Expression and effect of NAMPT (visfatin) on progesterone secretion in hen granulosa cells

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Abstract

In mammals, nicotinamide phosphoribosyltransferase (NAMPT) is an adipokine produced by adipose tissue that is found in intracellular and extracellular compartments. The intracellular form of NAMPT is a nicotinamide phosphoribosyltransferase, whereas the extracellular form is considered an adipokine. In humans, NAMPT regulates energy metabolism and reproductive functions, such as ovarian steroidogenesis. To date, no study has investigated the role of NAMPT in hen ovaries. We investigated whether NAMPT is present in hen ovarian follicles and its role in granulosa cells. Using RT-PCR, western blotting and immunocytochemistry, we detected mRNA transcripts and proteins related to NAMPT in theca and granulosa cells from pre-ovulatory follicles. Using RT-PCR, we demonstrated that mRNA NAMPT levels were higher in granulosa cells than they were in theca cells and that during follicle development, theca cell levels decreased, whereas levels remained unchanged in granulosa cells. NAMPT protein quantities were significantly higher in theca cells than they were in granulosa cells, but they were unchanged during follicular development. Plasma NAMPT levels, as determined by ELISA and immunoblotting, were significantly lower in adult hens than they were in juveniles. *In vitro*, treatment with human recombinant NAMPT (100 ng/ml, 48 h) halved basal and IGF1-induced progesterone secretion, and this was associated with a reduction in STAR and HSD3B protein levels and MAPK3/1 phosphorylation levels in granulosa cells. These effects were abolished by the addition of FK866, a specific inhibitor of NAMPT enzymatic activity. Moreover, NAMPT had no effect on granulosa cell proliferation. In conclusion, NAMPT is present in hen ovarian cells and inhibits progesterone production in granulosa cells.

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Introduction

Nicotinamide phosphoribosyltransferase (NAMPT), also known as a pre-B cell colony-enhancing factor (PBEF), is a 52 kDa mammalian protein that is constitutively synthesised by adipose tissue and many other tissues, including reproductive tissue (Reverchon *et al.* 2014). NAMPT has been shown to have several intra- and extra-cellular functions, and two isoforms, intracellular and extracellular, have been reported. Intracellular NAMPT plays a critical role in maintaining the activity of nicotinamide adenine dinucleotide (NAD)-dependent enzymes (Revollo *et al.* 2004). It contributes to the biosynthesis of NAD by acting to convert nicotinamide into nicotinamide mononucleotide (NMN), and it represents the limiting factor for this enzyme reaction (Rongvaux *et al.* 2002). Intracellular NAMPT activity is inhibited by a pharmacological competitive inhibitor,

FK866, which binds to the active site formed by the dimer (Khan *et al.* 2006). This means that FK866 can be used to assess the physiological function of NAMPT in cells. Extracellular NAMPT is secreted by various cell types (Revollo *et al.* 2007a), and its biological role is still not entirely understood. Although the findings remain controversial, several studies have indicated that NAMPT has glucose-lowering and insulin-mimicking or insulin-sensitising effects. No NAMPT receptor has yet been identified, and the molecular mechanism by which NAMPT acts remains unknown. Several researchers, including Revollo and Moschen (Revollo *et al.* 2007a,b), failed to detect NAMPT binding to insulin receptors (INSRs), although other studies have shown that INSRs play an important role in the actions of NAMPT in peripheral blood mononuclear cells (Dahl *et al.* 2007) and in human osteoblasts (Xie *et al.* 2007).

In mammals, there is some evidence to suggest that NAMPT is directly involved in regulating reproductive functions. NAMPT is present in human ovarian follicles and increases insulin-like growth factor 1 (IGF1)-induced steroidogenesis in primary human granulosa cells (Reverchon *et al.* 2013a). Furthermore, in women who are undergoing controlled ovarian stimulation, there is a correlation between the concentration of NAMPT in the follicular fluids and the number of oocytes retrieved (Shen *et al.* 2010). In rats, the administration of NAMPT during superovulation has been shown to play an important role in the regulation of oocyte quality, and it can improve oocyte quality and fertility in aged female mice (Choi *et al.* 2012). NAMPT is expressed in the testes of male rats, more specifically in Leydig cells, spermatocytes and sperm (Gurusubramanian & Roy 2014). In cultured Leydig cells, NAMPT has been shown to increase testosterone production (Hameed *et al.* 2012). In humans, NAMPT levels are significantly higher in seminal plasma than in serum, which suggests that testicular cells produce NAMPT (Thomas *et al.* 2013).

The full-length cDNA of the NAMPT gene has been cloned and sequenced in chickens (Li *et al.* 2012). Chicken NAMPT has high amino acid sequence identity with human and rat NAMPT (94%) (Li *et al.* 2012). It has been shown to be expressed primarily in skeletal muscle rather than in visceral adipose tissue, and it was therefore postulated that NAMPT might be a myokine rather than a cytokine (Krzysik-Walker *et al.* 2008). In chickens, NAMPT gene expression is sex- and tissue-dependent. There is more NAMPT mRNA in the adipose tissue of female chickens than in that of male chickens (Ons *et al.* 2010). In chicken reproductive tissue, NAMPT is expressed at the gonadotrope axis level, but most research has looked at NAMPT in the testes, and it has been suggested that NAMPT may play an important role in testicular steroidogenesis and spermatogenesis (Ocón-Grove *et al.* 2010). However, to the best of our knowledge, there have been no reports of NAMPT in hen ovaries, and the present study is the first to elucidate the role of NAMPT in hen granulosa cells *in vitro*. We also investigated the potential modulation of signalling pathways by NAMPT in these cells.

Materials and methods

Ethics

All procedures were approved by the Loire Valley Animal Experimentation Ethics Committee (CEEA VdL, protocol registered as 01607.02) and were carried out in accordance with the guidelines of the French Council for Animal Care.

Animals

Fifty-two-week-old laying breed hens ($n=50$, ISA Brown, egg layer type, Institut de Selection Animale, Saint Briec, France)

were housed individually in laying batteries with free access to feed and water and a 15 h light:9 h darkness cycle. Individual lay patterns were monitored daily. Hens were killed by exsanguination following electronarcosis (Sanofi-santé, Libourne, France) between 10 and 12 h before the next oviposition, and the ovaries were immediately removed and placed in ice-cold sterile 1% NaCl saline solution for immediate use. Granulosa and theca cells from the first (F1), second (F2) and third (F3) largest hierarchical follicles were dissected as previously described (Chabrolle *et al.* 2007a, Tosca *et al.* 2007). Tissues (adipose tissue, skeletal muscle and ovarian cortex) and cells (granulosa and theca) were immediately snap-frozen in liquid nitrogen and stored at -80°C until use for RNA and protein extraction. Granulosa cells were also used directly for culture.

Blood samples were taken from prepubertal hens (3- and 9-week-old, $n=8$ for each stage) and 52-week-old laying breed hens ($n=8$, ISA Brown, egg layer type, Institut de Selection Animale). All of the animals were fasted overnight before sampling.

Isolation and culture of granulosa cells

Granulosa cells were prepared for culture by being dispersed in 0.3% collagenase type A (Roche Diagnostic) in F12 medium containing 5% fetal bovine serum (FBS). Cells were recovered by centrifugation, washed with fresh medium, and counted in a haemocytometer. The viability of F1–F4 granulosa cells was estimated by trypan blue exclusion at about 95%. The culture medium was DMEM supplemented with 100 U/ml penicillin, 100 mg/l streptomycin, 3 mmol/l L-glutamine and 5% FBS. Cells were initially cultured for 24 h with no treatment, then incubated in fresh culture medium with or without test reagents for the appropriate time. All of the cultures were maintained in a water-saturated atmosphere of 95% air:5% CO_2 at 37°C .

Hormones and reagents

Human recombinant IGF1 was obtained from Sigma. Purified ovine luteinising hormone (LH) (lot 26) and ovine follicle stimulating hormone (FSH)-20 (lot no. AFP-7028D) were obtained from Dr AF Parlow and the National Hormone and Pituitary Programme (Bethesda, MD, USA). Human recombinant full-length adiponectin and human recombinant NAMPT were obtained from R&D Systems (Lille, France). The NAMPT inhibitor, FK866, was obtained from Sigma.

Antibodies

Rabbit polyclonal antibodies to PRKAA1 (AMPK α 1) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Rabbit polyclonal antibodies to phospho-MAPK3/1 (Thr202/Tyr204), phospho-MAPK14 (Thr180/Tyr182), phospho-AKT1 (Ser 473), AKT1 and phospho-PRKAA Thr172 were obtained from New England Biolabs, Inc. (Beverly, MA, USA). Mouse MAB to vinculin (VCL) were obtained from Sigma. Rabbit polyclonal antibodies against P450 side chain cleavage, or P450SCC (CYP11A1), STAR and 3β -hydroxysteroid dehydrogenase (HSD3B) were generously provided by

Dr Dale Buchanan Hales (Southern Illinois University, Carbondale, IL, USA) and Dr Van Luu-The (CHUL Research Center and Laval University, Quebec, Canada) respectively. Rabbit polyclonal antibodies to MAPK1 (C14) and MAPK14 (C20) were purchased from Santa Cruz Biotechnology. All antibodies were used at 1:1000 dilution in western blotting.

The production of antibodies against chicken NAMPT by AgroBio Co. (AgroBio, Orleans, France)

One peptide corresponding to the SYSFDEVRQNARLNSELETAC (amino acids 471–491) carboxy-terminal residues of chicken NAMPT was conjugated to keyhole limpet haemocyanin using sulphhydryl chemistry. Two hundred micrograms of a mixture of two conjugated peptides were emulsified with an equal volume of complete Freund's adjuvant and injected into two New Zealand White rabbits. Secondary immunisations were performed on post-immunisation days 14, 28, 42, 56, 84 and 112, followed by bleeding on day 127 post-immunisation.

RNA extraction and RT-PCR

Total RNA was extracted from hen granulosa and theca cells of hierarchical follicles (F3 to F1), ovarian cortex and small white follicles (SW) on ice using an ultraturax homogeniser in TRIzol reagent according to the manufacturer's instructions (Invitrogen by Life Technologies). The total RNAs were treated with DNaseI using a DNA-free Kit (Ambion by Life Technologies). The quantity of RNA was then assessed using a NanoDrop spectrophotometer. RT of total RNA (1 µg) was performed for 1 h at 37 °C in a 20 µl mixture as previously described (Coyral-Castel *et al.* 2010). Single-strand cDNAs of NAMPT and β actin (*ACTB*) were amplified with the following specific primers (Invitrogen by Life Technologies): NAMPT forward: 5'-CGT-TCA-GCC-CAT-TTG-GTG-A-3', reverse: 5'-AGT-GGT-GCC-TCT-GGA-CTT-CG-3'; and ACTB forward: 5'-ACG-GAA-CCA-CAG-TTT-ATC-ATC-3', reverse: 5'-GTC-CCA-GTC-TTC-AAC-TAT-ACC-3'. The PCR was carried out in a previously described mixture (Coyral-Castel *et al.* 2010) for 30 (*ACTB*) or 35 (*NAMPT*) PCR cycles (1 min at 94 °C, 1 min at 58 °C, 1 min at 72 °C), with a final extension step of 7 min at 72 °C. PCR products were visualised in 1.5% (w/v) agarose gel stained with ethidium bromide. β actin was used as a positive control. Finally, DNA was extracted from the agarose gel using the EZNA microelute gel extraction kit (VWR, Fontenay-sous-Bois, France) according to the manufacturer's instructions. DNA was sequenced by Beckman Coulter Genomics (Grenoble, France). RT and PCR consumables were purchased from Promega.

Real-time quantitative PCR

Targeted cDNAs were quantified by real-time quantitative PCR using SYBR Green Supermix (Bio-Rad) and 250 nM of specific primers (Invitrogen by Life Technologies, Table 1) in a total volume of 20 µl in a MyiQ Cycle device (Bio-Rad). Samples were tested in duplicate on the same plate; PCR amplification with water instead of cDNA was performed systematically as a negative control. After incubation for 2 min at 50 °C followed by denaturation for 10 min at 95 °C, samples were subjected to

Table 1 Oligonucleotide primers sequences.

Name	Forward 5'–3'	Reverse 5'–3'
NAMPT	CGTTCAGCCCATTT-GGTGA	AGTGGTGCCTCTGGACTTCG
ACTB	ACGGAACACAGTT-TATCATC	GTCCAGTCTTCAACTATACC
RPL-15	TGTGATGCGTTTCC-TCCTTGG	CCATAGGTTGCACCTTTTGGG
EEF1A	AGCAGACTTTGTGA-CCTTGCC	TGACATGAGACAGACGGTTGC

40 cycles (30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C), and then the melting curve was acquired. Primer efficiency (E) was assessed from serial dilutions of a pool of cDNA obtained from the samples and ranged from 1.8 to 2.0. Three reference genes were used: *ACTB*, *RPL15* and *EEF1A1* (Table 1). Gene expression was quantified separately for each gene using the formula E^{-Cq} . The expression of NAMPT was measured relative to the geometric mean of the expression of the three reference genes.

Protein extraction and western blot

Lysates of tissues (adipose tissue and muscle) and cells were prepared on ice with an ultraturax homogeniser in lysis buffer as previously described (Coyral-Castel *et al.* 2010). Proteins extracts (80 µg) were denatured, subjected to electrophoresis in a 12% (w/v) SDS–PAGE, transferred onto nitrocellulose membrane and then incubated with specific antibodies as previously described (Coyral-Castel *et al.* 2010). Proteins were detected by enhanced chemiluminescence (Western Lightning Plus-ECL, PerkinElmer, Courtaboeuf, France) using a G:Box SynGene (Ozyme, Montigny-le-Bretonneux, France) with the GeneSnap software version 7.09.17. Signals were quantified with the GeneTools software version 4.01.02.

Measurement of progesterone secretion by granulosa cells

Progesterone concentration in hen granulosa cells was determined in serum-free media after culturing for 24 h in the presence or absence of 76 ng/ml NAMPT ± IGF1, 300 ng/ml FSH or 300 ng/ml LH. Initially, granulosa were grown in 48-well dishes (1.25×10^5 viable cells/250 µl medium per well) in DMEM and 5% FBS for 24 h. After overnight serum starvation, granulosa cells were incubated with the appropriate treatment for 48 h. The concentration of progesterone derived from hen granulosa cells, in the culture media was measured using an ELISA protocol as previously described (Canepa *et al.* 2008). The intra-assay coefficient of variation (CV) averaged <10%. The results are expressed as the amount (mean ± s.e.m.) of steroid (ng/ml) secreted per 48 h. There were four replicates for each treatment within each culture, and we performed four cultures. In each culture, we used four hens, and consequently we used a pool of granulosa cells from four follicles of the same category (granulosa cells from one follicle per hen).

Thymidine incorporation into granulosa cells

After 18 h of serum starvation, the culture medium was removed and 1 µCi/ml of [3 H]thymidine (Perkin-Elmer) was

added in the presence or absence of 10 or 100 ng/ml NAMPT $\pm$ 76 ng/ml IGF1 in enriched McCoy's 5A. After culturing for 24 h, excess thymidine was removed by washing twice with PBS 1 $\times$. The cells were then fixed using cold 50% (v:v) trichloroacetic acid for 10 min and lysed with 0.5 M NaOH. Radioactivity was determined in scintillation fluid by counting in a β -photomultiplier. Values are expressed as count per min (CPM). There were four replicates for each treatment within each culture, and we performed five cultures. In each culture, we used four hens, and we consequently used a pool of granulosa cells from four follicles of the same category (granulosa cells from one follicle per hen).

Immunocytochemistry

Hen granulosa cells were collected from hierarchical follicles (F1). They were then fixed with 4% paraformaldehyde in PBS (pH 7.4) for 10 min and washed three times with PBS. The fixed cells were then permeabilised with sodium citrate (0.1%) in 0.1% Triton X-100 for 2 min at 4 °C. Endogenous peroxidases were blocked in methanol with 3% H₂O₂ for 10 min at room temperature. Non-specific background was eliminated by blocking in PBS with 5% horse serum for 20 min, followed by incubation overnight at 4 °C with PBS containing rabbit primary antibody raised against NAMPT (1/100). Sections were washed twice for 5 min in a PBS bath, then incubated for 30 min with a second antibody (HRP anti-rabbit antibody). After this, the sections were again washed twice for 5 min in a PBS bath. Staining was revealed by incubation at room temperature with DAB. Immunospecific staining was brown if NAMPT was present. Finally, the sections were counterstained with haematoxylin. Slides were observed under an Axioplan Zeiss transmission microscope.

NAD⁺ and NADH quantification

The concentrations of NAD⁺ and NADH in cell lysates were evaluated using a NAD⁺/NADH Quantification Kit (Bio Vision, Lyon, France) according to the manufacturer's instructions. The amount of NAD⁺ in each sample was normalised to the protein content for each test sample.

Hen NAMPT ELISA

Blood samples were collected from the occipital venous sinus in heparin blood collection tubes. Plasma samples were obtained by centrifugation (3000 *g* for 15 min at 4 °C) and stored at –20 °C until analysed. Before the visfatin ELISA assay, plasma samples were again centrifuged (10 000 *g* for 10 min at 4 °C) to remove most of the lipids (observed mainly in the sexually mature hen). Chicken plasma NAMPT levels were determined using a commercially available chicken NAMPT ELISA (Hözel Diagnostika, Köln, Germany; reference no. E12V0003, 96 tests) according to the manufacturer's protocol, with an intra-assay CV of <6%. Briefly, 100 μ l of standard or sample was added to the appropriate well in the antibody pre-coated microtitre plate. Then, 50 μ l of conjugate enzyme was added to each well and the plate was incubated for 1 h at 37 °C. After washing, 50 μ l of substrate A and 50 μ l of substrate

B were added, and the plate was again incubated for 15 min at 37 °C. At this point, the reaction was stopped using 50 μ l of stop solution, and the optical density was immediately determined at 450 nm.

Statistical analysis

All experimental data are presented as means $\pm$ S.E.M. One-way ANOVA was used to test for: differences in plasma NAMPT at different ages in progesterone secretion in response to different concentrations of rhVisf and in response to rhVisf (100 ng/ml) in the presence or absence of IGF1, FSH and LH; in NAD⁺ concentration in response to different doses of FK866; in STAR/VCL HSD3B/VCL and CYP11A1 in response to rhVisf in the presence or absence of IGF1 or FK866; in phospho-MAPK1/MAPK1 in response to different times of stimulation of rhVisf or in response to rhVisf in the presence or absence of IGF1 or FK866; and in tritiated thymidine incorporation in response to different doses of rhVisf in the presence or absence of IGF1. Two-way ANOVA was used to test for differences in NAMPT expression in theca and granulosa cells at different follicle developmental stages and in progesterone secretion in the presence or absence of rhVisf and IGF1 with or without FK866. The level of statistical significance was set at $P < 0.05$. Statview software (Cary, NC, USA) was used for all statistical tests.

Results

NAMPT expression in hen ovarian cells

We determined the expression of NAMPT in ovarian cortex, theca and granulosa cells from each type of hierarchical follicle studied (F3 to F1) and in SW. As shown in Fig. 1A, we used RT-PCR to amplify one cDNA fragment of 302 bp, which corresponds to NAMPT. The specificity of the amplified product was assessed by sequencing. Using a chicken NAMPT antibody, we revealed the presence of NAMPT (52 kDa) by immunoblotting protein extracts from theca and granulosa cells derived from hierarchical follicles (F3 to F1, Fig. 1B). The chicken NAMPT antibody was produced by immunising two rabbits with a 21-amino acid peptide that corresponded to a highly variable region of chicken NAMPT located at the carboxy-terminal end of the mature protein (Supplementary Figure 1A, see section on supplementary data given at the end of this article). When it was used in western immunoblotting, the antiserum detected an ~52 kDa protein in chicken plasma, white abdominal adipose tissues and skeletal muscle lysates that were resolved by reducing with SDS-PAGE (Supplementary Figure 1B). We demonstrated the specificity of the antiserum by showing that the 52 kDa signal in chicken plasma, white adipose tissue and muscles lysates were eliminated when immunoblotting was performed in the presence of pre-immune rabbit serum or in the absence of chicken NAMPT antiserum (Supplementary Figure 1B). Immunocytochemistry with hen granulosa cells from F1 hierarchical follicles

Figure 1 NAMPT expression in ovarian cells. (A) Amplification by RT-PCR of *NAMPT* (302 bp) in ovarian cortex, small white follicles and granulosa (G1 to G3) and theca (T1 to T3) cells of the third largest hierarchical follicles (F3 to F1) in hens. (B) Protein expression determined by immunoblotting of NAMPT in granulosa (G1 to G3) and theca (T1 to T3) cells. (C) Immunocytochemistry of NAMPT in granulosa cells from F1 hierarchical follicles. The pictures are representative of results obtained in four animals.

confirmed the results obtained by immunoblotting (Fig. 1C). We therefore concluded that NAMPT is present in ovarian cortex, granulosa and theca cells from hierarchical follicles.

The evolution of NAMPT expression with follicular development

Developmental changes in the expression of NAMPT mRNA and protein during follicle development were investigated in both granulosa and theca cells from hierarchical follicles (F3 to F1) using real-time RT-PCR and western blotting respectively. As shown in Fig. 2A, *NAMPT* mRNA expression was significantly higher in granulosa cells than it was in theca cells in F1 and F2 hierarchical follicles, but in F3 hierarchical follicles, the level of expression was similar in the two cell types. In theca cells, there was a clear decrease in the expression of *NAMPT* mRNA as the follicle developed, whereas it remained stable in granulosa cells (Fig. 2A). Using immunoblotting, we demonstrated that the NAMPT protein content of theca cells was almost twice that of granulosa cells. NAMPT protein levels were unaffected by follicular development in both theca and granulosa cells.

Quantification of plasma NAMPT levels by ELISA and immunoblotting in young prepubertal and adult hens

We next determined plasma NAMPT levels in young prepubertal (3- and 9-week-old) hens and in laying adult

(52-week-old, the same age as the hens used for *in vitro* culture of granulosa cells) hens. NAMPT levels in the plasma collected from the adult animals were significantly lower than those in the plasma from young prepubertal (3-week-old) hens (Fig. 3A). Similar results were found using western blotting (Fig. 3B).

Figure 2 NAMPT expression during follicular maturation. (A) Relative expression of *NAMPT* mRNA in theca and granulosa cells from the third largest hierarchical follicles in hens, based on real-time RT-PCR. Total RNA from all types of ovarian cells and hierarchical follicles was treated with DNase and reverse transcribed as described in the Materials and methods. cDNAs were used in separate real-time quantitative PCR analyses of *NAMPT* mRNA or reference genes, and SYBR green was used as the dye. *NAMPT* expression was measured relative to the geometric mean of the expression of three reference genes as described in the Materials and methods. (B) Western blotting was used to determine the protein levels of NAMPT in theca and granulosa cells from the third largest hierarchical follicles of hens. Ponceau staining was used as a loading control. The data represent mean values from seven hens for each ovarian cell and hierarchical follicle. Different capital letters indicate a significant effect ($P < 0.05$) of cell type (granulosa vs theca cells), whereas lowercase letters indicate a significant effect of follicular developmental stage.

Figure 3 Plasma NAMPT levels determined by ELISA (A) and western-blot (B) in hens at 3, 9 and 52 weeks. (A) Plasma NAMPT levels in prepubertal (3- or 9-week-old) and adult (52-week-old) hens based on ELISA. All serum samples that were used in western blotting (B) contained equal amounts of protein; this was confirmed by staining the nitrocellulose membrane with Ponceau. Blots were quantified using an arbitrary scale. The letters above each bar indicate significant differences ($P < 0.05$). Data are represented as means $\pm$ S.E.M. ($n = 8$ /age group).

The effects of recombinant human NAMPT on progesterone secretion in granulosa cells from hierarchical follicles

To determine whether NAMPT is involved in steroidogenesis, we investigated the effect of human recombinant NAMPT (rhVisf) on progesterone production in granulosa cells from F1 (Fig. 4A and B), F2 and F3/4 (data not shown) hierarchical follicles. We used human recombinant NAMPT because chicken NAMPT protein is highly homologous to human NAMPT (93% amino acid identity; Krzysik-Walker *et al.* 2008). Granulosa cells from F1 hierarchical follicles were incubated in serum-free medium with different concentrations of rhVisf (0, 1, 10, 100 or 500 ng/ml; Fig. 4A) or with 100 ng/ml rhVisf in the presence or absence of IGF1 (76 ng/ml), FSH (300 ng/ml) or LH (300 ng/ml) for 48 h (Fig. 4B). The secretion of progesterone was inhibited by NAMPT treatment in a dose-dependent manner (Fig. 4A). As expected, IGF1 (76 ng/ml), FSH (300 ng/ml) and LH (300 ng/ml) significantly increased the production of progesterone by

granulosa cells (Chabrolle *et al.* 2007a). RhVisf produced a more than twofold reduction in IGF1-induced progesterone secretion but did not affect the response to LH or FSH (Fig. 4B). Similar results were observed in granulosa cells from F2 and F3/4 hierarchical follicles (data not shown).

The effects of FK866 on rhVisf-inhibited production of progesterone in hen granulosa cells

We confirmed the effect of NAMPT on IGF1-induced steroid production using FK866, a specific inhibitor of NAMPT enzyme activity in granulosa cells from F1 hierarchical follicles. First, we demonstrated that 10 nM FK866 inhibited NAMPT activity, which thus caused a significant decrease in NAD⁺ levels (Fig. 5A). Next, we checked that the same concentration of FK866 did not induce apoptosis using the trypan blue exclusion method and annexin V labelling in hen granulosa cells (data not shown). FK866 completely abolished the inhibitory effect of NAMPT on IGF1-induced progesterone secretion (Fig. 5B). Similar results were obtained using granulosa cells from F2 and F3/4 hierarchical follicles (data not shown). We therefore conclude that NAMPT treatment inhibits IGF1-stimulated production of progesterone in hen granulosa cells.

We next used hen granulosa cells from F1 hierarchical follicles to examine whether the inhibitory effect of NAMPT on progesterone production was a result of its effects on an important cholesterol carrier, STAR, or on two enzymes involved in steroidogenesis, HSD3B and CYP11A1. NAMPT treatment (100 ng/ml) in the

Figure 4 Effects of recombinant human NAMPT (rhVisf) on progesterone secretion in granulosa cells from F1 hierarchical follicles. Granulosa cells from the largest pre-ovulatory follicles were cultured for 24 h in medium with serum and then in serum-free medium for 48 h with different concentrations of rhVisf (0, 1, 10, 100 or 500 ng/ml) (A) or with 100 ng/ml rhVisf in the presence or absence of IGF1 (76 ng/ml), FSH (300 ng/ml) or LH (300 ng/ml) (B), as described in Materials and methods. The culture medium was then collected, and its progesterone content (A and B) was determined by ELISA. Results (means $\pm$ S.E.M.) from four independent experiments are expressed in ng/ml. There were four replicates for each treatment within each experiment. In each experiment, we used four hens, and we consequently used a pool of granulosa cells from four follicles of the same category (granulosa cells from one follicle per hen). Bars with different letters are significantly different ($P < 0.05$).

Figure 5 The effect of FK866 on NAMPT activity (A) and on the inhibitory effect of rhVisf on progesterone secretion (B) in granulosa cells from hierarchical follicle F1. (A) Granulosa cells from the largest hierarchical follicles were cultured for 24 h in medium with serum and then in serum-free medium for 48 h in the presence of FK866 (1 or 10 nM), an enzymatic inhibitor of NAMPT. Granulosa cells were then lysed, and the amount of NAD⁺ was determined as described in Materials and methods and represented as NAD⁺ production per ng of total protein. Results are means $\pm$ S.E.M. from four independent experiments. Bars with different letters are significantly different ($P < 0.05$). (B) Granulosa cells from the largest hierarchical follicles were cultured for 24 h in medium with serum and then in serum-free medium for 48 h in the presence or absence of rhVisf with or without IGF1 (76 ng/ml) and FK866 (10 nM), as described in Materials and methods. The culture medium was then collected, and its progesterone content was analysed by ELISA. Results (means $\pm$ S.E.M.) from four independent experiments are expressed in ng/ml. There were four replicates for each treatment within each experiment. In each experiment, we used four hens, and we consequently used a pool of granulosa cells from four follicles of the same category (granulosa cells from one follicle per hen). Bars with different letters are significantly different ($P < 0.05$).

presence or absence of IGF1 halved the amount of STAR (Fig. 6A) and HSD3B (Fig. 6B), but it did not affect CYP11A1 protein expression (Fig. 6C). Furthermore, these effects were eliminated in the presence of FK866 (Fig. 6A and B). The results indicate that the decrease in progesterone secretion following NAMPT treatment may be a result of the reduced production of the STAR and HSD3B proteins.

Signalling pathways involved in the NAMPT effects in hen granulosa cells

It is well known that NAMPT treatment activates various signalling pathways, including PRKA, AKT1, MAPK3/1 and MAPK14, in different cell types (Cheng *et al.* 2011, Brandauer *et al.* 2013, Reverchon *et al.* 2013a, Song *et al.* 2014). We showed that in hen granulosa cells from F1 hierarchical follicles, NAMPT treatment decreased MAPK3/1 phosphorylation after 30 or 60 min of stimulation (Fig. 7A). Consistent with previous research using chicken cells (Tosca *et al.* 2006), phosphorylated MAPK3/1 appeared as a single band (~ 42 kDa) and probably corresponded to MAPK1. Under the same conditions, PRKA, AKT1 and MAPK14 phosphorylation levels were unchanged (data not shown). We next assessed the effects of NAMPT

treatment on IGF1-induced MAPK3/1 phosphorylation (Fig. 7B). Hen granulosa cells from F1 hierarchical follicles were incubated in serum-free medium supplemented with recombinant human NAMPT (100 ng/ml) for 48 h (the procedure used to measure progesterone production) and were then treated with or without IGF1 (76 ng/ml) for 5 min. As expected, IGF1 treatment

Figure 6 The effect of rhVisf on the amounts of the STAR (A), HSD3B (B) and CYP11A1 (C) proteins in granulosa cells from hierarchical follicles F1 in the absence or presence of FK866. Protein extracts from hen granulosa cells from F1 hierarchical follicles were cultured for 48 h with rhVisf in the presence or absence of IGF1 (76 ng/ml) with or without FK866 (10 nM), then subjected to SDS-PAGE as described in Materials and methods. They were then transferred onto nitrocellulose membranes and incubated with antibodies raised against the STAR (A), HSD3B (B) and CYP11A1 (C) proteins. We verified the similarity of protein loads by re-probing the membrane with an anti-vinculin antibody. Results are representative of at least four independent experiments. Blots were quantified, and the STAR, HSD3B and CYP11A1/VCL ratios are shown. The results are expressed as means $\pm$ S.E.M. from three independent experiments. Bars with different letters are significantly different ($P < 0.05$).

Figure 7 The effect of rhVisf on basal (A) and IGF1-induced (B) levels of MAPK3/1 phosphorylation in granulosa cells from F1 hierarchical follicles. Granulosa cell lysates were prepared from cells incubated for 48 h with rhVisf (100 ng/ml) for varying periods of 0, 1, 5, 10, 30 or 60 min (A) or rhVisf (100 ng/ml) in the presence or absence of FK866 (10 nM) (B) and then stimulated with IGF1 (76 ng/ml) for 5 min. Lysates (80 µg) were resolved using SDS-PAGE, transferred to nitrocellulose and probed with anti-phospho-MAPK3/1 and anti-MAPK3/1 total. Representative blots from three independent experiments are shown. Bands on the blots were quantified, and the ratio of phosphorylated protein:total protein is shown. The results are reported as means $\pm$ S.E.M. Bars with different letters are significantly different ($P < 0.05$).

alone significantly increased phosphorylation of MAPK3/1. Treatment with recombinant human NAMPT alone did not affect basal phosphorylation of MAPK3/1, although it completely eliminated IGF1-induced phosphorylation of MAPK3/1. Furthermore, this effect was abolished when the cells were incubated with 10 ng/ml FK866 (Fig. 7B). In summary, acute (30 or 60 min) NAMPT treatment reduces MAPK3/1 phosphorylation and inhibits IGF1-induced MAPK3/1 phosphorylation in hen granulosa cells.

The effects of NAMPT on granulosa cell proliferation and viability

We also investigated whether or not treatment with NAMPT affected the number of granulosa cells in culture by inducing mitosis or altering cell viability by assessing the incorporation of [3 H]thymidine into hen granulosa cells from F1 hierarchical follicles after culturing for 24 h in the presence or absence of NAMPT (10 or 100 ng/ml) and/or IGF1 (76 ng/ml) in serum-free medium. Treatment with IGF1 (76 ng/ml, 24 h) produced a roughly fivefold increase in [3 H]thymidine incorporation ($P < 0.05$) (Fig. 8). NAMPT treatment (10 or 100 ng/ml) did not affect the level of [3 H]thymidine incorporation regardless of the presence or absence of IGF1. Staining with trypan blue revealed that NAMPT treatment (10 or 100 ng/ml for 24 or 48 h) had no effect on cell viability regardless of the presence or absence of IGF1 (data not shown). Similar results were obtained in granulosa cells from F2 and F3/4 hierarchical follicles. We therefore concluded that NAMPT does not alter cell proliferation in primary hen granulosa cells.

Discussion

The present study has provided the first evidence that NAMPT is present in hen ovarian follicle cells. NAMPT (mRNA and protein) is expressed in granulosa and theca cells from hierarchical follicles. Plasma NAMPT levels are higher in young hens than they are in adults. We showed that in primary hen granulosa cells, treatment with recombinant human NAMPT inhibited basal and IGF1-induced levels of progesterone secretion, and this effect was associated with a reduction in STAR and HSD3B protein levels and in MAPK3/1 phosphorylation.

Figure 8 Effects of NAMPT (rhVisf) on hen granulosa cell proliferation. The incorporation of thymidine was evaluated in hen granulosa cells from F1 hierarchical follicles. Hen granulosa cells were cultured for 24 h with various doses of rhVisf (0, 10 or 100 ng/ml) in the presence or absence of IGF1 (76 ng/ml) in serum-free medium, as described in Materials and methods. Results are representative of four independent experiments. There were four replicates for each treatment within each experiment. In each experiment, we used four hens, and we consequently used a pool of granulosa cells from four follicles of the same category (granulosa cells from one follicle per hen). The results are expressed as means $\pm$ S.E.M. Bars with different letters are significantly different ($P < 0.05$).

Furthermore, all of these effects were abolished when cells were incubated with FK866, which indicates that they are dependent on the enzymatic function of NAMPT.

The present study demonstrated that NAMPT is present in granulosa and theca cells from hen hierarchical follicles. NAMPT is also expressed in human and mouse ovarian cells, including oocyte, granulosa and theca cells (Park *et al.* 2011, Reverchon *et al.* 2013a). It has also been shown that in the human granulosa cell line KGN, NAMPT is induced by another adipokine named INTELECTIN 1 (Reverchon *et al.* 2013a). In chickens, NAMPT mRNA transcripts have been detected in various tissues, including male and female gonadal tissue (ovaries and testes) (Ons *et al.* 2010). In chicken testes, the levels of NAMPT mRNA expression are higher in adults than they are in prepubertal animals. In adult chickens, NAMPT has been localised to several types of testicular cells (Leydig cells, Sertoli cells, germinal cells with the exception of spermatogonial cells) as well as seminal plasma and sperm. The present study is the first to show that levels of NAMPT mRNA expression are higher in granulosa cells than they are in theca cells; however, we observed the opposite pattern at the protein level, which suggests that there is translational regulation of NAMPT mRNA or differential turnover of NAMPT protein and RNA in granulosa and theca cells. The differential regulation of mRNA and protein levels of NAMPT has already been demonstrated in chickens; Ocón-Grove *et al.* (2010) showed that testicular NAMPT mRNA levels were higher in adult chickens than they were in prepubertal birds, whereas NAMPT protein levels were similar at both developmental stages. Previous research has shown that in hens, other adipokines, including ADIPOQ (adiponectin) protein, are also expressed at higher levels in theca cells than they are in granulosa cells (Chabrolle *et al.* 2007a), and similar patterns of results have been reported in human subjects (Chabrolle *et al.* 2009) and rodents (Chabrolle *et al.* 2007b). In the present study, we detected a reduction in plasma NAMPT levels as hens aged; however, it has been reported that in broiler chickens, plasma NAMPT levels increase between 4 and 8 weeks of age (Krzysik-Walker *et al.* 2008), and higher NAMPT plasma levels have been detected in adult (29-week-old) chickens than in prepubertal (4- and 14-week-old) male chickens (Ocón-Grove *et al.* 2010). These results suggest that the regulation of NAMPT levels during development varies according to sex and breed (meat vs laying) of the chickens. These hypotheses need to be confirmed in larger samples.

In the present study, we observed *in vitro* that recombinant human NAMPT inhibited basal and IGF1-induced progesterone secretion by granulosa cells. We used recombinant human NAMPT because the deduced amino acid sequence of full-length chicken NAMPT is 92–93% homologous with human NAMPT (Krzysik-Walker *et al.* 2008). Furthermore, we stimulated primary hen granulosa cells with a dose of 100 ng/ml NAMPT,

which is close to the level observed in plasma. The effect of NAMPT on hen granulosa cell steroidogenesis *in vitro* appears to be different from that observed in human granulosa cells. Our laboratory has previously shown that NAMPT stimulates IGF1-induced progesterone secretion (Reverchon *et al.* 2013a). This difference between species could be related to the peculiarities of chicken glucose metabolism. In spite of having 'normal' levels of circulating insulin, chickens have high glycaemia levels and show lower sensitivity to exogenous insulin as compared to humans (Simon 1989, Akiba *et al.* 1999, Dupont *et al.* 2004, Braun & Sweazea 2008). The inhibitory effect of NAMPT on IGF1-induced progesterone production is dependent on the intracellular enzymatic activity of NAMPT as it is abolished by the pharmacological competitive inhibitor FK866 (10 nM). The results presented here are consistent with recent data showing that extracellular NAMPT has NAMPT enzymatic activity in mice (Zhao *et al.* 2014). Moreover, the present results suggest that NAMPT may play a role in ovulation in hens; it has already been shown that progesterone triggers ovulation in birds, which causes a surge in LH production (Sauveur & de Reviers 1988). One hypothesis for this is that a decrease in NAMPT plasma levels before the increase in progesterone secretion is responsible for the pre-ovulatory LH surge; however, cyclical changes in the plasma levels of NAMPT in hens have yet to be determined. We also demonstrated that FK866 (10 nM) significantly decreased levels of NAD⁺ in hen granulosa cells. NAMPT is the rate-limiting enzyme in NAD biosynthesis, and it plays a critical role in several mammalian physiological processes (Revollo *et al.* 2004, Imai 2009). In mammals, NAMPT regulates the activity of the sirtuin 1 (SIRT1) histone deacetylase (Revollo *et al.* 2004), and it has been shown to be involved in the regulation of steroidogenesis (Morita *et al.* 2012, Wu *et al.* 2012). Sirtuins have not yet been identified in hen ovaries, but they may play a role in the effects of NAMPT on hen granulosa cell steroidogenesis.

In the present study, we showed that recombinant human NAMPT significantly reduced protein levels of the cholesterol carrier STAR, which regulates steroidogenesis within pre-ovulatory follicles (Stocco 2001) and HSD3B in primary hen granulosa cells. These effects could explain the inhibitory effect of rhVisf on progesterone production. We also observed that acute treatment with rhVisf reduced MAPK3/1 phosphorylation but did not affect PRKA, AKT1 or MAPK14. Treatment with rhVisf also reduced IGF1-induced MAPK3/1 phosphorylation. Several studies in mammals and chickens have shown that the MAPK3/1 signalling pathway is associated with events which contribute to the regulation of STAR expression and steroidogenesis in granulosa and Leydig cells (Johnson & Bridgham 2001, Martinelle *et al.* 2004, Tosca *et al.* 2005, 2006). The decrease in IGF1-induced MAPK3/1 phosphorylation in

response to NAMPT may thus help explain the reduction in progesterone production by hen granulosa cells. We did not investigate the effect of rhVisf on IGF1 receptor activation in the present study. The identity of the NAMPT receptor is still unknown; however, we can speculate that rhVisf binds to IGF1R and decreases IGF1 receptor tyrosine phosphorylation and that this accounts for the inhibition of IGF1-induced MAPK3/1 phosphorylation and progesterone production. In mammals, NAMPT modulates INSR signalling pathways, including tyrosine phosphorylation of the INSR β subunit and its substrates, insulin receptor substrate 1 (IRS1) and IRS2, in human osteoblasts (Xie *et al.* 2007). Other adipokines, such as resistin and chemerin, have also been shown to reduce IGF1R signalling in human granulosa cells (Reverchon *et al.* 2012, 2013b).

Concluding remarks

In summary, NAMPT is expressed in hen ovarian follicles and, more specifically, in granulosa and theca cells of hierarchical follicles. In hens, plasma NAMPT levels are significantly lower in adults than they are in young birds. *In vitro* recombinant human NAMPT decreases basal and IGF1-induced production of progesterone by primary hen granulosa cells. This effect is associated with a reduction in STAR and HSD3B protein levels and MAPK3/1 phosphorylation. Taken together, these data suggest that NAMPT plays a role in hen folliculogenesis and, more precisely, in the control of steroidogenesis.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-15-0021>.

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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