expression of visfatin in fresh hCGs and in the human granulosa tumour cell line, KGN. As shown in Fig. 1A, we amplified by RT–PCR one cDNA fragment of 228 pb, corresponding to visfatin. The specificity of the amplified products was assessed by sequencing. Visceral and subcutaneous adipose tissues (Vis AT, Sc AT) were used as a positive control. We found that the transcript of visfatin is strongly expressed in GCs and as expected in the adipose tissues (Fig. 1A). Immunoblotting of protein extracts revealed the presence of visfatin (52 kDa) in fresh hCGs and in KGN cells (Fig. 1B). Immunohistochemistry with human ovarian follicle sections confirmed the results obtained by immunoblotting. Visfatin was detected in not only GCs but also in cumulus cells (CC) and oocyte and less abundantly in theca cells (Fig. 1C). Thus, visfatin is expressed in human ovarian follicles and more particularly in GCs.

**Effect of MetF and rosiglitazone on visfatin mRNA and protein expression in hGCs**

We next investigated the effect of two insulin sensitizers, MetF and rosiglitazone, on visfatin expression in KGN (Fig. 2) and primary hGCs (data not shown). Overnight starved cells were incubated for different times (24 and 48 h) with different concentrations of MetF.

![Figure 2](https://academic.oup.com/molehr/article-abstract/19/5/313/1227784)  
**Figure 2** Effect of MetF and rosiglitazone (Rosi) on visfatin expression at the mRNA (A) and protein (B) level in KGN cells. (A) Visfatin gene expression was measured by quantitative real-time-PCR in KGN cells after 24 and 48 h of stimulation with or without different doses of rosiglitazone (Rosi, 0.1, 1 and 10 μM) or MetF (0.1, 1 and 10 mM). β-actin was used as a reference gene. Similar results were obtained using two other reference genes, RPL19 and cyclophilin A. (B) Protein levels of visfatin were analysed after 24 and 48 h of stimulation with or without rosiglitazone (Rosi, 10 μM) or MetF (10 mM). VLC was used as a loading control. Results are representative of at least eight cultures of KGN cells obtained at different passages. In each culture, each treatment (in the presence or in the absence of MetF or rosiglitazone) was applied in duplicate. Results are represented as mean ± SEM. Different letters indicate significant differences at P < 0.05.
(0, 0.1, 1 and 10 mM) or rosiglitazone (0, 0.1, 1 and 10 μM). By real-
time quantitative PCR, we showed that after 24 h of stimulation, MetF increased visfatin mRNA expression in a dose-dependent manner, whereas rosiglitazone increased it only at the 10 μM concentration (Fig. 2A, left panel). As shown in Fig. 2A, right panel, we observed opposite effects after 48 h of incubation. Similar results were obtained using two other reference genes (RPL19 and Cyclophilin A). We next examined whether these effects of MetF and rosiglitazone on visfatin mRNA were also observed at the protein level. As shown in Fig. 2B, MetF treatment (10 mM) for 24 h increased the level of visfatin protein by more than 2-fold, whereas it halved it after 48 h in KGN cells (Fig. 2B). Rosiglitazone treatment (10 μM) did not affect visfatin protein expression at any time points that were studied (Fig. 2B). Similar results were observed in hGCs (data not shown). We next investigated the molecular mechanisms involved in the regulation of visfatin expression in response to MetF.

Involvement of AMPK in the MetF effects on visfatin expression in hGCs

It is well known that MetF treatment activates AMPK in various cell types, including hGCs (Richardson et al., 2009). In KGN cells (Fig. 3A) and in hGCs (data not shown), we showed that MetF treatment (10 mM) increased AMPK phosphorylation on Thr172 residue after 60 and 120 min of incubation. Similar results were observed after 12 or 24 h of stimulation, whereas MetF treatment for 48 h significantly inhibited AMPK phosphorylation (Fig. 3B). We also indirectly assessed the AMPK activity by measuring the phosphorylation of its downstream target, Acetyl CoA carboxylase (ACC) (data not shown). ACC phosphorylation paralleled that of Thr172 AMPK in response to MetF (data not shown). To determine whether AMPK phosphorylation is involved in the regulation of visfatin expression in response to MetF in KGN cells, we investigated the effects of Comp

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**Figure 3** Effect of MetF on AMPK phosphorylation (A, B) and effect of compound C on visfatin expression in response to MetF in KGN cells (C, D). (A and B) Cell lysates were prepared from cells incubated in serum-free medium in the presence of MetF (10 mM) for various times: 0, 5, 10, 30, 60 or 120 min (A) or for 12, 24 or 48 h (B). Lysates (50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose and probed with anti-phospho-AMPK and anti-AMPK (A and B). Results are representative of at least four cultures of KGN cells obtained at different passages. In each culture, each treatment (visfatin in the presence or absence of MetF) was applied in duplicate. Results are represented as mean ± SEM. Different letters indicate significant differences at P < 0.05. (C) KGN cells were pre-incubated in the serum-free medium in the presence or absence of Comp C (10 μM) for 60 min and then stimulated or not with 10 mM MetF for 120 min in serum-free medium. Cells were then lysed and subjected to western blotting with antibodies against phospho-AMPK (Thr 172) and AMPK. Representative blots for the three experiments are shown. Blots were quantified, and the phosphorylated protein:total protein ratio is shown. The results are mean ± SEM. Different letters indicate significant differences with P < 0.05. (D) KGN cells were pre-incubated in the serum-free medium in the presence or absence of Comp C (10 μM) for 60 min and then incubated or not with 10 mM MetF for 24 h or 48 h in serum-free medium. Visfatin gene expression was measured by quantitative real-time-PCR as indicated in Fig. 2. Results are representative of at least eight cultures of KGN cells obtained at different passages. In each culture, each treatment (in the presence or in the absence of Comp C and/or MetF for 24 or 48 h) was applied in duplicate. Results are represented as mean ± SEM. Different letters indicate significant differences at P < 0.05.
C, a well-known inhibitor of AMPK. After 2 h of MetF stimulation in serum-free medium, immunoblot analysis confirmed that Comp C treatment (10 μM) significantly decreased AMPK phosphorylation when compared with the control treated with dimethyl sulphoxide (Fig. 3C). Comp C treatment (10 μM) totally eliminated the effect of MetF on visfatin expression after 24 and 48 h of incubation (Fig. 3D). Similar results were observed on the visfatin protein levels (data not shown). The viability of KGN cells as determined by trypan blue staining was not affected by Comp C treatment (data not shown). Thus, Comp C inhibited AMPK phosphorylation and eliminated the effects of MetF on visfatin expression. To confirm that AMPK is involved in the regulation of visfatin expression in hGCs, we incubated cells with Aicar, an activator of AMPK in the absence or presence of Comp C treatment, and investigated visfatin mRNA and protein expression. As shown in Fig. 4A, Aicar (1 mM) increased AMPK phosphorylation on Thr172 residue after 30, 60 and 120 min of incubation. Aicar treatment (10 μM) increased visfatin mRNA expression by about 3-fold after 24 h of incubation (Fig. 4B), whereas it reduced it by about 3-fold after 48 h of incubation (Fig. 4C). Comp C treatment (10 μM) totally abolished the effect of Aicar on visfatin expression after 24 and 48 h of incubation (Fig. 4B and C). Similar results were observed on the visfatin protein levels (Fig. 4D and E).

Involvement of SIRT1 in MetF-induced visfatin expression in hGCs

MetF is also known to activate the NAD+-dependent histone/protein deacetylase SIRT1 (Caton et al., 2010). Furthermore,
numerous studies have shown that AMPK activation can also lead to increased SIRT1 activity (Fulco et al., 2008; Canto et al., 2009; Caton et al., 2010), with two of these studies demonstrating that MetF increases the NAD+/NADH ratio and SIRT1 abundance and activity (Canto et al., 2009; Caton et al., 2010). As shown in Fig. 5A, we observed that MetF treatment for 12 and 24 h increased SIRT1 activity in KGN cells. Similar results were obtained in hGCs (data not shown). To determine whether SIRT1 mediates the positive effects of MetF on visfatin expression, we treated cells with Sirtinol (a SIRT1 inhibitor). As expected, Sirtinol inhibited SIRT1 activity as compared with basal state (no stimulation) or in response to MetF (Fig. 5A). Furthermore, as shown in Fig. 5B, treatment of KGN cells with Sirtinol significantly reduced visfatin expression in response to 24 h MetF treatment. Similar results were observed on the visfatin protein levels (Fig. 5C). The viability of KGN cells as determined by trypan blue staining was not affected by sirtinol pretreatment (data not shown). Thus, MetF-induced visfatin expression is not only AMPK- but also SIRT1-dependent.

**Effect of human recombinant visfatin on basal and IGF-1 or FSH-induced progesterone and estradiol production by KGN and hGCs cells**

We next determined the effects of visfatin treatment on steroidogenesis in KGN and primary hGCs (Fig. 6). Cells were incubated in serum-free medium with human recombinant visfatin (Rh visfatin) (10 ng/ml) for 48 h in the presence or absence of IGF-1 (10 nM) or FSH (10 nM). As expected, IGF-1 or FSH treatment alone increased progesterone and estradiol secretion in KGN cells (Fig. 6A and B, Pierre et al., 2009) and primary hGCs cells (Fig. 6C and D, Chabrolle et al., 2009). In KGN cells, in basal state and in the presence of IGF-1, visfatin treatment (10 ng/ml) increased by at least 2-fold secretion of progesterone (Fig. 6A, $P < 0.001$) and estradiol (Fig. 6B, $P < 0.001$), whereas no effect of visfatin was observed in the presence of FSH. In primary hGCs, similar effects were observed except that no effect of visfatin was observed in the basal state (Fig. 6C and D).

We next confirmed the effect of visfatin on IGF-1-induced steroid production using FK866, a specific inhibitor of enzymatic activity of vis-
fatin/NAMPT (Fig. 7). Cells were incubated in serum-free medium with human recombinant visfatin (Rh visfatin) (10 ng/ml) for 48 h in the presence or absence of IGF-1 (10 nM) and/or FK866 (10 nM). As a preliminary experiment, we showed that the dose of FK866 (10 nM) inhibited NAMPT activity causing a significant decrease in the NAD\(^+\) levels (data not shown). As shown in Fig. 7A and B, FK866 totally eliminated the effect of visfatin on progesterone and estradiol production in basal state (no stimulation) and in response to IGF-1. In primary hGCs, FK866 also abolished IGF-1-induced progesterone and estradiol concentration (ng/ml)/cellular protein concentration/well. Results are mean ± SEM of the six cultures of primary hGCs and four independent experiments (at four different passages) for KGN cells. For primary hGCs, each culture was performed using cells obtained from different follicles from one patient. In each culture, each treatment was applied in quadruplicate. Bars with different letter indicate significant differences (P < 0.05).

**Effect of human recombinant visfatin on human granulosa cell proliferation**

We next examined the effect of rh visfatin on the DNA synthesis of primary hCGs (Fig. 8A) and KGN cells (data not shown). \(^{[3]H}\)-thymidine incorporation by hCGs was determined after 24 h of culture in serum-free medium in the presence or absence of rh visfatin (10 ng/ml) and FK866 (10 nM) ± IGF-1 (10 nM). Visfatin did not affect basal proliferation, whereas IGF-1 treatment alone increased DNA synthesis significantly by about 5-fold in hCGs (P < 0.05, Fig. 8A). In these latter cells, visfatin treatment significantly increased \(^{[3]}H\)-thymidine incorporation induced by IGF-1 by about 1.3-fold (P < 0.05). As shown in Fig. 8A, FK866 eliminated the positive effect of visfatin on the IGF-1-induced cell proliferation (Fig. 8A). Similar results were observed in KGN cells (data not shown). Thus, rh visfatin increased IGF-1-induced DNA synthesis of primary hGCs.

**Effect of human recombinant visfatin on signalling pathways in primary hGCs**

Because adipokines like adiponectin and resistin can modulate phosphorylation of MAPK ERK1/2 and p38, AKT and AMPK, we studied the pattern of these signalling pathways in response to visfatin in fresh primary hCGs cells. Cells were serum starved overnight and incubated with human recombinant visfatin (10 ng/ml) for different
times (1, 5, 10, 30 and 60 min). As shown in Fig. 8B–D, rh visfatin activated quickly (at 1 min of stimulation, \( P < 0.05 \)) the MAPK ERK1/2, p38 and AKT signalling pathways. However, it did not affect AMPK phosphorylation (data not shown). Thus, rh visfatin activates Akt and MAPK-ERK1/2 and -p38 signalling pathway in primary hGCs.

**Discussion**

In this study, we report for the first time that visfatin is present in human ovarian follicles and in the human ovarian granulosa-like tumour cell line, KGN. In primary hGCs and KGN cells, visfatin expression is regulated by MetF through the AMPK and SIRT1 signalling pathways. Furthermore, recombinant human visfatin increased IGF-1-induced thymidine incorporation and steroid production, suggesting that visfatin could affect ovarian folliculogenesis.

We have also shown the presence of visfatin in human oocytes. Other adipokines, including leptin and adiponectin, have already been found in oocytes of various species, including man (Chabrolle et al., 2007; Madeja et al., 2009). Their role in oocytes is still unclear. Energy homeostasis of the oocyte is a crucial determinant of fertility. It depends on numerous enzymes that are dependent on NAD biosynthesis. Because visfatin is the rate-limiting enzyme in the NAD biosynthesis pathway from nicotinamide, it could influence oocyte quality. Visfatin is a secreted cytokine-like protein (Ognjanovic et al., 2005). Thus, it will be interesting to know if the ovarian cells are able not only to produce but also secrete visfatin and if this local production has a real role in the ovarian functions. Several studies found a similar concentration of visfatin in follicular fluid and plasma, suggesting that ovarian cells do not secrete much visfatin (Shen et al., 2010).

In our study, we have shown that two insulin sensitizers, rosiglitazone and MetF, known to regulate human granulosa cell steroidogenesis, modulated visfatin expression in primary hGCs and KGN cells. They increased it after 24 h of stimulation and inhibited it after 48 h of stimulation. These effects were confirmed at the protein level only for MetF. Rosiglitazone affects visfatin mRNA expression only at the higher concentration used (10 \( \mu M \)), whereas a dose–response effect was
observed for MetF. Thus, we cannot exclude that the rosiglitazone effect is non-specific. However, rosiglitazone is known to increase visfatin expression in visceral fat of Otsuka Long Evans Tokushima Fatty rats (Choi et al., 2005) and in human macrophages (Mayi et al., 2010). The antidiabetic thiazolidinedione rosiglitazone activates the peroxisome proliferator-activated receptor gamma (PPARγ) that, once activated, forms a heterodimer with the retinoic X receptor (Semple et al., 2006). The binding of this heterodimer to specific DNA sequences, called PPAR response elements (PPRE), results in the regulation of its target genes (Semple et al., 2006). In primary human macrophages, a functional PPRE was identified within the visfatin promoter. In hGCs, PPAR gamma is strongly expressed. Thus, rosiglitazone could increase visfatin expression through the PPRE. We observed no effect of rosiglitazone on visfatin protein levels, suggesting that these effects are only transcriptional. Conversely, MetF treatment regulated mRNA and protein visfatin levels in hGCs. Indeed, it increased visfatin expression after 12 (data not shown) or 24 h of stimulation and decreased it after 48 h of stimulation. A dose–response of MetF was observed for these effects. MetF treatment has already been shown to increase visfatin expression in other cell types than ovarian cells. For example, in mice, administration of MetF increased visfatin expression in white adipose tissue (Caton et al., 2010).

**Figure 8** Effect of recombinant human visfatin on thymidine incorporation (A) and signalling pathway activation (B, C, D) in primary hCGs. (A) Thymidine incorporation was determined in hGCs cultured for 24 h in the presence or absence of human recombinant visfatin (10 ng/ml) + IGF-1 (10 nM) and FK866 (10 nM) as described in Materials and Methods. Results are representative of at least four cultures of GCs. Each culture was performed using cells obtained from different follicles from one patient. In each culture, each treatment (visfatin in the presence or in the absence of IGF-1 or FK866) was applied in triplicate. The results are expressed as mean ± SEM. Different letters indicate significant differences (P < 0.05). (B, C, D) Cell lysates were prepared from primary hGCs incubated with visfatin 10ng/ml for various times: 0, 1, 5, 10, 30 or 60 min. Lysates (50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-phospho-MAPK ERK1/2 (A), anti-phospho-Akt (B), anti-phospho-p38 (C) and then with anti-ERK2, Akt or P38 protein antibodies. Representative blots from four different cultures are shown. Each culture was made using cells obtained from different follicles from one patient. In each culture, each treatment (different time of visfatin stimulation) was applied in duplicate. Blots were quantified, and the phosphorylated protein/total protein ratio is shown. The results are represented as mean ± SEM. Different letters indicate significant differences (P < 0.05).
We investigated the molecular mechanisms involved in the regulation of visfatin expression. The search for the specific intracellular targets of MetF is still ongoing. However, AMPK is one of potential targets of MetF’s action on glucose and steroid metabolism (Tosca et al., 2006, 2007). It is a serine/threonine protein kinase that has emerged as a master sensor of cellular energy balance in mammalian cells. In GCs, AMPK is expressed and its activation modulates steroid production in different species (Tosca et al., 2005, 2006, 2007). As previously shown (Richardson et al., 2009), we have observed that MetF activates AMPK in hGCs. Furthermore, in our study, we showed that an inhibitor of AMPK, Compound C, abolished the MetF effects on visfatin mRNA and protein expression. In contrast, an activator of AMPK, Aicar, increased and decreased visfatin expression after 24 and 48 h of stimulation, respectively, suggesting that MetF regulates visfatin expression through AMPK in hGCs. We also studied the involvement of the histone deacetylase, SIRT1, in the MetF effects on visfatin expression. Like AMPK, SIRT1 regulates energy homeostasis and is expressed in the ovary (Monta et al., 2012). Furthermore, MetF has already been shown to activate SIRT1 in skin cells (Lee et al., 2010). To our knowledge, our study is the first to show that MetF activates SIRT1 activity in hGCs. Furthermore, we have observed that a specific inhibitor of SIRT1, sirtinol, abolished MetF-induced visfatin mRNA and protein expression, suggesting that not only AMPK but also SIRT1 is involved in this process. Previous studies have reported that AMPK can activate SIRT1 in skeletal muscle and cultured myocytes (Fulco et al., 2008; Canto et al., 2009). Thus, it remains to be determined if MetF-induced visfatin expression through AMPK is SIRT1 dependent and what are the effects on the granulosa cell functions.

Our study is the first to demonstrate that recombinant human visfatin (10 ng/ml) significantly increased IGF-1-induced progesterone and estradiol secretion in both primary hCGs and KGN cells. Furthermore, this effect disappeared when cells were incubated with FK866, a specific inhibitor of visfatin activity. In contrast, we observed no effect of visfatin on the steroid production induced by FSH in hGCs. Plasma visfatin concentration is about 3–30 ng/ml (Shen et al., 2010; Olszanecka-Glinianowicz et al., 2012), suggesting that the dose that we used was not pharmacological. However, we obtained similar results with visfatin 100 ng/ml (data not shown). This effect of visfatin on IGF-1-induced steroid production is in good agreement with the literature. Indeed, studies on human osteoblasts showed that visfatin exerted insulin-mimetic effects through stimulated phosphorylation of insulin receptor, insulin receptor substrate-1 and -2 (IRS-1 and IRS-2) (Xie et al., 2007). It remains to be seen whether visfatin can phosphorylate insulin receptor or IGF-1R in hGCs. In the basal state (no stimulation with IGF-1), we have shown that visfatin activates MAPK ERK1/2 and Akt. However, it is not known whether visfatin increases IGF-1-induced steroid secretion through IGF-1R phosphorylation and/or MAPK ERK1/2 or Akt. We also investigated the effect of rh visfatin on steroid production in response to FSH in primary hGCs, and we observed no effect of visfatin (data shown). This result suggests a specific effect of visfatin on the IGF-1-induced steroidogenesis. In basal state (no IGF-1 stimulation), we detected no effect of visfatin on steroid production in primary hGCs, whereas visfatin treatment increased progesterone and estradiol secretion in KGN cells. In contrast, in both primary hGCs and KGN cells, visfatin increased IGF-1-induced cell proliferation, and this effect was totally eliminated when cells were incubated with the potent NAMPT catalytic inhibitor, FK866. Previous reports have shown that visfatin is a survival factor for various cell types. Indeed, several studies have revealed that visfatin inhibits apoptosis in different cell types (Lim et al., 2008; Rongvaux et al., 2008; Cheng et al., 2011). Furthermore, NAMPT activity of visfatin seems to be required for cell protection because FK866 prevents visfatin-mediated cell protection (Yang et al., 2007). Further studies are needed to elucidate the molecular mechanism involved in the visfatin effect on the IGF-1-induced cell proliferation in hGCs.

In conclusion, we have shown the presence of visfatin in human ovarian follicles, including oocyte and CC. In primary hGCs and KGN cells, visfatin expression (mRNA and protein) is regulated by MetF through AMPK activation and SIRT1 activity. Furthermore, we observed that rh visfatin increased IGF-1-induced steroid secretion and cell proliferation through NAMPT activity in hGC and KGN cells. These findings significantly increase our understanding of the role of visfatin in hGCs. However, further investigations are needed to understand the role of visfatin on other human ovarian cells, including theca cells and oocytes and also its potential involvement in PCOS.

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Authors’ roles

M.R. participated together with J.D. in the design of the study. The experiments were carried out by M.R., M.C., L.C., F.G., D.R. and J.D. Data analysis was performed by M.R., M.C., L.C. and J.D. The manuscript was written by M.R. and J.D. All authors have read and approved the final manuscript.

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Conflict of interest

None declared.

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